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Polycatechol-Functionalized Gelatin Bioadhesives
for Sutureless Wound Closure

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Bioengineering

by

Hossein Montazerianoliaee

2023

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2023

ABSTRACT OF THE DISSERTATION

Polycatechol-Functionalized Gelatin Bioadhesives for Sutureless Wound Closure

by

Hossein Montazerianoliaee

Doctor of Philosophy in Bioengineering

University of California, Los Angeles, 2023

Professor Nasim Annabi, Co-Chair

Professor Paul S. Weiss, Co-Chair

Bioadhesive hydrogels provide promising solutions for bleeding wound management to replace invasive wound closure methods such as suturing and stapling. Adhesive substances based on naturally occurring biological materials from living organisms, such as mussels and Boston ivy plants, harness phenolic compounds for their attachment to wet surfaces. Recent efforts have centered on catechol groups as effective functionality to leverage tissue adhesion in biomaterials. Bioadhesion performance in catechol-integrated biomaterials is a strong function of polyphenolic structure and the processing approach for their integration into hydrogel networks.

One of the major hurdles in catechol-functionalization chemistries is insufficient phenolic uptake due to their low yield and inherently limited functionalization capacity of

proteins. Polyphenols not only enable stronger attractions with various substrates, but also act as crosslinking points, strengthening polymer network cohesion. Polyphenolic compounds derived synthetically from phenolic small molecules as well as those occurring naturally, such as tannins, have formed a large library of additional functionality such as antimicrobial and photothermal responsiveness, calling for further development for applications in wound management.

Here, different strategies undertaken for developing bioadhesives are first introduced in terms of constitutive polymers and their crosslinking mechanisms. Then, procedures for introducing hemostatic efficacy to hydrogels are discussed in detail to enable their function for sealing bleeding wounds. Next, a mechanistic study is described that highlights current pitfalls in the design of catechol-functionalized biomaterials in free-radical-based photocrosslinking processes. Then, *in situ* polymerization of catecholic compounds is introduced to enable robust bioadhesion of gelatin methacryloyl (GelMA) hydrogel patches. Given the significant demand for injectable bioadhesives, a chemical procedure is proposed for high-efficacy loading of catecholic compounds covalently to gelatin backbones. Here, simple oxidative polymerization is implemented on catechol-carrying molecules (caffeic acid) prior to standard coupling amidation reactions to boost catechol attachment and their function for wound closure. Further, photothermal responsive function and rapid thermal and covalent gelation enabled by larger molecular weight polymers of polyphenolic structures (*i.e.*, L-3,4-dihydroxyphenylalanine, L-DOPA) are obtained following the same procedure. Finally, I discuss the implication of these studies in clinical settings and potentials for further development and additional tissue regenerative functions.

The dissertation of Hossein Montazerianoliaee is approved.

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University of California, Los Angeles

2023

To my family, mentors, and friends

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CHAPTER 1: INTRODUCTION

Bioadhesives are garnering substantial interest due to their utility in sutureless wound closure and their applications in the integration of biomedical devices with the tissues.¹ Surgical sealants are effective tools for the sealing and management of surgical incisions and bleeding wounds as alternatives to conventional suturing and stapling processes.² In addition to their wound-sealing capacity, bioadhesives can be designed for various functions such as drug delivery,³ wound healing,⁴ and tissue regeneration.⁵ Current commercial tissue adhesives, however, are restricted by poor wet adhesion *in vivo*.⁶ Fibrin-based sealants suffer from limited human sources and carry the risk of viral transmission.⁶ Natural protein-based biomaterials, such as gelatin and bovine serum albumin constitute major portions of material compositions in commercially available sealants due to their availability, low cost, biocompatibility, and biodegradability.⁷ Gelatin can be modified through various chemical procedures in a tunable manner enabling its applications in tissue regeneration, drug delivery, and wound healing. Bioadhesive design strategies are evolving to impart tough bioadhesion to gelatin through various chemical methodologies.⁸ These approaches aim to establish stronger interactions between the biomaterials and tissue substrates at the molecular scale under wet conditions to address more complicated scenarios such as adhesion on bleeding wounds.¹

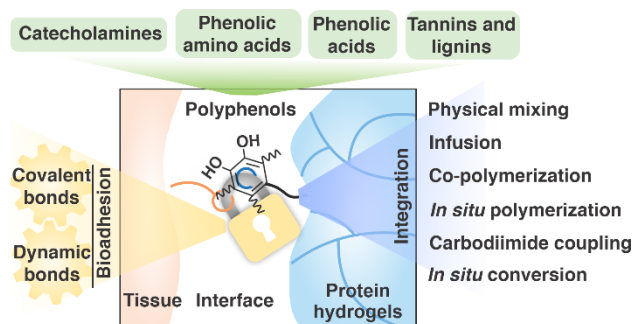
Bioadhesion in aqueous conditions is challenged by the interference of water molecules with the hydrogel-tissue interactions. Therefore, chemical functionality is required to enable dehydration of the interfacial layers at the molecular level. Charged phenolic structures such as catechol groups are able to remove the hydration film enabling

direct interactions of the functional groups with the wet tissue substrates.⁹ Phenolic structures have received significant interest as adhesive building blocks, particularly because they play major roles in wet adhesion of natural organisms, such as mussel byssal threads,¹⁰ and plants, such as Boston ivy.¹¹ Mussel byssal threads secrete mussel foot proteins (mfp), which facilitate their adhesion due to the abundance of catechol-rich amino acid residues in their structure. Catechol groups enable adhesion through various covalent and non-covalent bonds (hydrogen-bonding and π - π interactions). Covalent adhesion is driven primarily *via* Schiff base formation, Michael-type addition, and di-catechol coupling. These reactions occur when catechol groups are converted into reactive quinones under the oxidative conditions of ocean water. In addition, metal ions can form ionic complexes between catechol groups and hydroxyl groups on the adherent substrates. The strong affinity of phenolic compounds towards biological tissues has inspired incorporation of their polymeric derivatives into hydrogels for bioadhesion. Next-generation polyphenolic bioadhesives are emerging in the form of injectable biogluers¹² and hydrogel patches, and microneedles,¹³ enabling new therapeutic functions compared to other bioadhesive types, as discussed in previous reviews. However, there are still many unknown aspects in developing polyphenolic bioadhesives that needs to be understood before they can be translated for clinical applications.

In this thesis, I introduce chemical principles and the working mechanism of polyphenolic compounds as adhesive building blocks in the modification of functional biomaterials (Chapter 2). Then, I provide a broad overview of the bioadhesive design roadmap where I introduce materials and processing techniques that provide tough bioadhesion in Chapter 2. Subsequently, In Chapter 4, I discuss the procedures implemented

for introducing hemostatic function in biomaterials for efficient bleeding control and to promote post-incision wound healing. In Chapters 2-4, the major breakthroughs in the field are discussed in detail and the current research gaps are identified. Here, we highlight the controversy in the literature on the bioadhesion function of polyphenolic compounds in free-radical crosslinking hydrogels. Therefore, in Chapter 5, I develop experimental procedures to address the role of catechol functional groups in photocrosslinkable hydrogel systems. One major problem in the fabrication of degradable bioadhesives for internal use is the limited polyphenolic loading capacity in both chemical conjugation- and physical mixing-based approaches. Therefore, in the following chapters, biomaterial design strategies are developed to enhance the effects of catechol function by boosting their loading capacity in gelatin-based hydrogel systems. In chapter 6, I propose an *in situ* polymerization of catecholic compounds where polydopamine chains are grown onto the methacryloyl sites of gelatin methacryloyl (GelMA) backbones. The resulting hydrogels are served as bioadhesive patches that can be used as wound dressings and as bridging elements at the human-machine interfaces. Then, in Chapters 6 and 7, I focus on amplifying catechol grafts conjugated to gelatin backbone using standard coupling chemistries (*i.e.*, carbodiimide coupling) for injectable bioadhesion using caffeic acid, and L-3,4-dihydroxyphenylalanine (L-DOPA), respectively. In these chapters, I propose pre-conjugation polymerization of catechol-carrying small molecules where an array of catechol groups is conjugated per carbodiimide reaction. By this means, not only a more robust bioadhesion is attained, further improvements in the rheology of prepolymer and more efficient crosslinking are indicated. Finally, in Chapter 9, I highlight current challenges and future opportunities for taking polyphenolic gelatin bioadhesives closer to clinical translation.

CHAPTER 2: POLYPHENOLIC GELATIN-BASED BIOADHESIVES



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Abstract

Polyphenols play important roles in covalent and non-covalent interactions with functional groups in biological substrates, including keratins, connective tissue, or soft internal tissues in areas such as the heart. Here, we review several recent breakthroughs in polyphenol-integrated gelatin that have been analyzed in the context of their use as bioadhesives. We consider different polyphenol-carrying compounds for modification including catecholamines, phenolic amino acids, tannins, and lignins. We then discuss how these polyphenolic materials can be fabricated to mimic naturally derived bioadhesives through infusion, physical mixing, and co-polymerization. This analysis includes

implications for how the rheological properties might affect their delivery. We discuss the implications of using these bioadhesives, questioning their viability and prospects.

2.1 Introduction

Bioadhesives are important for the future of medicine in their roles in wound closure and as measures to enhance wound healing. These adhesives are more effective and less invasive than conventional wound closure methods, such as surgical sutures or staples. Adhesive substances based on naturally occurring biological materials from living organisms harness phenolic compounds for their attachment to wet surfaces. For example, plants, such as Boston ivy, or animals, such as mussels, have evolved tissues that create optimal adhesion under a variety of challenging conditions, including in aqueous and saline environments. Current research aims at using biomimetic strategies to create a new generation of bioadhesives that will be better suited for medical use. Biomaterials design has evolved around integration of phenols with protein backbones among which gelatin has received particular attention due to its excellent bioactivity, biocompatibility, biodegradability, low cost, facile chemical tunability, and tissue-mimetic properties. Bioadhesion performance in these biomaterials is a strong function of polyphenolic functionality and the processing approach for their integration into hydrogel networks. A number of studies have used phenolic small molecules to modify biomacromolecules chemically for bioadhesion. One of the major hurdles in these studies is insufficient phenolic uptake due to low-yield modification chemistries and inherently limited functionalization capacity of proteins.

Phenolic structures have received significant interest as adhesive building blocks, particularly because they play major roles in wet adhesion of natural organisms, such as

mussel byssal threads¹⁰ and plants, such as Boston ivy (Figure 2.1A,B).¹¹ Polyphenolic compounds can be obtained *via* simple chemical processes such as oxidative polymerization and metal ion coordination.^{14,15} Phenols and their derivatives interact with functional groups present on tissue surfaces through various covalent and non-covalent chemical interactions, as illustrated in Figure 2.1C. The outermost layer of skin epidermis, *i.e.*, the stratum corneum, is covered by dead cells filled with densely packed keratin proteins. Keratins consist of hydrophobic (*e.g.*, alanine and valine) and hydrogen-bond donor amino acids (*e.g.*, glutamic acid and serine),¹⁶ which can establish non-covalent π - π interactions and hydrogen bonding with phenolic compounds.¹⁷ In addition, covalent attachment can occur through Michael addition reactions of oxidized phenol (quinones) with the thiol and amino side chains in amino acid residues, such as cysteine and lysine. Such combinations of amino acids are present on the surfaces of internal soft tissues such as the fibrous pericardium layer of the heart¹⁸ and the visceral pleura layer of the lungs.¹⁹ These layers are composed of connective tissues from collagen- and elastin-based fiber proteins, which are rich in electrostatically charged, hydrophobic, and hydrophilic nucleophile amino acids enabling chemical interactions with phenols.²⁰ The physical and chemical characteristics of polyphenolic molecules significantly affect the possible processing methods for their integration with biomaterials. Molecular weights of polyphenolic compounds synthesized from various phenolic small molecules can vary from dimers and trimers to larger molecular weight nanoparticles. Polyphenolic moieties generated from chemically similar structures can lead to significantly different biological responses.²¹ These differences can restrict potential applications of the synthesized biomaterials.

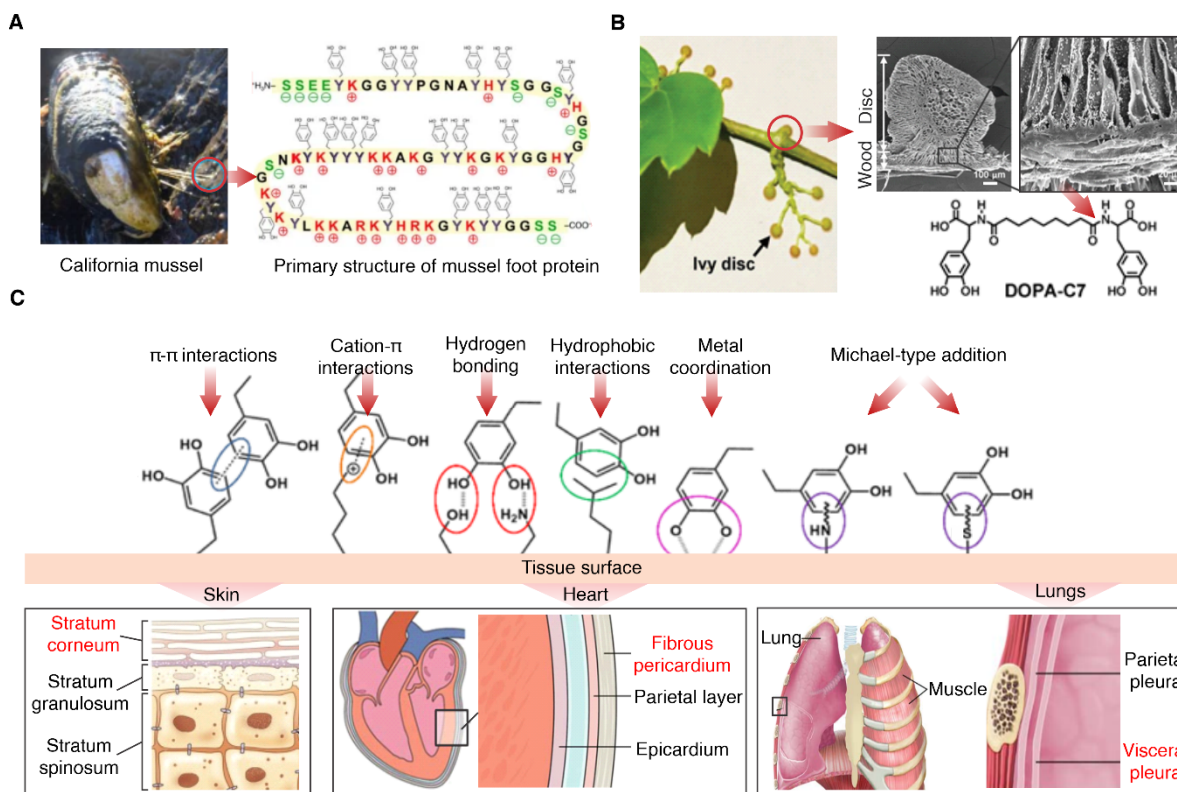


Figure 2.1. Process of bioadhesion *via* phenolic groups. (A) Phenolic compounds in mussel foot proteins (mfp) secreted from mussel byssal threads. Reproduced with permission from reference ¹⁰. Copyright 2015 Nature Publishing Group. (B) Plant adhesion to surfaces through 3,4-dihydroxyphenylalanine bolaamphiphile (DOPA-C7) in ivy discs. Reproduced with permission from reference ¹¹. Copyright 2020 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Chemical interactions driving bioadhesion to tissue surfaces. Reproduced with permission from reference ¹⁷. Copyright 2021 Elsevier. Reproduced with permission from reference ²². Copyright 2013 XanEdu Publishing Inc. Download for free at <https://openstax.org/details/books/anatomy-and-physiology>.

Polyphenols are an attractive toolbox for bioadhesive design as they not only enable stronger interactions with various substrates, but also act as crosslinking points, strengthening polymer network cohesion. In addition, the crosslinking mechanism used for gelation of bioadhesives should be compatible with polyphenolic moieties as, for instance, free-radical polymerization in the presence of phenolic compounds is compromised by their free-radical scavenging effects. Polyphenolic compounds derived synthetically from

phenolic small molecules as well as those occurring naturally, such as tannins, have added a large library of additional functionality, such as antimicrobial and photothermal responsiveness, calling for further developments for applications in wound management. In this chapter, we introduce phenolic compounds, their natural origins, and processes for their conversion into polyphenolic molecules. Then, the chemical and physical processes for incorporation of polyphenols into gelatin-based hydrogels are highlighted in terms of processing parameters, their limitations, and implications on bioadhesion capacity. Subsequently, we discuss the rheology of hydrogel precursors, their crosslinking kinetics, and mechanical properties post-crosslinking to elucidate the interrelations between polyphenolic moieties and hydrogel network characteristics.

2.2 Polyphenolic compounds for bioadhesion

Below, the most common phenol-carrying agents introduced into gelatin-based biomaterial systems for bioadhesion are discussed.

2.2.1 Catecholamines

Several *in vivo* physiological processes involve catecholamines including dopamine, epinephrine, and norepinephrine.²³ These molecules are produced predominantly in the adrenal gland on the kidneys and the hypothalamus in the brain.²³ Among catecholamines, dopamine has received the most attention for development of bioadhesives.²⁴ Dopamine is a monoamine neurotransmitter enabling chemical communications in the central nervous system (CNS).²³ Polydopamine nanoparticles, a product obtained by oxidized dopamine intermediates (*i.e.*, quinones) have been the most popular polyphenol used to improve adhesion in hydrogels.⁹ In addition, polydopamine imparts photothermal responsiveness to

near-infrared (NIR) light for cancer therapy and serves as a coating agent on implants for improved osteogenic properties.²⁵ Polydopamine chains are composed of covalently linked dopamine, indolinedione, and dihydroxyindole units.²⁶ Dopamine units provide polydopamine nanoparticles with free amine groups that serve as conjugation sites for coupling with biomacromolecules.

2.2.2 Phenolic amino acids

Aromatic amino acids, which also exist in the protein structures at the interface of tissue adherents, are another phenol-carrying means to endow bioadhesion. Tyrosine, phenylalanine, tryptophan,²⁷ and L-3,4-dihydroxyphenylalanine (L-DOPA) are examples of phenolic amino acids, among which L-DOPA has been studied extensively²⁸ as they are responsible for adhesion of mfp.¹⁰ In addition, L-DOPA, known otherwise as Levodopa, is a U.S. Food and Drug Administration (FDA)-approved drug for slowing the progression of Parkinson's disease. L-DOPA is decarboxylated to dopamine *in vivo* through various metabolic pathways; *e.g.*, *via* aromatic L-amino acid decarboxylase (AAAD) enzymes in the liver, kidney, gut, and nerve terminals of the brain striatum.²⁹ Recently, L-DOPA was shown to have antioxidative effects.³⁰ Similar to polydopamine, oxidative conditions lead to polymerization of L-DOPA to poly(L-DOPA).³¹ As opposed to polydopamine, where its nucleophilic amine groups are converted into non-reactive secondary amines in most of its repeating units, the carboxyl nucleophiles in the structure of L-DOPA remain intact in the repeating unit of poly(L-DOPA). This structure facilitates conjugation with other biomolecules.

2.2.3 Phenolic acids

Polyphenolic acids are most widely found in plants and food products. Hydroxycinnamic acid derivatives are a major class of such structures.³² Caffeic acid is one of the most available hydroxycinnamic acids with strong antioxidant effects and anti-inflammatory properties.³² Caffeic acid is found most abundantly in coffee (although it also exists in other foods such as fruits, *e.g.*, apples). Gallic acid is also extracted from fruits and plants, such as blackcurrant leaves and the ripe pulp of mangoes.³³ Caffeic³⁴ and gallic acids³⁵ have been studied previously as functional groups for improving biomolecular adhesion. They both form oligomeric phenol derivatives under oxidative conditions as opposed to catecholamines, which form higher molecular weight polymers.³⁶ These oligomers preserve and repeat the carboxyl conjugate sites present in monomers, rendering their suitability for polyphenolic modification. Natural hydroxycinnamic acids involve more species that are rarely studied for their bioadhesion effects. Some of these agents include cinnamic acid, coumaric acid, rosmarinic acid, chlorogenic acid, and ferulic acid.

2.2.4 Tannins and lignins

The richest sources of tannins and lignins are plant components including leaves, fruits, seeds, and tree bark. Tannic acid (TA) is the most commonly used tannin for bioadhesion, which can be incorporated in hydrogels non-covalently because they have no functional groups in their structure for nucleophilic conjugation to biomaterials.³⁷ Tannic acids form strong chemical complexes with proteins and metal ions. Lignin is another similarly structured molecule used to introduce bioadhesion and is mostly integrated physically with the hydrogel networks.³⁸

2.3 Processing methods for incorporating polyphenols in hydrogels

Various approaches are followed to incorporate polyphenolic moieties into gelatin-based proteins. These processes are designed based on factors such as: (1) solvent compatibility and water solubility of polyphenol-carrying agents, (2) availability of reactive functional groups on protein backbone and polyphenol-carrying agents, (3) molecular interactions, (4) target crosslinking mechanism, and (5) intended form of use, *i.e.*, topical *versus* implantable applications.¹ In protein-based hydrogels, backbones made of gelatin derivatives have been of particular interest due to their availability, low cost, and extracellular matrix-mimetic structure rendering them biocompatible and biodegradable.¹ Routine processing techniques for introducing polyphenolic functionality to gelatin-based hydrogels are highlighted below. Given the variety of adhesion assessment methods in the literature, we focus mainly on the implications of each processing method on bioadhesion.

2.3.1 Physical mixing

Physical mixing provides flexibility on the amounts of polyphenols one can load in hydrogels enabling amplification of their bioadhesive effects.²⁴ Although, the maximum loading can be limited by the crosslinking approach and potential cytotoxicity of polyphenolic functionality.²⁴ For example, the addition of dopamine at higher concentrations of >2 %w/v to 20 %w/v gelatin methacryloyl (GelMA) pre-polymers compromised their free-radical crosslinking due to free-radical scavenging effects of catechol groups.²⁴ In addition, in a physically mixed phenolic hydrogel, the additives interact non-covalently with the hydrogel chains making them susceptible for leaching out *in vivo* raising the risk of toxicity and immunogenic effects.²¹

Physical integration of polyphenols in hydrogels is one of the most straightforward methods to impart bioadhesion.²⁴ A recent report proposed incorporation of oxidized TA into gelatin solutions followed by crosslinking using borax (Figure 2.2A).³⁷ The hydrogels adhered to various tissue types (adhesion strength of up to ~100 kPa), which improved further with increasing TA content (Figure 2.2B,C). Bioadhesion in these hydrogels was found to be pH-responsive where increased acidity triggered hydrogel dissociation and aided in controllable deactivation of bioadhesion (Figure 2.2D). In another study, bioadhesive electroconductive sutured-nitrocatecholic strands (S-nCAT) were developed, which could be incorporated into gelatin solutions for injectable bioadhesion (Figure 2.2E,F).³⁹ The results of wound closure tests suggested significant improvements in adhesion strength, reaching ~40 kPa compared to <5 kPa commercial sealants (Figure 2.2G). Oxidatively polymerized gallic acid physically complexed with GelMA was copolymerized with poly(ethylene glycol diacrylate) (PEGDA) hydrogels.⁴⁰ This approach enabled temperature-sensitive bioadhesion for sensitive and fragile tissues (*i.e.*, infant skin and diabetic patients).

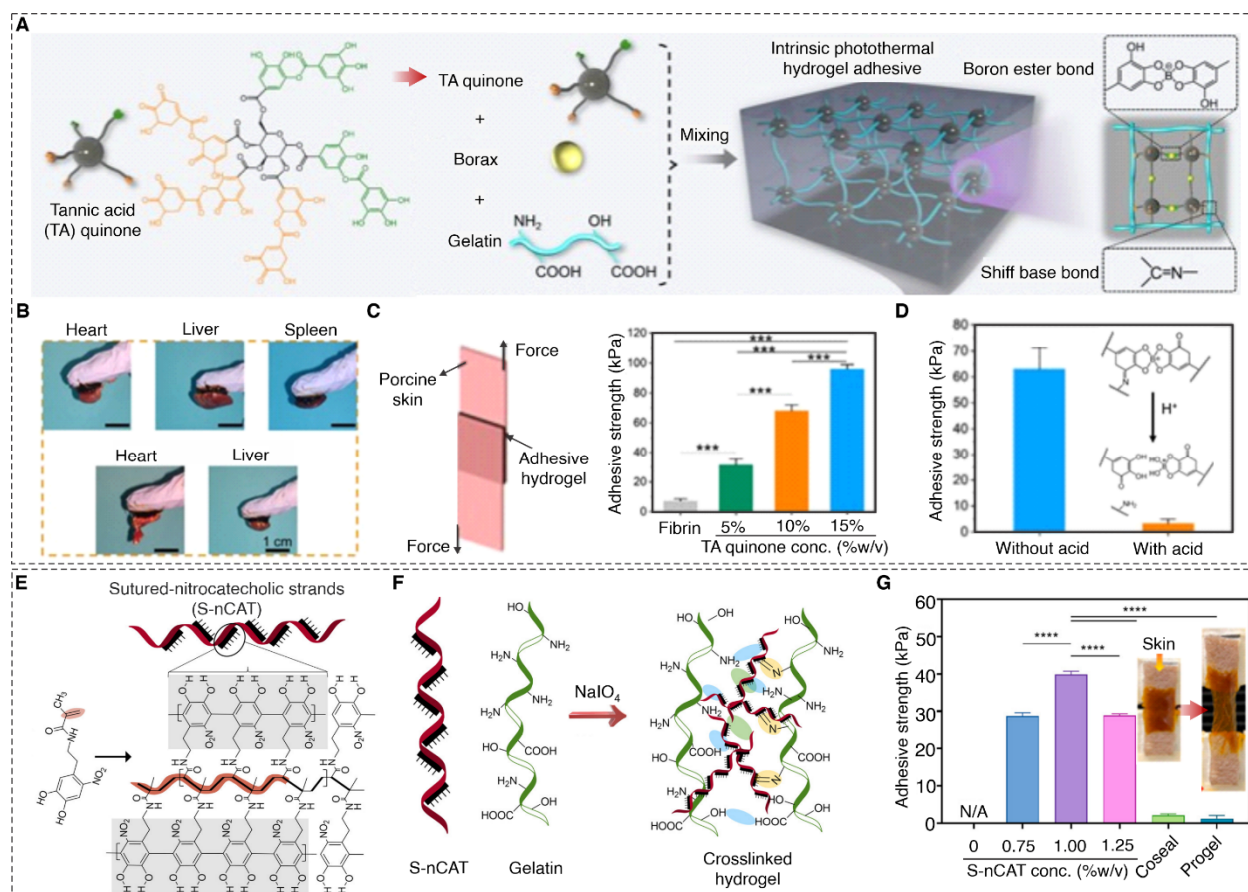


Figure 2.2. Physical mixing of phenolic compounds for introducing bioadhesion in hydrogels. (A) Tannic acid (TA) quinones obtained by NaIO₄ oxidation incorporated in a gelatin matrix. (B) Adhesion of hydrogels to different organs harvested from rats. (C) Lap shear adhesion test of gelatin-TA quinone in hydrogels supplemented with different TA concentrations. (D) Responsiveness of adhesion to pH. Reproduced with permission from reference ³⁷. Copyright 2023 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (E) Synthesis of electroconductive sutured-nitrocatecholic strands (S-nCAT). (F) Incorporation of S-nCAT in gelatin hydrogels followed by oxidative crosslinking. (G) Wound closure test on gelatin/S-nCAT hydrogels showing improved bioadhesion compared to commercial sealants. Reproduced with permission from reference ³⁹. Copyright 2022 American Chemical Society.

2.3.2 Infusion

Polyphenolic moieties can be incorporated within crosslinked hydrogels by *in situ* polymerization/association of phenolic compounds infused *via* soaking in phenolic

solutions.⁴¹ Hydrogels can uptake water-soluble phenolic small monomers (*e.g.*, dopamine) where their polymerization can be triggered oxidatively. Water-soluble polyphenolic moieties that strongly bind to the gelatin and protein backbones such as TA can be infused without the need for post-*in situ* polymerization (Figure 2.3A).⁴¹ This process led to significant improvements in adhesion, from <5 kPa to ~80 kPa. A washing step is required to remove the unassociated TA molecules (to prevent their burst release). The ionic association of infused phenols, such as TA, enabled the formation of a secondary dynamic network within covalent hydrogels leading to interpenetrating polymer networks (IPNs) with superior toughness in addition to bioadhesion.⁴¹ Bioadhesion of TA-infused GelMA hydrogels increased with the TA concentration in diffusive solution (Figure 2.3B). The resulting hydrogels also had self-healing properties (Figure 2.3C). Note that polyphenol infusion is only applicable to bioadhesive patches and not to injectable sealants because this method requires soaking crosslinked hydrogels in polyphenol solutions. Bioadhesive hydrogel patches can serve as wound dressings and as interfaces with bioelectronic devices.

2.3.3 Co-polymerization

A non-oxidative mechanism of phenol polymerization that enables their covalent incorporation within gelatin-based chains is free-radical co-polymerization of acrylated phenols with methacryloyl-modified gelatin.⁴² Phenolic compounds, such as dopamine, can be reacted with methacrylic anhydride (MA) to yield dopamine methacryloyl monomers (DMA), as shown in Figure 2.3D.⁴³ Hydrogels obtained through co-polymerization of DMA and GelMA were secondarily crosslinked using antimicrobial Zn²⁺ ions leveraging their application for wound healing (Figure 2.3E).⁴³ One major drawback associated with such a co-polymerization system originates from the radical scavenging effects of phenols. Free

radicals lead to phenolic oxidation and quinone formation. With increased acrylated phenols, the consumption of free radicals through phenolic side chains could leave behind more unreacted monomers, raising biosafety concerns, and limiting the possible number of acrylated phenols for gel formation. As a result, polyphenolic compounds are prone to diminishing free-radical crosslinking density implying low hydrogel cohesion and thereby poor bioadhesion strength. We note that bioadhesion of photocrosslinkable proteins (*i.e.*, GelMA) co-polymerized with phenolic monomers has not been well studied and remains to be explored.

2.3.4 *In situ* conversion

Most natural proteins including gelatin contain amino acid residues with side chain functionality that can undergo chemical reactions themselves and turn into phenolic compounds.⁴⁴ An example of this reaction is conversion of tyrosine residues on the gelatin backbone to catechol groups using tyrosine enzymes (Figure 2.3F).⁴⁴ The hydrogels obtained through this process were ionically crosslinkable using Fe^{3+} , promoting hemostatic effects in hydrogels (Figure 2.3F). We note that this approach is used for introducing catechol functions; polyphenol formation in these systems requires further oxidation of the newly formed catechol groups through coupling reactions. There is a paucity of data on bioadhesion capacity of such hydrogels. The available results on hemostatic effects were mainly attributed to the Fe^{3+} ions, as opposed to catechol groups, which showed no active hemostatic efficacy. Metal ion coordination *via* Fe^{3+} has been a frequently used approach for gelation of polyphenolic bioadhesives due to its biocompatibility *in vivo* in addition to its hemostatic effects.⁴⁵ Note that this finding contradicts other work⁴⁵ that assigned blood coagulating effects to conjugated catechol motifs.

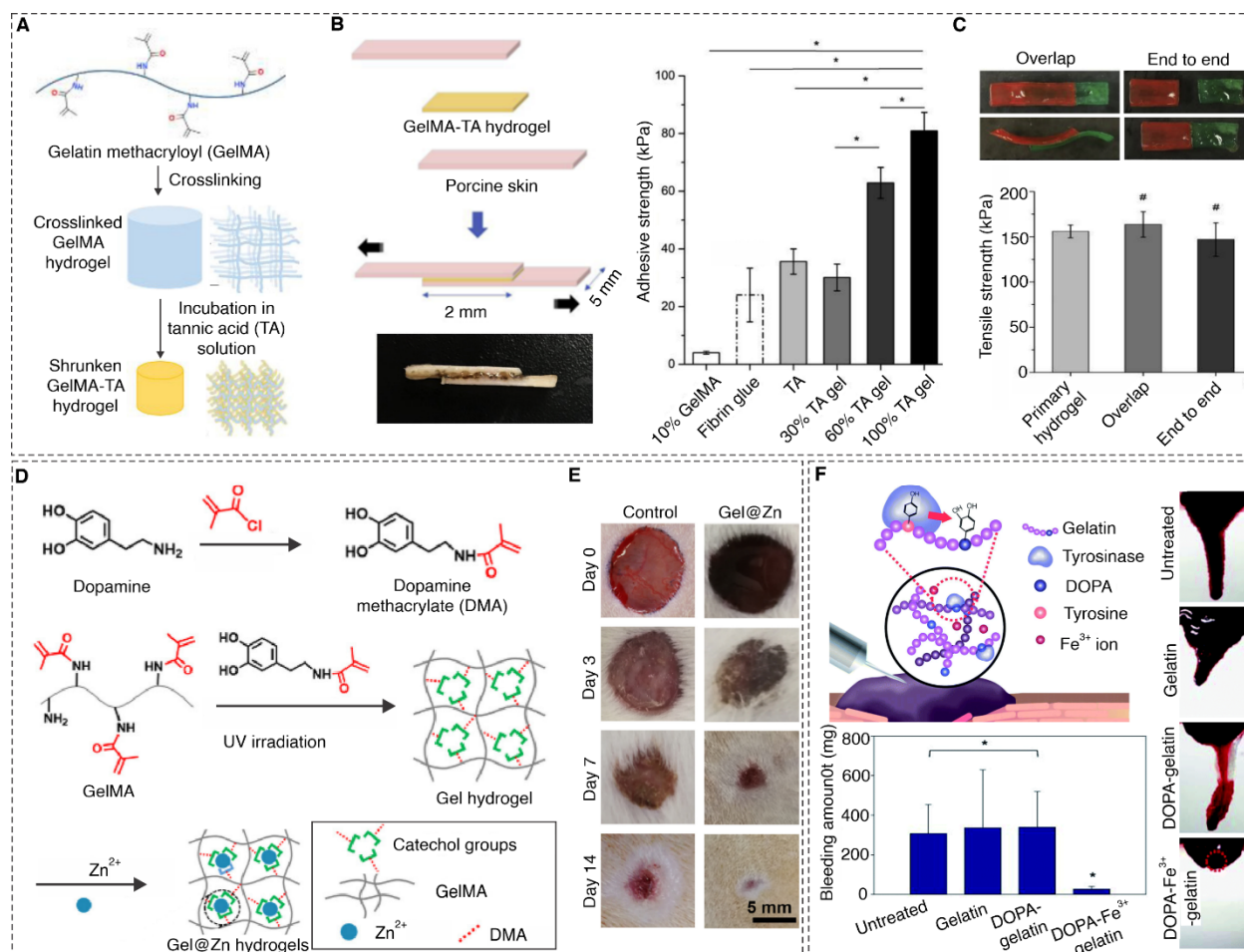


Figure 2.3. Different approaches undertaken for development of polyphenolic bioadhesives. These methods involve (A-C) infusion, (D, E) co-polymerization, and (F) *in situ* conversion. (A) Tannic acid (TA) infused-gelatin methacryloyl (GelMA) developed by soaking crosslinked GelMA in TA solutions. (B) Lap shear test on GelMA-TA hydrogels showing improved adhesion at higher TA contents. (C) Self-healing of GelMA-TA hydrogels. Reproduced with permission from reference ⁴¹. Copyright 2018 Elsevier. (D) Synthesis route of copolymerized dopamine methacrylate (DMA) with GelMA followed by crosslinking *via* antimicrobial Zn^{2+} ions (Gel@Zn hydrogels). (E) Promoted wound healing through treatments with Gel@Zn hydrogels. Reproduced with permission from reference ⁴³. Copyright 2022 Elsevier. (F) Process of converting tyrosine residues to catecholic moieties (DOPA) through tyrosinase enzymes. Hydrogels showed hemostatic effects after crosslinking with Fe^{3+} ions. Reproduced with permission from reference ⁴⁴. Copyright 2014 Royal Society of Chemistry.

2.3.5 Carbodiimide conjugation

Carbodiimide coupling is one of the most well-adapted processes for polyphenol modification *via* amidation through carboxyl and amino groups.⁹ According to the amino acid composition of gelatin, the contents of amino and carboxyl side groups are estimated to be on the order of 0.3 and 1.2 mmol per gram of gelatin, respectively.²⁰ Conjugation of catecholamines requires 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) activation of carboxyl groups in gelatin proteins. In this case, homocoupling of gelatin during EDC activation reaction is a trivial drawback limiting the conjugation efficacy. This issue, however, is not of concern for the conjugation of phenolic acids since carboxyl activation is performed separately, in the absence of gelatin.³⁴ Phenolic acids are mostly water-insoluble, requiring optimization of a hybrid reaction medium for their reaction with water-soluble gelatin.³⁴ Carbodiimide reactions require controlled pH and their overall reaction efficacy is relatively low (*i.e.*, <10%),^{34,46,47} resulting in limited instantaneous adhesion, on the order of ~9 kPa lap shear strength.⁴⁶

The low yield and limited available conjugation sites present major obstacles for polyphenol adhesion. Previously, we showed that GelMA hydrogels supplemented with ~650 μ M catechol content (dopamine) per gram of GelMA enabled significant bioadhesion in GelMA patches,²⁴ whereas the conjugation capacity of dopamine to gelatin *via* carbodiimide coupling was *ca.* 25 nM per gram gelatin.⁴⁶ Recently, we proposed oxidative polymerization of phenolic compounds (*e.g.*, L-DOPA) before their coupling reaction to gelatin (GelDOPA).²⁸ This approach resulted in significant improvements in catechol uptake adhesion from ~4 kPa to ~12 kPa. Here, in lieu of converting each individual conjugation site available on the gelatin to a phenolic group, they were each coupled with an array of phenolic

chains. In addition, oxidized phenols (quinone species) reacted with nucleophilic amino groups through Michael addition reactions, further strengthening the degree of conversion. The proposed reaction scheme resulted in accelerated thermal gelation in gelatin and the hydrogels achieved wet stability through covalent crosslinking efficiently by diffusion of NaIO_4 oxidizing agents. Further, this modification enabled photothermal responsiveness to NIR light for cancer therapy applications and antimicrobial properties due to the release of diffused IO_4^- ions back into the media. Although some studies emphasized that oxidized catechol loses its potential for adhesion, oxidatively polymerized catechol motifs enhanced adhesion strength (~ 8 kPa in *ex vivo* studies), beyond those of many commercial sealants, which typically have burst pressures of 2-4 kPa. We note that although quinones are less capable of non-covalent hydrogen bonding, they become much more covalently reactive towards nucleophiles (such as primary amines on the tissue surfaces) through *e.g.*, Michael addition reactions, explaining the observed tissue adhesion.

2.3.6 *In situ* polymerization

Oxidative polymerization of polyphenolic compounds is accompanied by generation of free radicals. Therefore, *in situ* polymerization of phenolic compounds in the presence of methacryloyl-modified proteins leads to their growth and attachment onto gelatin backbones at the methacryloyl sites.²⁴ Depending on the degree of unreacted methacryloyl groups, GelMA prepolymers can form adhesive hydrogels through free-radical polymerization, such as photo-crosslinking. However, previous studies showed that photo-crosslinking bioadhesives with *in situ* polydopamine polymerization have tissue adhesion only if used as a hydrogel patch and not when used as an injectable sealant (*i.e.*, *in situ* crosslinking). Although hydrogel patches showed improved tissue adhesion by $\sim 3\times$,

crosslinking time is prolonged and crosslinking density is reduced with the increase in PDA content.

In situ polymerization can be also implemented for grafting polyphenols to gelatin with no methacryloyl motifs. This attachment occurs primarily through Schiff base and Michael addition between quinone radicals (oxidized phenols) formed during propagation and primary amine nucleophiles present on the gelatin chains. Tannic acid (TA) quinones were attached to gelatin through this methodology.⁴⁸ The resulting hydrogels demonstrated adhesion strength $\sim 3\times$ higher than standard commercial sealants.

2.4 Rheological implications of polyphenols in bioadhesive solutions

Rheometric aspects of pre-gel solutions play critical roles in their handling during their application on tissues *via* injection. Nevertheless, the rheology of pre-gels has not received as much attention as the mechanical properties *e.g.*, tensile and adhesion strength after crosslinking. Low-viscosity pre-gel solutions are prone to dripping under gravity on the time scale between their application and crosslinking.³⁴ This issue will become particularly problematic in bleeding wounds, which are common during cardiac surgeries such as aortic replacement procedures.³⁴

Polyphenolic moieties can alter the behavior of gelatin in solution, depending on their integration process.^{24,34} Based on this effect, further biomaterial strategies can be undertaken to tune pre-gel viscosity. For instance, shear-thinning biomaterials.⁴⁹ The effects of phenolic compounds in gelatin solutions are discussed below with regard to their integration chemistry.

2.4.1 Non-covalent integration

Physical mixing of dopamine in gelatin and GelMA solutions has been tested to understand the effects of dopamine-gelatin interactions on its rheological characteristics.²⁴ Dopamine additives were found to disrupt the thermoreversible sol-gel transition in gelatin solutions. Gelatin's phase transition is due to hydrogen-bond-driven helix formation. Non-covalent attraction of catechol groups to gelatin through hydrogen bonding and π - π interactions can occupy hydrogen-bonding donor sites of gelatin, hindering the self-association of chains at low temperatures. Such weakened interchain associations suggest thinning effects of dopamine in gelatin solutions. The effects of free polyphenolic compounds in the absence of small phenolic molecules were not investigated, so it remains uncertain whether they can act as dynamic crosslinkers (having the opposite effect of strengthened interchain association) or whether their steric hindrance has similar impact on gelatin solutions as dopamine.

2.4.2 Covalent integration

As opposed to non-covalent addition of dopamine, covalent attachment of polyphenolic moieties has been shown to strengthen protein-protein interactions in gelatin solutions.^{28,34} We recently oligomerized caffeic acid and then conjugated it to gelatin, enabling high catechol conjugate content.³⁴ This modification resulted in rapid thermal gelation upon exposure of pre-gel solution to room temperature, targeting hemorrhage control in bleeding wounds. This effect was due to shifting the sol-gel transition temperature to higher temperatures with increasing oligomerization degree of caffeic acid. This increase in gelation temperature is primarily attributed to the added temperature-sensitive hydrogen

bonding between the gelatin chains due to oligocatechols accelerating their transition into the double helix conformation. A similar trend was observed when polymers of L-DOPA were used to modify gelatin.²⁸ Fast thermal gelation of gelatin at room temperature facilitated controllable injectability and prevented pre-gel dripping when applied on vertical surfaces.

2.5 Synergies of polyphenols with crosslinking and cohesion

Cohesion is a primary requisite for bioadhesion.⁸ In other words, for biomaterials to hold tissues together, they should first be able to accommodate their own mechanical integrity. Recently, many efforts have been directed at imparting bioadhesion to tough hydrogel matrices such as IPNs.⁸ Toughness is referred to as the material's ability to dissipate deformation energy and by definition, either high stretchability or strength enhances toughness (assuming no change in stiffness).^{24,45} In most cases of integrating polyphenolic compounds in hydrogels, however, changes in stretchability and strength have been inversely proportional. In this scenario, we believe that softer but stretchable matrices are preferred over hard but strong hydrogels, because in the former case, less deformation energy is transferred to the tissue/biomaterial interface (reducing the likelihood of adhesion detachment). In addition to toughness, injectable bioadhesives should solidify rapidly, through a crosslinking mechanism, to control bleeding more efficiently. Introducing polyphenolic compounds in hydrogels affects their crosslinking reactions and mechanical properties, depending upon their mechanism of crosslinking. Below, the most popular types of crosslinking in polyphenolic bioadhesives are assessed in terms of phenolic effects on crosslinking and mechanical properties.

2.5.1 Photocrosslinking in methacryloyl-modified gelatin

Modification of gelatin and elastin⁵⁰⁻⁵³ with methacryloyl groups enable free-radical photocrosslinking for on-demand hydrogel formation in injectable applications, *e.g.*, corneal repair,⁵⁴⁻⁵⁷ as shown in Figure 2.4A-C. Methacryloyl-modification of gelatin is characterized by rapid gelation, and robust and tunable mechanical properties (Figure 2.4D). This approach has become an effective means for developing injectable bioglues with on-demand gelation.⁵⁸ Photocrosslinking methacryloyl-modified gelatin and elastin⁵⁰⁻⁵³ on tissue substrates has resulted in adhesion strengths exceeding those of commercially available tissue sealants (Figure 2.4E). Tissue adhesion in photocrosslinked bioadhesives is driven by covalent adhesion due to Michael addition reactions between the radicals generated during free-radical polymerization and functional groups such as amines on tissues.⁵⁹ This bioadhesion has been reported for various initiator systems triggered with UV as well as visible light.⁶⁰⁻⁶¹

Several studies have explored polyphenolic modification of photocrosslinkable biomaterials to improve bioadhesion.^{45,62-64} However, one major reservation about these studies is hybridizing free-radical photocrosslinking of methacryloyl groups with secondary oxidative crosslinking mechanisms *via* phenolic grafts.⁴⁵ Whether catechol groups can effectively impart bioadhesion to photocrosslinkable biomaterial systems remains a question mark in these studies due to the interference of catechol oxidative crosslinking on hydrogel cohesion and adhesion. We introduced polyphenolic compounds, such as catechol groups to methacryloyl-modified proteins, which not only did not improve bioadhesion but also degraded cohesion and crosslinking kinetics.³¹ We attributed this effect to radical scavenging by phenols acting against free-radical crosslinking reactions, thereby

compromising hydrogel cohesion. Additionally, poor gelation of dopamine-incorporated GelMA hydrogels at the interfaces of the pre-polymer with tissue and air was most likely due to increased oxygen sensitivity in crosslinking. This evidence renders free-radical crosslinking an ineffective and incompatible approach in gelation of polyphenolic biomaterials. In essence, the majority of previous studies that integrated phenolic compounds with free-radical-based crosslinking systems⁴⁵ have required additional oxidative crosslinking through phenolic groups to compensate for the compromised cohesion in these hydrogels.

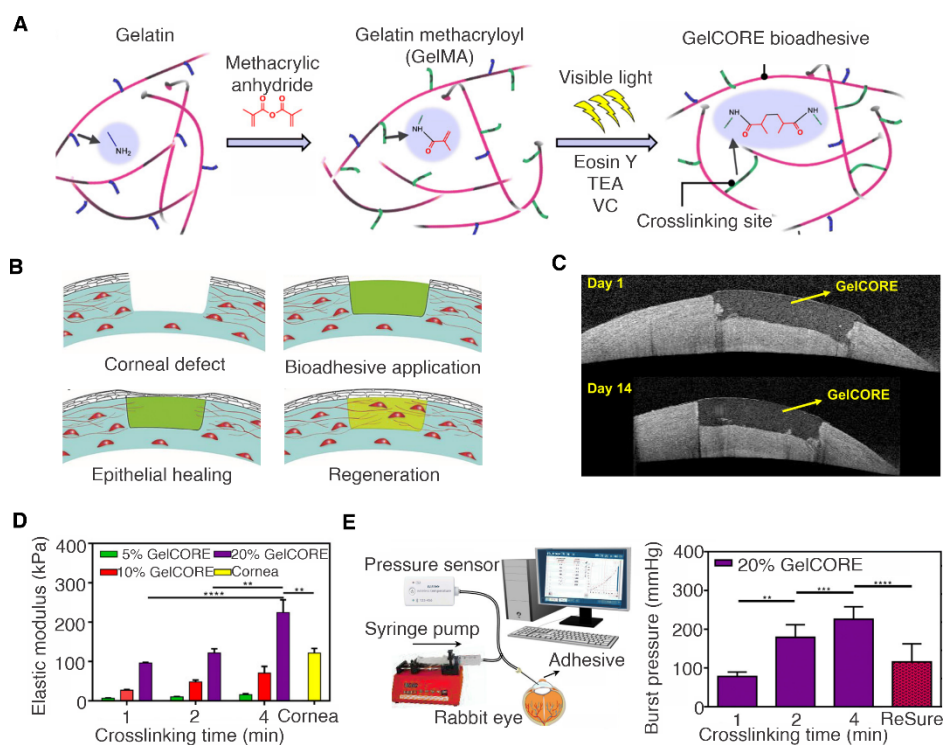


Figure 2.4. Bioadhesion in methacryloyl-modified photocrosslinkable gelatin. (A) Synthesis and crosslinking of visible light crosslinkable gelatin methacryloyl hydrogels (GelCORE). *N*-vinylcaprolactams (VC); triethanolamine (TEA). (B) Application of GelCORE for corneal repair. (C) Cross-section of GelCORE hydrogels after 1 and 14 days of application of rabbit cornea. (D) Elastic modulus of GelCORE hydrogels at different crosslinking times and concentrations (%w/v). (E) Comparison of burst pressure results with commercial sealants. Reproduced with permission from ref⁶⁵.

2.5.2 Oxidative crosslinking of phenol-modified gelatin

Polyphenolic compounds can undergo self-association through covalent coupling under oxidative conditions and metal ion complexation as shown in Figure 2.5A,B, respectively.⁹ As shown in Figure 2.5B, pH-responsive bioadhesives were developed *via* ion complexation type of crosslinking.⁹ Oxidative conditions turned catechol groups into quinone intermediates through which di-catechol coupling reactions progress. Some of the most widely used crosslinking agents triggering these reactions include Fe^{3+} , borate ions, periodates, and H_2O_2 +horseradish peroxide system.⁹ Although ionic coordination is among the fastest associations generally, we showed that NaIO_4 provided the most water-stable crosslinked networks.²⁸ These crosslinking agents can be either mixed with pre-polymers or applied topically (diffusive crosslinking).²⁸ The advantage of the latter is that the phenolic compounds at the interface are not consumed for crosslinking allowing their participation in adhesive interactions with the underneath tissues. The diffusive crosslinking deactivates hydrogel bioadhesion over the diffusion interfaces. As a result, bioadhesion becomes selective to the underneath tissue preventing unwanted adhesion to the adjacent tissues.³⁴ Note that diffusive crosslinking can be impractical for low-viscosity pre-gel solutions, in which case a two-part mixture with oxidant should be undertaken.

Oxidative crosslinking applies mostly to biomacromolecules modified chemically with polyphenols. Larger amounts of grafted polyphenols provide faster crosslinking and require lower concentrations of oxidant solutions.^{2,28} For instance, larger molecular weight motifs of polyphenolic conjugates such as oligo(caffeic acid)³⁴ and poly(L-DOPA)³¹ enabled more efficient covalent catechol crosslinking in gelatin compared to their caffeic acid and L-DOPA counterparts, respectively. As compared with photocrosslinked GelMA, oxidatively

crosslinked GelDOPA hydrogels showed significantly better tensile properties including toughness, compliance, and stretchability compared to GelMA controls.

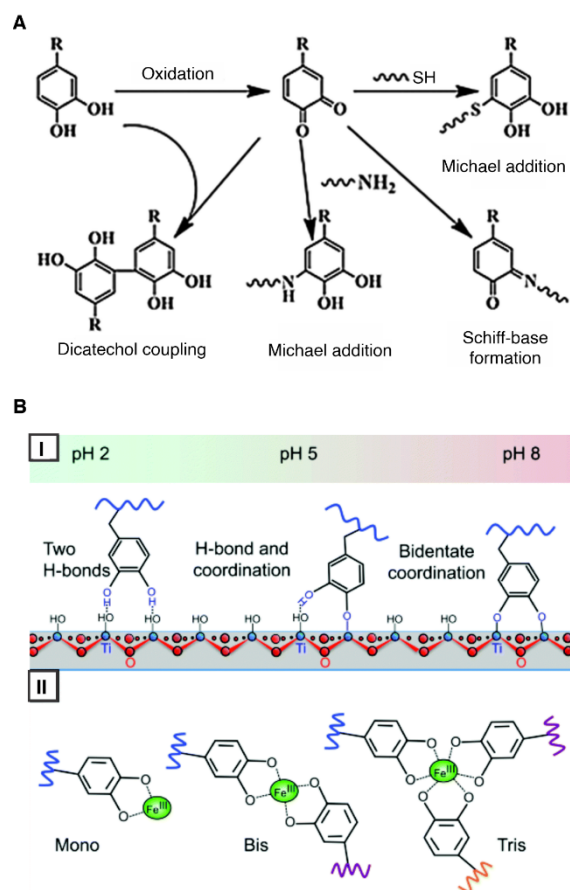
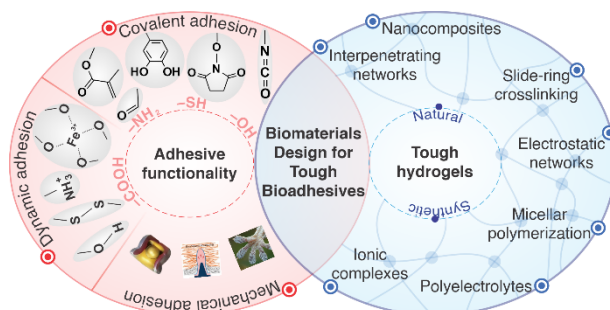


Figure 2.5. Oxidative crosslinking of polyphenol-modified gelatin bioadhesives. (A) Chemical reactions driving covalent crosslinking of gelatin *via* quinone intermediates. (B) Metal coordination crosslinking through catechol groups and pH sensitivity of crosslinking in these crosslinking systems. Reproduced with permission from reference ⁹. Copyright 2020 Royal Society of Chemistry.

CHAPTER 3: BIO-MACROMOLECULAR DESIGN ROADMAP TOWARDS TOUGH BIOADHESIVES



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Abstract

Emerging sutureless wound-closure techniques have led to paradigm shifts in wound management. State-of-the-art biomaterials offer biocompatible and biodegradable platforms enabling high cohesion (toughness) and adhesion for rapid bleeding control as well as robust attachment of implantable devices. Tough bioadhesion stems from the synergistic contribution of cohesive and adhesive interactions. This chapter provides a biomacromolecular design roadmap for the development of tough adhesive surgical sealants. We discuss a library of materials and methods to introduce toughness and adhesion

to biomaterials. Intrinsically tough and elastic polymers are leveraged primarily by introducing strong but dynamic inter- and intramolecular interactions either through polymer chain design or using crosslink regulating additives. In addition, many efforts have been made to promote underwater adhesion *via* covalent/noncovalent bonds, or through micro/macro-interlock mechanisms at the tissue interfaces. The materials settings and functional additives for this purpose and the related characterization methods are reviewed. Measurements and reporting needs for fair comparisons of different materials and their properties are discussed. Finally, future directions and further research opportunities for developing tough bioadhesive surgical sealants are highlighted.

3.1 Introduction

Over ~129 million surgical procedures were performed in the US alone in 2018 (with a market value of over \$14 billion); this figure is expected to rise to ~144 million procedures per year by 2023 according to MarketsandMarkets™.⁶⁶ Current techniques used for surgical wound closure rely heavily on suturing and stapling. These methods are time-consuming, require technical skills, cause traumatic tissue damage, and cannot immediately and effectively seal wounds.⁶⁷ In particular, in organs with dynamic movements, such as the heart, wound closure is more difficult to perform.⁶⁸ As such, there are increased risks of post-operation complications, such as infection. Due to the limitations associated with existing wound-closure techniques, there are unmet needs for developing new methods to manage wounds⁶⁹ and lacerations of various types (*i.e.*, surgical, incident, *etc.*).⁷⁰

Tissue sealants and bioadhesives have gained tremendous attention due to their potential for effective wound closure and reducing post-surgery complications.⁷¹ In general,

bioadhesives consist of a polymeric matrix (a hydrogel network) that are available either as a wound dressing patch,⁷² dry cryogel,⁷³ or as an injectable glue. Bioadhesive patches are growing rapidly as substrates for smart bandages.⁷⁴⁻⁷⁸ Hydrogel-based sealants maintain moisture on the skin lacerations.^{79,80} They can offer biodegradable platforms for wound-closure purposes.⁸¹ Injectable bio-glues are fit-to-shape sealants that can conform easily to the double-curved tissue surfaces.^{82,83} They can interlock mechanically with the tissue in addition to providing adhesion. They can be loaded with antimicrobial and hemostatic agents, to prevent infection, to promote tissue healing, and to control hemorrhage.² In addition, the gelation times of injectable bioadhesives are tuned simply by processing parameters based on the relevant surgical time scales. Despite the numerous advantages offered by current surgical sealants, there remain many obstacles to overcome. Recent Food and Drug Administration (FDA)-approved commercial sealants based on fibrin (Tisseel®, Evicel®, Vitagel™, *etc.*)⁸⁴ are used for lung incisions,⁸⁵ hernia repair,⁸⁶ treatment of refractory chylous ascites,⁸⁷ skin grafts in burn surgery,⁸⁸ and cleft palate repair.⁸⁸ Most of commercial sealants lack sufficient adhesion strength and durability, and they are prone to induce immune responses *in vivo*.⁸⁹

Achieving strong adhesion to wet tissue surfaces using hydrogels is a major challenge and requires deep understanding of the mechanisms involved. In general, bioadhesion in hydrogels can fail, either due to rupture in their crosslinking network (cohesive failure) or detachment at the tissue interface (adhesive failure).⁷ A number of studies have focused on improving cohesion in adhesive networks in terms of toughness, to prevent failure in bioadhesives.⁹⁰ Bioadhesives are required to mimic the mechanical properties of native tissues and are therefore required to be strong yet stretchable and elastic. For instance,

photocrosslinked biodegradable hydrogels based on gelatin derivatives such as gelatin methacryloyl (GelMA) can stretch only ~30%, whereas this figure reaches ~150-200% for collagen in skin tissue.⁹¹ Failure of bioadhesion in hydrogels can be exacerbated in wet environments due to significant loss of mechanical strength caused by water absorption.⁹² Several strategies have been used to toughen hydrogel networks, but they have yet to be adapted for applications in wounds.

Adhesion failure due to insufficient interactions between the hydrogel and tissue surfaces is a major obstacle in the development of bioadhesives. Many attempts have been made to endow hydrogels with durable wet adhesiveness.^{8,93} The efforts thus far have been focused primarily on taking advantage of mechanical interlocks as well as introducing covalent and/or noncovalent bonds with the underlying substrates.⁷ Nevertheless, most studies have reported adhesive strengths on the order of 10^1 – 10^2 kPa for hydrogel-based bioadhesives,⁹⁴ which is significantly lower than the intrinsic strength of most native tissues in the human body (*e.g.*, up to 15 MPa for skin).⁹¹ The lack of appropriate adhesion strength in recently developed bioadhesives has motivated research to explore novel biomaterials for stronger adhesion.¹⁷

Conflicting bioadhesive design requirements result in compromising one property when optimizing others.⁹⁵ For instance, a high swelling ratio is desired to induce hemostasis, and thereby, stop hemorrhage rapidly,^{96,97} whereas the augmented swelling in hydrogels usually deteriorates adhesion strength.^{2,98} In many cases, enhancing cohesion decreases adhesion and *vice versa*.⁹⁹ Hence, to choose the proper polymer network, crosslinking strategy, and adhesion mechanism for a cohesive (tough) and adhesive network, a rational design map is required to meet the specific requirements based on the intended application.

As the field progresses, additional capabilities, such as monitoring biological function will be built into bioadhesive materials.^{100,101}

In this chapter, we discuss a broad range of biomaterials and fabrication methods that serve to enhance cohesion and adhesion, along with their advantages and shortcomings. As illustrated in Figure 3.1, the design of bioadhesives begins with the selection of a polymer backbone and a crosslinking mechanism that supports cohesion and toughness in the material. Hence, we first cover the macromolecular strategies and various crosslinking chemistries used to engineer tough and stretchable hydrogels. Then, a variety of chemical and physical mechanisms can be used to introduce interfacial interactions for adhesion. Therefore, the subsequent sections are devoted to biomaterial types and modification procedures leveraging their bioadhesion. We discuss the measurements used to compare materials and their properties. Finally, current challenges and targets for future research and development in tough bioadhesives are highlighted.

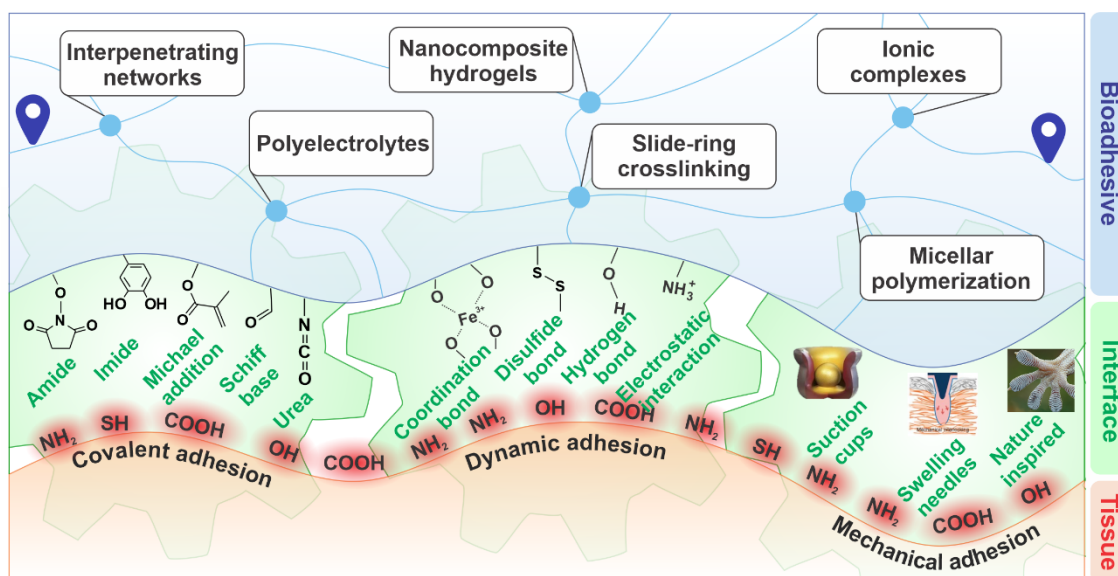


Figure 3.1. An overview of the material design roadmap for tough bioadhesives. Development of bioadhesive materials involves: (i) design for cohesion where a combination of the polymer backbone and crosslinking strategy is selected to ensure mechanical durability in a tough hydrogel,

and (ii) design for bioadhesion where a mixture of covalent and dynamic interactions, as well as mechanical interlocks, are incorporated into the material design.

3.2 Cohesive biomaterials: Tough stretchable polymers

Tuning intra- and intermolecular chemical and physical interactions are essential for bioadhesives, to enhance their cohesion and adhesion. Cohesive failure refers to the early failure of polymer matrix that occurs before the tissue/hydrogel interface is damaged.¹⁰² The mechanical mismatch between the sealant and tissue is a major cause of cohesive failure. This issue is particularly observed in brittle hydrogels, as they fail to comply with the tissue deformation.²⁴ To address this issue, bioadhesives with high stretchability and strain recovery are favored. These characteristics are reflected in so-called “tough” hydrogels.¹⁰³ Most organs and tissues in the body, such as the myocardium, undergo continuous dynamic deformation. However, hydrogels, if not processed and designed properly, are rather brittle (mainly due to uncontrollable and inhomogeneous crosslinking distributions)^{104,105} and are thereby susceptible to failure in response to dynamic loads. Therefore, they fail mostly at much lower fracture energies than natural tissue (*e.g.*, two orders of magnitude lower than cartilage).¹⁰⁶ Hence, considerable effort has been devoted to tuning the mechanical properties of bioadhesives to minimize mechanical mismatches with underlying tissue.¹⁰⁷ Soft, compliant hydrogels are of great interest to avoid early adhesion failure.²⁴ Failure in adhesion stems primarily from the deformation resistance in stiff hydrogels. The advances made for creating tough, yet compliant and stretchable hydrogels are inspired largely by efforts made in the field of flexible electronics and stretchable wearable devices¹⁰⁸⁻¹¹⁰ such as ultrasonic devices.¹¹¹ Similar principles can be implemented for the development of

mechanically robust tissue adhesives. Below, methodologies to fabricate tough and stretchable hydrogels are highlighted.

3.2.1 Interpenetrating multi-network hydrogels

One common approach to enhance toughness in hydrogels is to support covalent polymer networks with secondary networks that can dissipate the deformation energy *via* non-covalent reversible attractions. In these so-called “interpenetrating polymer networks (IPN)”, reversible dynamic bonds reform continuously once polymer chains start to break off under tensile mechanical loads.¹⁰² These double networks can also be formed *via* crosslinking two separate polymers with different chain lengths.¹¹² In these networks, when mechanical loads are applied, initially, the short-chain bridging networks are first ruptured irreversibly under tension, and thereafter, the long-chain macromolecule-based networks bear larger deformations.¹¹³ Alginate, hyaluronic acid (HA), and chitosan are among the natural biomolecules used to introduce such short-chain energy dissipative networks in these hydrogel systems. Poly(acrylamide) (PAM) and poly(ethylene glycol)diacrylate (PEGDA) are the most commonly used long-chain polymer backbones used as elastic covalent networks.^{106,113,114}

Incorporating chemical functionality that enables different crosslinking possibilities, such as covalent and ionic interactions, has leveraged tough hydrogels. The ionic crosslinks between the chains are broken reversibly during tensile deformation, whereas the covalently crosslinked network provides additional support by bridging the cracks through the dynamic molecular rearrangements.¹¹² For example, a maximum stretchability of 23× was obtained in a PAM-alginate hydrogel due to the synergistic effects of covalent and ionic crosslinked networks. This stretchability was significantly higher than that of its constitutive

components (alginate and PAM with $1.2\times$ and $6.6\times$ stretchability, respectively).¹¹² Similarly, the addition of ionically crosslinked alginate within a poly(ethylene glycol) (PEG) network enhanced the stretchability of the hydrogel from $\sim 300\%$ to $\sim 400\%$ compared to control PEG samples.¹¹⁵ One possible drawback associated with tough double network hydrogels is the permanent rupture of the short chains and thereby high mechanical hysteresis in response to cyclic loads.¹¹⁶ This issue was addressed in the alginate-PEG crosslinked network, where alginate re-associates after removing the load, enabling the hydrogel to self-heal after each loading cycle.

In combination with covalent networks, hydrogen bonding between two polymers also improves toughness. This effect was demonstrated in a PAM and poly(ethylene oxide) (PEO)-based hydrogel (see Figure 3.2A).¹¹⁷ The stretchability of the composite material was controlled by changing the concentration of PEO. At 8 wt.% PEO, approximately $5\times$ improvement in stretchability was observed, compared to pure PAM. This effect was more prominent for lower molecular weight PEO due to the fewer polymer entanglements. In addition, the hydrogels were also characterized by high tear resistance (Figure 3.2B-D).

Copolymerization of different monomers is another simple approach to design multi-network tough hydrogels. Recently, a polymeric matrix with a pH-sensitive swelling was prepared through copolymerization of acrylamide (AAm) and acrylic acid (AA) monomers within an agar network.¹¹⁸ Agar is a thermosensitive linear polysaccharide that forms a gel below $35\text{ }^{\circ}\text{C}$. Integration of agar within poly(AAm-*co*-AA) network led to a pH-sensitive tough hydrogel (stretchability on the order of 1500%) with tunable mechanical properties.

Interactions between two oppositely charged polymers drive another class of double-networked stretchable and tough hydrogels. For instance, strong electrostatic complexation

of positively charged poly(diallyldimethylammonium chloride) (PDDA)/branched poly(ethylenimine) (PEI) and negatively charged polyelectrolyte mixture of poly(sodium 4-styrenesulfonate) (PSS)/poly(acrylic acid) (PAA), led to tough and stretchable (the maximum strain at the break of ~2400%) hydrogels (Figure 3.2E-G).¹¹⁹ The stretchability of the hybrid hydrogel was tunable by modifying the concentrations of the chemical constituents (*i.e.*, the PDDA-PSS to PEI-PAA ratio). In addition to electrostatic interactions, *in situ* formation of PDDA-PSS nanoparticles within the PEI-PAA crosslinked chains introduces dynamic hydrogen bonding, which improves the mechanical properties of the synthesized biomaterial (Figure 3.2G). In another study, coacervation of natural bio-polyelectrolytes, such as the mixture of chitosan and HA in NaCl solution (due to syneresis, the expulsion of liquid) led to highly stretchable hydrogels.¹²⁰ The hydrogels were developed by desalting the mixture of chitosan and HA under different dialysis pH conditions. Changing the pH from the pK_a of amine groups in chitosan (6.3) to the pK_a of carboxylic acid in HA (2.9) during the dialysis process led to improvement in hydrogel stretchability (Figure 3.2H). At low pH (<3), HA molecules fold due to strong intra- and intermolecular interactions, while chitosan chains form extended conformations (Figure 3.2I,J). Thus, hydrogen-bonding interactions within the folded network of HA acted as energy-dissipating components improving the cohesive strength of the hydrogel.

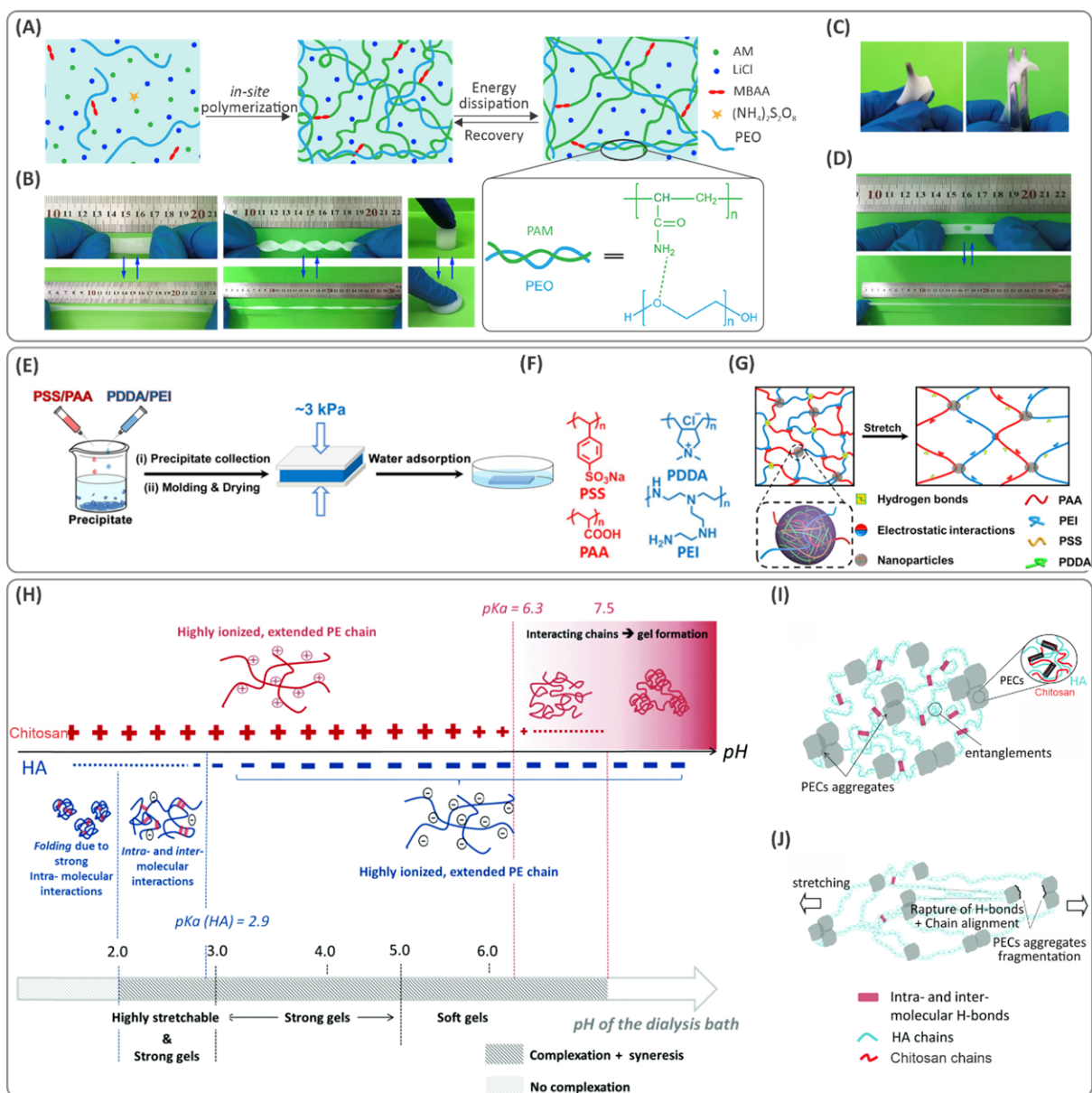


Figure 3.2. Examples of interpenetrating hydrogel networks (IPN) with improved toughness. (A) Schematic of components and interactions in an IPN of poly(acrylamide) (PAM)/poly(ethylene oxide) (PEO) hydrogel crosslinked by *N,N'*-methylene-bis(acrylamide) (MBAA) toughened due to the synergy between hydrogen-bonding and covalent networks. (B) Hydrogel resistance against tensile, compressive, and torsion deformations. The hydrogels were able to stretch by $\sim 8.8\times$. (C) Hydrogels showed anti-puncturing characteristics. (D) The punctured hydrogels still maintained their stretchability. Reproduced with permission from ref ¹¹⁷. Copyright 2019, Elsevier. (E) Schematic of processing poly(diallyldimethylammonium chloride) (PDDA)-poly(sodium 4-styrenesulfonate) (PSS)/branched poly(ethylenimine) (PEI)-poly(acrylic acid) (PAA) hydrogels. (F) Chemical structure

of PSS, PAA, PEI, and PDDA components. (G) Toughening mechanism of the hydrogels: the effect of electrostatic interactions and hydrogen bonding. Reprinted with permission from ref ¹¹⁹. Copyright 2019 American Chemical Society. (H) Effect of pH on the conformation of polyelectrolytes (PE). At low pH, chitosan is ionized resulting in polyelectrolyte repulsion. Hyaluronic acid (HA), on the other hand, tends to fold due to intramolecular interactions. As the pH increases (above pK_a of chitosan), a random coil conformation is formed by chitosan. Any further increase above pK_a of HA leads to its extended conformation. Schematic of molecular reformation of the chains before (I) and after (J) the application of stretching loads. The dissipative process stems from the interchain hydrogen bonding and crosslinking through the polyelectrolyte complex (PEC) aggregates and hydrogen bonding. Reproduced with permission from ref ¹²⁰. Copyright 2017, Royal Society of Chemistry.

3.2.2 Ionic hydrogels

Electrolytes have shown improvements in the stretchability of hydrogels by introducing dynamic ionic and dipole-dipole interactions. Such ionic hydrogels are electrically conductive, in addition to their mechanical stretchability, which is important in wearable and implantable devices.¹²¹⁻¹²³ Improved stretchability in ionic hydrogels is attained due to dynamic ionic interactions.¹²⁴ For example, poly(vinylidene fluoride-*co*-hexafluoropropylene) (PVDF)-*co*-hexafluoropropylene (HFP) polymers with high HFP content (*i.e.*, 45 wt.%) served as highly polar polymers that can be easily crosslinked upon the addition of an ionic liquid (*i.e.*, imidazolium).¹²⁴ The strong, reversible interactions between the polar groups on the polymeric chain and the ionic salt resulted in polymeric matrices with stretchability on the order of 5500%, while the lower ionic liquid concentrations showed stretchability of *ca.* 1000%. Here, the deformation of the material was fully reversible for strains smaller than 50%. Higher levels of strain reversibility (up to 100% strain) were obtained in ionic hydrogel systems composed of physically crosslinked poly(vinyl alcohol) (PVA) in NaCl solution.¹²⁵ In this hydrogel, hydroxypropyl cellulose

(HPC) drew in Na⁺ and Cl⁻ ions, leading to high ionic conductivity. The addition of HPC (16 wt.%) led to lower PVA crosslinking density. Hence, an ion-rich, porous PVA network with improved stretchability up to ~800% (from ~550%) was achieved. Variation of the NaCl concentration, from 0 to 5 M, increased the hydrogel stretchability from 300% to 850%. Stretchable hydrogels enabled by ion-dipole and dipole-dipole interactions were synthesized by using an ionic liquid (*i.e.*, 1-ethyl-3-methylimidazolium dicyanamide ([EMIm][DCA])) that also led to high electrical conductivity in the hydrogels.^{126,127} The ionic liquids composed of [EMI][DCA] were used to develop a new type of hydrogel matrix where polymerization of 3-dimethyl and AA monomers was propagated in the presence of methacryloyloxyethyl ammonium propane sulfonate (DMAPS). This process resulted in dipole-dipole interactions between pendant zwitterionic functional groups and resulted in stretchability on the order of ~800%. Copolymerization of AAm and AA in the presence of CoCl₂ demonstrated a similar effect (see Figure 3.3A).¹²⁸ In this case, the hydrogel showed stretchability of greater than 1200%. Additionally, increasing the Co²⁺ concentration led to increased stretchability, from 300% to 700% (see Figure 3.3B,C). This stretchability was due to strong ionic interactions between Co²⁺ ions and the carboxylic groups present in the polymeric backbone that act as dynamic crosslinking points. The addition of Co²⁺ also enhanced the self-healing of the hydrogels (Figure 3.3D). Integration of other ionic liquids (*e.g.*, 1-ethyl-3-methylimidazolium ethyl sulfate) with PAA and a polyzwitterionic macromolecule, poly(3-dimethyl(methacryloyloxyethyl) ammonium propane sulfonate) (PDMAPS) has been shown to form strong hydrogel networks.¹²⁹ In this study, the hydrogel was reinforced by the ion-dipole interactions due to the ionic liquid, leading to stretchability ranging from 2000% to 100,000%, at different ionic-liquid-to-PDMAPS ratios. We attribute

the low stretchability and the high elastic modulus in the absence of IL to dipole-dipole interactions (due to the ion-rich sites of the polyelectrolytic chain). Here, the addition of ionic liquids reduced Coulombic interactions, which results in enhanced stretchability (at 1:2 molar ratio of ionic liquid:PDMAPESS). However, excess ionic liquid content leads to imperfect binding and uncontrolled interactions between the ionic liquid and polyelectrolytic macromolecules, degrading mechanical properties.

Recently, a physically crosslinked PVA-based hydrogel was crosslinked through hydrogen bonding between hydroxyl groups on the polymer chains.¹³⁰ Here, crosslinking in the presence of H₂SO₄ increased stretchability up to 380%. To enhance the stretchability of the synthesized PVA-based hydrogel, salts of a weak acid (*e.g.*, borax), were introduced to the system to interact with the tetrafunctional borate ions and hydroxyl groups of PVA macromolecular chains (Figure 3.3E,F).¹³¹ The hydrogels showed stretchability of up to 1000% at 4 wt.% PVA concentration (Figure 3.3G).

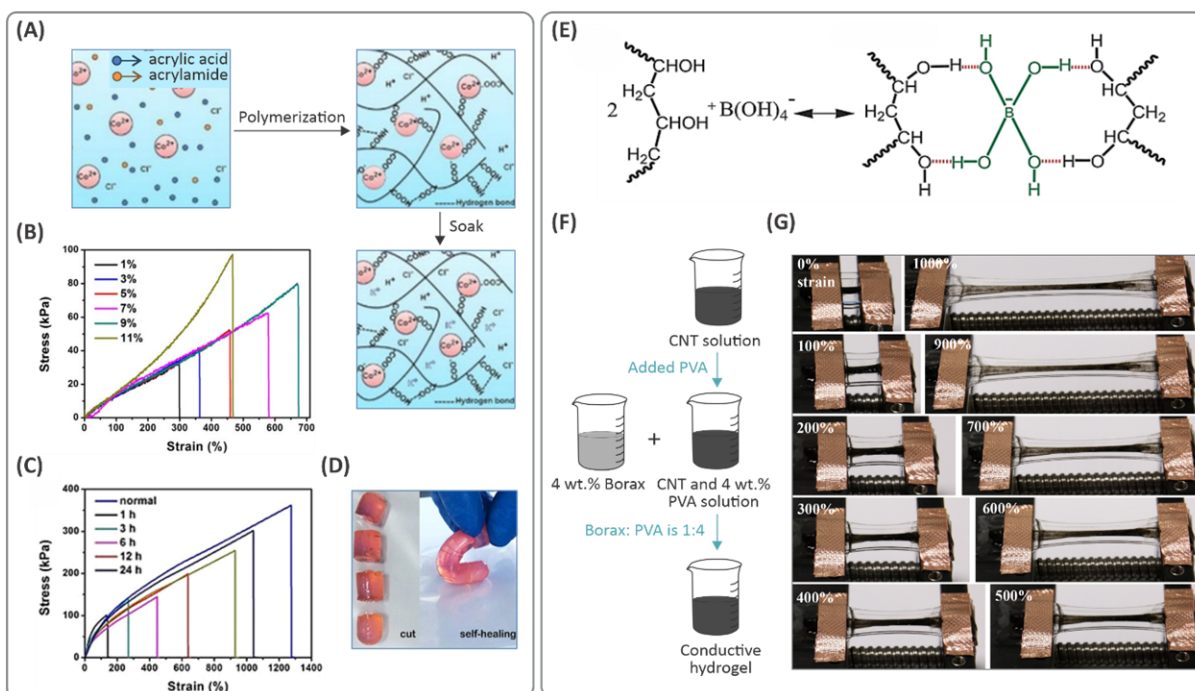


Figure 3.3. Engineering tough and stretchable ionic hydrogels. (A) Processing scheme for the ionic hydrogels based on poly(acrylic acid-*co*-acrylamide)/CoCl₂ composition. (B) Effect of the Co²⁺ concentration on the tensile mechanical properties of the hydrogels. (C, D) Self-healing properties of the hydrogels and mechanical properties of the healed hydrogels at different time points. Reprinted with permission from ref ¹²⁸. Copyright 2019 American Chemical Society. (E) Fabrication procedure of the carbon nanotubes (CNT)/poly(vinyl alcohol) (PVA) hydrogels with borax to form the PVA complex through (F) attraction of borax to the hydroxyl groups of the PVA chain. (G) Tensile deformation of the hydrogel by 1000%. Reproduced with permission from ref ¹³¹. Copyright 2017, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

3.2.3 Macromolecular modulation of crosslinkers and monomers

The chemical structures of the monomer units in single-component hydrogel systems play key roles in their mechanical properties. Many studies have examined the effects of polymerization processing parameters and monomer structure on mechanical properties. Chemical functionalization of the macromolecules with *e.g.*, allyl and methacryloyl groups enables crosslinking through radical polymerization reactions in the presence of chemical

initiators.¹³² For instance, modification of cellulosic biomaterials with allyl glycidyl ether led to crosslinkable allyl cellulose.¹³³ Polymerization of allyl cellulose in NaOH/urea solution using an ammonium persulfate (APS) initiator led to the stretchability of 126% (as opposed to <100% for those of unmodified cellulose hydrogels).¹³⁴

The tertiary amine groups present in aliphatic amines such as *N,N,N',N'*-tetramethylethylenediamine (TEMED) can catalyze the chemical reaction with APS through electron transfer, producing free radicals for polymerization at room temperature. Redox polymerization enabled by introducing the tertiary amine on the monomer itself enhances stretchability.¹³⁵ By conjugating aliphatic amine functionality on the monomer backbone or the crosslinker, the crosslinking sites can be distributed more homogeneously, leading to tough hydrogels. Figure 3.4A shows a polymeric network containing polyetheramine (PEA) and a linear epoxy with covalently grafted tertiary amines. The grafted tertiary amines enable the polymer molecule to act as an initiator during the radical polymerization process. Finally, polymerization of PEA along with PAM leads to a highly stretchable (up to 2000%) hydrogel and strain recovery for strains smaller than 1000% (Figure 3.4B, C), which was significantly larger than that of PAM hydrogels.

As discussed above, the chemical structure of the crosslinker is a key factor in the hydrogel toughness and elasticity. The lengths of polymer chains and crosslinkers can significantly change the stretchability of hydrogels. Stretchable PAM-based hydrogels crosslinked using PEGDA with different chain lengths show significant increases in swelling and stretchability when longer chains of PEGDA were used.¹¹⁸ Similarly, a linear epoxy polymer with multiple tertiary amine sites (*i.e.*, PEA) was designed (initiating polymerization) to synthesize a PAM-based hydrogel with stretchability as high as

2000%.¹³⁵ Figure 3.4D illustrates a PAA polymer-based crosslinked hydrogel network synthesized with Pluronic F127 (F127DA) molecules.¹²⁶ Both stretchability and strength were enhanced with increased crosslinker concentration. Although at concentrations higher than 36 mg/ml, the stretchability was found to decrease with F127DA, the strength continued to increase.

Liquid metals are employed as additives for tuning the polymerization rates and mechanical properties of hydrogels.¹³⁶ Liquid metals act as redox catalysts and therefore accelerate the radical generation in APS-based radical-generation systems.^{137,138} Gallium indium eutectic (EGaln) (composed of 75 v/v% gallium and 25 v/v% indium) is an example of a gallium-based liquid metal, which can be introduced in hydrogel systems to enhance toughness. Encapsulation of EGaln in graphene oxide (GO) nanoparticles through coordination with Ga^{3+} led to crosslinking complexes that can form hydrogen-bonding and covalent interactions with polymer matrices composed of alginate and polyacrylamide. These interactions resulted in over 4× improvement in stretchability.¹³⁷ Solutions of liquid metals are generally unstable in water; however, when mixed with polar monomers (*i.e.*, AAm and 2-hydroxyethyl acrylate, HEA), they can enhance surface interactions and form stable dispersions that favor polymerization of tough hydrogel materials (Figure 3.4E).¹³⁹ Co-gelation of the above-mentioned monomers occurred within 20 s, once potassium persulfate (KPS) was added at room temperature. Tensile tests demonstrated that the hydrogels could stretch up to 1500%, as shown in Figure 3.4F-H (~4× higher than the control samples with no liquid metal content), confirming the potential use of liquid metals as an effective strategy for toughening the hydrogel network.

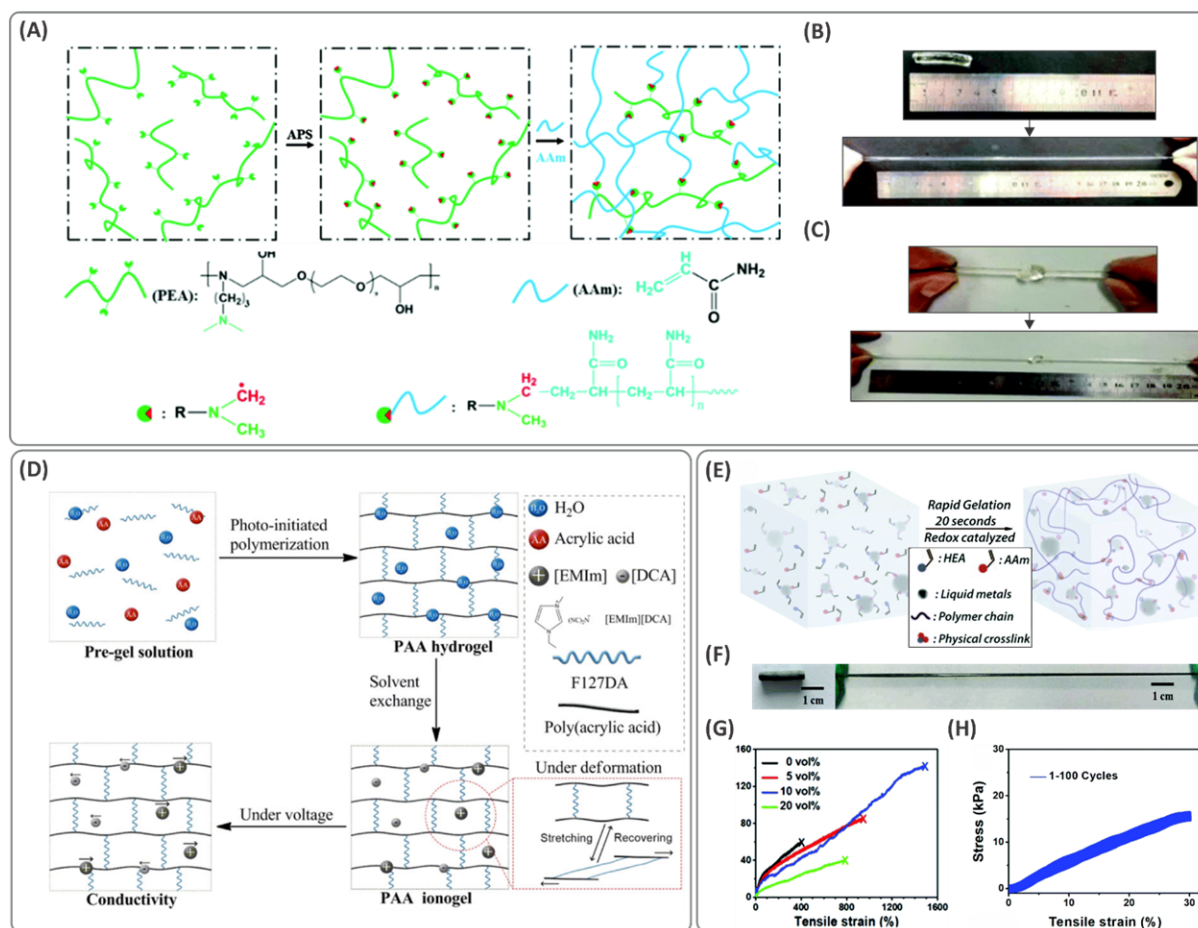


Figure 3.4. Creating tougher hydrogels through functionalization of the crosslinker and polymeric backbone. (A) Functionalization of polyetheramine (PEA) with tertiary amines allowed the polymeric backbone to act as both the initiator and crosslinker during the radical polymerization in the presence of poly(acrylamide) (PAM) network. (B, C) Tough and stretchable hydrogels were obtained with stretchability of up to 2000%. Reproduced with permission from ref ¹³⁵. Copyright 2016, Royal Society of Chemistry. (D) Schematic illustration of the synthesis and deformation mechanism of poly(acrylic acid) (PAA) hydrogels crosslinked with Pluronic F127 (F127DA). Ionogels were fabricated by adding ionic liquid 1-ethyl-3-methylimidazolium dicyanamide ([EMIm][DCA]) through a solvent exchange process. The hydrogel showed high fatigue resistance for strains of up to 850%. Reprinted with permission from ref ¹²⁶. Copyright 2019 American Chemical Society. (E) Rapid synthesis procedure of the liquid metal-based hydrogels acrylamide (AAm) and 2-hydroxyethyl acrylate (HEA) formed *via* a redox catalyzed reaction. (F) Elasticity of the hydrogel and (G) the stress-strain curves showing stretchability at the optimized concentration of liquid metal (up to ~1500%). (H) Cyclic stress-strain curves of the hydrogel for 100 cycles. Reproduced with permission from ref ¹³⁹. Copyright 2019, Royal Society of Chemistry.

3.2.4 Slide-ring crosslinking

Slide-ring crosslinking is a relatively new chemical platform for introducing toughness into hydrogels.¹⁴⁰ These hydrogels are formed of a series of pulley-shaped molecules that pass through linear polymeric chains, which can slide freely along those chains.¹⁴¹ This crosslinking approach reduces the stress concentration at the crosslinking sites when external deformations are applied.¹⁴¹ Polyrotaxane (PR) derivatives, composed of cyclodextrin polysaccharide units, are one such moiety that can slide freely along linear chains, such as PEG.¹⁴¹ Low concentrations (2-3 wt.%) of these pulley-shaped cyclodextrin molecules improve the stretchability of PVA hydrogels (up to ~1600% elongation at break).¹⁴²

One of the main limitations of the unmodified PR however, is its poor water solubility, due to the hydroxyl-induced aggregation of the cyclodextrin molecules limiting the sliding effect of cyclodextrin along the chains.¹⁴¹ High pH can be used to ionize the hydroxyl groups to avoid cyclodextrin aggregation; however, it is not always practical to polymerize hydrogels at high pH. Recently, a tough slide-ring hydrogel with enhanced water solubility was synthesized using carboxyl-functionalized hydroxypropylated PR (HPR) crosslinkers.¹⁴¹ In this ionic PR-based crosslinker, PR molecules are in their expanded form at the neutral pH. As shown in Figure 3.5A,B, PR was modified with isocyanates (to form bonds with carboxyl groups) and vinyl groups to crosslink a poly(*N*-Isopropyl acrylamide) (PNIPAM) and PAA copolymer. This approach led to the slide-ring hydrogels with a maximum stretchability of ~1500%, which was significantly higher than the hydrogels crosslinked conventionally using *N,N'*-methylene-bis(acrylamide) (MBAA), (29% elongation at failure) as seen in Figure 3.5C,D.

Different types of pulley-shaped molecules have been designed to tune their toughening effects. In one example, a molecule with a hydrophobic cavity and inward-facing complementary polar groups formed slide-rings with hydrophilic linear chains, such as PEG.¹⁴³ The pseudo-polyrotaxane molecules can crosslink PEG networks with Cu(II) metal ions due to their chelation with carboxyl groups (Figure 3.5E-G).¹⁴⁴ Gelation was triggered upon vigorous shaking (for ~30 s) because of the applied shear forces. However, the gel was transformed into the sol state for hours or days after formation, depending on the compositions of the different components. These shear-induced hydrogels showed fast self-healing and excellent stretchability (~25-30× more), when compared to controls (Figure 3.5H).

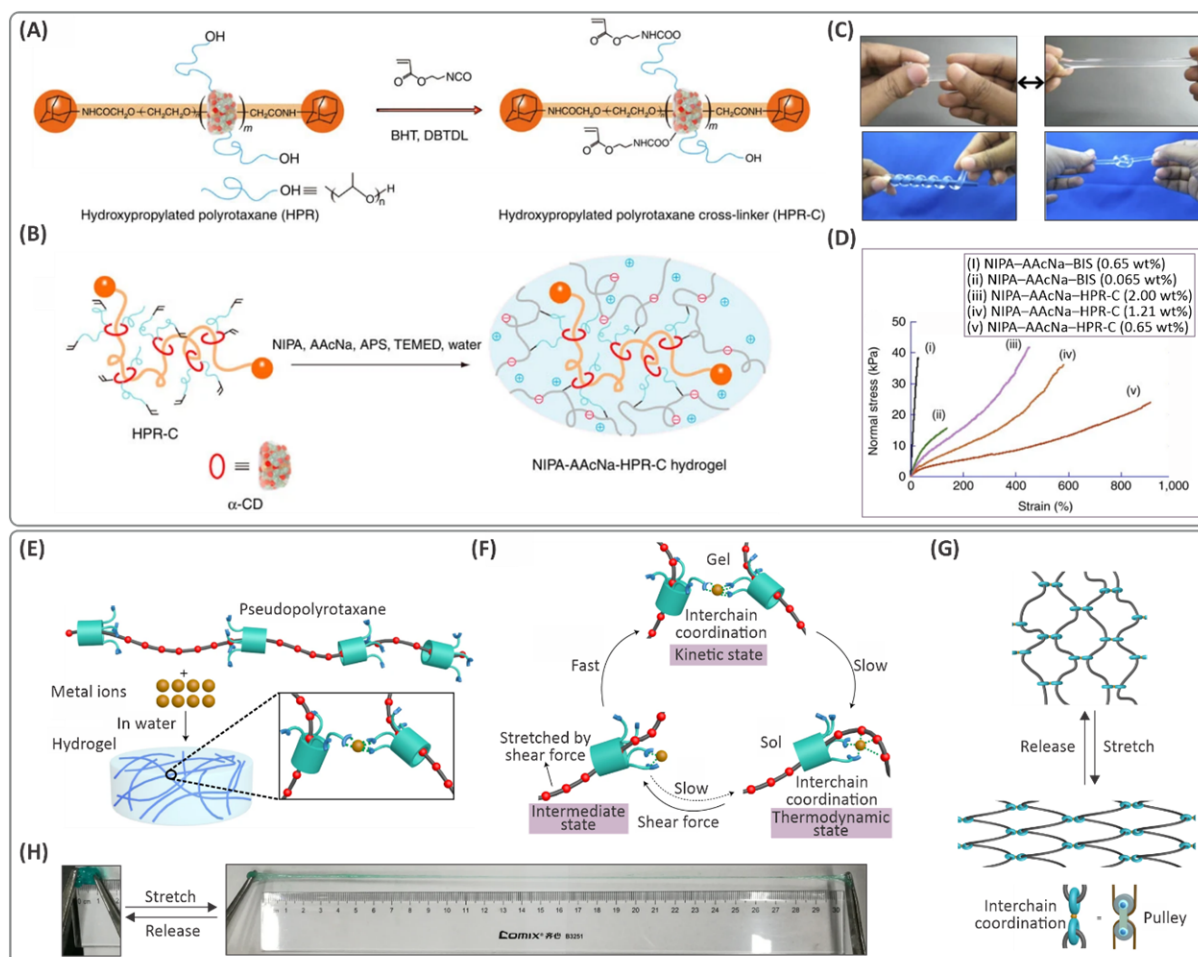


Figure 3.5. Examples of slide-ring polymers to form hydrogels with improved toughness. (A) Molecular design of a hydroxypropylated polyrotaxane (HPR) crosslinker (HPR-C) based on α -cyclodextrin (α -CD) and (B) schematic of free radical copolymerization of *N*-isopropyl(acrylamide) (NIPA) and sodium acrylic acid (AAcNa) using the developed crosslinker. (C) Demonstration of highly stretchable and deformable hydrogels. (D) Stress-strain curves for (i) NIPA-AAcNa-*N,N'*-methylene-bis(acrylamide) (BIS) (0.65 wt%), (ii) NIPA-AAcNa-BIS (0.065 wt%), (iii) NIPA-AAcNa-HPR-C (2.00 wt%), (iv) NIPA-AAcNa-HPR-C (1.21 wt%) and (v) NIPA-AAcNa-HPR-C (0.65 wt%) shows the hydrogels containing the same amount of crosslinkers but different amounts of HPR-C crosslinker can stretch up to 912%, which is significantly higher than that of BIS crosslinker, (*i.e.*, 29%). Reprinted by permission from ref ¹⁴¹. Nature Publishing Group, Copyright 2014. (E) Schematic illustration of the gelation *via* metal coordination through pseudo-polyrotaxanes. (F) The mechanism proposed for the thermal relaxation and shear-induced gelation effects. (G) Chain conformation changes with stretching the hydrogel, and (H) digital photographs of the hydrogel stretched by $\sim 30\times$. Reprinted by permission from ref ¹⁴⁴. Nature Publishing Group, Copyright 2019.

3.2.5 Micellar polymers

Micellar copolymerization has provided new opportunities to develop different types of stretchable hydrogels where the dynamic hydrophobic interactions between the surfactant micelles and polymer chains define crosslinking points.¹⁴⁵ Reversible bond formation in micelles can deform, break, and reform continuously during mechanical deformation. These dynamic interactions in covalent networks lead to high stretchability in micellar polymers.¹⁴⁶ Stable dispersions of micelles in aqueous solutions are another advantage, as they provide homogeneous and uniform crosslinking distributions throughout the polymeric backbones. Recently, highly stretchable PAM-based hydrogels (with higher than 10000% failure strain) were fabricated using a crosslinker based on hydrogen-bonding and hydrophobic associations (Figure 3.6A).¹⁴⁷ The synthesized crosslinker consisted of a hydrophobic alkyl spacer that bridged a 2-ureido-4-pyrimidone (UPy) tail to an acrylic head. The crosslinker molecules were encapsulated into sodium dodecyl sulfate (SDS) micelles enabling micellar polymerization of acrylamide through the SDS emulsion. The obtained hydrogels exhibited stretchability over $\sim 100\times$ (Figure 3.6B).

Other studies have revealed the potential mechanical improvements enabled by a combination of hydrophobic interactions and secondary dynamic networks, *e.g.*, using additives such as GO¹⁴⁸ and PVA.¹⁴⁹ The addition of GO in a hydrophobically associated hydrogel increased the crosslinking density through the formation of new hydrogen bonds, which further improved the toughness of the hydrogel matrix as demonstrated in Figure 3.6C.¹⁴⁸ The synthesized hydrogel showed high stretchability, on the order of $\sim 30\times$, and could undergo extensive deformations, (*i.e.*, bending, knotting, *etc.*, see Figure 3.6D,E).

2,4,6-Trimethylbenzoyl-diphenylphosphine oxide (TPO) is a highly efficient

photoinitiator for UV crosslinking of polymers. However, the poor water solubility of TPO has limited its use. To address this issue, SDS was used to form stable TPO nanoparticle dispersions in water.¹⁵⁰ The TPO emulsion was further used for 3D printing highly stretchable hydrogels (~1300% stretchability) from PAM-PEGDA hydrogels, which were far larger than conventionally processed hydrogels (*e.g.*, PEGDA with ~150% stretchability). In this study, the TPO nanoparticle-dispersed emulsion improved the polymerization kinetics significantly as compared to commercially available photoinitiator, *i.e.*, Irgacure 2959 (12959).

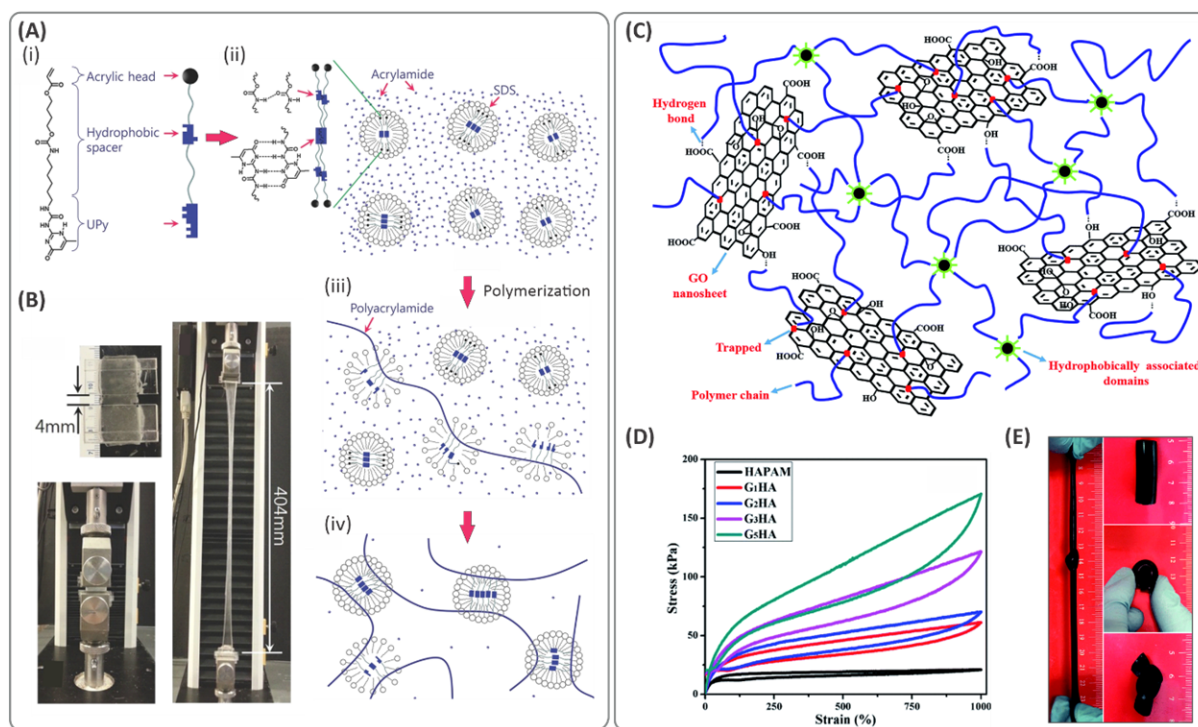


Figure 3.6. Examples of tough hydrogels created by micellar polymers. (A) (i) Schematic illustration of the crosslinker based on hydrophobic interactions. (ii) A crosslinker that consists of an acrylic head, a hydrophobic alkyl spacer connected by carbamate, and a 2-ureido-4-pyrimidone (UPy) tail (UPyHCBA) and the micelles loaded with UPyHCBA in an acrylamide solution. (iii, iv) micellar copolymerization of acrylamide using the developed hydrophobic crosslinkers. (B) Demonstration of the stretchability of tough acrylamide hydrogels stretched by 100×. Reproduced with permission.¹⁴⁷ Copyright 2016, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Dual crosslinking

mechanism in the hydrophobically crosslinked polyacrylamide (PAM)/GO composite hydrogel. Hydrophobic domains formed by the interactions between hydrophobic sides of stearyl methacrylate (SMA) and sodium dodecyl benzene sulfonate (SDBS), which led to abundant dynamic crosslinking points well dispersed within the polymer network. (D) The stress-strain curves under cyclic loads demonstrating elasticity and strain-recovery of the hydrogels. (E) Digital photographs of the hydrogel under bending, knotting, and stretching conditions. Reproduced with permission from ref ¹⁴⁸. Copyright 2015, Royal Society of Chemistry.

3.2.6 Nanocomposite hydrogels

The incorporation of inorganic nanoparticles can enhance dynamic crosslinking and thereby, toughness in hydrogels.¹⁵¹ The high specific surface area of nanoparticles and the presence of active functional groups on their surface, facilitate their dynamic interaction with the polymer matrix. The stretchability of the polymeric nanocomposite hydrogels depends on the concentration and dispersibility of the nanoparticles, as well as the particle sizes and their chemical structures.¹⁵² The main challenge in the synthesis of nanoparticle-reinforced hydrogels is poor dispersion stability during polymerization.¹⁰⁵ For instance, GO has a large surface area with exposed hydroxyl, carboxyl, and epoxide functional groups that facilitate various physiochemical interactions with the PAM molecules.¹⁰⁴ Recently, hybrid nanocomposite hydrogels based on traces of GO/PAM were observed to exhibit maximum elongation at failure of greater than 3400% (*i.e.*, a 10-fold increase compared to unmodified PAM hydrogels).¹⁰⁴ This improvement is attributed to a variety of molecular interactions, including the combination of the hydrogen-bonding, ionic bonds, and physical interactions between the nanocomposite components. A similar combination of interactions was observed in a PAA and reduced graphene oxide (rGO)-based nanocomposite, where GO sheets functionalized with polydopamine (PDA) introduced a secondary strong ionic

crosslinking point upon the addition of Fe^{3+} ions.¹⁵³ The mechanical properties of the hydrogel were highly dependent on the concentration of Fe^{3+} and GO. For example, increasing the concentrations of Fe^{3+} and GO to 0.25 and 0.05 wt.%, respectively, resulted in reduced stretchability of the materials from a maximum of ~1250% to ~500%.

Hydrophobic interactions and plastic deformation of the micellar particles play important roles in dissipating deformation energy. Recently, a tough hydrogel was synthesized where the deformation energy dissipated through the frictional forces between the micelles (Figure 3.7A-D).¹⁵⁴ In this case, casein additives, a milk-based protein molecule, formed micellar-structured nano/microparticles in water where the negatively charged hairy layer present on the surface caused repulsion and thereby a steric stabilization of the particles (Figure 3.7D). Polymerization of these AAm molecules in the presence of casein showed significant improvement in stretchability of the hydrogels, from 1600% to over 3500%, and increased both stiffness and strain recoverability (Figure 3.7C).¹⁵⁴ In a similar study, a casein-reinforced hydrogel containing dopamine was synthesized to improve the adhesion of the material.¹⁵⁵ Similarly, high stretchability, on the order of 1600-2900%, was obtained when different concentrations of casein (up to 37.5 wt.%) were added to the hydrogel. In contrast to the previous study, although casein additives enhanced the mechanical strength, they compromised the overall stretchability of the hydrogels. Although nanoparticle reinforced hydrogels have shown a great promise in robust mechanical properties, their release can induce cytotoxicity and thrombosis, which should be paid attention particularly for internal use.

3.2.6.1 Hydroxide nanoparticles

The incorporation of hydroxide nanoparticles during the synthesis of hydrogels has

proven to be a robust means to toughen hydrogels. Hydroxide nanoparticles introduce dynamic interactions within the polymeric macromolecules and produce reversible crosslinks within polymer networks. For instance, in a recent study, traces of $\text{Ca}(\text{OH})_2$ nanospherulites (40 ppm) were used as dynamic crosslinkers for synthesizing PAM hydrogel matrixes.¹⁵⁶ These particles were less than 5 nm in size and prepared through the hydration of Ca_3SiO_4 . Here, the Ca^{2+} ions from the $\text{Ca}(\text{OH})_2$ nanoparticles interacted with the $\text{S}_2\text{O}_8^{2-}$ ions released from APS (Figure 3.7E). Therefore, the persulfate initiator molecule (capping the chain end through free radical polymerization) favored the spontaneous ionic binding of the polymeric chains at the surface of $\text{Ca}(\text{OH})_2$ particles, enabling the formation of a hydrogel network. The crosslinked hydrogel showed remarkable elongation at the break, up to ~12,100% (Figure 3.7F), along with excellent strain recovery. The improved mechanical properties of these hydrogels were related to (1) the aggregation-free, homogeneous dispersibility of the $\text{Ca}(\text{OH})_2$ nanoparticles, (2) increased surface area resulting from single-digit size of $\text{Ca}(\text{OH})_2$ nanoparticles, and (3) optimized concentrations of the nanospherulites, which lowered the formation of connective filaments and thereby resulted in higher pore sizes and enhanced deformability.¹⁵⁶ In another study, the use of Portland cement, instead of Ca_3SiO_4 nanoparticles, enhanced the mechanical properties of the hybrid hydrogel with the maximum stretchability of PAM network, up to ~11,200%.¹⁵⁷ In this case, similar $\text{Ca}(\text{OH})_2$ nanoparticles (<5 nm) were synthesized through the hydration reaction of $\text{Ca}(\text{OH})_2$ in the presence of a polycarboxylate-ether superplasticizer (PCE) that restricted the precipitation of the cement. The addition of 2-3 nm-sized $\text{Al}(\text{OH})_3$ particles instead of $\text{Ca}(\text{OH})_2$ nanoparticles resulted in similar properties in the materials. In this case, the presence of inorganic nanoparticles with hydroxyl groups on the surface enhanced hydrogen

bonding in PAM molecules, and 2-acrylamide-2-methylpropane sulfonic acid (AMPS)-based hydrogel materials,¹⁵⁸ and resulted in improved mechanical properties. The synthesized hydrogels were observed to have the highest stretchability, on the order of 2090% when 3 wt.% Al(OH)₃ was added.

3.2.6.2 Nanoclays

One approach to improve toughness in hydrogels is to reinforce them with various nanoclays with strong surface charges. Recently, the addition of exfoliated Montmorillonite (MMT) clay particles ($[(\text{Al,Mg})_4\text{Si}_8\text{O}_{20}(\text{OH})_4]\text{Na}_{0.66}$) during the polymerization of PAM (Figure 3.7G) led to a remarkable increase in stretchability of hydrogels, on the order of 11,800%, compared to ~40% for the PAM hydrogels crosslinked by MBAA using APS/TEMED.¹⁵⁹ Another hydrogel with a similar chemical composition showed significantly reduced stretchability of 1290% when the polymerization reaction was carried out without TEMED.¹⁶⁰ The chemical reaction conditions play critical roles in the hydrogel mechanical properties. Laponite® nanoclay particles, ($[\text{Mg}_{5.34}\text{Li}_{0.66}\text{Si}_8\text{O}_{20}(\text{OH})_4]\text{Na}_{0.66}$), enhanced the mechanical properties of hydrogels. *In situ* polymerization of PAM with Laponite® at ~4 wt.% improved the elongation at break (stretchability up to ~5000%).¹⁶¹ Similar improvements were achieved through crosslinking PAM linear chains in the presence of a 60 nm double-layer hydroxide (DLH) with a chemical formula of $[\text{Mg}_{2.52}\text{Al}(\text{OH})_7](\text{HO}(\text{CH}_2)_2\text{SO}_3) \cdot 1.27\text{H}_2\text{O}$.¹⁶² In this study, ionic interactions between nanoparticles and initiators led to the formation of clay-brush particles, connecting the linear polymeric chains in an APS/TEMED crosslinking system. The electrostatic interactions involved in DLH/PAM nanocomposites were responsible for the improved mechanical properties (~3000% stretchability) of the developed hydrogel moiety at the optimum ~5 wt.% clay content. The

hydrogen bonding between the clay and the initiator/catalyst system contributed to this improvement. In combination with dopamine, talc particles were well dispersed in water and facilitated the polymerization of dopamine molecules over the talc surface through partial oxidation.¹⁶³ The PDA-coated talc nanoparticles were embedded within the PAM network and enabled several dynamic interactions including physical attractions between the PDA and PAM molecules, π - π stacking, and hydrogen bonding between PDA and PAM. In this case, at dopamine:AAm and talc:AAm ratios of 0.5 and 0.75%, respectively, a maximum stretchability of over 1500% was reported.

3.2.6.3 Surface-functionalized nanoparticles

Apart from the non-covalent interactions discussed above, covalent bonds between the polymer molecules and various functionalized nanoparticles can also improve toughness in hydrogels. Copolymerization with these crosslinking regulating nanoparticles is achieved primarily by nanoparticle surface functionalization with reactive functional groups such as methacrylates. For instance, silica nanoparticles were modified chemically with vinyltriethoxysilane (VTES) through a sol-gel process and covalently crosslinked with PAM chains (Figure 3.7I,J);¹⁶⁴ as a result, stretchabilities over 3400% (Figure 3.7K,L) were reported.¹⁶⁵ A similar approach was demonstrated to polymerize AAc monomers. In this study, the incorporation of Fe^{3+} ions induced reversible ionic interactions and promoted interchain associations through the chelation of carboxylic groups.¹⁶⁶ These reversible interactions were further supported by hydrogen bonding and resulted in strain at break of 2300%,^{166,167} while the control PAA crosslinked material showed stretchability of up to ~1500%. Similarly, gold nanoparticles were used to serve as a vehicle to carry surface-attached double bonds across the polymeric network. In this case, *N,N*-

bis(acryloyl)cystamine (BAC) molecules with polymerizable alkyl groups were grafted chemically onto the gold nanoparticles. Because of chemical affinity between Au and S atoms, covalently crosslinked hydrogels were formed where crosslink points were cleavable due to the disulfide (S-S) bonds.¹⁶⁸

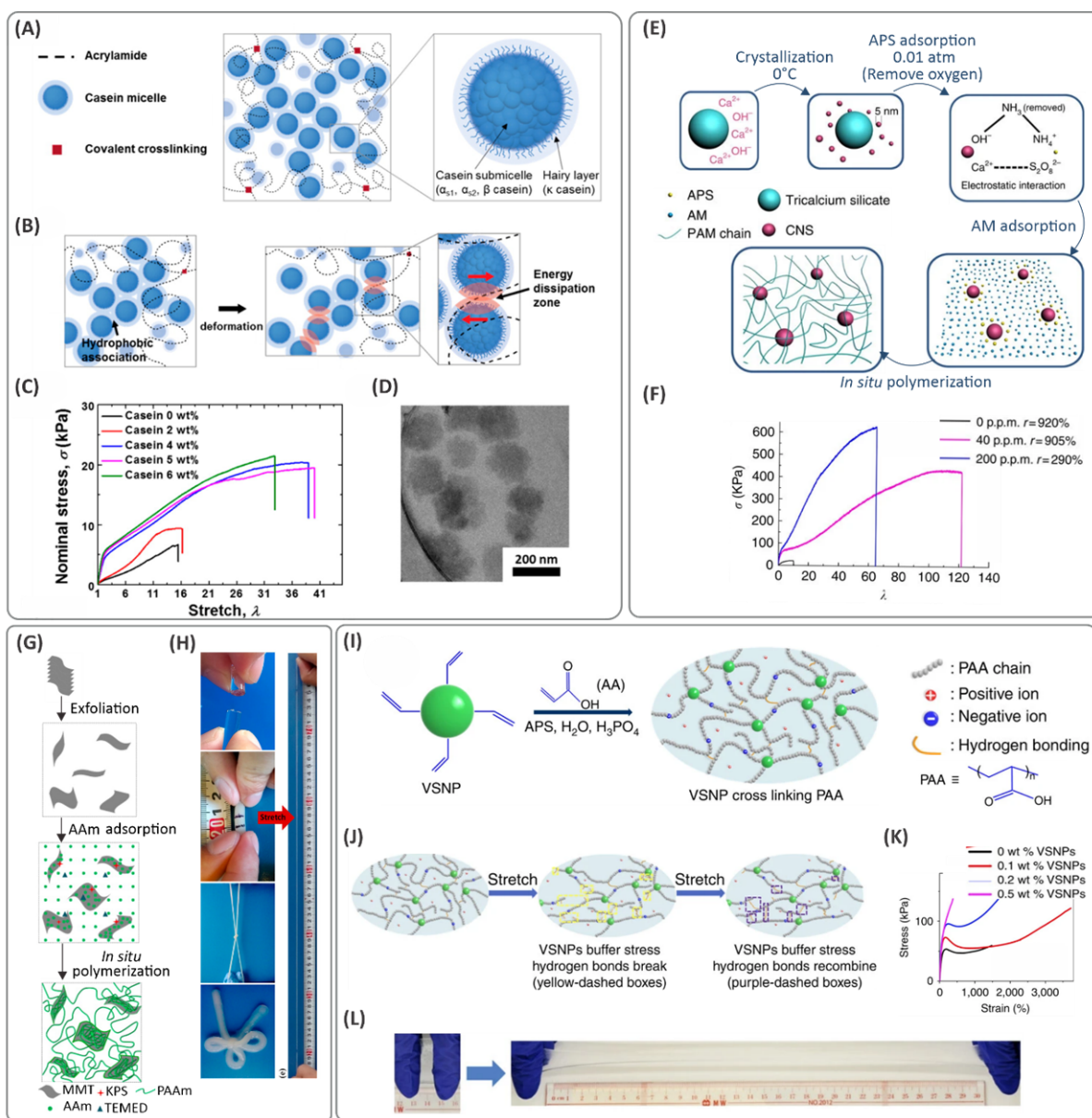


Figure 3.7. Examples of nanocomposite (NC) hydrogels. (A) Internal components of a casein-reinforced polyacrylamide (PAM) hydrogel, and (B) schematic illustration of the toughening mechanism of casein additives due to energy dissipation through hydrophobic interactions. (C) The

tensile mechanical properties, and (D) transmission electron microscopy (TEM) images of casein micelles in the hydrogels (reprinted with permission from ref ¹⁵⁴). (E) Fabrication process of tough nanocomposites mediated by incorporating calcium hydroxide ($\text{Ca}(\text{OH})_2$) nano-spherulites (CNS) in a PAM network. The Ca_3SiO_5 releases Ca^{2+} and OH^- in a hydration process during which the small-sized CNS particles (<5 nm in size) are crystallized at 0 °C. The persulfate ions from the ammonium persulfate (APS) initiator are attracted electrostatically to CNS and act as crosslinkers. (F) Significant improvement of stretchability in PAM networks with small amounts of CNS. Reprinted by permission from ref ¹⁵⁶. Nature Publishing Group, Copyright 2016. (G) Fabrication steps of montmorillonite (MMT)/PAM composite hydrogels. (H) Digital photographs of bow-tied hydrogel, and tensile stretching of the hydrogels over 12000%. Reprinted with permission from ref ¹⁵⁹. Copyright 2015 American Chemical Society. (I) Preparation of vinyl functionalized hybrid silica nanoparticles (VSNPs)-poly(acrylic acid) (PAA) hydrogels where VSNP nanoparticles act as crosslinking points. (J) The mechanism explaining the improved hydrogel stretchability and molecular mechanism of deformation. (K) Stress-strain characteristics of the hydrogels with different amounts of VSNP nanoparticles, and (L) illustration of manually stretched hydrogels. Reprinted by permission from ref ¹⁶⁷. Nature Publishing Group, Copyright 2015.

3.3 Bioadhesive materials: Synthesis and characterization

After tough polymer networks are designed with a proper selection of backbone and crosslinking strategy through the methods discussed above, a variety of approaches can be employed to introduce adhesion (Figure 3.1). Long-lasting and strong adhesion to tissue surfaces is an essential requirement in bioadhesive sealants. The mechanical stability of the sealants depends on the synergy between the adhesion and cohesion.⁸ Typically, adhesive failure occurs when the inter- and intramolecular interactions within the hydrogel network are stronger than the interactive forces between the tissue and sealant material.⁷ This failure becomes even more of a concern when it comes to wet tissue surfaces. Furthermore, the gradual swelling of hydrogels, due to the absorption of biofluids, presents another challenge that needs to be taken into account.⁹²

Although the underlying mechanisms of adhesion are not fully understood, adhesion is being explored rigorously through chemical covalent and non-covalent (such as hydrogen-bonding^{169,170} and cation- π ^{171,172}) interactions as well as physical (mechanical interlocks, tissue fusion, and topological chain entanglement¹⁷³) pathways. Nucleophilic functional groups present on the tissue surfaces drive chemical interactions of the bioadhesives to the underlying tissue substrates.^{174,175} From a mechanical viewpoint, interlocking between the bioadhesives and uneven tissue surfaces can also favor bioadhesion.¹⁷⁶ In this regard, ultrasound-induced cavitation in hydrogels are emerging as a means to enhance polymer entanglement with tissues for tough bioadhesion.¹⁷⁷ In the following sections, we give a detailed overview of recent advances made in creating bioadhesive materials, and methods involved in their development. First, we introduce the testing methods used to characterize and to assess adhesion. Standard testing methods of key properties are critical to fair comparisons of materials prepared by different laboratories. Then, we discuss the chemistries involved in creating synthetic, natural,⁵⁹ and bio-inspired adhesives to enhance wet adhesion in bioadhesives.

3.3.1 Experimental characterization of adhesive strength

Several methods have been reported to quantify the adhesive strength of hydrogels. Despite having standardized procedures to demonstrate tissue adhesion, the lack of consistency seen in the testing instrumentation and its implementation makes it difficult to compare and to evaluate the adhesive characteristics of different hydrogels. Adhesion strength values are influenced by several handling and testing factors, including the loading rate, environmental temperature, humidity and dryness (which affects the hydrogel dimensions overtime), and substrate type.⁸ Depending on the application and curing

mechanism, a proper testing method should be selected to characterize adhesion strength. For example, photopolymerization requires illumination with light (with a certain wavelength), which may not be possible in testing adhesives that are applied between light-absorbing adherents (*i.e.*, in a lap shear test).

Axial adhesion strength, one of the simplest methods to test the adhesion of the material, defines the maximum load that an adhesive can tolerate under tensile loading.¹⁷⁸ In this case, the axial detachment of the adherents determines the failure point. Peeling tests reflect the normal adhesion of the materials, which can be carried out in 90° or 180° configurations.¹⁷⁸ Another method is the burst pressure test, which simulates the seals on tissue incisions while subject to liquid pressure. Here, the stability of the sealant under increasing liquid/air pressure is measured. This test determines the origin of failure, which can be related to cohesion, adhesion, or a combination of both. The lap shear test evaluates the adhesion under shear deformations. The wound-closure test consists of filling the gap between two separate pieces of tissues or filling an incision on an integral tissue piece with an adhesive material. In this test, the tensile load applied to the tissue ends is transferred to bioadhesive in both shear (adhesive) and tensile (cohesive) modes.⁵⁸

The sealing capacity of the sealants can be evaluated *ex vivo* by polymerizing the prepolymer on punctured tissue while flowing biological fluids at the tissue/adhesive interface to mimic the real-time sealing performance under heavy bleeding conditions.¹⁷⁹ Given the dynamic loading applied to the tissue adhesives, looking at the cyclic decays in the above-mentioned testing platforms can be of importance. However, the cyclic response of bioadhesives has not been as thoroughly investigated as the static tests in the literature.

3.3.2 Synthetic bioadhesives

Synthetic polymers are synthesized using organic compounds to develop bioadhesives with the desired functionality.¹⁸⁰ Synthetic hydrogels provide a versatile platform for introducing functional bioadhesives with thermo-responsive, electrical conductivity, and stimuli-responsive properties. For example, a thermo-responsive sealant was engineered based on the copolymerization of physically crosslinked PNIPAM and butylacrylate molecules for occluding open globe injuries of the eyeball.¹⁸¹ Being liquid at room temperature, the resulting copolymer enabled facile injection, which then transitioned to a solidified occlusion at (higher) body temperature. This sealant showed reversible sealing with adhesive strength comparable to cyanoacrylate glue, with no neurotoxicity nor significant inflammatory responses. Thermosensitive coacervate complex formation for bioadhesion was also demonstrated in a PNIPAM-based hydrogel containing charged polyelectrolyte moieties.¹⁸² The precursor solution was in the liquid state at room temperature and could form a nonflowing adhesive coacervate complex at temperatures above the lower critical solution temperature (LCST) (with lap shear strength on the order of ~ 7 kPa). Another example of temperature-driven coacervate formation with LCST was proposed by Narayanan *et al.*¹⁸³ A protein-like polyester statistical copolymer composed of a tropoelastin-mimicking unit responsible for coacervate formation (*i.e.*, bis(2-methoxyethyl)succinimide pendant), a catechol containing unit for adhesion, and a light-activatable mechanical strengthener was incorporated into a hydrogel for underwater adhesion over a wide pH range, 3-12. The highest adhesion strength was measured to be ~ 100 kPa for the optimal conditions in lap shear tests.

Synthetic approaches have leveraged bioadhesive electronics for monitoring

chemical and physical signals.¹⁸⁴⁻¹⁸⁶ Implantable sensors and wireless devices¹⁸⁷ should be adhered firmly to their target tissue to be able to function for long periods to monitor diseases.¹⁸⁸⁻¹⁹⁰ Several bioadhesive materials have been proposed for the attachment of bioelectronic devices to tissue. Zhao and coworkers fabricated a stretchable adhesive dry patch based on PAA¹⁹¹ that adheres to wet tissue surfaces. Here, hydrogels were formed by absorbing tissue moisture,¹⁹¹ as a result of which the reactive functionalities in the designed materials could interact readily with the biological surfaces to form covalent and noncovalent linkages. The PAA was functionalized with *N*-hydroxysuccinimide (NHS) to stabilize long-term adhesion through covalent attachment to the amine groups present on the tissue.¹⁹¹ A similar patch was used as a platform to develop graphene nanocomposite-based conducting adhesive patches with high electrical stability.¹⁹² These dry patches were fabricated based on origami architectures to enable their application in minimally invasive surgical tissue sealing.¹⁹³ Another example involves an adhesive supercapacitor based on polyaniline (PANI), rGO, and MXenes embedded in a hybrid hydrogel based on PVA and NHS functionalized PAA.¹⁹⁴ Here, hydrogel electrodes can bind efficiently to the biological surfaces for bio-integration of electronic systems. Synthetic patches were also designed with stimuli-responsive functionality to fabricate on-demand detachable bioadhesives.^{190,195-197}

Many synthetic adhesives demonstrated in the literature require the use of cytotoxic procedures and hence, may not be used in biomedical applications;¹⁹⁸ however, studies of these materials can lead to insight into the design of biocompatible medical adhesives. For instance, a creeper sucker-inspired adhesive was synthesized utilizing poly(hydroxyethyl methacrylate) (PHEMA) containing crystallizable 1-ethyl-3-methylimidazolium bromide ([EMIM]Br) solvents.¹⁹⁹ This study highlighted that hydrogen bonding has tremendous

potential to transmit adhesion stress robustly, to enable high adhesive strengths of ~ 10 MPa (in lap shear test using glass substrates). Strong hydrogen-bonding-driven bioadhesives were demonstrated *via* coacervate formation²⁰⁰ as well as the incorporation of triple hydrogen-bonding clusters (THBC) in other examples.²⁰¹ For the latter, a copolymer composed of *N*-(3-aminopropyl)methacrylamide hydrochloride (APMA) decorated with *N*-[tris(hydroxymethyl)methyl]acrylamide (THMA) (a branched hydroxyl-capped component) was synthesized. A so-called “load shearing” effect in the high-density hydrogen-bonding network explained the high maximum adhesion strength of ~ 120 kPa in a lap shear test using glass slides.

Other synthetic copolymers were developed for bioadhesion with specific functionality for a wide variety of tissues. In a recent study, a copolymer composed of AAm (for network cohesion), methyl acrylate (for hydrophobicity), AA (for mucoadhesion), and MBAA (for crosslinking) was synthesized.²⁰² This hydrogel system was obtained through a photocrosslinking process either as an *ex situ* patch or *in situ* glue for the prevention of intestinal anastomotic leakage. Adhesion strength (lap shear test) for *ex situ* (~ 7 kPa) was significantly lower than *in situ* application (~ 12 kPa). The study found that AA plays a major role in adhesion force as increasing the AA acid content from 0 to 25 wt.% led to increases in adhesion force by over $\sim 700\%$. Further increases in acrylic acid content, however, deteriorated mechanical properties. Bioadhesive powders²⁰³ were introduced for rapid hemostasis *e.g.*, using a synthetic/natural combination of PEI, PAA, and quaternized chitosan (QCS).²⁰⁴ The powders were characterized by self-gelling properties (gelation in 4 s) upon contact with and absorption of blood plasma. Due to the strong adhesion (burst pressure ~ 240 mmHg), bleeding could be arrested in 10 s. Thermoplastic biomaterials such as

polycaprolactone (PCL) have also shown potential for use as bioadhesives.²⁰⁵ These materials could be applied topically at elevated temperatures (40-50 °C, depending on the PCL molecular weight) using a glue gun.²⁰⁶ The star-PCL structures with carboxyl end groups were activated using amine-reactive NHS ester for bioadhesion. The proposed bioadhesive achieved ~50% of the Dermabond®'s adhesion strength (a commercial cyanoacrylate-based glue). Elastomeric bioadhesives are emerging as promising backbones due to their intrinsic toughness and stretchability. Poly(glycerol sebacate) (PGS) is one example of a degradable elastomer synthesized through condensation reactions between sebacic acid and glycerol.²⁰⁷ A modified PGS involving PEG chain expanders and dihydrocaffeic acid additives for adhesion was demonstrated in combination with a secondary gelatin-based network.²⁰⁸ Here, gelatin was modified with UPy to endow self-assembly and self-healing properties to the biomaterial. Significant improvements in adhesive strength were achieved by increasing the gelatin-UPy content (by over ~10×).

Synthetic bioadhesives, in many cases, are designed to mimic the structures of adhesive materials found in natural organisms.²⁰⁹ The strong hydrogen-bonding interactions in DNA molecules inspired the design of a nucleobase-modified polyphosphoester hydrogel adhesive (Figure 3.8A-C).²¹⁰ This monomer consists of purine rings and pyrimidine functionality that introduce hydrophobic reactions as well as hydrogen bonding to the hydrogel. The resulting hydrogel is degradable under alkaline conditions; therefore, these adhesive hydrogels (maximum lap shear ~40 kPa) can be removed on demand with exposure to high pH, without leaving behind any glue residues on the tissue surface. In another study, barnacle-inspired adhesive pads were synthesized using a copolymer consisting of hydrophobic aromatic 2-phenoxyethyl acrylate and positively charged 2-

(acryloyloxy)ethyl trimethylammonium chloride (ATAC).²¹¹ Mechanical toughness was achieved due to the dynamic π - π and cation- π interactions, leading to stretchability of over $\sim 700\%$. The high maximum adhesion strength of 180 kPa was obtained in the axial adhesion tests for samples with hydrophobic monomer (*i.e.*, 2-phenoxyethyl acrylate) molar fractions of 0.85, which was $\sim 7\times$ larger than those with 0.7 aromatic content ratio. In general, synthetic adhesives result in higher adhesion strength (in the MPa range) compared to most natural bioadhesives. In a recent study, a waterborne, synthetic yet biocompatible, and strong (~ 6 MPa adhesive strength) bioadhesive was proposed for dental tissues.²¹² Here, inspired by insect sclerotization, a cost-effective procedure based on phenol-polyamine reaction served to seal skin wounds within a few seconds.

Combinations of synthetic networks with natural hydrogels are used frequently to enable biodegradation and to improve bioadhesion performance in tough hydrogel platforms.^{213,196} Recently, a mixture of PAM and alginate macromolecules was used to design tough hydrogel materials, which were applied to the tissue surfaces in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and NHS coupling reagents. *Ex vivo* adhesion energy for these materials was in the range of 300-700 J/m².²¹⁴ In another report, an adhesive hydrogel patch was developed using a covalent network containing energy dissipative alginate (tough IPN matrix) as shown in Figure 3.8D-F.²¹⁵ This patch was treated with coupling chemicals that diffused through the hydrogel matrix and acted as a bridging layer. The bridging polymer, comprised of amine-rich macromolecules, enabled the covalent bonding between hydrogel and tissue surface through substitution reactions. Active functional groups present on the tissues favored covalent adhesion and responded spontaneously upon the application of gentle pressure on the hydrogel patch. The materials

synthesized with polyallylamine and chitosan (bridging polymers) possessed the strongest adhesion when compared to PEI, collagen, and gelatin with adhesion energy on the order of 10^3 J/m^2 . This adhesion energy was significantly higher for tough PAM-alginate-based double network hydrogels when compared to the corresponding adhesion energy of individual constitutive components, indicating the substantial roles of cohesion in bioadhesion.

Topological adhesion has recently emerged as a potential mechanism for bioadhesion. In this process, polymer chains of two hydrogel networks are stitched together at their interfaces through a stitching polymer chain that can entangle within the hydrogel networks through diffusion. The diffused and entangled chains of stitching polymer are then associated together *via* an external trigger such as pH.¹⁷³ This concept was demonstrated using a variety of synthetic hydrogels and different stitching polymers (*i.e.*, chitosan, alginate, and cellulose) that can associate strongly and crystallize at higher pH than their pK_a at the adhesion interface. Stitching (gluing) polymers can also be used to attach *ex situ* crosslinked hydrogels to tissues. An example of a gluing polymer developed by Gao *et al.* involves a PAA modified with catechol groups where entanglement and crosslinking with the backing hydrogel are triggered by NaIO_4 solution.²¹⁶ This bioadhesive system was able to adhere PAM hydrogels to tissue surfaces robustly (with peel-off adhesive energies of $\sim 150\text{--}200 \text{ J/m}^2$). Overall, synthetic approaches provide more versatility when it comes to designing multifunctional and stimuli-responsive bioadhesives.

3.3.2.1 Cyanoacrylates

Since the 1960s, cyanoacrylate-based polymers were used extensively as surgical sealants due to their high adhesion strength to wet surfaces. However, the heat generated

during polymerization and the resulting toxic degradation byproducts have limited their use for internal tissues.²¹⁷ Moreover, complications associated with exothermic reaction-induced tissue damage, granulomatous keratitis, glaucoma, and cataract formation have further caused concerns for the application of such biomaterials in clinical settings.²¹⁸ These complications have motivated researchers to design biosafe alternatives with similar adhesive strengths. One common example of such materials includes the chemical synthesis of cyanoacrylate adhesives that express minimal toxicity. For instance, a PEG biscyanoacrylate-based bioadhesive hydrogel was used as a crosslinker in an octyl cyanoacrylate adhesive polymer.²¹⁹ Here, anthracenyl cyanoacrylic acid was esterified while anthracene protected the vinyl groups. This copolymerization resulted in a cytocompatible strong and adhesive (over 200 kPa peel-off strength) material. Polymerization of allyl 2-cyanoacrylate (ACA) in mixture with poly(L-3,4-dihydroxyphenylalanine), poly(L-DOPA), demonstrated biocompatible adhesives for medical applications.²²⁰ Recently, ACA was polymerized in the presence of hydroxyapatite and bisphenol-A glycidyl methacrylate (bis-GMA) to improve the biocompatibility and physical properties of the adhesive material.²²¹ Moreover, an adhesive based on the mixture of poly(L-lactic acid) (PLLA) and pre-polymerized ACA molecules promoted wound healing and showed better biocompatibility.²²² The healed tissues treated with this adhesive exhibited better tensile tearing strength compared to control samples treated with commercially available adhesives.

3.3.2.2 Polyurethanes

Polyurethane (PU) is a tough polymer, with inherent flexibility, making it a suitable candidate for sealing wounds.²²³ The PU macromolecules are synthesized through the

formation of covalent carbamate linkage between the isocyanate functionalized molecules and a polyol (with two or more hydroxyl groups) in the presence of a catalyst or upon UV light activation.²²⁴ The flexibility and elasticity of the polymer depend on the long-chain monomers. Polyurethane and acrylate-modified PU can be designed for *in vivo* biodegradation.²²⁵ For this purpose, a two-step reaction, catalyzed with dibutyltin dilaurate, was designed using isophoronediiisocyanate (IPDI), polycaprolactonediol (PCLD), and hydroxyethyl acrylate (HEA), where a faster biodegradation rate was attained by incorporating PCLD molecules into the PU acrylate backbone.²²⁶ The adhesion strength and gel fraction for the biodegradable polyurethane adhesive were reported to be 9 MPa and 93%, respectively. In a further study, IPDI was also polymerized with castor oil and PEG at 70 °C for synthesizing a PU-based bioadhesive material.²²⁷ The monomer ratio (-NCO/-OH) was optimized to attain strong chemical bonding interactions between the material and tissue by tailoring the distribution of -NCO groups. These groups could later couple with the amine groups of the tissue surface for covalent adhesion. Here, the curing time at room temperature was ~7-25 min and *in vitro* degradation of the hydrogel occurred over 7 weeks. In this study, a maximum lap shear strength of ~40 kPa and burst strength of ~30 kPa were reported for samples containing 12 v/v% castor oil. These figures dropped to ~30 and ~15 kPa when the castor oil content was reduced to 3 v/v%. Recently, hydroxyl-rich xylose monosaccharide molecules were mixed with diisocyanate molecules, 4,4'-methylenebis(cyclohexyl isocyanate) (MCI), in combination with PEG and triethylamine as a catalyst to synthesize muscle tissue adhesive (Figure 3.8G).²²⁸ The bioadhesive was left for 1 to 24 h in the air before the adhesion tests. Hydrogel materials had maximum lap shear strengths of 94 kPa and showed biodegradation of 20% upon incubation for 8 weeks.

Inspired by mussel adhesion, catechol groups have also been introduced into urethane-based chains for improved adhesion. In a recent study, dopamine was incorporated into a polyurethane backbone.²²⁹ As a result, mechanical strength was improved from <0.05 MPa (no dopamine addition) to ~1.9 MPa. The improved cohesion also favored adhesion strength: lap shear strength was increased to ~70 kPa compared to ~30 kPa in the absence of dopamine.

3.3.2.3 Poly(ethylene glycol)

Poly(ethylene glycol) adhesives are well-established and already commercialized for use as tissue sealants (*e.g.*, CoSeal™ and DuraSeal™). Apart from biocompatibility, PEG hydrogels are nonimmunogenic and bioresorbable. However, PEG by itself is a poor adhesive;⁸² and further chemical modifications are required to improve its adhesion.²³⁰ Hence, PEG polymers are processed in composite form with other components or modified chemically to tailor their physical properties for bioadhesive applications. For instance, a covalent network of polymerized PEGDA was formed in the presence of giant PEG chain networks to develop bioadhesives that could entangle spontaneously and penetrate within the substrate tissues.²³¹ The hydrogels also promoted wound healing and reduced the immune response. Lap shear tests indicated that the use of high molecular weight PEG as a secondary network leads to adhesion strength and toughness close to those of cyanoacrylate glues. In terms of stretchability, the double network PEGDA hydrogels with high molecular weight PEG molecules exhibited a *ca.* two-fold increase as compared to the low molecular weight PEG.

To reduce the high swelling of PEG for bioadhesion, hyperbranched PEG-polyester polymers demonstrated robust adhesion with low swelling and improved

biodegradability.⁹⁸ Multi-arm PEG molecules can also improve adhesion and be used as crosslinkers in bioadhesives. For instance, cyclic succinyl ester functionalized tetra-PEG was reported to be highly reactive to amine functional groups and established covalent adhesion to the underlying tissue in a mixture with amine-capped tetra-PEG.²³² Gelation in this material system occurred within 5 min compared to fibrin glue, which took over 20 min to form a gel.²³² The synthesized hydrogels showed cohesive strength of ~ 20 kPa and burst pressure strength of up to 300 mmHg. In another study, the end group modification of an 8-arm PEG molecule with amine and aldehyde functionalities enabled an adhesive PEG polymer. Schiff base formation between the active chemical sites facilitated both crosslinking of the material and its adhesion to the tissue surface through reactions with nucleophilic amine groups (Figure 3.8H,I). While the gelation time was measured to be in the range of 30-75 s, the adhesion strength in the lap shear test was $\sim 0.2\times$ of the cyanoacrylate adhesive (Figure 3.8J,K). Recently, a 4-arm methacrylate capped pentaerythritol molecule was polymerized in presence of dopamine and PEGDA using Michael addition (in a dimethyl sulfoxide, DMSO, medium).²³³ This procedure resulted in branched hydrophobic polymers with abundant catechol end groups. Upon contact with water, these polymers form coacervates and thereby trigger adhesion to the surface (lap shear adhesion strengths of ~ 100 -200 kPa, depending on the substrate material). This bioadhesive was effective for application in bone fracture and bleeding prevention from deep wounds.

Hybrid PEG hydrogels containing poly(lactic-*co*-glycolic acid) (PLGA) have shown potential for use as bioadhesives. A mixture of PEG, PLGA, and silica particles in acetone was solution blow spun onto the tissue surface (Figure 3.8L). The resulting sealant showed strong tissue adhesion, comparable to that of cyanoacrylate adhesives (160 mmHg burst

pressure strength), as shown in Figure 3.8M.²³⁴ Increasing the silica particle size (from 20 to 620 nm) led to a 2-fold increase in the burst strength. Here, the larger silica particles (620 nm) suppressed crack formation and propagation, which led to improved stretchability and adhesive strength. In addition, lower swelling of hydrogel compared to CoSeal™ was reported. In another study, a mixture of PEG and PLGA polymers (both at 5 wt.%) dissolved in acetone was solution blow spun to form temperature-responsive fiber mats (~0.5-2.5 μm diameter).⁷⁰ The fibers retained their fibrous form at temperatures below 31 °C. Once applied to the tissue using an airbrush, the fiber mats transitioned to an adhesive film layer, which could conform to the shape of tissues at body temperature. The pull-off tests demonstrated increased adhesion strength with the PEG concentration at body temperature, with a maximum of ~120 kPa.

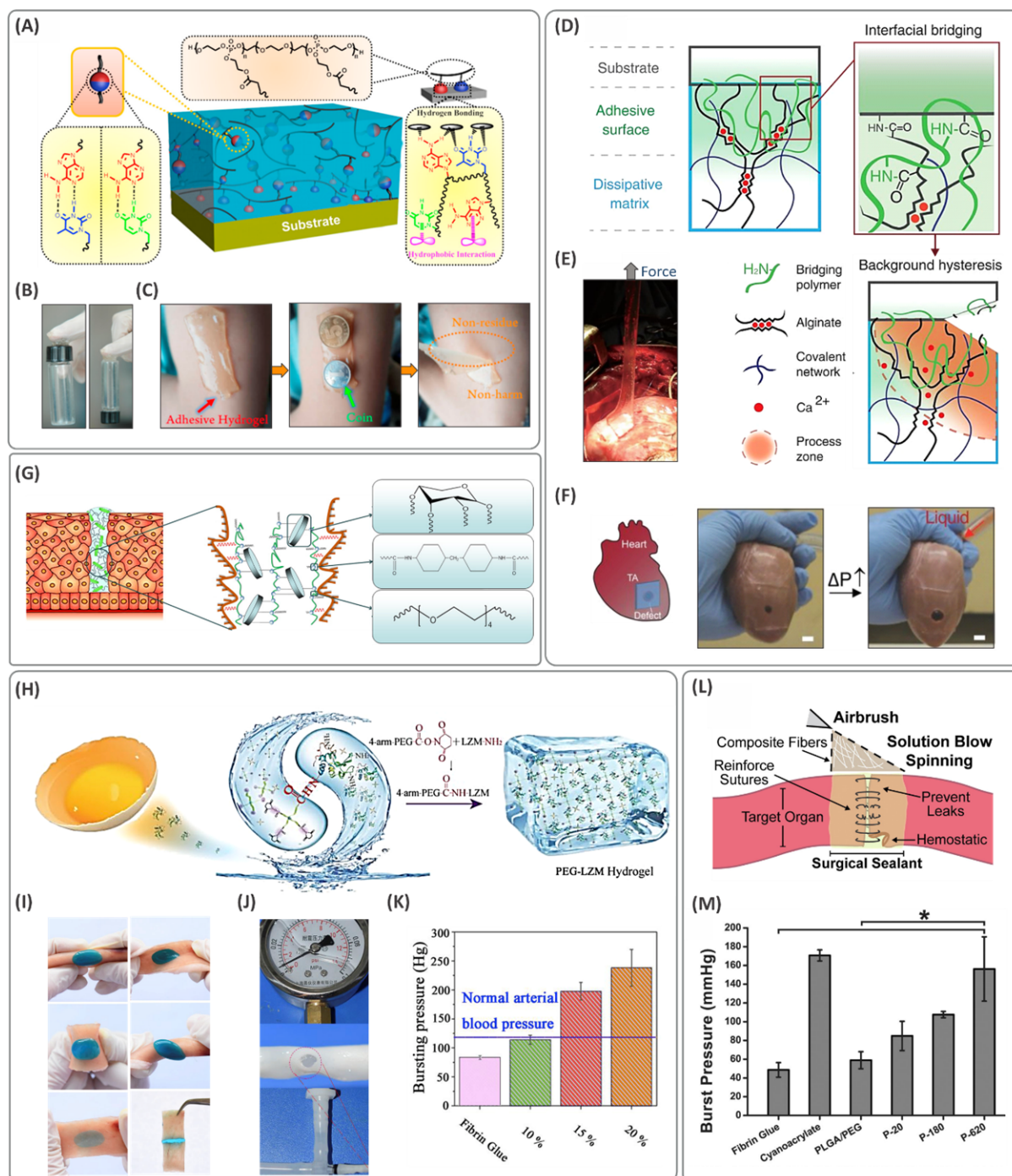


Figure 3.8. Methods and procedures for developing synthetic bioadhesives. (A) Crosslinking mechanisms and the molecular interactions between the substrate leading to adhesion in nucleobase materials. (B) Demonstration of adhesion of polyphosphoesters to glass vials and (C) human skin. Reprinted with permission from ref²¹⁰. Copyright 2019 American Chemical Society. (D) Schematic of the tough hydrogels comprised of a dissipative layer matrix and bridging polymers containing primary amines, which can diffuse into the substrate and the sealant. Propagation of a crack at the

tissue interface is inhibited by the energy absorbed through the dynamic ionic bonds between the calcium ions and alginate chains. (E) Illustration of the tough adhesive adhered to the myocardium tissue while peeling off, and (F) under internal pressure. Reprinted with permission from ref ²¹⁵. Copyright AAAS. (G) Schematic of the xylose-based polyurethane (PU) sealant and their mechanism of adhesion. Reprinted with permission from ref ²²⁸. Copyright 2016 American Chemical Society. (H) Chemistry of adhesion in the polyethylene glycol (PEG)-lysozyme (LZM) hydrogels formed *via* the amidation reaction between the egg-derived lysozyme protein and 4-arm-PEG-*N*-hydroxysuccinimide. (I) Demonstration of the conformation of hydrogels onto the tissue under different deformation scenarios. (J) *In vitro* analysis of the burst tests on the porcine vessels, and (K) the burst pressure results showing that the strength values are greater than those of the normal arterial blood pressure. Reproduced with permission from ref ⁸². Copyright 2019, Elsevier. (L) Schematic of the composite fiber deposition on the wounded tissue using an airbrush acting as a surgical sealant. (M) Burst pressure data for the sealants show enhanced strength with increasing silica particle size in PEG/poly(lactic-co-glycolic acid) (PLGA)-based hydrogel. Reproduced with permission from ref ²³⁴. Copyright 2019, Elsevier. *N*-hydroxysuccinimide, NHS.

3.3.3 Natural bioadhesives

One of the main advantages of using natural polymers for the synthesis of adhesive sealants is their innate biocompatibility and biodegradability. Hence, a surge of interest has been directed at the addition of selective chemical functionalities to natural polymeric backbones to enhance their adhesion. Although inflammatory responses induced by natural backbones such as chitosan and gelatin have raised concerns over their internal use, current research is striving to mitigate those effects while enabling their widespread as biodegradable tough bioadhesives. Below, we discuss different classes of natural polymeric backbones used for the development of adhesive hydrogel materials.

3.3.3.1 Polysaccharide-based bioadhesives

Polysaccharides are formed from monosaccharide repeating units connected through

glycosidic linkages.²³⁵ Various types of natural polysaccharide-based backbones are used in bioadhesive materials, including chitosan,²³⁶ alginate,²³⁷ HA,²³⁸ carboxymethyl cellulose,²³⁹ *etc.* Active functional groups present in the polysaccharide backbones enable different chemistries leading to adhesive characteristics through intra- or intermolecular interactions. One of the common procedures both to functionalize with chemical moieties and to establish adhesion with tissues is to oxidize the vicinal diol groups present in their monosaccharide units that enable Schiff base formation with tissue surfaces.¹⁷⁹ Other nucleophiles present in some polysaccharides can also be employed to graft adhesive functionalities. Below, examples of approaches undertaken to modify polysaccharide-based biomaterials for tissue adhesion are reviewed.

3.3.3.1.1 Hyaluronic acid

Hyaluronic acid is an immunoneutral polysaccharide that exists in the human body comprised of repeating β -1,4-d-glucuronic acid and β -1,3-N-acetyl-d-glucosamine disaccharide units.²⁴⁰ Many biological functions and cellular activities rely on HA in tissues such as the eye and cartilage. Hyaluronic acid has served as a critical building block to develop biomaterials with tissue regenerative properties.²⁴⁰ Modification of HA for bioadhesion through its abundant carboxyl conjugation sites has frequently been demonstrated.²⁴¹ For instance, serotonin is a neurotransmitter released from platelets; it can activate platelets to enable secretion of blood-clotting factors such as platelet factor IV, factor V, von Willebrand factor, and fibrinogen. Serotonin can react with the functional groups present on the tissue surfaces and also act as a crosslinking component for gel formation.²⁴² Serotonin conjugated to HA was shown to promote adhesion during gelation due to the free radicals generated that promote reaction with the underlying tissue substrate.²⁴² This

adhesion, however, is terminated upon completion of gelation (after 10 min) probably due to the full consumption of the oxidative intermediates. The adhesion-terminating character of the hydrogel after crosslinking was assigned to its anti-biofouling properties, which prevent unwanted and abnormal adhesion to the neighboring tissues. Catechol modification of HA is another approach to achieve bioadhesion. A two-component HA hydrogel involving HA-catechol mixed with HA modified with thiourea groups (HA-NCSN) was developed for healing gastric wounds.²⁴³ The pre-polymer solution was crosslinked using a NaIO₄ spray. The thiourea groups in this formulation prevented auto-oxidation of catechol groups that cause adhesion loss. As opposed to HA-catechol bioadhesives, the HA-catechol/HA-NCSN composition led to stable adhesion in an acidic environment (pH ~2) making the gel a suitable candidate for sealing gastric wounds. In other examples,^{179,244} glycosaminoglycan HA was functionalized with a photoreactive functional group *N*-(2-aminoethyl)-4-(4-(hydroxymethyl)-2-methoxy-5-nitrosophenoxy) butanamide (NB), which generated aldehyde groups upon UV light irradiation. Aldehyde groups were able to form Schiff bases with the primary amine groups on the tissue surface for adhesion (Figure 3.9A). The NB-modified HA was reinforced with GelMA. High cohesion and adhesion led to burst pressure of up to 290 mmHg, higher than most commercial bioadhesives. The sealant rapidly stopped heavy bleeding from incisions in carotid arteries and the heart *in vivo* in a pig model (Figure 3.9B,C) due to its strong adhesion.

3.3.3.1.2 Chitosan

Chitosan is obtained from one of the most abundant polysaccharides in nature (*i.e.*, chitin from marine organisms such as shrimp, crab, *etc.*)²⁴⁵ and consists of β -(1→4)-linked *N*-acetyl-d-glucosamine homopolymers.²⁴⁶ Primary amine groups present on the chitosan

backbone provide positive charges leading to its antimicrobial²⁴⁷ and hemostatic²⁴⁸ properties. Positive charges can be further enhanced by the quaternization of chitosan (QCS).²⁴⁹ The mixture of QCS with Fe³⁺-associated protocatechualdehydes, in a recent example, produced a strong antibacterial, photothermal, and bioadhesive hydrogel for the treatment of infected wounds.¹⁴ Note that both humoral and cell-mediated immune responses are associated with chitosan.²⁵⁰ Nevertheless, chitosan has shown promise in bioadhesives and has been used to treat bleeding and diabetic wounds.^{251,252}

Amine groups on the chitosan backbone can be used to modify chitosan for improving adhesion *via* substitution reactions.²⁵³⁻²⁵⁵ For instance, the NB functional groups were grafted on chitosan backbones to enable photo-triggerable injectable bioadhesives in a mixture with unmodified chitosan.²⁵⁶ The optimized composition achieved a maximum adhesion strength of ~100 kPa (~5× larger than fibrin glue). Inspired by trichome in tunicates, pyrogallol groups were grafted chemically onto the chitosan backbone to improve both its solubility under physiological conditions and wet adhesion beyond that of commercial adhesive fibrin glue.²⁵⁷ Recently, a gallic acid-modified chitosan hydrogel was electrospun to form microfiber structures mimicking the natural tunicate's body armor.²⁵⁸ Chitin derivatives have also shown promising adhesion strength. Water-soluble 2-hydroxy-3-methacryloyloxypropylated carboxymethyl chitin (HMA-CM-chitin) was polymerized using hydrogen peroxide.²⁵⁹ The addition of chitin nanofibers (CNFs) and surface-deacetylated chitin nanofibers (S-DACNFs) significantly enhanced adhesive strength, comparable to the cyanoacrylate adhesives. The lap shear strength of the material was close to 3 kPa, and the burst pressure was measured to be ~170 kPa. Schiff base formation in the chitosan hydrogel system using PEG-dialdehyde in the presence of reductive agents was

investigated.²⁶⁰ Here, chitosan was functionalized initially with PEG molecules to enhance their water solubility. Further, the chain length of the PEG molecules was engineered to lower the gelation time down to ~1 min. *Ex vivo* lap shear tests on porcine skin showed a two-fold increase compared to the low molecular weight PEG chains (750 versus 2000 g/mol).

3.3.3.1.3 Alginate

Alginate is a major component of algae and is composed of alternative units of 1→4 linked β -d-mannuronic acid and α -l-guluronic acid monosaccharides.²⁶¹ Abundant carboxyl groups result in negative charges and enable ionic crosslinking with multivalent cations (such as Ca^{2+}) as well as functionalization through coupling reactions. In one example, to develop alginate-based bioadhesives, methacrylated alginate molecules were oxidized to introduce aldehyde functionality for bioadhesion.²⁶² The hydrogels made of non-oxidized methacrylated alginate molecules exhibited larger burst strength values compared to the oxidized methacrylated alginate-based hydrogels. While non-oxidized molecules resulted in delamination failure, material failure was observed for the oxidized methacrylated alginate molecule in burst pressure tests. Even though the oxidation of alginate molecules introduced aldehyde groups to improve the adhesion of the hydrogel material, it lowered mechanical cohesion somewhat. The higher degree of methacrylation in this study was associated with larger adhesive strength, which is attributed to both improved cohesion (due to increased crosslink density) as well as covalent adhesive bonds (through Michael addition between acrylate groups and primary amines on the tissue). Modification of alginate with boronic acid is another method to improve the adhesion strength of alginate.²³⁷ Boronic acid conjugates enable pH-triggerable crosslinking at slightly alkaline conditions. They play similar roles to

catechol groups in terms of bioadhesion mechanism. Peel-off tests using mouse intestine adherents suggested adhesive strength of ~22 kPa for the crosslinked samples while no significant adhesion force was recorded for unmodified alginate.

3.3.3.1.4 Cellulose

Cellulose nanocrystals (CNCs) are being investigated as additives to promote adhesion in hydrogels. To promote adhesive and mechanical properties in PAA-based hydrogels, CNCs were coated with tannic acid (TA).²⁶³ In this approach, the abundant catechol groups on TA were utilized to create mussel-inspired adhesives. The TA-coated cellulose nanocrystals were embedded in the PAA network, which was further crosslinked ionically with Fe³⁺ ions through the catechol functionalities. The fabricated hydrogels showed self-healing properties and tensile adhesive strength of 5-15 kPa to various substrates (*e.g.*, aluminum and hog skin), and the stretchability was enhanced by 5× (strain at rupture ~3000%) compared to pristine PAA.

3.3.3.2 Polypeptide-based bioadhesives

Protein and polypeptides such as silk fibroin (SF),²⁶⁴ albumin,²⁶⁵ and decellularized tissues²⁶⁶ have become attractive candidates to design adhesive backbones mainly due to their excellent biodegradation, biocompatibility, and abundance in nature.^{267,268} Many proteins are inherently glutinous. One of the promising examples of such materials is the recombinant human protein tropoelastin, which was shown to provide an extensible matrix upon crosslinking.²⁶⁹ Methacryloyl-substituted tropoelastin molecules (MeTro) showed excellent sealing when applied on lung and artery leakages (Figure 3.9D-H).⁵¹ When the pre-polymer was synthesized *in situ*, the hydrophobic interactions led the macromolecules to

coacervate at body temperature and to form physical crosslinks. This process can aid in initial conformation to tissue shape. Later, the hydrogel was attached covalently to the tissue surface by UV curing. The mechanical properties were readily tunable with the degree of methacryloyl-modification as well as the prepolymer composition. In terms of adhesion, the lap shear, burst pressure, and wound-closure tests all suggested that the adhesion strength increased with the degree of methacryloyl-modification and MeTro concentration. Here, the maximum values related to these experiments were 172.1 kPa, 11.9 kPa, and 75.9 kPa, respectively, which were significantly higher than commercial Evicel® and CoSeal™ counterparts (Figure 3.9E). An extracellular matrix (ECM) mimicking glycopolyptide, *i.e.*, poly(l-lysine) (PLL) was functionalized with catechol groups²⁷⁰ and glucose²⁷¹ to develop bioadhesives for hemostasis and wound-healing applications. This study²⁷¹ revealed that metal ion coordination crosslinking using Fe³⁺ resulted in stronger adhesion force compared to those of crosslinked enzymatically using horseradish peroxidase (HRP)/H₂O₂ systems. Another study revealed the adhesion of a double network PLL modified with 4-hydroxyphenylacetic acid in mixture with agarose (enzymatically crosslinked *via* HRP/H₂O₂).²⁷² The hydrogels were characterized by self-healing properties. Schiff base formation between oxidized phenols and amine groups on the tissue led to adhesion strength of ~34.5 kPa in burst pressure tests, which is larger than arterial blood pressure (16 kPa). Lysozyme protein, derived from eggs, with excellent antibacterial properties, has also been used to design adhesive sealants.^{82,273} *In situ* crosslinking between the amine groups present in lysozyme molecules and reactive NHS capped 4-arm-PEG (4-arm-PEG-NHS) crosslinker was recently proposed as a bioadhesive.¹⁹⁸ Covalent adhesion through bonding with the tissue amine groups led to stable covalent adhesion (with burst pressure strengths up to

250 mmHg) and sealing incisions (such as blood vessel lacerations, repairing the tracheal trauma, and heart wall defects) effectively. In another recent example, injectable bioadhesives were made from *Bacillus subtilis* biofilms, containing catechol-functionalized amyloid and hydrophobin-like proteins.²⁷⁴ Metal ion solutions (*i.e.*, CaCl₂, MgCl₂, and FeCl₃) were applied to crosslink the hydrogel precursors, among which FeCl₃ led to the strongest adhesion strength (*i.e.*, ~250 kPa). Among the polypeptide-based bioadhesives, gelatin and fibrin have received particular attention due to their superior biocompatibility and biodegradability. Next, the latest advances in gelatin and fibrin-based bioadhesives are discussed.

3.3.3.2.1 Gelatin

Gelatin is an ECM-mimicking protein obtained by hydrolysis of collagen from animals. Along with its excellent biocompatibility and biodegradability, gelatin-based hydrogels have shown intrinsic adhesion that promotes their application as sealing materials.⁴⁶ For instance, gelatin forms crosslinked hydrogels in mixtures with oxidized chondroitin sulfate for thermosensitive bioadhesion.²⁷⁵ Thermosensitive gelation of gelatin at low temperature (20 °C) results in lower adhesion when compared to higher temperature (37 °C). Hence, bioadhesive patches with body temperature-triggered adhesion were achieved where on-demand removal of the bioadhesive is possible by treating the adhesion site with cold water.

Various chemical modifications are implemented to improve bioadhesive strength and durability of gelatin for bioadhesion. Catechol functionalization of gelatin followed by crosslinking through metal ion (Fe³⁺) coordination is an example of injectable bioadhesives developed for sealing internal leaks.²⁷⁶ In another study, gelatin-catechol conjugates were crosslinked through Michael addition reactions, *i.e.*, crosslinking reactions through thiol and

amine groups of keratin, as well as biaryl formation (coupling the catechol groups).²⁷⁷ Mixtures of keratin and gelatin-catechol led to gelation times as low as 20 s. These bioadhesives led to ~30-40% improvement in wound breaking strength, compared to cyanoacrylate and fibrin glue. In a recent study, gelatin was functionalized with 3,4-hydroxyphenylpropionic acid (HPA) (Gtn-HPA) where crosslinking was achieved *via* HPA groups upon irradiation of visible-light in the presence of sodium persulfate and $[\text{RuII}(\text{bpy})_3]^{2+}$ within ~30 s.²⁷⁸ A second component, *i.e.*, carboxymethyl cellulose-tyramine (CMC-Tyr) conjugates, was added to the bioadhesive material formulation to introduce porous structures within the hydrogel network. The hydrogels had tensile adhesive strengths of ~57 kPa and ~87 kPa for the Gtn-HPA and Gtn-HPA/CMC-Tyr hydrogels, respectively, which were significantly higher than that of fibrin glue (~7 kPa). The bioadhesive was tailored for *in situ* 3D printing of adhesive sealants.

Methacryloyl-modification of gelatin enables both crosslinking and *in situ* adhesion on wet tissues.^{41,63,279-280} For example, injectable GelMA pre-polymers (containing 20 wt.% GelMA) crosslinked on corneal tissue using a visible light exhibited significant improvements in terms of adhesion (*e.g.*, up to more than 15× burst pressure compared to commercial sealants).⁶⁵ Bioadhesion of *in situ* crosslinked GelMA is attributed to the free radical polymerization of methacryloyl groups *via* Michael addition reaction, coupling pre-polymer backbone to the tissue surface nucleophiles (*e.g.*, primary amines).²⁸¹ Composites of GelMA with ECM protein derivatives such as MeTro have also shown promise for the treatment of chronic non-healing wounds (Figure 3.9I,J).⁵¹ In this case, the GelMA/MeTro hydrogel was crosslinked using a visible light source. In addition, antimicrobial peptide (AMP) Tet213 (KRWWKWWRRRC) peptide was conjugated to gelatin to endow the hydrogels with

antimicrobial properties. The mechanical properties of the hydrogel were tunable by changing the concentrations of the constitutive components (*e.g.*, increasing the MeTro content resulted in a lower elastic modulus and higher stretchability). Adhesion strength measured with lap shear, burst pressure, and wound-closure tests suggested that increasing the GelMA:MeTro ratio as well as the total hydrogel concentration results in better adhesive strength. Gelatin methacryloyl could also be functionalized with dopamine and dual-crosslinked through Fenton reaction.²⁸² Fenton chemistry generates hydroxyl radicals from ferrous salts (Fe^{2+}) and H_2O_2 , both of which promote the rapid polymerization of the vinyl-containing macromers. The normal adhesive strength obtained by applying the bioadhesive between the tissue layers was on the order of ~ 15 kPa, which was 2- and 4-fold higher than fibrin glue and GelMA, respectively. In addition, a wide range of mechanical strengths (100-5000 kPa) was obtained by controlling the initiator components in the Fenton reaction. Other catechol-integrated GelMA polymers are developed for bioadhesion. *In situ* polymerization of dopamine in the presence of GelMA enabled growth of PDA moieties at the methacryloyl sites.²⁴ Photocrosslinking of the resulting pre-polymer introduced stickiness to the GelMA patches (up to $\sim 7\times$ increase in adhesion force), and improved stretchability of GelMA hydrogels by $\sim 6\times$. In another study, GelMA chains were complexed *via* oxidized gallic acid species and polymerized in the presence of PEGDA using APS/TEMED radical polymerization system.⁴⁰ The hydrogels adhered reversibly to fragile skin tissues of infants as well as diabetic patients *via* body temperature trigger and could be detached painlessly by cooling the adhesive using an ice bag.

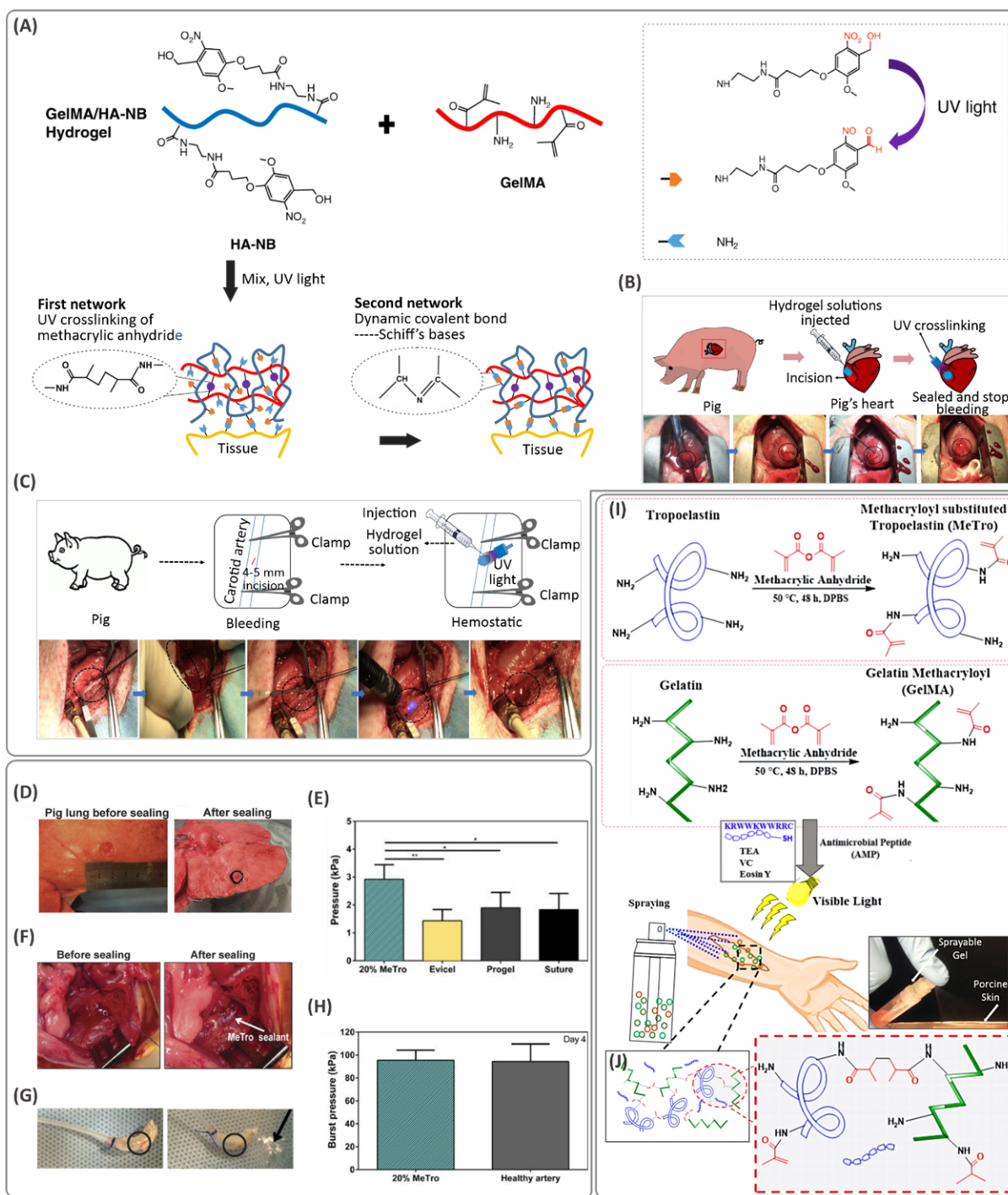


Figure 3.9. Different chemical modification strategies used to create gelatin-based bioadhesives. (A) Chemical synthesis route for the development of highly adhesive and strong hydrogels based on modified hyaluronic acid (HA) and gelatin methacryloyl (GelMA) and its application in sealing (B) heart and (C) arterial-related incisions. Reproduced with permission from ref ¹⁷⁹. Copyright 2019, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (D) *Ex vivo* evaluation of the methacryloyl-

substituted tropoelastin (MeTro) sealant for sealing pig lung. (E) The sealing pressure was increased significantly using a MeTro hydrogel at 20 wt.% pre-polymer concentration. (F, G) Application of MeTro for sealing rat artery, and (H) the burst pressure suggesting the MeTro hydrogel is on the same order as a healthy artery. From ref ⁵¹. Reprinted with permission from AAAS. (I) Synthesis of the MeTro and (J) GelMA pre-polymers for application on the wound site. (J) MeTro/GelMA hydrogels in a crosslinked network. Reproduced with permission from ref ⁵¹. Copyright 2017, Elsevier.

3.3.3.2.2 Fibrin

Fibrin is a protein formed from fibrinogen that plays important roles in hemostasis, thrombosis, wound healing, and several other major pathological conditions. The enzymatic cleavage of fibrinogen by thrombin leads to the formation of fibrin monomers and thereby its polymerization by factor XIII in the presence of calcium ions.²⁸³ Fibrin facilitates enzymatic self-crosslinks as well as bonding with tissue.²³⁴ Fibrin-based materials are commercialized for tissue adhesive applications (such as Evicel®); they offer biodegradability, but they pose the risk of disease transmission and have short life spans (~2 weeks).²⁸³ Fibrin composites with other types of bioadhesives have been used to improve the physical properties of hydrogel materials. For example, fibrin microparticles were integrated into NHS-capped multi-arm PEG matrices for the synthesis of an injectable hydrogel. The results showed improved adhesion to wet tissue.²⁸⁴ Further improvement in adhesion was observed when nitric oxide (NO), a wound-healing reagent, was added to the material. The lap shear strength was ~3.5 kPa compared to control PEG-NO with 1 kPa strength. In general, fibrin bioadhesives lead to weak bioadhesive strength.

3.3.4 Mussel-inspired bioadhesives

Various living organisms in nature, such as mussels²⁸⁵ and ivies,¹¹ establish strong

adhesion under wet conditions. The physical and chemical mechanisms behind their adhesion have inspired researchers to develop bioadhesives with similar strong wet adhesion. Mussels produce abundant catechol-containing proteins involving L-DOPA amino acids that are responsible for their adhesive functionality, acting alongside positively charged^{286,287} and hydrophobic amino acid residues present on the protein backbone to establish adhesion in wet environments.²⁸⁸ Hence, catechol groups play important roles in adhesion. Catechol-based adhesives were first found in the byssal threads of mussels,²⁸⁹ and obtained through purification of precursors produced from *Escherichia coli*.²⁹⁰ The catechol group in L-DOPA undergoes an oxidation process and produces reactive quinone species. These reactive quinones couple with the functional groups present on the wet surfaces of the tissues (*i.e.*, amines, thiols, *etc.*) through a variety of chemical mechanisms, resulting in covalent attachment.^{291,292} Underwater adhesives are mostly supported by the synergistic contribution of catechol-induced reactions, hydrophobic, electrostatic, and hydrogen-bonding interactions.²⁹³ Similar phenolic hydroxyl functional groups are seen in nature, such as TA²⁹⁴ and lignin²⁹⁵⁻²⁹⁸ that are extracted from the plants and used for the synthesis of different adhesive hydrogels.

During the past decade, over 100,000 articles have been published on mussel-inspired hydrogels.²⁹⁹ Although several attempts were made to mimic the strong underwater adhesion of marine living organisms, current research remains unable to replicate and to explain the chemical mechanisms leading to such strong adhesion. The adhesion mechanisms of mussels do not rely solely on the catechol groups present in their secreted proteins; rather, it is a complex process that involves the synergistic contribution of L-DOPA, lysine, and oppositely charged amino acid residues (such as those comprising of phosphates

and amines).^{300,301} Lysine aids in removing the hydrated cations from the surfaces and thereby facilitates the adhesion of catechol to the oxide groups present on the substrate.³⁰² It has been discovered that before mussel feet are engaged with the substrate surface underwater, the local pH of the protein is maintained under acidic conditions, at pH below ~2. By this means, catechol groups are protected from oxidation. Once the mussel adhesive proteins (MAPs) are secreted into the alkaline pH of seawater (pH~8), *in situ* oxidation of catechol moieties enables their reaction with the substrate underneath as they turn into their reactive quinone form.³⁰³ The MAPs secreted by the underwater living organisms are insoluble in the ocean (due to electrostatic coacervate formation of oppositely charged proteins), and their curing is timed accurately to avoid clogging the adhesive ducts (*e.g.*, in the case of sandcastle worms, this protection is ensured by the low pH in their secretory granules).³⁰¹ In addition, liquid–liquid phase separation of MAPs driven by L-DOPA enables mussel fiber formation.³⁰⁴ Inspired by natural adhesion mechanisms, ongoing research strives to engineer catechol-functionalized polymers for the development of biomedical adhesives.³⁰⁵ Next, chemistries and examples of mussel-inspired bioadhesives are reviewed and the latest methods to incorporate mussel-inspired adhesive functionality into polymer backbones are discussed.

3.3.4.1 Crosslinking mechanisms *via* catechol groups

In addition to adhesion, catechol moieties enable polymerization of hydrogel networks.⁹ Catechol groups also introduce dynamic intra/intermolecular interactions to covalent networks to enhance their toughness.⁹ Catechol-catechol and catechol-nucleophilic covalent bonding form through oxidative crosslinking under certain chemical conditions. These conditions include (i) basic pH (pH > 8), (ii) oxidants such as persulfates, periodates,

and HRP/H₂O₂, (iii) enzymes such as tyrosinase or laccase, and (iv) catalysts such as hematic.^{9,306} All these chemical mechanisms trigger the transformation of catechol functionality to highly reactive quinone intermediates.

Catechol groups can generate crosslinked networks in hydrogels through various chemistries. The Michael addition reaction between catechol and thiol groups can lightly crosslink the hydrogel networks at neutral pH.¹⁷ Recently, chitosan³⁰⁷ and HA³⁰⁸ were functionalized with catechol-carrying dopamine molecules to crosslink their hydrogels using thiol-capped Pluronic F127 (Figure 3.10A).³⁰⁸ The catechol-functionalized HA showed excellent tissue adhesion and reversible thermo-sensitivity, enabling its injection as a liquid solution at room temperature *in vivo*, and solidification at body temperature.

Reversible dynamic interactions, enabled by catechol groups, are introduced through hydrogen-bonding, π - π stacking, π -cation interactions, and metal-ion coordination.³⁰⁹ Catechol groups can form pH-sensitive metal ion coordination complexes *via* their hydroxyl sites, in the presence of multivalent metal cations such as Fe³⁺, V³⁺, Cu²⁺, *etc.* The number of catechol groups chelated per multivalent cation increases with pH.³¹⁰ Note that catechol oxidation through metal ion coordination, however, turns the reversible attractions into covalent bonds. To avoid this oxidation, which consumes catechol groups that promote adhesion, electron-withdrawing substituents such as -CN, -NO₂, and -Cl can be introduced.³¹¹ Recently, a 3-hydroxy-4-pyridinone polymer was designed to avoid fast catechol oxidation in mussel-inspired polymers by lowering pK_a of the phenolic ring.³¹²

Metal-ligand chemistry can produce free radicals that can drive gelation in the polymer network.³¹² The low [vanadium]/[catechol] molar ratio led to the generation of organic radicals in catechol-modified chitosan, where the addition of excess vanadium ions

inhibited the gelation process (Figure 3.10B-D).³¹³ The polymerization of dopamine molecules is accompanied by generation of stable free radicals.³¹⁴ Dopamine-initiated polymerization with the acrylate monomers on solid surfaces with high molecular weight was demonstrated for anti-fouling coating applications.³¹⁴ Oxidization of dopamine is accelerated in the presence of oxygen.³¹⁵ Irradiation with UV light promoted polymerization of dopamine due to the generation of reactive oxygen species (ROS, *i.e.*, hydrogel radicals, superoxide radicals, and singlet oxygen).³¹⁵

Catechol groups can form hydrogen-bonding-driven hydrogels. Tough hydrogels based on composites of SF and TA were developed, as shown in Figure 3.10E-G.³¹⁶ Tannic acid has abundant hydroxylated benzyne rings that can form strong hydrogen bonds with proteins and peptides, leading to their instant gelation. An optimal concentration of 0.3 g/ml TA enabled the gelation process. Moreover, the addition of TA to the SF backbone resulted in SF conformational changes from coil to β -sheet structures, leading to the formation of nanofibrillar structures that provided high toughness (123 kJ/m^3) and strong binding affinity to wet tissue surfaces (lap shear strength of 134 kPa). A similar SF-based hydrogel system with hydroxyapatite and TA additives was developed for the fixation of bone fractures.³¹⁷ Hydroxyapatite additives introduced additional coordination bonds with TA. As a result, significant improvements in both toughness (from below 50 kJ/m^3 to $\sim 450 \text{ kJ/m}^3$) and adhesion strength (from below 20 kPa to above 600 kPa in the presence of blood) were achieved. Fractured rat femur bones treated with the developed bioadhesives were tested after two weeks of healing *in vivo*. The treated bone was reported to be approximately twice as strong as those of non-treated groups.

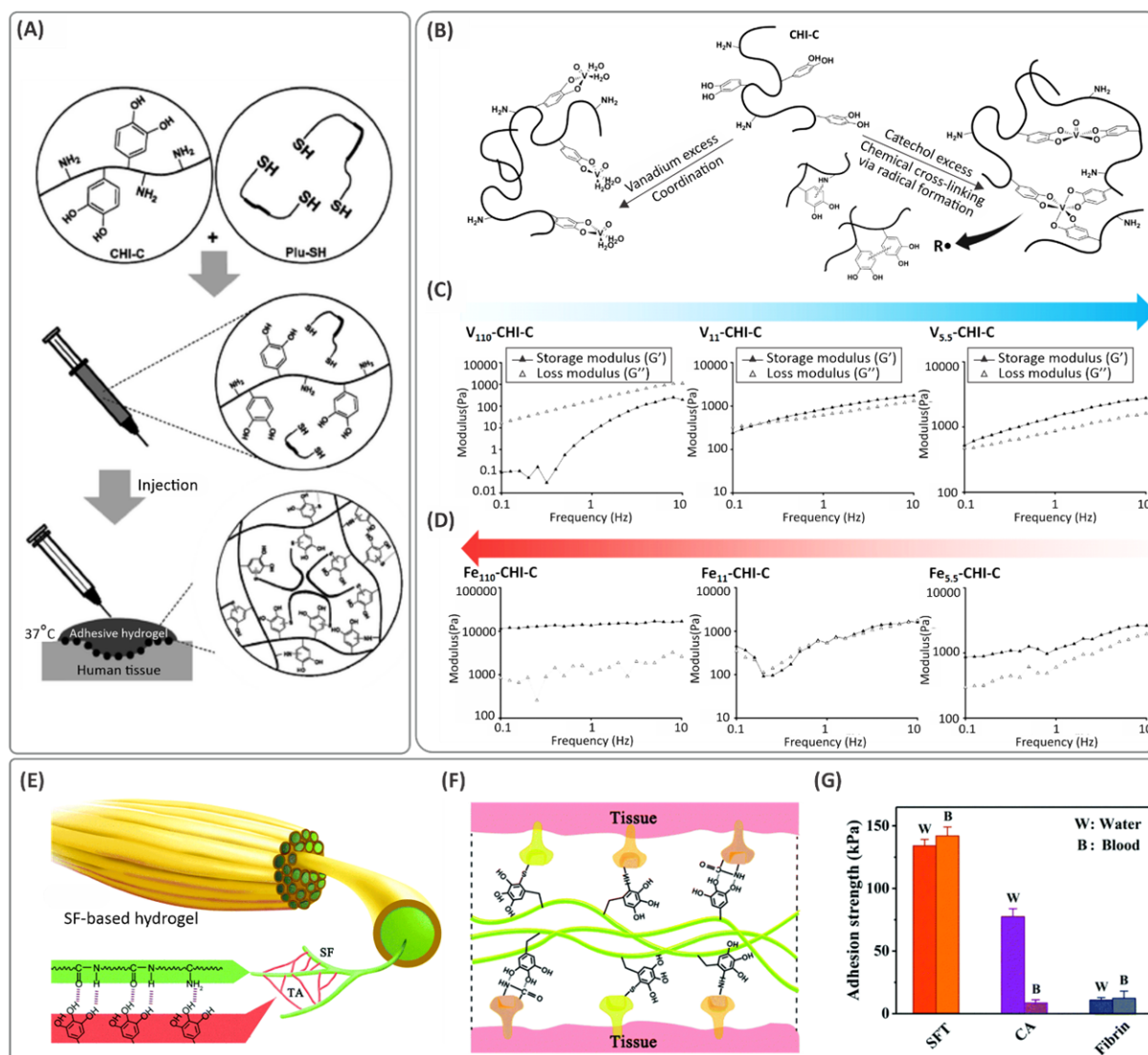


Figure 3.10. Mechanisms of introducing covalent and non-covalent interactions in catechol-terminated hydrogels. (A) The crosslinking scheme of catechol functionalized chitosan (CHI-C) and thiolated Pluronic F127 (Plu-SH), resulting in thermosensitive *in situ* crosslinking at body temperature. Reprinted with permission from ref³⁰⁷. Copyright 2011 American Chemical Society. (B) Schematic of the crosslinking mechanism of catechol conjugated chitosan *via* vanadium. Rheological characterization of a pre-polymer containing 4 wt.% CHI-C with different compositions of (C) vanadium and (D) Fe³⁺ ions. Reprinted with permission from ³¹³. Copyright 2014 American Chemical Society. (E) Schematic of the mussel-inspired silk fibroin (SF)-based hydrogels, and (F) the different mechanisms of hydrogel-tissue adhesion. (E) Lap shear adhesive strength of the SF hydrogel sealant (SFT) is significantly higher than that of commercially available fibrin sealant. Reproduced with permission from ref³¹⁶. Copyright 2019, Royal Society of Chemistry.

3.3.4.2 Chemistry of catechol adhesion

Catechol groups can generate non-covalent (*e.g.*, hydrogen-bonding) and covalent interactions with the nucleophilic functional groups on tissue surfaces. These covalent interactions involve: (i) Schiff base formation, wherein amine groups from the polymeric macromolecule react with the oxidized catechol (*i.e.*, quinone), (ii) Michael-type addition, where amine groups couple to the benzyl, as a result of which a secondary amine is formed to bridge the polymer chains, and (iii) di-catechol formation, in which C-C bonds form between two quinone moieties.²⁹² Theoretical analyses have indicated that catecholic moieties can repel water molecules aside to facilitate their direct bonding to the underlying surfaces.³¹⁸ Note that the structure of the polymer backbone plays key roles in its binding affinity.³¹⁹ For instance, hydrophobic backbones are repelled from hydrophilic surfaces. Therefore, the catechol-modified hydrophobic backbones may fail to bind to the hydrophilic surfaces effectively. On the other hand, there should be a trade-off between hydrophilicity and solubility. High solubility in water is associated with lower underwater adhesion strength mainly due to the compromised cohesion when exposed to aqueous media.⁷

The adhesion of catechol-conjugated polymers is highly sensitive to parameters such as processing factors and whether the hydrogel is used as an injectable bioadhesive or in the form of a patch. The adhesion environment is another limiting factor.³¹⁹ For example, catechol-containing polyoxetane-based polymer that is crosslinked with FeCl₃ exhibited a strong adhesive strength to dry surfaces (5.6 MPa lap shear strength on a stainless steel surface); however, adhesion strength was much lower when placed on wet surfaces (0.41 MPa on a porcine skin).³²⁰

The charged units and hydrophobic residues in the secreted proteins of mussels have

motivated researchers to study their synergistic effects on adhesion.³²¹ A mussel-inspired bioadhesive consisting of zwitterionic surfactant molecules (having charged quaternary amine and phosphate groups) and hydrophobic alkyl tails was developed, as shown in Figure 3.11, to study the roles of different interactions in wet adhesion.¹⁰ In this study, similar composition ratios of charged residues, hydrophobic moieties, and catechol content as in the mussel foot proteins (mfp) were used to mimic mfp structures in synthetic polymers. Strong wet adhesion ($\sim 20 \text{ mJ/m}^2$) was attributed to the hydrophobic interactions between the alkyl tails as well as the electrostatic interaction through the phosphate and amine functionalities. In a similar approach, to mimic the catechol content and hydrophobic residues of mpf, copolymerization of styrene and 3,4-dimethoxystyrene followed by deprotection of methoxy groups (using BBr_3) led to an adhesive polymer (*i.e.*, poly[(3,4-dihydroxystyrene)-*co*-styrene]) that had a maximum lap shear adhesion strength of 11 MPa on dry aluminum plate surfaces. Although the developed material could not be used as a bioadhesive due to the presence of organic solvents,³²² the results demonstrated the importance of hydrophobic residues in adhesion strength. Similarly, a copolymer with an additional component containing positive charges was synthesized, which had lap shear adhesion strengths of 2.8 MPa (dry) and 0.4 MPa (wet), suggesting the role of positive charges in catechol adhesion.³²³

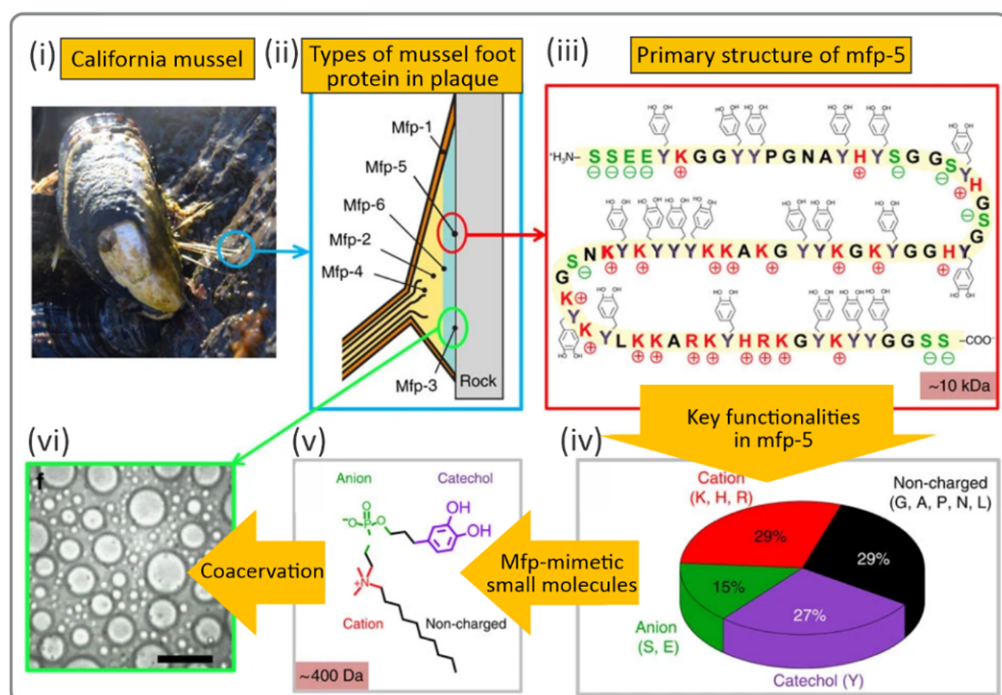


Figure 3.11. Mussel foot proteins (mfp)-inspired adhesive biomaterials. (i) A mussel anchored by plaques and threads to a rock. (ii) Illustration of the mussel adhesive protein distribution within a plaque. (iii) The primary sequence of the mussel foot protein (mfp)-5, and (iv) distribution of the functionalities in mfp-5. (v) A polymer design based on small molecules to mimic the functionalities inside mfp-5, and (vi) micrograph photo of the liquid phase-separated zwitterionic surfactant (scale bar = 200 μm). Reprinted by permission from ref 10. Nature Publishing Group, Copyright 2015.

3.3.4.3 Catechol functionalization of biomolecules for bioadhesion

A wide variety of methodologies has been implemented to graft catechol groups on different polymer backbones. These approaches are primarily based on the conjugation of catechol-carrying small molecules that possess nucleophile functional groups, and hence, can be coupled to biopolymer backbones. Some reagents carrying catechol groups used for polymer conjugation involve dopamine (conjugation through amine groups),^{306,324} 3,4-dihydroxyhydrocinnamic acid,³²⁵ caffeic acid (conjugation through carboxylic groups),³²⁶ and 3,4-dihydroxybenzaldehyde (conjugation through aldehyde groups).²¹

Various coupling chemistries are used to introduce catechol functionality to biopolymers. For instance, a catechol-coupled poly(vinyl pyrrolidone) (PVP) was synthesized *via* click chemistry.³¹⁹ The catechol-grafted polymer solution was later crosslinked with Fe³⁺. Interestingly, a high wet adhesion lap shear strength (1.13 MPa) was obtained (using glass slides), which was twice as high as the dry adhesion strength (0.56 MPa). The higher adhesion strength under wet conditions was explained by (i) easier Fe³⁺ ion diffusion compared to the dry condition, and (ii) the lower water solubility of the polymer as a result of catechol conjugation. In another study, low-cost and scalable synthesis of catechol-containing PVA-based dry adhesive materials was demonstrated *via* an acid-catalyzed acetal formation reaction. In that case, 3,4-dihydroxybenzaldehyde (DBA) molecules were linked chemically to the PVA molecules (in DMSO using *p*-toluenesulfonic acid (TsOH) as catalysts).³²⁷ This mixture was applied to the adherent surfaces and cured *via* solvent evaporation. The excellent lap shear adhesion strengths (as high as 17.5 MPa) obtained here were far higher than those of commercially available adhesives (*e.g.*, Krazy glue, 7.25 MPa).

A common approach to add catechol functionalities is to infuse catechol-carrying molecules within pre-crosslinked hydrogels (primarily for wound-closure patches). For example, a mixture of quaternary ammonium-modified chitosan and PEGDA was photocrosslinked and soaked in a TA solution to allow infusion within the hydrogel material.³²⁸ This process resulted in increased stretchability of the chitosan-based hydrogel from less than 200% to ~700% in addition to the significant improvement in adhesion (*i.e.*, with burst pressure up to ~60 mmHg). A similar TA infusion process for GelMA hydrogels was demonstrated by Liu *et al.*⁴¹ Tannic acid acted as a secondary network to toughen GelMA,

leading to a 4-fold increase in tensile strength. In addition, the swelling ratio was reduced by 1/3 and the adhesion strength in lap shear tests improved by more than 10× compared to GelMA (maximum adhesive strength was measured ~80 kPa).

3.3.4.3.1 Copolymerization with catechol-containing monomers

Copolymerization of monomers carrying catechol moieties is one of the most popular and the easiest approach to incorporate catechol groups within the hydrogel networks.³²⁹ As an example, methacrylated dopamine (DMA) molecules were oligomerized in an alkaline Tris-HCl solution (pH ~8).⁴² The DMA oligomers were copolymerized within the GelMA polymer to reduce the physical chain entanglement between GelMA macromolecules, which thereby enhanced the mechanical deformability and strain recoverability of the hydrogel. A temperature-responsive bioadhesive was obtained using a host-guest macromolecular configuration using slide-ring chemistry.³³⁰ A guest chain was synthesized by copolymerization of DMA molecules (responsible for adhesion) with adamantane motifs (acting as host receivers) and methoxyethyl acrylate (enabling hydrophobic interactions). The host molecule was based on PNIPAM copolymerized with β -cyclodextrin (hosting adamantane in the guest chain). Here, at temperatures lower than LCST (*i.e.*, 25 °C), water molecules easily infuse within the polymer chains, reducing adhesive strength; whereas, at higher temperatures, the hydrogen bonding between water molecules and PNIPAM breaks down, resulting in collapsed chain conformations and thereby exposing the catechol groups to adhere freely to the substrate. Underwater adhesion strength changed reversibly with temperature so that a 20-fold larger adhesive strength was measured at 40 °C compared to 25 °C. In another study, adhesion in a shear-thinning, injectable, and fit-to-shape sealant was demonstrated *via* copolymerization of DMA with maleic-modified chitosan (MCS),

dibenzaldehyde-terminated poly(ethylene glycol) (PEGDF), and PEGDA monomers (Figure 3.12A).⁸³ The shear-thinning properties were observed due to the reversible Schiff base formation between dibenzaldehyde-terminated poly(ethylene glycol) (PEGDF) and the chitosan backbone. The adhesion stability of the designed hydrogels even at pH~1 demonstrated the applicability of the material in acidic environments (such as the stomach). The lap shear test results indicated improved adhesive strength of 46 kPa, which was almost 8× greater than that of commercially available fibrin glue.

Copolymerization of catechol-functionalized PEG has also gained interest in creating mussel-inspired bioadhesives. Catechol-modified PEG chains were copolymerized in a solvent and initiator-free setup with polymer units that (i) promoted hydrophobic interactions and reduced the glass transition temperature, and (ii) enhanced cohesive strength through a UV light-sensitive side chain for crosslinking (Figure 3.12B,C).³³¹ In this study, by tuning the polymerization feeding ratio, a variety of pre-gel solutions with different viscosity levels were obtained. The adhesion increased for low viscosity polymers. Hydrophobic groups play key roles in achieving high levels of adhesive strength. The ester bonds present in the polymer backbone provided rapid biodegradation and biocompatibility. In a further study, a set of branched PEG-catechol was polymerized with strong cohesion and a thermo-responsive negative swelling.³³² Introducing the catechol moieties significantly improved the adhesive strength of the PEG-based polymers (with maximum lap shear strength of 209 kPa).

3.3.4.3.2 Carbodiimide coupling

Carbodiimide EDC/NHS chemistry is a standard coupling reaction between carboxyl and primary amines, implemented extensively to conjugate catechol-carrying moieties to

large biomacromolecules. The catechol loading capacity in this approach is limited by the available conjugation sites and depends on the reaction yield. For successful reaction and to achieve a maximum yield, it is important to control the pH of the conjugation process (pH~5.5-6). Dopamine molecules, containing amine groups, were conjugated to the carboxylic-rich HA at pH 5, resulting in 47% substitution.³⁰⁸ The synthesized catechol-functionalized HA polymer was then crosslinked chemically through covalent interactions between catechol and thiol-capped Pluronic crosslinkers. The adhesion strength for the modified HA-Pluronic hydrogels was improved by 150% compared to each of the constitutive components.³³³

A wide variety of natural biomolecules, including protein/peptides and polysaccharides, have been functionalized with catechol groups using EDC/NHS coupling. For instance, gelatin was coupled with caffeic acid and crosslinked using oxidative agents (*i.e.*, sodium meta-periodate) to form catechol-catechol crosslinks (Figure 3.12D).³³⁴ The synthesized hydrogel demonstrated enhanced wound healing, radical scavenging, antimicrobial, and antioxidant properties as compared to unmodified gelatin. In another study, dopamine was grafted to gelatin using EDC/NHS, followed by double-crosslinked with Fe³⁺ (catechol coordination), and genipin (a covalent crosslinker for gelatin) as illustrated in Figure 3.12E,F.⁴⁶ The adhesion of the material characterized by the lap shear test using porcine skin, showed an adhesive strength of 24.7 kPa (Figure 3.12G,H). Here, the bioadhesion was attributed to the infusion of the macromer solution into the skin.

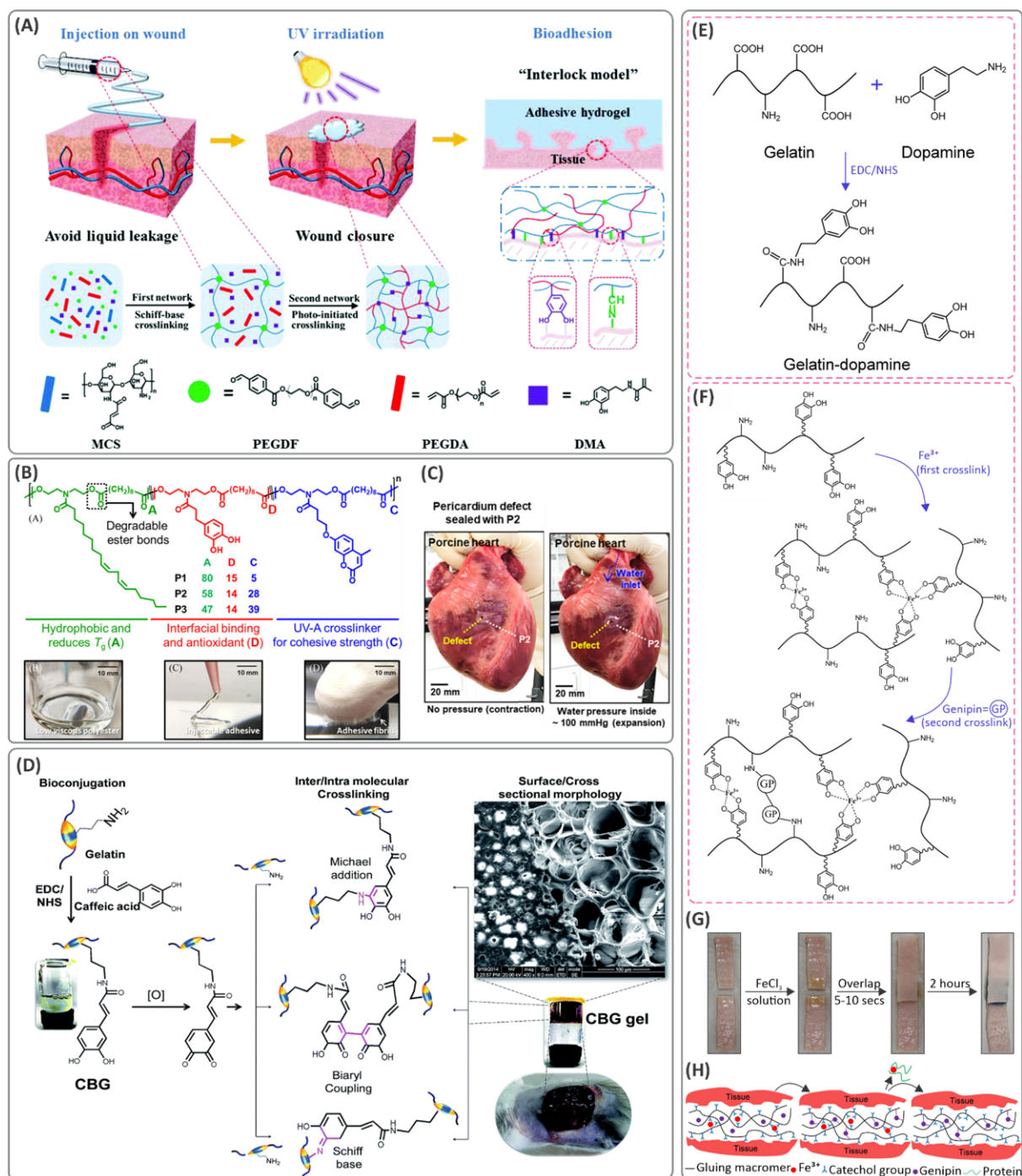


Figure 3.12. Examples of copolymerization of catechol-containing monomers, and conjugating catechol groups to the hydrogel polymer backbones for enhancing adhesion. (A) Schematic of the fit-to-shape photocurable sealants and underlying mechanisms of crosslinking and adhesion to the tissue in wound-closure applications. The hydrogel is composed of PEGDF, maleic-modified chitosan (MCS), dopamine methacrylate (DMA), and poly(ethylene glycol)diacrylate (PEGDA) components. Reproduced with permission from ref⁸³. Copyright 2019, Royal Society of Chemistry. (B) Molecular

design of statistical co-polymer adhesives consisting of hydrophobic, interfacial bonding region, and crosslinking portions. (C) Application of the hydrogel for sealing incision and defects on the myocardium tissue. Reprinted with permission from ref ³³¹. Copyright 2019 American Chemical Society. (D) Carbodiimide coupling of the caffeic acid to the gelatin backbone as a wound dressing material. The caffeic-acid-bioconjugated gel (CBG) was crosslinked through Michael addition, Biaryl coupling, as well as Schiff base formation mechanisms. Reproduced with permission from ref ³³⁴. Copyright 2015, Royal Society of Chemistry. (E) Chemical conjugation of the dopamine to the gelatin backbone using carbodiimide chemistry. (F) Mechanism of dual ionic and covalent crosslinking of the catechol conjugated gelatin using Fe³⁺ ions and genipin. (G) The lap shear adhesion test and (H) the molecular mechanisms of adhesion to the tissue substrates. Reproduced with permission from ref ⁴⁶. Copyright 2016, Elsevier.

3.3.4.3.3 Reductive amination

Different reductive agents such as NaBH₄ and NaCNBH₃ can be used to couple aldehyde-containing catechol derivatives, such as 3,4-dihydroxybenzaldehyde, to amine groups on the monomer backbone.³³⁵ This approach leads to high and controllable synthetic yield (substitution of 18-80% in less than 5 h).²⁸⁸ One example is the reaction between amine-functionalized star PEG and dextran aldehyde, wherein imine bonds between aldehyde and amine groups) drive the crosslinking process.³³⁶ To circumvent the detrimental effect of swelling on adhesion, Shazly *et al.* conjugated L-DOPA to a PEG-dextran network through a reductive amination process and optimized the biomaterial to minimize swelling of the hydrogel by tuning the concentration and thereby improved the durability of the wet adhesion.³³⁷ The *ex vivo* burst pressure tests with the L-DOPA-conjugated polymers on the intestinal puncture wounds did not reduce the adhesion strength, whereas 1 h soaking of neat (control) samples led to a 39% decrease in the burst strength.

3.3.4.3.4 Enzymatic coupling

With the aid of enzymes such as tyrosinase or laccase, one can catalyze the oxidization of catechol groups that are later reacted with nucleophiles. Amine groups bind chemically to the carbonyl groups of the quinones through the Schiff-base reaction and thereby form stable conjugates.⁹ Recently, a tyrosine-containing mfp-3S mimetic peptide with a similar protein composition was engineered with the aim of recapitulating natural mfp. Here, positive charges were introduced to enable coacervate formation due to electrostatic interactions as they occurred in natural mfp.³³⁸ Further, tyrosine was hydroxylated enzymatically to L-DOPA using mushroom tyrosinase to enhance adhesion by replacing surface-bound water molecules. Using a similar approach, the tyrosine residues of gelatin derived from the human adipose tissue were converted to L-DOPA to develop tissue-adhesive hydrogels.⁴⁴ Complexation crosslinks were formed by the addition of Fe^{3+} ions within seconds after mixing. The hydrogels were adhesive and stable at body temperature.

3.3.4.3.5 Catechol-based nanoparticles

Catechol-coated nanoparticles can improve adhesive and toughness in hydrogels simultaneously.^{339,340} Tough nanocomposite PAM-based hydrogels enabled by mesoporous silica nanoparticles (MSNs) and PDA additives were proposed as an example of bioadhesive patches for transdermal drug delivery.³⁴¹ This study revealed the critical roles of MSNs in leveraging hydrogel toughness and adhesion strength. Typically, the synthesis of catechol-coated nanoparticles (*e.g.*, coating with PDA) is carried out by dispersing nanoparticles in an alkaline solution in the presence of catechol-containing molecules (mostly dopamine).³⁴² Clay particles release ions in water and hence provide the alkaline conditions required for

catechol polymerization.³⁴³ In this case, polymerization occurs in the nanoscale space between the nanoclay sheets. In one study, dopamine molecules were intercalated into Hectorite nanoclay sheets and polymerized along with the clay particles.³⁴³ Then, the AAm monomers were added to the solution and polymerized along with the PDA-coated clay sheets. The hydrogels produced were tough and adhesive, with a maximum adhesion strength of over 30 kPa and toughness over 6000 J/m², which is significantly higher than that of the human skin (2000 J/m²). However, adhesion was reduced when the hydrogel was applied under wet conditions.

Catechol groups can be loaded onto different chemical moieties before being coated on the nanoparticle surface. For instance, dopamine-modified PEG interacts with Laponite® nanoclays through a combination of dynamic (dopamine-Laponite® reversible bonding *via* the hydroxyl groups) and covalent bonding (dopamine-dopamine covalent crosslinking), as shown in Figure 3.13A,B.³⁴⁴ Increasing the Laponite® content leads to higher hydrogel toughness. Further, PEG branching was associated with stronger burst pressure. Hydrogels were stretchable and adhesive, as shown in Figure 3.13C-E.

Carbon-based nanoparticles can also be coated with PDA to promote adhesion. In addition, such chemical modifications enhance the dispersibility of the carbon-based nanoparticles in the pre-gel solution by altering their surface properties/energy. Coating with PDA also deactivates the van der Waals interactions between the carbon nanomaterials, which are responsible for their agglomeration.⁸⁰ Recently, PDA-coated carbon nanotubes (CNTs) were embedded within the PAM-PAA hybrid hydrogels (Figure 3.13F). Polymerization was carried out in a binary solvent mixture containing water and glycerol to maintain hydrogel moisture in the long term. The synthesized hydrogels were introduced as

highly adhesive materials with a tensile adhesive strength of up to 57 kPa and characterized with long-lasting stable physical properties even at extremely cold and hot temperature ranges (-20 to 60 °C).⁸⁰ The high adhesion of the hydrogel is attributed to the chemical linkages with the tissue substrates through catechol moieties. Moreover, improved cohesion was related to the additional strong hydrogen bonds introduced by glycerol. In another approach, a pre-polymerized dopamine solution was used to reduce GO nanoparticles partially³⁴⁵ during the polymerization of AAm monomers. Apart from strong adhesion (~25 kPa tensile adhesive strength), the hydrogels exhibited electrical conductivity, self-healing properties, and high stretchability (up to ~37×). Similar properties were also reported for PDA-functionalized single-wall carbon nanotube (SWCNT)/PVA hydrogels.³⁴⁶ Apart from electrical conductivity and high self-healing efficiency of 99%, tensile adhesion strength of ~4.5 kPa was reported for the porcine skin substrates. Huang *et al.* incorporated PDA-coated rGO nanoparticles in a polymer matrix composed of poly(*N,N*-dimethylacrylamide-stat-3-acrylamidophenylboronic acid) (PDMA-stat-PAPBA), and poly(glycerol monomethacrylate) (PGMA).³⁴⁷ The PDA-modified rGO played important roles in both the crosslinker and the reinforcing filler. The addition of nanoparticle fillers showed significant improvements in the mechanical properties and adhesion to different substrates. Despite the improvements seen in the adhesion of hydrogels containing catechol-coated core/shell nanoparticles, some studies have reported conflicting trends of adhesion performance with these additives. For instance, PDA-coated silica particles embedded within a blend of PLGA and PEG failed to improve the burst pressure strength on the material, whereas the noncoated particles were associated with significant enhancements in adhesion.²³⁴ Chemical mechanisms and hydrogel system types are important factors that

should be considered for design of nanocomposite bioadhesives.

Metal nanoparticles are other candidates for modification with catecholic components. In one study, lignin-coated Ag nanoparticles were synthesized through the reduction of silver ions (Ag^+) by the phenolic and methoxy groups present on lignin molecules.³⁴⁸ The resulting core-shell nanoparticles were incorporated within a PAA hydrogel, where quinone functionalities enabled durable adhesion. Pectin molecules were introduced to this hydrogel to promote the mechanical properties and toughness (stretchability over 2500%) through the formation of the hydrogen bonds between the carboxyl and hydroxyl groups. The Ag-lignin hydrogels showed a tensile adhesive strength of 27 kPa, which was higher than that of the native PAA (17 kPa). The adhesion of the material was attributed hydrogen-bonding, metal complexation, hydrophobic interactions, and covalent-bonding interactions between catechol and tissue surfaces.

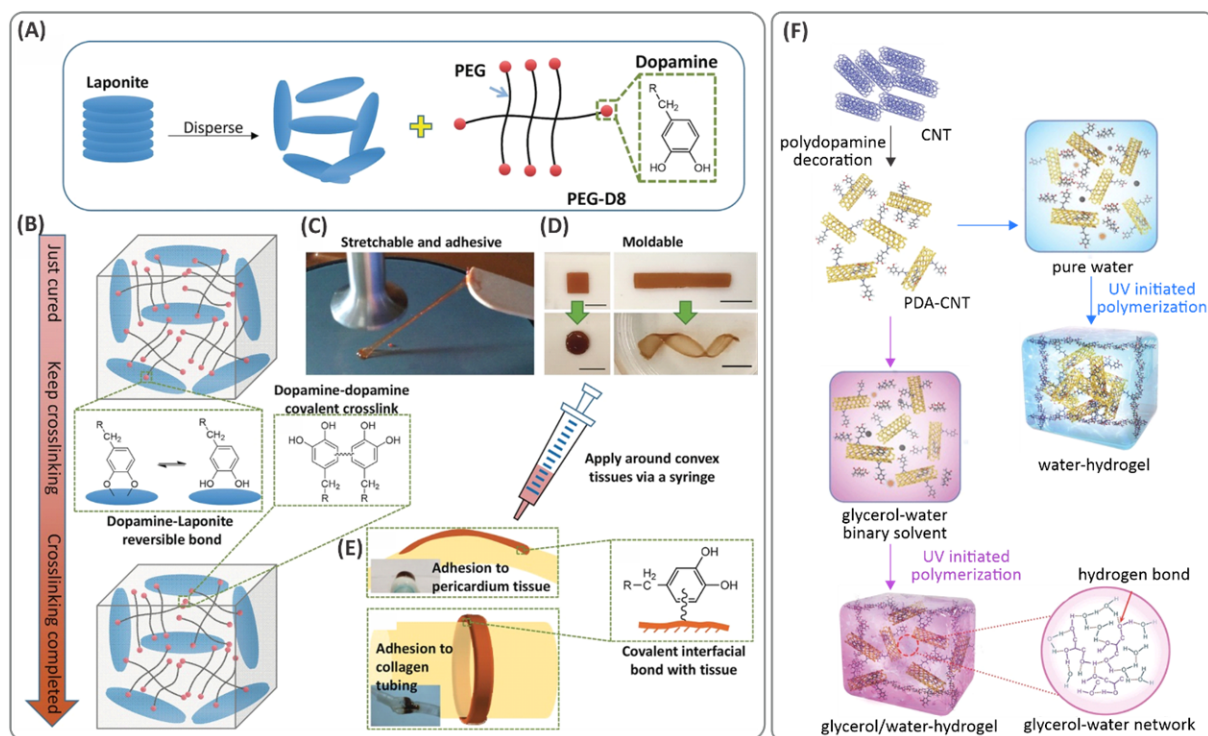


Figure 3.13. Coating nanoparticles with polydopamine (PDA) for nanocomposite (NC) hydrogel development and improvement in adhesion and cohesion. (A) Design and processing of adhesive nanocomposite hydrogels based on Laponite® nanoclays. Multi-arm poly(ethylene glycol) (PEG) was capped with the catechol groups. (B) Curing occurred *via* a mixture of dynamic interactions and covalent bonds between the catechol-functionalized PEG and Laponite®. (C) Reversible and loose crosslinking as well as adhesion through catechol groups, and (D) moldability of the cured hydrogels. (E) Application of the adhesive hydrogel to the convex pericardium and collagen tubing and the mechanism of covalent bonding explaining adhesion. Reproduced with permission from ref ³⁴⁴. Copyright 2017, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (F) Schematic of nanocomposite hydrogels based on PDA-coated carbon nanotubes (CNT). The UV-assisted crosslinking of the hydrogel network and internal strong hydrogen-bonding interactions between the polymer molecules led to gelation in the hydrogels. Reproduced with permission from ref ⁸⁰. Copyright 2018, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

3.4 Nature-inspired mechanical bioadhesives

Adhesion in hydrogels based on chemical and physical molecular attractions suffers from poor durability in wet environments, especially when subjected to various harsh

conditions due to the effects of pH and many other factors related to the media and chemical constituents. In addition, it is difficult to avoid toxic chemicals during synthesis of most bioadhesives. There are many examples of mechanical adhesion systems in nature that have inspired adhesive patches with engineered surface micro- nanostructure for on-demand application on wet tissues. Surface features of such adhesives mimic living organisms including octopus, gecko, tree frogs, clingfish,³⁴⁹ and mayfly larvae.³⁵⁰ Micro- and nanoscale surface features in these examples promote mechanically driven adhesion through physical mechanisms such as suction, capillary forces, mechanical interlocking, van der Waals attractions, and friction.³⁵¹ Below, we discuss adhesive patches developed to mimic adhesion in biological systems.

3.4.1 Octopus-inspired suction cup arrays

A cluster of suction cups present on the tentacles³⁵² of octopi enables adhesion to a variety of rough surfaces.³⁵³ The suction cups (a few-centimeters thick) contain an orifice (or acetabulum) and a dome-shaped protuberance that contracts to produce negative pressure at the tissue-surface interface. These architectures can be reproduced by filling an array of micro-holes partially on a silicon mold with a PU acrylate pre-polymer, as shown in Figure 3.14.³⁵⁴ During the casting process, the trapped bubbles inside the holes along with the circumferential capillary effects mimic the dome-shaped protuberance. An adhesive patch was developed where the backpressure activates the adhesion of the material to wet/dry surfaces with adhesion strengths proportional to the applied pressure. Peeling tests indicate adhesion forces as high as 170 kPa. Adhesion in these materials arise from a set of capillary actions, moving the trapped liquids inside the chamber. The cohesive attractions between the liquid molecules in the internal chambers originate from the compressive

elastic deformation of the pad. Following a similar concept, octopus-like micro tips were fabricated in micropillars by manipulating the meniscus of the pre-polymer in mold features. The results indicated the same order of magnitude peel-off adhesion strength (110 kPa) as reported in the previous study.³⁵⁵ Octopus-inspired adhesives have leveraged the application of smart artificial skins and flexible electronics. Adhesive patches are designed to monitor body temperature, activity, tremor, respiration, electrocardiogram (ECG), *etc.* continuously.³⁵⁶ The octopus-inspired adhesive pads exhibited durable conformations to match moving human joints during finger bending, as opposed to flat polydimethylsiloxane (PDMS) and “scotch tape.”³⁵⁷ Adhesion in the adhesive pads stems from the capillary interactions with the wet substrate due to the bottom wrinkles. These adhesive pads enable efficient sealing of heart and liver lacerations (Figure 3.15). Temperature-responsive adhesive pads were also developed by placing a PNIPAM hydrogel layer on the top of an elastomeric PDMS pad comprising an array of microcavities.³⁵⁸ Due to the thermal expansion of PDMS, adhesion was switched reversibly by changing the temperature between 22 and 61 °C. The highest normal adhesion strength was reported to be 94 kPa. In another work, pads with nanosuction cups were templated by monolayer non-close-packed silica colloidal crystals (250 nm diameter).³⁵⁹ The pads effectively adhered to wet tissues such as myocardium as well as microrough surfaces.

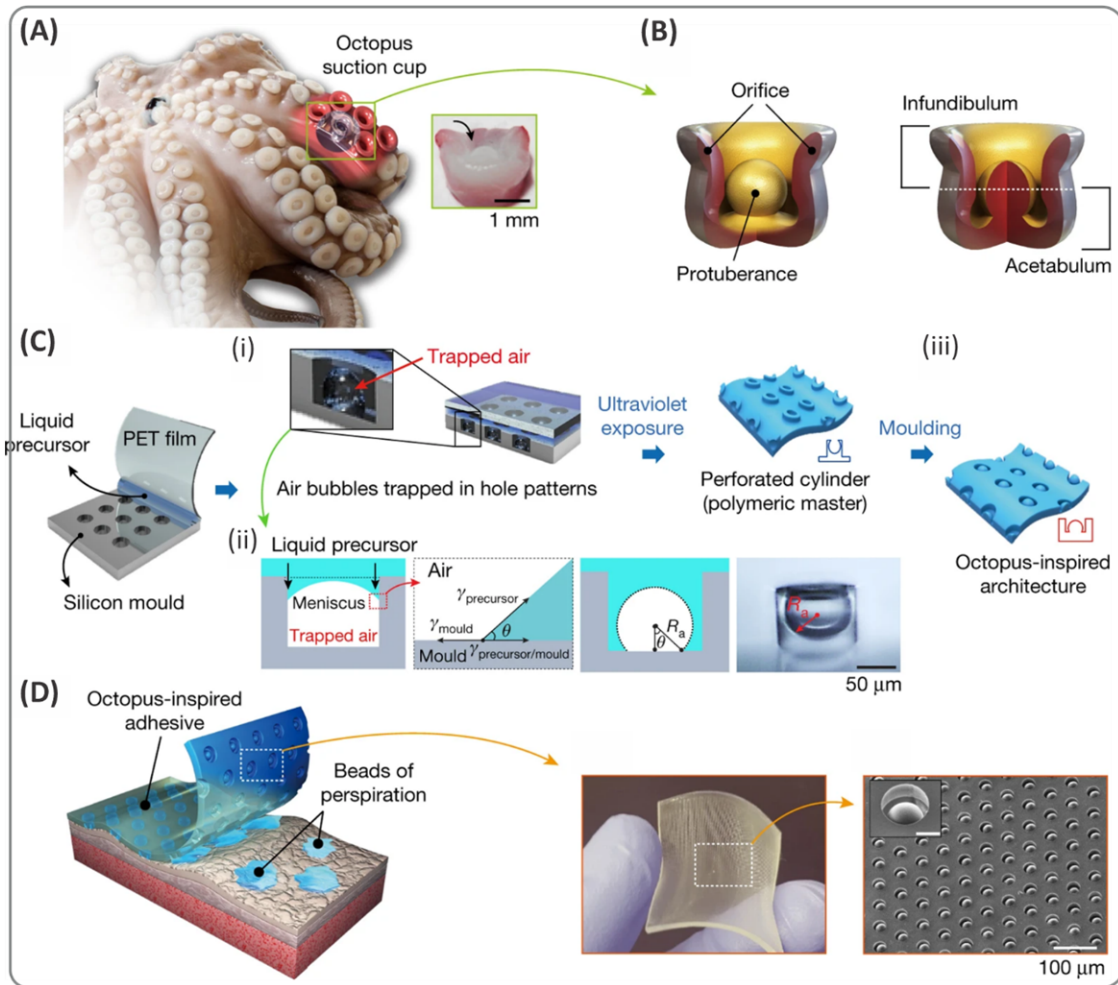


Figure 3.14. Illustration of octopus-inspired mechanical bioadhesives. (A) Anatomical structure of *O. vulgaris* tentacles. (B) Suction cups on the *O. vulgaris* tentacles. (C) The fabrication process of the octopus-inspired adhesive tapes. (D) Illustration of the attachment mechanism of adhesive layers with octopus suction-inspired surface architecture. Reprinted by permission from ref ³⁵⁴. Nature Publishing Group, Copyright 2017.

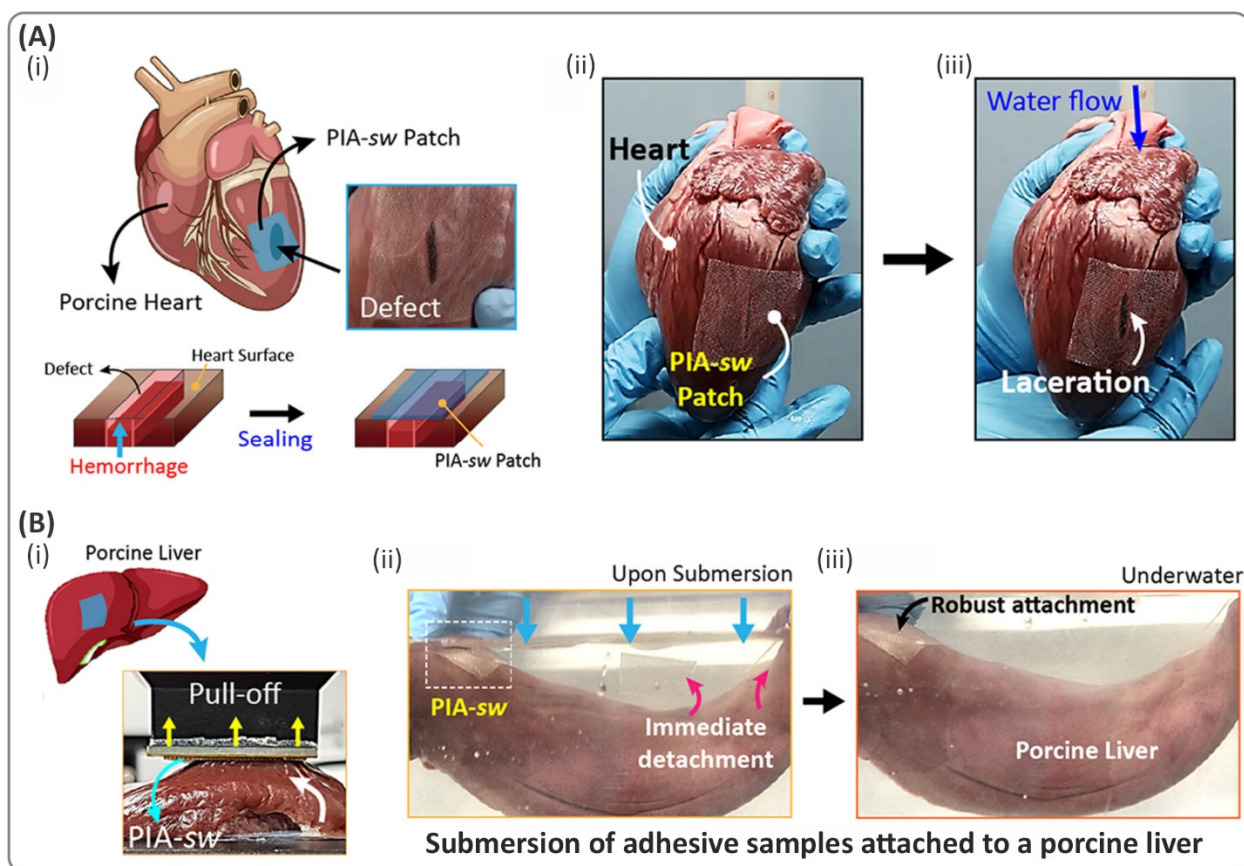


Figure 3.15. The application of organ-attachable wrinkled octopus-inspired adhesives for sealing incisions. Demonstration of sealing wounds in (A) heart and (B) liver tissues using protuberance-inspired architectures (PIA) with soft wrinkles (sw). Reprinted with permission from ref ³⁵⁷. Copyright 2019 American Chemical Society.

3.4.2 Tree-frog-inspired polygonal patterns

The 'soft' hexagonal arrays of the epithelial cells on the toe pads of tree frogs are surrounded by oriented 'hard' keratin nanofibrils. Due to this microstructural design, tree frogs adhere to dry/wet surfaces regardless of surface roughness. Such adhesion stems from the synergistic contributions of the capillary forces (enabled by secreted mucus), mechanical interlocking (friction), and van der Waals forces.³⁶⁰ This mechanism of adhesion has motivated the development of fabrication processes by incorporating hard/soft microphases

on the adhesive surfaces. A micropatterned surface consisting of PDMS (soft) micropillars was prepared recently in which polystyrene (PS) nanopillars (hard) were templated using anodic aluminum oxides.³⁶¹ Embedded PS pillar arrays enabled adhesion and friction to different surfaces. In another study, toe-like hexagonal micropatterns were inscribed inside the octopus-inspired convex suction cups using photolithography.³⁶² The rGO nanoparticles were coated on the microstructured surface to enable their application in flexible electronics. The adhesive pads had peeling strength on the order of ~ 0.8 N (maximum peeling energy was ~ 45 J/m²). The application of these pads as stretchable electrodes for long-term vital sign monitoring and real-time recording of the electrophysiologic patterns of heart motion was demonstrated for wearable devices. A comprehensive parametric study on the design factors was conducted to maximize the adhesion strength.³⁶³ It was found that elongated hexagonal patterns improve the anisotropic friction force due to the large pillar deformability as well as the edge density in the direction of sliding.

3.4.3 Gecko-inspired nanopillars

Gecko toes are decorated with millions of epidermally derived adhesive ~ 200 nm setae. This architecture enables fast switching of adhesion (within 15 ms) by rotating the setal shafts by 30° .³⁶⁴ Adhesion in gecko toes is driven mainly by van der Waals interactions.³⁶⁵ However, recent studies revealed the contributions of capillarity to adhesion due to the effects of humidity.³⁶⁶ A number of studies have been conducted on gecko-inspired adhesives for developing graspers in soft robotics applications.^{367,368} The fabrication of gecko-mimetic high-aspect-ratio structures is difficult and requires complicated engineering designs. In one study, a sacrificial template-mediated nanoimprinting approach was employed to develop gecko-inspired hierarchical arrays on flexible polycarbonate sheets

with the aid of micropillar and anodic aluminum oxide sacrificial templates.³⁶⁹ The shear strength of the adhesives was measured to be ~ 119 kPa, which decayed by 20% when subjected to a cyclic adhesion test over 200 cycles. In a later study, a microfabrication approach was implemented to fabricate 20 μm circular pillars on a PDMS surface.³⁷⁰ This study showed that surface roughness can significantly alter the adhesion strength due to changes in the contact area due to mechanical interlocking. A similar fabrication process was demonstrated to develop asymmetric-pillared architectures.³⁷¹ In this approach, gripping-direction switchable adhesive pads resulted in an adhesive force of 12.5 kPa (when actuated), which decreased to 3.4 kPa when deactivated. Patterned silicon nanowires were also used as a template to fabricate swellable nanofibrillar surfaces where the friction coefficient was augmented with the water uptake content for wet bioadhesion.³⁷² In another study, a simplified one-step fabrication scheme was introduced resulting in a chitosan film covered with nanofibril structures of 100-600 nm diameter and 70 nm height.³⁷³ The adhesive pad was laser-bonded to the tissue to establish full adhesion through van der Waals and electrostatic interactions, with a wet tissue adhesion strength of ~ 8 kPa. Recently, high-resolution 3D printing using two-photon polymerization was used to fabricate flexible springtail-gecko-inspired adhesive patches from acrylated urethanes.³⁷⁴ The patches consisted of pillar microstructures that repel liquids from the sides to establish wet adhesion. It was further demonstrated that modifying the pillars by adding octopus-inspired suction cups on the micropillars can significantly improve adhesion compared to those of patches with simple pillar architectures. A similar design concept was reported by Wang and Hensel in a recent study on PU-based patches architected with cupped micropillars.³⁷⁵ The patches were fabricated by two-photon lithography and demonstrated maximum peel-off

adhesive strengths of ~ 200 kPa, which was almost $7\times$ larger than that of micropillars without cups.

3.4.4 Needle-interlocking adhesives

Mechanical adhesives rely primarily on interlocking surface features patterned on the adhesive materials with the roughness of adherents. Animal parts such as the quills of North American porcupines (*Erethizon dorsatum*), where their backward-facing barbs result in strong adhesion of their stingers, have inspired microneedle array designs for efficient bioadhesion of patches to the tissues.³⁷⁶ To fabricate microneedle arrays with backward-curved barbs, a 4D printing approach was undertaken using digital light processing (DLP). A graded through-thickness crosslinking density was obtained in the synthetic barb structure *via* directional UV irradiation, which enabled backward bending after dissolving the uncured polymers in solvents. Pull-out tests demonstrated $\sim 3.5\times$ increases in adhesion force for the barbed microneedles compared to barbless microneedle patches.

Integration of interpenetrating interlocks and their swelling upon exposure to biofluids can be used to strengthen adhesion. Using a photolithographic approach, an array of swellable microhooks was patterned on a PEGDA hydrogel surface.³⁷⁷ The interlocking microhooks resulted in maximum adhesion strengths of 799 kPa after a 20 h swelling period, which was significantly (730%) greater than in dry conditions. In another study, microneedle patches with swellable needle tips (for interlocking adhesion) inspired by the adhesion mechanism in endoparasites were reported.¹⁷⁶ One way to create these surfaces is to copolymerize a layer of soft swellable PS-*b*-PAA block copolymers on top of a non-swellable stiff PS microneedle platform (Figure 3.16).³⁷⁸ Normal adhesion tests on the mucosal surfaces of intestinal tissue measured 45.3 kPa adhesion strength (compared to

9.3 kPa for dry skin), which is attributed to the strong chemical interactions between the material and mucin. This strong adhesion is attributed to swelling, which promotes interlocking with the tissue. In addition, the flexible backing material facilitated adhesion to mucosal surfaces. In a further study, double-layered microneedles with similar working mechanisms were designed with the soft swellable mussel-inspired protein phase integrated with a non-swelling SF on a microneedle core.¹⁷⁶ The *ex vivo* wound sealing of the microneedle in wet environments (18.6 kPa) was found to be comparable to sutured tissue controls (20.1 kPa).

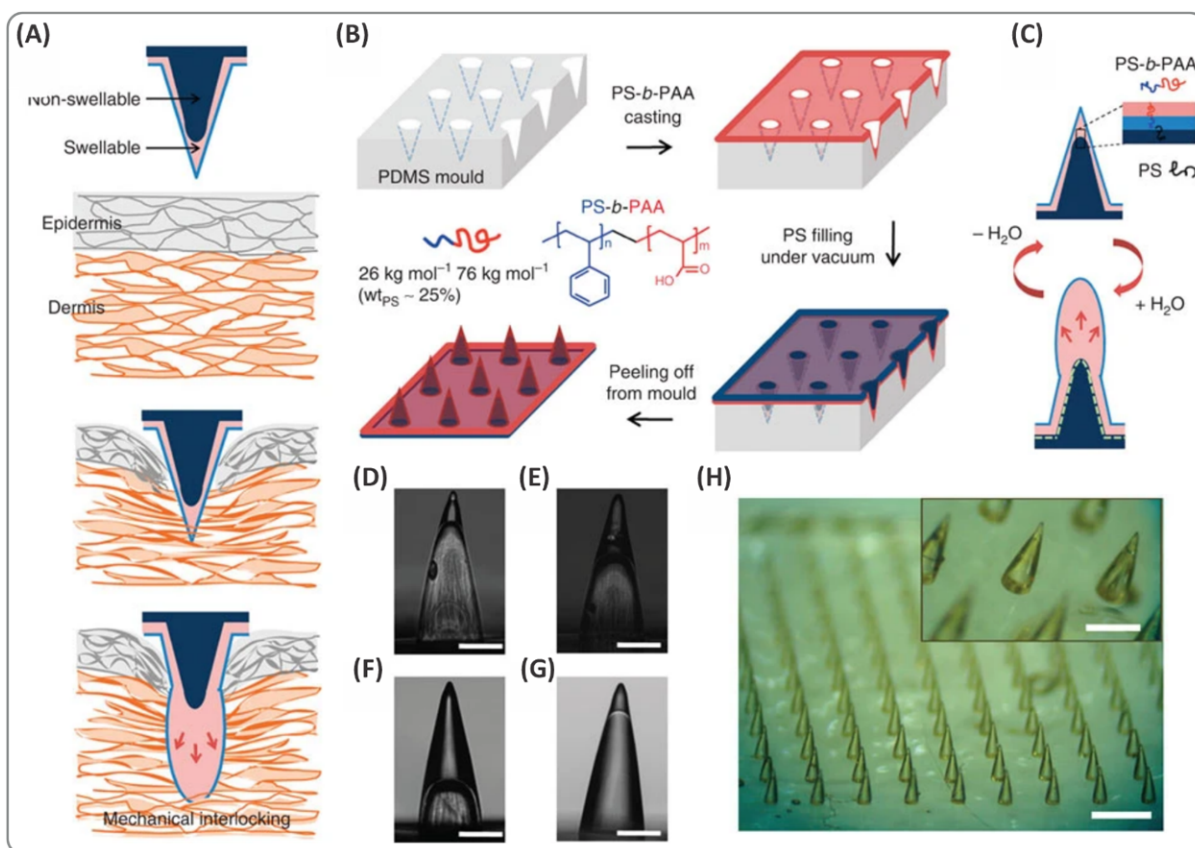


Figure 3.16. Swelling-mediated adhesive microneedles. (A) Illustration of mechanical interlock adhesion enabled by swelling of the needle tip in the microneedle patches. (B) The fabrication process of the microneedle array using a polydimethylsiloxane (PDMS) mold and double-layered deposition of stiff PS core and swellable polystyrene-block-poly(acrylic acid) (PS-*b*-PAA) coating. (C) The mechanisms showing the swelling effect on the improved interlock with the tissue. (D-H)

Magnified images of the fabricated needle tip on the microneedle arrays. Reprinted by permission from ref ³⁷⁸. Nature Publishing Group, Copyright 2013.

3.5 Conclusions and Prospects

The fast-paced progress made towards designing bioadhesives has paved the way for advancing sutureless wound-closure techniques. Hydrogels have demonstrated enormous potential in this regard; however, the state of research identifies the need for highly elastic and tough bioadhesives before they can be brought to the market on larger scales. Tough bioadhesion is a result of synergistic contribution of cohesion and adhesion. While the lack of sufficient cohesion entails poor tissue adhesion, strong cohesion does not necessarily imply tough bioadhesion. Hence, a rational bioadhesive design begins with engineering of cohesive forces followed by integration of adhesive functionality in a closed-loop optimization process. The interrelations between adhesion and cohesion have been ignored and need further attention. For instance, introducing adhesion *via* catechol functionality (which acts as radical scavengers) in photocrosslinkable injectable bioadhesives can counteract polymerization reaction thereby impairing cohesion. Bioadhesive requirements differ from tissue to tissue, most studies thus far have focused on designing bioadhesives for soft tissues (such as liver, heart, lung, and skin). Other tissues such as bone and nerves need further attention.

In terms of intrinsic mechanical properties, improvements in elasticity and toughness are necessary. Such properties have been explored primarily by introducing dynamic interactions to covalent hydrogel networks. Stretchability in hydrogels is achieved mainly by synthetic hydrogels that express intrinsic stretchability such as PAM and PAA. More efforts

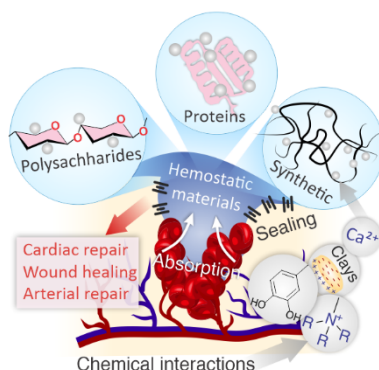
towards natural polymers such as gelatin derivatives, which are more common in biological settings, are in demand. Characterization of toughness and stretchability in hydrogels is measured by tensile tests, whereas other mechanical aspects, such as fatigue and impact performance, are neglected largely. Given the dynamic loading required, gaining a deeper understanding of the cyclic response of such hydrogels is essential for evaluating their use as biomedical sealants. The lack of standardized methods used for measuring adhesion presents further obstacles in comparing observations reported in different studies.

Wet adhesion in hydrogels is a primary challenge. The main approaches undertaken to attain adhesion include mechanical interlocks with the uneven surface of tissues and covalent and noncovalent bonds. One goal of the ongoing research is to move the adhesive strength of the natural hydrogels to levels comparable to strong synthetic hydrogels to circumvent the biocompatibility and biodegradability issues. Despite the advances made thus far, there are major aspects of bioadhesive performance that need to be understood. First, understanding the time-dependent evolution of adhesion is necessary. Water content in the hydrogels varies over time, with significant consequences in terms of dry *versus* wet conditions and at different humidity levels. Environmental conditions such as local pH differ in organs (such as the stomach or lungs) and therefore need to be addressed in testing processes. In addition, due to the dynamic deformations in most organs inside the body, more attention needs to be paid to the characterization of adhesion under cyclic loads.

Future work is also encouraged in adding electrical conductivity to bioadhesives to enable applications as nerve glues and heart sealants. We imagine embedded sensing elements that can report the properties of the bioadhesive materials in action, both for scientific understanding and also as diagnostics of the state of the tissue repair. Stimuli-

responsive and smart adhesives (such as those of thermoresponsive hydrogels having LSCT temperature between room and body temperature) are attractive candidates for the development of injectable sealants that solidify at body temperature. A large number of studies have revealed the potential brought to the field by the mussel-inspired bioadhesives introducing catechol groups into different hydrogel systems. However, their adhesion still fails to mimic the strong adhesion in mussels since multiple complex aspects are yet to be recapitulated in synthetic and hybrid materials designs. To address the high sensitivity of adhesion to environmental and testing parameters, more robust and controllable testing protocols need to be developed for better consistency in the analyses of these materials and comparisons of results between laboratories.

CHAPTER 4: ENGINEERED HEMOSTATIC BIOMATERIALS FOR SEALING WOUNDS



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Abstract

Hemostatic biomaterials show great promise in wound control in the treatment of uncontrolled bleeding associated with damaged tissues, traumatic wounds, and surgical incisions. A surge of interest has been directed at boosting hemostatic properties of bioactive materials *via* mechanisms triggering the coagulation cascade. A wide variety of biocompatible and biodegradable materials have been applied to the design of hemostatic platforms for rapid blood coagulation. Recent trends in the design of hemostatic agents emphasize chemical conjugation of charged moieties to biomacromolecules, physical

incorporation of blood-coagulating agents in biomaterials systems, as well as super-absorbing materials in either dry (foams) or wet (hydrogel) states. In addition, tough bioadhesives are emerging for efficient and physical sealing of incisions. In this chapter, we highlight the bio-macromolecular design approaches adopted to develop hemostatic bioactive materials. We discuss the mechanistic pathways of hemostasis along with the current standard experimental procedures for characterization of the hemostasis efficacy. Finally, we discuss the potential for clinical translation of hemostatic technologies, future trends, and research opportunities in terms of the development of next-generation surgical materials with hemostatic properties for wound management.

4.1 Introduction

Excessive bleeding and hemorrhage present major challenges to and burdens on the healthcare system.³⁷⁹ Many underlying conditions cause bleeding-related morbidity and mortality. The reported death rate associated with trauma is ~30-40%.³⁸⁰ In cardiac surgery, as well as percutaneous coronary interventions, 7% hemorrhaging has been reported.³⁸⁰ The mortality rate in elective vascular patients is up to 20%.³⁸⁰ Current techniques to treat surgical and trauma wounds, including compression of open wounds with absorbable textiles, fail to stop heavy bleeding efficiently.³⁸¹ To prevent complications associated with bleeding, current surgical toolboxes offer solutions for wound management based on hemostatic gauzes, tissue adhesives, and sealants.³⁸² Surgical materials promote blood coagulation using hemostatic biomaterials that can also provide adhesion to tissue surfaces.²³⁴ Apart from bleeding wounds, hemostatic materials could provide improved treatment strategies for vascular hemorrhage *via* endovascular embolization, where blood

vessels are filled with a biomaterial to avoid tissue rupture. The current standard coil embolization method involves the insertion of a coil-shaped metal wire to induce blood clotting, which is inefficient in the case of patients suffering from coagulopathic diseases.³⁸³ It is of the utmost importance to understand the hemostasis mechanisms and suitable materials platforms for the design of functional hemostatic biomaterials.

Hemostasis can be achieved through different mechanisms including: (i) strong physical adhesion to the tissue, (ii) chemical (such as electrostatic) interactions and bonding with coagulation factors, and (iii) absorption of plasma. Physical sealing of bleeding wounds with strong bioadhesives²⁴ without the need to trigger the coagulation cascade is one of the highly favored approaches for hemostasis.^{384,385} Hemorrhage control using bioadhesives may lower the risk of thrombus in blood veins. However, most of the strongest bioadhesives are made of synthetic polymers. Synthetic tissue adhesives such as cyanoacrylates, are prone to cytotoxicity and triggering immune responses.^{386,387} Other drawbacks and complications reported for the cyanoacrylate-based adhesives involve exothermic polymerization,^{388,389} which leads to tissue burn, high stiffness³⁹⁰ (mismatching the soft skin or other tissue), and poor resistance to dynamic deformation.³⁹¹ Natural bioadhesives are less prone to cytotoxicity and immune response. However, examples such as fibrinogen bioadhesives suffer from limited donor sources and pose the risk of viral transmission.^{379,392}

In addition to physical sealing, hemostatic properties can be achieved by introducing hemostatic agents within the hydrogel matrices to trigger the blood coagulation cascade and accelerate blood clot formation.³⁸¹ One example of this approach involves adding charged functionalities and additives such as nanoclays that trigger the coagulation cascade.^{393,394} Incorporation of such hemostatic agents, however, raises the risk of release into the vascular

system, which can result in the uncontrolled formation of blood clots in distant blood vessels.³⁹⁵ Therefore, it is essential to employ materials design strategies to avoid unwanted systematic release into circulatory system.

Fluid absorption is another driving factor to establish hemostasis in wound control. As a traditional example, gauzes are extensively used as wound dressings to treat bleeding wounds, due to their high absorptivity. The use of gauzes, however, is limited to topical wounds and in many cases, they lead to unwanted adhesion to the wound site. Therefore, gauzes are associated with delayed wound healing compared to their hydrogel counterparts. Swellable hydrogels can absorb blood plasma to concentrate blood clotting factors locally and to trigger the coagulation process.³⁹⁶ Injectable hydrogels provide versatile platforms for chemical functionalization and mechanical tunability.³⁹⁷ However, the blood clotting timescale for dry wound dressings is generally shorter than hydrogels.^{398,399} As a result, ongoing research seeks effective biomaterials design strategies for bleeding management using injectable hydrogels with particular attention to their wet tissue adhesive properties.

This chapter provides insights into designing novel hemostatic materials, with a focus on the selection of materials, their syntheses, and the mechanisms by which they trigger coagulation (Figure 4.1). We include in-depth discussions of the materials and methods used in hemostasis and in introducing hemostatic properties into biomaterials. First, we provide a brief overview of the natural coagulation cascade and experimental methods related to the measurement of the hemostatic properties. Then, we describe the materials and their corresponding molecular mechanisms for blood coagulation. We introduce the commercially available hemostatic agents along with their limitations and discuss their hemostatic applications in surgical settings. Finally, we describe the challenges and opportunities ahead

for further research in hemostatic wound control.

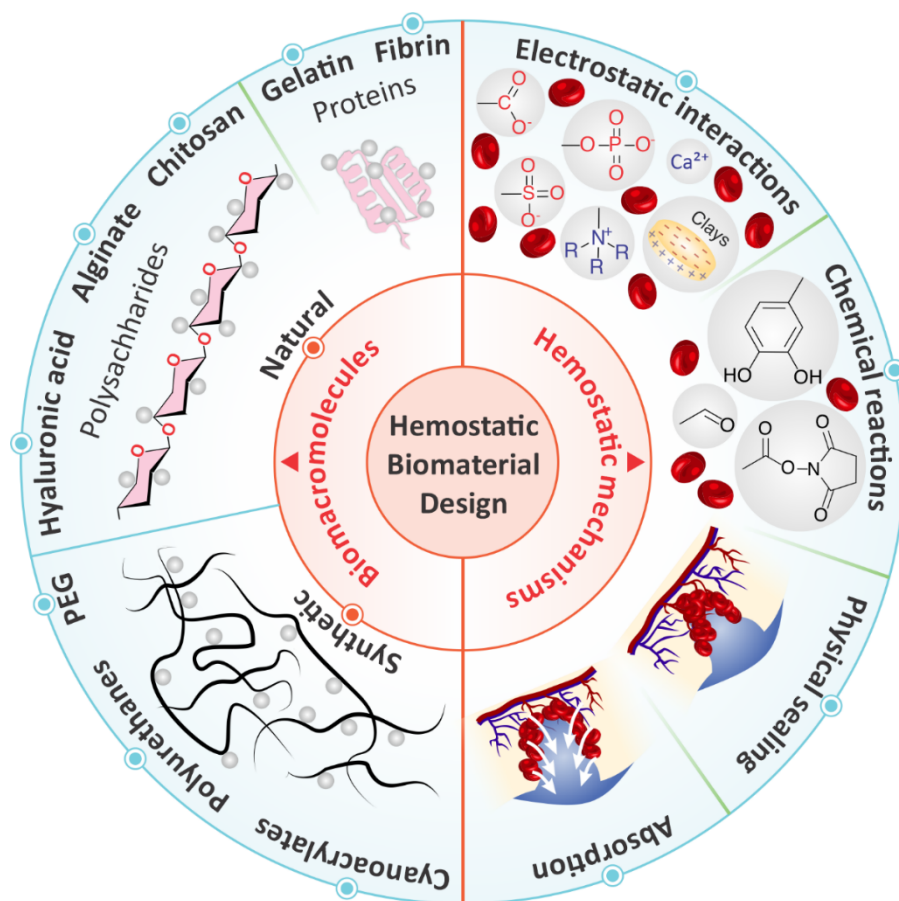


Figure 4.1. The process of hemostatic biomaterials design. Natural and synthetic macromolecular backbones have intrinsic hemostatic properties. They can also be functionalized with hemostatic agents/functional groups (gray circles). Hemostatic properties can be established *via* active mechanisms, where chemical interactions and reactions with blood components can trigger the coagulation cascade. Hemostasis can also be achieved by physical adhesion and the sealing properties of adhesive hydrogels as well as by body fluid uptake in hemostatic absorbents, which concentrate blood components.

4.2 Fundamentals of hemostasis

4.2.1 Coagulation cascade

Blood clot formation is a spontaneous and natural response of the body during bleeding.⁴⁰⁰ This process starts with initial hemostatic activation (primary hemostasis) where platelets aggregate at the bleeding site to form a platelet plug. Subsequently, as represented in Figure 4.2, coagulation factors are released to augment blood clot adherence to the subendothelial matrix (secondary hemostasis). At this stage, both intrinsic and extrinsic coagulation pathways are collaboratively activated to form the primary platelet plug. The extrinsic pathway is triggered by exposure to tissue factors, due to vascular damages or trauma, where factor VII is first activated in the presence of calcium ions.⁴⁰¹ The intrinsic pathway is triggered through the activation of factor XII due to surface damage through negative charges present on the damaged tissue surface. Both pathways follow a complex series of chemical activation of the coagulation factors that eventually merge to activate the factor X. Finally, the activated factor X cleaves prothrombin into thrombin, which turns fibrinogen into fibrin and initiates fibrin polymerization at the injury site while calcium ions and factor XIII precipitate to reinforce the platelet plug. Sometimes, this process fails to stop the bleeding in patients with regeneration disabilities, *e.g.*, due to the aging process⁴⁰² and hemophilia,⁴⁰³ or infections, such as those reported in COVID-19 patients.⁴⁰⁴ As a result, an external agent is sometimes required to control bleeding and to aid the healing process.

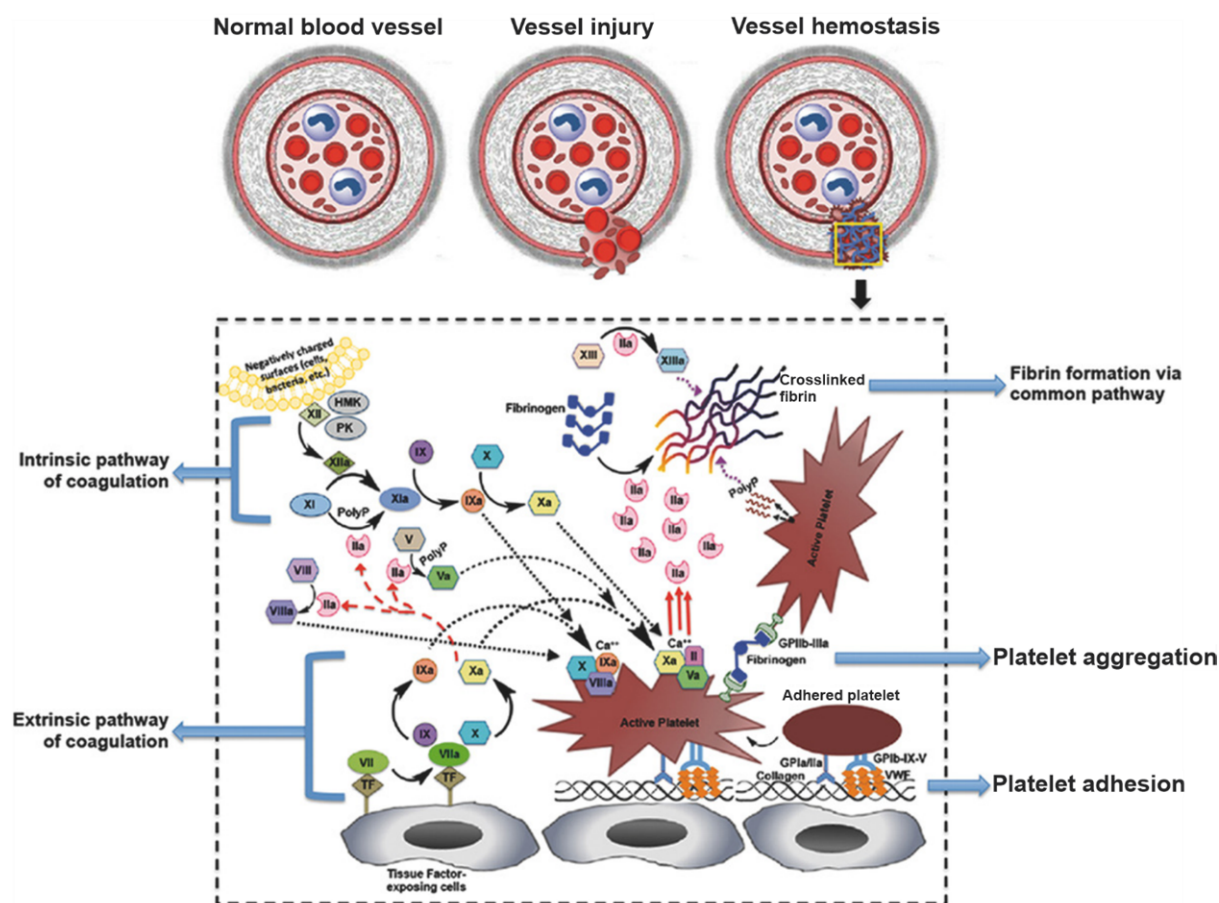


Figure 4.2. Schematic representation of the complex blood coagulation cascade. Upon the formation of an injury to blood vessels, the coagulation cascade is triggered by activation of the intrinsic and extrinsic pathways of coagulation. Damaged tissue exposes tissue factor to the blood that leads to activation of factor VII and platelets. This process causes sequential activation of the other factors that eventually result in the polymerization of fibrin initiated by the activated thrombin in the blood and finally aggregation and adhesion of platelets at the site of injury. Reproduced with permission.³⁷⁹ Copyright 2017, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.2.2 Mechanisms of action in hemostatic materials

Hemostatic materials actively influence either the initial or secondary blood clotting process (active hemostasis) as shown in Figure 4.1. Active hemostatic materials contribute to: (i) the coagulation cascade steps for activation of platelets, (ii) the activation of

coagulation factors, and/or (iii) aggregation of platelets and red blood cells (RBCs). Hemostasis is mainly achieved by using physical and chemical interactions established between the hemostatic material and these biomolecules.³⁸¹ Physical hemostasis can be achieved by rapidly and securely sealing off the bleeding before clot formation occurs (passive hemostasis).¹⁷⁹

Platelet adhesion/aggregation can be triggered through different material properties including hydrophobicity, crystallinity, and most commonly, surface charge and energy.^{379,405,406} Activation of platelets increases with negative charge density, such as with urethanes and inorganic metal oxides having negative charges on their surface (*e.g.*, silica). As an example, the negative charges of alginate biomolecules activate factor XII and can potentially promote fast blood clotting.⁴⁰⁷ Cationic charges also promote hemostatic activity (*e.g.*, non-sterically hindered tertiary amines)³⁸¹ by adhering to platelets. For instance, positive charges on chitosan (CS) macromolecules cause platelet adhesion through charge interactions.⁴⁰⁸ Releasing clotting factors is another means to achieve hemostasis. We note that active hemostasis is susceptible to systemic thromboses or emboli.⁴⁰⁵ Implementation of a passive mechanism of coagulation through surface modification with proteins and platelets is more useful and efficient for hemostatic applications.⁴⁰⁹ Chemical bonding with protein molecules, participating in the coagulation process, can be used to bind platelets or RBCs, or to activate coagulation factors. For example, oxidization of polysaccharides yields aldehyde groups; these aldehyde groups can chemically bind to the free amino groups present on proteins and cells in blood (leading to reactions such as Schiff base formation) to trigger coagulation.⁴¹⁰

Swelling of hydrogels can interfere with mechanical adhesion.⁷ However, swelling can

accelerate the formation of the blood clot through the concentration of the RBCs and blood coagulating factors as a result of blood plasma absorption.⁴¹¹ Plasma absorption is also the basic mechanism of action for mineral additives, such as silicates, alongside the abovementioned electrostatic contributions. Conventional cotton gauzes trigger blood coagulation by wicking of blood plasma fluid due to their hydrophilic porous structure.⁴¹² Excess blood absorption by medical gauzes however, is associated with significant blood loss and morbidity, and therefore, a balance should be made between hydrophilicity and hydrophobicity (*e.g.*, by introducing long alkyl chains onto the textile surfaces⁴¹²).

4.2.3 Experimental approaches for characterization of hemostatic materials

A diverse range of experimental methods has been used to characterize different aspects of the hemostatic properties in biomaterials. These aspects include cytotoxicity studies, hemostatic performance, physical characterizations (*e.g.*, adhesion, swelling, mechanical, and rheological properties), and studies to understand hemostasis mechanism.

As a part of cytotoxicity studies in addition to the standard live/dead assays, hemocompatibility is one of the basic assessments wherein the percentage of hemolysis is quantified. In this test, a diluted suspension of RBCs is exposed to the biomaterial, and following an incubation period, absorbance values (*OD*) are measured using a plate reader. Taking saline and Triton-X 100 solutions as negative and positive controls, respectively, the hemolysis percentage can be calculated using Eq. (1):⁴¹³

$$Hemolysis = \frac{OD_{sample} - OD_{negative}}{OD_{positive} - OD_{negative}} \quad (1)$$

A wide variety of testing procedures has been implemented to assess hemostatic

properties, from which suitable methodologies are selected based on the targeted form and application of hemostatic materials. Blood clotting assays can be used to quantify the time required for blood clot formation and to provide information on the hemostatic mechanisms. Blood clotting time is measured as the time when the blood and hemostatic material do not flow if both are tilted or flipped in the vial.⁴¹⁴ Real-time monitoring of blood clot formation is feasible by rheological measurements under oscillatory modes⁴¹⁵ as well as measuring the changes in the electrical conductivity of the blood.⁴¹⁶ One way to perform blood clotting assays is to expose the hemostatic biomaterial to a fresh whole blood source (which can be drawn from either human or animal sources) for a predetermined period to hemolyze the free erythrocytes. The hemostatic effect of the material can be measured by the absorbance of hemoglobin at a wavelength of 540 nm.^{417,418} A second approach is related to the ability of the material to induce thrombus by placing the biomaterial into well plates and adding the whole blood on top.⁴¹⁹ Then, at certain time increments, the blood is diluted and its absorbance is measured at 540 nm. The blood clotting index (BCI) is defined to describe the hemostasis effect according to:⁴²⁰

$$BCI = \frac{A}{B}, \quad (2)$$

where A and B are the absorbance of the blood after exposure to the sample and the citrated blood at 540 nm, respectively. The lower the BCI, the higher the blood coagulation activity. Platelet adhesion can be demonstrated to show hemostatic effect by exposing the hemostatic material to the extracted platelets from whole blood for a specified period. After that, the platelet-treated samples are washed, and the adhered platelets are subsequently lysed using Triton-X 100.⁴¹⁷ Collected platelets are then seeded to measure their lactate dehydrogenase activity at 450 nm. Platelet adhesion has also been characterized by scanning electron

microscopy (SEM) studies with the platelets adhered to the hydrogel material.⁴²⁰ Initially, the material is treated with the platelets extracted from the whole blood and cultured on the surface of the materials in an incubator. Later, the treated surfaces are washed to remove the non-adherent cells, and then, the released lactate dehydrogenase (LDH) is measured. Finally, the cells can be fixed for SEM imaging and visualization purposes. Protein absorption is another assay of thrombotic property, and can be measured through a batch contact method.⁴²¹ This assay also gives valuable information on the degree of intrinsic thrombosis and hemocompatibility of materials.⁴²² Samples are normally soaked in phosphate-buffered saline (PBS), allowing them to swell; then, they are soaked in a bovine serum albumin (BSA) solution.⁴²¹ Subsequently, the supernatants are analyzed using the absorbance at 280 nm to measure the residual BSA in the solution. The absorbed protein is calculated using Eq. (3):⁴²¹

$$Adsorbed\ BSA = \frac{C_0 - C_a}{w} V, \quad (3)$$

where w is the weight of swollen hydrogels, C_0 and C_a are the BSA concentrations before and after adsorption, respectively, and V is the volume of the BSA solution. Higher antithrombic effects are associated with lower BSA absorption values that correspond to greater BCI and lower levels of hemolysis.⁴²³ The amount of generated thrombin⁴¹⁷ as well as the fibrinogen adhesion is also measurable using the enzyme-linked immunosorbent assay (ELISA) method. For the former, thrombin generation is stopped using a sodium citrate solution after the sample is immersed in whole blood for a specified period. For the latter, the samples are placed in horseradish peroxidase (HRP)-conjugated goat antihuman fibrinogen to undergo an enzymatic reaction. Then, a stop solution is used at specific timepoints, ceasing the reaction; absorbance at 450 nm (OD) measures the human fibrinogen (Hfg) from Eq. (4):⁴²⁰

$$Adsorbed\ Hfg = 4.154 \times 10^{-3} OD + 4.404 \times 10^{-5} . \quad (4)$$

Care should be taken when choosing timepoint-based *in vitro* models as the lag time due to sampling processes may cause inconsistent blood exposure times when running multiple samples together. Furthermore, donor to donor variation of blood sources used for the current *in vitro* studies can be a major source of error leading to non-robust results, which should be carefully addressed in the experimental procedures. In general, the diversity of hemostatic tests and the lack of standardized methods have made it difficult to compare different studies in the literature.

Similarly, *in vivo* tests can be implemented for testing and understanding material hemostatic behavior. The bleeding experiment is a popular approach where the amount of blood collected after making an incision (into *e.g.*, the liver or an artery), followed by treating with the hemostatic material.²³² Evaluation of hemostatic properties *in vivo* is mostly limited to tissue incision models. Further expansion of the hemostatic characterizations on the animal disease models⁴²⁴ with hemostasis disabilities such as diabetes is required to elucidate hemostatic function under abnormal conditions.

Various physical characterizations have been implemented to quantify other aspects of hemostatic properties. For instance, swelling tests in the presence of water/blood media can elucidate the contributions of absorption towards the hemostatic behavior of the materials. Various adhesion tests can be defined to evaluate the capacity of biomaterials to seal open wounds. Furthermore, mechanical and rheological tests are essential to ensure sufficient cohesion, and matching with the target tissue.

4.3 Hemostatic materials and reagents

As described above, the physical and chemical properties of materials can be modified to add hemostatic properties to the materials. Although some hydrogels intrinsically encourage blood coagulation based on the chemistry of the constituent molecules, additives can also be used to tune their interactions with blood. To introduce such interactions, natural hemostats and synthetic materials have been engineered using different methods and processed through various fabrication procedures to shape them in specific forms. In general, hemostatic materials can be found in the form of injectable hydrogels, wound dressing patches, or particles.⁹⁶ Injectable hydrogels are based on *in situ* crosslinking of pre-polymer solutions upon application to the wound site,⁴²⁵ which enable filling complex-shaped voids, full coverage of wounds, and integration with minimally invasive surgeries.⁹⁶ Hydrogel beads also have shown the capacity for injectability⁴²⁶ and demonstrated potential for hemostatic applications.^{414,427,428} Fast gelation of injectable hydrogels is of great importance to seal the bleeding area physically.^{273,429-431} Wound dressings can be designed in the form of wet hydrogels,⁷² dry patches,¹⁹¹ particles.^{432,433} Hemostasis in wet hydrogels is mostly based on chemical interactions with blood plasma, whereas dry patches, in the form of membranes,¹⁹³ microneedle arrays,^{434,435} electrospun mats,⁴³⁶ textiles,⁴³⁷ sponges,⁴³⁸ cryogels,⁷³ as well as aerogels⁴³⁹ can promote hemostasis through both plasma absorption and interactions with blood plasma components similar to hemostatic particles. Solutions of hemostatic particles can be sprayed for easier application on the wound site.²³⁴ In the following sections, different chemical approaches and fabrication procedures implemented to develop hemostatic materials are discussed.

4.3.1 Natural hemostatic materials

Natural polymers can be classified into polysaccharide-based and protein- or peptide-based materials among which fibrinogen has received particular attention. Next, recent advances in designing natural hemostatic materials are reviewed.

4.3.1.1 Proteins and peptides

Peptide and protein molecules consist of amino acid units with different combinations of electrostatic charges and hydrophobicity, enabling their physical entanglements⁴⁴⁰ and chemical interactions⁴⁴¹ with platelets and blood cells for hemostasis. For instance, β -sheet amphiphilic peptides designed with various net charges showed that positively charged peptides could immediately coagulate platelet-poor plasma (PPP) whereas those of negatively charged retarded coagulation process.⁴⁴¹ One example of a peptide-based hemostatic material is RADA16-I, an ionic nanopeptide, comprising an alternating hydrophobic and hydrophilic amino acid sequence with both positively and negatively charged residues.^{442,443} The RADA16-I forms a hydrogel with a structured nanofibrous architecture under physiological conditions, which can entrap blood cells *in situ* and stop bleeding.⁴⁴⁰ The application of such nanopeptides for the prevention of bleeding from parenchyma wounds has been demonstrated in liver, spinal cord, brain, and femoral artery models.⁴⁴⁴ The hemostatic behavior of RADA16-I has also been studied for the ilium rabbit bone defect model. The advantages of using RADA16-I as compared with bone wax⁴⁴² are: (i) RADA 16-I allows osteoblast penetration and (ii) the concentrated RADA 16-I possesses higher biocompatibility due to its hydrophilicity. In addition, (RADA)₄ is another type of self-assembling peptides with biocompatibility, neutral net charge density (implying

hemocompatibility), and injectability. Complete hemostasis time for (RADA)₄ was ~15 s under severe hemorrhagic conditions without any auxiliary hemostatic agents.⁴⁴⁴ Longer (RADA)₄ fibers resulted in larger values of storage modulus, leading to more efficient control of bleeding.⁴⁴⁵ Hemostatic properties of (RADA)₄ have been attributed to the formation of high surface area nanofibers, leading to strong interactions with plasma proteins.⁴⁴⁶ The potential of short peptides for hemostatic applications was evaluated by a set of pseudo proteins based on poly(ester amide) (PEA)-conjugated with three different cationic short peptides, namely RGDK, RRRFK, and RRRFRGDK.⁴⁴⁷ The peptide RRRFRGDK conjugated to the hydrogel was demonstrated to promote hemostatic and antibacterial properties as well. An extracellular matrix (ECM)-like backbone (*i.e.*, poly(l-lysine) (PLL) glycopolypeptide) was modified with catechol and glucose groups and crosslinked using two different crosslinking agent, namely FeCl₃ (coordinated crosslinking) and horseradish peroxidase (H₂O₂/HRP) catalytic system (covalent crosslinking).²⁷¹ The results suggest superior hemostatic performance by each crosslinking system (~6% blood loss) compared to that of fibrin glue; the wounds treated by the developed hydrogels were fully regenerated with hair follicles within 14 days. The hemostatic performance was found to be significantly correlated to micropore size; larger pore sizes (16-18 μm) were associated with faster hemostasis. In another study, a PLL backbone was crosslinked with poly(ethylene glycol) diglycidyl ether (EPPE) in a mild ring-opening reaction.⁴⁴⁸ The extraordinarily high swelling ratio of 6300% resulted in significantly enhanced hemostatic properties compared to that of commercial gelatin sponges *in vivo* (rat tail amputation model).

Keratins are another class of natural proteins that are hemostatic biomaterials.⁴⁴⁹ Keratin can induce hemostasis *via* increasing fibril lateral assembly and decreasing plasma

clotting lag times.⁴⁵⁰ Kerateine nanoparticles (a type of keratin obtained from human hair through reductive conditions) reduced bleeding compared to kerateine extracts in liver wound and tail amputation models.⁴⁵¹ These nanoparticles form high viscosity gels on the wound area, whereas keratin-based hydrogels possess poor mechanical and rheological properties. To address this limitation, keratin-based materials were synthesized in combination with reinforcing components, as shown in Figure 4.3A.⁴⁵² Keratin-catechin nanoparticles with ~40 nm diameters were integrated into a cellulose-based hydrogel to improve adhesion and hemostatic properties for fast blood clot formation (Figure 4.3B). The hemostatic effect originated from the effects of keratin, which activated thrombin and platelet aggregation. Complete cessation of bleeding was observed within 2 min, in the *in vivo* liver puncture models. The hemostatic capacity of keratin, along with its wound healing properties, was strengthened by conjugating insulin moieties using the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemistry.⁴⁵³ Insulin is known to trigger cell migration and accelerates re-epithelialization *via* enhanced angiogenesis.⁴⁵⁴ The insulin-conjugated keratin formed a gel when added to water through the formation of hydrophobic interactions and disulfide and hydrogen bonds. *In vivo* liver bleeding models showed significant decreases in clotting time for the insulin-conjugated keratin compared to keratin.

The hemostatic capacity of decellularized ECM as a biocompatible hemostatic agent has been explored.⁴⁵⁵ Decellularized ECM-based hydrogel with a swelling ratio of ~90-95% was prepared using EDC crosslinkers. Hemostasis time in rat tail and artery models was reduced to ~120 s (controls with no crosslinking showed ~350 s clotting times). Hemostatic properties were attributed to high absorption and large amount of collagen in decellularized

ECM, leading to thrombin formation and platelet activation. In a recent study, BSA was used as a backbone to develop a mussel-inspired hemostatic adhesive hydrogel (Figure 4.3C).²⁶⁵ First, to augment the carboxyl sites needed to graft dopamine (DA) onto the BSA backbone, citrate acid was used to convert primary amine groups to carboxyl moieties. This process blocked the primary amines to avoid self-crosslinking during the EDC/NHS-induced conjugation of DA to BSA. Finally, hydrogels were crosslinked by adding Fe^{3+} ions and subsequently OH^- ions through the catechol-catechol interactions (Figure 4.3D). The rheological behavior showed a 20-30 min time-to-plateau until full gelation was obtained, which represents a favorable curing time for practical surgical conditions. Although it provides weak bonding initially, it allows the surgeon to adjust the tissue properly before the full curing time. The potential applicability of the hydrogel to act as an adhesive hemostatic material was demonstrated by 10-fold improvement in adhesive strength compared to fibrin glue. *In vivo* experiments using the liver bleeding model showed that blood loss in the models treated with the hemostatic material was reduced to 4% of that of control models (Figure 4.3E).

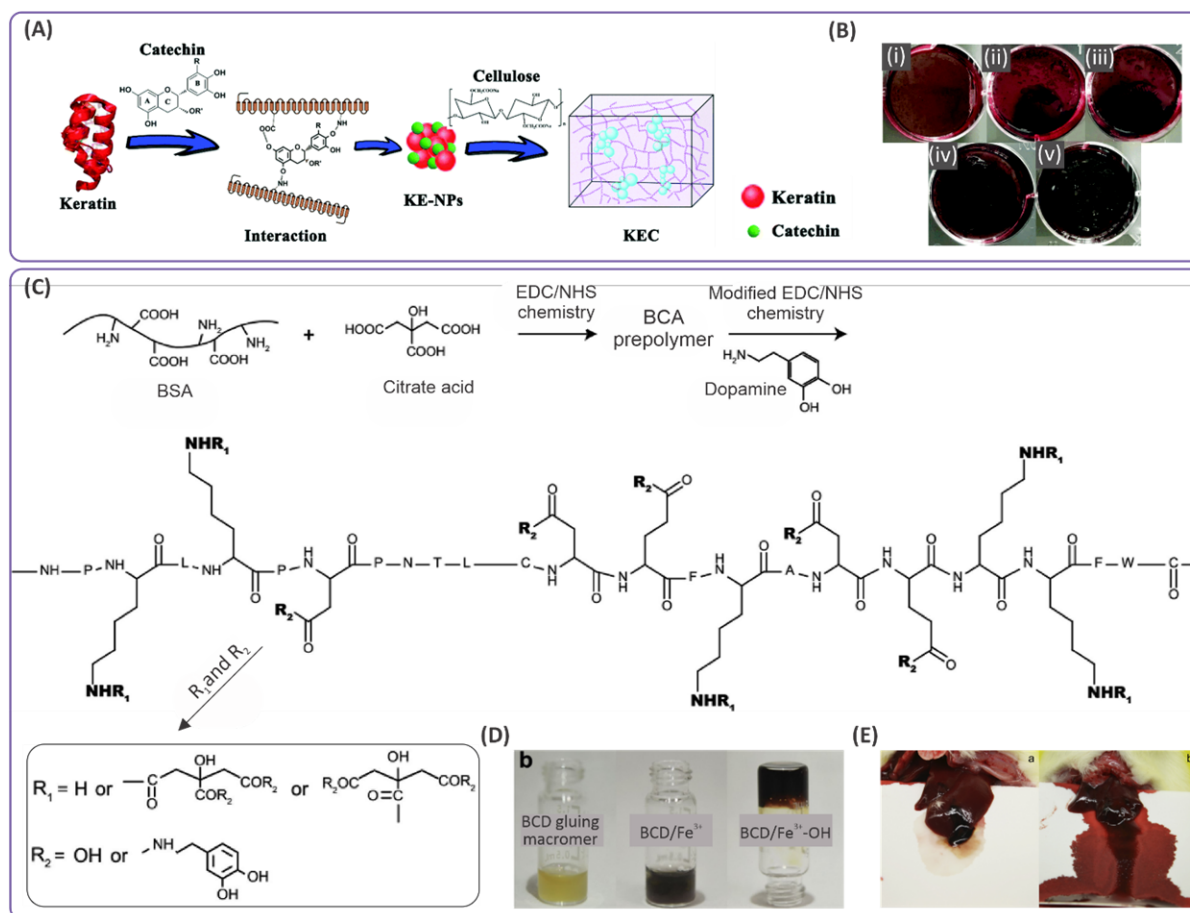


Figure 4.3. Examples of hemostatic material designs based on natural polypeptides. (A) Schematic representation of the molecular assembly for keratin/catechin nanoparticles (KE-NPs) incorporated within a cellulose matrix (i.e., KEC hydrogels). (B) Illustration of blood clot formation in (i) blank control, (ii) cellulose hydrogel, with (iii) 0.5, (iv) 1, and (v) 1.5 wt.% keratin content. Reproduced with permission.⁴⁵² Copyright 2012, Royal Society of Chemistry. (C) The synthesis process of a tissue adhesive based on carboxylated bovine serum albumin (BSA) and citrate acid (BCA) conjugated with dopamine (referred to as "BCD" hydrogels). (D) Representation of the gelation behavior of the BCD with the addition of Fe^{3+} and OH^- ions. (E) Rat liver bleeding model for the wounds treated with and without BCD hydrogels. Reproduced with permission.²⁶⁵ Copyright 2017, Elsevier. EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide.

4.3.1.1.1 Silk-fibroin

Silk fibroin (SF) derived from *Bombyx mori* is a heavy protein connected through

disulfide bonds. Due to their biocompatibility and minimal inflammatory responses, SF-based hemostats have attracted much attention.⁴⁵⁶ The SF hydrogels can be physically crosslinked as they transition to their secondary structures, *i.e.*, hydrophobic β -sheet formation *via* pH, temperature, and solvent composition.⁴⁵⁷ Formation of such secondary structures is associated with stronger crosslinking and adhesion, as well as low aqueous swelling, making SF a good candidate for internal wound control.⁴⁵⁸ A powder-based low molecular weight SF hemostat processed by hydrolysis of SF at 50 °C significantly promoted blood clotting *in vitro*.⁴⁵⁹ Composites of SF with cellulose covalently crosslinked in urea and NaOH solutions were developed and followed by a pH neutralization process using a CO₂ gas injection process (to aggregate cellulose chains *via* hydrogen bonding).⁴⁶⁰ The obtained hydrogel was characterized with low hemolysis and proposed for hemostatic applications as per the significant drop (~75%) in blood loss in mouse liver bleeding models.

4.3.1.1.2 Gelatin derivatives

Gelatin derivatives have shown hemostasis through rapid and strong physical sealing of the bleeding in the artery and heart.¹⁷⁹ Gelatin offers promising biodegradability and cell adhesive properties and has been used in different forms in hemostatic applications. Erythropoiesis is augmented by gelatin molecules, which lead to increased concentrations of RBC and platelets.^{417,461} Gelatin sponges are currently available commercially (*e.g.*, Gelfoam®, Pfizer) and are approved to stop bleeding from surgical wounds. Recently developed gelatin nanofiber sponges fabricated *via* electrospinning were proposed for arterial injuries due to their superior hemostatic capabilities, compared to conventional medical gauzes and gelatin hemostatic sponges.⁴⁶² Blood clots form due to gelatin's ability to activate platelets and to trigger coagulation.^{463,464} Many studies have focused on gelatin to

strengthen its hemostatic effects and bioadhesion. For instance, chemically crosslinked gelatin *via* EDC/NHS coupling in combination with other macromolecules, such as hyaluronic acid (HA), have been characterized and used as hemostatic biomaterials.³⁸⁵ Gelation of gelatin (reported to take ~90 s) was reduced to ~60 s with the addition of HA. Gelatin-based hybrid hydrogels have been demonstrated in multiple examples. In a recent study, gelatin grafted with DA (GT-DA) was mixed with a chitosan-based polymer matrix and incorporated with polydopamine (PDA)-coated carbon nanotubes (CNT-PDA) to develop multifunctional hemostatic hydrogels with adhesive, conductive, and antioxidant properties.⁴⁶⁵ Antimicrobial properties were introduced to the hydrogel by the addition of doxycycline. The hydrogel system was crosslinked using the hydrogen peroxide/horseradish peroxidase (H_2O_2 /HRP) catalytic system. The catechol content in the hydrogel improved tissue adhesion, which enables effective sealing of the wound area. In another example, gelatin was modified with 3-carboxy-phenylboronic acid and crosslinked with poly(vinyl alcohol) (PVA).⁴⁶⁶ The hydrogels were encapsulated with vancomycin-conjugated silver nanoclusters as well as nimesulide-loaded micelles to impart antibacterial and anti-inflammatory properties to the hydrogel for diabetic wound treatment. Experiments demonstrated considerable improvement in hemostatic properties (almost an order of magnitude lower blood loss *in vivo*) compared to blank controls. Gelatin-based cryogels were developed *via* cryopolymerization of DA-modified gelatin in the presence of $NaIO_4$ followed by freeze-drying.⁴⁶⁷ The crosslinked network of gelatin-DA cryogels was characterized with injectability, shape-memory properties, near-infrared (NIR)-responsive photothermal activity, and antibacterial properties. In addition, blood cells and platelets could be activated more effectively compared to the commercial wound

dressings as demonstrated in the porcine subclavian artery and vein incision models.

The strong adhesion offered by gelatin methacryloyl (GelMA) alongside its mechanical strength, make it a strong candidate for physical hemostasis *in vivo*.¹⁷⁹ The GelMA hydrogels were used in combination with a *N*-(2-aminoethyl)-4-(4-(hydroxymethyl)-2-methoxy-5-nitrosophenoxy) butanamide (NB) conjugated to the HA (HA-NB) matrix to reinforce and to promote adhesion in hemostatic hydrogels to stop bleeding rapidly, as shown in Figure 4.4. The NB-functionalized HA enabled on-demand generation of aldehyde groups upon irradiation with UV light, facilitating Schiff base formation with nucleophiles present on the tissue surface for better adhesion strength. Strong adhesion led to hemostatic sealants with the ability to resist 250 mmHg blood pressure *in vitro* (Figure 4.4B,C), seal cardiac wounds with up to ~6 mm diameter, and carotid arteries with ~4-5 mm long incisions. Hemostatic capabilities of GelMA can be enhanced *via* snake venom extract additives.⁴⁶⁸ A visible light crosslinkable GelMA hydrogel containing snake venom extracted from *Bothrops atrox* led to activation of platelets and thereby reduced clotting time to ~45 s (from ~5-6 min controls, with no treatment). The snake venom extracts did not affect the adhesive strength of the hydrogels. In addition, successful wound-sealing performance was reported in abdominal aorta as well as liver injury models. In another study, GelMA hydrogels were supplemented with tannic acid (TA), gallic acid (GA)-modified silver nanoparticles (AgNPs), and polyphosphate to develop thermo-responsive hemostatic hydrogels with photothermal antimicrobial properties.⁴⁶⁹ Apart from the excellent hemostatic properties due to the charge interactions (phosphate groups), methicillin-resistant *Staphylococcus aureus* (MRSA) and *E. coli* could be removed by ~98% and ~96%, respectively, *in vitro*. A similar study investigated addition of

TA to the pristine gelatin for potential hemostatic applications and skin wound healing due to the pH-sensitive release of TA promoting antioxidant, antibacterial, as well as anti-inflammatory properties at the wound site.⁴⁷⁰

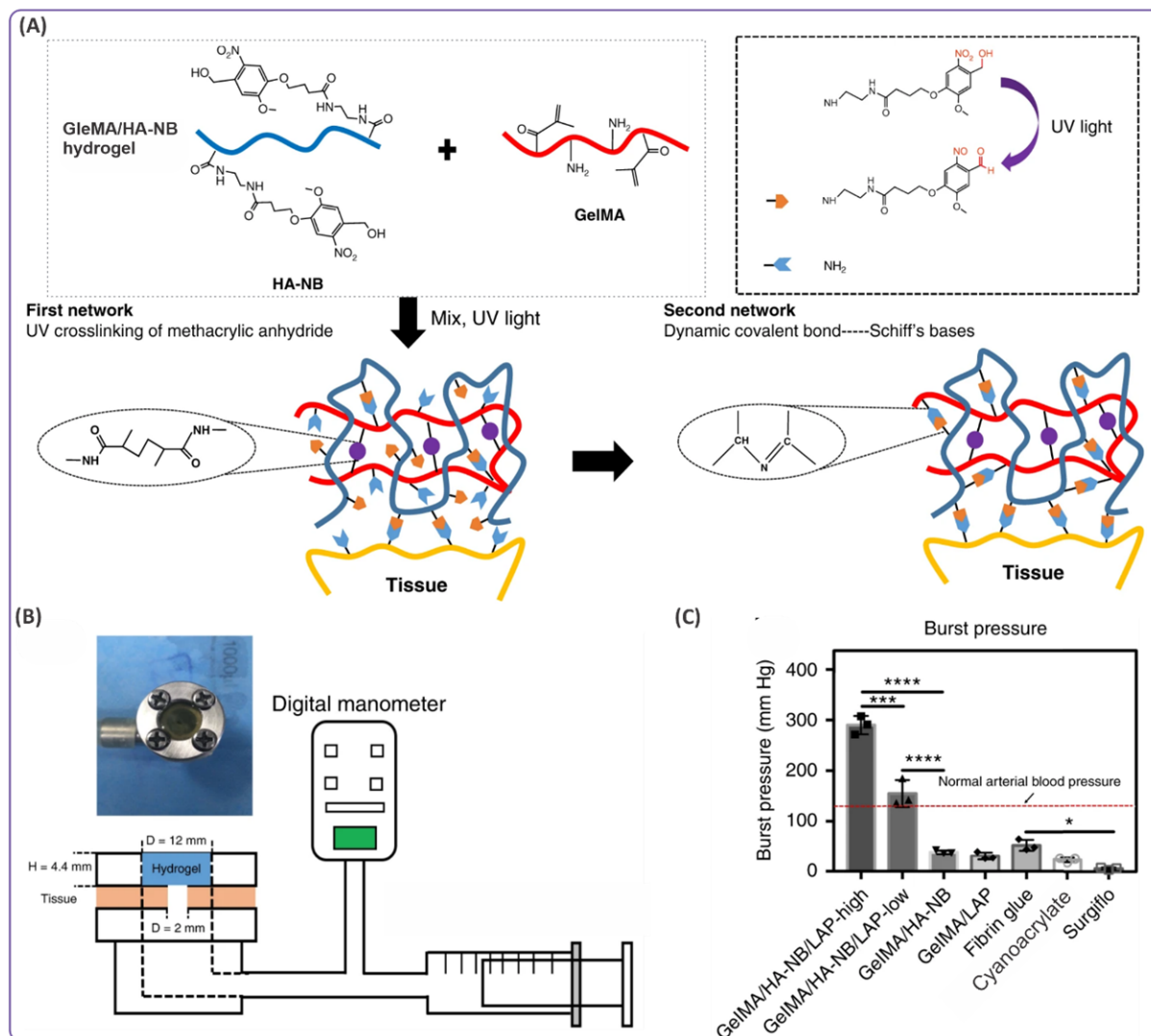


Figure 4.4. Gelatin-based materials used for adhesive hemostatic hydrogels. (A) Chemical structure and two-step crosslinking mechanism of the sealant developed based on gelatin methacryloyl (GelMA) and *N*-(2-aminoethyl)-4-(4-(hydroxymethyl)-2-methoxy-5-nitrosophenoxy) butanamide (NB) conjugated to the hyaluronic acid (HA-NB) and (B) The experimental setup for measurement of adhesion strength. (C) The results of the burst pressure test confirm the adhesion strength beyond normal arterial blood pressure. Reprinted from Ref 179 LAP, lithium phenyl-2,4,6-trimethylbenzoylphosphinate.

4.3.1.1.3 Fibrinogen

Fibrin-based sealants have been used for more than two decades, as they can mimic the final stages of the blood coagulation process. It has been used for hemostasis in many different surgical settings, including neurogenic,⁴⁷¹ cardiac,⁴⁷² thoracic,⁴⁷³ reconstructive,⁴⁷⁴ and plastic surgeries⁴⁷⁵. Previously, fibrin sealants were based on animal antifibrinolytic proteins, such as bovine aprotinin, and have been associated with anaphylactic reactions.⁴⁷⁶ Next-generation fibrin sealants were designed with concentrated clot-activating proteins or so-called biological active components (BAC) consisting of human fibrinogen and α -thrombin.⁴⁷⁷ Here, plasminogen contents were eliminated through chromatographic separations. Plasminogen removal helped to remove the antifibrinolytic agents, *i.e.*, aprotinin or tranexamic acid, which are known to be neurotoxic.⁴⁷⁸ Recently, a mixture of fibrinogen and thrombin, a composite material used as a sealant material, led to the formation of stable clots *via* covalent crosslinking of fibrin meshes.⁴⁷⁹ Different brands of commercial fibrin-based sealants have different formulations. For instance, the lack of antifibrinolytic agents in Evicel® diminishes the biodegradability of fibrinogen.⁴⁷⁹ Clinical studies have assessed the hemostatic capabilities of fibrin sealants.^{480,481} The data indicate hemostatic efficacy due to more patients achieving full hemostasis within as short a time as 4 min, in vascular surgeries.⁶ Despite the widespread use of fibrin sealants, research continues to find ways to improve the poor adhesion and infection issues of these materials. On this basis, a hemostatic and antibacterial *in situ* sealant was developed by the combination of chitin (CH), fibrin (FB), and tigecycline-loaded gelatin nanoparticles (tGNPs), as shown in Figure 4.5A-C.⁴⁸² The gel was applied with a dual syringe, one containing chitin, fibrinogen, and tGNPs, and the next one containing thrombin, chitin, and tGNPs. The

synthesized gel showed excellent hemostasis (Figure 4.5D,E) and adhesive properties with a gelation time of ~ 1 min and acceptable hemolysis behavior (Figure 4.5F). *In vivo* hemostatic evaluations revealed rapid blood clot formation within 84 s and 154 s in liver and artery bleeding models, respectively. This hemostasis timescale makes the materials composition suitable for cardiac surgery where mediastinitis is common due to deep bleeding and infections. In another study, the hemostatic efficacy of fibrin-based hydrogel materials, composed of nanostructured fibrin and agarose, was evaluated in a rat hepatic resection model.⁴⁸³ Significantly enhanced hemostatic effects were observed compared to commercial counterparts where the time to hemostasis was reduced to ~ 30 s.

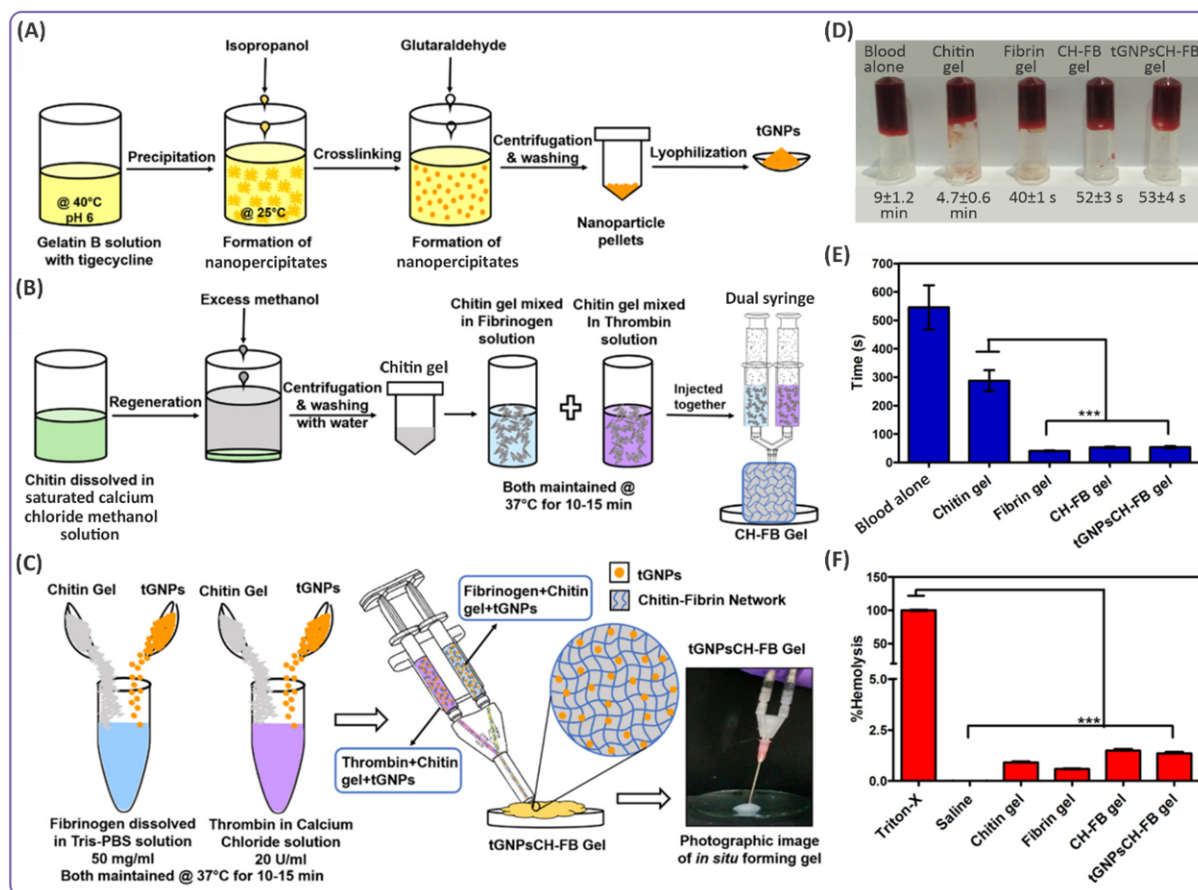


Figure 4.5. Synthesis and hemostatic characterization of the hydrogel composites based on chitin (CH)/fibrin (FB) networks and tige cycline-loaded gelatin nanoparticles (tGNP). (A) Synthetic route

for preparing the tigecycline-loaded gelatin nanoparticles (tGNP), and (B) representation of CH/FB nanocomposite processing procedure. (C) Final mixing of chitin gel and tGNP resulting in the final hydrogel solution ready for application as a hemostatic biomaterial. (D) Representation of coagulated blood for different samples. (E) Coagulation time and (F) hemolysis results for hydrogels with different components. Reprinted with permission from ref ⁴⁸². Copyright 2018 American Chemical Society.

4.3.1.2 Polysaccharides

Polysaccharide building blocks are attractive candidates for designing hemostatic materials due to their biocompatibility, biodegradability, and intrinsic hemostatic characteristics.^{254,484} Polysaccharides are chemically modifiable to enhance their hemostatic activity.⁴⁰⁹ For example, chondroitin sulfate conjugated with serotonin (mediating the blood clotting process) was developed as a hemostatic bioadhesive for wound healing applications.⁴⁸⁵ Here, serotonin groups were oxidized using the horseradish peroxidase (H₂O₂/HRP) catalytic system to crosslink the hydrogels. In this material system, aloe vera powder was used as an additive for improvements in wound healing function. In the following sections, the major classes of polysaccharide-based backbones modified for improved hemostatic function are discussed.

4.3.1.2.1 Cellulose

Cellulose is composed of a homopolysaccharide of d-glucopyranose and acts as a hemostatic material due to its absorbable properties.^{409,486} Cellulose in oxidized form has been well explored and reported for effective hemostatic properties *via* absorption and activation of platelets leading to thrombus formation.⁴⁸⁷ Recently, nanocellulose fiber was mixed with gelatin and amine-functionalized silver nanoparticles to form physically

crosslinked hydrogels for wound dressings, as shown in Figure 4.6A.⁴⁶¹ *In vitro* blood clotting assays with freeze-dried hydrogels revealed the roles of gelatin molecules and silver (Ag) nanoparticles in the augmented platelet adhesion and blood clotting, as shown in Figure 4.6B.⁴⁶¹ Mechanistically, the protonated free amine groups on the Ag surface and gelatin backbone are capable of adsorbing negatively charged moieties present on the RBC membranes leading to hemagglutination. *In vitro* blood clotting results indicate that higher Ag content is associated with lower hemostatic effects, which was explained by fast and dense crosslinking. In another study, a hybrid hydrogel sponge based on quaternized hydroxyethyl cellulose (HEC) and mesocellular silica foam (MCF) aimed to activate the coagulation cascade in addition to contributing its antibacterial effects (Figure 4.6C).⁴⁸⁸ In this study, the hydrogels were crosslinked by free radical copolymerization of (methacryloyloxy)-ethyltrimethylammonium chloride (MATMA), poly(ethylene glycol) diacrylate (PEGDA), and HEC *via* ammonium persulfate (APS)/tetramethylethylenediamine (TEMED). Figure 4.6D shows the main mechanisms triggering blood coagulation in the proposed materials design. The hemostatic effects are strengthened by the superabsorbent capacity leading to adhesion of RBCs and platelets. The absorption of the cells in blood was further facilitated by electrostatic interactions (initiating hemagglutination) *via* the MCF that activates coagulation factor XII. This process led to decreased blood clotting time, down to ~1 min *in vitro*. Another cellulose-based hemostatic sponge was obtained by incorporation of skin secretion of *Andrias davidianus* (SSAD) in a composite of cellulose nanocrystals (CNCs) and cellulose nanofibers (CNFs).⁴⁸⁹ The sponges were reported to be injectable and engineered for high resilience and strength in the swollen state. Rapid fluid absorption, within 3 s, led to excellent hemostatic properties compared to gelatin sponges, as confirmed

in *in vivo* studies.

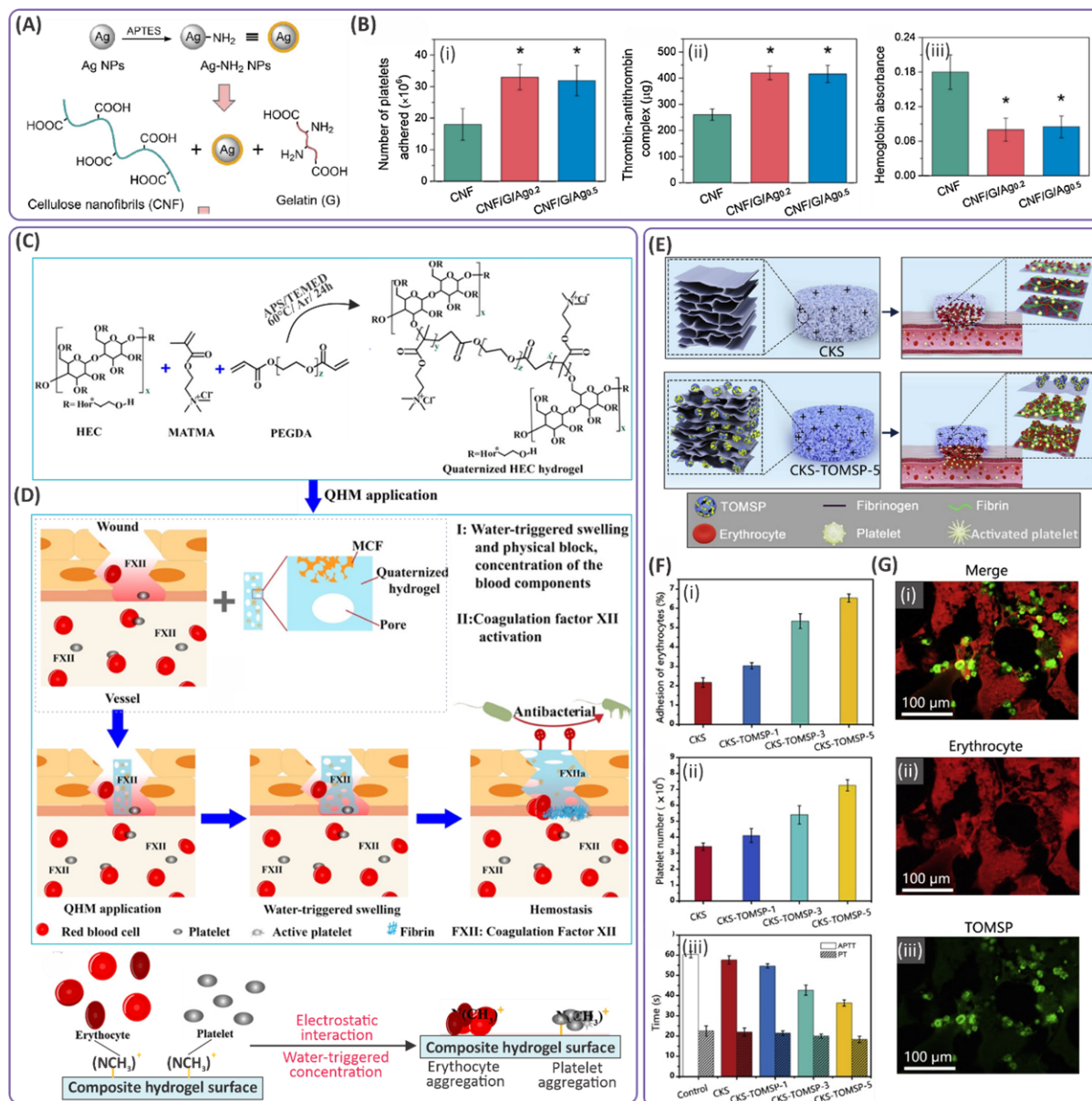


Figure 4.6. Examples of polysaccharide-based hemostats. (A) Schematic of the hydrogels based on amino-functionalized silver (Ag) nanoparticles and the crosslinking of the modules in combination with cellulose nanofibrils (CNF) and gelatin (G). APTES, (3-aminopropyl)triethoxysilane. (B) *In vitro* hemostatic test results in terms of (i) hemoglobin absorbance, (ii) number of adhered platelets, and (iii) thrombin generation studied *via* thrombin-antithrombin complex. Reproduced with permission from ⁴⁶¹. Copyright 2018, Elsevier. (C) Chemical synthesis of the hemostatic sponge hydrogels based on quaternized hydroxyethyl cellulose (HEC) embedded in a co-polymer based on 2-(methacryloyloxy)-ethyltrimethylammonium chloride (MATMA) and poly(ethylene glycol)

diacrylate (PEGDA) and (D) mechanisms of action triggering the coagulation cascade in quaternized hydroxyethyl cellulose/mesocellular silica foam (MCF) hydrogel sponges (QHM). Reprinted with permission from ref⁴⁸⁸. Copyright 2019 American Chemical Society. (E) Hemostatic mechanism and properties of the chitosan/konjac glucomannan subtract (CKS) and CKS-thrombin-occupied microporous starch particles (TOMSP) hydrogels, respectively. (F) Results of (i) erythrocyte aggregation, (ii) platelet adhesion, and (iii) clotting time measurements *in vitro*. (G) Analysis of fluorescent staining for CKS-TOMSP. Erythrocytes were stained with mouse anti-IL12B monoclonal antibodies and goat anti-mouse (IgG-RBITC) antibodies (red), where TOMSP was stained with fluorescein isothiocyanate (green). Reproduced with permission from ref⁴⁹⁰. Copyright 2020, Elsevier.

4.3.1.2.2 Starch

Starch is one of the most abundant and low-price polysaccharides and has also been used in hemostatic materials by incorporating appropriate functional groups through chemical modification.⁴⁹¹ For instance, blends of starch with acrylamide and acrylic acid monomers were polymerized together through free-radical polymerization and studied for their hemostatic applicability.⁴⁹² The synthesized materials showed excellent hemocompatibility along with a rapid hemostasis, originating from the fast swelling rate (18,000% over 30 min). Complete hemostasis was observed within 10 s when tested with the femoral artery wound model. In this case, the cells present in blood aggregate with the hydrogel material and establish an efficient sealing. Composites of konjac glucomannan and chitosan with thrombin-infilled microporous starch particles were recently introduced for initiating hemostasis (Figure 4.6E).⁴⁹⁰ The composites exhibited high absorption as well as desirable activated partial thrombin time (APTT), thrombin time (PT), and whole blood clotting indices (*e.g.*, BCI). The hemostatic mechanism in this material relied on combinatorial effects of absorption and interactions between the material and platelets,

erythrocytes, and coagulation factors. Positive surface charges led to blood cell aggregation. Then, the porous and rough interfaces favored flow of the blood plasma into the material. Fibrin network formation occurred due to the presence of thrombin leading to further adhesion of the RBCs and thereby clot formation. *In vitro* analysis results shown in Figure 4.6F confirmed the hemostatic effectiveness of the hydrogel in inducing hemostasis.

4.3.1.2.3 Agarose

Agarose-based hydrogels have also shown potential for hemostatic applications. The mixture of agarose ethylenediamine conjugate (AG-NH₂) and dialdehyde capped poly(ethylene glycol) (DF-PEG) were demonstrated to form stable gels within ~10 s *via* Schiff base formation that eventually led to tissue adhesiveness.⁴⁹³ The short hemostasis times, down to 10 s, with total bleeding amounts of only ~0.2 g confirmed the promising hemostatic capacity of the developed hydrogel. The hydrogels showed self-healing, pH-responsiveness, and biocompatibility, demonstrating their potential to be used as wound dressings.

4.3.1.2.4 Dextran

Dextran molecules are highly water soluble and have advantages in their hemostatic behavior over other polysaccharide molecules.⁴⁰⁹ Dextran has been modified in different ways to introduce hemostatic properties. For example, dextran was oxidized to yield active aldehyde groups to form an *in situ* crosslinked hydrogel with chitosan. This material was used as a hemostatic agent with rapid gelation, minimal cytotoxicity, and low swelling.⁴⁹⁴ This hydrogel system showed hemostatic effects comparable to commercial BioGlue® in *in vivo* liver bleeding models. Oxidized dextran was also used in combination with

hydrophobically modified chitosan for wound healing applications, as a hemostatic agent.^{495,496} Oxidized aldehyde dextran sponges were characterized as having high absorption (blood absorption of 47.5 g/g) and adhesion properties (~100 kPa adhesive strength), reducing blood loss in femoral artery, ear vein, and rabbit liver model injuries.⁴⁹⁷

4.3.1.2.5 Chitosan

Chitosan (CS) is one of the most extensively studied biomaterials for hemostatic properties due to the positively charged amine groups on its backbone.^{498,499} Chitosan has been used in mixtures with synthetic backbones to elevate the hemostatic effects.^{500,501} For example, in combination with gelatin and PVA, it had hemostatic characteristics where a BCI index of 0.032 was reached within 5 min. Methacrylated chitosan was copolymerized with *N*-hydroxymethyl acrylamide (NMA) in the presence of DA using redox-free radical polymerization (APS/TEMED).²⁵⁵ The hydrogels reduced liver bleeding *in vivo* from ~266 mg to ~32 mg. An adhesive hydrogel composite, synthesized with quaternized chitosan grafted with polyaniline and benzaldehyde-functionalized poly(ethylene glycol) (PEG) showed excellent hemostatic and antibacterial properties (Figure 4.7A-C).⁵⁰² The blood loss mass in the *in vivo* experiment was under 215 mg compared to the control with over 2000 mg blood loss. The natural hemostatic properties of the chitosan molecules were augmented with the synergistic contributions of polyaniline segments and the positively charged quaternary ammonium groups. Rapid gelation of the hydrogel also formed an efficient barrier against blood flow. Quaternized chitosan was mixed with benzaldehyde-terminated poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PF127-CHO) in another study where Pluronic F-127 (PF-127) served as a crosslinker, facilitating both the Schiff base reaction and the formation of micelles through

dynamic self-assembly in water (Figure 4.7D).⁵⁰³ Here, positive charges on the modified chitosan molecules, along with the adhesive properties of the hydrogel, synergistically led to hemostatic properties (~80 mg *versus* ~620 mg reported blood loss for the treated and control samples, respectively) (Figure 4.7E,F). The use of carboxymethyl chitosan grafted poly(acrylic acid) (CMCS-*g*-PAAc) was demonstrated as another potential solution to stop bleeding.⁵⁰⁴ The polymers were developed through solvent precipitation and showed a high swelling capacities (~10 times in less than 20 min). The hemostatic effects were attributed to the low crosslinking density of the material originating from the low reactivity of sodium acrylate during polymerization. For optimized samples, complete hemostasis was achieved within ~20 s, which is associated with both protonated amine groups of chitosan molecules and physical swelling of the hydrogel during bleeding. A catechol-modified chitosan (Chi-C) was crosslinked with a dialdehyde cellulose nanocrystal (DACNC), and formed a bioadhesive hydrogel that effectively filled the defective bone *in vivo* and stopped bleeding under wet conditions.²⁵³ The rabbit ilium bone defect model showed ~75% decrease in blood loss when using this hydrogel, compared to untreated controls, due to rapid sealing of the wound site. The hydrogels demonstrated self-healing properties and full bone regeneration along with material degradation was observed after four weeks. Modification of chitosan with thiol groups was also demonstrated to enhance its hemostatic properties significantly, as the blood loss was lowered by over ~75%.⁵⁰⁵ In another example, carboxymethyl chitosan (CMCS) in mixture with TA was developed as a hemostatic hydrogel, as illustrated in Figure 4.8A.⁵⁰⁶ To reduce the gelation time and enhance the mechanical properties, 1,4-benzenediboronic acid (BDBA) was added. The gelation was driven mainly by hydrogen bonds, and boronate ester bonds as quickly as 10 s. *In vivo* liver bleeding test results

indicated the amount of bleeding was reduced from 240 mg to 55 mg in the samples treated with adhesive hydrogels *versus* untreated controls (Figure 4.8B). The impact of TA in enhancing hemostatic effects comes from the fact that charged polyphenol moieties can play roles as contact activators where they can activate factor XII in the coagulation cascade. In essence, as represented in Figure 4.8C, the strong interactions with blood components originating from π -cation interactions, hydrogen bonds, Schiff base formation, as well as Michael addition could explain the mechanisms of hemostatic properties in the polyphenolic components such as TA, catechol groups, *etc.* Phenolic compounds, therefore, play important roles in triggering blood coagulation due to their multiple interaction pathways with blood components. Modification of CMCS with *o*-nitrobenzyl alcohol functional groups (*i.e.*, NB) has enabled on-demand generation of aldehyde groups upon UV irradiation which can participate in crosslinking reactions.²⁵⁶ In essence, the NB groups are converted to *o*-nitrosobenzaldehyde groups in response to UV illumination leading to Schiff base formation with amino groups present on the tissue surfaces. In addition to antibacterial properties, the liver bleeding model demonstrated that the material could halve blood loss compared to blank controls. Coating with PDA has been a popular means of introducing such phenolic components to the hydrogel for hemostatic applications. A mixture of chitosan and calcium pyrophosphate coated with PDA (PDA@CS-CaP) resulted in a hydrogel wherein the hemostatic properties were enabled by hydrophilicity, interactions *via* amino and phenol groups present in PDA, as well as a flower-like hierarchical structure of the granules that aids in plasma absorption (Figure 4.8E,F).⁵⁰⁷ The study suggests that coating the chitosan (CS)-CaP with PDA played an effective role in reducing the *in vitro* clotting time from ~175 to ~125 s (Figure 4.8G). Conjugation of chitosan with catechol groups enabled hemostatic

swabs that could effectively stop bleeding in coagulopathic animal models with diabetes. The hemostatic property was attributed to the intermolecular interactions between chitosan-catechol conjugates and whole blood proteins.⁵⁰⁸ A reversibly thermosensitive version of chitosan with hemostatic properties was obtained by the synthesis of catechol-hydroxybutyl chitosan (HBCS-C), as illustrated in Figure 4.9A.⁵⁰⁹ The lower critical solution temperature (LCST) was found to be tunable and could fall within the range between room and body temperatures. The hydrogels strongly adhered to the tissue surfaces and gelation occurred within 30 s after injection *in situ*. The strong adhesion driven by the hydrogen bonding, π - π stacking, and catechol-amine reactions led to full hemostasis *in vivo* within the gelation time period, whereas for untreated samples, it took approximately 2 min until the bleeding was stopped completely. We note that there are ongoing discussions as to whether chitosan may induce allergic responses in some individuals.^{510,511}

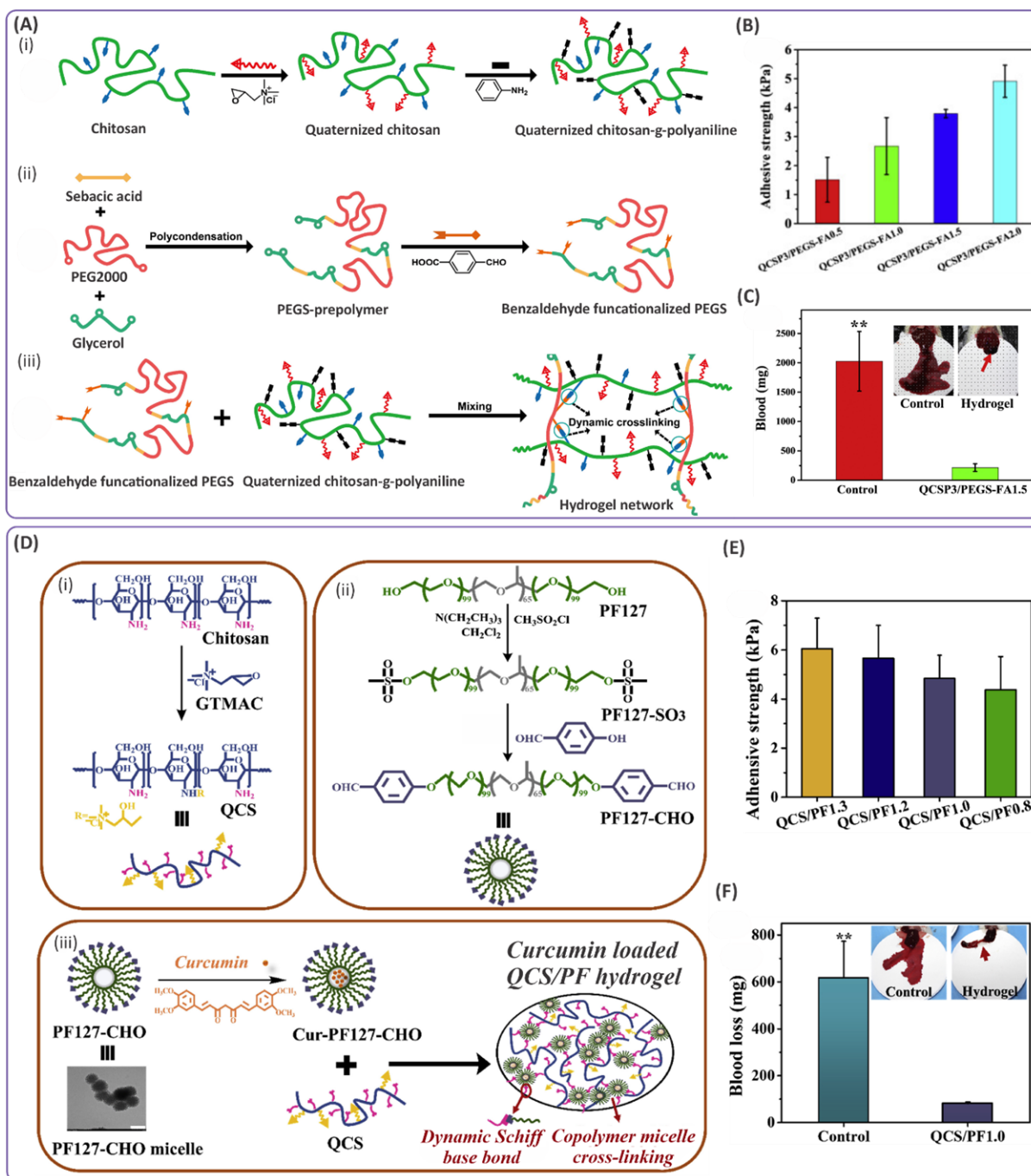


Figure 4.7. Chemical modification of the chitosan backbone to enhance its hemostatic effects. (A) Schematic illustration of (i) quaternized chitosan-g-polyaniline and (ii) benzaldehyde-functionalized poly(ethylene glycol) (PEG) synthesis. (iii) Crosslinking mechanism between benzaldehyde-functionalized PEG and quaternized chitosan-g-polyaniline. (B) Adhesion strength and (C) hemostatic efficacy of the developed hydrogels in the liver wound models in terms of blood loss. Reproduced with permission from ref ⁵⁰². Copyright 2017, Elsevier. (D) Synthesis of

curcumin-loaded quaternized chitosan (QCS) and Pluronic F-127 (PF-127) or (PF) hybrid hydrogels (Cur-QCS/PF). (i) Modification of chitosan with glycidyltrimethylammonium chloride (GTMAC) reagent, (ii) functionalization of PF-127 with aldehyde end groups, and (iii) crosslinking the components with curcumin drug added to the QCS/PF hydrogel. (E) The results of adhesive strength at different QCS to PF-127 ratios and (F) blood loss in the *in vivo* liver bleeding model confirming hemostatic effects of the curcumin-loaded QCS/PF hydrogel. Reproduced with permission from ref ⁵⁰³. Copyright 2018, Elsevier.

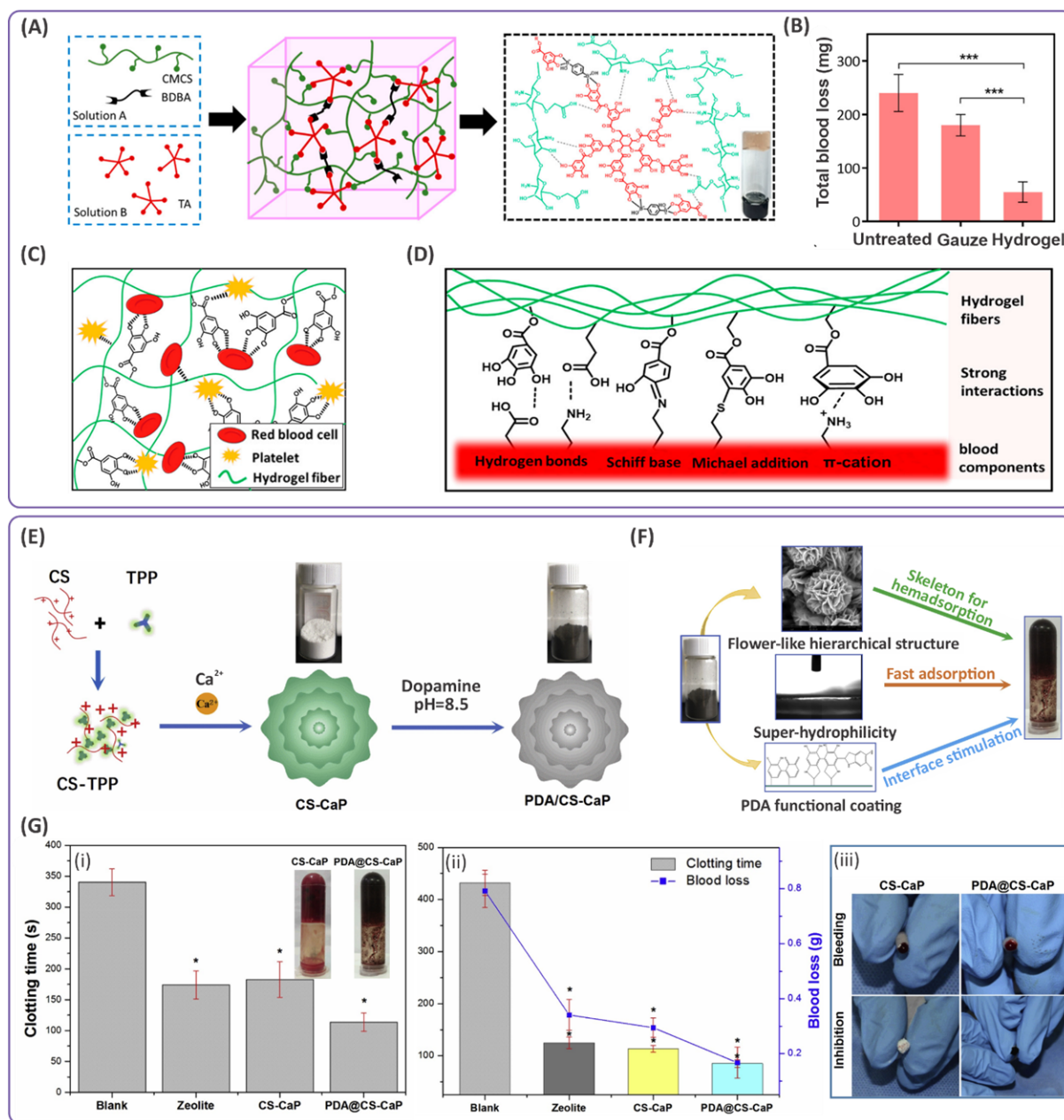


Figure 4.8. Hybrid hydrogels based on chitosan and its derivatives for hemostatic applications. (A) Materials design and crosslinking mechanisms of the carboxymethyl chitosan (CMCS)/tannic acid (TA)/benzenediboronic acid (BDDBA) hydrogels and (B) *in vivo* liver bleeding test for characterization of hemostatic behavior. (C, D) Schematic of the interactions between hydrogel components and blood ingredients. Reprinted with permission from ref ⁵⁰⁶. Copyright 2020 American Chemical Society. (E, F) A hemostatic mixture of chitosan (CS) and pentasodium tripolyphosphate (TPP) leading to the formation of chitosan/calcium pyrophosphate hybrid micro flowers (CS-CaP) in presence of the calcium ions (Ca^{2+}). Then, the coating of the powders was performed by alkaline polymerization of

dopamine enables fast absorption and interface stimulation for hemostatic effects. (G) (i) *In vitro* and (ii, iii) *in vivo* hemostatic test results showing significant effects of polydopamine (PDA), in reducing the clotting time. Reproduced with permission from ref⁵⁰⁷. Copyright 2019, Elsevier.

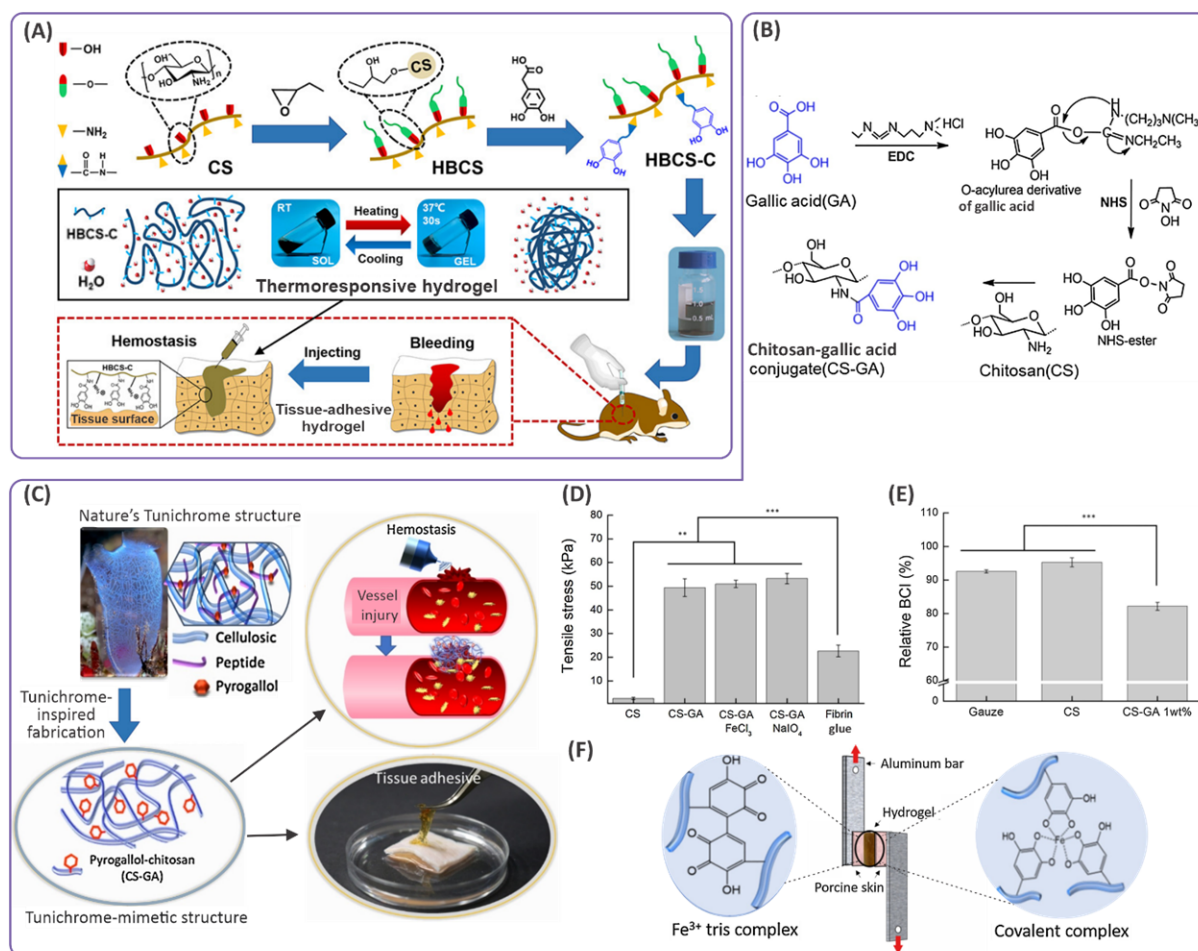


Figure 4.9. Chitosan (CS) macromolecules conjugated with polyphenols for high adhesion and hemostatic effects. (A) Schematic representation of synthesis route, and mechanisms of action in thermo-responsive hemostatic catechol-hydroxybutyl chitosan (HBCS), *i.e.*, HBCS-C hydrogels. Reprinted with permission from ref⁵⁰⁹. Copyright 2020 American Chemical Society. (B) Synthesis of gallic acid (GA)-conjugated chitosan (CS-GA) hemostatic hydrogels *via* *N*-hydroxysuccinimide (NHS)/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling. (C) Tunichrome-inspired chitosan-based hydrogels for adhesion-enabled hemostasis in chitosan-based hydrogels. (D) Mechanical properties of the hydrogels, (E) *in vitro* characterization of blood clotting index (BCI) confirming the significant effects of GA in improving the hemostatic properties of chitosan. (F) Lap shear test setup for measurement of adhesive strength for CS-GA and the crosslinking mechanism *via*

covalent bonding and non-covalent interactions. Reproduced with permission from ref²⁵⁷. Copyright 2019, Elsevier.

In another study, a tunichrome-inspired chitosan-based hydrogel was developed to enhance hemostatic effects by pyrogallol functionalization (Figure 4.9B-F).²⁵⁷ Oxidation of tunicates, which is accompanied by covalent bond formation and crosslinking, is known to promote hemostatic properties. The improved hemostatic ability of the hydrogel due to the conjugation of GA was quantitatively confirmed by with BCI data (Figure 4.9E). Instant coagulation at the interface with blood was observed once it dropped onto the hydrogel surface. The significant improvement of hemostatic properties was attributed to the high adhesiveness of the hydrogel, resulting in the pyrogallol groups (Figure 4.9F). In another study, the hemostatic properties were enhanced with the addition of catechol groups to chitosan composite hydrogels.⁵¹² Addition of DA to chitosan not only did not cause any hemocytotoxicity but also introduced antibacterial properties to the hydrogel. Blood clotting time assays showed that addition of 2 wt.% DA to chitosan could lower hemostasis time from 83 to 20 s.

Nanoscale assembly of chitosan can enable facile loading and promote the hemostatic behavior of chitosan. In a recent study, a hydrolytically degradable hydrogel was developed based on chitosan assembly within a macrocyclic oligosaccharide (β -cyclodextrin) crosslinked network. The host-guest interactions aided the loading of chitosan into the polyester-linked (dianhydride) hydrogel enabling its hydrolytic degradation *in vivo*. The liver bleeding model *in vivo* confirmed a significant reduction in time to hemostasis as well as blood loss for the chitosan/ β -cyclodextrin hydrogels as compared to commercially available controls. Other approaches to enhance the hemostatic properties of chitosan

involve the addition of CaCO_3 to acetate chitosan that is commercially available for wound-healing applications.⁵¹³ First, the addition of CaCO_3 eliminated the allergic potential of acetate chitosan (due to its acidity). Secondly, the released calcium ions aided crosslinking of chitosan *via* electrostatic interactions that enhanced hydrogel toughness. Finally, CaCO_3 was associated with enhanced blood coagulation and thereby promoted wound healing. Chitosan-based coatings (*i.e.*, dodecyl-modified chitosan, DCS) could also enable the fabrication of hemostatic microneedles with strong tissue fixation and blood coagulation capabilities.⁴³⁴ A pagoda-inspired microneedle substrate was fabricated by molding with PEGDA hydrogels, on the top of which DCS solutions were dripped for coating. The patches obtained could effectively stop bleeding in rabbit liver, spleen, and kidney bleeding models. Chitosan-based aerogels are able to uptake large amounts of biofluids, leading to strong hemostatic properties. Chitosan aerogels containing diatom biosilica and DA (for crosslinking) were fabricated by alkaline precipitation and *tert*-butyl alcohol replacement.⁵¹⁴ High water absorption on the order of ~ 300 g/g%, along with strong charge interactions resulted in hemostatic function *in vitro* with clotting times below ~ 70 s.

4.3.1.2.6 Alginate

The release of the calcium ions from ionically crosslinked calcium alginate has demonstrated its promising potential as a hemostatic material. The hemostatic effect is mainly due to the calcium ions activating the coagulation process through the aggregation of platelets.⁴⁰⁵ Further, the hemostatic activity of alginate molecules can be augmented by chemical modifications. For instance, sodium alginate (SA) molecules, thiolated using cysteine methyl ester, readily enabled gelation through the formation of disulfide bonds within less than 20 s.⁵¹⁵ Composites of alginate with PVA and collagen were mixed with

Tween80 surfactant to develop mechanically stable wound dressings with outstanding hemostatic behavior.⁵¹⁶ Swelling of up to ~2400% for the optimized composite materials led to time to hemostasis as low as 17 s with the lowest blood loss of 66 mg. Apart from biocompatibility, biodegradability, and high mechanical strength, the thiolated alginate reduced the hemostasis time in a rat tail amputation model from 8.3 to 3.2 min. Alginate-based supramolecular hydrogels involving peptide conjugates (Pept-1) have also led to hemostatic properties (Figure 4.10A).⁵¹⁷ Such hemostatic behavior was assigned to (i) the dense 3D co-assembled network of alginate macromolecules and Pept-1 with negative zeta potential, providing a physical barrier to entrap blood cells, (ii) release of calcium ions, and (iii) platelet adhesion enabled by RGD moieties in Pept-1, which led to downstream platelet activation. Clotting times for the alginate/Pept-1 alone were ~28 times longer than the mixture of both components. The clotting time decreased to 41 s, leading to over 80% reduction in bleeding compared to the control in the *in vivo* liver model. Alginate has been modified in several different ways to introduce adhesive and hemostatic functionality. Recently, oxidized alginate molecules were mixed with CMCS and gelatin, which facilitated the formation of the hydrogel through Schiff base chemistry. This hydrogel showed fast gelation times as low as 30 s⁵¹⁸ and reduced the hemostasis time and blood loss by 84.2% and 82.2%, respectively. In another study, alginate was oxidized and conjugated with catechol groups and used in a mixture with a polyallylamine (PAA) polymer to form gels *via* Schiff base formation quickly, within 5-10 s (Figure 4.10B).⁵¹⁹ Evaluation of the hemostatic effect *in vivo* using a liver bleeding model indicated a statistically significant decrease in the bleeding weight for the modified alginate compared to alginate with no modification. Hybrid hydrogels consisting of oxidized alginate and *N*-carboxyethyl chitosan (CEC) containing CuS

nanoparticles were characterized with photothermal hemostatic properties.⁵²⁰ The hydrogels significantly reduced blood loss in the liver bleeding models *in vivo*.

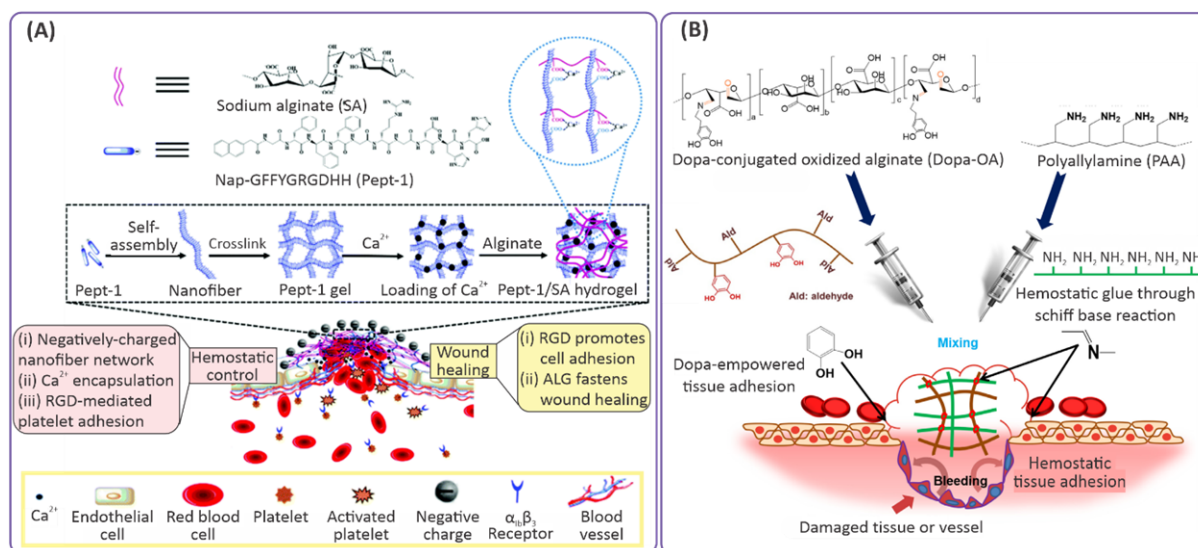


Figure 4.10. Alginate-based examples of the hemostatic materials. (A) The materials design, crosslinking process, and hemostatic mechanism of the alginate-based hydrogels incorporated with Pept-1. Reproduced with permission.⁵¹⁷ Copyright 2019, Royal Society of Chemistry. (B) Application of hybrid hydrogels based on polyallylamine (PAA) and oxidized, catechol-functionalized alginate for improving adhesion and thereby hemostatic effects. Reprinted with permission from ref ⁵¹⁹. Copyright 2019 Springer.

4.3.1.2.7 Hyaluronic acid

Recently, a HA-based hydrogel system containing PDA-grafted graphene oxide and DA-grafted HA molecules has been synthesized through a crosslinking step governed by the oxidative coupling of catechol groups *via* H_2O_2 /HRP catalysts; it showed both antioxidant and hemostatic characteristics (Figure 4.11A).⁵²¹ The antioxidant properties of catechol groups promote wound healing by balancing the oxidative pressure in the wound area. Also, inspired by mussels, catechol groups drive strong adhesion (Figure 4.11B) and thereby physically seal the wounds. The hydrogels had high swelling ratios and adhesive capabilities,

which resulted in efficient hemostasis by reducing the blood loss from ~400 mg (for control) to ~74 mg (Figure 4.11C). In another study, HA was also grafted with serotonin, a blood-clotting mediator factor activating platelet attraction to facilitate hemostasis (*i.e.*, factor V, factor IIV, von Willebrand factor, and fibrinogen) as shown in Figure 4.11D,E.²⁴² The serotonin-conjugated HA, crosslinked *via* HRP/H₂O₂, locally concentrated blood serum components. In addition to bonding to the nucleophiles in the biomolecules during oxidative crosslinking, they led to promising hemostatic behavior. The liver bleeding model indicated blood coagulation within 30 s, with lower blood loss compared to commercial controls. The results showed significant increases in the levels of released factors from the platelet alpha-granules, *i.e.*, platelet factors IV and V, for the HA-serotonin hydrogels compared to HA (Figure 4.11F,G). The hemostatic effect of HA-serotonin hydrogels was also observed, based on the blood clot mass measurements, which were obtained by exposing whole blood to different hydrogel samples (Figure 4.11H). Compositions of hyaluronan and polyphosphate were examined for potential use for bleeding conditions (Figure 4.11I).⁵²² The HA molecules were initially modified with aldehyde groups and mixed with another set of hydrazine-modified HA-conjugated polyphosphates that lower the gelation time below 5 min but at the expense of lowering the swelling capacity. The hydrogel was fully degraded in 3 weeks and showed comparable hemostatic behavior to fibrin glue when tested in an *in vivo* mouse liver bleeding model. In addition, the hemostatic properties of HA were reinforced by the addition of zinc oxide (ZnO). The HA was crosslinked by 1,4-butanediol diglycidyl ether (BDDE) crosslinker and ZnO nanobelt-like particles were formed *in situ via* the mechanism shown in Figure 4.11J,K.⁵²³ The results suggested that the addition of ZnO to HA can reduce clotting time from 360 s (in HA hydrogels) to 160 s without compromising

hemocompatibility. The data showed hemolysis lower than 1.3% across all ZnO concentrations. Modification of HA with a von Willebrand factor-binding peptide as well as a collagen-binding peptide resulted in a hemostatic agent that could selectively bind to the active platelets.⁵²⁴ Blood loss and bleeding time in the tail vein amputation model both decreased by ~97% compared to no treatment controls. Enhanced survival rates of ~284% were found for the rat inferior *vena cava* traumatic model.

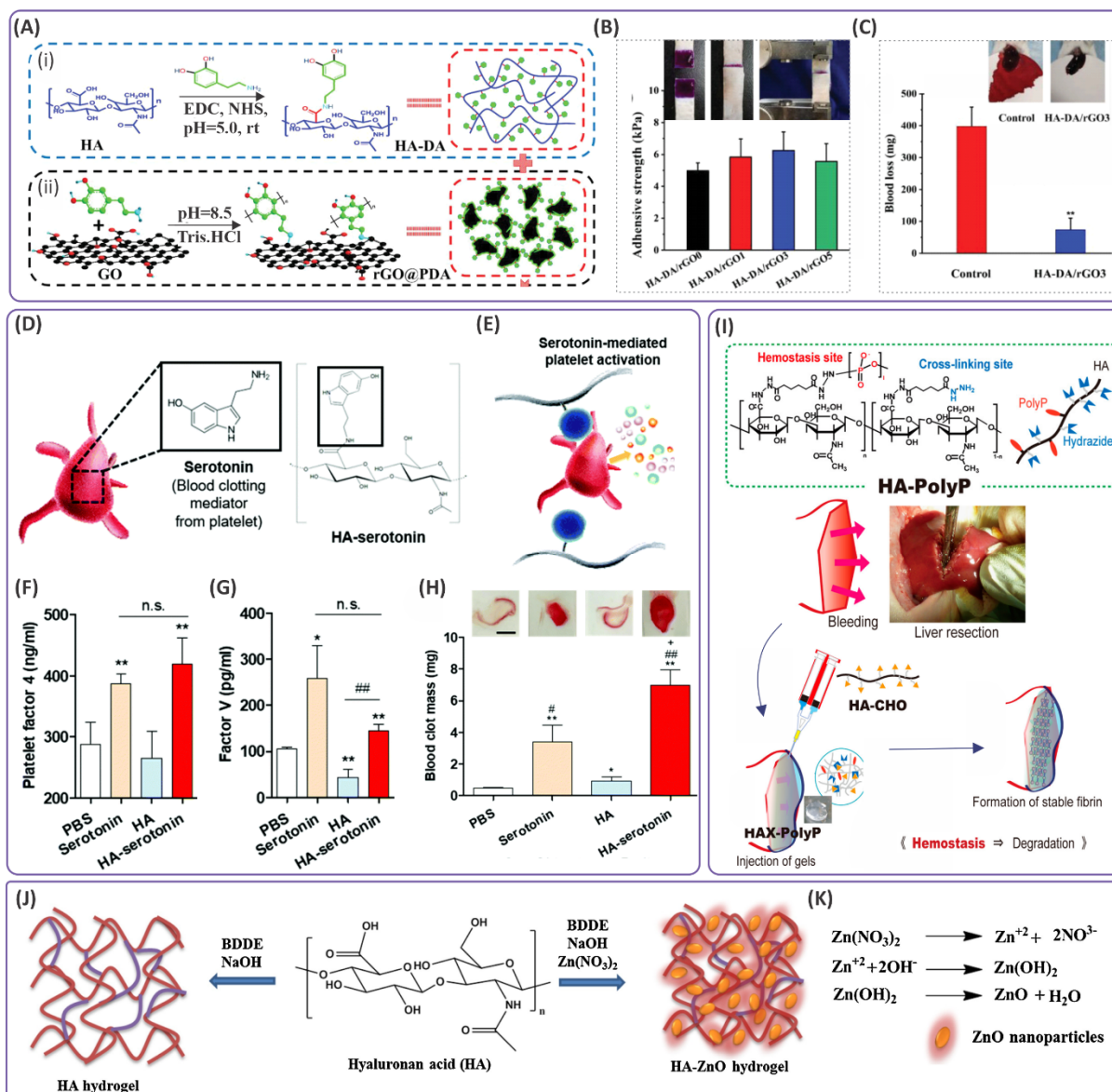


Figure 4.11. Hyaluronic acid (HA) and its derivatives for applications as hemostatic materials. (A) Schematic of dopamine (DA)-conjugated hyaluronic acid (HA-DA) and coating of reduced graphene oxide (rGO) as a potential material for hemostatic applications. EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide. (B) Adhesive strength and (C) hemostatic potential of the developed material in a liver bleeding model. Reproduced with permission from ref ⁵²¹. Copyright 2019, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (D) Chemical structure of serotonin-conjugated HA where (E) serotonin plays a role in activating platelet adhesion. (F, G) Enzyme-linked immunosorbent assay (ELISA) analysis of coagulation factors IV, and V, respectively. (H) The mass of blood clots formed due to exposure of whole blood to different hydrogel compositions. Reproduced with permission.²⁴² Copyright 2019,

Royal Society of Chemistry. (I) Representation of the molecular modification of HA with an inorganic polyphosphate (PolyP), *i.e.*, HA-PolyP, aldehydes, and hydrazides to introduce hemostatic effect. Reprinted with permission from ref⁵²². Copyright 2018 American Chemical Society. (J) Development of HA-based hydrogels with *in situ* zinc oxide (ZnO) and, 1,4-butanediol diglycidyl ether (BDDE) for hemostatic applications and (K) the reactions leading to the ZnO formation. Reproduced with permission from ref⁵²³. Copyright 2019, Elsevier.

4.3.2 Synthetic hemostatic materials

Synthetic approaches, where materials are developed with molecularly designed macromolecular backbones, enable control over the physical and chemical properties of the hydrogel such as swelling, charge, *etc.* Synthetic hydrogel-based internal hemostatic sealants for cardiac reconstruction and pulmonary repair have been commercialized and developed based on PEG (*e.g.*, CoSeal[™],⁵²⁵ Progel[™],²³² and Tridyne[™]⁵²⁶) and cyanoacrylates (Dermabond[®]⁵²⁷) as well as natural components such as gelatin (FloSeal[®]⁵²⁸), BSA (BioGlue[®]⁵²⁹), and fibrinogen (TISSEEL[®]⁵³⁰ and EVICEL[®]⁵³¹).⁷ Recently, Quan *et al.* reported strategies to impart hemostatic properties to the material through crosslinking 2,3-diaminopropionic acid (DapA) with a graphene oxide (GO) sponge, as shown in Figure 4.12.⁵³² Unlike ethanediamine-crosslinked materials, the synthesized hydrogel exhibited less plasma absorption and more efficient activation of the erythrocytes and platelets. Also, negatively charged carboxyl groups along with increased oxidation and fast absorption led to strong hemostatic behavior in rat tail amputation tests. Positive charge has been employed in synthetic hydrogel designs for hemostasis. An epoxy-amine-based sponge was obtained by an aqueous reaction between polyetheramine and butadiene diepoxide agents wherein the abundant amine groups contributed to positive charges.⁵³³ *In vitro* hemostatic tests demonstrated an almost 50% decrease in hemostasis time compared to the

blank controls.

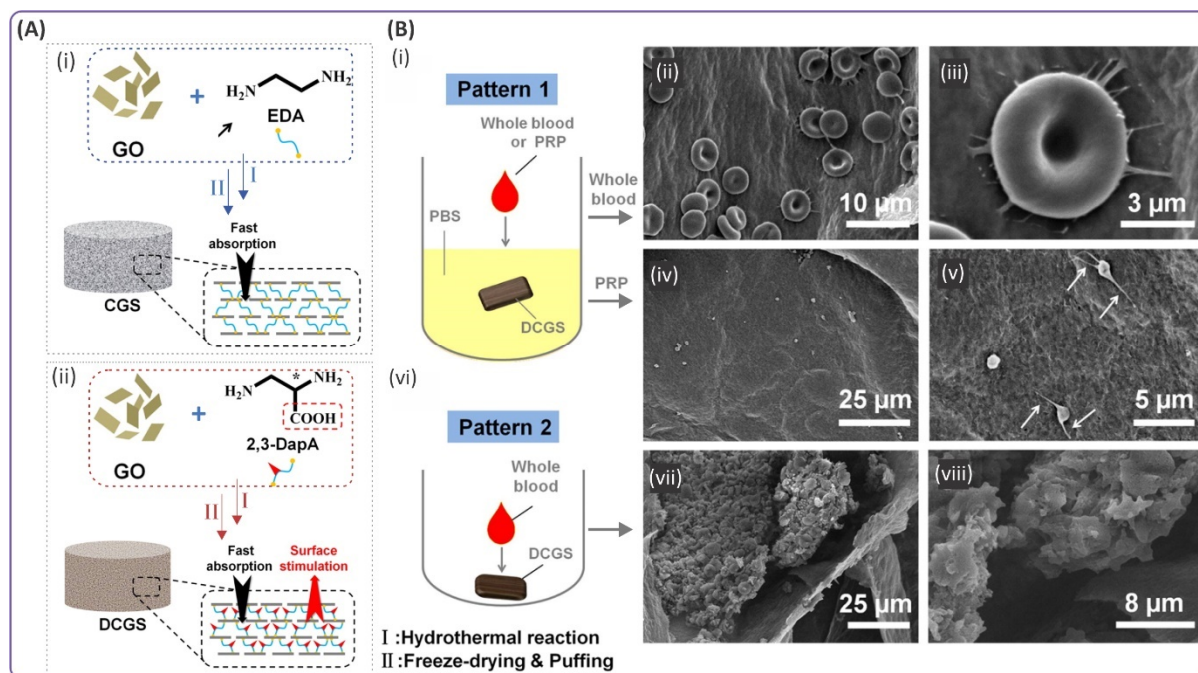


Figure 4.12. The synthetic examples of hemostatic materials. (A) Schematic of 2,3-diaminopropionic (DapA) crosslinked graphene sponge (DCGS) preparation and its hemostatic mechanisms. (i) Combination of graphene oxide (GO) and ethanediamine (EDA) resulting in crosslinked graphene sponge (CGS) capable of fast absorption. (ii) addition of surface stimulation by replacing the EDA with 2,3-DapA for improved hemostatic abilities. (B) Schematic of the interactions between the red blood cells (RBCs) and DCGS hydrogel treated with whole blood and platelet-rich plasma (PRP) under two different experimental approaches, *i.e.*, (i-v) Pattern 1, and (vi-viii) Pattern 2. The magnified scanning electron microscope images demonstrate selective adhesion of the erythrocyte and platelets to the hydrogel. Reprinted with permission from ref ⁵³². Copyright 2016 American Chemical Society.

Due to the biodegradability and biocompatibility issues associated with many synthetic hemostats, hybrid systems combining natural and synthetic components have been developed. For instance, composites of PDA, SA, and polyacrylamide (PAM), *i.e.*, PDA-SA-PAM, were tailored for skin tissue engineering and wound healing applications (Figure 4.13A).⁵³⁴ To prepare the hydrogels, DA was first polymerized in the presence of SA

to form a PDA-SA complex. Next, acrylamide was polymerized *via* a redox free-radical polymerization in the presence of PDA-SA to form the final hydrogel (PDA-SA-PAM). The polymers were characterized in terms of cell and tissue adhesion, hemostatic behavior, and biodegradability, and mechanical properties. The results of *in vitro* blood clotting assays, adhesion tests, and mechanisms of adhesion are shown in Figure 4.13A-C. Briefly, the experimental results revealed that the addition of PDA to the SA-PAM network not only improved the tissue adhesion by over 50%, but it also almost halved the time to hemostasis, aside from the improvement in blood compatibility. Catechol groups enabled hemostatic effects along with tissue adhesion in another hydrogel materials, based on oxidized alginate and poly(lactic-*co*-glycolic acid) (PLGA).⁵³⁵

In other work, it was shown that the hydrogen bonding between a poly(*N*-hydroxyethyl acrylamide) (PHEAA) polymer and the TA formed coacervate hydrogels with self-healing, antibacterial, strongly adhesive, and hemostatic properties (Figure 4.13D-F).⁵³⁶ The high intrinsic adhesive strength of PHEAA (compared to other hydrogels such as PEG and PVA) was further strengthened by the addition of TA. The adhesion, alongside the interactions of TA with the proteins present in the blood, resulted in improved hemostatic activities, as evaluated in the rat liver bleeding model (as indicated by the significant reduction in blood loss). Polyacrylamide (PAM)-based hydrogels mixed with chitosan and Matrigel also showed potential for use as wound dressings due to their hemostatic effects and wound healing properties.⁵³⁷ The fabricated hydrogels showed blood loss below 200 mg, as compared to controls at ~600 mg. Rapid absorption of polyethyleneimine (PEI)/poly(acrylic acid) (PAAc)/quaternized chitosan(QCS) powders applied on the bleeding wounds resulted in effective hemostasis due to the absorption and

aggregation of platelets and blood cells.⁵³⁸ *In vivo* studies on various bleeding models showed that bleeding was stopped within 10 s, suggesting promising potential for full-thickness skin wounds. A Michael-addition-associated network comprised of branched poly(glycerol ethylenimine) (PGE) and glycerol triacrylate (GTA) was mixed with oxidized HA and PDA-coated MXene ($\text{Ti}_3\text{C}_2\text{T}_x$) and characterized with hemostatic properties.⁵³⁹ Although it was not clear whether MXene components play any role in hemostasis, the hydrogel scaffolds could reduce bleeding by over 75%. Metal-organic hydrogels (MOG) have recently emerged as a new class of hemostatic materials. The MOGs were processed following a phase-transfer method using zinc ions (metal nodes) and 4,5-imidazole dicarboxylic acid (organic ligands).⁵⁴⁰ The hemostatic properties of the material were realized through *in vivo* liver bleeding models, where the blood loss was reduced by a factor of 1/3. Moreover, the MOGs could carry cargoes that delivered drugs *via* glucose and pH stimuli.

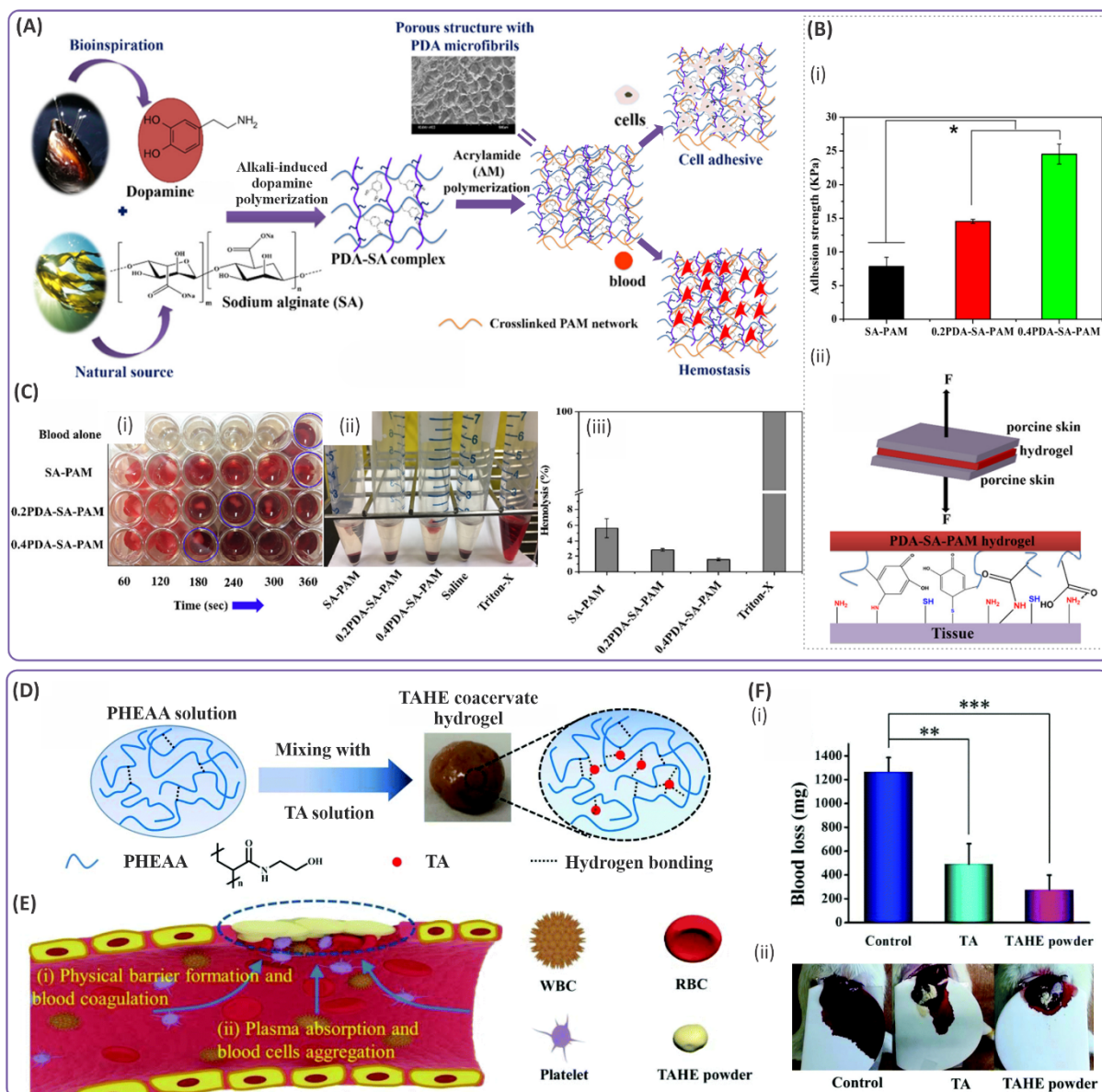


Figure 4.13. Demonstration of hybrid synthetic and natural hydrogel components for use as hemostatic agents. (A) Synthesis and preparation for the hemostatic hydrogels based on polydopamine (PDA), sodium alginate (SA), and polyacrylamide (PAM). (B) (i) Characterization of normal tensile adhesion to porcine skin tissue, and (ii) mechanisms of adhesion. (C) The results of blood clotting and (d) hemolysis assay showing the hemostatic effect of catechol moieties while maintaining hemocompatibility. Reprinted with permission from ref ⁵³⁴. Copyright 2019 American Chemical Society. (D) Hemostatic hydrogels based on the mixture of poly(*N*-hydroxyethyl acrylamide) (PHEAA) and tannic acid (TA) with gelation driven by hydrogen bonding forming TA coacervated hydrogels (TAHE). (E) The mechanisms of hemostasis in the PHEAA/TA hydrogels involving interactions with red blood cells (RBCs) and white blood cells (WBCs) and (F) (i) the results

of *in vivo* rat liver bleeding model in terms of blood loss and (ii) the snapshots from the liver bleeding experiments. Reproduced with permission from ref ⁵³⁶. Copyright 2020, Royal Society of Chemistry.

4.3.2.1 Poly(ethylene glycol) derivatives

As a well-known synthetic backbone, PEG has been widely applied in tissue engineering and drug delivery applications due to its chemical versatility,⁵⁴¹ biocompatibility, non-immunogenicity,⁵⁴² antifouling properties,⁵⁴³ and tissue-mimetic mechanical properties.⁵⁴⁴ Due to the lack of hemostatic properties in neat PEG hydrogels, many studies introduced the hemostatic capacity to PEG-based hydrogels through chemical approaches. For example, Schiff-base-mediated crosslinking of hydrogels combining 4-arm-PEG molecules, capped with amines, NHS, and aldehyde groups resulted in gelation times of less than 30 s.⁵⁴⁵ The hemostatic effect was attributed to the materials ability to provide efficient physical barriers to stop bleeding. In this study, absorption of blood serum through the porous network of the hydrogel assists hemostasis by concentrating RBCs. Application of the tetra-PEG hydrogels for visceral hemostasis was also studied with similar functionalized materials, where hemostasis was achieved within 30 s.²³² To support the physical hemostatic capabilities of PEG hydrogels, polylysine, with inherent hemostatic properties (through aggregating and gelation of fibrinogen),⁵⁴⁶ was grafted on a PEG-based polymer and crosslinked with an enzyme-catalyzed method.⁵⁴⁷ Significantly lower amounts of blood loss (62.2 vs 211.5 mg) compared to controls were observed when the sealant material was tested in a rat hepatic hemorrhage model. Multi-arm PEG is used in many hemostatic hydrogel platforms as crosslinkers. For example, a hydrogel with hemostatic properties was developed based on a combination of four-arm benzaldehyde-terminated

(BAPEG) and DCS (Figure 4.14A-C).⁵⁴⁸ In this hydrogel, a reversible aromatic Schiff base was formed between the amino groups of chitosan and benzaldehydes, leading to self-healing properties. The dodecyl groups on the chitosan backbone were able to anchor onto the cell membrane lipid bilayer, enabling strong adhesion as well as hemostatic effects. *In vivo* vessel and liver bleeding models confirmed the rapid coagulation of blood. In another study, an antimicrobial hemostatic wound dressing material was developed with a mixture of methacrylated HA, PEG (through enzyme degradation), and chlorhexidine diacetate-loaded nanogel, as shown in Figure 4.14D.⁵⁴⁹ The hybrid hydrogel was photocrosslinked and tested by *in vitro* BCI and *in vivo* mouse liver bleeding response to demonstrate its hemostatic properties (Figure 4.14E). While the BCI index for the hydrogel reduced to almost half, compared to the gauze control, *in vivo* bleeding time decreased to 100 s, as compared with a bleeding time of 150 s for the gauze. The hemostatic mechanism was related to the initial physical barrier to the wound supported by swelling of and/or absorption by the hydrogel.

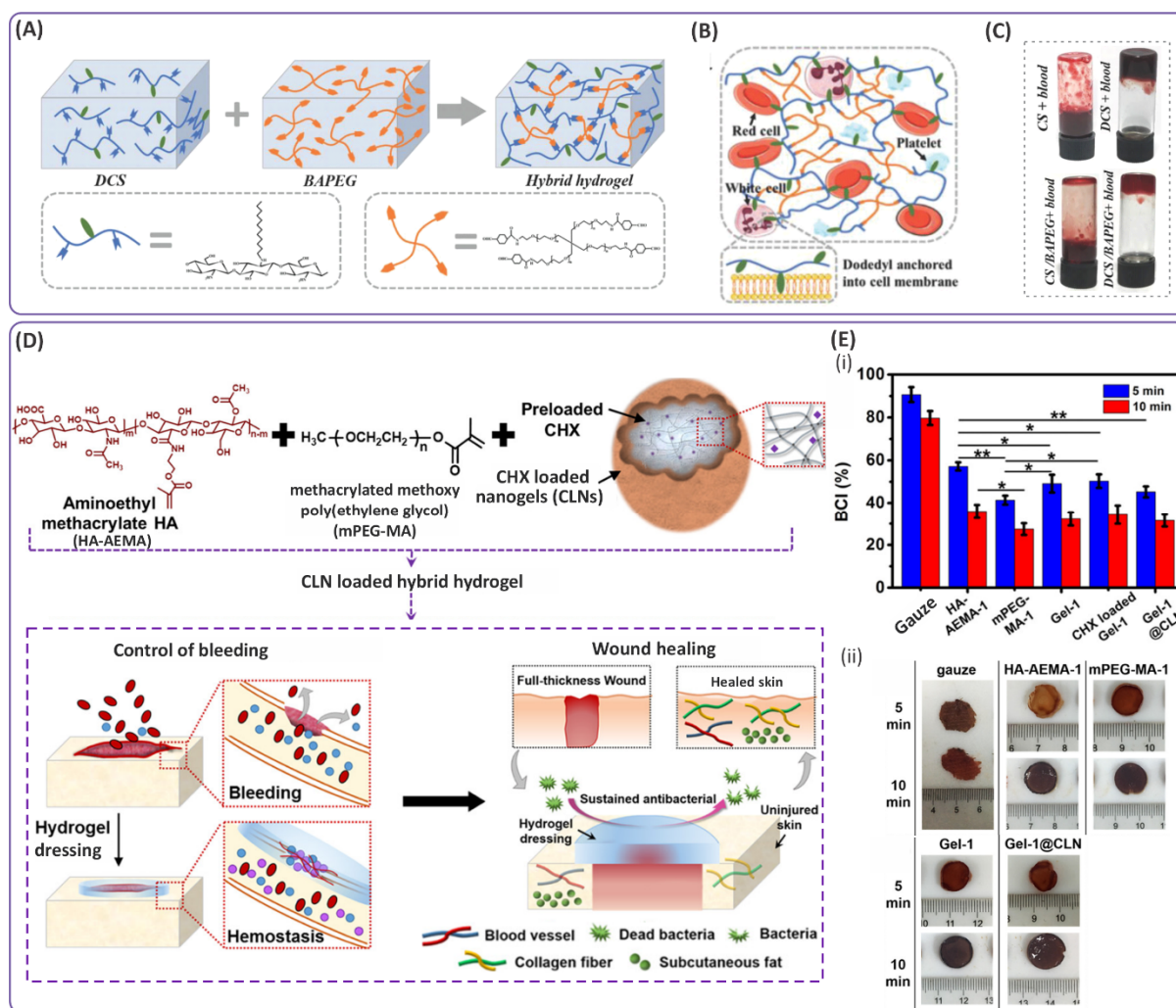


Figure 4.14. Application of poly(ethylene glycol) (PEG)-based hydrogels in hemostatic agents. (A) Schematic of the Schiff base association of dodecyl-modified chitosan (DCS) and four-arm benzaldehyde-terminated PEG (BAPEG) in hybrid hydrogels for hemostatic applications. (B) Schematic of the interactions between blood cells and hemostatic hydrogel leading to hemostatic effects, and (C) effects of chitosan (CS), DCS, and BAPEG on the heparinized blood from the mouse source. Reproduced with permission.⁵⁴⁸ Copyright 2018, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (D) The synthesis route of the hemostatic hyaluronic acid (HA)-based material containing aminoethyl methacrylate hyaluronic acid (HA-AEMA), methacrylated methoxy polyethylene glycol (mPEG-MA), and chlorhexidine diacetate (CHX) loaded nanogels (CLNs), abbreviated as Gel-1, and the mechanism of action for its hemostatic effects. (E) The results of blood clotting index (BCI) for different hydrogels within 10 min, and (ii) digital images of the hydrogels after 5 and 10 min. Reprinted with permission from ref ⁵⁴⁹. Copyright 2018 American Chemical Society.

The polyurethane (PU) foams synthesized from PEG-based diisocyanates also exhibit rapid hemostatic properties. In a recent study, a PEG-diisocyanate solution was reacted with 4,4'-diaminodicyclohexylmethane (PACM) to obtain PU-urea foam (PUUF), which was used as a wound dressing after freeze-drying.⁴²⁰ Rapid water uptake (over 1200% in 2 min) during the swelling test confirmed the efficient and rapid hemostasis effect of the engineered material compared to commercial PU dressings. The BCI for this material was measured to be 21.9% after 10 min of incubation, while the values for the commercial control PU wound dressings were up to 50.6%. In addition, the PUUF showed better biocompatibility and enhanced re-epithelialization.

4.3.3 Mineral hemostatic agents

Mineral granular agents are often used in combination with other hemorrhage control tools such as impregnated fabrics⁵⁵⁰ and gauzes⁵⁵¹ to promote their hemostatic behavior. The hemostatic behavior of this category of materials relies on both absorption and surface charge interactions.⁵⁵² In the following sections, the most common mineral candidates used to promote the hemostatic effects in hydrogels are discussed.

4.3.3.1 Silica

Silica plays an important role in the activation of the coagulation factor XII⁵⁵³ and calcium ions as well as different activation steps of the coagulation cascade through the intrinsic pathway.⁵⁵⁴ Phosphate ions also promote the coagulation process through an extrinsic pathway and stabilizing the blood clot.⁵⁵⁵ Similarly, nano-Bioglass (nBG), causes blood protein aggregation and plays vital roles in the activation of coagulation factors.⁵⁵⁶ A multi-component composite material was developed with chitosan, nBG, silica, phosphate,

and calcium ions where each component played roles in controlling bleeding (Figure 4.15A).⁴¹⁹ The blood-clotting assay shown in Figure 4.15B demonstrated that hydrogels with 2 wt.% chitosan reduced whole blood clotting time by 24%. Further addition of nBG at 5 wt.% almost halved the blood clotting time *via* the mechanism of action shown in Figure 4.15C. Positive charges released from the material were found to be responsible for the hemostatic properties. The hemostatic capability of the hydrogel is due to the synergistic contributions of (i) protonated amines in chitosan (leading to blood cell aggregation), (ii) silica ions released from the nBG that activate coagulation factor XII, (iii) phosphate ions that trigger the extrinsic coagulation cascade, and (iv) calcium ions that activate the coagulation factors. Silica nanoparticles coated with PDA showed potential for antibacterial hemostatic applications. The porous network of the PDA/silica nanoparticles formed by lyophilization along with phenol hydroxyl, amino groups, as well as hydrophobicity was reported to promote the hemostatic effects of the developed material.⁵⁵⁷

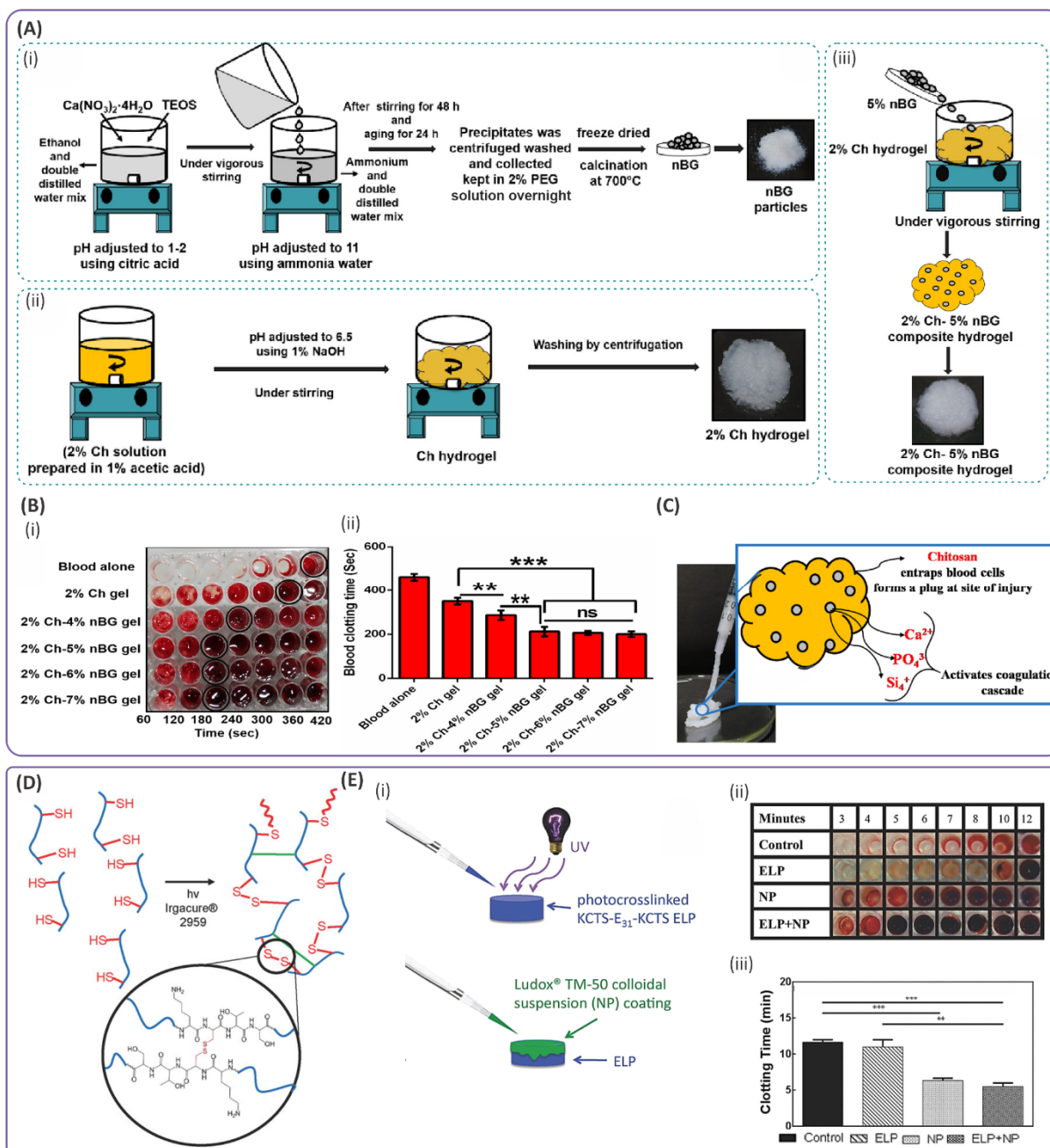


Figure 4.15. Examples of nanocomposite hemostatic hydrogels. (A) Fabrication process for composite hydrogels based on chitosan and preparation procedure of nano-bioglass (nBG): (i) nBG synthesis procedure, (ii) chitosan (Ch) preparation, and (iii) mixing step with tetraethyl orthosilicate (TEOS) to obtain homogeneous final composite hydrogel. (B) *In vitro* blood clotting assay for different component compositions. PEG, poly(ethylene glycol) (B) Results of *in vitro* blood clotting assay and (C) mechanism of action in hemostatic injectable hydrogels based on nBG and chitosan. Reproduced with permission from ref ⁴¹⁹. Copyright 2019, Elsevier. (D) (i) Chemical structure of

photocrosslinkable elastin-like polypeptides (ELPs) macromolecules containing cysteine groups (KCTS-E31-KCTS). Disulfide bonds (red) and crosslinks (green) drive gelation. The ELP solutions are mixed with a photoinitiator and then reduced by tris (2-carboxyethyl) phosphine hydrochloride (TCEP) reagent. (E) (i) Schematic of coating silica with the photocrosslinked elastin-like peptide for improved hemostatic effects. (ii, iii) Blood clotting assay showing clotting time, which can be reduced by ~50% by adding silica nanoparticles. Reproduced with permission from ref ⁵⁵⁸. Copyright 2015, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. Irgacure 2959, 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone.

Diatom silica structures, synthesized from natural diatomite, were coated with chitosan to boost the hemostatic properties of the materials. Chitosan-coated silica showed desirable hemocompatibility (hemolysis below 5%) with a hemostasis time of ~250 s at 10 mg/ml. Here, hemostatic activity was attained through the absorption and aggregation of erythrocyte along with the intrinsic clotting pathway where activation of the coagulation factors happened *via* the polar framework of silanol groups present in the diatom core. A coagulation time of ~100 s was achieved in the *in vivo* test using a rat tail amputation model, which was comparable to the commercial QuikClot® zeolite. Diatom biosilica was coated with PDA and immobilized with thrombin for hemorrhage control.⁵⁵⁹ Here, the thrombin activity was reported to be ~67% after 30 days. In another study, crosslinked elastin-like polypeptide (ELP) polymer coated with a colloidal solution of silica nanoparticles lowered clotting times down to 5 min, from over 10 min for the control, in an *in vitro* blood clotting assay, as shown in Figure 4.15D,E.⁵⁵⁸ The coated ELP hydrogels efficiently stopped bleeding and established hemostasis in lethal liver wound models within 2 min. As opposed to the synthetic silica, diatom frustule silica is highly hydrophilic, rendering its superhemophilicity.⁵⁶⁰ Surface wettability of diatom silica stems from nanoporous structure as well as silanol anions present on their surface. The hemophilicity of natural diatom silica

led to strong hemostatic properties, while hemostatic effect was much less in synthetic silica with hemophobic structure.

4.3.3.2 Silicates

4.3.3.2.1 Zeolite and kaolin

Zeolite and kaolin are aluminosilicates known for rapid water absorption as well as *in situ* calcium release.^{561,562} Hemostatic function of zeolite originates by concentrating the platelets and clotting factors, which occurs through exothermic hydration reactions⁵⁶³ that locally damage the tissues. The kaolin reactions are less exothermic. Kaolin activates factor XII in the blood-clotting cascade.⁴¹⁴ To minimize the cytotoxicity and exothermic hydration issues associated with zeolite, it was blended with alginate and chitosan to enhance biocompatibility and swelling.⁴¹⁴ As seen in Figure 4.16, the alginate/zeolite bead hydrogels were prepared by stirring the solution while adding calcium chloride. Coagulation tests using the hydrogel beads with optimized compositions decreased the blood clotting time from over 9 min (untreated control) to ~2 min. In another study, the addition of kaolin to the synthetic hydrogels based on PAAc and TA was reported to enhance physical crosslinking, to act as a factor XII activator to promote hemostasis, and to enhance tissue adhesion strength.⁵⁶⁴

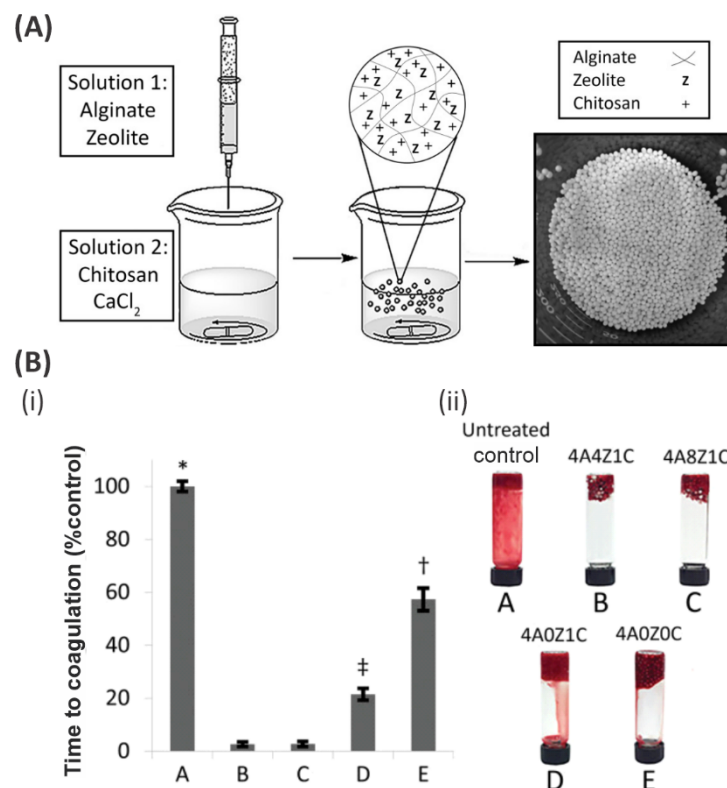


Figure 4.16. Silicates as additives for introducing hemostatic properties. (A) Preparation of alginate/zeolite nanocomposite bead hydrogels for hemostatic applications and (B) (i) results of blood clotting assay with (ii) photographs of blood gelation for the samples treated with different alginate and zeolite compositions (A: alginate, Z: zeolite, C: chitosan). Reproduced with permission from ref ⁴¹⁴. Copyright 2018, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.3.3.2.2 Laponite®

Laponite® is a synthetic silicate comprising of nontoxic components (Na^+ , Mg^{2+} , $\text{Si}(\text{OH})_4$, Li^+) and is used extensively to endow tunable shear-thinning properties to hydrogels, which improves the injectability and delivery of biocomponents. Laponite®-based nanocomposites have been extensively investigated. Having both positive and negative charges on the edge and the surface, respectively, Laponite® particles readily establish strong electrostatic interactions with macromolecules such as polyampholytic gelatin.⁵⁶⁵

Although gelatin molecules are hygroscopic and can absorb fluid components, they are ineffective in initiating the coagulation process. Hence, blends of exfoliated nanosilicate or Laponite®, with gelatin (with concentrations ranging from 3-9 wt.%), were introduced to promote the hemostatic behavior of gelatin, and reduced the clotting time up to 77% (down to 2.5 min).³⁹³ In a Laponite®/PAM nanocomposite hydrogel, incorporation of gelatin avoided nonspecific protein adsorption due to its hydrophilicity and wettability, resulting in an anti-thrombogenic effect. This anti-thrombogenic effect led to enhanced hemolysis and thereby prolonged coagulation time.⁴²³ Another hybrid hydrogel was synthesized with Laponite®, PVA, and alginate molecules where Laponite® particles not only played important roles in increasing hemostasis, they also significantly improved the mechanical strength of the hydrogels.⁴²¹ The hemolysis ratio increased with Laponite® content, but remained within the acceptable limit of 5% for wound closure. The addition of Laponite® was also found to decrease the blood clotting time from 135 min to below 20 min. The hemostatic effect was a consequence of the Laponite®'s effect on the denaturation of fibrinogen. In essence, the negatively charged Laponite® activated the intrinsic pathway of the coagulation cascade by triggering factor XII. Injectable, polysaccharide-based nanocomposites of κ -carrageenan (κ CA) were developed and showed improved hemostatic behavior through the introduction of Laponite® particles (Figure 4.17A).⁴¹⁵ Blood clotting time for the κ CA-nano silicate hydrogels in the range of 100-200 s, which was almost half of those of the control blood samples (Figure 4.17B,C). Blood clot formation was monitored in real time by rheological analysis of the blood fluid (Figure 4.17E). The results confirmed increases in storage modulus, suggesting blood clot formation.

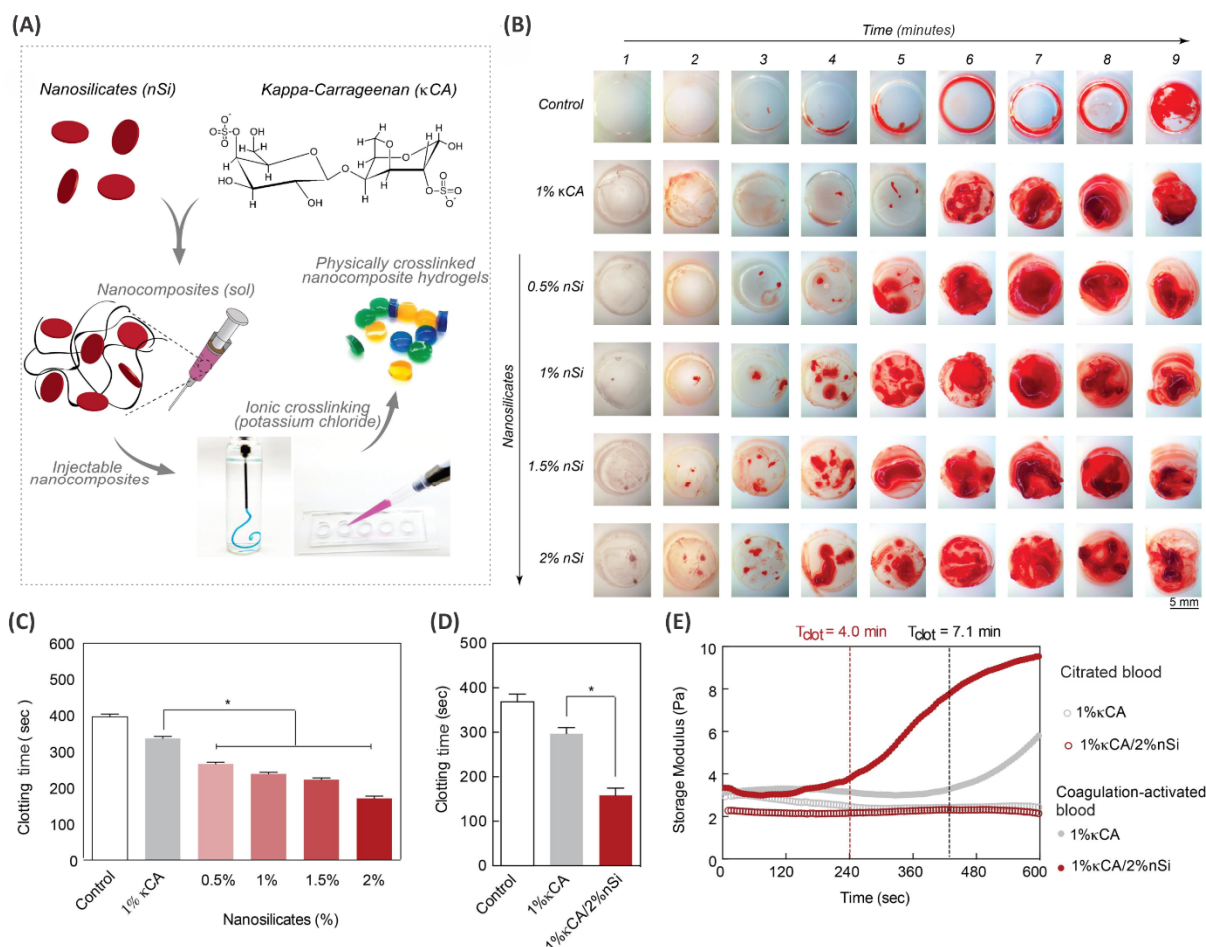


Figure 4.17. Shear-thinning hydrogels enabled by Laponite® nanoclays with hemostatic properties. (A) Schematic fabrication route for the Kappa Carrageenan (κ CA)-nanosilicate (nSi) hydrogels and (B) blood clotting assay confirming promoted blood clotting upon the addition of Laponite®. (C) Clotting time was reduced by ~50% by adding 2 wt.% Laponite®. (D) activated clotting time demonstrating the reduced clotting time. (E) Blood clotting was confirmed through the rheological characterization of storage modulus. Reproduced with permission from ref ⁴¹⁵. Copyright 2018, Elsevier.

4.3.3.2.3 Montmorillonite

Montmorillonite (MMT) is one of the most effective hemostatic candidates to reinforce the hemostatic property in hydrogel systems. The hemostatic effect of MMT is related to its superior plasma absorption capacity among natural phyllosilicates. However,

MMT released in blood vessels may cause thrombosis, and thus, managing the release of hemostatic clay particles remains a challenge.⁵⁶⁶ To avoid this issue, MMT particles were incorporated within GO-based crosslinked composite sponges, as shown in Figure 4.18. This approach resulted in significant reductions in the amount of MMT platelets that were released into the blood and thus, reduced thrombosis. The results suggest that the addition of <15 wt.% MMT could stop bleeding within 84 s in a rabbit artery wound model through the activation of the Hageman factor (Factor XII) and rapid absorption of plasma. The gelatin/alginate hydrogels loaded with MMT and Kaolin, crosslinked with EDC/NHS chemistry, showed excellent adhesion and hemostatic properties.⁵⁶⁷ Compared to Kaolin, at the same concentration, MMT particles potentially had better adhesion to the hydrogel.⁵⁶⁸

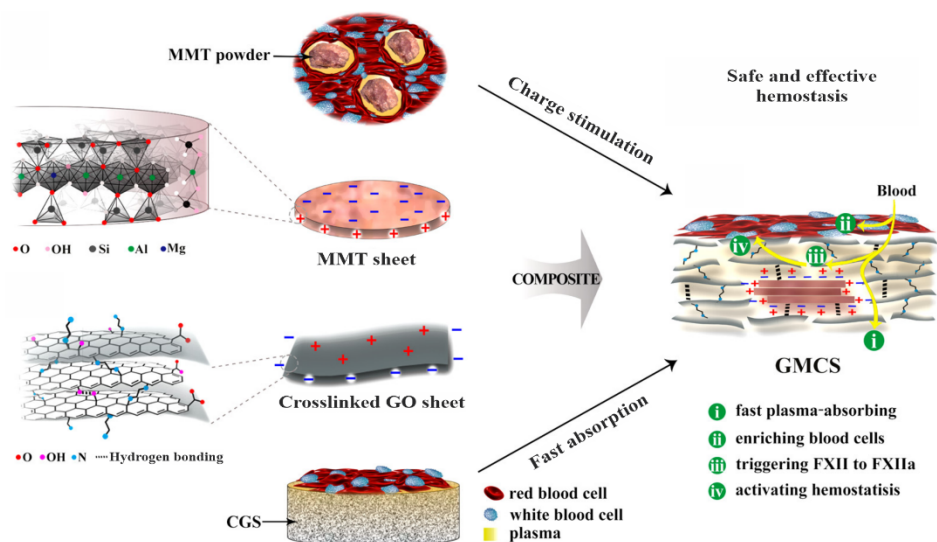


Figure 4.18. Schematic of graphene oxide (GO)/montmorillonite (MMT) composites (GMCS) forming crosslinked graphene sponges (CGS) for hemostasis and wound healing applications. Reprinted with permission from ref ⁵⁶⁶. Copyright 2016 American Chemical Society.

4.3.3.2.4 Whitlockites

Nanowhitlockites ($\text{Ca}_{18}\text{Mg}_2(\text{HPO}_4)_2(\text{PO}_4)_{12}$) have been incorporated in different hydrogel systems, such as chitosan, to induce hemostatic properties. Mechanistically, their mechanism of action is based on the release of Ca^{2+} , Mg^{2+} , and PO_4^{3-} ions that activate the coagulation cascade in multiple ways, as shown in Figure 4.19. Nanowhitlockites are synthesized through precipitation and the resulting nanocomposite hydrogels were found to be cytocompatible and hemocompatible.⁵⁶⁹ Addition of nanowhitlockites significantly reduced both blood clotting time *in vitro* and blood loss *in vivo* by approximately 50% compared to sham controls.

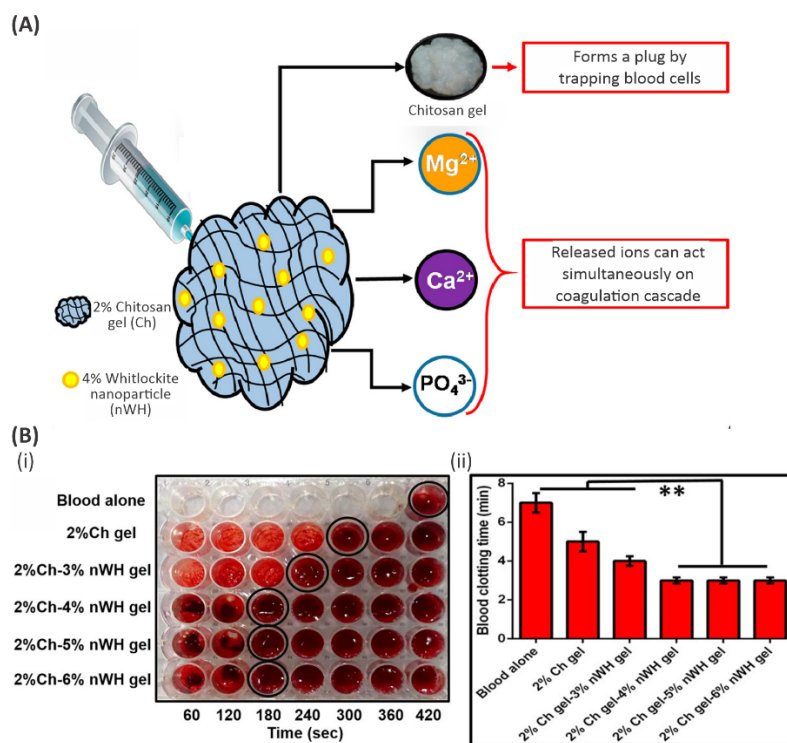


Figure 4.19. Hemostatic properties of nanocomposite hydrogels based on chitosan (Ch) and nano whitlockites (nWh). (A) Schematic of the mechanisms of hemostasis and (B) the *in vitro* blood clotting time assay showing the significant hemostatic effect upon addition of whitlockites. Reprinted with permission from ref ⁵⁶⁹. Copyright 2016 American Chemical Society.

4.3.3.3 Self-propelling particles

Self-propelling hemostatic agents have recently emerged as potential candidates for rapid hemostasis. A recent study developed self-propelling particles that can migrate deep into the wounds and deliver hemostatic agents for rapid blood coagulation.⁵⁷⁰ In this study, natural starch was converted to Janus particles using enzymatic hydrolysis and esterification (using amylase) and negatively charged with phosphate groups (using sodium trimetaphosphate, STMP, reagent) to strengthen hemostatic effects. The negatively charged mesoporous starch (MMS) was combined with CaCO_3 crystals and protonated tranexamic acid (TXA-NH_3^+) to make it self-propelling *via* a bubble detachment mechanism. The particles successfully stopped deep liver and femoral artery bleedings within ~50 s and 3 min, respectively. Layer-by-layer assembly of quaternized starch and TA double layers coated on microporous starch microparticles were developed as hemostatic agents for bone repair.⁵⁷¹ Mouse bone defect models were used to test the potential for bone regeneration, reducing immune response, and effective control of bleeding. A similar approach was undertaken to fabricate self-propelling chestnut-like particles that were able to puncture RBCs for activation of hemostasis.⁵⁷² These biocompatible particles were fabricated by a macro-acanthosphere coated with CaCO_3 and TXA-NH_3^+ , and demonstrated sufficient momentum to travel into deep wounds. As a result, liver and arterial bleeding *in vivo* could be arrested within 90 s and 4 min, respectively, which was significantly lower than commercial Celox particle controls (*e.g.*, ~6 min in arterial bleeding model).

4.4 Potential for clinical translation

Hydrogels and dry biomaterials have demonstrated enormous potential in wound

control by facilitating the first stage of wound healing process, *i.e.*, hemostasis. Wound dressings have also accelerated subsequent stages of wound healing (*i.e.*, inflammation, proliferation, and remodeling) due to their intrinsic wound healing promoting effects and capacity to load various drugs.⁵⁷³ Hemostatic materials and smart hemostatic devices are rapidly being developed to address various clinical needs such as cardiac, arterial, and gastrointestinal hemorrhage.^{430,425} Blood coagulation during implantation of indwelling needles and bleeding after their withdrawal are the main problems with the current needles. A smart indwelling with switchable hemostasis was developed wherein the hemostatic properties could be controlled using a magnetic field.⁵⁷⁴ Here, the outer surface of the needle was coated with a hemostatic material (*i.e.*, catechol-conjugated chitosan), and the internal surface was loaded with an anticoagulant magnetic material (*i.e.*, iron nanoparticles coated with heparin-DA conjugates, FeNP-HepDA). While exposed to a magnetic field, the FeNP-HepDA acts as an anticoagulatory agent to prevent coagulation. Upon the on-demand removal of the magnetic field, the FeNP-HepDA coating is released, and thereby, the chitosan-catechol coating triggers hemostasis to prevent bleeding during needle withdrawal. Self-sealing hemostatic needles were also developed by coating injection needles with catechol-modified chitosan.⁵⁷⁵ Studies suggest that coating temperature plays a key role in arresting bleeding at the hemorrhaging sites. High-temperature ($\sim 60^\circ\text{C}$) coating led to Michael addition dominating the oxidation process of catechol groups, and thereby weaker attachment forces between chitosan-catechol conjugates *versus* low-temperature coating (*i.e.*, 4°C where Schiff base formation dominates). High-temperature coating more efficiently releases hemostatic materials and stops bleeding within <5 s.

In situ fiber deposition using electrospinning technology has been proposed for the

treatment of minimally invasive surgical wounds due to its potential for visceral hemostasis.⁵⁷⁶ A recent study demonstrated electrospinning-based deposition of CuS composite nanofibers for ablation of the *Pseudomonas aeruginosa* (PA) superbacteria using a portable device.⁵⁷⁷ This approach is biocompatible and easy to implement. However, the risk of creepage and short circuits are limiting factors that could be addressed by using the gas-blowing spinning method as a safer alternative.⁵⁷⁸ This technology allows the deposition of a wide range of polymer solutions, such as those based on poly(vinyl pyrrolidone) (PVP), poly(caprolactone) (PCL), poly(methyl methacrylate) (PMMA), and poly(vinyl butyral) (PVB).

As for endovascular embolization, an injectable gel embolic material formulation (GEM) involving gelatin with laponite® and tantalum powders was recently developed to trigger hemostasis for the treatment of vascular hemorrhage.³⁸³ This material can be delivered using catheters and was found to be visible using clinical imaging methods. Furthermore, rapid hemostasis was achieved even in the case of coagulopathic states. Tantalum additives in combination with glass fibers result in strong hemostatic properties, as well (due to their negative charge).⁵⁷⁹

Another translational application of hemostatic materials is in the prevention of tumor recurrence after the tumor resection surgery. Post-operative bleeding is a major cause of tumor metastasis and therefore requires hemostatic materials. In a recent study, a zeolitic imidazolate framework (ZIF-8)-based hemostatic cryogel network with antibacterial and shape memory properties was developed to prevent tumor recurrence in hepatocellular carcinoma.⁵⁸⁰ Dopamine-modified ZIF-8 was incorporated within the polymer matrices based on hematoporphyrin monomethyl ether (HMME), HA-DA, and glycidyl

methacrylate-functionalized quaternized chitosan (QCSG). The obtained material was able to kill cancer cells by generating reactive oxygen species (ROS) and arresting bleeding at the tumor site.

Hemostatic materials have paved the way towards efficient management of surgical and traumatic wounds. For instance, gelatin modified with phenyl isothiocyanate was mixed with GelMA hydrogels to form bioadhesive hemostats and has been proposed for dental applications.⁵⁸¹ These materials have been tailored to address the needs of repairs to a wide range of tissues and organs. Next, the applications of hemostatic hydrogels treatment of bleeding in different tissue types are summarized.

4.4.1 Wound healing

Hemostatic materials not only aim to stop bleeding at the early stages of the wound-healing process, but also to aid in accelerating wound healing. Improvements in wound healing are due to the latest advances in biocompatible materials, which encapsulate the desired drugs and functional components and enhance immune response. One of the main issues of commercially available hemostatic agents is the instability of the materials during blood flow at the wound site. To address such limitations, hemostatic materials with adhesive properties are being explored. For instance, the serotonin-conjugated HA (HA-serotonin) hydrogel, which was already engineered for enhanced hemostatic abilities *in vivo* in a mouse liver hemorrhage model (both normal and hemophilic injuries), exhibited superior wound-healing efficacy compared to a commercially available fibrin-based hemostatic agent.²⁴² Detailed histological analyses also confirmed the formation of blood clots even in the wound area in hemophilic mice. Importantly, the biomaterial prevented abnormal tissue adhesion through anti-biofouling effects. Another multifunctional

bioadhesive engineered by Zhu *et al.*, based on polysaccharides and peptide dendrimers, was found to have high biocompatibility and antimicrobial properties,⁵⁸² which are essential for efficient wound healing. The aldehyde/amine-mediated bioadhesive reduced blood loss, when compared to CoSeal™, a commercially available sealant, in a rat liver hemorrhage model. In this study, hydrogels based on CMCS and peptide dendrimers (*i.e.*, G3KP) had superior and accelerated wound-healing efficacy when tested *in vivo* utilizing a full-thickness rat dorsum incision model. Although the histology data with hematoxylin and eosin (H&E) staining showed initial acute inflammation both for the material and control, the engineered material promoted complete wound healing, in addition to formation of blood vessels and dermis, negligible cell infiltration, and lack of any visible fibrosis. Therefore, the ability to form rapid covalent bonding with tissue surfaces at the incision site made this adhesive biomaterial an excellent candidate for clinical wound closure applications. Recently, an injectable multifunctional composite hydrogel composed of HA-DA and reduced graphene oxide (rGO) engineered by Liang *et al.* exhibited excellent hemostasis along with superior adhesive, antioxidant, and conductive properties.⁵²¹ Interestingly, rGO and HA-DA-based nanocomposites possessed native human skin tissue-like mechanical properties and were used for full-thickness skin regeneration during wound healing. Conductivity of hydrogel enabled excitation of electrical signals between cells across the wound site, and thereby aided wound healing process.⁵⁸³ The HA-DA/rGO adhesive conductive hydrogels were found to have good adhesive strength *in vivo*, comparable to, or even better than, commercial Tegaderm™ film dressings. To evaluate the performance of the hybrid hydrogels on real tissue surfaces and homeostatic conditions, HA-DA/rGO hydrogels were tested in a liver bleeding mouse model and showed good hemostatic capacity. Although strong adhesion is

of needed for efficient wound healing, wound dressings are also required to be detachable on demand.¹⁴ A pH-sensitive crosslinker comprised of a protocatechualdehyde complexed by ferric ions (pH ~10) through their catechol groups was designed to crosslink QCS hydrogels. In this material, aldehyde end groups of protocatechualdehyde formed Schiff base with QCS to fabricate dual-crosslinked adhesive hydrogels. This hydrogel could dissociate by reducing pH to ~8 to facilitate post-wound-closure care in the infected skin lacerations. The hydrogels were further characterized with antibacterial, hemostatic, and NIR-responsive properties.

Diabetic wound healing has been addressed by the wound healing capability of hemostatic materials.⁵⁸⁴ Compared to commercial wound dressings (*i.e.*, Hydrosorb® Gel), modified gelatin had excellent hemostatic and diabetic wound-healing properties.⁴⁶⁶ In this system, gelatin was conjugated with 3-carboxy-phenylboronic acid and crosslinked with PVA. The hydrogel was further supplemented with vancomycin-conjugated silver nanoclusters and nimesulide-loaded micelles to enhance the antibacterial and anti-inflammatory properties. The excellent photothermal antibacterial property, as well as bioactivity of PDA, make this material a promising bioactive wound dressing candidate. The efficacy of mussel-inspired hydrogels and the role of catechol groups in triggering blood coagulation was evaluated by Kim *et al.*⁵⁸⁵ They assessed chitosan-catechol conjugates in terms of hemostatic effects in pre-clinical animal models as well as in human clinical trials for hepatectomies. The materials generated strong interactions with the blood plasma *in situ* and formed a barrier layer within a few seconds due to the synergistic effects of charged chitosan and catechol groups.

4.4.2 Cardiac and arterial reconstruction

Wet adhesion and rapid hemostasis are required in open procedures such as those involving heart and arteries. On the one hand, blood pressure is an obstacle to sealant adhesion; on the other hand, the application of the hemostatic hydrogels poses the risk of release and formation of clots in the blood vessels. The dynamic deformations of these tissues require hydrogels with superior fatigue performance both in terms of adhesion and material cohesion. Many studies have attempted to address the application of hemostatic materials for rapid heart and artery wound closure. Recently, Cui *et al.* developed a highly adhesive hemostatic sealant based on pentaerythritol tetra-acrylate (PETEA), PEGDA, and DA.²³³ The resulting hydrogels rapidly stopped visceral bleeding while strongly adhering to the wound site. This sealant material consisted of a hydrophobic backbone and hydrophilic adhesive catechol side branches, which trigger rapid adhesion upon contact with water. Two distinct models, femoral artery bleeding and liver bleeding in rats were used to study the hemostatic effects of the hydrogels and concluded that the bleeding was quickly and completely stopped with almost no detectable bloodstain. Subcutaneous implantation in rat models showed complete degradation of the material without any indication of thrombosis. Meanwhile, the material was found to be useful for irregular and deep wounds due to its easy injectability. Similarly, Hong *et al.* designed another highly biocompatible adhesive hemostatic hydrogel based on GelMA and glycosaminoglycan HA, *i.e.*, HA-NB, specifically for the repair of the injured arterial and heart bleeds.¹⁷⁹ The GelMA/HA-NB hydrogel rapidly sealed the bleeding site upon photocrosslinking with UV irradiation. A series of *in vivo* experiments were performed to study the efficacy of hydrogels in both rabbit (liver and artery) and pig (carotid arteries and heart) models. Burst pressure tests indicated that the

repaired sites could withstand blood pressures up to 290 mmHg, which is significantly higher than systolic blood pressures in most clinical settings. The GelMA/HA-NB adhesive exhibited good biocompatibility and diminished cytotoxicity when subcutaneously implanted in a rat model. Altogether, the GelMA/HA-NB matrix hydrogels are significantly improved and promising hemostatic sealants. Recently, a silk-based and bioinspired hemostatic sealant was designed and developed by Bai *et al.*³¹⁶ *In vivo* hemostatic capacity of the SF-based sealant (SFT) hydrogels was tested in a rat liver and heart bleeding models. The results demonstrated the SFT hydrogel's instant hemostatic capacity (within 30 s) and tough adhesion in wet and dynamic conditions. This material adhered slightly more strongly to tissue surfaces in the presence of blood compared to in water. Due to their rapid co-assembly and tough adhesion to mechanically robust bleeding tissues, the SFT hydrogels are strong candidates for wound closure and surgical sealing applications. One major challenge in current bioadhesives for sealing cardiac and arterial wounds is non-selective adhesion to other tissues through the backside of bioadhesive.¹⁹³ Asymmetric bioadhesives have been proposed as a solution for postoperative antiadhesion for internal applications.⁵⁸⁶ In one example, injectable gelatin bioadhesives crosslinked with PEG-NHS linkers were treated with alkaline solutions after crosslinking to hydrolyze adhesive NHS groups and deactivate bioadhesion on the hydrogel surface.⁵⁸⁷ The PEG content in this bioadhesive led to antifouling properties and prevented cell adhesion. Although *in vivo* testing demonstrated reduced hepatic and cecum-sidewall adhesion, further application of asymmetric bioadhesives in cardiac and arterial reconstruction is yet to be explored.

4.5 Commercialized hemostatic sealants

Several commercial products have been developed to meet the rising demand for hemostatic sealants in clinical applications. These FDA-approved products are available for the indications noted in Table 4.1. Although some of these products offer advantages compared to the traditional treatments, limited elasticity, inadequate stiffness, and low tissue adhesion, particularly in wet and highly dynamic physiological environments in the presence of blood, are some of their limitations. For example, cyanoacrylate-based sealants possess high adhesive strength but are much stiffer than native tissues; they also have cytotoxicity issues, limiting their utility in internal use.^{588,589} A few of the commercially available hemostatic agents (*e.g.*, Tisseel® and Evicel®) rely on the use of gelatin or fibrin as a sealing matrix while incorporating thrombin and coagulating factors (factor XIII) to achieve hemostasis. Although highly biocompatible, these hemostatic agents do not possess adequate mechanical and adhesive properties.⁵⁹⁰ Adhesives with thrombin⁵⁹¹ as a hemostatic agent rely on the patient's innate coagulatory response, which in some cases is compromised. Moreover, hemostatic sealants containing thrombin and fibrinogen pose the risk of introducing strong coagulation activators into the bloodstream.⁵⁹² Current commercially available products often fail to provide appropriate sealing capabilities and hemostatic properties simultaneously. As such, other hemostatic sealants are under development to fulfill these requirements.

Table 4.1. Commercial hemostatic adhesive products

Product Name	Materials	Indicated Application	Curing Time	Application Method	Most Common Adverse Reactions Observed in Clinical Trials
Tisseel®	Human fibrinogen Thrombin Bovine fibrinolysis inhibitor	Cardiopulmonary bypass	2 min	Spray	Increased D-dimer levels in cardiovascular surgery (but did not exceed values reported in the literature occurring after this type of surgery)
Evicel®	Human fibrinogen Thrombin	Liver/HPB procedures Orthopedic surgeries	10 min (to achieve hemostasis)	Spray Drip	Bradycardia Nausea Hypokalemia Insomnia Hypotension Pyrexia, graft infection Vascular graft occlusion Peripheral edema Constipation
BioGlue®	Bovine serum albumin Glutaraldehyde	For open surgical repairs of aorta, femoral, and carotid arteries	2 min	Extrude	Hemorrhage Infection Myocardial infarction Infection Inflammatory, immune systemic allergic reaction Ischemia Death
ArterX, PreveLeak	Bovine serum albumin Polyaldehyde	Vascular reconstruction	10 min	Extrude	Infection Thrombosis/Thromboembolism Ischemia Myocardial infarction Hypotension Respiratory failure / dysfunction Steal syndrome Pleural Effusion Death
Tridyne	Human serum albumin Poly(ethylene glycol)	Aortic sealing	10 min (to achieve hemostasis)	Spray	Pleural Effusion Anemia Atelectasis Peripheral Edema Thrombocytopenia Nausea Hypotension Pulmonary Edema Hypoxia Hypokalemia Tachycardia
CoSeal	Glutaryl-succinimidyl ester Thiol terminated Poly(ethylene glycol)	Vascular reconstruction	10 min (forms a gel in <3 s)	Extrude	Edema Elevated temperature Erythema Infection Hematoma

					Non-healing wound
Ethicon OMNEX	2-Octyl cyanoacrylate Butyl lactoyl cyanoacrylate	Vascular reconstruction	2 min	Extrude	Bleeding and coagulopathy Erythema Wound infection Dehiscence Lymphocele / lymph Fistula

4.6 Conclusions and prospects

The fast-paced progress in medical sealants has paved the way for advances in sutureless wound-closure techniques. Flexibility of the hydrogel processing techniques has enabled integrated components to promote the required functionalities such as antimicrobial properties. The current state of research indicates the need for hemostatic and adhesive properties as the basic requirements to be met before they can be used for clinical applications for effective sealing of open wounds. The underlying mechanisms behind each of these characteristics are known; however, the materials and processing techniques need to be tailored to achieve further improvements. Due to the unmet needs for safe, fast, and effective blood coagulation at wound and incision sites, more research is required to address a number of key aspects of hemostatic materials.

One major challenge is to prevent the release of blood-clotting agents into the blood downstream particularly for those of hemostatic hydrogels with nanocomposite bases. The effects of the release of hemostatic agents on local coagulation has not yet been sufficiently addressed. From this perspective, moving from active hemostasis methods to passive hemostatic mechanisms may be advantageous. Swelling and adhesion-driven hemostasis may be preferred over other approaches, due to their increased safety over those of composite hydrogels involving blood-coagulating components.

There have been successes in achieving rapid hemostasis in terms of the enhanced

adhesiveness in state-of-the-art hydrogels. The development of strong adhesive hydrogels with engineered mechanical properties matching those of host tissue should be a priority. From a mechanical standpoint, the ideal sealant should impose minimal resistance against natural deformations of the tissue. Soft but elastic hydrogels with high adhesion and stretchability are the main target for this requirement. These properties lead to better conformability to underlying tissue and mechanically favor natural tissue adhesion. Dynamic tissues such as the heart and skin (that may undergo up to ~50% strain during daily activities) impose significant constraints in this regard. Current hemostatic bioadhesives mostly adhere non-selectively on both sides. More efforts are required to leverage one-sided bioadhesion to the target tissue to avoid unwanted adhesion. In addition, further developments to fabricate on-demand detachable bioadhesives are highly encouraged. Adhesive hemostats are susceptible to detachment due to fatigue. Fatigue failure of hemostat hydrogels may affect either the material or the interfacial adhesion and needs to be addressed experimentally. Although superabsorbent hydrogels have been developed with significant attention to hemostasis effects, the negative impact of hydrogel swelling on hydrogel tissue adhesion is a major concern that should be addressed. A compromise could be made in future material designs between wet adhesion and hydrogel swelling behavior in hemostatic materials.

Note that the required adhesive properties differ from tissue to tissue. Most studies thus far have been biased towards tissues such as liver, heart, lung, and skin, whereas other soft tissues, such as nerve, and hard tissues, such as bone, need further attention. In more challenging tissues, such as nerves, where apart from adhesion, electrical conductivity and elasticity must be addressed, there is plenty of room for translational developments. The lack

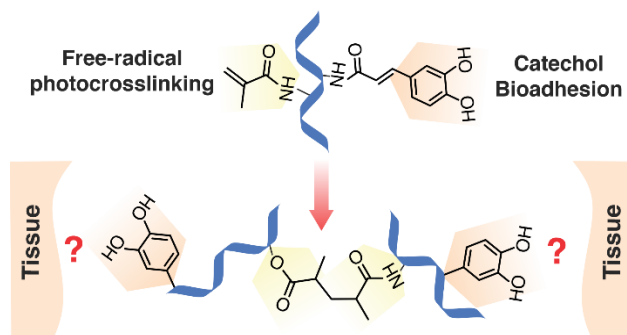
of standardized methods for measuring hemostatic characteristics presents obstacles in comparing and contrasting the observations in the literature, given the varying material properties identified. Hence, development of standardized testing devices and methods are needed for materials comparisons and further development.

With the advent of smart hydrogels, many other possibilities open up for wound control. For instance, temperature-responsive hydrogels with LCST behavior allowing gelation at body temperature can enable the application of the hemostatic materials in minimally invasive surgeries and therapeutics where photocrosslinking would be challenging. Smart hydrogels, responsive to external stimuli, may turn on and off the hemostatic properties in the hydrogels to control the local hemostatic behavior and avoid unwanted downstream blood clotting. The role of electrical excitation and material conductivity in hemostasis and wound healing processes remain unexplored and require further attention.

For commercialization, the precise application (*i.e.*, type of tissue and wound) and context of use (*i.e.*, self-administration, surgery, emergency use) are the key factors in the development of novel hemostatic constructs. Responsive hydrogels may be promising for surgical applications, where trained operators can induce the desired response. However, such smart materials may lack controllability in the case of difficult to access wounds and require extra training for their use, which may not be practical in emergencies such as where frontline workers deal with accidents. Complex state-of-the-art materials might not be appropriate for broad use, where lower quality hemostats with easier handling, storage, and accessibility would be indicated. The development of smart, auto-responsive hemostatic materials that adapt to all wounds is nonetheless an idealized target. Prolonged shelf life,

enhanced biocompatibility, as well as non-immunogenicity are amongst other needs to be met in current commercial hemostatic materials. Future research will further explore different materials and techniques in terms of their hemostatic behavior, to provide better and more efficient solutions for wound management.

CHAPTER 5: CATECHOL CONJUGATION FOR BIOADHESION IN PHOTOCROSSLINKABLE BIOMATERIALS



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Abstract

Inspired by mussel adhesion, catechol functionalization of different biomacromolecules has become a popular technique to impart adhesion to biomaterials. However, the bioadhesion capacity of catechol motifs in hydrogels that crosslink through free-radical-based systems is debated. Here, we revisit the bioadhesion efficacy of catechol functionalization in methacryloyl-modified photocrosslinkable biomaterials. For this purpose, gelatin biomolecules functionalized with caffeic acid (CA) and methacryloyl groups

are crosslinked using UV light to assess their crosslinking and bioadhesion. Despite the typical emphasis on wet adhesion of catecholic biomaterials, samples with larger catechol content showed lower adhesion strength, particularly when bioadhesives are crosslinked *in situ* as injectable sealants. Crosslinking was found to decay toward the interface with the substrates as more catechol groups are introduced to the hydrogels, which further undermines hydrogel integrity and adhesive bonds. Finally, cytotoxic and immunostimulatory effects associated with the catechol groups are evaluated for their translational potential in clinical applications.

5.1 Introduction

Bioadhesive hydrogels have emerged as an attractive toolbox for sealing wounds,^{43,2} for integration of bioelectronic devices,^{41,192} and artificial tissue constructs^{13,593-595} with living tissues. Current biomaterial design methodologies for bioadhesion are primarily based on hydrogel-tissue molecular interactions at their interface.^{8,7} Multiple functional groups capable of forming covalent and non-covalent associations with tissue surfaces have been exploited to endow hydrogels with adhesion through chemical modification procedures.¹⁷⁰ Catecholic residues present in ocean organisms such as mussel foot proteins are responsible for their strong adhesion underwater due to their ability to form chemical bonds to a wide range of substrates.^{9,1} Inspired by mussel adhesion, integrating catecholic moieties with biomaterial backbones has therefore become a gold-standard technique for introducing adhesion to natural biomacromolecules such as gelatin.⁴² Gelatin in particular, has received extensive attention due to its extracellular matrix-mimicking properties including favorable biodegradability, biocompatibility, and cell adhesion.⁵⁹⁶

One of the critical requirements of injectable bioadhesives for sealing surgical wounds is the ability to crosslink their pre-gel solutions *in situ* at the wound site. Physical mixing of catechol-functionalized polymers with oxidative agents can initiate crosslinking through di-catechol coupling reactions.²⁷⁶ However, in addition to cytotoxicity concerns due to oxidative reagents such as ammonium persulfate, catechol groups at the tissue interface are consumed for intermolecular crosslinking rather than participating in covalent interactions with tissue surfaces.¹⁰ Hence, free-radical photocrosslinking through methacryloyl motifs using on-demand triggers such as light has become a popular approach for gelation of hydrogels.⁵⁹⁷ Crosslinking of hydrogels *via* methacryloyl groups not only leads to robust mechanical properties but also enables covalent adhesion bond formation with biological tissues.⁵⁹⁷ In essence, during polymerization propagation reactions, free-radical-activated methacryloyl groups at the vicinity of wet tissues can covalently bond to nucleophilic groups present on the tissue surface through Michael addition reactions.^{59,65}

Recent efforts towards photocrosslinkable bioadhesives aim at a more robust bioadhesion, which has been pursued through co-modification of polypeptide backbones such as gelatin with both methacryloyl and catechol groups. However, most of these approaches seem to be associated with major limitations. For instance, He *et al.*⁶² and Xuan *et al.*⁶³ characterized bioadhesion of catechol-conjugated Gel-MA bioadhesives; however, the results were not compared to Gel-MA controls with no catechol conjugation thereby the roles of catechol conjugates in their bioadhesion remains inconclusive. In another study,⁵⁹⁸ tissue-adhesive constructs based on catechol-modified Gel-MA were bioprinted and crosslinked oxidatively *via* catechol groups. However, the methacryloyl groups were left uncrosslinked and therefore the need for methacryloyl modification remains unclear.

A number of studies that developed catechol-functionalized Gel-MA proposed dual crosslinking processes involving a photocrosslinking step in combination with an oxidative catechol coupling process.^{45,64,599} Although these studies have reported improvements in adhesion strength of GelMA-catechol as compared to Gel-MA controls, it remains unclear whether this improvement is due to the strengthened network cohesion (because of the secondary crosslinking *via* catechol groups) or the adhesive bonds established through catechol groups. In other words, the roles of catechol groups in adhesion of hydrogels in the absence of the secondary oxidative crosslinking in Gel-MA-catechol bioadhesives is unknown.

We note that conflicting evidence has been reported on the bioadhesion capacity of catechol motifs for photocrosslinkable sealants. For example, Baghdasarian *et al.* reported no significant difference in burst pressure between Gel-MA and Gel-MA-catechol conjugates (both crosslinked solely by visible light), whereas adhesion strength was found to be significantly higher for Gel-MA-catechol in wound closure tests. In addition, while *in situ* photocrosslinking of dopamine-modified Gel-MA was associated with improved adhesion to skin tissue⁶⁰⁰ and titanium surfaces,²⁵ in lap shear tests, recent findings²⁴ suggest a negative correlation between catechol content and burst pressure. Therefore, we believe that greater understanding of the roles of catechol moieties in bioadhesion is required to assess the clinical benefits and relevance of catechol modification for introducing bioadhesion to injectable surgical sealants.

Here, we aim to provide a balanced assessment of catechol-conjugation effects on bioadhesion of photocrosslinkable biomaterials. For this purpose, gelatin biomacromolecules are grafted with catecholic moieties (*i.e.*, caffeic acid). Then, a

methacryloyl-modification reaction is executed to introduce methacryloyl groups, and thereby to enable free-radical photocrosslinking *in situ* by UV light for injectable bioadhesion. After the chemical characterization of gelatin modification, a rheological approach is undertaken to study crosslinking of hydrogels. Subsequently, the effects of chemical modifications on mechanical and adhesion properties are investigated. Finally, these effects are studied in terms of biological responses of living cells through *in vitro* biocompatibility and immunoactivity studies.

5.2 Results and Discussion

5.2.1 Synthesis of methacryloyl-modified gelatin-catechol conjugates

The synthesis route for modification of gelatin with catechol and methacryloyl groups is shown in Figure 5.1a. To functionalize gelatin with catechol groups, the standard carbodiimide reaction (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC/*N*-hydroxysuccinimide, NHS) was performed to couple carboxyl groups of caffeic acid (CA) catechol-carrying molecules to the primary amine groups present on the gelatin backbone (resulting in gelatin-caffeic acid conjugates, Gel-CA, as shown in Figure 5.1aI). We design this reaction with excess caffeic-acid-to-gelatin weight ratio to maximize the grafted catechol amount and thereby to magnify its effect on bioadhesion. However, the catechol loading capacity remains limited to the number of available conjugation sites on the gelatin backbone as well as the low yield of carbodiimide coupling (reported to be <10% based on previous publications).⁴⁷ To increase catechol loading efficiency beyond its capacity in carbodiimide coupling, a recently reported protocol⁴⁰ was implemented where catechol groups are oxidized into higher molecular oligo-catecholic species prior to the amidation

reaction. For this purpose, a set of gelatin-catechol conjugates was prepared *via* conjugation of oxidatively oligomerized CA moieties (Figure 5.1aII): The CA oligomers (CAo) were synthesized using NaIO_4 prior to the EDC/NHS conjugation reaction with gelatin, resulting in gelatin-caffeic acid oligomer conjugates (Gel-CAo). Both Gel-CA and Gel-CAo products were then subjected to a methacryloyl-modification using different concentrations of methacrylic anhydride (MA) to graft methacryloyl groups *via* the hydroxyl and unreacted primary amine groups (Gel-CA-MAx and Gel-CAo-MAx final products, respectively, where x represents the concentration of MA in %v/v). Due to the rising interest in injectable bioadhesives with on-demand triggerable crosslinking, this methacryloyl-modification reaction was implemented to enable their free-radical photopolymerization using 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure2959) photoinitiators under the trigger of UV light.

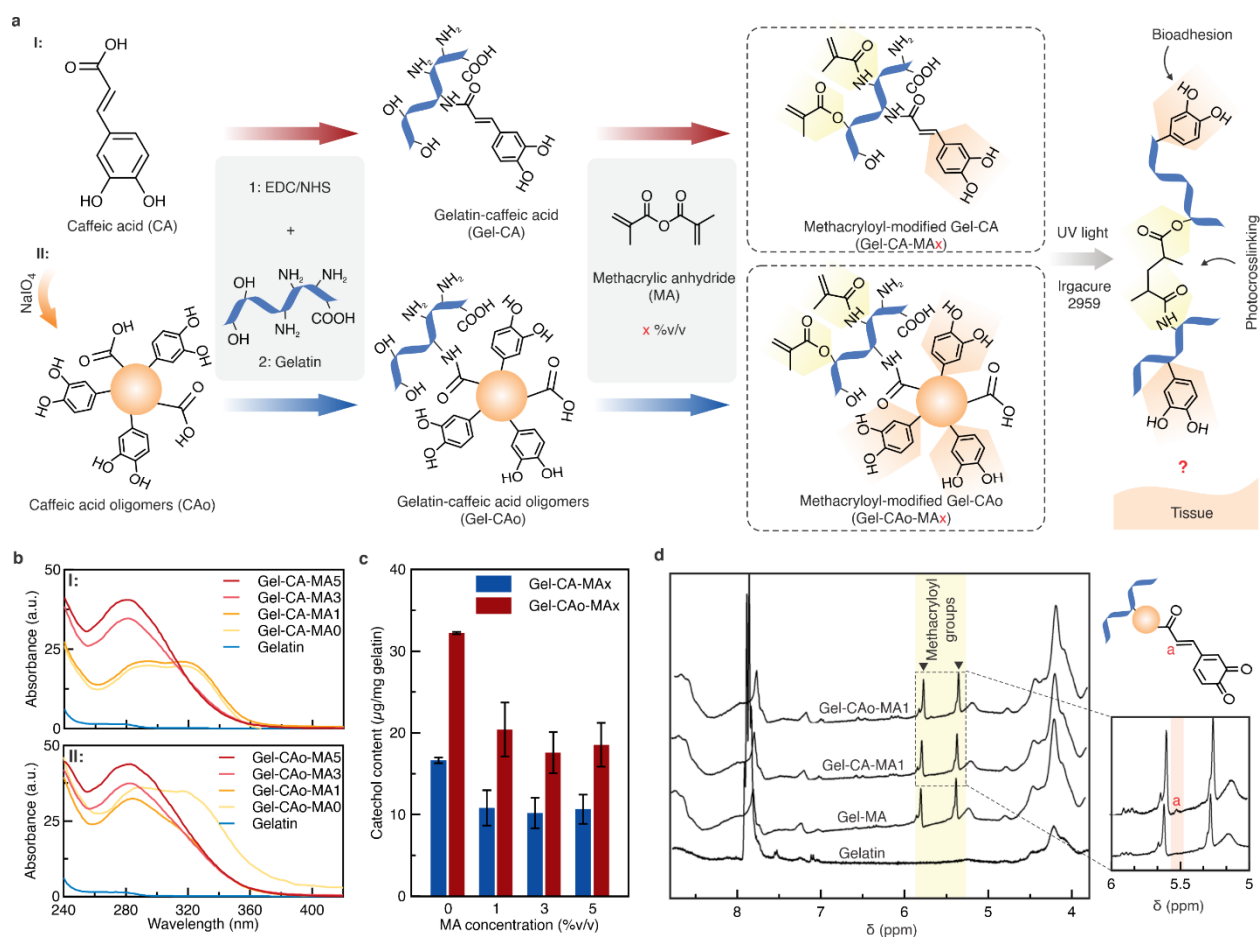


Figure 5.1. The biomaterial design approach for assessment of catechol effects on adhesion of photocrosslinkable methacryloyl-modified biomaterials. (a) The synthesis route of bioadhesives starts from I: caffeic acid (CA) catechol-carrying molecules, which couple to gelatin backbone *via* carbodiimide coupling reaction (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC/*N*-hydroxysuccinimide, NHS) to yield gelatin-caffeic acid (Gel-CA) conjugates. The Gel-CA components are modified with methacryloyl groups using $x = 1, 3, 5$ v/v% concentration of methacrylic anhydride (MA), resulting in methacryloyl-modified Gel-CA (Gel-CA-MAx). Bioadhesives with a larger amount of catechol groups were synthesized *via* II: pre-coupling oxidative oligomerization of caffeic acid (*i.e.*, CA oligomers, CAo). Gelatin-CAo conjugates (Gel-CAo) were then methacryloyl-modified to produce methacryloyl-modified Gel-CAo (Gel-CAo-MAx, x is the concentration of MA in %v/v). Pre-polymer solutions photocrosslinked using 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure2959) photoinitiators using UV light. (b) UV-vis spectrum of Gel-CA-MAx and Gel-CAo-MAx solutions. (c) Results of catechol content (Arnow assay) grafted to gelatin backbone. (d) Proton nuclear magnetic resonance spectroscopic (¹H NMR) assessment of methacryloyl modification and catechol conjugation to gelatin. Gel-MA, gelatin methacryloyl.

We note that to be able to study the effect of catechol content (*via* pre-conjugation oxidative polymerization approach presented here), the catechol-carrying small-molecule candidate for conjugation is preferred to be carboxyl end-capped (as is the case with CA) because carboxyl groups are preserved in the repeating units of polymerized components, *i.e.*, CAo.³⁶ Otherwise, *e.g.*, in the case of amine end-capped catecholic molecules such as dopamine (DA), primary amine conjugation sites are mostly turned into secondary amines in polydopamine moieties,⁶⁰¹ which are incapable of binding to NHS-activated carboxyl groups of gelatin. In addition, conjugation of catecholamines requires NHS activation of carboxyl in gelatin, which promotes homocoupling reaction and further reduces the reaction efficiency. Therefore, catechol conjugation was performed through primary amine groups of gelatin molecules. Since the methacryloyl-modification reaction is non-selective towards both primary amines and hydroxyl groups of gelatin,²⁰ the catechol conjugation step was performed prior to the methacryloyl-modification reaction.

5.2.2 Chemical characterization

To characterize the chemical effects of the conjugation reaction, UV-vis spectra of modified gelatin components are studied in Figure 5.1b. As seen, no significant absorption peak was found in the unmodified gelatin between 250 and 400 nm. In the gelatin-catechol conjugates without methacryloyl modification (*i.e.*, Gel-CA-MA0 and Gel-CAo-MA0 in Figure S5.1a,b, respectively) two absorption peaks at 280 and 320 nm were observed, which corresponded to the peaks found in CA indicating conjugation of CA moieties to gelatin backbone. These absorption peaks are attributed to the π - π^* transition from the aromatic ring and n - π^* transition due to the —OH groups connected to the aromatic ring in CA structure, respectively.⁶⁰² As shown in Figure S5.1, the values of absorbance at these

wavelengths are significantly larger in the group of Gel-CAo-MA0 compared to Gel-CA-MA0, supporting that a larger amount of CA-based moieties grafted to the Gel-CAo-MA0 group. Correspondingly, the absorbance band at wavelengths larger than 320 nm in Gel-CAo-MA0, which was absent in Gel-CA-MA0 samples is assigned to the formation of o-quinone structures and larger molecular weight CAo with oxidation of CA species (*e.g.*, see 400 nm in Figure 5.1bI *versus* Figure 5.1bII). Methacryloyl-modification of both groups of gelatin-catechol conjugates (*i.e.*, Gel-CA and Gel-CAo) was accompanied by an attenuation of absorbance peak at 320 nm while a peak intensification at 280 nm was observed. Based on the functional groups associated with those peaks,⁶⁰² the former can be explained by the reaction of MA with the hydroxyl groups in some of the conjugated catechol groups. The latter are due to the π conjugates of methacryloyl groups grafted to gelatin-catechol molecules. These chemical reactions are studied in Figure 5.1c,d, respectively, as detailed below.

The amount of catechol groups conjugated to gelatin was studied *via* Arnow assay as shown in Figure 5.1c. In the samples with no methacryloyl modification, significant increases in catechol content were attained with pre-conjugation oxidation of CA (*i.e.*, Gel-CAo-MA0 compared to Gel-CA-MA0). This increase is attributed to (i) the larger number of catechol groups and conjugation sites per molecule in CAo and (ii) the Schiff base coupling of oxidized CA to primary amine groups of gelatin in addition to carbodiimide coupling. Methacryloyl groups of gelatin-catechol components, however, contained a less amount of catechol groups, possibly due to the reaction between MA and the —OH of catechol groups, which is in accord with UV-vis peak decay at 320 nm as discussed above (in Figure S5.1b). Although methacryloyl groups could impact the number of free catecholic moieties on the gelatin

backbone, catechol groups remained more abundant in the Gel-CAo-MAx samples compared to Gel-CA-MAx.

Molecular characteristics of synthesized samples were further investigated *via* proton nuclear magnetic resonance (^1H NMR) as shown in Figure 5.1d and Figure S5.2. In the group of Gel-CAo-MAx samples, a new peak at ~ 5.5 ppm was observed (Figure 5.1d), which was absent in their Gel-CA-MAx counterparts. We assign this peak to the oxidized caffeic acid quinones due to the chemical shift of protons at the double-bond conjugates of CA side chains.⁶⁰³ A similar peak was reported previously in the corresponding proton of pyrrole group in 5,6-indolequinone moieties.⁶⁰⁴ Conversion of primary amine groups due to catechol conjugation (measured based on the consumed lysine peaks) was $\sim 8\%$ and 43% for Gel-CA-MA0 and Gel-CAo-MA0, respectively, according to our previous study.³⁴ As far as catechol conjugation, a larger degree of amine consumption (DoC) in Gel-CAo-MAx compared to Gel-CA-MAx corroborates its larger catechol content, which is in accord with the results of the Arnow assay (Figure 5.1c). The DoC for all methacryloyl-modified samples, which represents both catechol functionalization and methacryloyl modification was found to be in the same order between $\sim 82\%$ and 86% with no significant differences across different MA concentrations (Figure S5.2). Note that in addition to amine groups, MA can also react with hydroxyl side chains,²⁰ thereby its conversion degree can cause a difference in overall methacryloyl content of samples synthesized at different MA concentrations. Assuming a similar level of hydroxyl conversion at the same MA concentration, Gel-CA-MAx is expected to carry a larger amount of methacryloyl groups compared to Gel-CAo-MAx because more unreacted available primary amines were left for methacryloyl modification after the initial catechol conjugation reactions.

5.2.3 Rheological analysis of photocrosslinking kinetics

To study the crosslinking characteristics of hydrogels, the rheological evolution of pre-polymer solutions in response to UV irradiation was monitored in real time (Figure 5.2a). Variations of storage (G') and loss (G'') modulus with UV light exposure are shown in Figure 5.2b and Figure S5.3, based on which the reaction kinetics were calculated (slope of G' with crosslinking time) in Figure S5.4. A sharp increase in G' followed by crossing G'' corresponded to the gelation point. In both highly methacryloyl-modified samples of Gel-CA-MA5 and Gel-CAo-MA5, the gelation point was reached before UV exposure due to thermal gelation of pre-polymer solution. Note that the tests were performed using a custom-made add-on setup (**Figure 5.2a**) at room temperature to simulate operating conditions. In these samples, the reaction kinetic profiles provide better understanding of their gelation time, as covalent crosslinking proceeded at higher rates than thermal gelation (Figure S5.4) based on which the onset of covalent crosslinking could be determined. As opposed to Gel-MA, where decay in the G' rate was observed after the peak of the reaction rate (due to saturation of polymerization), catechol-functionalized samples reached a plateau at lower rates, at least one order of magnitude smaller than the maximum reaction rate of Gel-MA samples.

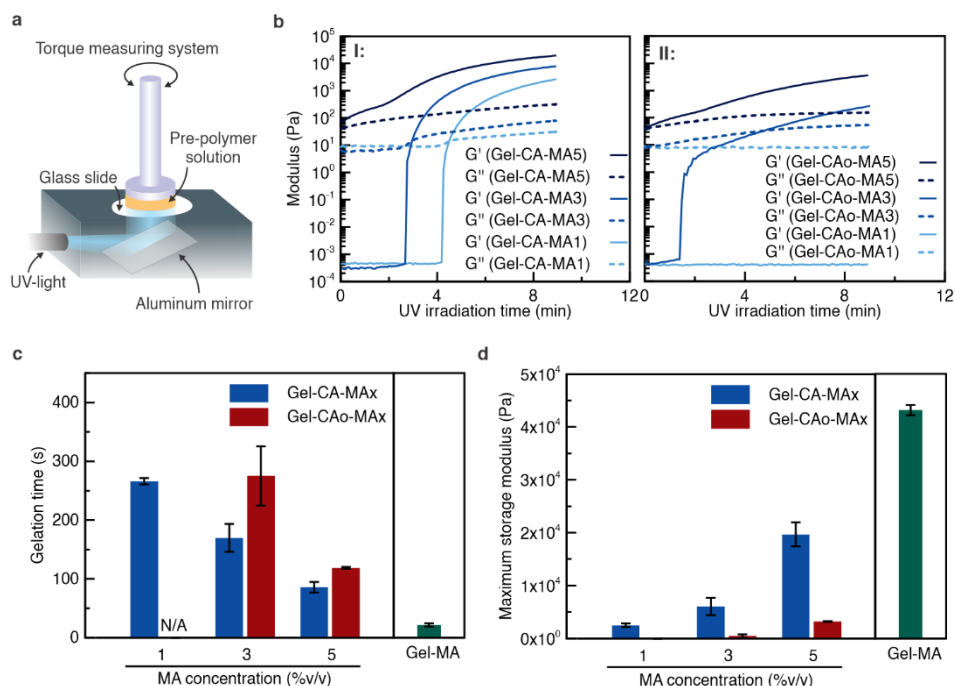


Figure 5.2. Rheological investigation of photocrosslinking kinetics using UV light. (a) Experimental setup for exposure of bioadhesives to UV light during real-time rheological measurements. (b) Variations of storage (G') and loss (G'') modulus with UV irradiation time for I: methacryloyl-modified gelatin-caffeic acid (Gel-CA-MAx) and II: methacryloyl-modified gelatin-caffeic acid oligomer (Gel-CAo-MAx) conjugates, where x represents the amount of methacrylic anhydride used during synthesis in %v/v. Results of (c) Gelatin time and (d) maximum storage modulus obtained from the rheological photocrosslinking studies. Gel-MA, gelatin methacryloyl.

The effects of catechol-modification and degree of methacryloyl-modification on crosslinking are shown in Figure 5.2c,d in terms of gelation time and maximum G' ($G'_{\max}$, measured at 10 min), respectively. Longer gelation times were attained for samples synthesized at the lower MA concentrations as well as for those with greater catechol content (*i.e.*, Gel-CAo-MAx group). Note that the long gelation times of greater than 4 min in hydrogels with the lowest methacryloyl modification (*i.e.*, Gel-CA-MA1 and Gel-CAo-MA1) render these conditions impractical for use in surgical settings; therefore they were not characterized further. For other samples, G' reached close to the $G'_{\max}$ ($G'/G'_{\max}$ ratio of $\geq 20\%$ and $\geq 35\%$

for MA concentrations, $x = 3$ and 5 %v/v, respectively) at UV irradiation times of greater than 5 min based on which crosslinking time was determined for hydrogel preparation (Figure 5.2c). Correspondingly, $G'_{\max}$ after crosslinking (which is correlated to crosslinking density based on the Flory–Rehner theory of elasticity)⁶⁰⁵ increased with the concentration of MA used in the synthesis process. The values of $G'_{\max}$ were inversely proportional to the catechol content in the hydrogels, suggesting that catechol groups have an inhibitory influence on the free-radical polymerization of hydrogels. This effect is in agreement with previous reports^{45,24} and is attributed to the radical scavenging property of catechol groups,⁶⁰⁶ which leads to lower crosslinking density. Chain transfer reactions to catechol groups can be another factor leading to weak free-radical polymerization.⁶⁰⁷ According to a previous study⁶⁰⁷ such termination reactions are reported to be more applicable to polymerization of vinyl-carrying monomers compared to those of methacrylic monomers. Nevertheless, the result of this study suggests significant sensitivity of methacryloyl monomers to catechol groups in presence of catecholic compounds. We note that the lower number of methacryloyl motifs in samples with larger catechol content (as discussed above) is also responsible for their slower polymerization kinetics.

According to the above results, radical polymerization of catecholic monomers requires protection of catechol groups.^{323,608} However, catechol deprotection after gelation is impractical for injectable *in situ* crosslinking bioadhesives in clinical settings rendering catechol conjugation an incompatible approach for bioadhesion in free-radical-based photocrosslinking systems.

5.2.4 Physical and mechanical properties

Hydrogel cohesion is an important criterion in the design of bioadhesives; hence,

tensile mechanical tests were performed to evaluate hydrogel network integrity under mechanical loads (see Figure 5.3a). The trends in elastic modulus (Figure 5.3aI) were consistent with those of G' values after crosslinking (Figure 5.2d). In general, the lower elastic moduli of catechol-modified samples compared to Gel-MA are representative of the enhanced compliance of hydrogels on tissues. In terms of tensile strength, no significant differences were observed across all conditions except Gel-CAo-MA5, which showed ~53% improvement compared to Gel-MA controls (Figure 5.3aII). As shown in Figure 5.3aIII, strain at failure in both groups of catechol-containing hydrogels methacryloyl-modified using 3 and 5 %v/v MA was significantly improved when larger amounts of catechol motifs were grafted. Superior stretchability and tensile strength of Gel-CAo-MA5 among other samples led to the highest toughness, ~33.4 kJ/m³ (see Figure 5.3aIV), which was ~130-140% larger than that of Gel-MA controls and Gel-CA-MA5 samples. This remarkable toughness is attributed to the interpenetrating network (IPN) formation within the hydrogel due to the synergistic contribution of strong covalent bonds and strengthened dynamic interactions (*i.e.*, hydrogen bonding and π - π associations) *via* catecholic moieties.⁶⁰⁹

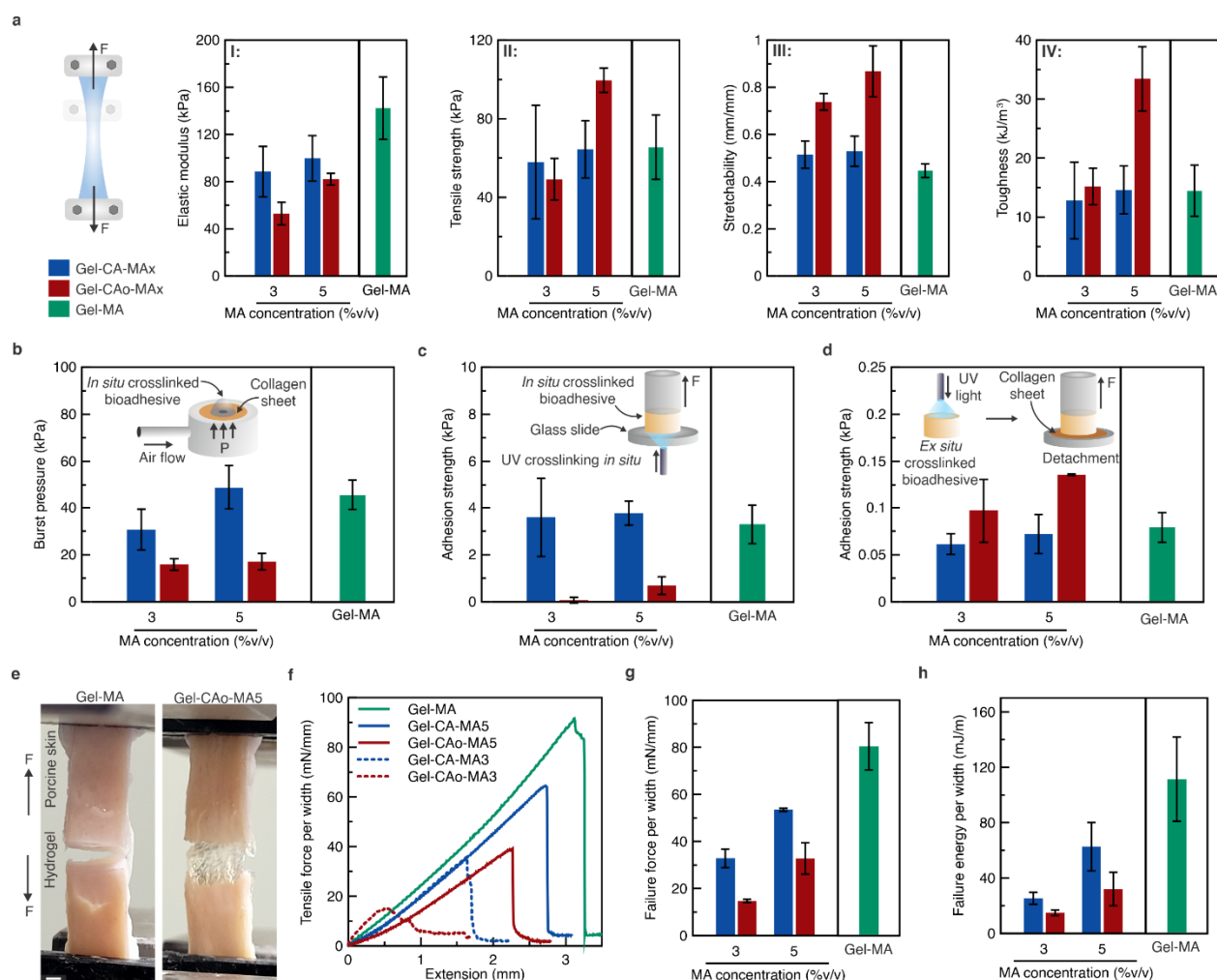


Figure 5.3. Mechanical properties and adhesion performance of the methacryloyl-modified gelatin-caffeic acid (Gel-CA-MAX) and methacryloyl-modified gelatin-caffeic acid oligomer (Gel-CAo-MAX) conjugates (x shows the %v/v concentration of methacrylic anhydride during their synthesis). (a) Tensile test results comparing I: elastic modulus, II: tensile strength, III: stretchability, and IV: toughness of Gel-CA-MAX and Gel-CAo-MAX hydrogels with methacryloyl (Gel-MA) controls. (b) Burst pressure tests quantified bioadhesion capacity hydrogels crosslinked *in situ* on collagen sheet substrates. (c) Axial adhesion (tack) tests performed on hydrogels crosslinked *in situ* between a metal and glass surface. (d) Bioadhesion of hydrogel crosslinked *ex situ* to collagen sheet as a tissue substrate model for potential application as wound dressing patches. (e) Failure mechanism of bioadhesives in the *ex vivo* wound closure test setup using porcine skin. Scale bar = 2 mm. (f) Force per width *versus* displacement results of wound closure tests. (g) Failure force and (h) failure energy per unit of width calculated based on force-displacement data obtained from wound closure tests.

Although the synergy between covalent and noncovalent bonds improved the energy absorption capacity of hydrogels, we note that the toughness values obtained here, as well as those reported in the majority of previous studies, are from tensile tests performed at room temperature. These properties can significantly change under physiological conditions. For instance, the contribution of dynamic hydrogen bonds can be weakened at the elevated temperatures of *in vivo* conditions. To assess this effect, wet stability of hydrogels at body temperature was studied (Figure S5.5). We find that as catechol content increases, the crosslinking depth is reduced (samples tested were 2 mm thick); thereby, solidification of the pre-polymer at the interface with the substrate was primarily because of physical gelation. Hence, as opposed to Gel-MA, which swelled in aqueous media and gained weight, catechol-modified samples partially dissociated and lost weight. No significant difference in weight change was observed between different degrees of methacryloyl modification.

5.2.5 Bioadhesion performance

Catechol groups have long been identified as adhesion-inducing motifs for the modification of biomacromolecules.⁹ Here, we evaluate the impact of catechol-conjugation to photocrosslinkable gelatin on its bioadhesion using various test setups including burst pressure, axial adhesion test *in situ* and *ex situ*, and wound closure tests. Figure 5.3b shows the burst pressure results where punctured collagen sheets (representative model of skin surface) sealed with the *in situ* crosslinked hydrogels are subjected to air pressure. The results not only showed no improvement in burst pressure with conjugation of catechol motifs (in Gel-CA-MAX group), but also the increase in catechol content had a detrimental effect on burst pressure values (in Gel-CAo-MAX group) compared to Gel-MA controls.

However, the fact that burst pressure in Gel-CA-MAx group was larger in the samples synthesized at higher MA concentrations corroborates the stronger tissue binding capacity of methacryloyl groups compared to catechol groups. The groups of Gel-CAo-MAx hydrogels failed due to delamination (adhesion failure) while cohesive failure was observed in Gel-MA and Gel-CA-MAx groups. Burst pressure followed a decreasing trend with the concentration of NaIO₄ used for synthesis of CAO conjugates (Figure S5.6).

We conclude that better adhesion of methacryloyl-modified samples with lower catechol content can be due to:

1. higher density of covalent attachment (*via* Michael addition) to tissue compared to catechol groups having less stronger dynamic interactions (hydrogen bond and π - π interactions) and slow covalent binding affinity (*via e.g.*, Schiff base formation) towards tissue surfaces.⁹
2. negative effects of catechol-conjugation on free-radical-based adhesive reactions of methacryloyl groups (*i.e.*, Michael addition reactions) at the interface with tissue because of radical scavenging characteristic of catechol groups, which is exacerbated by lower methacryloyl content in catechol-rich samples as discussed above.
3. through-thickness decay of free-radical stability in catechol-rich samples as discussed above for Figure 5.2b, results in an insufficient crosslinking of hydrogel in the vicinity of the tissue surface, despite the overall enhanced mechanical toughness of catechol-rich samples (*i.e.*, Gel-CAo-MA5 compared to GelMA, as shown in Figure 5.3aIV).

Similar trends for bioadhesion strength were observed in axial adhesion tests

performed by *in situ* crosslinking (Figure 5.3c). A significant drop in adhesion strength was seen in the samples with the highest catechol content (*i.e.*, Gel-CAo-MAx). The fact that catechol motifs in Gel-CAo-MAx are oxidized into quinones (as opposed to Gel-CA-MAx hydrogels) is not expected to underly the lower adhesion of Gel-CAo-MAx group as quinones are also potent adhesive functionality according to the previous studies.^{24,34} Quinones are in essence, mediators of catechol's covalent adhesion to biological tissues. Furthermore, given that Gel-CA-MA5 samples synthesized at their maximum conjugation capacity, and did not show significant difference from Gel-MA, changing the graft ratio by varying the CA-to-gelatin ratio is not anticipated to improve adhesion.

Therefore, despite the well-known bioadhesion capacity of catechol groups, described in the literature, catechol-conjugation can also compromise bioadhesion in the injectable bioadhesive backbones based on free-radical crosslinkable methacryloyl-functionalized biomacromolecules (such as Gel-MA). These results highlight the roles of crosslinking compatibility considerations in bioadhesive design, which should be taken into account with regard to target applications.

Despite the poor performance of catecholic backbones in the *in situ* photocrosslinking settings for injectable applications, slight improvements in axial adhesion were attained when hydrogels (Gel-CAo-MA5) crosslinked *ex situ* compared to Gel-MA controls (Figure 5.3d). Therefore, we infer that catechol chemical conjugation can introduce *limited* adhesion to pre-formed patches, *e.g.*, for topical applications such as wound dressings and bioelectronic interfaces where an easily detachable bioadhesive is in demand (in patients with fragile skin such as for diabetics and infants).⁴⁰ These results are in agreement with previous works on Gel-MA-catechol conjugates where the molded prepolymer was

crosslinked *ex situ* for application as adhesive patches.^{610,611,612}

To test the bioadhesion of hydrogels to biological tissues, a wound closure test was conducted on porcine skin tissues (Figure 5.3e-h). As illustrated in Figure 5.3e, while the failure in Gel-MA samples stemmed from hydrogel rupture, adhesive failure was registered for Gel-CAo-MA5 samples, which we attribute to the poor free-radical-based adhesion at the interface (as discussed for Figure S5.5). The force-extension characteristics followed a linear trend up to an abrupt drop corresponding to the failure point Figure 5.3f. The values of failure force and failure energy per unit width (Figure 5.3g and h, respectively) agreed well with the results of *in vitro* adhesion tests where introducing catechol molecules correspond to the decrease in adhesion performance.

5.2.6 Biocompatibility *in vitro*

Bioadhesive hydrogels and the products released should be cytocompatible before they can be used in clinical applications. *In vitro* biocompatibility assays in many studies are limited to indirect cell exposure to the components released from hydrogels while direct hydrogel contact with biological elements can provide a more realistic understanding of cell-hydrogel interactions. Furthermore, the use of small sample sizes relative to culture media such as thin films⁴⁵ and procedures such as pre-dialysis of hydrogels for sterilization processes³¹⁶ are among the factors that can underestimate the cytotoxic effects of synthesized biological materials. Here, we study the effects of catechol-conjugation on viability of C2C12 mouse myoblast cell lines by directly seeding the living cells on the hydrogels. Cell morphology and viability after 4 days of culture were examined by live/dead assay as shown in Figure 5.4a,b. As opposed to Gel-MA samples where the cells were spread on the hydrogel, a round cell morphology along with lower viability and more sparse cell

distribution was observed in samples with high catechol content. Additionally, metabolic activity of cells, evaluated *via* PrestoBlue™ assay (Figure 5.4c), further supported the potential toxicity of catechol motifs (*e.g.*, Gel-CA-MA5 and Gel-CAo-MA5 compared to Gel-MA). The toxicity was exacerbated in samples with a lower degree of methacryloyl-modification (*i.e.*, Gel-CAo-MA3) potentially due to the greater amount of release from the hydrogel.

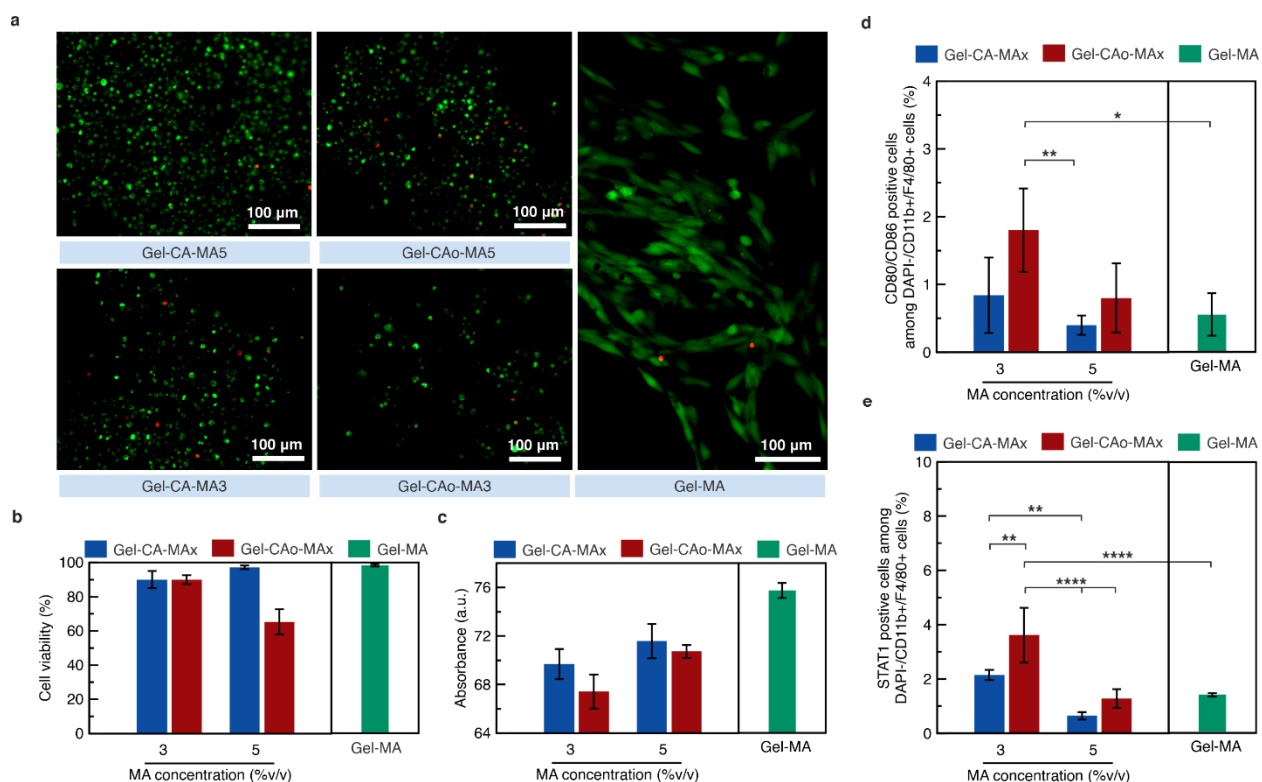


Figure 5.4. *In vitro* biocompatibility and immunoactivity of bioadhesives based on methacryloyl-modified gelatin-caffeic acid (Gel-CA-MAX) and methacryloyl-modified gelatin-caffeic acid oligomer (Gel-CAo-MAX) conjugates (x is the %v/v concentration of methacrylic anhydride for their synthesis). (a) *In vitro* live/dead assays and results of (b) cell viability after 4 days post-culture. (c) Metabolic activity of cells cultured on crosslinked hydrogels on day 4. The expression of (d) CD80/CD86 and (e) STAT1 positive macrophages on day 1. Gel-MA, gelatin methacryloyl.

5.2.7 Cellular immune function

The immunostimulatory effects of hydrogels were characterized by measuring co-stimulatory molecules expressed from the macrophage cells (Figure 5.4d,e and Figure S5.7) to evaluate the risk of immune system activation, organ damage, and autoimmune diseases through co-culturing macrophages with the crosslinked hydrogel samples in trans-well plates. In general, the average of inflammatory macrophages after exposure to hydrogels for one day slightly raised with catechol content in both 3% and 5% MA groups although this correlation was not statistically significant in the group of 5% MA. Therefore, the macrophage response to catechol content is much less significant at higher degrees of methacryloyl modification, likely due to higher crosslinking density and thereby lower release of uncrosslinked chains. Although immune response can be mitigated by increasing the crosslinking density in mussel-inspired hydrogels, this result suggests the risk of immune system activation in response to the degradation products of gelatin backbone, which should be investigated further in future studies. We note that potential inflammatory activation by catecholic compounds such as poly(levodopa) has been reported in the literature²¹ due to the increased aggregation and attachment of macrophages to catechols, as well as high expression of pro-inflammatory factors (*i.e.*, TNF- α and IL-1 β).

5.3 Conclusions and Prospects

The rising need for on-demand crosslinkable sealants with tough wet adhesion and cytocompatibility has motivated the research toward photocrosslinkable mussel-inspired bioadhesives. Recent approaches in biomaterial design have focused largely on introducing methacryloyl and catechol moieties to natural biomolecules. The results of this study,

however, underpin the incompatibility of catechol motifs in photocrosslinking systems based on methacryloyl groups for injectable wound sealing applications. Interference of catechol-conjugates with free-radical polymerization of methacryloyl groups reduces crosslinking density, thereby compromising overall cohesion and adhesion in hydrogels crosslinked *in situ*. Therefore, the choice of crosslinking systems (such as oxidative crosslinking through catechol motifs) is essential for effective bioadhesion in catecholic biomaterials. Accordingly, we infer that the detrimental effects of catechol modification on cohesion of free-radical crosslinkable biomaterials require the incorporation of secondary oxidative crosslinking mechanisms in most previous studies.^{64,599,610,613,611,612} Under these circumstances, the addition of potential toxicity and prolonged curing times, along with the need for light sources for photocrosslinking should be considered in assessing the clinical benefits of dual crosslinking mechanisms. Likewise, improving adhesion strength in photocrosslinkable injectable bioadhesives requires procedures that enhance hydrogel cohesion without negatively impacting free-radical polymerization during photocrosslinking (*e.g.*, through radical scavenging or termination reactions). Although catechol conjugation reduces bioadhesion of *in situ* photocrosslinkable injectable bioadhesives, we note that for applications in wound dressings and bioelectronic interfaces, catechol conjugation provides limited improvement in adhesion of pre-formed hydrogel patches photocrosslinked *ex situ*.

Although we use gelatin as a backbone for evaluation of catechols' bioadhesive capacity, further mechanistic studies using more defined biomacromolecules, such as chitosan are recommended. In addition, we encourage further exploration of toxicity and immunostimulatory effects, particularly in hydrogels with poor crosslinking density, to

increase the potential for clinical translation of mussel-inspired biomaterials. Therefore, further research on biosafe and catechol-compatible crosslinking mechanisms for mussel-inspired bioadhesives are of urgent importance and require additional attention.

5.4 Experimental Methods

5.4.1 Materials

Gelatin from porcine skin (type A), caffeic acid (CA), methacrylic anhydride (MA), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959), sodium nitrite (NaNO_2), sodium molybdate (Na_2MoO_4), sodium periodate (NaIO_4), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were supplied by TCI Chemicals, USA. Dulbecco's phosphate-buffered saline (DPBS) was obtained from Thermo Fisher Scientific.

5.4.2 Synthesis

For methacryloyl-modified gelatin-caffeic acid hydrogels (Gel-CA-MA_x, x represents the amount of MA used for methacryloyl-modification) conjugates, 212 mg CA was dissolved first in 5 ml 50 v/v% DMSO solution in deionized (DI) water. Then, the EDC/NHS coupling agent was prepared separately by adding 191 mg EDC and 115 mg NHS in a 50 v/v% DMSO solution, and the final volume was adjusted to 20 ml. The CA activation was initiated by adding the EDC/NHS solution to the CA solution, and the mixture was stirred for 2 h at room temperature. Subsequently, 1 g gelatin was dissolved in 75 ml 50 v/v% DMSO solution in water at 50 °C and added to the activated CA solution. The coupling reaction proceeded for

24 h at room temperature while stirring. Then, various amounts of MA were added to the reaction mixture (*i.e.*, 1, 3, and 5 ml), and the solution was kept under stirring for ~19 h at 40 °C. Next, the reaction byproducts were removed through a dialysis step against DI water (dialyzing water was refreshed 3 times per day), followed by freeze-drying for 4 days. The final foam products were stored at room temperature until further use.

Methacryloyl-modified gelatin-caffeic acid oligomers (Gel-CAO-MA_x, x represents the amount of MA used for methacryloyl-modification) were synthesized through the same procedure as described as for Gel-CA-MA_x; except for the CA was oxidatively oligomerized in the CA solution prepared at the beginning (before EDC/NHS activation), by adding 7.5 mg NaIO₄ (1.5 mg/ml) followed by vortex agitation for 2 h.

Gelatin methacryloyl (Gel-MA) was prepared according to the procedure described previously. In brief, MA (8 v/v%) was added to a gelatin solution (10 %v/v) in DPBS, and the solution was left at 40 °C for 3 h. Then, the reaction was stopped by adding an equal volume of DPBS. Subsequently, dialysis and freeze-drying steps were performed as explained above.

5.4.3 Hydrogel preparation

To prepare the pre-polymer solutions, first, a 0.5 %w/v Irgacure 2959 photoinitiator solution was prepared in DI water by heating at 80 °C in the dark. Then, the freeze-dried modified-gelatin foams were dissolved at 20 %w/v in the above 0.5 %w/v Irgacure 2959 solution in an 80 °C oven. The solutions were pipetted, followed by UV light irradiation for 5 min (unless noted otherwise), at the light intensity of ~150 mW/cm² using an OmniCure S2000 spot UV curing system.

5.4.4 UV-vis spectroscopy

The effect of chemical modifications on UV-vis spectra of gelatin was studied using a NanoDrop OneC Microvolume UV-vis spectrophotometer model 840-274200 (Thermo Scientific). Bioadhesives were dissolved in DI water at 3 %w/v concentration. Then, 2 μ l samples were pipetted onto the measurement surface and relative absorbance was measured across the UV-vis wavelengths between 190 to 750 nm.

5.4.5 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (^1H NMR) spectroscopy was performed to study the chemical modification of gelatin backbones. The samples were prepared by dissolving 10 mg freeze-dried foams in $\text{DMSO-}d_6$ and loaded to a 400 MHz Bruker AV400 spectrometer. The ^1H NMR spectra were corrected with phase and baseline. Then, the degree of conversion of amine groups at the lysine residues was calculated based on the integration of the peaks associated with methacryloyl groups.

5.4.6 Arnow catechol content assay

Arnow assays were performed to evaluate the conjugated catechol content to gelatin, based on the procedure described in previous studies.⁴⁶ Bioadhesives were dissolved in 0.5 M HCl at a final concentration of 0.15 %w/v. Then, the solutions were mixed with an equal volume of 10 %w/v sodium molybdate and 10 %w/v sodium nitrite in DI water. After shaking for 5 min, pH increased to 7 using 1 M NaOH, and subsequently, the final volume was adjusted to 5 ml. The absorbance of 200 μ L samples taken in a 96-well plate was measured at 520 nm using a plate reader (BioTek Synergy 2, USA). The amount of conjugated catechol was calculated based on calibration curves obtained from a diluted series of dopamine

solutions.

5.4.7 Rheological analysis of photocrosslinking

Photocrosslinking kinetics of hydrogels were evaluated by real-time monitoring of storage and loss modulus in response to UV light irradiation using an Anton Paar MCR 302 rheometer (USA, CA). For this purpose, 80 μ L pre-polymer solutions were pipetted onto an in-house-made sample holder, which guided the UV light transversally so that the entire sample was exposed to UV light from underneath. The measurements were performed using an 8 mm torque measuring system at 1 mm gap size, 10% strain, and 1 Hz frequency. Irradiation of the UV light started after 1 min of stabilization.

5.4.8 Tensile tests

Tensile mechanical properties of the hydrogels were evaluated using Instron 5943 universal testing machine equipped with a 100 N load cell (USA, MA). In order to prepare samples, 200 μ L of the pre-polymer solution was pipetted into a rectangular 4 mm $\times$ 25 mm mold, followed by UV irradiation as described above. Then, the hydrogels were glued to rectangular plain papers using Krazy glue (OH, USA) through which they were gripped using tensile test grippers. The tests were started at 8 mm/min displacement rate until the samples failed.

5.4.9 *In vitro* burst pressure

Burst pressure tests were carried out using 3 mm-punctured wet collagen sheet substrates, sealed with 100 μ L of bioadhesive pre-polymer crosslinked *in situ*. The 25 mm diameter collagen sheets were then placed and fixed onto a pressure chamber, which was connected jointly to a pressure sensor and syringe pump. The air was injected into the

pressure chamber at 20 ml/min, while the pressure sensor recorded the internal pressure. The maximum pressure recorded during the test up to the failure point was registered as burst pressure for each sample.

5.4.10 Axial detachment (tack) tests

The adhesion strength of hydrogels for applications as an injectable sealant and wound dressing patches were examined through *in situ* and *ex situ* detachment tests along the axial direction, respectively. For *in situ* crosslinking, the tests were performed using a rheometer equipped with an 8 mm flat torque measuring system. Here, 80 μ L pre-polymer solutions were added to the 1 mm gap between the metallic torque measuring device and underneath the glass surface, under which UV light could be irradiated to crosslink the sample. After crosslinking, the torque measuring device was lifted at the cross-head displacement rate of 0.5 mm/s while the axial force along the torque measuring device was recorded. The maximum load at the detachment point was used to calculate the adhesion strength.

5.4.11 *Ex vivo* wound closure tests

Wound closure tests were performed on porcine skin tissue cuts of 8 mm $\times$ 30 mm. Two tissue cuts were contacted through their 8 mm faces and their interface was treated with the pre-polymer solution over a length of $\sim$ 10 mm centered at the interface. The amounts of 100 μ l of pre-polymers were then photocrosslinked and the tissues were subjected to axial tensile displacement at a rate of 4 mm/min. The force at rupture point as well as the failure energy (calculated based on the area under the force-displacement curves) was normalized to the sample width.

5.4.12 Weight change in wet media

The stability of photocrosslinked hydrogels was evaluated by measuring the ratio of weight change after incubating in water for 24 h at 37 °C. Samples were prepared in an 8 mm mold by adding 80 μ L pre-polymer solution, followed by photocrosslinking. The weight before (w_0) and after (w_s) exposure to wet media was recorded, and the weight change ratio was calculated based on Equation (5):

$$\text{Weight change ratio} = \frac{w_s - w_0}{w_0} \quad (5)$$

5.4.13 *In vitro* immunization studies

The bone marrow-derived macrophage cells (BMDMs) were extracted from the tibia and femur bones of 5-6 weeks old C57BL/6 mice. Cells were isolated and seeded (10^6 cells per dish) in Dulbecco's modified eagle medium (DMEM) supplemented with 10 %w/v fetal bovine serum (FBS), 55 μ M β -mercaptoethanol, 100 U ml^{-1} penicillin, and 5 ng ml^{-1} of macrophage colony-stimulating factor (MCSF). Following 8 days of culture, the BMDMs were seeded at 2.5×10^5 cells per well in a 24-well Transwell® plate. After incubation overnight, macrophages were co-cultured with hydrogels crosslinked in disks of 100 μ L volume for one day. Then, the cells were collected and macrophages were stained for markers to determine their polarization and activation using flow cytometry (Attune, Thermo Fisher Scientific, USA).

5.4.14 *In vitro* biocompatibility

Cytocompatibility of the hydrogels was evaluated towards C2C12 murine myoblasts

cell lines (ATCC). The cells were incubated in an incubator (Thermo Fisher Scientific, USA) under standard conditions (*i.e.*, 37 °C temperature and 5% CO₂) using Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. To perform the live/dead assays, 75 µL of pre-polymer solutions were crosslinked in a 48-well plate and placed under a UV flood lamp for 15 min for sterilization. Then, 0.5 ml of cell suspension prepared in media was added to each well (resulting in 15000 cells per well). At different time intervals, 12 µL ethidium homodimer-1 (red/dead stain) and 8 µL calcein (green/live stain) were added to each well, followed by incubation for 15 min. Next, an Axio Observer 5 (Zeiss, Germany) fluorescence microscope was used to image the cells. The images were taken at the excitation/emission wavelengths of ~494/515 nm for calcein and ~528/617 nm for ethidium homodimer-1. Cell viability was calculated based on the ratio of the number of live cells to the total number of cells as determined using ImageJ software. Metabolic activity was also measured using PrestoBlue™ assay according to the following procedure. A PrestoBlue™ reagent was prepared in media by dilution at a 1:9 ratio. The cells were treated with this reagent while incubated for 2 h. The metabolic activity was quantified by measuring the fluorescence intensity at the excitation wavelength of 560 and emission wavelength of 590 nm using a BioTek Synergy 2 plate reader.

5.4.15 Statistical analyses

All experiments were repeated at least three times unless otherwise noted. The quantities were reported as the mean ± standard deviation. A one-way analysis of variance (ANOVA) was performed to test the significance of differences between parameters where $P < 0.5$ was considered significant.

5.5 Supporting Information

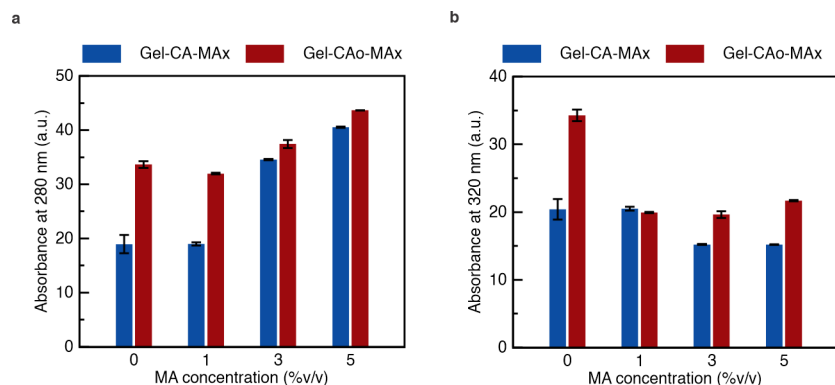


Figure S5.1. The UV-vis absorbance of methacryloyl-modified gelatin-caffeic acid (Gel-CA-MAx) and methacryloyl-modified gelatin-caffeic acid oligomer (Gel-CAo-MAx) conjugates (x is the %v/v concentration of methacrylic anhydride used for their synthesis) at (a) 280 and (b) 320 nm.

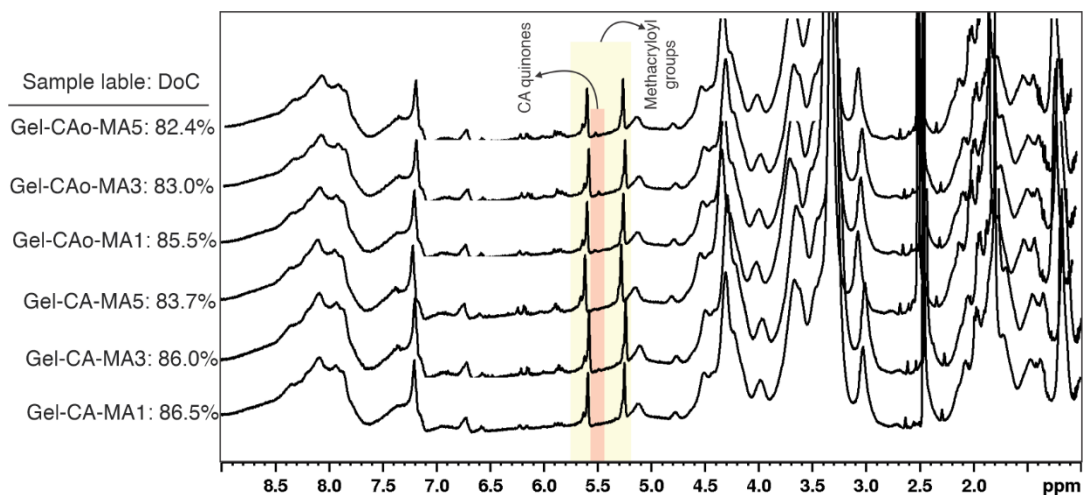


Figure S5.2. Proton nuclear magnetic resonance (¹H NMR) spectra of methacryloyl-modified gelatin-caffeic acid (Gel-CA-MAx) and methacryloyl-modified gelatin-caffeic acid oligomer (Gel-CAo-MAx) conjugates (x is the %v/v concentration of methacrylic anhydride used for their synthesis), based on which degree of amine conversion (DoC) was calculated. CA, caffeic acid.

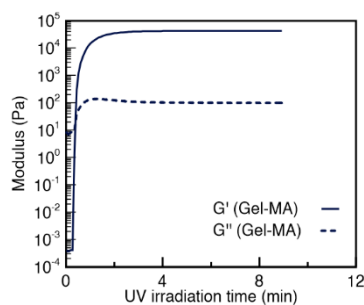


Figure S5.3. Rheological responses (*i.e.*, storage modulus, G' , and loss modulus, G'') of gelatin methacryloyl (Gel-MA) pre-polymers during UV polymerization.

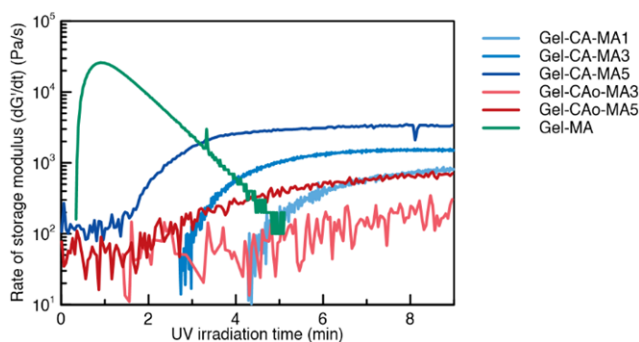


Figure S5.4. Photocrosslinking kinetics in terms of variation rate of storage modulus for methacryloyl-modified gelatin-caffeic acid (Gel-CA-MAx), methacryloyl-modified gelatin-caffeic acid oligomer (Gel-CAo-MAx) conjugates (x is the %v/v concentration of methacrylic anhydride used for methacryloyl-modification), and gelatin-methacryloyl (Gel-MA) hydrogels.

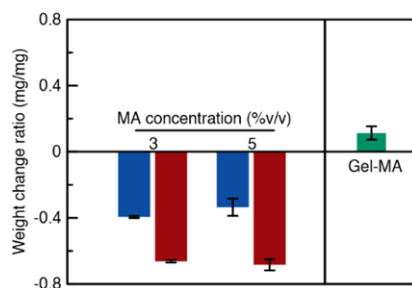


Figure S5.5. Weight change ratio of photocrosslinked methacryloyl-modified gelatin-caffeic acid (Gel-CA-MAx) and methacryloyl-modified gelatin-caffeic acid oligomer (Gel-CAo-MAx) conjugates (x is the %v/v concentration of methacrylic anhydride used for methacryloyl modification) after 24 h incubation in 37 °C. Gel-MA, gelatin methacryloyl.

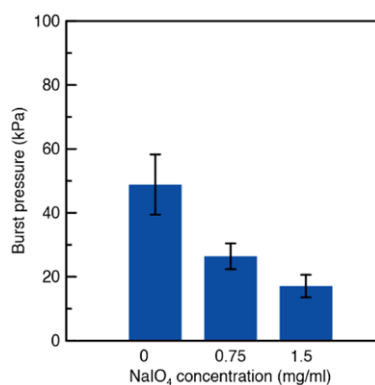


Figure S5.6. Comparison of burst pressure values for gelatin conjugated with caffeic acid oligomers (CAO) synthesized at different concentrations of NaIO₄.

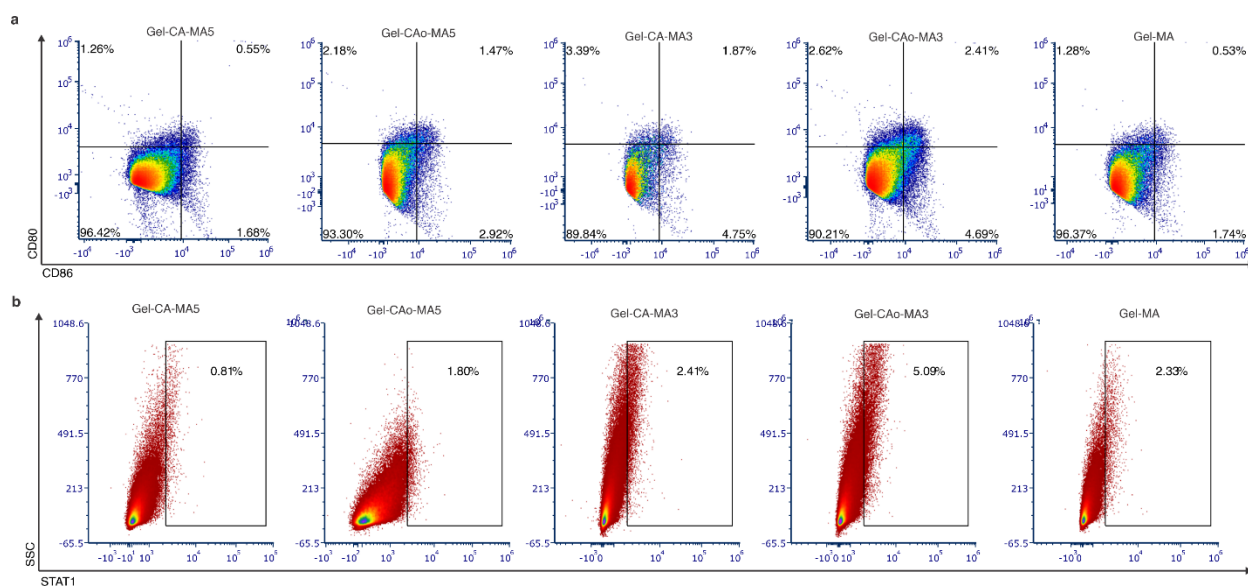
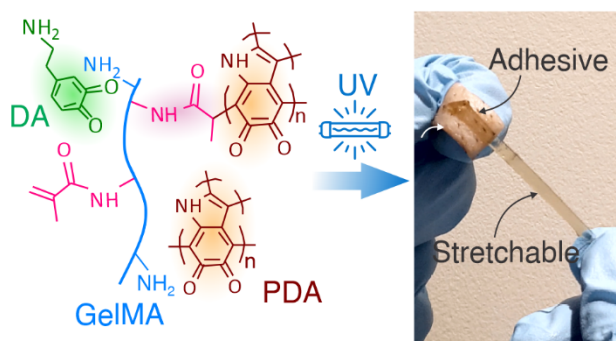


Figure S5.7. Flow cytometry scatter plots showing hydrogel immunoactivity: inflammatory marker (a) CD80/CD86 and (b) STAT3 expressions. SSC, side scatter.

CHAPTER 6: STRETCHABLE AND BIOADHESIVE GELATIN METHACRYLOYL-BASED HYDROGELS ENABLED BY *IN SITU* DOPAMINE POLYMERIZATION



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Abstract

Hydrogel patches with high toughness, stretchability, and adhesive properties are critical to healthcare applications including wound dressings and wearable devices. Gelatin methacryloyl (GelMA) provides a highly biocompatible and accessible hydrogel platform. However, low tissue adhesion and poor mechanical properties of crosslinked GelMA patches (*i.e.*, brittleness and low stretchability) have been major obstacles to their application for sealing and repair of wounds. Here, we show that adding dopamine (DA) moieties in larger

quantities than those of conjugated counterparts, to the GelMA pre-polymer solution followed by alkaline DA oxidation could result in robust mechanical and adhesive properties in GelMA-based hydrogels. In this way, crosslinked patches with $\sim 140\%$ stretchability and $\sim 19,000 \text{ J/m}^3$ toughness which correspond to ~ 5.7 and $\sim 3.3\times$ improvement, respectively, compared to that of GelMA controls, were obtained. The DA oxidization in the pre-polymer solution was found to play an important role in activating adhesive properties of crosslinked GelMA patches (~ 4.0 and $\sim 6.9\times$ increase in adhesion force under tensile and shear modes, respectively) due to the presence of reactive oxidized quinone species. We further conducted a parametric study on the factors such as UV light parameters, the photo-initiator type (*i.e.*, lithium phenyl-2,4,6-trimethylbenzoylphosphinate, LAP, *versus* 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, Irgacure 2959), and alkaline DA oxidation to tune the crosslinking density, and thereby hydrogel compliance for better adhesive properties. The superior adhesion performance of the resulting hydrogel, along with *in vitro* cytocompatibility demonstrated its potential for use in skin-attachable substrates.

6.1 Introduction

Tough hydrogel substrates with adhesive properties have brought many opportunities to the biomedical applications, including bioadhesives sealants,^{316,179} and skin-attachable wearable and implantable devices.^{614,42} The physical properties of the hydrogels are tunable by manipulating their macromolecular characteristics and polymer network structures, motivating research towards the development of hydrogels with high mechanical and adhesive properties. In order to establish strong adhesion, the hydrogel must be mechanically stable and tough. Toughening hydrogels has been pursued by

incorporation of both covalent and non-covalent interactions, to dissipate the deformation energy and to enable strain reversibility.³⁴⁹ To toughen hydrogels, depending on how the stiffness changes, either mechanical strength or stretchability is increased to enable greater energy absorption.⁶¹⁵ In addition, an ideal hydrogel patch, must comply with the natural deformations of the tissue with minimal mechanical resistance to avoid discomfort on dynamic and deformable tissue surfaces.⁶¹⁶⁻⁶¹⁸ This criterion can be met by the design of elastic hydrogels with lower elastic moduli. Therefore, stiff hydrogels, even though tough, may fail to meet the mechanical requirements for bioadhesives. Hence, in order to toughen hydrogels, improvements in hydrogel compliance while ensuring high stretchability are the key requirements for tissue-attachable adhesive hydrogels.²⁶³

Aside from improvements in cohesive properties of the hydrogels, several approaches have been demonstrated to promote adhesion to wet tissue substrates.⁶¹⁹ These methods are primarily based on the covalent and non-covalent bond formation between the hydrogel and nucleophilic groups present on the underneath tissue substrate.^{174,620} In addition to these methods, compliance of the hydrogel can favor adhesion by minimizing the mechanical resistance against the intrinsic tissue movements. In this regard, the mussel-inspired hydrogels account for a major class of bioadhesives wherein catechol groups play the dominant role in establishing adhesion.⁶²¹ Adhesion is a sophisticated process in mussel bypass threads where a combination of charged and a certain ratio of hydrophobic amino acids in mussel adhesive proteins (MAPs) work towards underwater adhesion.⁶²² Despite significant research efforts directed at understanding catechol-driven adhesion, the mechanism of action in such hydrogels is not fully understood.²⁹⁹ State-of-the-art mussel-inspired bioadhesives primarily involve chemical grafting of catechol groups onto different

natural and synthetic backbones to introduce adhesive properties to the polymers.⁶²³ However, the reaction yield and potential grafting sites to conjugate catechol groups in such approaches are often limited. The activation of the adhesive function in the mussel-inspired hydrogels differs from polymer backbone to backbone and requires specific processing.³⁰⁶ Although reports have shown overall advantageous physical properties for synthetic hydrogels, natural counterparts have received particular attention due to their biosafety and biodegradability.⁶⁵

Gelatin derivatives, in particular gelatin methacryloyl (GelMA) hydrogels, are attractive candidates for biomedical use due to their outstanding biocompatibility.⁵⁹⁷ The GelMA hydrogels, however, are brittle due to their heterogeneous crosslinking density and extensive entanglements.⁴² In addition, the lack of sufficient energy-dissipating non-covalent intramolecular interactions has limited their stretchability to ~30%, which is well below the natural deformations of most soft tissues.^{624,625} Although *in situ* crosslinking of GelMA hydrogel has demonstrated the potential to seal wounds and injuries effectively by the formation of stable adhesion,^{59,626} crosslinked GelMA hydrogel patches are rather slippery, hindering their adhesion to wet surfaces. Modification of gelatin-based macromolecules with catechol groups for higher adhesion has been previously demonstrated for wound care applications. For instance, a tissue adhesive was developed for *in situ* wound sealing and hemostasis based on dopamine (DA) conjugated gelatin *via* a dual-crosslinking with (i) dynamic complex formation by Fe^{3+} and (ii) covalent crosslinking by genipin.⁴⁶ The hydrogels showed slight improvement in tissue adhesion compared to commercially available fibrin glue. Hemostatic effects are improved by conjugation of catechol groups, as they are capable of triggering the coagulate cascade.^{627,506} Flexible thin GelMA films were

grafted with catechol groups and reinforced with polycaprolactone (PCL) to support the fabricated structures mechanically.⁶³ The hydrogels were characterized with hemostatic properties (by addition of Ca^{2+} ions) and showed promise in healing acute trauma. However, the simultaneous incorporation of mechanical toughness, stretchability, and adhesion properties in GelMA-based hydrogels remains a challenge.

Here, we aim to develop stretchable, tough, and adhesive photo-crosslinkable GelMA hydrogels by adding DA additives in larger quantities than those of conjugated examples to the GelMA precursor solution. Then, the DA-integrated GelMA (DA@GelMA) pre-polymers were oxidized/polymerized to polydopamine (PDA) in the presence of GelMA (PDA@GelMA) in an alkaline environment by adding NaOH to impart adhesive properties to the crosslinked hydrogels. In addition, we explored the effects of processing factors such as UV light parameters and photo-initiator type (*i.e.*, lithium phenyl-2,4,6-trimethylbenzoylphosphinate, LAP, and 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, Irgacure 2959) on the hydrogel physical properties to facilitate adhesion process. Finally, we demonstrated the cytocompatibility of the hydrogels for potential use in biomedical applications.

6.2 Results and discussion

6.2.1 Effect of dopamine on physical gelation of gelatin methacryloyl

Understanding the interactions between DA molecules and gelatin derivatives can give fundamental clues for explaining hydrogel behavior at the molecular level. Gelatin and its derivatives (modified with methacryloyl groups, see Figure 6.1a) are known to form hydrogels due to the hydrogen-bond-driven association of single/multi-stranded

polypeptides at room temperature.⁶²⁸ However, as shown in Figure 6.1b,c, we observed that mixing 2 wt.% DA molecules with GelMA prepolymer disrupted GelMA gelation at room temperature; mixing the same concentration of DA with gelatin did not affect its gelation at room temperature. To understand this effect, we performed temperature sweep tests and quantified the phase transition temperature changes of gelatin and GelMA in response to DA additives (Figure S6.1a,b). The gelation and dissolution temperatures (see Figure S6.1c,d, respectively) were significantly decreased with both functionalization as well as addition of DA, so that gelation temperature for GelMA containing 2 wt.% DA was reduced to ~ 11 °C. We attribute the reduced phase transition temperature with methacryloylation of gelatin to the consumption of hydrogen bond donor groups, such as primary amines and hydroxyls, due to their conversion to hydrophobic methacryloyl groups. Note that DA additives can interact with and aggregate around GelMA macromolecules *via* hydrogen bonding and π - π interactions, as shown schematically in Figure 6.1b. According to the results shown in Figure S6.1c,d, we infer that DA can disrupt helix strand formation⁶²⁹ in gelatin and GelMA. This disruption may be due to DA interactions with the gelatin backbone, blocking helix-forming hydrogen bonds (between the carbonyl oxygen of one amino acid and amide protons of another amino acid).⁶³⁰

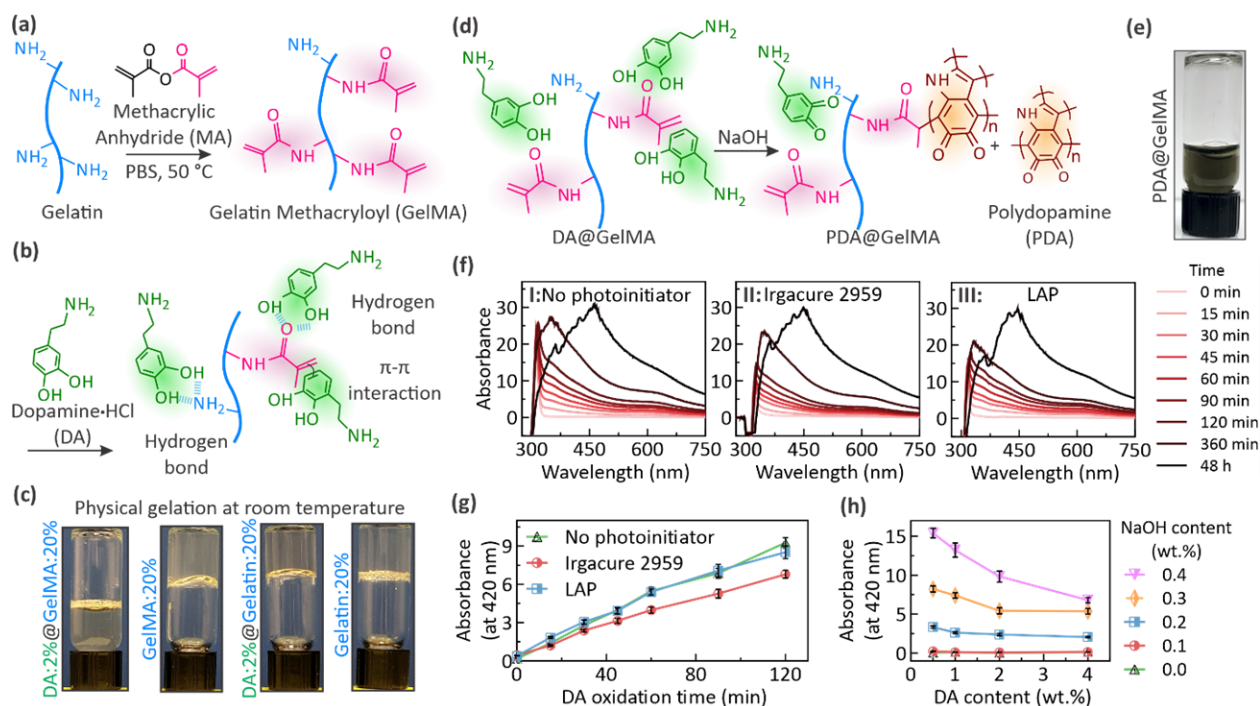


Figure 6.1. Formulation, chemical interactions, and oxidation in dopamine (DA) and polydopamine (PDA) incorporated gelatin methacryloyl (GelMA) hydrogels. (a) Synthesis route for the modification of the gelatin with methacryloyl groups. (b) Physical addition of DA to GelMA with aggregation around the GelMA macromolecules is driven by hydrogen bonding as well as π - π interactions. (c) Vial tilt assay demonstrating the roles of DA additives on physical gelation of gelatin *versus* GelMA at room temperature. (d) Alkaline oxidation reaction of DA in the presence of GelMA macromolecules, leading to DA polymerization or PDA formation.⁶³¹ The PDA can also be grafted onto the GelMA backbone during oxidation. (e) Optical image of the PDA@GelMA prepolymer solution after 1 h incubation at 37 °C (material compositions: GelMA: 20 wt.%, DA: 2 wt.%, NaOH: 0.2 wt.%). (f) Time-dependent UV-visible spectra of the hydrogel solutions in presence of different photo-initiators. (g) Time variation of absorbance at 420 nm wavelength for the pre-polymer solutions containing different photo-initiator types. (h) Variation of absorbance with different amounts of DA molecules at different NaOH levels.

6.2.2 Oxidation of dopamine in the presence of gelatin methacryloyl

It has been previously shown that oxidation of DA is accompanied by the generation of free radicals.³¹⁴ Free radicals can lead to PDA reaction with the methacryloyl groups on

GelMA (Figure 6.1d,e). This reaction was confirmed by the UV-vis spectra of the PDA@GelMA solutions after dialysis in DI water and centrifugation, as shown in Figure S6.2. The UV-vis spectra of PDA@GelMA were found to be identical to those prior to the dialysis step (Figure 6.1f), suggesting covalent attachment of PDA to GelMA.

We also measured the UV-vis spectra of the solutions with and without two different photo-initiators (*i.e.*, Irgacure 2959 and LAP, both at 0.5 wt.%) to quantify their effects on the alkaline-triggered oxidation of DA by assessing the color changes in PDA@GelMA prepolymer solutions. The UV-vis spectra of the PDA@GelMA (DA: 2 wt.%, NaOH: 0.3 wt.%) were monitored over time as shown in Figure 6.1f. The DA-free GelMA solutions with similar photo-initiator contents were used as blanks. The absorbance-wavelength spectra were found to follow the same trends across three different conditions of no photo-initiator, Irgacure 2959, and LAP. At time zero, a distinct absorbance peak at 280 nm was attributed to DA in its reduced form.⁶³² Once NaOH was added, the DA polymerization reaction led to gradual blackening of the solution due to the alkaline-induced oxidation of DA.⁶³³ This process led to the appearance of a broad shoulder at wavelengths in the range of 300 to 600 nm. This result is indicative of a polydisperse transition of DA to catechol-quinone oxidative intermediates as well as PDA species.⁶³⁴ According to the literature, the peaks at 280 nm, 395 nm, the spectra at 300-475 nm, and 420 nm correspond to DA, yellow chromophore dopamine *o*-quinone, aminochrome, and early stages of PDA formation, respectively.^{635,636} The gradual shift of the absorbance peak towards the larger wavelengths after 48 h is indicative of the polymerization of the majority of DA molecules. This wavelength shift to the red occurs due to the oxidative addition of benzene rings to the larger molecule PDA moieties, which reduce the bandgap.

The absorbance at 420 nm (representative of PDA formation)⁶³⁷ was measured over time and shown in Figure 6.1g. As can be seen, oxidation of DA proceeded at a slower rate in the presence of Irgacure 2959 photo-initiator, whereas the oxidation rate for LAP was similar to the samples with no photo-initiator. We attribute this result to the anti-oxidative effects of Irgacure 2959. Note that as opposed to LAP, Irgacure 2959 molecules contain strong electron donor groups, such as phenolic alkoxy groups, which can act as free radical scavengers⁶³⁸ and thereby lead to their antioxidative properties.

We also evaluated the effects of DA and NaOH concentrations on the DA oxidation in the pre-gel solution by characterizing the UV-vis absorbance at 420 nm (Figure 6.1h) after 1 h incubation at 37 °C (in the absence of photo-initiator). The addition of NaOH led to the alkaline oxidation of DA. The addition of DA, however, was associated with a lower oxidation rate at constant NaOH concentration. As the DA form was the hydrochloride (*vide infra*), we attribute this result to the HCl component in DA, which made the pH of the original pre-polymer solution more acidic.

6.2.3 Analysis of the photo-crosslinking reaction

Gelation of the hydrogels was explored to elucidate the roles of DA, DA oxidation in the pre-polymer, as well as the photo-initiator type in the crosslinking process using the setup shown in Figure 6.2a. For this purpose, the rheological characteristics of the pre-polymer solution (storage modulus, G' , and loss modulus, G'') were monitored in real time in response to UV irradiation. As shown in Figure 6.2b, G' rapidly increased after a period of UV irradiation, overtaking G'' at the gelation point before reaching a plateau. Figure 6.2cI,II illustrates the results of gelation time and G' after 5 min of UV irradiation for the pre-polymer solutions cured by using 0.5 wt.% Irgacure 2959 (at an UV light intensity of 35 mW/cm²).

Dopamine and NaOH concentrations were found to affect the crosslinking reaction kinetics and the final rheological characteristics. Gelation time increased with both DA and NaOH concentrations. In fact, no gelation was detected during the course of measurements for the samples containing more than 2 wt.% DA or 0.2 wt.% NaOH. The addition of 2 wt.% DA (even under acidic conditions) was associated with increases in gelation time from ~ 10 s to ~ 150 s. Commensurate with the observed changes in gelation time, the final storage modulus decreased with higher concentrations of both DA and NaOH.

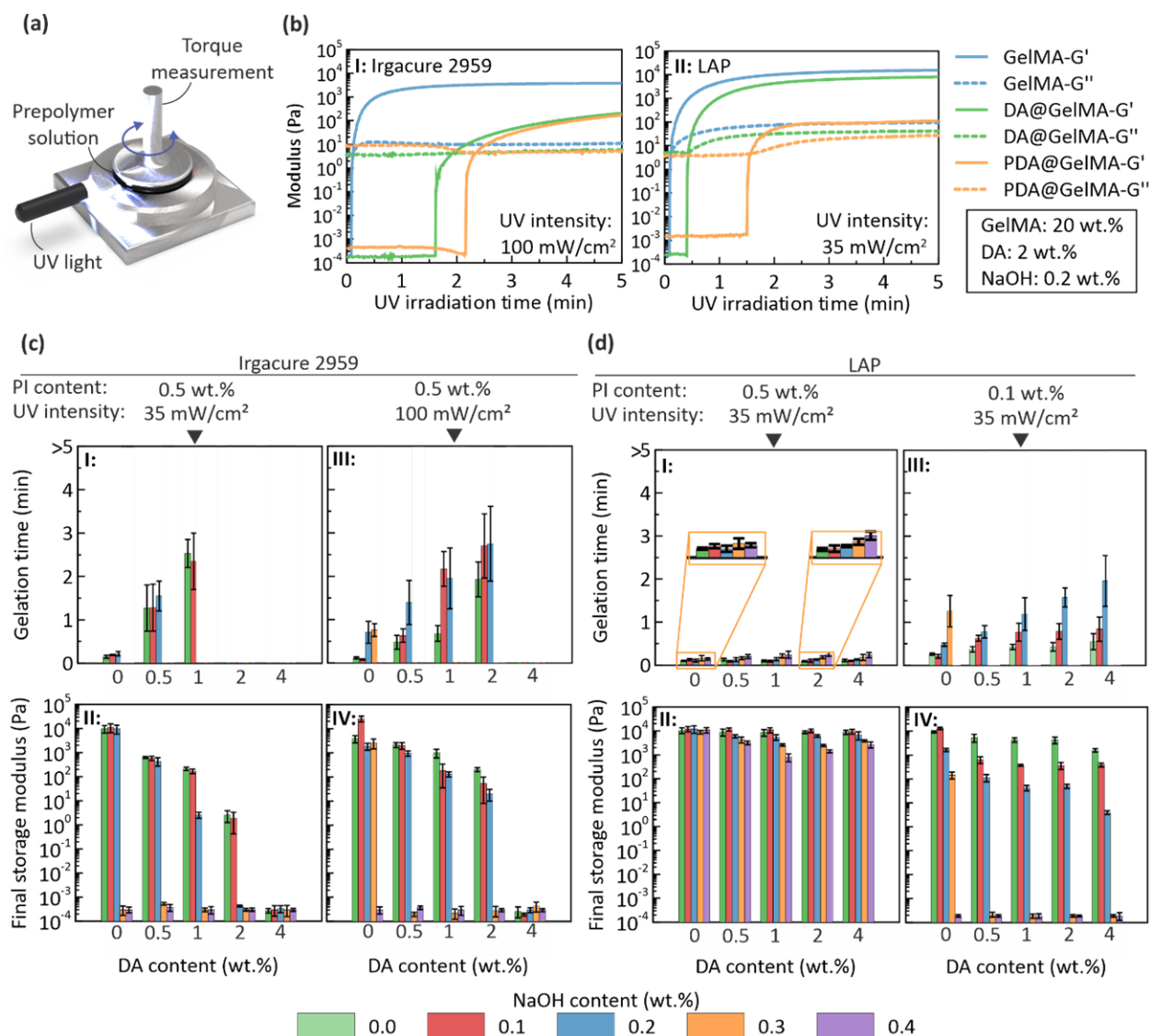


Figure 6.2. Real-time characterization of photo-crosslinking reaction by the rheological analysis of gelation. (a) The experimental setup for the rheological tests. (b) Storage (G') and loss modulus (G'') as a function of ultraviolet (UV) light irradiation time for the dopamine (DA) and polydopamine (PDA) containing gelatin methacryloyl (GelMA) prepolymers containing I: Irgacure 2959, and II: lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photo-initiators. (c) The effects of dopamine (DA) and NaOH concentrations on the gelation time and final storage modulus for 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) photo-initiator (0.5 wt.%) crosslinked using I, II: 35 mW/cm² UV light intensity, and III, IV: using 100 mW/cm² UV light intensity. (d) The effect of DA and NaOH concentrations on the gelation time and final storage modulus for LAP (UV light intensity of 35 mW/cm²) for the prepolymers containing I, II: 0.5 wt.% LAP and III, IV: 0.1 wt.% LAP.

For the group of hydrogels crosslinked using Irgacure 2959, the effect of DA was found to be more significant than that of NaOH on gelation time and final storage modulus. Addition of NaOH disabled polymerization above a threshold of 0.3 wt.%. We attribute the NaOH effect to the quenching of the free radical polymerization process by OH⁻ ions, according to the lack of gelation in the samples without DA. For the samples containing it, DA can act as an initiator with the addition of NaOH, because DA oxidative polymerization is triggered by alkaline conditions and is accompanied by free radical generation.³¹⁴ This result indicates the possible growth of PDA at the methacryloyl sites of the GelMA biomacromolecules (Figure 6.1d), hindering photo-polymerization in the oxidized solutions, as shown in Figure 6.2c. Note that PDA polymerization can proceed from different reactive sites on DA, resulting in polydisperse PDA formation.⁶³¹ The decreases in the final storage modulus with DA indicate reduced crosslinking density. This effect can arise from multiple possible crosslink-density-reducing interactions during both the initiation and propagation steps. First, DA is known to be a free radical scavenger.³³⁴ It can, therefore, quench the generated radicals and lower the crosslinking density. The free radical scavenging effects of DA are further intensified by the aggregation of DA around the methacryloyl groups, as shown in Figure 6.1b.

We evaluated the rheology of crosslinking at elevated UV light intensity (*i.e.*, 100 mW/cm²), for the samples crosslinked with Irgacure 2959 photo-initiator, as represented in Figure 6.2cIII,IV. Decreases in gelation time correspond to increases in final storage modulus, particularly at higher DA concentrations, as observed for the polymerized samples. Increases in UV light intensity did not lead to gelation for most of the samples that did not polymerize (no gelation observed) at the lower UV light intensities (see Figure 6.2cII,IV). However, the

final modulus for the polymerized samples reached the same order, $\sim 10^5$ Pa, regardless of DA concentration. Therefore, the effects of more intense UV intensity were most important in increasing the reaction kinetics and increasing the crosslinking density towards the saturation limit, which we attribute to the higher levels of stable free radicals.

Given the failure of Irgacure 2959 in polymerization at several DA and NaOH compositions, further studies were conducted on an alternative photo-initiator, *i.e.*, LAP. Figure 6.2dI,II demonstrates the gelation time and storage modulus post-crosslinking at 0.1 and 0.5 wt.% LAP photo-initiator, respectively, under 35 mW/cm² UV light intensity. The addition of either DA or NaOH to the hydrogel pre-polymer was associated with increases in DA gelation time and decreases in the final storage modulus, consistent with the results for Irgacure 2959. However, the effects of DA and NaOH on the crosslinking reaction were different than for Irgacure 2959. While gelation time and storage modulus for the hydrogels containing Irgacure 2959 photo-initiator were correlated with the DA concentration, for the case of LAP, the NaOH content played a key role in determining the crosslinking kinetics. In addition, gelation was detected for a wider range of material compositions when LAP was used as a photo-initiator. For instance, the samples were crosslinked with LAP even at maximum DA concentration (4 wt.%) and NaOH concentrations up to 0.2 wt.% with gelation times of ~ 2 min; however, the hydrogels were not formed under these conditions when Irgacure 2959 was used. Increasing the photo-initiator content to the same level as Irgacure 2959 (*i.e.*, 0.5 wt.%) led to gelation and crosslinking for the entire precursor solutions and reduced the gelation times for all samples to below 15 s (see Figure 6.2dI,II). Accordingly, the final storage modulus markedly increased to that for GelMA with no additives, suggesting the formation of a dense crosslinked network due to the large numbers of generated free

radicals.

The results of gelation time and modulus at both LAP concentrations demonstrated that DA concentration had only minor impact on the rheological characteristics in the crosslinking reaction. For the samples with no DA content, reducing the storage modulus was only evident at low LAP concentration (0.1 wt.%), consistent with the quenching effect of the OH⁻ ions discussed above for the case of Irgacure 2959. The generated free radicals, however, were large enough at 0.5 wt.% LAP to reach the maximum crosslinking density across all NaOH concentrations. At 0.1 wt.% LAP, all samples with DA showed significant decreases in storage modulus with NaOH due to the synergistic contribution of quenching OH⁻ ions, as well as blocked methacryloyl groups (due to alkaline-triggered polymerization of DA before the crosslinking reaction). The weaker initiation of Irgacure 2959 compared to LAP may also be the result of the radical scavenging functionality present in Irgacure 2959, discussed above.

6.2.4 Chemical characterization of crosslinking density

Nuclear magnetic resonance (¹H NMR) analysis was performed to evaluate the crosslinking density as well as methacrylation degree of gelatin. The degree of methacrylation for GelMA was found to be ~55% (Figure S6.3a). Peaks in the shaded area reflect the vinyl C-H bonds upon methacrylation of the parent gelatin. Photo-crosslinking of GelMA with Irgacure 2959 under UV illumination (100 mW/cm²) resulted in ~85% crosslinking density. This value reduced to ~55% and ~45% for the DA@GelMA samples (DA: 2 wt.%) and PDA@GelMA samples (DA: 2 wt.%, NaOH: 0.2 wt.%), respectively, crosslinked under the same conditions, as seen in the ¹H NMR spectra. This observation is in accord with the lower storage modulus with the addition of DA (Figure 6.2c,d) and consistent

with the correlation between the reduced crosslinking density and the final storage modulus. As discussed above, the reduced crosslinking density was largely due to the free-radical scavenging of catechol groups that was strengthened by the π - π aromatic interactions between the aromatic ring and unsaturated acrylate functionalities. The reduced crosslinking density in PDA@GelMA samples is linked to the increased probability of π - π interactions and covalent/non-covalent blockade of methacryloyl groups by PDA.

6.2.5 Evaluation of the hydrogel tensile properties

The tensile mechanical properties of the hydrogels were measured for the compositions and crosslinking parameters that led to gelation based on the rheological data. Illumination with UV light with an intensity of 35 mW/cm² and irradiation time of 2.5 min was selected for the LAP crosslinked hydrogels whereas, for the samples crosslinked with Irgacure 2959, the UV light parameters were set to 100 mW/cm² and 5 min, respectively. The effects of DA content, oxidation of DA (PDA formation), and photo-initiator type on the mechanical properties are illustrated in Figure 6.3. The stress-strain patterns (Figure 6.3a) were similar to those of typical hydrogels with linear elastic behavior up to the failure point (the stretched hydrogels are shown in Figure 6.3b).

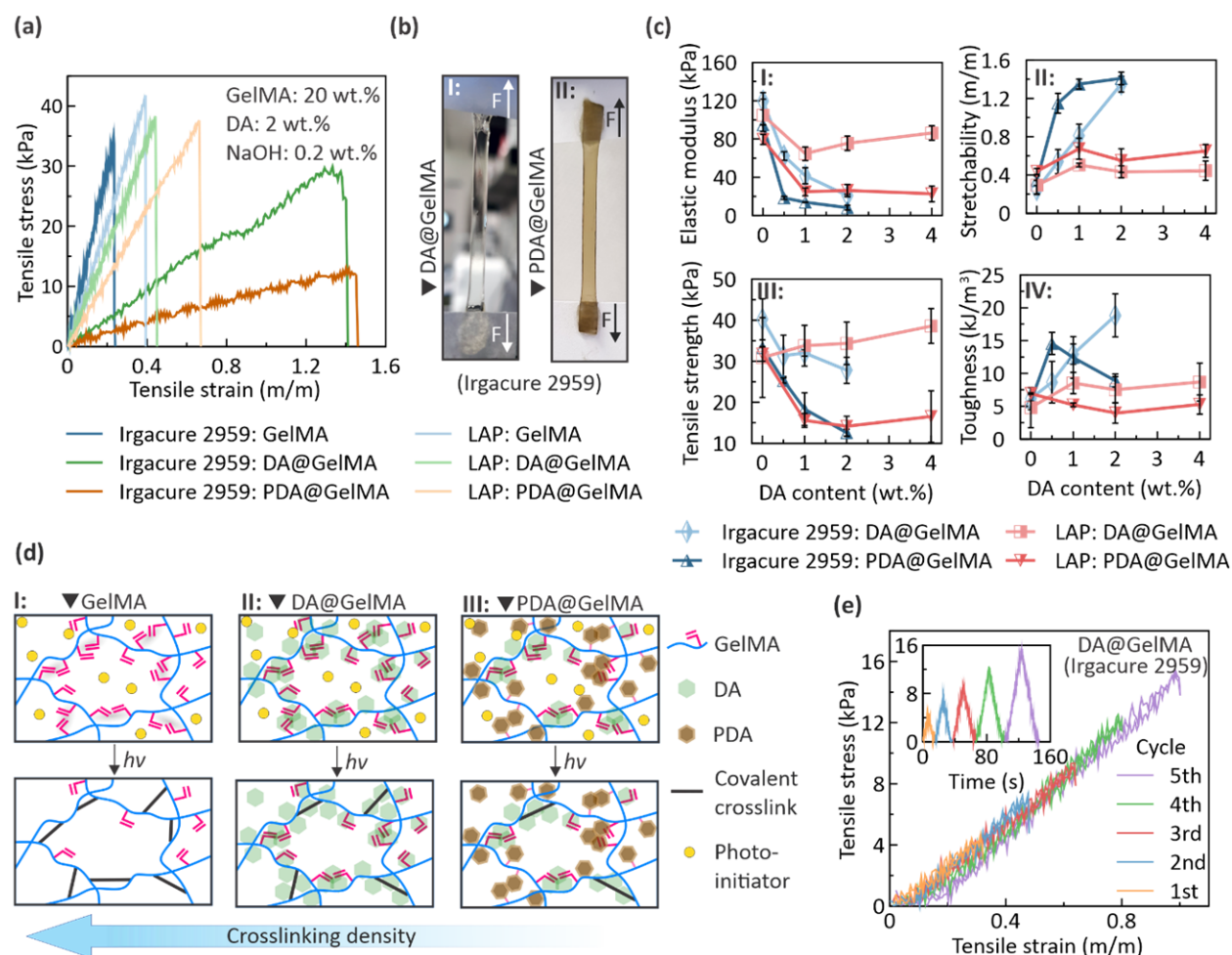


Figure 6.3. Tensile properties of the hydrogels. (a) The tensile stress-strain curves (0.5 wt.% photo-initiator at 100 mW/cm² UV light intensity was used for the 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) group and 0.1 wt.% photo-initiator at 35 mW/cm² UV light intensity was used for the lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP) group. (b) Optical images of I: dopamine (DA) @gelatin methacryloyl (GelMA) and II: polydopamine (PDA) @GelMA under extensive tensile deformations of over ~100% in the hydrogels crosslinked using 0.5 wt.% Irgacure 2959 photo-initiator. (c) The effects of the photo-initiator type, DA content, as well as DA oxidation on the tensile mechanical properties including I: elastic modulus, II: stretchability, III: tensile strength, and IV: toughness. (d) The molecular-scale mechanisms toughening the hydrogels due to the synergistic effects of reduced/homogeneous crosslinking density as well as non-covalent intramolecular interactions. (e) Stress-strain behavior of the DA@GelMA hydrogel under cyclic

loading confirming the hydrogel's elasticity by showing no hysteresis and permanent deformation at deformations up to 100%.

The mechanical properties of the hydrogels including elastic modulus, stretchability, tensile strength, and toughness are quantified in Figure 6.3cI-IV, respectively. The elastic modulus consistently decreased with the addition of DA (Figure 6.3cI). This reduced stiffness was more prominent for the samples polymerized with Irgacure 2959. Oxidation of DA in the presence of GelMA (PDA@GelMA samples) also led to further decreases in elastic moduli for both photo-initiator types. These observations are consistent with the results of the storage modulus (Figure 6.2c,d) and reflect the lower crosslinking density observed in the ^1H NMR results (Figure S6.3a). The higher compliance in the DA@GelMA and PDA@GelMA hydrogels can increase the conformability of the hydrogel on dynamic and elastic substrates, such as skin tissue, as it complies better with the deformation of the underlying tissues.

We also observed significant improvement in the hydrogel stretchability in both the DA@GelMA and the PDA@GelMA groups. The GelMA hydrogel formed at high concentration (20 wt.%) is brittle where the strain at failure does not typically exceed ~ 0.2 - 0.3 , no matter which photo-initiator was used (Figure 6.3cII). We observed $\sim 5.7\times$ improvement in the stretchability of GelMA ($\sim 140\%$) with the addition of 2 wt.% DA in hydrogels crosslinked *via* Irgacure 2959, whereas the stretchability varied over the range of 30-50% in the case of LAP. Alkaline oxidation of DA was also found to be effective in improving stretchability; however, the maximum stretchability for the PDA@GelMA group did not exceed those of non-oxidized DA@GelMA samples. High stretchability ($>140\%$) and low elastic modulus ($\lesssim 35$ kPa) facilitates hydrogel compliance with the natural deformations of tissue substrates (*e.g.*, up to $\sim 50\%$ strain in skin tissue),⁶²⁵ which requires lower adhesive resistance at the tissue

interface. We attribute the enhanced stretchability of the hydrogels to the reduction of the crosslinking density as well as dynamic interactions enabled by the large amount of DA acting as dynamic crosslinking points (see the proposed mechanism presented in Figure 6.3d). On the other hand, the low and likely more homogeneous crosslinking densities in DA@GelMA and PDA@GelMA composites are attributed to the radical-scavenging DA molecules that consumed the free radicals generated upon UV irradiation as well as steric blocking of the methacryloyl groups by aggregated DA at the methacryloyl sites, the effect of which was seen in rheological analysis of Figure 6.2c,d. On this basis, the stronger ability of LAP in generating free radicals under UV illumination underlies the lower sensitivity of the stretchability to DA concentration for samples crosslinked by LAP due to the denser crosslinking state in these hydrogels (despite the presence of DA-enabled noncovalent interactions). Comparing the LAP- to the Irgacure-2959-cured hydrogels indicates the more prominent contribution of crosslinking density to the observed improvement in stretchability. We attribute further enhancement of stretchability with NaOH treatment (for both LAP and Irgacure 2959) to alkaline polymerization of DA around and/or at the methacryloyl groups of the GelMA macromolecules *via* strengthening the steric hindrance and reducing the crosslink density.

Variations of tensile strength (Figure 6.3cIII) were found to be in accord with the observed elastic moduli, where in most cases, the addition of DA or NaOH either reduced the strength or had minimal impact. Analysis of toughness (a parameter that involves both stretchability and strength) shown in Figure 6.3cIV demonstrated that DA content has no effect on hydrogel toughness for the LAP group, whereas an average improvement of $\sim 3.3\times$ in toughness was achieved with the addition of 2 wt.% DA to hydrogels crosslinked by

Irgacure 2959 (toughness up to $\sim 19,000 \text{ J/m}^3$). Despite the current trends towards toughening hydrogels *via* introducing dynamic bonds, the results presented here suggest that optimizing the initiation step in photo-polymerization and tuning the crosslinking density can be effective in achieving extraordinary mechanical behavior (*e.g.*, stretchability and toughness) and deserves greater attention.

To demonstrate the strain recoverability and elastic behavior of the hydrogels, the most stretchable sample, *i.e.*, DA@GelMA (DA: 2 wt.%) with Irgacure 2959 was subjected to cyclic loading and unloading with increasing deformation amplitude. The samples were found to recover completely upon unloading at each loading cycle until the failure point, which indicated the lack of permanent deformation after the removal of extreme tensile loads. The stress-strain curves did not show any evidence of significant hysteresis, confirming full elastic energy recovery during unloading.

6.2.6 Characterization of adhesion

A large number of studies have been directed towards mussel-inspired adhesives serving catechol groups to establish covalent and noncovalent adhesion towards different substrate materials. Initially, we made attempts to explore the potential of the DA based GelMA composites as an injectable, *in situ* photo-crosslinkable adhesive biomaterial using a standard burst pressure test setup (Figure 6.4a). Our results demonstrated that regardless of the type of photo-initiator, any addition of either DA or NaOH reduced the burst strength (Figure 6.4b) This result is attributed partially to the compromised cohesion of the hydrogels, as explained in the tensile test results. On the other hand, the hydrogel composites were poorly crosslinked at the surrounding interfaces (possibly due to the oxygen inhibition effect in free radical polymerization), leaving behind a portion of uncrosslinked pre-polymer

between the hydrogel and substrate. This residue was evident when curing the DA@GelMA in polydimethylsiloxane (PDMS) molds, as some degree of pre-polymer was left in the mold after UV light irradiation and hydrogel removal (see Figure S6.4). As a result, the failure mechanism in burst pressure tests changed from cohesive failure (in GelMA) to adhesive failure (in DA@GelMA or PDA@GelMA samples, see Figure 6.4c). This observation indicates that the composite hydrogels are not suitable for *in situ* crosslinking as an injectable bioadhesive. On the contrary, GelMA hydrogels formed stable adhesion *in situ* in which no observable uncrosslinked pre-polymer was evident after UV light crosslinking (Figure S6.4). Besides, for the GelMA samples, the results represent larger burst strengths for the samples crosslinked with LAP than those with Irgacure 2959. Note that the adhesion of the photocrosslinked GelMA hydrogels (with the strongest adhesion forces) is known to be driven by covalent coupling reactions (*e.g.*, Michael addition) between GelMA and the substrate in the presence of free radicals. Therefore, the larger burst strength for LAP can be attributed to the stronger free radical generation ability in LAP than Irgacure 2959.⁶³⁹

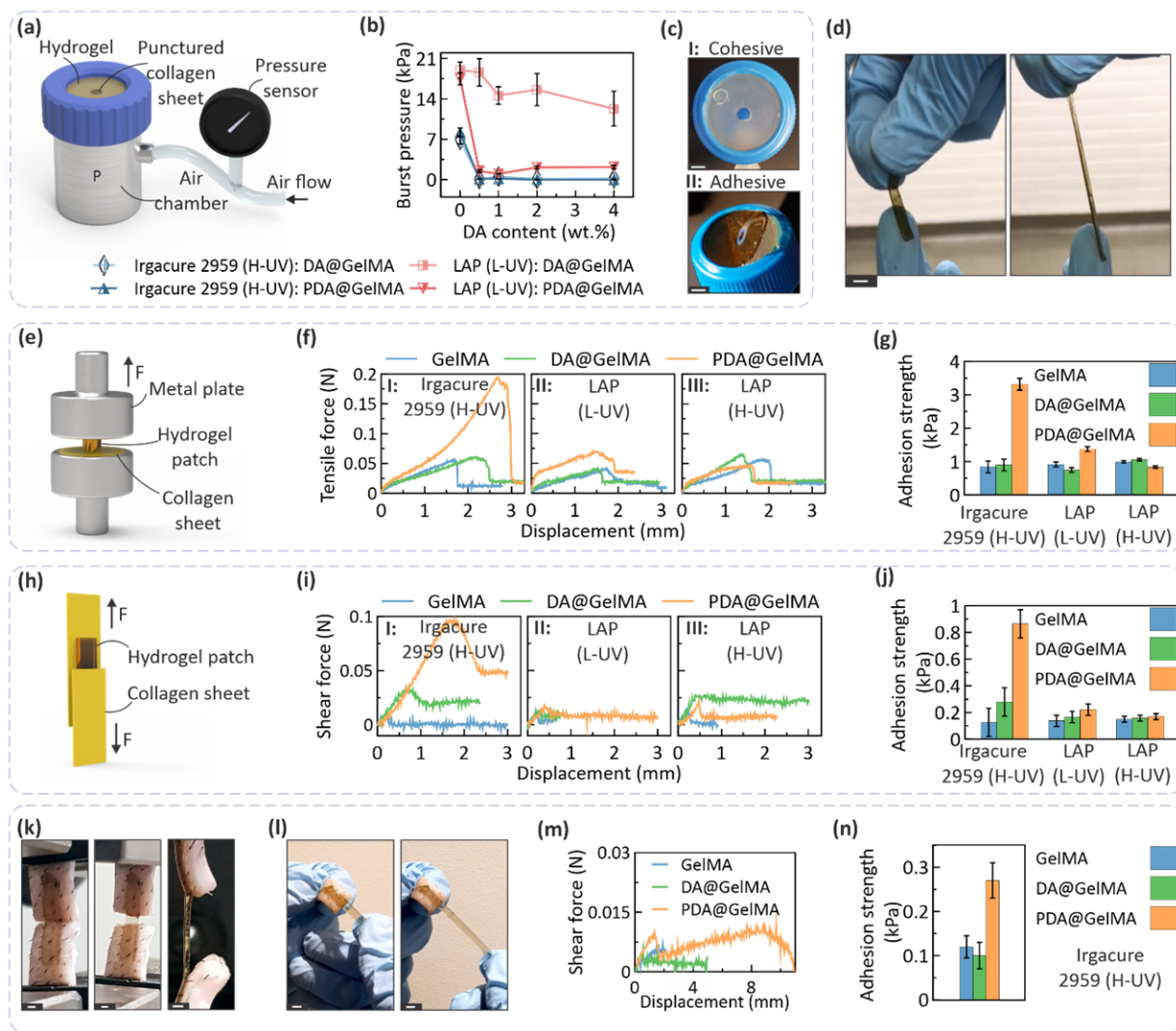


Figure 6.4. Characterization of the hydrogel adhesive properties. (a) Burst pressure setup used to evaluate the adhesion of hydrogels to collagen sheet substrates for assessing the hydrogels capability for *in situ* crosslinking applications. (b) The results of burst pressure tests performed on the hydrogels with different photo-initiator types, dopamine (DA), and sodium hydroxide (NaOH) concentrations. (c) Illustration of the I: cohesive, and II: adhesive failure mechanisms (scale bar = 3 mm). (d) Demonstration of the combination of adhesion and stretchability in the polydopamine (PDA) @gelatin methacryloyl (GelMA) hydrogels (dopamine (DA): 2 wt.%, NaOH: 0.2 wt.%) crosslinked using the 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) photo-initiator towards nitrile gloves (scale bar = 3 mm). (e) The schematic representation of the experimental setup used for evaluating the adhesion under tensile mode. (f) Tensile force-displacement results for the hydrogels photo-crosslinked using I: Irgacure 2959 at H-UV (*i.e.*,

100 mW/cm² UV light intensity for 5 min), II: lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) at L-UV (*i.e.*, 100 mW/cm² UV light intensity for 5 min), III: LAP at H-UV (*i.e.*, 35 mW/cm² UV light intensity for 2.5 min). (g) Adhesion strength in the tensile adhesion tests. (h) Experimental setup to study the results of adhesion tests under shear loads while slipping. (i) The shear force-displacement data for the hydrogels photo-crosslinked using I: Irgacure 2959 at H-UV (*i.e.*, 100 mW/cm² UV light intensity for 5 min), II: LAP at L-UV (*i.e.*, 100 mW/cm² UV light intensity for 5 min), III: LAP at L-UV (*i.e.*, 35 mW/cm² UV light intensity for 2.5 min). (j) The results of adhesion strength under shear loading mode. (k) Representation of the PDA@GelMA sample crosslinked with Irgacure 2959 in the *ex vivo* wound closure test setup (scale bar = 2 mm) and (l) adhesion of the hydrogel towards porcine skin (scale bar = 4 mm). (m) Shear-force versus displacement results and (n) adhesion strength data in wound closure test.

At the next stage, we characterized the adhesion for the already photo-crosslinked hydrogels to evaluate their potential for applications as tissue-attachable patches. To investigate the adhesion of the hydrogels, the hydrogels (100 mm²) were crosslinked separately and then placed between the collagen sheet substrates within lightly pre-loaded plates (~0.02 N). The adhesion stress was measured while the substrates were axially displaced away (Figure 6.4e) The results of adhesion force-displacement and tensile adhesion strength for GelMA, DA@GelMA (DA: 2 wt.%), and PDA@GelMA (DA: 2 wt.%, NaOH: 0.2 wt.%) samples using different photo-initiators (*i.e.*, Irgacure 2959 and LAP) crosslinked at different UV energy levels for the case of LAP (*i.e.*, L-UV at 35 mW/cm² intensity for 150 s and H-UV at 100 mW/cm² intensity for 5 min) is shown in Figure 6.4f,g. The adhesion force for all the samples was continuously increased with displacement. At this stage, the interfacial adhesion was dominant enough that imposed tensile displacement to the hydrogel material until the samples were fully detached from either collagen sheet substrates (corresponding to a sharp drop in load). Despite the current literature focus on the main hydrogel components and their resulting interactions to design adhesive

hydrogels,^{170,640-641} the results of tensile adhesion strength tests demonstrate the critical role of processing parameters such as UV light intensity and the initiation mechanism that typically receive less attention for adhesive hydrogel development. Even though these processing parameters had no significant effects in the cases of GelMA and DA@GelMA, they play large roles in the presence of NaOH (*i.e.*, for the PDA@GelMA hydrogels). Interestingly, the adhesion force dramatically increased $\sim 4\times$ in hydrogels with NaOH pre-oxidized DA (when Irgacure 2959 was used as the photo-initiator) compared to GelMA and DA@GelMA. These results are in contrast to previous studies emphasizing that catechol oxidation in mussel-inspired adhesives should be prevented in order for the catechol groups to be able to participate in adhesion driving interactions with the substrate.²⁹⁹ This hypothesis has come from the fact that MAPs are known to be produced at acidic pH, before deposition in nature (to protect catechol groups) so that after deposition and exposure to mildly alkaline ocean environments (pH ~ 8), catechol oxidation can drive mussel adhesion to substrates. Our experimental observations suggest high complexity and sensitivity of adhesion in catechol-incorporated hydrogels to processing parameters that can differ from polymer backbone to backbone and/or depend on the other components present (such as photo-initiators).

The force response of the hydrogels was also studied in shear mode to evaluate the slipping behavior of hydrogels placed between oppositely moving collagen sheet layers used as a biological substrate (Figure 6.4h). For this purpose, rectangular hydrogel samples (25 mm $\times$ 4 mm) were subjected to shear loads transferred *via* the large facets. The shear force-displacement results (Figure 6.4i) followed similar trends as friction test results where each started with a linear increase in load (representative of static friction) up to a maximum

peak. Thereafter, the load dropped to a plateau with displacement corresponding to dynamic friction. The adhesive strength results in shear mode (Figure 6.4j) were consistent with the tensile adhesion test results (Figure 6.4g). The maximum shear stress increased $\sim 6.9\times$ for the PDA@GelMA as compared to DA@GelMA or GelMA. Figure 6.4d illustrates the stretchability and stickiness of the top candidate, *i.e.*, PDA@GelMA hydrogels (DA: 2 wt.%, NaOH: 0.2 wt.%, Irgacure 2959 as photo-initiator, H-UV) towards nitrile gloves. From the results above, we interpret the improved adhesion as the reactive quinone intermediates as well as dynamic interactions between PDA species within the hydrogel. We attribute the low adhesion in DA@GelMA samples to the lack of strong covalent bonds between DA and GelMA, because the attractions between DA and the tissue surface functional groups do not constrain GelMA molecules to bind to the substrate surface. In addition, the “just-right” crosslinking density that enables superior stretchability and elasticity also synergistically favors adhesion due to the facile conformation and compliance of the hydrogel on yet dynamic substrates such as skin tissue. These characteristics make this hydrogel a promising candidate for skin-attachable substrates for applications such as wound dressings and stretchable devices and sensors. Similar trends were observed in the *ex vivo* wound closure test on the pig skin substrates (Figure 6.4k–n).

Note that DA has previously been conjugated to gelatin-based backbones, but the concentrations of grafted DA to gelatin have been limited. For instance, previous examples of catechol-conjugated gelatin have shown equivalent ranges of $\sim 0.1\%$ dopamine added to the hydrogel system,^{46,642} whereas in the present study, the incorporation of 2% dopamine is associated with significant improvement in adhesive and mechanical properties. The remarkable improvement in mechanical properties (*i.e.*, stretchability, toughness), as well as

adhesion performance, in this work has been enabled by the incorporation of higher levels of chemically active DA to the GelMA solution in the proposed one-pot mixing process in the pre-polymer preparation. We further synthesized a dopamine-conjugated GelMA (GelMA-DA) following a carbodiimide coupling protocol (EDC/NHS chemistry) reported in the literature²⁷⁶ and crosslinked the hydrogel *via* a similar approach as PDA@GelMA samples using an Irgacure 2959 photo-initiator. We observed that the PDA@GelMA samples at their optimum processing condition demonstrated significantly larger stickiness compared to those of conjugated GelMA samples, according to the normal adhesion test results (see Figure S6.6).

6.2.7 Biocompatibility of the adhesive patches

The *in vitro* biocompatibility of GelMA, DA@GelMA, and PDA@GelMA samples at the optimized conditions (*i.e.*, DA: 2 wt.%, NaOH: 0.2 wt.%, Irgacure 2959 as photo-initiator, H-UV) was tested to evaluate their potential for use in biomedical applications. The results of fluorescent live/dead images and cell viability are demonstrated in Figure S6.6a,b. We attribute the low cell viability for DA@GelMA to the acidic pH of the hydrogel, as the GelMA was originally treated with hydrochloric acid (HCl) to regulate the solution pH to ~5 (to prevent unwanted oxidation), which is further exacerbated by the HCl content of the dopamine itself. The cell viability, however, recovered to the order of GelMA controls in the PDA@GelMA samples, where added NaOH increased the pH to ~8, which is closer to physiological conditions. The cell viability for the stretchable and adhesive hydrogels (PDA@GelMA) was ~90% and the metabolic activity was in the range of GelMA, indicating that the samples supported cellular proliferation Figure S6.6c. The biocompatibility data suggest that the proposed material formulation can be used in biomedical settings as no

significant toxicity was observed by the DA or NaOH additives.

6.3 Conclusions and Prospects

The present study reveals the capability of DA additives and their oxidized species to enhance stretchability, toughness, and adhesion in GelMA hydrogels. Simply with physical one-pot mixing, and no need for secondary conjugation reactions, incorporation of larger DA content in GelMA hydrogels enabled stronger mechanical properties and adhesion in bulk hydrogels. We demonstrated the critical roles of processing parameters (such as photo-initiator type as well as UV light parameters) in activating adhesion and improving stretchability and toughness. According to our results, we draw the following conclusions: The DA-methacryloyl interactions introduce dynamic energy-dissipating components and lead to lower/more homogeneous crosslinking densities. These changes lead to significant improvements in stretchability (up to $\sim 5.7\times$) and toughness (up to $\sim 3.3\times$). Those mechanical improvements are achieved only when Irgacure 2959 is used as photo-initiator (not LAP). The physical properties in mussel-inspired hydrogels are not only a function of materials chemistry but also the processing parameters such as photo-initiation. UV photocrosslinking reaction kinetics and rheological properties of crosslinked hydrogel can be tuned by DA (for Irgacure 2959) and NaOH (for LAP) concentrations. The stickiness of GelMA-based patches can be significantly enhanced (increased adhesion strength up to $4.0\times$ under tension and $\sim 6.9\times$ under shear loads) when DA is first pre-oxidized using NaOH in the pre-polymer solution before UV light irradiation (*i.e.*, PDA@GelMA) *and* when proper polymerization conditions are implemented.

The improved adhesion is primarily due to the synergistic contributions of enhanced

compliance, stretchability, and interactions with the reactive quinone species. We find that outstanding adhesive properties are enabled by incorporating larger amounts of catechol groups than those of conjugated chemistries (*e.g.*, gelatin-dopamine); however, the release of dopamine inhibits its application for internal implantation. We are continuing research on improving the catechol loading with coupling chemistries. The abovementioned take-home messages provide valuable insight into rational processing procedures to use catechol groups for activating toughness and adhesion in GelMA-based hydrogels. The adhesion and stretchability of the hydrogel patches support their applications as skin-attachable substrates to develop wearable devices as well as for wound dressings.

6.4 Experimental methods

6.4.1 Materials

Gelatin from porcine skin (type A), methacrylic anhydride, and dopamine hydrochloric were supplied by Sigma Aldrich, USA. Sodium hydroxide (NaOH) pellets and dimethylsulfoxide (DMSO) were purchased from Thermo Fisher Scientific, USA. Photo-initiators including 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate were provided by Sigma Aldrich. Dulbecco's phosphate-buffered saline (DPBS) was supplied by Thermo Fisher Scientific, USA.

6.4.2 Hydrogel preparation procedure

Gelatin methacryloyl (GelMA) was synthesized following a previously described protocol.⁵⁹⁷ In brief, gelatin was dissolved in DPBS at 55 °C. After full dissolution, MA was

added dropwise to the gelatin solution (8 v/v%) and allowed the reaction to proceed for 3 h at the same temperature. Then, the reaction was stopped by adding an equal volume of DPBS to the reaction solution. The final solution was dialyzed against DI water to remove unreacted species for 7 days followed by lyophilizing. To prepare the pre-gel mixture, first, a stock solution of 40 wt.% GelMA was prepared (final concentration of GelMA after mixing other components was 20 wt.%) and the pH was set to ~5. Photo-initiator (Irgacure 2959 or LAP) solutions were prepared separately at stock concentrations of 2.5 wt.% and 0.5 wt.% for the samples with final photo-initiator concentrations of 0.5 wt.% and 0.1 wt.%. The photo-initiator solutions were kept at 80 °C oven until fully dissolved. DA stock solutions at 0, 2, 4, 8, and 16 wt.% (corresponding to final DA concentrations of 0, 0.5, 1, 2, and 4 wt.%) and NaOH solutions of 0, 2, 4, 6, and 8 wt.% (corresponding to the final NaOH concentrations of 0, 0.1, 0.2, 0.3, and 0.4 wt.%) were prepared, respectively. The composites of DA in GelMA in the absence and presence of NaOH (alkaline triggered DA polymerization) were labeled as DA@GelMA and PDA@GelMA, respectively. To reach the desired final concentrations, the GelMA, photo-initiator, DA, and NaOH were mixed with a volume ratio of 1:0.4:0.5:0.1. The pre-gel mixtures for the PDA@GelMA samples were kept at 37 °C for 1 h before performing the tests to allow DA oxidation unless otherwise noted. The hydrogels were UV-cured using an Omnicure System S2000 series at the designated intensities for the pre-defined exposure times.

6.4.3 UV-vis characterization

UV-vis spectra were measured for characterizing the oxidation of DA in the presence of GelMA macromolecules using a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, model 840-274200). For this purpose, the pre-gel solutions containing 0

wt.% DA was used as blanks. For each measurement, 2 μ L of the solution was pipetted onto the designated measurement surface and relative absorbance was registered across the UV-vis wavelengths at different time points up to 48 h. To evaluate permanent attachment of PDA to the GelMA, the PDA@GelMA prepolymer was diluted by 4 $\times$ in DI water (to 2 ml final volume) and dialyzed in DI water for 2 days using a 12-14 kDa dialysis membrane to remove free small-molecule residues. The solution was then centrifuged using a minicentrifuge for 5 min at 3500 rpm. The supernatant was pipetted for UV-vis spectroscopy.

6.4.4 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (^1H NMR) spectroscopy was used for the chemical characterization of the biopolymers and crosslinked hydrogels under different chemical conditions. ^1H NMR spectra were recorded for all samples at room temperature on a 400 MHz Bruker AV400 spectrometer. All spectra were corrected with phase and baseline and solvent peak was fixed before quantifying the degree of methacrylation. To quantify the degree of methacrylation, GelMA was dissolved in DMSO- d_6 at a concentration of 10 mg/mL, and the degree of substitution was calculated according to previously reported procedures.^{643,644} To assess the crosslinking degree of hydrogels, GelMA, DA@GelMA, and PDA@GelMA samples (GelMA: 20 wt.%, DA: 2 wt.%, NaOH: 0.2 wt.%) were photocrosslinked with Irgacure 2959 at 100 mW/cm². The samples were then immersed in DMSO for 7 days at 37 °C and sonicated to partially dissolve the hydrogels prior to conducting the ^1H NMR spectroscopy. The degree of crosslinking of hybrid hydrogels were calculated by integrating double bond protons (vinyl) at $\delta=5.28$.

6.4.5 Rheological characterization

Physical gelation of the hydrogels at room temperature was assessed by tilting the hydrogels in glass vials. To monitor the photo-crosslinking reaction of the prepolymer solutions, oscillatory rheological tests were performed for real-time characterization of the storage modulus (G') and loss modulus (G''). An 8 mm torque measurement system was used in a modular compact rheometer (MCR 302, Anton Paar) to implement the tests for which an accessory fixture was fabricated to hold the UV light source perpendicular to the measuring system axis which enabled transverse radiation of UV light during the test. The rheological tests were conducted on 100 μ L pre-gel solution applied between a 1 mm gap between the measurement system and a fixed base surface. Oscillatory runs were defined as a time sweep with 10% strain and 1 Hz as test parameters. The G' and G'' measurements were recorded for 4 min once the UV light irradiation was activated (after 1 min stabilization period). Gelation time was calculated as the UV light exposure time at which G' overtakes G'' and the storage modulus after reaching to the plateau was assigned to the final modulus. Temperature sweep tests were performed at similar strain and frequency values used for the crosslinking tests. The hydrogel prepolymer solutions cycled from 50 °C to 10 °C and heating back to 50 °C at a rate of 1 °C/min to quantify their phase transition temperature. Gelation and dissolution temperatures were defined as the crossover of G' and G'' during cooling and heating phases.

6.4.6 Mechanical tensile tests

The tensile properties of the hydrogels at different compositions were obtained by stretching the hydrogels glued to the frame-shaped papers (for facile gripping). To prepare

the tensile test samples, 200 μL prepolymer solution was cast in polydimethylsiloxane (PDMS) molds followed by UV light curing. The cured hydrogels with final dimensions of 4 mm $\times$ 25 mm were subjected to tensile loads. To do this measurement, an Instron 5943, USA universal testing machine equipped with a 100 N load cell was used. The crosshead displacement rate was defined as 4 mm/min and the tests continued until the samples fully ruptured. The force-displacement data were recorded during the tests from which the stress ($\sigma = F/A$) and strain ($\varepsilon = \delta/L$) curves were generated. The stretchability and strength were defined as strain and stress at the failure point, respectively. Toughness was calculated by the area under stress-strain curves ($U = \int \sigma d\varepsilon$) up to the failure point. To perform the cyclic tests, a higher loading rate of 40 mm/min was used.

6.4.7 Adhesion tests

The *in vitro* adhesion tests were conducted under different loading scenarios to wet collagen sheet (representative of skin tissue). Burst pressure tests were conducted. Collagen sheets already immersed in DI water were dried using Kimwipes and cut in ~ 25 mm diameter at the center of which a 3 mm hole was punctured. The collagen sheets were soaked in DPBS for 1 h. Wet collagen sheets were obtained by Kimwipe drying to remove the excess water; then they were fixed onto the fixture under which there was an air chamber jointly connected to a pressure sensor and syringe pump. The surface of the punctured collagen sheet was filled with 100 μL pre-gel solution followed by curing with UV light. The samples were tightly fixed to the outlet of an air chamber and then, the air was pumped to increase the air pressure inside the chamber *via* a syringe pump (at 20 ml/min) while the sensor recorded the air pressure. The adhesion strength was defined as the air pressure at the point where the hydrogel failed, and the air leaked. Tensile adhesion tests were performed using

the Instron 5943 testing device. The collagen sheets were attached to the surface of the top and bottom compression test plates. The crosslinked hydrogels (100 mm² and 1.5 mm thickness) were placed on the bottom plate and the top plate was moved down to touch the sample with a slight pre-load of ~0.02 N. The load and displacement data were recorded while the top plate was moved up until the hydrogel was fully detached from either surface. Tensile adhesion test data were recorded with a 1 mm/min crosshead displacement rate. The adhesion and slipping behavior of the hydrogels on collagen sheets were evaluated under shear loading mode. The tests were performed by placing the crosslinked hydrogels of (4 mm × 25 mm) in between two collagen sheet layers followed by a tensile displacement moving the sheets away from each other. The displacement rate was set to 5 mm/min for the shear slip tests.

The *ex vivo* adhesion test of wound closure was performed on pig skin tissues provided by the UCLA animal facility. Two skin cuts of 8 mm × 30 mm were placed adjacent to each other (at their 8 mm faces). Then, the crosslinked hydrogels were placed and gently pressured on the epidermis layer, bridging the interface between the two skin tissues. Finally, the tensile displacement was applied axially to the tissue substrates according to the procedure explained above. The shear strength was calculated by dividing the maximum force (first observed extremum) to half of the total hydrogel contact surface area (*i.e.*, 4 mm × 12.5 mm).

6.4.8 *In vitro* biocompatibility analysis

A 2D cell culture process was conducted to evaluate the cytocompatibility of the hydrogels towards a mouse fibroblast cell line (NIH/3T3, ATCC). The media used for cell culture was DMEM containing 1% penicillin-streptomycin and 10% FBS and the cell culture

was performed in an incubator (Thermo Fisher Scientific, USA) at standard 37 °C and 5% CO₂ conditions. To grow the cells, 75 mm² cell culture flasks were used, and the media was refreshed every two days. The cells were passaged upon confluency at ~90%. To evaluate the hydrogel cytotoxicity, four replicates of the samples (i) GelMA (20 wt.%), (ii) DA@GelMA (GelMA: 20 wt.%, DA: 2 wt.%), and (iii) PDA@GelMA (GelMA: 20 wt.%, DA: 2 wt.%, NaOH: 0.2 wt.%) were fabricated with dimensions 6 mm diameter and 1.5 mm height. The hydrogels were kept under UV light for 2 h for sterilization and then soaked in 2 ml media in 24-well plates containing 10,000 cells per well, so the cells were seeded on the top of the hydrogels. Live/dead assays were performed using viability kits provided by Invitrogen, USA on days 1, 3, and 7 post-culture. Briefly, the staining solution was prepared by adding 20 µl ethidium homodimer-1 and 5 µl calcein in 10 ml PBS in which the hydrogels were soaked for 30 min in an incubator. Then, the samples were imaged using a fluorescent microscope (Axio Observer 5, Zeiss, Germany) at excitation/emission wavelengths of ~494/515 nm (green) for calcein and ~528/617 nm (red) for ethidium homodimer-1, respectively. ImageJ software was used to analyze the images and to calculate the numbers of live cells. Cell viabilities were reported as the fraction of live cells (green) to the sum of live and dead (red) cells. Furthermore, the metabolic activities of the cells were evaluated on the same days using a 1:9 PrestoBlue reagent (Invitrogen) solution in PBS. A volume of 500 µL PrestoBlue solution was added to the media containing hydrogels, followed by 2 h incubation. A plate reader (vis Synergy 2, BioTek) was then used to measure the fluorescence intensity at excitation and emission wavelengths of 560 and 590 nm, respectively. The fluorescence intensity values were normalized with respect to the media with no cells.

6.5 Supporting information

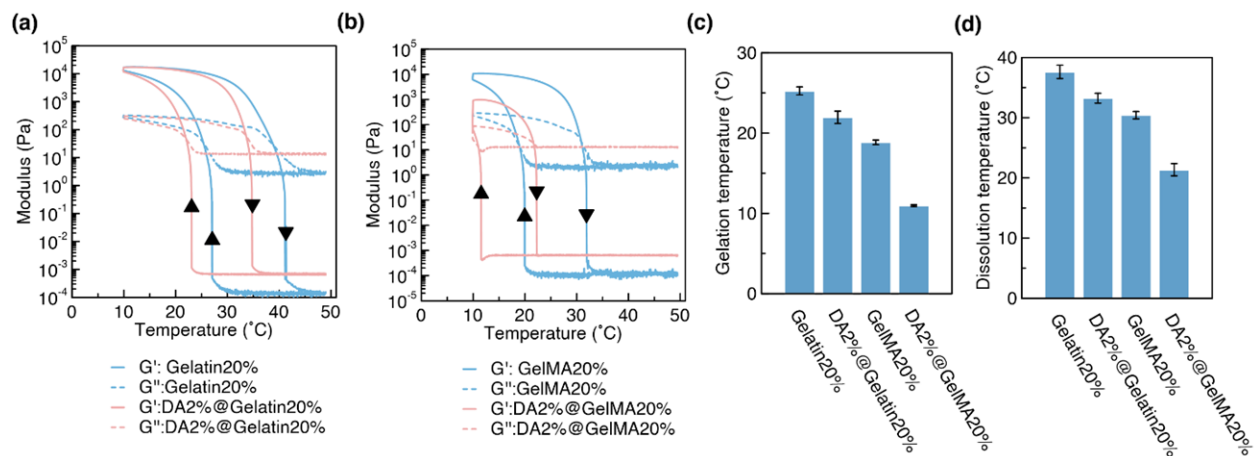


Figure S6.1. Rheological testing of the effect of dopamine (DA) on the sol-gel transition temperature of (a) gelatin and (b) gelatin methacryloyl (GelMA) solutions. (c) Gelation temperature and (d) dissolution temperature demonstrating both methacrylation and addition of DA lead to significant drop in phase transition temperatures.

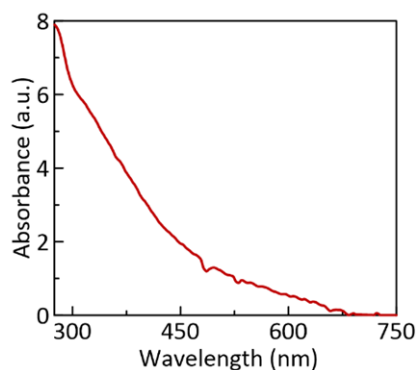


Figure S6.2. UV-vis absorption spectrum of polydopamine (PDA)@gelatin methacryloyl (GelMA) solution demonstrating *in situ* polymerization of dopamine (DA) in the presence of GelMA, which results in permanent attachment of PDA to the GelMA backbone.

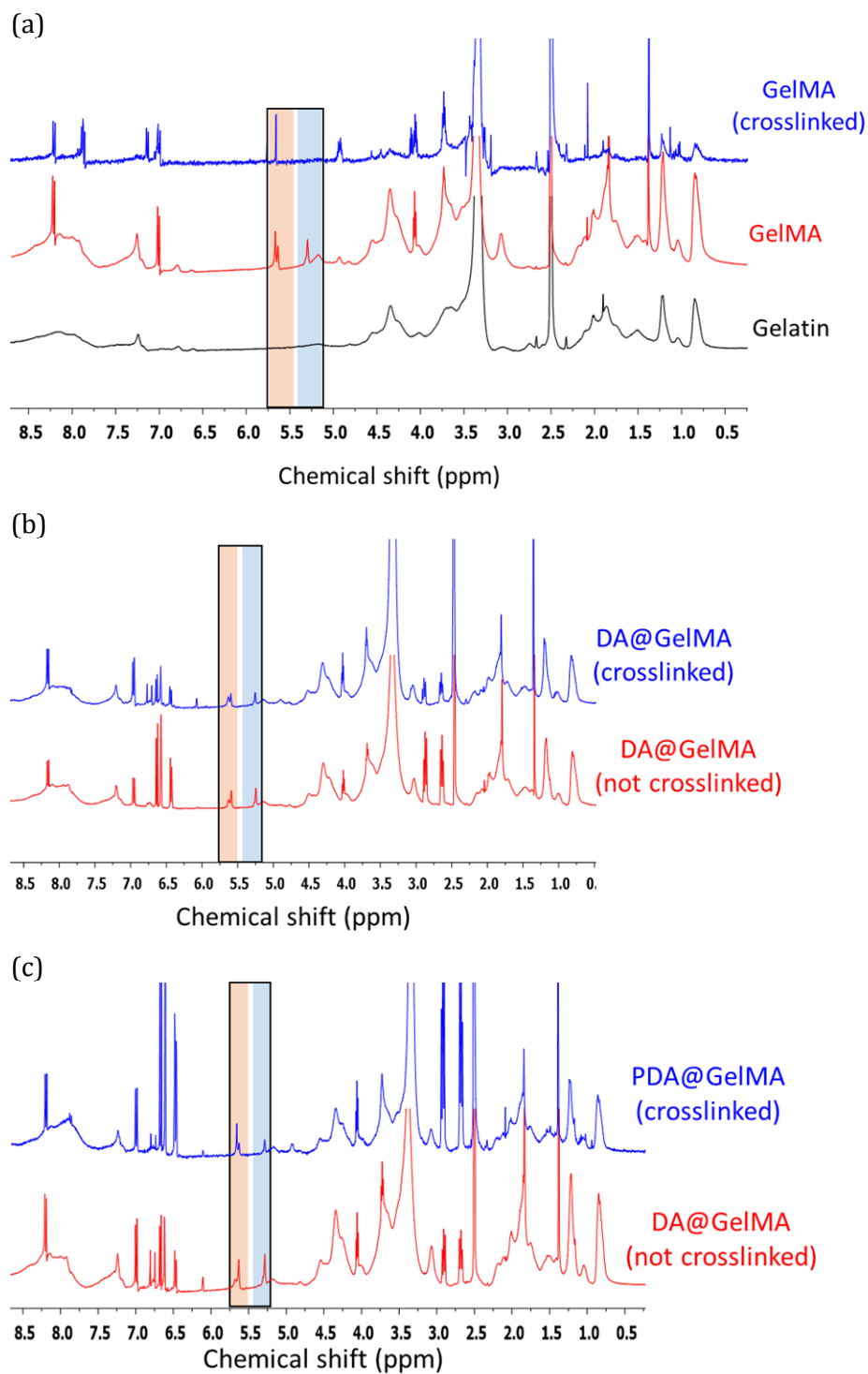


Figure S6.3. Nuclear magnetic resonance (^1H NMR) spectra of (a) gelatin (black), gelatin methacryloyl (GelMA) (red), and crosslinked GelMA (blue), (b) dopamine (DA) @GelMA and (c) polydopamine (PDA) @GelMA samples. Degree of GelMA methacrylation was *ca.* 55%. The degree of crosslinking

for GelMA, DA@GelMA, and PDA@GelMA samples were calculated as ~85%, ~55%, and 45%, respectively.



Figure S6.4. Digital photograph of the polydimethylsiloxane (PDMS) molds used to crosslink rectangular (25 mm $\times$ 4 mm) samples. I: Crosslinked dopamine (DA)@GelMA before removal, II: DA@GelMA and III: GelMA samples after removal. Some degree of pre-polymer residue are left behind when DA is added to the hydrogels. Scale bar = 2 mm.

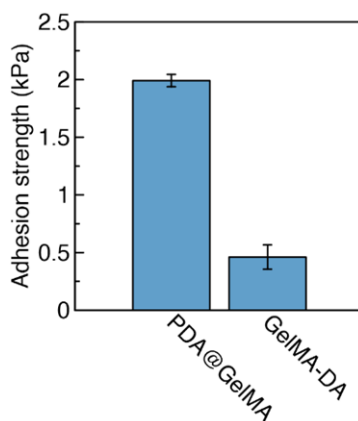


Figure S6.5. Normal adhesion test comparing polydopamine (PDA)@gelatin methacryloyl (GelMA) with DA-conjugated GelMA (GelMA-DA).

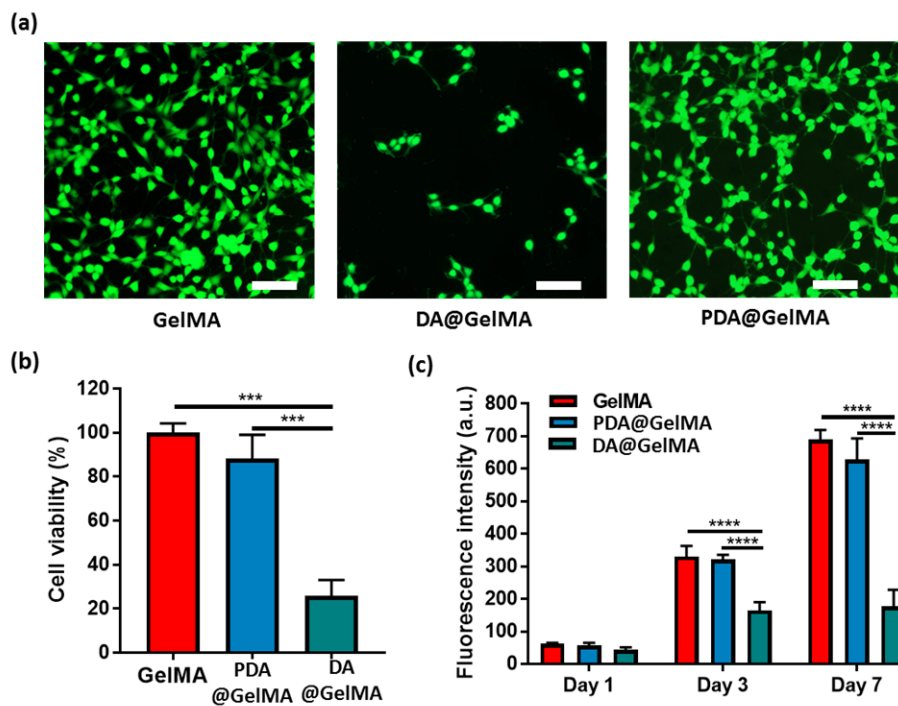
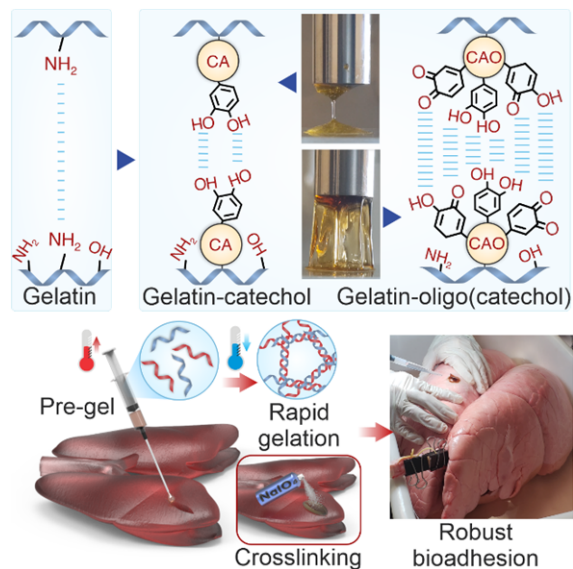


Figure S6.6. *In vitro* biocompatibility tests. (a) Live/dead fluorescence images (scale bar = 100 μm), (b) cell viability data, and (c) fluorescence intensity results for study of metabolic activity in gelatin methacryloyl (GelMA), dopamine (DA)@GelMA, and polydopamine (PDA)@GelMA samples.

CHAPTER 7: INJECTABLE GELATIN-OLIGO-CATECHOL CONJUGATES FOR TOUGH THERMOSENSITIVE BIOADHESION



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Abstract

Extracellular matrix (ECM)-derived biomaterials such as gelatin-based hydrogels are attractive candidates for sealing internal leakages due to their excellent bioactivity. However, gelatin derivatives are brittle and suffer from poor adhesion. Modifications of gelatin with adhesive catechol moieties have been limited to low degrees of substitution.

Here, we propose oxidative oligomerization of catecholic compounds (namely, caffeic acid, CA) prior to the coupling reaction, to augment the number and availability of grafted catechol groups per carbodiimide conjugation reaction, thereby achieving robust bioadhesion. Bioadhesion of injectable gelatin-caffeic acid (GelCA) hydrogels in two different crosslinking scenarios are examined: (i) in mixtures with gelatin methacryloyl (GelMA) for photo-crosslinking, and (ii) oxidative crosslinking *via* surface infusion of NaIO₄. As opposed to photo-crosslinkable GelCA, oxidatively crosslinked GelCA bioadhesives offer wet bioadhesion stability and one-sided adhesion selectivity to tissue. *Ex vivo* adhesion tests on pig lungs suggests $\sim 3\times$ improvement in adhesion strength compared to other gelatin derivatives (*i.e.*, GelMA controls), which we attribute to their enhanced cohesion (*i.e.*, $\sim 5.3\times$ and $\sim 11.5\times$ improvements in stretchability and toughness, respectively). Furthermore, as opposed to gelatin solutions that easily dissociate *in vivo*, aqueous solutions of GelCA self-associate at body temperature. Functionalization of gelatin with CA oligomers (CAO) enables rapid formation of physical gels upon exposure to room temperature, tunes the viscosity of the GelCA pre-gel solution for controllable injectability onto vertical and multiply curved tissue surfaces, and boosts the antioxidant effects of CA. The robust bioadhesion and biocompatibility of the GelCA hydrogels suggest that GelCA bioadhesives are promising candidates for sutureless closure of internal surgical wounds.

7.1 Introduction

Suturing and stapling have led to frequent cases of failure in sealing surgical wounds, particularly in the case of friable tissues such as pulmonary lacerations.⁶⁴⁵ Surgical sealants are increasingly being used in place of conventional wound closure methods.¹⁷ In this

respect, shape-fitting injectable bioadhesives have received tremendous attention, as they reduce blood loss and surgical time while preventing additional trauma.⁸ Ideal bioadhesives are nontoxic and establish rapid tough adhesion to wet tissues on timescales compatible with surgical settings.^{646,1} Natural adhesives offer biocompatibility and biodegradability.^{647,648} However, among natural sealants, fibrin-based bioadhesives are rather costly, they suffer from poor adhesion, and they pose the risk of viral transmission.⁶⁴⁹ Gelatin-based biomaterials are low cost and form glutinous aqueous solutions at high concentrations. In addition, gelatin-based commercial products such as Gelfoam® are U.S. Food and Drug Administration (FDA) approved for hemostatic applications; however, they have poor adhesion strength.^{276,2} Thermosensitive gelation of gelatin occurs at temperatures below body temperature.⁶⁵⁰ Hence, gelatin readily dissociates in wet media and its solutions can easily spread over the surrounding tissues due to its low viscosity, leading to unwanted inflammatory reactions.⁵²⁰ Physical hydrogels made of gelatin are also brittle²⁰⁸ and have poor mechanical strength. Hence, near-term goals include enhancing adhesion and cohesion in gelatin hydrogels for bioadhesive applications.^{278,626,642,651-652}

Modification of gelatin with methacryloyl groups has led to photo-crosslinkable gelatin methacryloyl (GelMA) bioadhesives.^{4,59-61} Michael addition reactions during polymerization of GelMA drive covalent adhesive reactions with tissue surfaces.⁵³ In contrast to soft and expandable tissues, such as lung, GelMA hydrogels are rather brittle (~30% stretchability)⁴¹ and therefore, adhesion failure occurs as they do not conform to tissue deformation. Inspired by adhesion in marine organisms,⁴² many studies have explored catechol functionalization of gelatin *via* carbodiimide coupling by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) reagents⁶⁵³

using catechol-carrying small molecules such as dopamine.^{46,62-63,276,654} Conjugation efficacy in such reactions is low (below ~10%) and the number of conjugation sites on gelatin are limited.^{47,24} Previous studies report that *in situ* polymerization of dopamine in the presence of GelMA imparted bioadhesion to crosslinked hydrogel patches when high catechol content was used (*i.e.*, 1:10 dopamine to GelMA weight ratio).^{24,655} Unwanted gelatin homocoupling reactions are also inevitable, which can further reduce conjugation efficacy. In addition, crosslinking in catechol-modified backbones mainly relies on metal-ion coordination^{46,656} and coupling reactions in the presence of oxidants^{48,657-660} wherein catechol groups are consumed to interact with themselves instead of tissues. Adhesion in most injectable bioadhesives is irreversible and non-selective (as the bioadhesive can adhere to tissues through both sides).¹⁹¹ Therefore, it remains challenging to achieve robust bioadhesion using gelatin-based biomaterials for their application in surgical wound management.

Among the phenolic compounds for catechol conjugation, caffeic acid (CA) is found in coffee, olive oil, *etc.*³⁶ and is characterized by radical scavenging, carcinogenic inhibition, and wound-healing³³⁴ properties. In this study, by means of CA molecules, we increase the number of coupled catechol motifs using EDC/NHS chemistry to reinforce the effects of catechol functionalization in gelatin-based injectable bioadhesives. For this purpose, CA is first oxidized to yield caffeic acid oligomers (CAO) prior to the conjugation reaction (Figure 7.1A). By means of chemical interactions introduced by quinone CAO components, thermosensitivity of gelatin-caffeic acid (GelCA) solutions is tuned to enable their fast thermal gelation after application to the wound (Figure 7.1B), and to enhance their stability at body temperature. Those interactions are further employed to toughen gelatin-based hydrogels and thereby to delay cohesive failures in adhesion. Comprehensive parametric

studies are conducted to optimize processing parameters, including CA oligomerization, gelatin source, and *in situ* crosslinking approach (*i.e.*, photo-crosslinking in GelCA/GelMA hybrid systems *versus* oxidative crosslinking *via* NaIO₄ solutions) to meet the requirements for internal bioadhesion (Figure 7.1A,B). Finally, we test the adhesion efficacy of GelCA hydrogels in *ex situ* tests on the pig tissue models by comparing the results with commercially available as well as under development sealants and studying their biocompatibility and antioxidant activity for applications in sealing internal wounds.

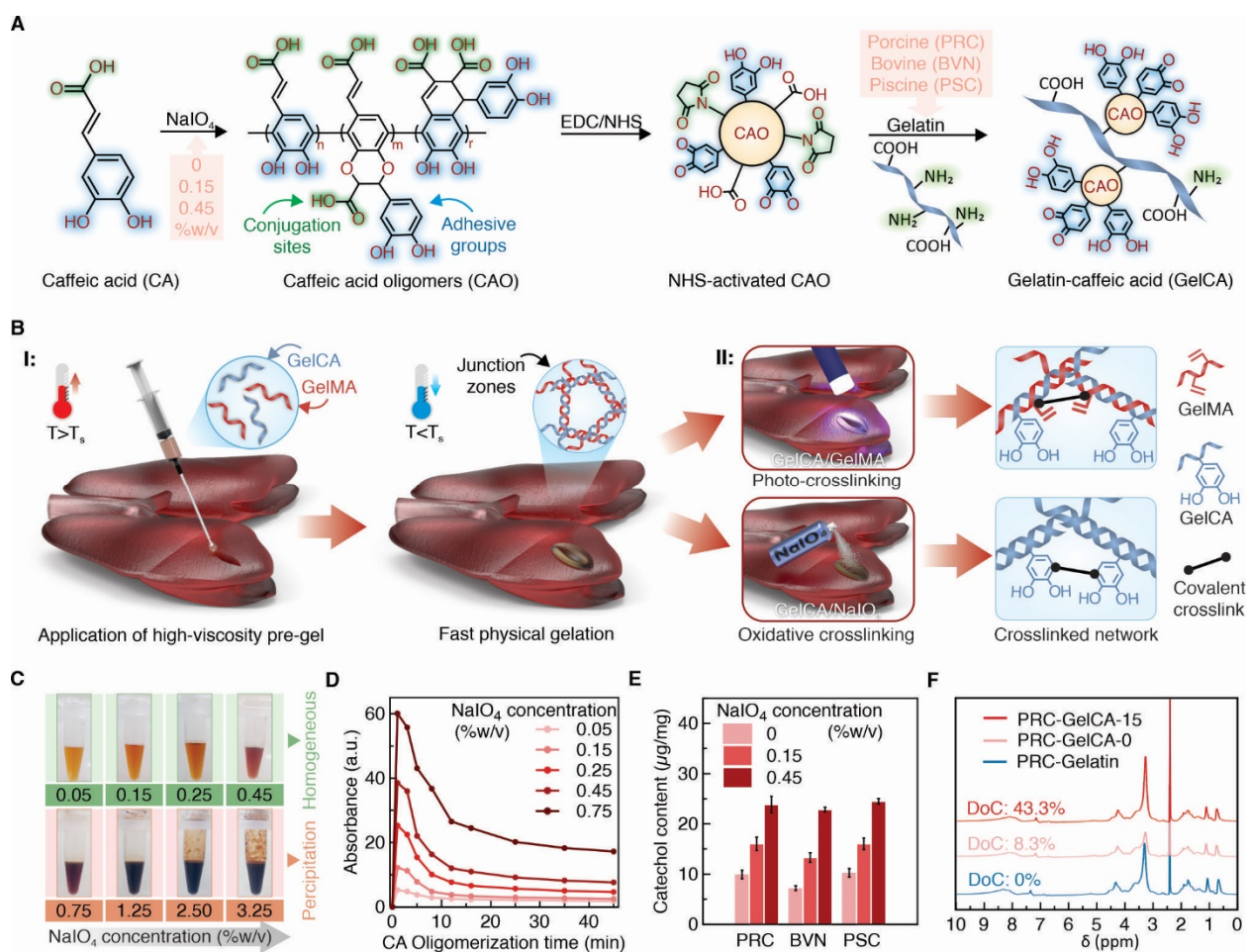


Figure 7.1. Bioadhesive design and chemical characterizations. (A) Synthesis scheme for the functionalization of gelatin with oligo-catechol groups. Caffeic acid (CA) is oxidized with NaIO₄ to yield caffeic acid oligomers (CAO) moieties containing an array of catechol groups for adhesion and carboxyl groups for 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide

(NHS) coupling reaction. The EDC/NHS coupling reaction results in gelatin-caffeic acid conjugates (GelCA). Gelatin was taken from different sources, *i.e.*, porcine (PRC), bovine (BVN), and piscine (PSC). (B) The process for application of injectable GelCA bioadhesives on surgical wounds in soft internal tissues, such as lungs. The pre-gel solution is applied at a temperature higher than the sol-gel transition temperature (T_s) followed by rapid physical gelation upon exposure to the operating room temperature. Subsequent on-demand crosslinking imparts robust stability *in vivo*. Crosslinking strategies studied here include photo-crosslinking in hybrid mixtures of GelCA with gelatin methacryloyl (GelMA) as well as oxidative crosslinking of GelCA using a NaIO_4 solution. (C) The effect of NaIO_4 concentration on oxidative polymerization of CA after 1 h. (D) UV-vis characterization of oxidation reaction kinetics in terms of absorbance at 500 nm (representative of CAO). (E) Results of catechol content per mg gelatin obtained *via* Arnow assay suggesting the larger amount of catechol conjugates due to pre-conjugation oxidative oligomerization of CA. The data are presented as means $\pm$ SD. (F) Proton nuclear magnetic resonance (^1H NMR) comparing the degree of primary amine consumption (DoC) in PRC-GelCA synthesized with different levels of CA oxidation.

7.2 Results and Discussion

7.2.1 Synthesis of gelatin-caffeic acid conjugates

Gelatin was modified with CA molecules and their oligomers, following the reaction scheme shown in Figure 7.1A. Note that, as opposed to other phenolic compounds such as dopamine where oxidative polymerization consumes their primary amine conjugation sites, the carboxyl conjugation sites of CA remain intact in their oxidized CAO moieties. Here, each molecule of CAO carries a larger number of catechol groups than CA. Therefore, we hypothesize that more catechol groups are grafted per EDC/NHS coupling reaction. In addition, NHS activation of CAO in the absence of gelatin reduces gelatin homocoupling compared to when gelatin is NHS-activated to conjugate with *e.g.*, dopamine.

To synthesize CAO, CA molecules were oxidized with different concentrations of

NaIO₄.³⁶ Oligomerization kinetics of CA were characterized *via* UV-vis spectroscopy (Figure S7.1A). Upon addition of NaIO₄ to CA, the solution color changed to dark brown due to CA oligomerization, as shown in Figure 7.1C. Absorbance at 500 nm corresponded to CAO formation (see Figure 7.1D).³⁶ The values of absorbance sharply increased upon addition of NaIO₄ during the first *ca.* 2 min, reaching a peak as CA coupling reactions progressed to form CAO (Figure 7.1B). Thereafter, the absorbance dropped to a plateau proportional to the concentration of NaIO₄ at ~1 h. The initial recovery of absorbance may be due to interactions between the primarily generated CAO with dimethyl sulfoxide (DMSO) present in the reaction solvent. According to a previous study,⁶⁶¹ DMSO establishes strong hydrogen bonds with phenolic compounds and thereby inhibits formation of high molecular weight aggregates. In addition, the UV-vis spectra of CA measured in 50 %v/v DMSO (peak at 380 nm) compared to water (peak at 320 nm) exhibited dissimilar peaks indicating interactions between DMSO and CA (Figure 7.1C). Increasing the NaIO₄ beyond 2.5 %w/v led to precipitation and forming a cloudy solution (Figure 7.1C). Similar aggregation occurred upon addition of EDC/NHS to the solutions containing 0.75 %w/v and higher NaIO₄ (Figure S7.2). Hence, we limited NaIO₄ content to 0.45 %w/v in subsequent experiments. The CAO products were then conjugated to gelatin from various animal sources (*i.e.*, porcine, PRC; bovine, BVN; and piscine, PSC) of gelatin. The experimental conditions used throughout this study along with their labeling format are summarized in Table S1. Note that the process variables including the gelatin type and concentration of NaIO₄ used for CA oligomerization, in addition to crosslinking parameters, were optimized to tailor the bioadhesives for injectable applications.

Removal of the unreacted small molecules during the dialysis step was monitored by

UV-vis analysis of the dialysis solution (Figure S7.3A,B) for PRC-GelCA-0 and PRC-GelCA-15 samples, respectively. The value of the absorbance at 320 nm (representative of CA content)^{662,663} reached a minimum plateau after four dialysis water replacement cycles (Figure S7.3C). Conjugation of CA-based moieties to gelatin was evaluated spectrometrically. The UV-vis spectra of GelCA with reference to unmodified gelatin are shown in Figure S7.4A. The characteristic peaks of GelCA samples at 320 and 290 nm in the CA spectrum (Figure S7.3C) are attributed to the π - π^* transition of the double bonds and benzene ring in CA,^{602,664-665} indicating conjugation of CA moieties to gelatin. The absorbance (at 320 nm) shown in Figure S7.4B increased with CA oligomerization level (NaIO_4 concentration), indicating higher amounts of catechol conjugated to gelatin. The source of the gelatin did not have a significant effect on the absorbance. These results are in agreement with the Arnow assay performed to determine the conjugated catechol content (Figure 7.1E). Oligomerization of CA prior to EDC/NHS coupling improved the overall conjugated catechol content. Larger numbers of catechol groups were covalently loaded by CAO compared to CA. Note that the ratio of catechol content in PRC-GelCA-15 and PRC-GelCA-45 to PRC-GelCA-0 (~ 1.6 and ~ 2.5 for concentrations of 0.15 and 0.45 %w/v NaIO_4 , respectively) was in accord with the number of CA repeating units in CAO ($N < 2-5$), as reported in the literature.³⁶ The results of proton nuclear magnetic resonance (^1H NMR) data also suggest significantly higher amine consumption rates for gelatin conjugated with CAO (PRC-GelCA-15) compared to CA (PRC-GelCA-0), as shown in Figure 7.1F.

7.2.2 Thermosensitivity of gelatin-caffeic acid pre-gel solutions

To maximize chemical interactions between GelCA pre-gel solutions and tissues, we sought to find the maximum pre-gel concentration of GelCA that allows injectability. For this

purpose, the viscosity of solutions with different pre-gel concentrations was assessed *via* a vial tilt assay at 50 °C (Figure 7.2A) for GelCA-15 samples. In contrast to unmodified gelatin, where highly concentrated solutions (40 %w/v) could still flow under gravity, the CAO-modified gelatin either transformed into the gel state upon hydration (PRC sample) or formed a highly viscous fluid (in the cases of BVN and PSC groups). The improved association of the GelCA network stems from the high density of junction zones, which was due to: (i) the reinforced hydrogen bonding and π - π interactions through CA (*via* phenolic hydroxyl groups),²⁹³ and to a larger extent, CAO conjugates (*via* phenolic hydroxyl and carboxyl groups), as depicted in Figure 7.2B,C; (ii) Michael addition and Schiff base formation between gelatin primary amine and carbonyl groups present in the oxidized CA-based moieties;⁶⁶⁶ and (iii) potential homocoupling of gelatin chains due to the EDC/NHS reaction⁶⁴² and potential quinone coupling reactions⁹ leading to high molecular weight gelatin species. The total pre-gel solution concentrations for the samples studied here were fixed at 20 %w/v, as they were found to be soft enough and therefore injectable in clinical settings across all gelatin sources (Figure 7.2A).

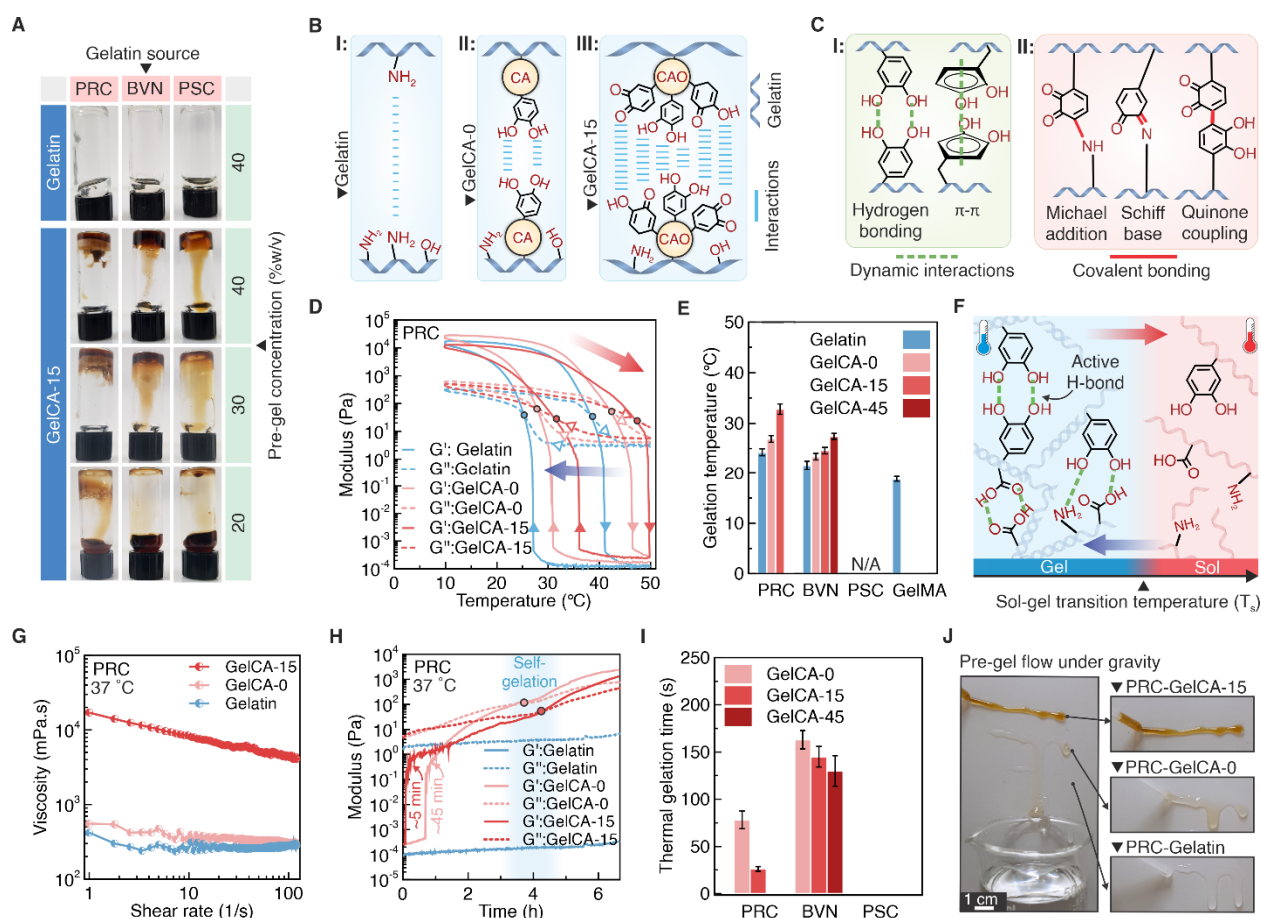


Figure 7.2. Thermosensitivity and injectability of gelatin-caffeic acid (GelCA) solutions. (A) Vial tilt assay investigating flow of pre-gel solutions with different concentrations under gravity at 50 °C. Conjugation of caffeic acid (CA) oligomers (CAO) led to significant increases in the viscosity and the integrity of solutions. (B) Augmented thermosensitive hydrogen-bonding interactions between gelatin macromolecules due to the conjugated catechol moieties, leading to increases in the gelatin phase transition temperature. (C) Dynamic (*i.e.*, hydrogen-bonding and π - π) as well as covalent interactions (*i.e.*, Michael addition, Schiff base formation, and quinone coupling) introduced *via* the catechol groups. (D) Rheological measurement (storage modulus, G' , and loss modulus, G'') of gelatin thermosensitivity in terms of CA oligomerization for porcine (PRC) GelCA samples over a cooling-heating temperature cycle. (E) Sol-gel transition temperature obtained from the gelation process for PRC, bovine (BVN), and piscine (PSC) GelCA. The data are presented as means $\pm$ SD. (F) The increase in gelation temperature due to the faster association of the gelatin backbone because of the increased density of temperature-sensitive hydrogen bonds with CA modification. (G) Variation of viscosity in CAO-conjugated PRC gelatin (GelCA-15) compared to their CA counterparts (GelCA-0) across different shear rates. (H) Self-gelation properties of PRC-GelCA samples. (I) Time to physical gelation

in response to a temperature step from 50 °C to room temperature at 25 °C. The data are presented as means $\pm$ SD. (J) Facilitated injectability and control over the pre-gel in CAO-conjugated gelatin (PRC-GelCA-15) for applications on non-horizontal surfaces.

We investigated the effects of CA modification on the thermosensitive physical gelation in 20 %w/v pre-gel solutions (see Figure 7.2D for the PRC group and Figure S7.5A for the BVN group). As opposed to the PSC group that remained in the sol state across different temperatures, the PRC and BVN groups demonstrated thermoreversible modulus changes with temperature in response to thermal cycles between 50 °C and 10 °C. As shown in Figure 7.2E and Figure S7.5B, sol-gel transition temperatures increased with both CA and more significantly with CAO conjugation. This increase was more prominent for the gelatin sources with higher molecular weight (*i.e.*, PRC>BVN>PSC). The PRC-GelCA with CAO conjugates at the highest oxidation level (PRC-GelCA-45) stayed in the gel state, even at 80 °C (Figure S7.6). This system was therefore not characterized further. We attribute the significant changes in the gelatin phase transition temperature to the augmented density of thermosensitive hydrogen bonds with CA-based modifications (Figure 7.2F).^{667,668} Likewise, we attribute the lower sol-gel transition temperature of GelMA, compared to gelatin, to the elimination of hydrogen bonds due to the conversion of hydroxyl and amine groups to methacrylate groups (Figure S7.7). The sol-gel transition temperature can be further tuned with the concentration of GelCA solution, as illustrated in Figure S7.7. Shifting the sol-gel transition temperature towards that of body temperature gives more physical integrity to the gelatin networks, as opposed to the unmodified gelatin, which readily dissociates at 37 °C.

The viscosity-shear rate characteristics for the PRC group at 37 °C suggest

approximately two orders of magnitude improvement in GelCA-15 (CAO-conjugated gelatin) *versus* GelCA-0 (CA-conjugated gelatin) across all shear rates, as shown in Figure 7.2G. The long-term rheological evolution of samples maintained at 37 °C (over 6 h) was monitored, as shown in Figure 7.2H. Due to the CA-induced covalent interactions shown in Figure 7.2C, spontaneous association of GelCA-15 and GelCA-0 led to continuing gelation that was only evident for PRC (Figure 7.2H and Figure S7.8). An initial jump in storage modulus of GelCA-15 was observed within ~5 min; this jump for GelCA-0 occurred after ~45 min (a gelation time was recorded to be the order of ~4 h). This behavior suggests body-temperature-driven self-association of PRC GelCA contingent on the pre-gel being maintained at high concentration (in other words, if not allowed to dissolve in aqueous media). Importantly, this self-gelation of PRC GelCA occurred on time scales that would allow pre-gel handling and injectability in surgical settings.

7.2.3 Injectability of pre-gel solutions

The declining trend of viscosity with shear rate (as discussed above for Figure 7.2G) demonstrated the shear-thinning properties of GelCA pre-gel solutions, indicating their injectability. We attribute this shear-thinning property to the shear-induced breaking of the gelatin network. As observed in Figure 7.2D,E and Figure S7.5, thermal hysteresis led to significantly larger solation than gelation temperatures. This thermal history effect results in a lag in thermal gelation during the cooling process, which allows injectability for a longer period after exposure to room temperature. As shown in Figure 7.2I, we assessed the time to thermal gelation in response to a step temperature change from 50 to 25 °C (Figure S7.9A,B). The results for different compositions of PRC and BVN GelCA:GelMA composites are displayed in Figure S7.9C. As for pure GelCA solutions, the shortest thermal

gelation times were attained for CAO-modified PRC GelCA (~30 s after reaching 25 °C). This time was longer for their CA-modified counterparts (~75 s) and for the BVN group (~130-160 s). The GelMA additives could further delay the thermal gelation time for all GelCA groups. The short thermal gelation times could provide rapid physical stability to the hydrogel on the wound, while secondary covalent crosslinking is initiated on-demand.

Accelerated thermal gelation kinetics along with the enhanced viscosity of CAO-modified PRC gelatin also facilitated application of GelCA solutions on non-horizontal surfaces and double-curved tissues, such as lungs. As demonstrated in Figure 7.2J, PRC gelatin and their CA-modified solutions drip under gravity, upon injection on a vertical plane, while the CAO-modified gelatin remains integral during injection. In this regard, Figure S7.9D compares the values of storage moduli 1 min after reaching 25 °C. The largest storage moduli were obtained for CAO-modified PRC GelCA. The steady storage modulus values (8 min after exposure to 25 °C) however, demonstrated no significant differences between CA- and CAO-modified groups (Figure S7.9E). In fact, higher catechol contents accelerate gelation; the steady modulus of physical gels however, remains consistent. Here, the large molecular weight gelatin sources (*i.e.*, PRC) possessed the highest gel stiffness, which decays with addition of GelMA components.

7.2.4 Kinetics of covalent crosslinking

As discussed above, in Figure 7.2H, PRC GelCA solutions exhibited continuous self-gelation in the absence of aqueous media. However, they dissolved if exposed to wet media before covalent crosslinking. Thus, an additional crosslinking step was required to provide wet stability to the hydrogels. For this purpose, we evaluated two different covalent network-forming strategies that could be triggered on demand for internal bioadhesion (see

Figure 7.1B): (i) a photo-crosslinking procedure based on free-radical polymerization was initiated by 0.25 %w/v lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) in GelCA/GelMA hybrid pre-gels with varying GelMA:GelCA ratios. In this system, GelMA provides a covalent hydrogel within which GelCA macromolecules were physically entrapped; (ii) oxidative polymerization of GelCA by applying NaIO_4 solution on the physical hydrogels after injection. In this approach, infusion of NaIO_4 solution through the hydrogel surface forms a cohesive topical crosslinked layer *via* catechol coupling reactions. This layer protects against hydrogel dissolution under wet physiological conditions. On the other hand, the catechol groups in the layer at the interface were left intact, so that they could be involved in adhesion, rather than being consumed for crosslinking.

Rheological evolution of pre-gel solutions under covalent crosslinking was investigated to assess the solidification (gelation) time scales required during surgery. As for the photo-crosslinkable group (Figure S7.10A), Figure S7.10B compares overall trends of storage (G') and loss (G'') modulus with crosslinking time for GelCA/GelMA hybrid hydrogels in response to UV light exposure (35 mW/cm^2 light intensity). For the PSC group, a clear gelation point was observed due to the absence of physical gelation (Figure S7.10BI). Gelation time in PSC GelCA/GelMA composites was significantly longer for the samples with higher catechol content (*i.e.*, CAO- compared to CA-conjugated). We attribute this effect to free radical scavenging by the conjugated catechol groups,²⁴ which can hinder free radical crosslinking of GelMA.⁶⁶⁹ At this point, we excluded further investigation for conditions that require more than 2 min UV exposure due to their potential UV-induced toxicity.⁶⁷⁰ All samples containing PRC- and BVN-GelCA underwent physical gelation prior to UV light irradiation. Hence, the time to physical gelation (Figure 7.2C) best describes their

solidification times (~ 25 -180 s for PRC and ~ 130 -330 s for BVN groups, where higher GelMA content is associated with longer gelation times). In these groups, free-radical polymerization of GelMA augmented G' values (after 2 min UV light irradiation) compared to those of physical hydrogels (Figure S7.11). Due to radical scavenging by catechol groups, lower crosslinking density, and thereby storage moduli, were obtained for samples with higher catechol content (*i.e.*, GelCA-15 and GelCA-45). To understand radical crosslinking in the GelCA/GelMA hydrogel system, the rate of variation in G' with crosslinking time (representative of crosslinking reaction kinetics) was plotted in Figure S7.12. Interestingly, the UV-triggered sharp increase in reaction rate was evident even in CA-conjugated PRC- and BVN-GelCA samples without GelMA additives. We tentatively attribute this effect to free-radical-sensitive double bonds present in unoxidized CA and amino acid residues on the PRC and BVN gelatin sources, which can undergo coupling reactions in the presence of free radicals.⁶⁷¹ The reaction rates were significantly slower in samples with high catechol content. In addition, higher molecular weight GelCA sources (PRC>BVN>PSC) exhibited greater rates of change in storage modulus.

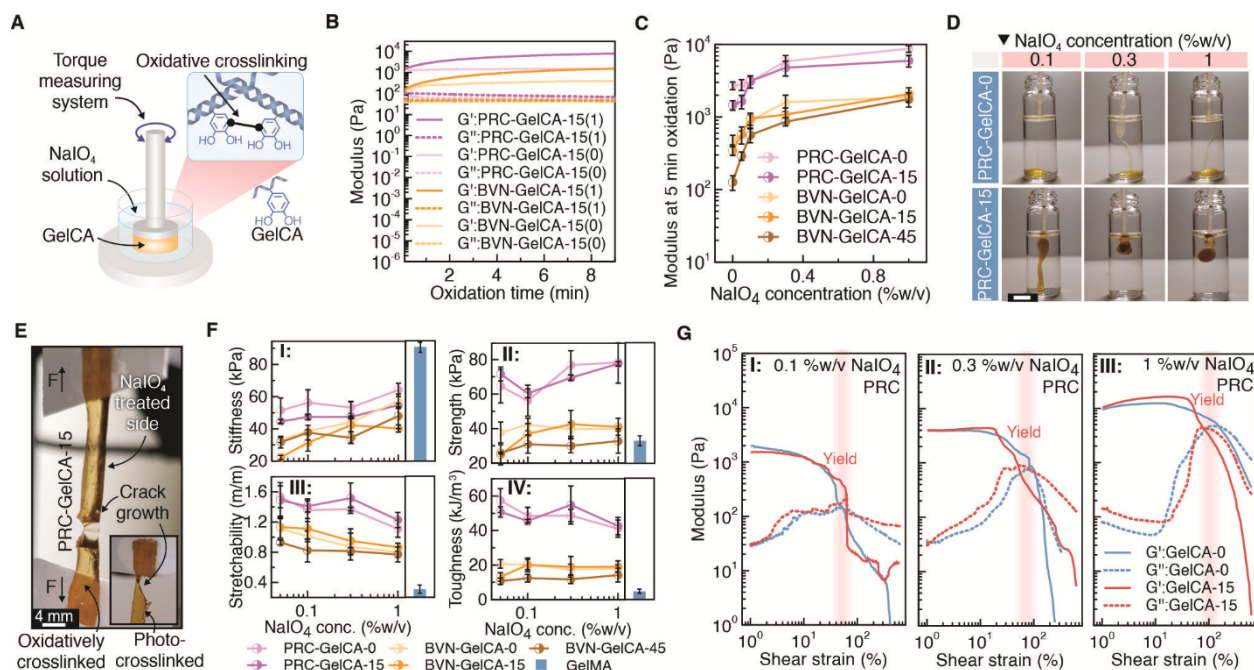


Figure 7.3. Characterization of crosslinking kinetics and mechanical properties of gelatin-caffeic acid (GelCA) hydrogels. (A) Experimental setup for characterization of oxidative crosslinking of GelCA using NaIO_4 solutions. (B) Variation of G' and G'' for porcine (PRC) and bovine (BVN) GelCA in response to peripheral NaIO_4 infusion at different concentrations (*i.e.*, 0.05, 0.1, 0.3, and 1 %w/v). (C) Storage modulus after 5 min crosslinking by exposure to different concentrations of NaIO_4 . The data are presented as means $\pm$ SD. (D) Rapid gelation of GelCA pre-gel solutions upon injection in NaIO_4 solutions. Gelation of GelCA with oligomerized caffeic acid (CAO) conjugates (PRC-GelCA-15) occurred at lower NaIO_4 concentrations than gelatin-caffeic acid conjugates (PRC-GelCA-0). (E) The failure mechanism of oxidatively crosslinked GelCA samples for the cases of photo-crosslinked samples, PRC-GelCA-15(11:8), and oxidatively crosslinked samples, PRC-GelCA-15(0.3) hydrogels in mechanical tensile tests. (F) The results of tensile tests for the group of oxidatively crosslinked GelCA in terms of I: elastic modulus, II: tensile strength, III: strain at failure, and IV: toughness. The data are presented as means $\pm$ SD. (G) Strain amplitude sweep tests on PRC-GelCA samples crosslinked with I: 0.1, II: 0.3, and III: 1 %w/v NaIO_4 solutions.

Gelation for oxidative crosslinking was performed by soaking the samples in a 25 °C NaIO_4 solution (Figure 7.3A). The PSC group readily dissociated once NaIO_4 solutions were added to the dish (Figure S7.13A). Hence, we did not further characterize the PSC group of

oxidatively crosslinked GelCA hydrogels. The PRC and BVN groups, however, improved their structural integrity as they were cooled, upon immersion in the 25 °C NaIO₄ solutions (Figure S7.13B,C). Diffusive oxidation crosslinking of PRC-GelCA-0 in the presence of NaIO₄ suggests the formation of a topical covalent crosslink layer. Figure 7.3B demonstrates the trends of G' and G'' with peripheral infusion of NaIO₄. Figure S7.14 also compares crosslinking kinetics in terms of the rate of variation in G' . An initial sharp increase in G' upon addition of NaIO₄ solution corresponds to synergistic contributions of physical gelation due to initial cooling by NaIO₄ solution and oxidative crosslinking. Values of G' reached a plateau within *ca.* 5 min for most samples as they reached thermal equilibrium at 37 °C while they were partially and circumferentially crosslinked through NaIO₄ infusion. Since the hydrogels have already transitioned to the sol state at this temperature, the increases in steady G' values reflect covalent crosslinking. Figure 7.3C compares the values of G' at 5 min crosslinking time for different crosslinking conditions. Overall, G' increased with NaIO₄ concentration and this increase was more prominent for PRC compared to BVN GelCA. Here, CA oligomerization did not have significant impact on the final storage moduli. Figure 7.3D demonstrate fast gelation of CAO-conjugated PRC gelatin upon exposure to NaIO₄ solution. Compared to CA-conjugated gelatin, the CAO-modified gelatin formed instant crosslinking at lower concentrations of NaIO₄.

7.2.5 Analysis of tensile mechanical properties

Mechanical properties of bioadhesives play pivotal roles in their adhesion to soft and mechanically dynamic tissue substrates. Low elastic moduli in bioadhesives result in better compliance with natural tissue deformation due to less adhesive resistance at the tissue interface. High toughness is also a critical requirement, toughening hydrogels while lowering

elastic moduli entails high stretchability. The tensile characteristics of the hydrogels are illustrated in Figure 7.3E,F and Figure S7.15. The failure mechanism for the photo-crosslinked group was accompanied by the propagation of lateral cracks leading to catastrophic failure (Figure 7.3E). The mechanical properties of photo-crosslinked and oxidatively crosslinked GelCA samples are depicted in Figure 7.3F and Figure S7.15, respectively. The elastic moduli (Figure S7.15A) were initially reduced with the addition of GelCA content for all gelatin sources. We attribute the decrease in elastic moduli with further addition of GelCA to be a result of reduced covalent crosslinking density due to the free radical scavenging effect of the catechol groups. As the GelMA content decreased further, this trend was reversed, more prominently in PRC group, likely because of the hydrogen-bonding-driven association of GelCA, as these experiments were performed below the gelation point, at room temperature. The tensile strengths in the PRC group far exceeded those of other counterparts. No significant difference was observed among the different catechol loadings, *e.g.*, PRC-GelCA-15 *versus* PRC-GelCA-0 (Figure S7.15B). Tensile strength increased with increasing GelCA content, presumably due to the contribution of physical associations at room temperature. The stretchability in GelCA hydrogels was improved by $\sim 6.5\text{-}7\times$ compared to the GelMA samples (Figure S7.15C). Furthermore, a ~ 6.5 -fold increase in toughness compared to GelMA in the PRC group (Figure S7.15D) was observed. We attribute the enhanced toughness and stretchability with increasing GelCA content to non-covalent interactions (*i.e.*, hydrogen-bonding and π -stacking interactions) introduced by catechol groups (forming a double-network with the covalent network of GelMA), as well as reduced covalent crosslinking density in the hydrogels with high catechol content.

The mechanical properties of oxidatively crosslinked hydrogels are shown in

Figure 7.3E,F. Elastic moduli increased with increasing concentration of the NaIO_4 solution, due to crosslinking *via* catechol coupling (Figure 7.3FI). Similar to that of photo-crosslinked hydrogels, stronger mechanical properties (stiffness, strength, stretchability, and toughness) were recorded for PRC compared to the BVN group, and the differences between the CA- and CAO-conjugated groups were insignificant. As can be seen in Figure 7.3FII, the maximum tensile strength for oxidative crosslinking was $\sim 60\%$ larger than that of oxidative polymerization in PRC group (and $\sim 2\times$ greater than GelMA). Overall, the values of stretchability were slightly lower than the photo-crosslinked group of GelCA samples. Nevertheless, the maximum stretchability in the oxidatively crosslinked PRC group was $\sim 5.3\times$ larger than GelMA controls (Figure 7.3FIII). Increased NaIO_4 concentration led to decreased hydrogel stretchability. As illustrated in Figure 7.3E, crack propagation initiated from the non-treated (hydrogen-bonding associated) side of the specimens and slowed down as the crack tip reached the NaIO_4 -treated (covalently crosslinked) side. The through-thickness crosslinking density gradient in the samples due to NaIO_4 infusion-postponed failure, because propagating cracks are hindered as they pass through layers with stronger crosslinking densities. As for toughness (Figure 7.3FIV), the PRC group was comparable to that of the photo-crosslinked groups. Values for toughness did not show significant sensitivity to NaIO_4 concentration. Toughness for the PRC group was improved by approximately $11.5\times$ compared to GelMA hydrogels. We attribute this improvement to the synergistic contributions of hindered crack propagation mechanism of failure as well as tough interpenetrating covalent and non-covalent (*i.e.*, hydrogen-bonding and π -stacking interaction) networks introduced by the conjugated catechol groups.

7.2.6 Rheological analysis of oscillatory amplitude sweep

The results of the tensile tests reflect the mechanical properties at room temperature in the absence of aqueous media and thus involve interchain physical gelation effects. In order to understand mechanical responses under hydrated conditions at body temperature, strain amplitude sweep tests were performed on the most mechanically robust GelCA, *i.e.*, PRC groups (Figure 7.3G and Figure S7.16) for the oxidatively crosslinked groups. In these tests, physically associated hydrogels were peripherally treated with NaIO₄ solution followed by thermal stabilization at 37 °C. The results again indicated that CA oligomerization did not have substantial impact on the hydrogel mechanical properties. In turn, the magnitude of G' significantly increased with increasing NaIO₄ concentration, in agreement with the results of tensile tests (Figure 7.3FI). Superstructure breakdown and micro-crack formation in the early stages led to the descending trend of G'' for NaIO₄ concentrations of 0.1 and 0.3 %w/v. In fact, the yield strain was found to increase overall with NaIO₄ concentration (Figure 7.3G), as opposed to the tensile test data where strain at failure decreased with increasing NaIO₄ concentration (Figure 7.3FIII). Likewise, the values of yield stress increased with increasing NaIO₄ concentration (Figure S7.16), a trend that was not seen in tensile tests. Therefore, in the absence of hydrogen bonding (physical association), larger oxidative strength can provide more deformability in the hydrogels. In essence, chain flexibility was improved as hydrogen-bonding associations were weakened by elevating the temperature to 37 °C.

7.2.7 Adhesion stability under wet conditions

Given the intended application of tissue adhesives for internal use, the stability of

adhesion was investigated by incubating hydrogels crosslinked on wet collagen sheets in phosphate-buffered saline (PBS) at 37 °C for 1 day. Figure 7.3A and Figure S7.17 represent the recorded state of adhesion for oxidatively crosslinked and photo-crosslinked hydrogels, respectively. Adhesive failure was due to: (i) delamination arising from hydrogel swelling, partial dissolution, or water-triggered disruption of bioadhesives/tissue interfacial interactions and (ii) dissolution of GelCA due to weak interchain associations. The results shown in Figure S7.17 indicate that among the photo-crosslinked hydrogels, only a few of the conditions and compositions maintain wet adhesion to the collagen sheet. Adding GelMA to GelCA (~3-9 %w/v) provided structural support to prevent full dissolution; however, delamination failure was frequently observed in these samples. Pure GelMA hydrogels also exhibited delamination failure in wet media. The results for oxidatively crosslinked samples (Figure 7.4A) suggest that treating the hydrogels with NaIO₄ solutions at 0.1-0.3 %w/v provide stable static wet bioadhesion. Further, we examined release of GelCA after crosslinking and incubation at 37 °C in aqueous media (Figure S7.18). Oxidatively crosslinked bioadhesives showed the highest stability in the PRC group (Figure S7.18a), revealing the significant role of gelatin molecular weight in the formation of interconnected 3D networks. Oligomerization of CA further enhanced the hydrogel stability in both the PRC and BVN groups, which we attribute to the larger catechol content and thereby higher crosslinking density in CAO- compared to CA-conjugated gelatin. Release of GelCA in the PRC group dropped significantly, to below ~3%, as the concentration of NaIO₄ in the treating solution was increased from 0.05 to 0.1 %w/v.

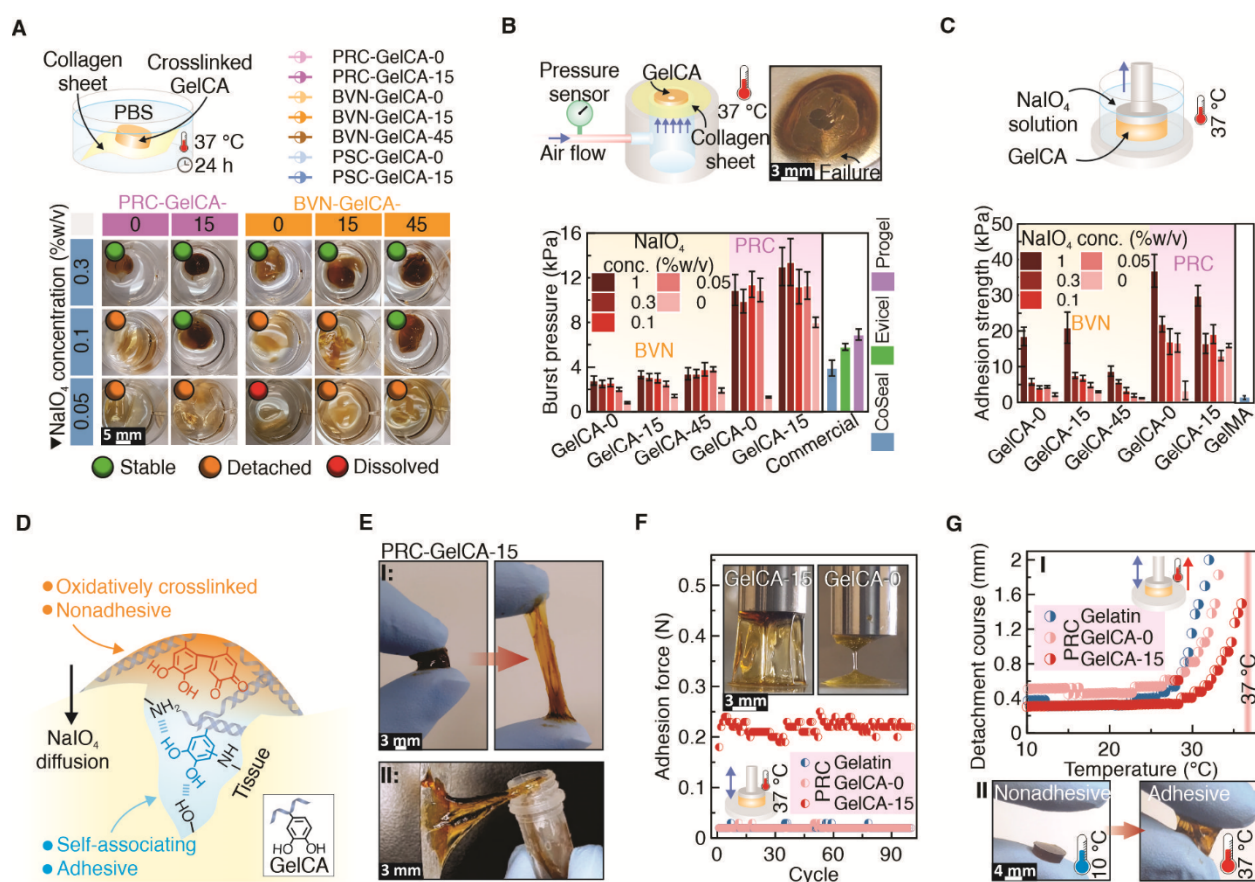


Figure 7.4. *In vitro* characterization of adhesion in gelatin-caffeic acid (GelCA) bioadhesives. (A) Experimental procedure and inspection of adhesion stability in wet conditions for oxidatively crosslinked GelCA (porcine, PRC; bovine, BVN sources) on collagen sheets incubated in water at 37 °C for 24 h. (B) The process and results of *in vitro* burst pressure tests. The data are presented as means ± SD. (C) Tack tests on different conditions of oxidatively crosslinked GelCA samples. The data are presented as means ± SD. (D) Schematic of the synergistic contributions of the cohesive and adhesive mechanisms in oxidatively crosslinked GelCA. A crosslinking gradient is formed by the infusion of NaIO₄ in the physical GelCA gels, resulting in a protective non-adhesive layer on the top reinforcing hydrogel cohesion and a self-associating hydrogel underneath contributing to adhesion to wet tissues. (E) Demonstrations of adhesion of PRC-GelCA-15 pre-gel solution to I: nitrile gloves and II: metal spatula at 37 °C (in the absence of crosslinking). (F) Cyclic tack test performed using a rheometer on the non-crosslinked pre-gel solutions, demonstrating that caffeic acid (CA) oligomer (CAO) conjugates, *i.e.*, GelCA-15, provided gelatin with structural integrity at 37 °C that leads to stable adhesion as opposed to gelatin and CA-conjugated gelatin, *i.e.*, GelCA-0, where the phase transition to sol state at 37 °C led to disruption of cohesion thus also adhesion. The insets compare the hydrogel stickiness of GelCA-15 and GelCA-0 between glass/metal substrates after lifting the metal adherent

following the cyclic tack tests. (G) I: Effect of temperature on pre-gel stickiness (PRC-GelCA-15). II: Characterization of the temperature effects on stickiness in terms of detachment positions in cyclic tack tests. GelMA, gelatin methacryloyl.

The results of the release study for photo-crosslinked hydrogels, however, demonstrated much greater instability compared to their oxidatively crosslinked counterparts (release ranging from ~10-100%), as shown in Figure S7.18B. These data explain the adhesion failures observed in Figure S7.17 and indicate that GelMA networks in the composition ranges studied here failed to establish sufficiently strong interactions with GelCA to remain stable within the GelMA networks. Note that CAO-conjugated samples containing more catechol groups exhibited greater dissociation compared to CA-conjugated samples. From the poor adhesion of the photo-crosslinked hydrogels in wet conditions, we infer that this mechanism of gelation may not be practical for internal wound-closure applications. Hence, we focused instead on oxidative crosslinking of single-component GelCA in the subsequent measurements.

Swelling of the CAO- and CA-conjugated PRC gelatin crosslinked at different concentrations of NaIO_4 solution followed by incubation in PBS are compared in Figure S7.19A,B, respectively. NaIO_4 concentrations over 0.3 %w/v led to the minimal equilibrium swelling ratio of ~0.2 in the CAO-conjugated gelatin. The converged swelling properties at NaIO_4 concentrations over 0.3 %w/v indicate a saturated infusion crosslinking. The minimal swelling below this concentration is in accord with the stable wet adhesion observed in Figure 7.4A. The effect of pH on swelling was also tested on PRC-GelCA-15 hydrogels crosslinked at 1 %w/v NaIO_4 , as shown in Figure S7.19C where no statistically significant difference was found between pH 5.0 and 7.4 (the common pH range in healing

wounds).⁶⁷²

7.2.8 Adhesion performance *in vitro*

Burst pressure tests were performed underwater to assess hydrogel sealing performance by applying bioadhesives to a collagen sheet (Figure 7.4B). The PRC group exhibited higher burst pressure ($\sim 2\text{-}3\times$ larger than that of commercial sealants); no significant differences were found between CA- and CAO-conjugated gelatin types. The burst pressure results demonstrated minor sensitivity to NaIO_4 concentration for most GelCA types. At the burst point, initially, adhesive failure developed from the punctured area, leading to cohesive failure at the edges of the sample. The trends observed in the burst pressure were consistent with those of mechanical tensile (Figure 7.3F) or strain sweep (Figure 7.3G), suggesting that the adhesion strength was primarily governed by the material's intrinsic toughness. Adhesion strength was also measured for bioadhesives using a tack test in wet media at 37 °C, as shown in Figure 7.4C. Increasing the adhesion strength with NaIO_4 concentration was more significant compared to those in burst pressure results where significant increases in adhesion strength were observed at 1 %w/v NaIO_4 . Note that although previous published work has emphasized the necessity of catechol protection against oxidation to enable their adhesion,²⁹⁹ no meaningful correlations were found between the adhesion strength in CA and CAO conjugates. We infer that interactions between oxidized catechol groups with nucleophiles can drive as strong adhesion as those with non-oxidized catechol groups. Similar to the burst pressure results, the PRC group, in general, excelled over BVN-GelCA in the tack test. Thus, the subsequent optimization studies only involved PRC-GelCA samples.

In the oxidative crosslinking of GelCA, NaIO_4 infusion through the physical hydrogels

leads to a through-thickness gradient crosslink where the densely crosslinked surface reinforces the hydrogel and protects the pre-gel underneath from being released (Figure 7.4D). Here, the catechol groups of non-crosslinked GelCA at the tissue interface are not consumed for crosslinking (as is the case when mixing with crosslinking agents) enabling them to interact with tissues and self-associate at body temperature. As discussed above, tackiness and burst strength in the non-crosslinked PRC GelCA (0 %w/v NaIO₄ in Figure 7.4B,C) were measured to be ~1.5-4× larger for CAO- compared to CA-conjugated gelatin. We attribute this improvement to the better chain integrity/cohesion and higher viscosity of the gelatin-CAO conjugates at 37 °C, as discussed for Figure 7.2.

Adhesion of GelCA pre-gel solutions is shown in Figure 7.4E-G. Stickiness of PRC-GelCA-15 pre-gel was demonstrated in Figure 7.4E. To reveal the roles of thermosensitivity in bioadhesion, a cyclic tack test followed by detachment was performed at 37 °C in axial mode (Figure 7.4F). The nearly zero detachment forces of gelatin and CA-modified gelatin were due to the thermosensitive phase transition to the sol state at 37 °C. Oligomerization of CA prior to conjugation, however, provided gelatin with a structural integrity at this temperature, leading to an adhesion force of ~0.2 N. The adhesion force remained consistent across 100 adhesion cycles, indicating the durability of adhesion. We further monitored adhesion in terms of material elongation at detachment *via* cyclic adhesion tests while changing temperature from 10 to 40 °C (Figure 7.4G). Elongation at detachment increased sharply as the pre-gel underwent the phase transition to the sol state. For gelatin and PRC-GelCA-0, the increase in elongation before detachment began at ~27 °C, and the material transitioned fully to the sol state before reaching 37 °C. Pre-conjugation oligomerization of CA (CAO conjugates) though, enabled structural integrity to the hydrogel

where elongation at detachment reached ~ 1.6 mm at 37 °C. As shown in Figure 7.4GII, the hydrogels at 37 °C exhibited more tackiness compared to 10 °C. This thermosensitivity of adhesion enables the application of molded physical hydrogel patches, wherein stickiness can be activated in response to exposure to body temperature.

Previous examples of bioadhesives could form unwanted adhesion from the back side of the bioadhesive.¹⁹¹ Here, oxidative crosslinking *via* NaIO₄ consumed the catechol groups on the back surface for catecholic crosslinking (Figure 7.4D), thereby deactivated surface stickiness *in situ*. Thus, oxidative crosslinking using NaIO₄ forms a slippery anti-adhesive topical surface with selective adhesion to the tissue underneath, as the interfacial catechol groups were preserved on the opposite “front” side to establish adhesion to tissue. As shown in Figure S7.20, PRC-GelCA-15 patches applied to gloves at 37 °C only adhered through their non-crosslinked side while the other side did not show adhesion.

7.2.9 Analysis of adhesion *ex vivo*

Adhesion of GelCA samples was studied *ex vivo* by measuring adhesion energy *via* wound-closure tests using pig lung and heart tissues, as shown in Figure 7.5A. No delamination was observed in GelCA specimens since adhesion failure originated from materials failure (Figure 7.5AI). Adhesion energy for lung tissue was comparable to that of heart tissue (see Figure S7.21 and Figure 7.5AII), however, adhesion was considerably larger than for commercial sealants (by *ca.* an order of magnitude). As observed in mechanical characterization, oligomerization of CA conjugates had no significant effect on adhesion *ex vivo*. Increasing NaIO₄ concentration from 0.1 to 0.3 %w/v led to increased average adhesion energy; further increases in NaIO₄ concentration to 1 %w/v did not have significant impact on adhesion strength. Hence, the *ex vivo* burst pressure test on pig lungs was performed on

GelCA samples crosslinked at 0.3 %w/v NaIO₄, as shown in Figure 7.5AI. In contrast to GelMA, where failure occurred in the early stages of lung tissue expansion (due to its limited inherent stretchability), GelCA samples remained stable and accommodated tissue expansions with no observable failure. The hydrogels did not burst, even after the lung was fully expanded with air pressure, at which point remote leakage spots were observed in the lung tissue. The measured burst pressure results demonstrated significant improvement in adhesion for both GelCA samples (with either CA or CAO conjugates) compared to commercial adhesives and GelMA hydrogels (Figure 7.5BII). Also, the burst pressure tests performed on pig bladder tissue suggested significant improvements in adhesion strength (Figure S7.22A); however, the improvement was more considerable in the case of lung tissue, suggesting the roles of tissue type in adhesion efficacy. The GelMA hydrogels were delaminated more rapidly with bladder expansion compared to GelMA, since they failed to comply with deformations of bladder tissue (due to the stiffness of GelMA); however, flexible GelCA hydrogels could accommodate bladder tissue expansion better, and the leak point was thereby shifted to higher values (Figure S7.22B,C). Bioadhesives applied on pig heart tissue were subjected to tap water pressure (at ~37 °C) for ~30 s, as shown in Figure S7.23. The CAO-modified gelatin resisted the harsh water pressure for a longer period than CA-conjugated gelatin, which we attribute to the stronger interactions and better thermal stability of gelatin-CAO conjugates. The bioadhesives applied to the heart tissue substrates did not detach after being subjected to harsh twist deformation. In addition, the bioadhesives could be detached with minimal residues and no visible damage to the lung model tissues.

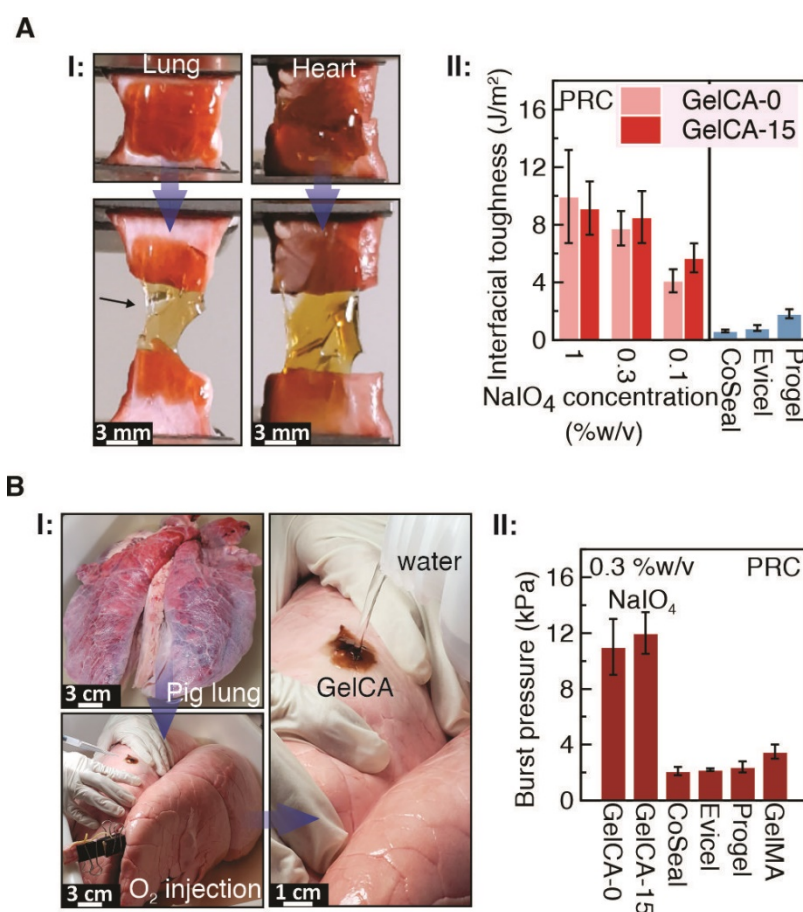


Figure 7.5. *Ex vivo* characterization of adhesion in gelatin-caffeic acid (GelCA) bioadhesives. (A) I: Deformation mechanism and II: results of wound-closure tests. The data are presented as means $\pm$ SD. (B) I: *Ex vivo* testing procedure and II: results of burst pressure tests on pig lungs. The data are presented as means $\pm$ SD. The results compare gelatin from porcine sources (PRC) modified with caffeic acid (GelCA-0), and caffeic acid oligomers (GelCA-15) *versus* commercial sealants and gelatin methacryloyl (GelMA).

7.2.10 *In vitro* and *in vivo* biocompatibility

The biocompatibility of oxidatively crosslinked group of CA- and CAO-conjugated PRC gelatin using 0.3 %w/v NaIO₄ solution was evaluated *in vitro* (Figure S7.24). The samples were exposed to cells seeded on a well plate on which live/dead assays were performed using human lung fibroblast (HLF) cells (Figure S7.24A). The results indicated viability

values of over ~80% after 7 days. Compared to blank controls, no significant viability drop was seen for GelCA-0 nor in GelCA-15 samples. PrestoBlue™ assays performed on C2C12 cells also suggested increases in cell proliferation over time, comparable to GelMA controls (Figure S7.24B). Furthermore, *in vivo* biocompatibility studies showed no evidence of toxicity in extracted tissues from different organs at weeks 1 and 4 (Figure S7.25). Therefore, functionalization of gelatin with CA or its oxidized moieties, as well as diffusive crosslinking with NaIO₄, are cytocompatible processes for using GelCA as an internal bioadhesive.

7.2.11 Drug release and antioxidant function

Bioadhesive hydrogels can be leveraged for wound healing applications by incorporating various drugs such as antibiotics and by introducing antioxidation effects. The effects of CAO conjugates on the drug release kinetics (using Rhodamine B as small molecule model drug) were compared *via* drug release from PRC-GelCA-15(0.3) *versus* photocrosslinked GelMA hydrogels (Figure S7.26). The results showed no significant differences between the two groups, suggesting that the crosslinking process in GelCA does not undermine the drug loading and release capacity of these hydrogels. Despite the additional phenolic groups introduced to the gelatin backbone, the release kinetics were not altered by the potential interactions between CAO and drug molecules.

Hydrogels with antioxidant function have been shown to accelerate chronic wound repair by reducing the oxidative stress at the wound site.⁶⁷³ Caffeic acid is known for its strong antioxidant effects⁶⁷⁴ and is used to impart antioxidant function to natural biomolecules.⁶⁷⁵ The antioxidant activity of the prepolymer solutions was examined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, as illustrated in Figure S7.27. Conjugation of CA raised DPPH radical inhibition from ~4% to ~75%. This

figure was increased to ~95% with conjugation of CAO to porcine skin, rendering the proposed conjugation scheme a promising approach to boost antioxidant effects of catecholic small molecules. The stronger antioxidant effect is attributed to the larger numbers of catechol groups grafted to gelatin backbone. We note that this result further supports the lower crosslinking kinetics and density of photocrosslinked hydrogels due to the radical scavenging effects of the catechol groups, as characterized in Figure S7.10.

7.3 Conclusions and Prospects

Taken together, we find that CA-based moieties and their oxidatively oligomerized components in the chemical modification of gelatin backbones are promising as thermosensitive, biodegradable, and tough bioadhesives for sealing internal surgical wounds. On this basis, processing parameters (*i.e.*, gelatin source, crosslinking approach, and CA oligomerization prior to conjugation) were optimized to attain robust bioadhesion. Oligomerization of CA prior to conjugation with gelatin enabled covalent grafting of a large numbers of catechol groups through standard EDC/NHS coupling reactions. In this way, in addition to introducing adhesion to the physical gels, gelatin's intrinsic thermosensitivity can be tuned so that not only the hydrogels remain integral, but they can also self-associate at body temperature. Modification of gelatin with CAO-enhanced injectability on non-horizontal tissue surfaces accelerated physical gelation and provided better pre-gel control due to increased viscosity. In contrast to photo-crosslinkable GelCA/GelMA hybrid hydrogels, aqueous stability and static adhesion were preserved more efficiently in the oxidatively crosslinked groups. In both cases, considerable improvements in tensile properties, *i.e.*, toughness (~11.5×) and stretchability (up to ~5-7×), were obtained

compared to GelMA hydrogels. Significant improvements in gelatin cohesion and thus adhesion strength were obtained *ex vivo* (by an order of magnitude relative to commercial sealants). The hydrogel tackiness improved and better resistance against delamination under harsh conditions was achieved in CAO- compared to CA-conjugated gelatin at body temperature. In addition, the presented approach can boost hydrogel functionality for chronic wound management by strengthening the antioxidant effects of catechol moieties as a result of their increased conjugation efficacy.

By increasing the limits of conjugation capacity through catechol modification and polymerization prior to conjugation, we are opening up new opportunities to enhance adhesion performance in natural biomaterials. In this regard, chemical modification of natural biomaterials with the other oxidized catechol-carrying molecules than caffeic acid (*e.g.*, 3,4-dihydroxy-L-phenylalanine) should be studied. We will explore implementation of this strategy with different backbones for other adhesive functionalities in upcoming studies. Finally, we anticipate further exploration of the mucoadhesion of conjugated polycatecholic compounds for potential drug delivery applications.

7.4 Experimental Procedures

7.4.1 Materials

Gelatin from porcine (PRC) skin type A, gelatin from bovine (BVN) skin type B, gelatin from cold water fish (PSC) skin, caffeic acid (CA), sodium periodate (NaIO_4), dimethylsulfoxide (DMSO), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), sodium molybdate, sodium nitrite, and methacrylic anhydride (MA) were purchased from Sigma Aldrich, USA. *N*-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-

ethylcarbodiimide hydrochloride (EDC) were provided by TCI Chemicals, USA. Porcine lung and bladder tissues were supplied by Sierra for Medical Science, USA. Dulbecco's phosphate-buffered saline (PBS) was purchased from Thermo Fisher Scientific, USA.

7.4.2 Synthesis of gelatin methacryloyl

Gelatin methacryloyl (GelMA) was synthesized according to the previously reported procedures.^{58,676} In brief, 10 g gelatin from porcine skin was dissolved in PBS at 10 %w/v. Then, 8 ml MA was added dropwise to the solution in dark and the reaction was continued while stirring for 3 h at 50 °C. The final solution was dialyzed against deionized (DI) water for 7 days and the dialysis solution was refreshed twice a day. The dialyzed solution was then filtered and freeze-dried for 4 days.

7.4.3 Modification of gelatin with caffeic acid-based moieties

To modify gelatin with CA-based motifs, first, 212 mg (4.25 %w/v) CA was dissolved in 5 ml 50 %v/v DMSO in de-ionized (DI) water solution. Note that in contrast to CA, gelatin is soluble in water and hardly soluble in organic solvents. Hence, a 50 %v/v DMSO solution in DI water was chosen for complete dissolution of reaction components. Then, various amounts of NaIO₄ (0, 7.5, and 22.5 mg corresponding to 0, 0.15, and 0.45 %w/v) were added to the CA solution and shaken for 1 h to produce CAO. Subsequently, 20 ml 50 %v/v DMSO in water solution containing 191 mg EDC (0.96 %w/v) and 115 mg NHS (0.58 %w/v) were added to the CAO solution, and the NHS activation reaction proceeded for 2 h. Then, 1 g gelatin from different sources including PRC, BVN, and PSC was dissolved in 75 ml 50 %v/v DMSO solution in water (1.33 %w/v gelatin) at 50 °C for 1 h. The gelatin solutions were added to the NHS activated CAO solution and the conjugation reaction was maintained at

room temperature for 24 h at room temperature. The resulting solutions were dialyzed against DI water for 3 days and the dialysis solution was refreshed three times a day. The GelCA products were then freeze-dried for 4 days and stored in a 4 °C refrigerator.

7.4.4 Hydrogel preparation and crosslinking procedure

Two groups of hydrogels with different crosslinking approaches were studied. Photo-crosslinkable groups of pre-gel solutions were prepared by dissolving GelCA/GelMA composites in 50 °C solutions of 0.25 %w/v LAP photo-initiator in 2 ml Eppendorf tubes. Composition ratios of GelCA:GelMA were 20:0, 17:3, 14:6, 11:9, 8:12 %w/v:%w/v in pre-gel solutions with total concentrations of 20 %w/v. The mixtures were vortexed and kept at 50 °C for about 1 h until full dissolution. To speed up the dissolution process, bubbles were removed *via* centrifugation for increments of ~30 s at 5400 rpm every ~10-20 min using a mini centrifuge. Crosslinking was carried out by irradiating UV light (365 nm wavelength) at 35 mW/cm² light intensity using an OmniCure S2000 Spot UV Curing System, USA for 2 min.

For oxidatively crosslinked hydrogels, crosslinking solutions of NaIO₄ were first prepared at 0.05, 0.1, 0.3, and 1 %w/v. The GelCA components were dissolved in DI water at 20 %w/v concentration, similar to the procedure described for the photo-crosslinkable group. The hydrogels were either cast in polydimethylsiloxane (PDMS) molds or pipetted on tissue substrates (depending on the experiment type) using a Pos-D™ positive-displacement pipette. The pre-gels were left at room temperature for ~45-60 s to form a physical gel. Then, the NaIO₄ solutions were pipetted on the hydrogel surfaces so that the physical gels were fully covered with the crosslinking solutions. The samples were exposed to NaIO₄ solution for 5 min to allow infusion-triggered crosslinking, and then washed with PBS solution.

7.4.5 UV-vis spectroscopy

To monitor elimination of small molecules during dialysis, before refreshing the dialysis solution each time, sample volumes of 200 μL were taken from the dialysis solution and added to a 48 well plate. Absorbance was measured at 320 nm wavelength (associated with CA) using a plate reader and the results were expressed with respect to DI water blanks.

Oligomerization reactions of CA in presence of NaIO_4 were monitored *via* UV-vis measurements. Different amounts of NaIO_4 (2.1, 6.3, 10.5, 18.9, 31.5, 52.5, 105.0, and 136.5 mg) were dissolved in 880 μL DI water Eppendorf tubes. In each tube, excess solution (792 μL) was discarded, leaving behind 88 μL of the NaIO_4 solution. Then, 334 μL of CA solution in 63 %v/v DMSO solution in DI water was added to each tube starting the oxidation reaction at the final NaIO_4 concentrations of 0.05, 0.15, 0.25, 0.45, 0.75, 1.25, 2.5, and 3.25 %w/v in presence of 4.25 %w/v CA and 50 %v/v DMSO. At different timepoints, 2 μL of each reaction solution was taken to measure absorbance at 500 nm wavelength using a NanoDrop OneC Microvolume UV-vis spectrophotometer. A solution of 50 %v/v DMSO in water was used as a blank.

In order to test conjugation of CA to gelatin, UV-vis spectroscopy was conducted as follows. Different types of synthesized GelCA samples were dissolved in DI water at 3.2 %w/v and absorbance values were measured across wavelengths. Corresponding solutions of unmodified gelatin sources were used as blanks.

7.4.6 Catechol content assay

Arnow assays were performed to study the concentrations of catechol groups in modified gelatin following procedures reported in the literature.⁴⁶ Briefly, 1 ml of 0.15 %w/v

GelCA dissolved in 0.5 M HCl was mixed with an equal volume of solution containing 10 %w/v sodium molybdate and 10 %w/v sodium nitrite prepared in water. The final solution was shaken for 5 min followed by increasing pH to 7 by adding 1 M NaOH and increasing the final volume to 5 ml. The values of absorbance for 200 μ L samples added to a 48-well plate were measured at 520 nm using a plate reader. Standard calibration curves were obtained by implementing the above procedure for various concentrations of dopamine based on which the conjugated catechol content was calculated.

7.4.7 Nuclear magnetic resonance

Proton nuclear magnetic resonance (^1H NMR) spectroscopy was conducted for chemical characterization of biopolymers. The ^1H NMR spectra were recorded for all samples at room temperature using a 400 MHz Bruker AV400 spectrometer. All spectra were corrected with phase and base line and solvent peak were fixed before quantifying the degree of conjugation. Modified gelatin samples were dissolved in DMSO- d_6 at a concentration of 10 mg/mL, and the degree of amine consumption was estimated based on reported literature.^{58,643-644,677}

7.4.8 Rheological measurements

All the rheological measurements were performed using an MCR 302, Anton Paar (Graz, Austria) rheometer equipped with an 8 mm diameter torque measurement system. An 80 μ L pre-gel solution was pipetted for each experiment at 1 mm gap size, unless noted otherwise. Where applicable, the gelation point was defined by the point where storage modulus (G') exceeded loss modulus (G'').

Prior to the tests, the samples were stabilized at 50 $^{\circ}\text{C}$ for 5 min. Analysis of

thermosensitivity was conducted by a temperature sweep starting from 50 °C to 10 °C at a rate of 1 °C/min followed by a 3 min isothermal period and then a heating cycle over the same temperature range. The values of G' and G'' were recorded at 10% strain and 1 Hz oscillation. Measurements of viscosity-shear rate were performed at 37 °C across shear rates from 1 to 100 s⁻¹ at 0.5 mm gap size.

Time sweeps were carried out for the study of physical and chemical gelation kinetics. For the photo-crosslinkable samples, a custom-made fixture setup was used to expose the pre-gel solutions to UV light vertically, by transferring the light from a horizontal optical fiber using a 45° aluminum reflector. Accordingly, oxidative crosslinking kinetics were evaluated by implementing the experiments in a petri dish where pre-gel solution was immersed in NaIO₄ solutions of different concentrations. Storage and loss moduli were recorded after 1 min stabilization period at 10% strain and 1 Hz frequency over 10 min. Time sweeps for photo-crosslinkable and NaIO₄-crosslinked hydrogels were performed at room temperature and 37 °C, respectively.

7.4.9 Mechanical tensile tests

Tensile properties of hydrogels were evaluated using Instron 5943 (Norwood, MA, USA) universal testing instrument equipped with a 100 N load cell. Specimens of 25 mm length, 5 mm width, and 2 mm thickness were molded and crosslinked according to the above-mentioned procedure. Next, the specimens were glued to a piece of plain paper used to grip the samples. Quasi-static tensile tests were performed at 4 mm/min while the force-displacement data were recorded. Stress-strain data were calculated by defining stress as $\sigma = F/A$ and strain $\varepsilon = \delta/L$ where F represents force, A is the cross-sectional area, δ is displacement, and L is the initial length of the samples. Tensile strength and strain at failure

(stretchability) were defined as the stress and strain at the failure point, respectively. Toughness was also characterized as the absorbed deformation energy per unit of material volume, in other words, the area under the stress-strain curves. Elastic modulus (stiffness) was measured from the slope of stress-strain curves.

7.4.10 Swelling tests

The swelling kinetics of the oxidatively crosslinked hydrogels were evaluated by casting 100 μ l pre-gel solutions in an 8 mm circular mold. The hydrogels were removed after forming a physical gel and were immersed in NaIO₄ solutions of different concentrations for 5 min, followed by washing with PBS. The hydrogels were weighed (w_0) and incubated in 2 ml PBS in 12 well plates. The weights of swollen hydrogels at different timepoints were registered (w_s) and the corresponding swelling ratio was defined by Equation (1):

$$\text{swelling ratio} = \frac{w_s - w_0}{w_0} \quad (1)$$

7.4.11 Antioxidant activity assay

To evaluate antioxidant activity of the prepolymer solutions, a DPPH assay was performed following the procedure reported by Spizzirri *et al.*⁶⁷⁸ with slight modifications. The samples were dissolved at 4 mg/ml in distilled water. Then, 200 μ l of the sample solution was mixed with 1 ml DPPH solution (200 μ M in ethanol) in a 48-well plate. The reaction was allowed to proceed for 30 min. Then, the absorbance of the DPPH was measured at 517 nm (A_s) using a NanoDrop OneC Microvolume UV-vis spectrophotometer. The absorbance of samples with no gelatin content was assigned to the blank sample (A_0). The DPPH radical scavenging activity was calculated according to Equation (2):

$$\%DPPH\ inhibition = \frac{A_0 - A_s}{A_0} \times 100 \quad (2)$$

7.4.12 Drug release tests

Hydrogel prepolymers were prepared by dissolving modified gelatin bioadhesives in 2 mg/ml Rhodamine B as a small-molecule model drug. Then, disks of 8 mm diameter and 2 mm height were crosslinked according to the described procedures. The hydrogel samples were incubated in 1 ml PBS. The absorbance of the PBS solution was measured every 30 min based on which the drug release was measured according to calibration curves obtained from Rhodamine B solutions at various concentrations.

7.4.13 Hydrogel static adhesion stability

To study hydrogel stability and pre-gel release in aqueous media, UV-vis measurements were first implemented on the non-crosslinked pre-gel solutions. For this purpose, 80 μ L pre-gel solution was diluted in 500 μ L DI water and vortexed before keeping it at 37 °C. Then, the crosslinked samples were prepared by crosslinking the same amount of pre-gel on wet collagen sheets followed by incubation in 500 μ L DI water in 12-well plates. The samples were maintained at 37 °C for 24 h. Next, the amount of release was calculated as the ratio of the absorbance of the solution containing crosslinked samples to their corresponding non-crosslinked pre-gel solutions at 320 nm wavelength. Furthermore, the state of adhesion was inspected visually at 24 h, and the observable detachments were registered.

7.4.14 Tack tests

The normal adhesion force produced by crosslinked hydrogels was measured using a rheometer according to the following procedure. An amount of 80 μ l pre-gel was pipetted onto a petri dish at 37 °C and contacted with an 8 mm diameter torque measurement system at 1 mm gap. Then, different concentrations of 25 °C NaIO₄ solutions were added to the petri dish to immerse the hydrogel completely from the peripheral edges. After 5 min, the torque measuring system was lifted while recording the normal force at 0.5 mm/min. Adhesion strength was reported as the maximum recorded reaction force divided by interfacial area.

To perform cyclic tack tests for evaluation of adhesion durability and variation with temperature for the pre-gels, disk-shaped poly(methyl methacrylate) (PMMA) sheets with 10 mm internal diameter and 1 mm thickness was first laser cut and closed on one side using a glass slide. The molds were attached to the sample holder and filled with pre-gel solutions. Then, the temperature was reduced to 10 °C to form a physical gel. Using an 8 mm diameter torque measurement system, a preload of 5 N was applied to determine the zero position on the surface of hydrogels. Then, the temperature was raised to 37 °C and samples were left for thermal stabilization for 10 min. Next, vertical motion profiles involving 100 cycles of vertical displacement were defined over of 400 s at 3 mm displacement amplitude. The temperature for the adhesion durability tests was kept constant at 37 °C. For study of temperature effect, the temperature was varied from 10 to 40 °C during the cyclic tests and the maximum reaction force due to adhesion was reported.

7.4.15 *In vitro* burst pressure test

Collagen sheets of 25 mm diameter were punched using a 3 mm diameter puncher

and soaked in PBS for ~1 h. The punctured collagen sheets were attached on a fixture and tightened to cover the air chamber completely. The excess water was removed using Kimwipes and 80 μ L pre-gel solution was pipetted to cover the incision completely. After ~45-60 s physical gelation at room temperature, the samples were treated with NaIO_4 solutions for 5 min and subsequently washed with PBS. Air was pumped into the air chamber using a syringe pump at 20 ml/min. A pressure sensor was used to monitor the air pressure inside the chamber. The pressure and mode of failure at the time of failure or leakage were registered.

7.4.16 Wound-closure test *in vitro*

Pig lung and heart tissue surfaces were harvested in strips of 10 mm width. Rectangular portions of 10 mm length were cut in the middle. The incision was glued symmetrically covering ~10 mm $\times$ 10 mm area by the pre-gels and crosslinked using NaIO_4 treatment for 5 min after physical gelation. The specimens were washed with PBS and incubated in DI water at 37 °C for at least 10 min. Tensile displacement was applied to the two ends of tissues at a rate of 25 mm/min. The work of adhesion was measured according to the area under the force-displacement curve until the break point. The adhesion toughness was reported by dividing the work of adhesion to the total surface area under the pre-gel.

7.4.17 Burst pressure *ex vivo*

For the *ex vivo* burst pressure tests on pig lungs, the tissue was connected to an oxygen tank and a pressure sensor using silicone tubes through the esophagus, which was tightened with clips. The lung tissue was immersed in 37 °C water. A 3-mm incision was

made on the tissue surface, which was sealed with pre-gel solutions followed by crosslinking. A similar procedure was applied to the pig bladder. Oxygen pressure was increased while pressure sensors monitored the internal pressure. A similar procedure was performed for burst pressure measurement for porcine bladder tissue, except that 37 °C water was pumped into the bladder tissue using a positive-displacement pump.

7.4.18 *In vitro* biocompatibility

The cytotoxicity of the bioadhesives was evaluated by two-dimensional (2D) cell culture procedures in 12-well plates. The hydrogels were prepared in disk shapes by adding 60 μ L pre-gel solutions to the 8 mm diameter PDMS molds. After physical gelation, the disks were soaked in NaIO₄ solutions for 5 min for crosslinking. Then, the hydrogels were transferred into fresh PBS solutions under UV light for 2 h for sterilization. To perform the live/dead assay, human lung fibroblast cells (HLFs) were seeded at $\sim 1 \times 10^4$ cell/well density using 2 ml of a FGM™-2 BulletKit™ fibroblast growth media (Lonza, Basel, Switzerland) for 24 h. The sterilized GelCA hydrogels were then added to the wells. At different time points, live/dead assays (Invitrogen, USA) were performed by treating the cells with 500 μ L of the live/dead reagent consisting of 5 μ L calcein and 20 μ L ethidium homodimer-1 prepared in 10 ml PBS. The cells were incubated for ~ 20 min, and fluorescence imaging was conducted using a fluorescence microscope (Zeiss Axio Observer 5, Germany) at the excitation/emission wavelengths of 528/617 nm for ethidium homodimer-1 (red) and 494/515 nm for calcein (green). Cell viability was calculated by the ratio of the number of live cells to the total number of cells where the cells were counted using ImageJ software. We further assessed the metabolic activity of the C2C12 murine myoblasts cell lines (ATCC) cultured at $\sim 2.5 \times 10^4$ cell/well density similar to the procedure explained for live/dead assay

using a Dulbecco's modified Eagle's medium (DMEM) media containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Here, a PrestoBlue™ reagent (Thermo Fisher Scientific, USA) was diluted in DMEM in 1:9 ratio and the cells were treated with a fresh 2 ml of this solution. The cells were then incubated for ~45 min and then, the fluorescence intensity at excitation and emission wavelengths of 560 and 590 nm, respectively, was measured at different time points using a BioTek Synergy 2 plate reader.

7.4.19 *In vivo* biocompatibility

Biocompatibility of hydrogels was evaluated *in vivo* by implanting ¼ cylindrical samples (8 mm diameter and 2 mm height) subcutaneously in C57B6 black mice supplied by The Jackson Laboratory, USA. These studies were carried out under an approved protocol (Institutional Animal Care and Use Committee, IACUC, approval #22747-01) at the Lindquist Institute, Torrance, CA, USA. The mice were fed with laboratory pellets and purified water and maintained at 25 °C in pathogen-free (SPF) facilities. Isoflurane (1.5 %v/v in O₂ gas) was used along with carprofen injection for anesthesia during the surgery. Subcutaneous implantation was conducted *via* a 15 mm surgical incision following sterilization. After 1 and 4 weeks, the mice were euthanized by inhalation of carbon dioxide. Then, sample extraction was performed from the implanted sites, adjacent tissues, and organs. The samples were fixed using formalin (10 %v/v), and after implantation, the samples were embedded in paraffin, cut into 5 µm slices, and then stained with hematoxylin and eosin (H&E) to analyze cell morphology.

7.5 Supporting Information

Table S1. Description of sample labeling and conditions studied for both cases of photo-crosslinking and oxidative crosslinking approaches.

Factors	Gelatin type	Concentration of NaIO ₄ for CA oligomerization ($\times 10^{-2}$ %w/v)	Crosslinking parameters	
			Photo-crosslinking	Oxidative crosslinking
			Concentration of GelCA:concentration of GelMA in pre-gel (%w/v)	Concentration of NaIO ₄ in spray solution (%w/v)
Code	G	0	C	
Levels	Porcine (PRC) Bovine (BVN) Piscine (PSC)	0 15 45	20:0 17:3 14:6 11:9 8:12	0 0.05 0.1 0.3 1
Label format	G-GelCA-O(C)			
Example			BVN-GelCA-45(17:3)	PRC-GelCA-0(0.3)

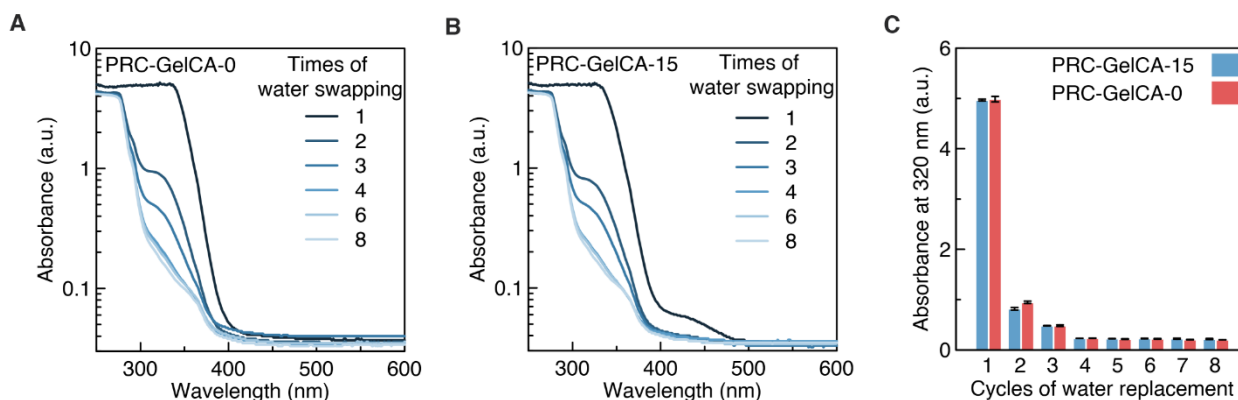


Figure S7.1. Characterization of caffeic acid (CA) oxidation kinetics using NaIO₄. (A) UV-vis characteristics of CA oxidation in 50 %v/v dimethylsulfoxide (DMSO) solutions. (B) Variation of absorbance with NaIO₄ concentration at 1 h and 24 h post-oxidation. (C) Comparing the UV-vis characteristics of CA in water and DMSO solution in de-ionized (DI) water.

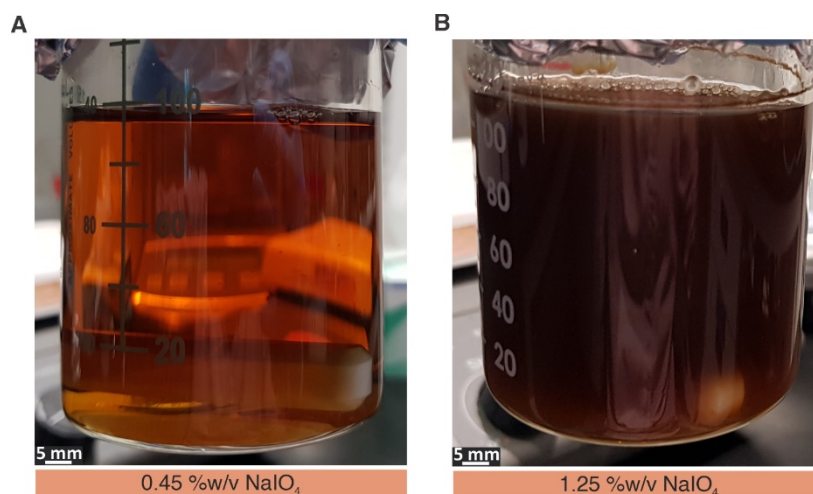


Figure S7.2. The appearance of reaction solutions after coupling reaction of caffeic acid oligomers obtained at (A) 0.45 and (B) 1.25 %w/v NaIO_4 . The reaction solutions remained transparent with no observable agglomeration at NaIO_4 concentrations of 0.45 %w/v and lower.

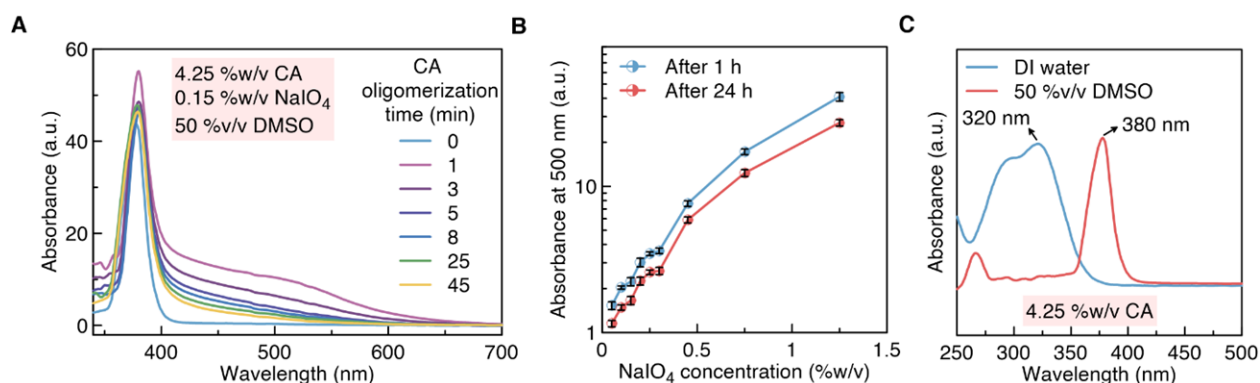


Figure S7.3. Monitoring removal of small molecules and reaction byproducts during dialysis in the synthesis of gelatin-caffeic acid (GelCA) conjugates. (A, B) UV-vis results of the samples taken from the dialysis solution after multiple cycles of swapping dialysis water for caffeic acid (CA)- and caffeic acid oligomer (CAO)-conjugated porcine (PRC) gelatin, respectively. (C) The values of absorbance at 320 nm, representing CA moieties in dialysis solution. The data are presented as means $\pm$ SD.

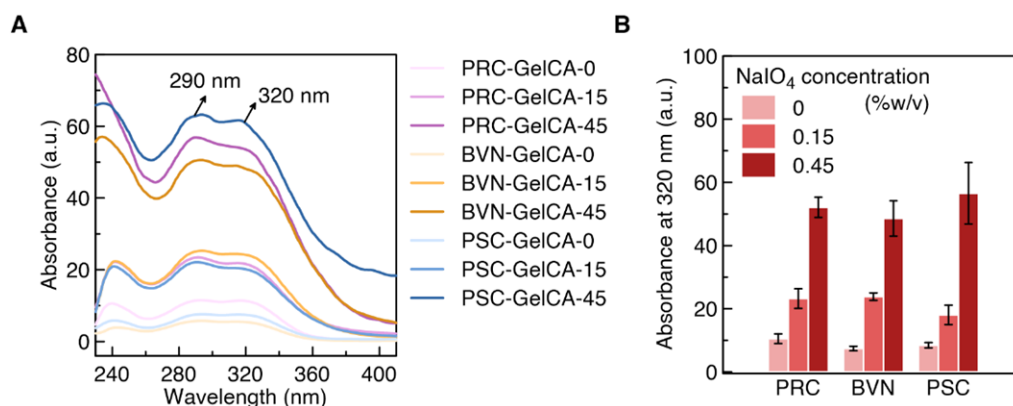


Figure S7.4. UV-vis study of caffeic acid (CA) conjugation to gelatin from different sources, *i.e.*, porcine (PRC), bovine (BVN), and piscine (PSC). (A) UV-vis spectra of gelatin-caffeic acid (GelCA) solutions showing a peak at 320 nm and 290 nm in gelatin-caffeic acid (GelCA) conjugates. (B) The absorbance of GelCA solutions at 320 nm for GelCA samples where CA was oligomerized at different NaIO_4 concentrations. The data are presented as means $\pm$ SD.

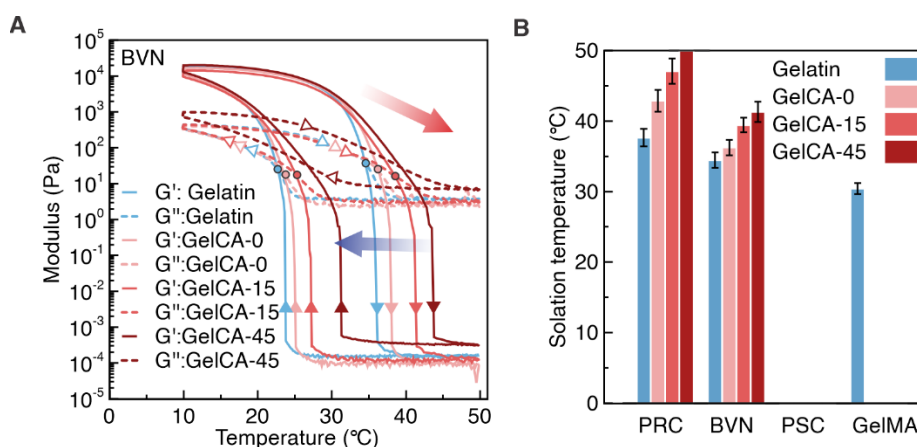


Figure S7.5. (A) Rheological study of temperature effect on storage modulus (G') and loss modulus (G'') for bovine (BVN) gelatin-caffeic acid (GelCA) conjugates. (B) The results of solation temperature for GelCA synthesized with different gelatin types, *i.e.*, porcine (PRC), BVN, and piscine (PSC), and various extents of caffeic acid (CA) oligomerization. The data are presented as means $\pm$ SD.

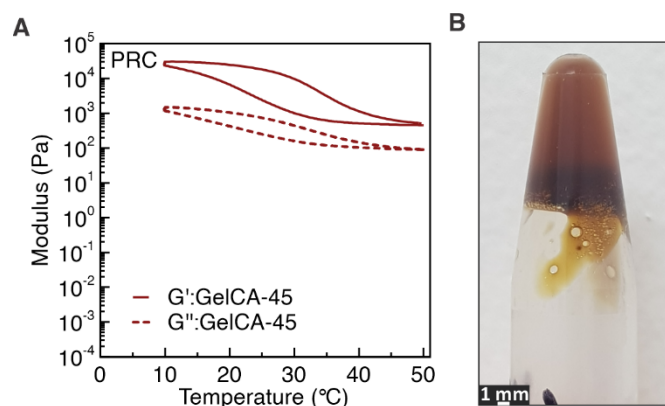


Figure S7.6. (A) Thermosensitivity of porcine (PRC) gelatin-caffeic (GelCA) conjugated with the caffeic acid (CA) oligomers (*i.e.*, PRC-GelCA-45 where CA was oxidatively oligomerized at the highest concentration of NaIO_4 tested here, namely 0.45 %w/v). (B) Demonstration of self-gelation of 20 %w/v PRC-GelCA-45 solution at 80 °C.

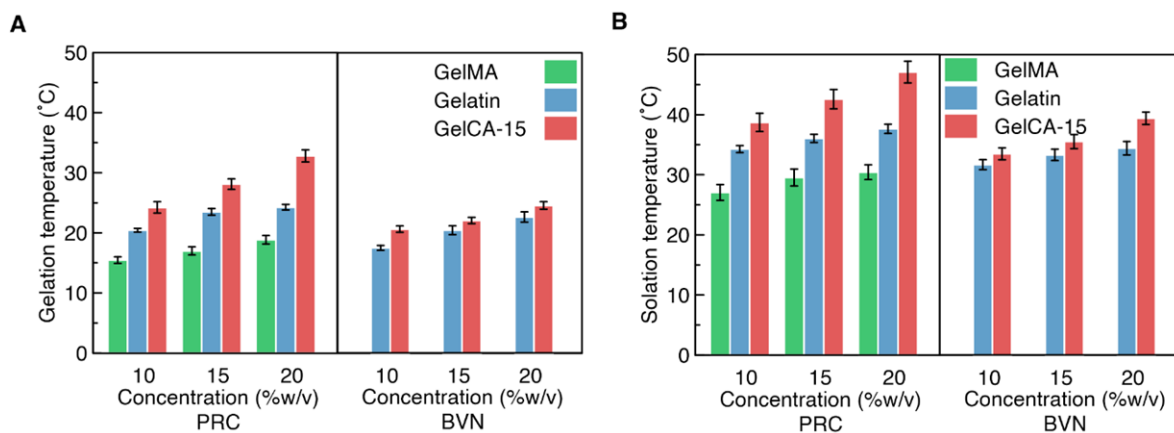


Figure S7.7. The effects of pre-gel concentration in caffeic acid-modified gelatin (GelCA) and gelatin methacryloyl (GelMA) on (A) gelation and (B) solation temperatures. The GelCA samples were obtained from different sources of porcine (PRC) and bovine (BVN) gelatin. The data are presented as means $\pm$ SD.

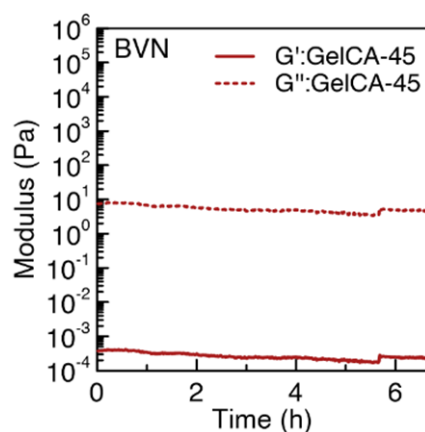


Figure S7.8. Rheological evolution of bovine (BVN) gelatin-caffeic acid (GelCA) solution at the highest degree of caffeic acid (CA) oligomerization studied here. No evidence of self-gelation was seen during the measurements.

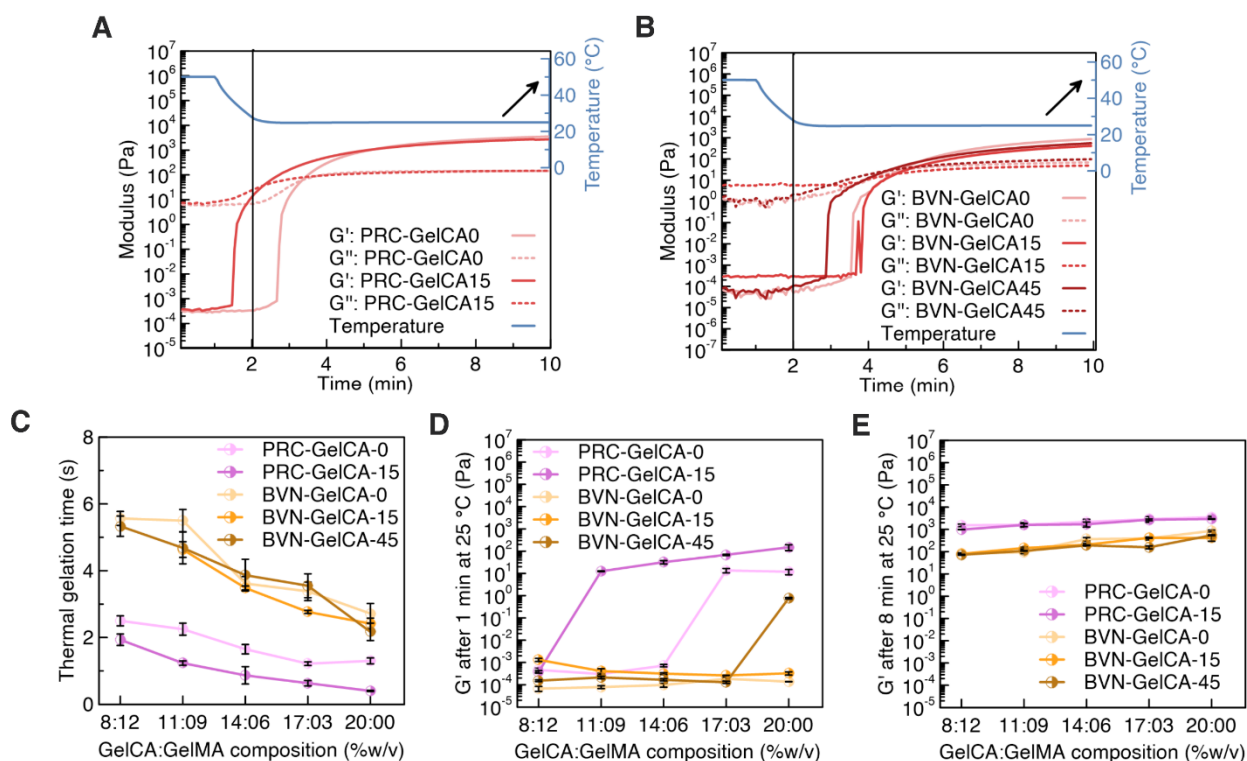


Figure S7.9. Evaluation of time to thermal gelation in response to temperature drop from 50 °C to 25 °C. (A, B) Variations of storage modulus (G') and loss modulus (G'') with cooling for gelatin-caffeic acid (GelCA) conjugated with porcine (PRC) and bovine (BVN) sources, respectively. (C) The results

of thermal gelation time for composites of GelCA and gelatin methacryloyl (GelMA) at different mixing ratios. The data are presented as means $\pm$ SD. (D, E) The values of G' at 1 and 8 min after reaching 25 °C, respectively. The data are presented as means $\pm$ SD.

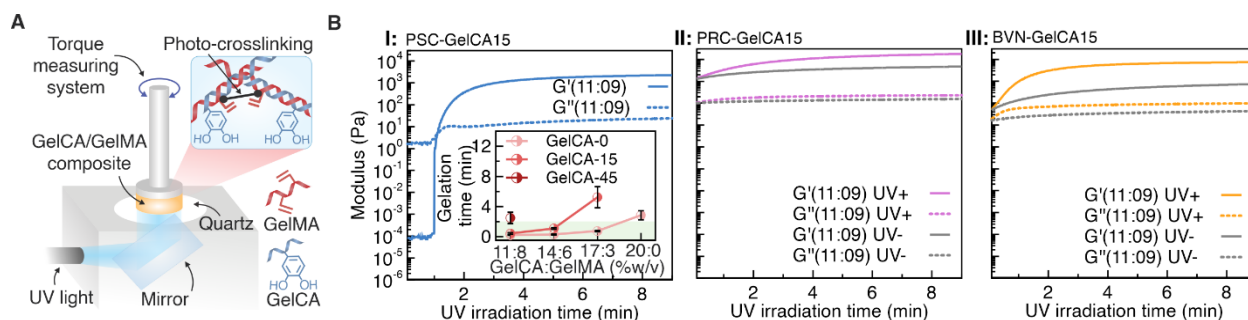


Figure S7.10. Crosslinking kinetics of photo-crosslinkable group of bioadhesives. (A) Experimental setup used for monitoring reaction kinetics in photo-crosslinkable gelatin-caffeic acid conjugate (GelCA)/gelatin methacryloyl (GelMA) hybrid hydrogels. (B) Storage modulus (G') and loss modulus (G'') for GelCA/GelMA hydrogels containing 11:9 %w/v GelCA:GelMA in response to UV light exposure for I: piscine (PSC) (inset shows the variation of gelation time at different GelCA:GelMA composition ratios), II: porcine (PRC), and III: bovine (BVN) GelCA. In the PRC and BVN groups, most samples underwent thermal gelation before UV irradiation (UV+: samples with UV exposure, UV-: samples without UV exposure).

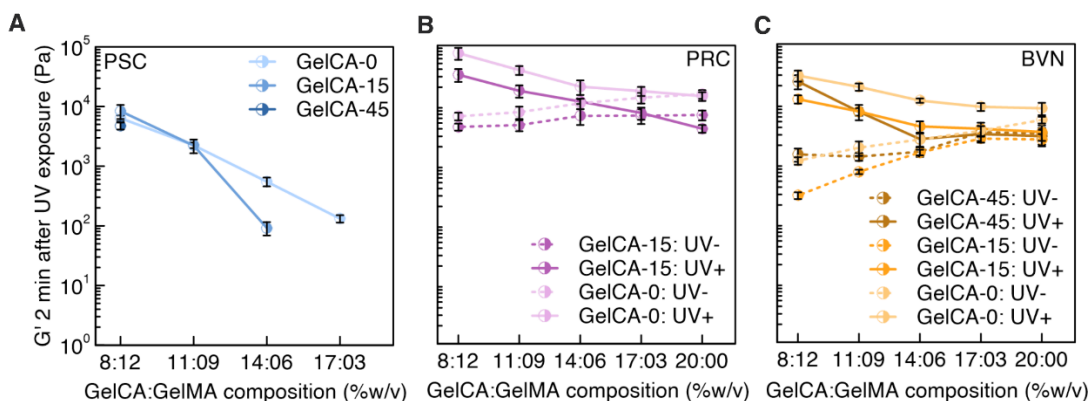


Figure S7.11. The values of storage modulus (G') after 2 min exposure to UV light in photo-crosslinkable hydrogels containing different weight ratios of gelatin-caffeic acid conjugates (GelCA) and gelatin methacryloyl (GelMA) for GelCA sources of (A) piscine (PSC), (B) bovine (BVN), and (C) porcine (PRC). UV+: samples with UV exposure, UV-: samples without UV exposure. The data are presented as means $\pm$ SD.

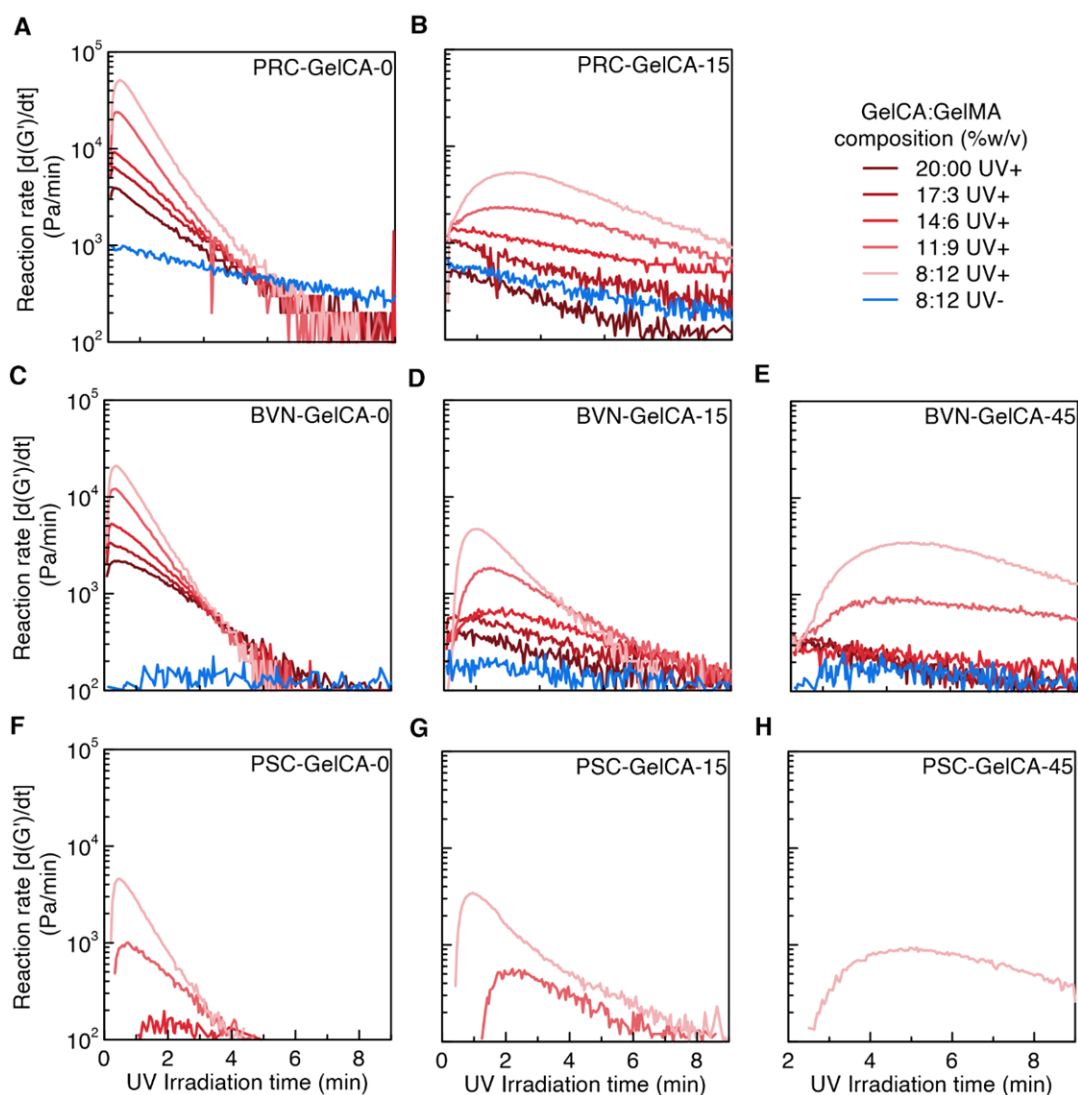


Figure S7.12. Kinetics of crosslinking reaction in photo-crosslinkable groups of gelatin-caffeic acid (GelCA)/gelatin methacryloyl (GelMA) composite hydrogels. Different sources of GelCA comprising of (A, B) porcine (PRC), (C-E) bovine (BVN), and (F-H) piscine (PSC) with different extents of caffeic acid (CA) oligomerization were studied. UV+: samples with UV exposure, UV-: samples without UV exposure.

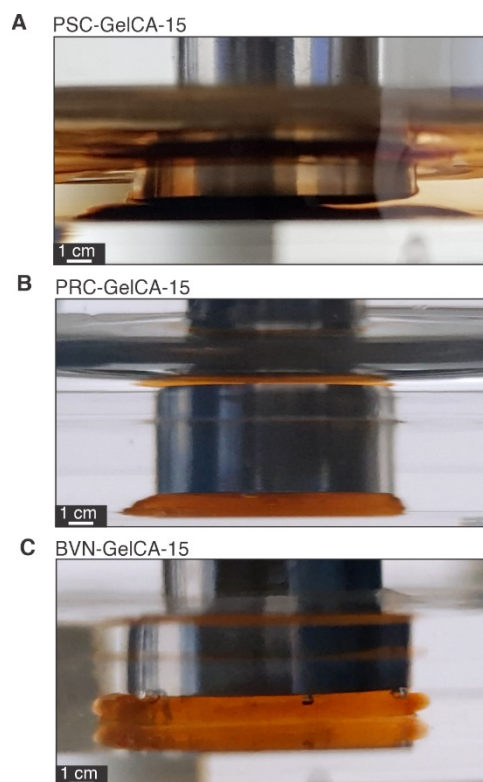


Figure S7.13. Stability of gelatin-caffeic acid (GelCA) pre-gel solutions under the rheometer torque measuring system upon immersion into 1 %w/v sodium hydroxide (NaIO_4) solutions for GelCA from (A) piscine (PSC), (B) porcine (PRC), and (C) bovine (BVN) sources. As opposed to PSC GelCA, which dissociated upon immersion in NaIO_4 solution, BVN and PRC GelCA retained their physical integrity enabling diffusive crosslinking.

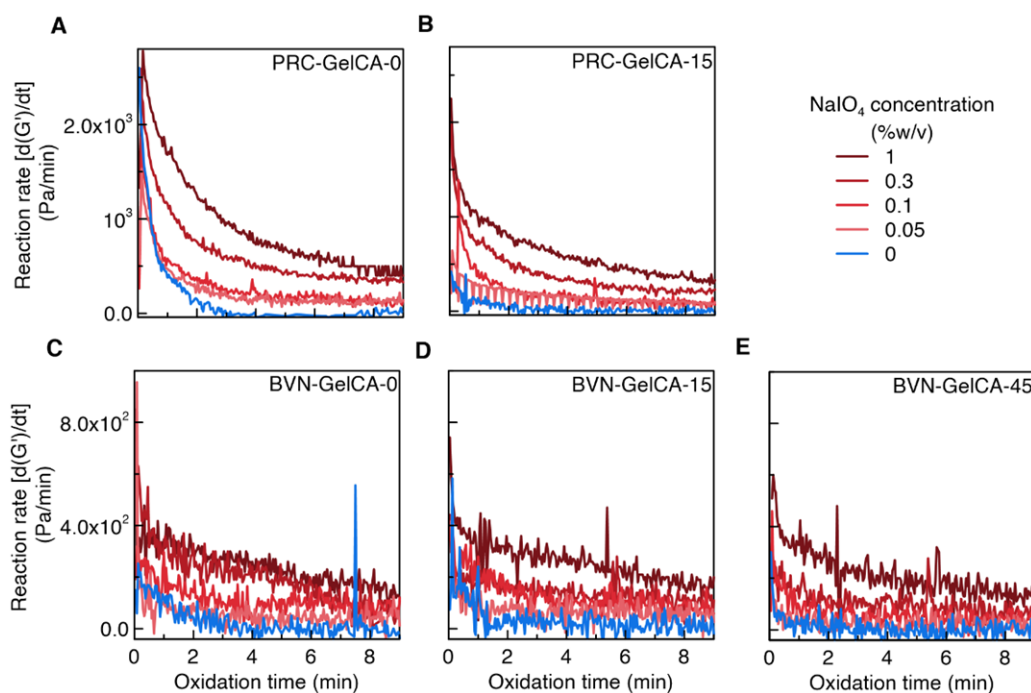


Figure S7.14. Kinetics of oxidation crosslinking reaction in gelatin-caffeic acid (GelCA) conjugates with gelatin from (A, B) porcine (PRC), and (C-E) bovine (BVN) sources at different caffeic acid (CA) oligomerization levels. Crosslinking was performed at different NaIO_4 concentrations (*i.e.*, 1, 0.3, and 0.1 %w/v).

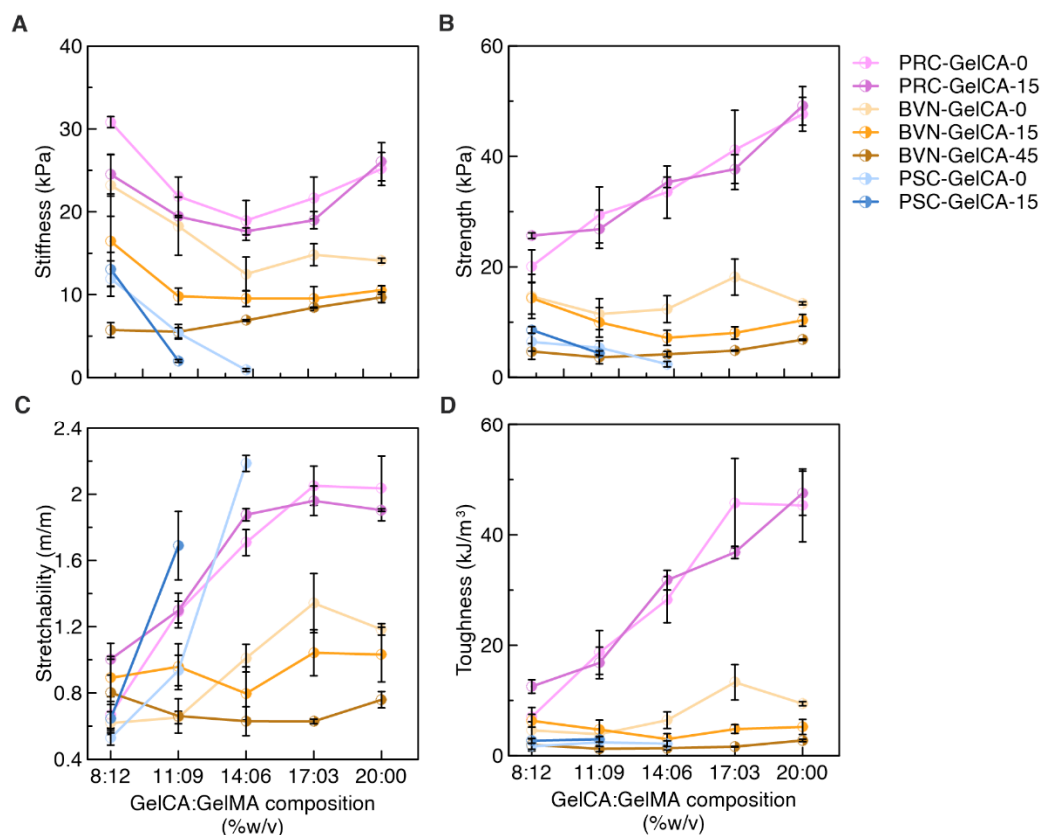


Figure S7.15. Tensile mechanical tests on gelatin caffeic acid (GelCA) conjugates from porcine (PRC), bovine (BVN), and piscine (PSC) sources in composition with photo-crosslinkable gelatin methacryloyl (GelMA) in terms of (A) elastic modulus, (B) tensile strength, (C) strain at failure and (D) toughness. The data are presented as means $\pm$ SD.

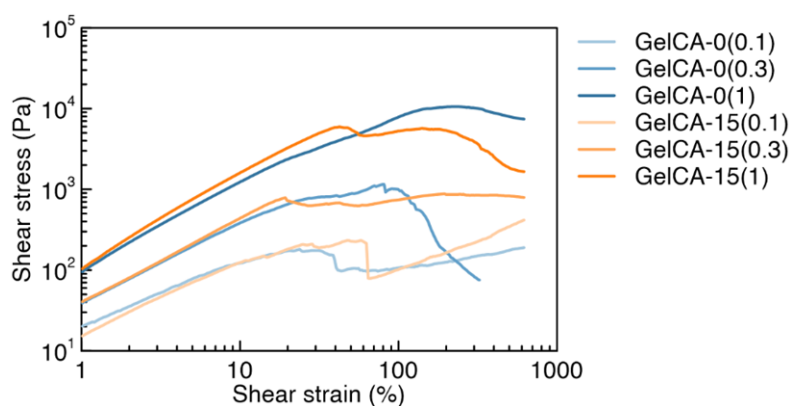


Figure S7.16. The results of shear stress-shear strain for oxidatively crosslinked gelatin-caffeic acid (GelCA) conjugates with gelatin from porcine (PRC) gelatin source crosslinked at different NaIO_4 concentrations (*i.e.*, 1, 0.3, and 0.1 %w/v).

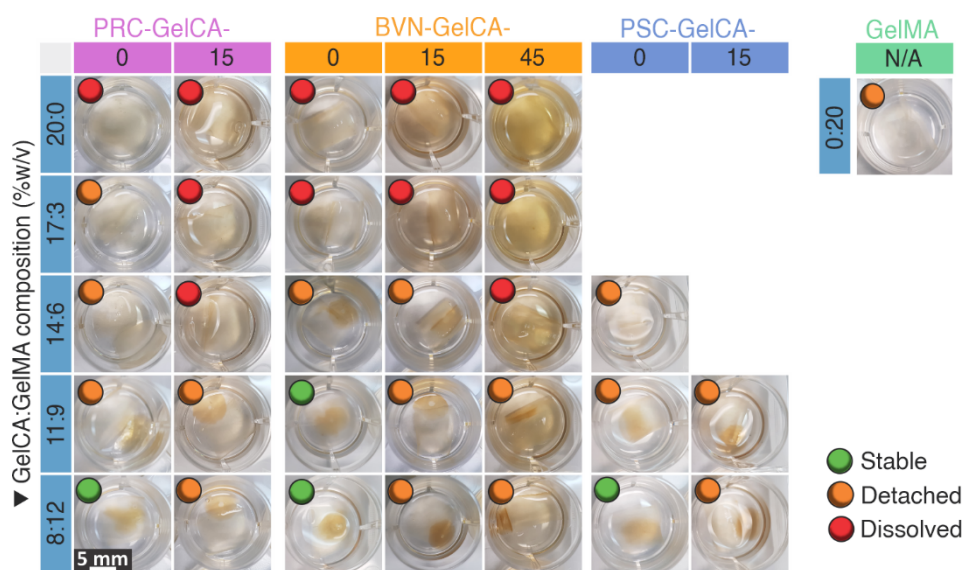


Figure S7.17. Inspection of wet adhesion stability under static incubation in water at 37 °C for the group of photo-crosslinkable hydrogels containing gelatin-caffeic acid (GelCA) and gelatin methacryloyl (GelMA). Here, GelCA was synthesized from porcine (PSC), bovine (BVN), and piscine (PSC) gelatin sources.

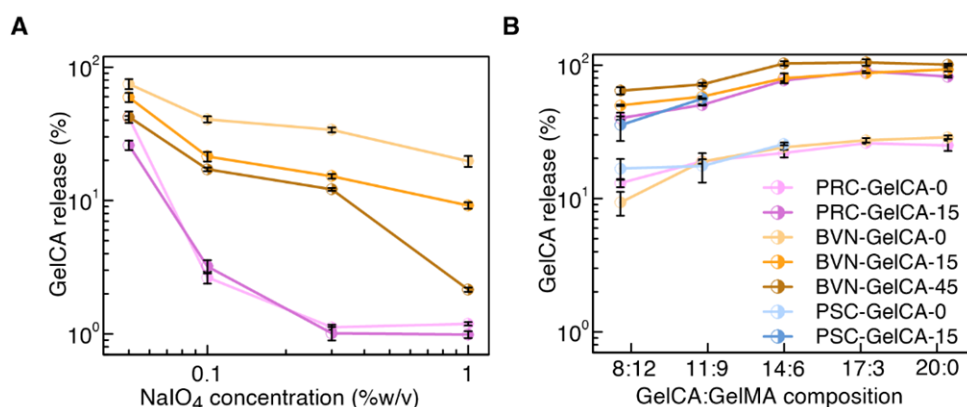


Figure S7.18. Comparing the results of gelatin-caffeic acid (GelCA) release over 24 h for the (A) oxidatively crosslinked group and (B) photo-crosslinked hybrid hydrogels synthesized from porcine (PRC) and bovine (BVN) sources. The data are presented as means $\pm$ SD.

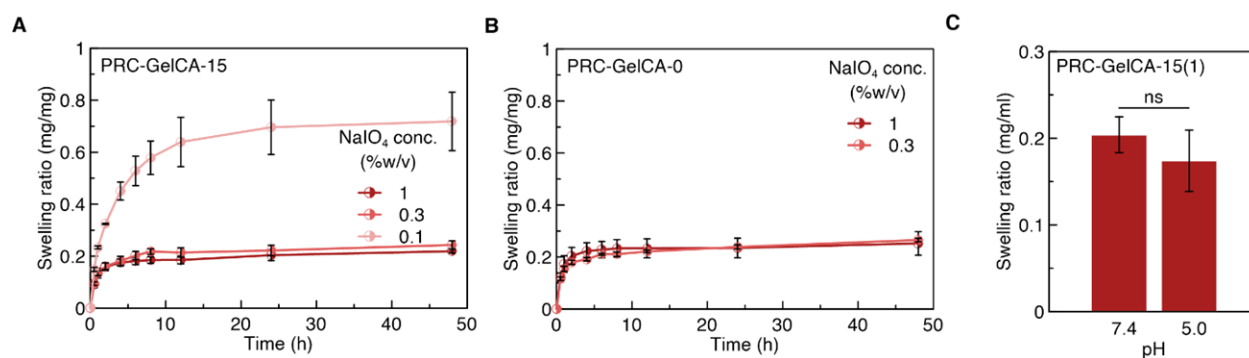


Figure S7.19. Swelling of gelatin-caffeic acid (GelCA) hydrogels from porcine (PRC) gelatin sources for (A) PRC-GelCA-15 (caffeic acid oligomer (CAO)-conjugated gelatin) and (B) PRC-GelCA-0 (caffeic acid-conjugated gelatin) bioadhesives. The data are presented as means $\pm$ SD. (C) Steady swelling of PRC-GelCA-15 hydrogels crosslinked in 1 %w/v NaIO₄ after 24 h incubation in phosphate-buffered saline (PBS) solutions with pH values of 7.4 and 5.0. The data are presented as means $\pm$ SD.

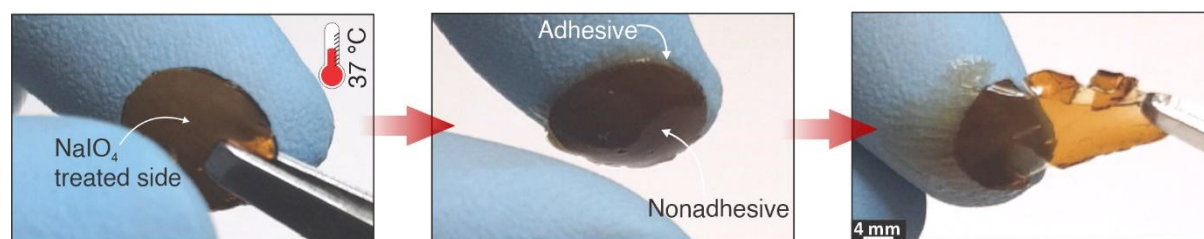


Figure S7.20. Side-selective adhesion of gelatin-caffeic acid (GelCA) patches from porcine (PRC) source, *i.e.*, PRC-GelCA-15, treated on one side with 0.3 %w/v NaIO₄ solution. The NaIO₄ treated surface showed no adhesion, whereas adhesion from the other side could be triggered by bringing the temperature to 37 °C.

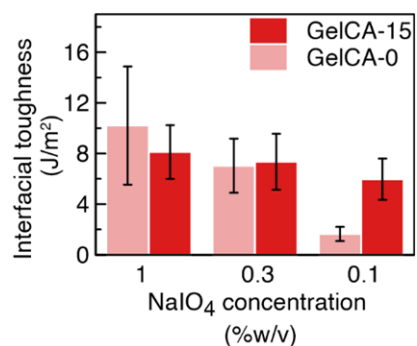


Figure S7.21. The results of interfacial toughness in wound-closure tests using oxidatively crosslinked porcine gelatin-caffeic acid (GelCA) conjugates for adhesion to pig heart tissue. The data are presented as means $\pm$ SD.

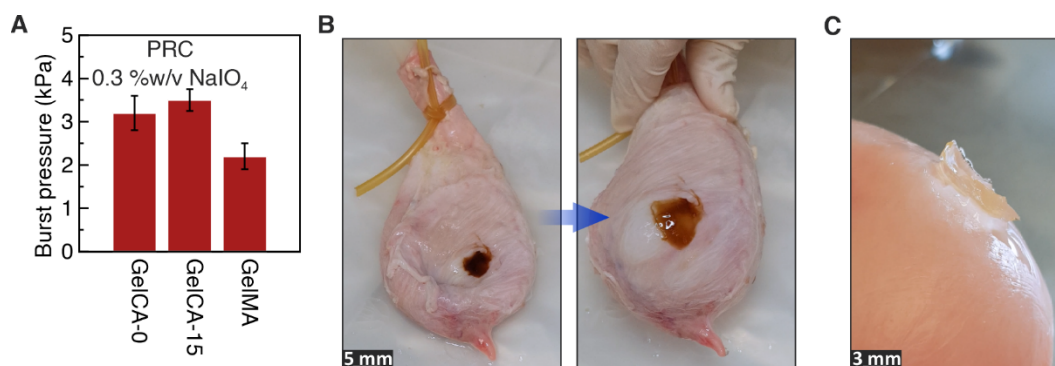


Figure S7.22. (A) The results of *ex vivo* burst pressure tests on pig bladder. The data are presented as means $\pm$ SD. (B) comparison of failure mechanism of gelatin-caffeic acid (GelCA) conjugates (sample PRC-GelCA-15 crosslinked at 0.3 %w/v NaIO₄) from porcine (PRC) sources with that of (C) gelatin methacryloyl (GelMA) hydrogels. While GelMA delaminated due to the mechanical mismatch with bladder tissue expansion, GelCA samples complied better with the mechanical deformations of the tissue substrates.

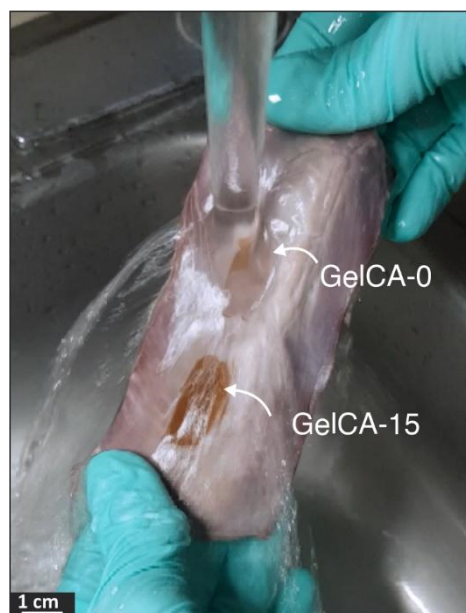


Figure S7.23. Demonstration of the robust and durable adhesion of porcine gelatin-caffeic acid (GelCA) with caffeic acid (CA) oligomer (CAO) conjugates, *i.e.*, GelCA-15, compared to that of GelCA with CA conjugates, *i.e.*, GelCA-0 samples under extreme tap water pressure at 37 °C after ~30 s.

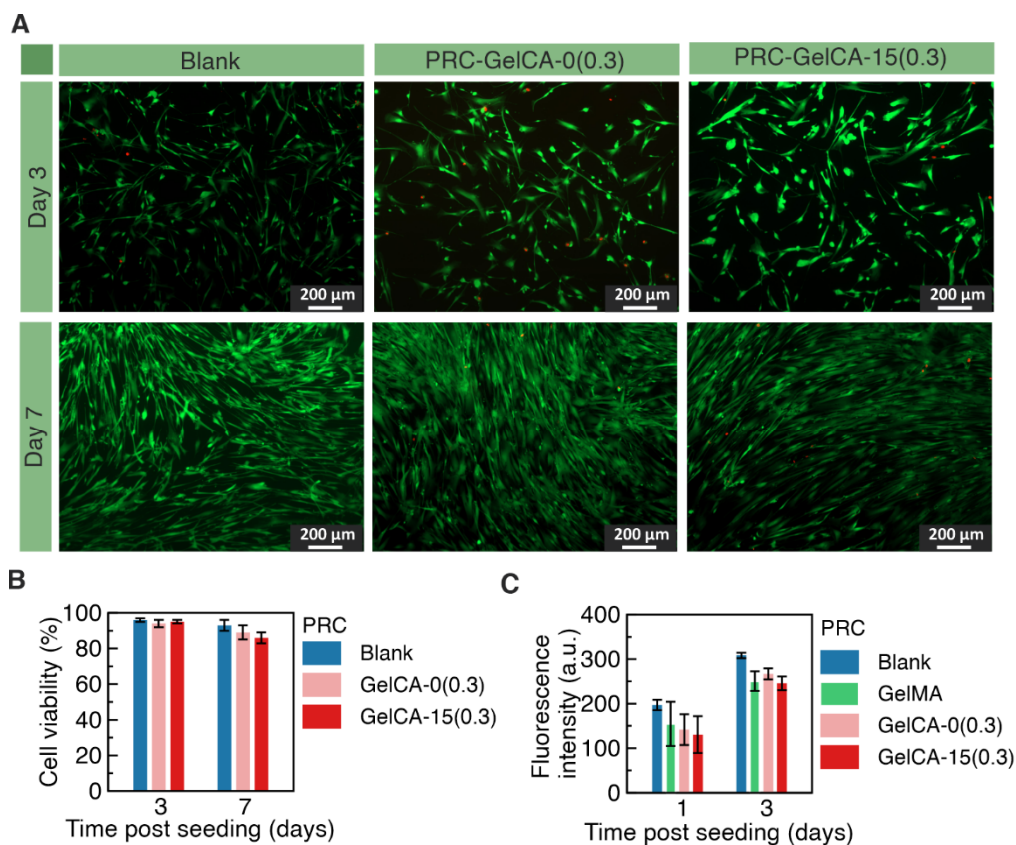


Figure S7.24. *In vitro* biocompatibility analysis of oxidatively crosslinked porcine (PRC) gelatin-caffeic acid (GelCA) conjugates. (A) The live/dead images of the human lung fibroblast (HLF) tests cultured in presence of NaIO_4 crosslinked hydrogel disks and (B) results of cell viability. The data are presented as means $\pm$ SD. (C) PrestoBlue™ metabolic activity assay studied on C2C12 cells in the presence of crosslinked GelCA hydrogels. The data are presented as means $\pm$ SD.

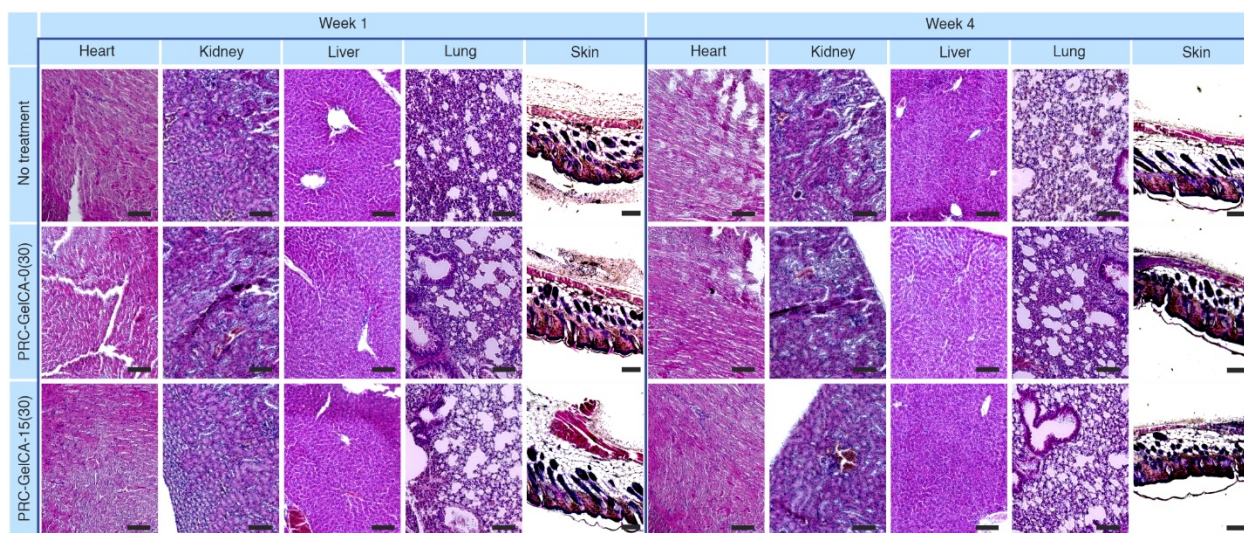


Figure S7.25. *In vivo* study of biocompatibility of oxidatively crosslinked porcine (PRC) gelatin-caffeic acid (GelCA) conjugates implanted subcutaneously in mice in terms of hematoxylin and eosin (H&E) staining images (scale bars for skin are 200 μ m and for all other tissues are 100 μ m).

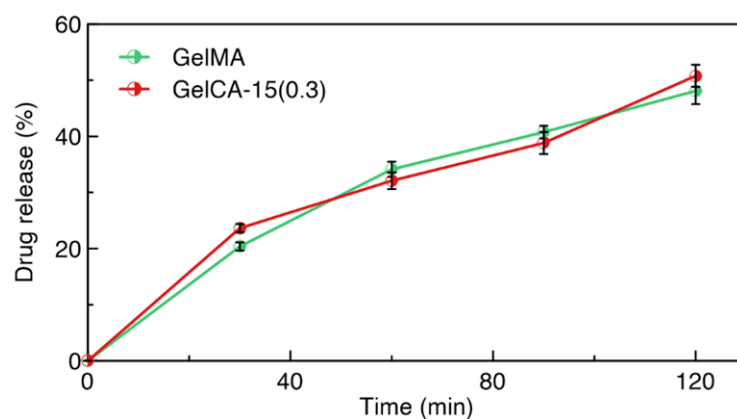


Figure S7.26. Release kinetics of a model drug (Rhodamine B) loaded into gelatin methacryloyl (GelMA) versus gelatin-caffeic acid oligomer conjugates crosslinked at 0.3 %w/v NaIO_4 solution, GelCA-15(0.3). The data are presented as means $\pm$ SD.

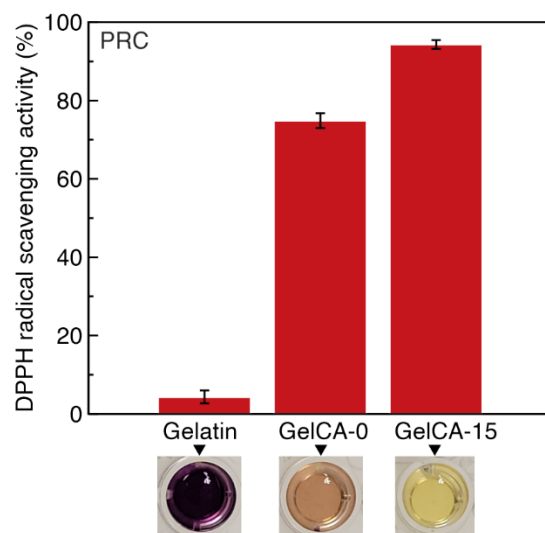
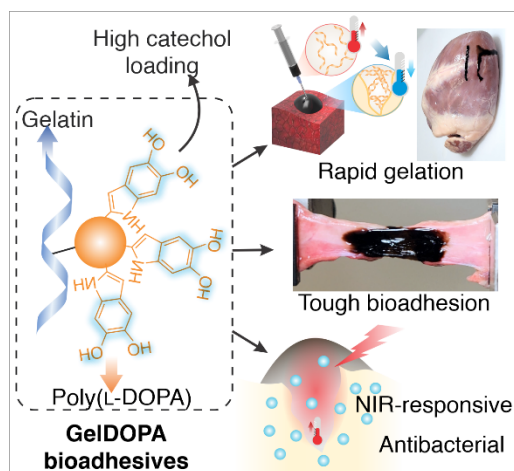


Figure S7.27. Antioxidant activity of porcine (PRC) gelatin-caffeic acid (GelCA-0) and gelatin-caffeic acid oligomer (GelCA-15) conjugates. The photos show the color changes of 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent as a result of incubation with modified gelatin solutions. The data are presented as means $\pm$ SD.

CHAPTER 8: POLY-CATECHOLIC FUNCTIONALIZATION OF BIOMOLECULES FOR RAPID GELATION, ROBUST INJECTABLE BIOADHESION, AND NEAR-INFRARED-RESPONSIVENESS



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Abstract

Mussel-inspired catechol-functionalization of degradable natural biomaterials such as gelatin has garnered significant interest as an approach to achieve bioadhesion for sutureless wound closure. However, conjugation capacity in standard coupling reactions such as carbodiimide chemistry is limited by low yield and lack of abundant conjugation

sites. Here, we propose a simple oxidative polymerization step before conjugation of catechol-carrying molecules (*i.e.*, 3,4-dihydroxy-L-phenylalanine, L-DOPA) as a potential approach to amplify catechol function in bioadhesion of natural biomaterials such as gelatin. Solutions of gelatin modified with poly(L-DOPA) moieties (GelDOPA) are characterized by increased viscosity, providing better control on double-curved tissue surfaces, and enabling fast thermal gelation *in situ* upon exposure to the tissue surface at room temperature compared to those of L-DOPA-conjugated gelatin. Physical hydrogels treated topically with low concentrations of NaIO₄ solutions were crosslinked on-demand *via* through-thickness diffusion. Poly(L-DOPA) conjugates enhance crosslinking density compared to L-DOPA-conjugated gelatin, resulting in lower swelling and enhanced cohesion in physiological conditions. Together with cohesion, more robust bioadhesive properties at body temperature were achieved by poly(L-DOPA) conjugates, exceeding those of commercial sealants. Further, poly(L-DOPA) motifs introduced photothermal responsive properties *via* near-infrared (NIR) irradiation for controlled drug release and potential applications in photothermal therapy. The above functionalities along with antibacterial activity render the proposed approach an effective biomaterial design strategy for wound closure applications.

8.1 Introduction

Bioadhesive hydrogels in various forms of patches^{13,191,193} and injectables⁶⁴ have leveraged sutureless wound closure and have served as wound dressings as well as smart bioelectronic interfaces.^{192,679} Among those bioadhesives, injectable tissue sealants are proven as effective alternatives or adjuncts to traditional wound closure techniques such as suturing and stapling in a wide range of surgical procedures.^{7-8,680} Clinical studies have

associated injectable sealants with lower serum formation following breast mastectomy,⁶⁸¹ faster hemostasis in vascular replacement surgery,^{6,2} shorter operation times in peripheral nerve reconstruction,⁶⁸² and lower risk of alveolar air leak following pulmonary resection surgery.⁶⁸³ Fibrin-based sealants are among the most common commercial wound sealing products because of their hemostatic properties.^{88,684-685} However, human-derived fibrin glues suffer from the risk of viral transmission and high cost.⁶⁴⁹ Further, failure of fibrin sealants, *e.g.*, in the prevention of postoperative cerebrospinal fluid leakage, has been reported due to the insufficient tissue adhesion strength of fibrin (*i.e.*, ~12 mmHg whereas the intraspinal pressure can reach up to ~45 mmHg).⁶⁸⁶ Hence, a safe, economical, and robust bioadhesive material is in high demand for wound closure in surgical settings.^{72,96}

Gelatin is a low-cost, well-accessible extracellular matrix-mimicking backbone, which offers superior biocompatibility and biodegradability.^{72,208,280,520} Multiple commercial wound care products are composed of gelatin components in whole (such as Gelfoam® sponge patches) or in part (such as injectable Floseal® sealants).⁷ These products generally lack bioadhesive functionality and lose their cohesion at the elevated temperatures of physiological conditions.²⁷⁶ Furthermore, injectable solutions of gelatin are characterized by low viscosity, rendering them difficult to control on double-curved tissue surfaces.¹ Hence, rheological characteristics of gelatin-based bioadhesives require tuning for facile clinical use. Many attempts have been made to introduce adhesive properties to gelatin.^{40-42,63,612} For instance, gelatin methacryloyl (GelMA)-based bioadhesives can be photopolymerized *in situ* to seal wounds through the bioadhesion driven by Michael addition reaction with nucleophilic groups on the tissue surface.^{4,59-60} Adhesive failure in GelMA hydrogels, however, stems from the lack of hydrogel compliance with deformations of soft tissue

substrates (especially when applied to dynamic tissues such as lungs and the heart).⁴¹ Inspired by sea mussels, catechol-functionalization of biomolecules has been extensively explored as another potential approach to introduce wet-adhesive properties to biocompatible materials.^{2,9,24,687} Integration of free catechol-capped molecules within pre-crosslinked GelMA hydrogels have resulted in high stretchability and bioadhesion to bioadhesive patches.^{24,41} However, this approach resulted in a detrimental impact on adhesion strength when bioadhesive crosslinked *in situ* as an injectable sealant.²⁴ Additionally, the burst release of catecholic moieties due to the insufficient interaction with the hydrogel polymer chains led to the increased risk of cytotoxicity.²⁴ Hence, catechol modification of natural biomolecules has been pursued as a potential means to endow adhesion to injectable biomaterials for surgical sealants⁴⁶ capable of integration with minimally invasive surgeries.⁶⁴

Conjugation of catechol hydroxyphenols is mostly performed *via* carbodiimide coupling (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC/*N*-hydroxysuccinimide, NHS) amidation reactions.^{179,170} One trivial drawback associated with this approach is the low conjugation efficacy, which is exacerbated by the limited availability of conjugation sites on the natural biomolecules such as gelatin.⁴⁷ For instance, the maximum degree of substitution for a gelatin-dopamine bioadhesive was $< \sim 10\%$.⁴⁷ This poor conjugation capacity hinders the effective function of catechol motifs.²⁴³ In terms of bioadhesion, gelatin-dopamine conjugates (crosslinked through metal coordination) have comparable adhesion strength to that of unmodified gelatin.²⁷⁶ In another example, GelMA-dopamine patches showed burst pressure values below that of arterial blood pressure.⁶³ Low amounts of catechol conjugates can also lead to weak oxidative crosslinking, which results in poor

cohesion and undesirably high swelling ratios (*e.g.*, on the order of 100×).²⁷⁶ Therefore, alternative strategies for efficient incorporation of catechol groups are in demand to enable mussel-inspired gelatin-based bioadhesives for clinical applications.

Here, we propose oxidative polymerization of catecholic small molecules prior to chemical coupling reactions to augment the loading capacity of catechol motifs on the natural biomacromolecules, thereby strengthening their effects for more robust bioadhesion. To test this hypothesis, a catechol-carrying amino acid (*i.e.*, 3,4-dihydroxy-L-phenylalanine, L-DOPA, a drug approved by The U.S. Food and Drug Administration to treat Parkinson's disease)²⁹¹ is first oxidatively polymerized to poly(L-DOPA) prior to EDC/NHS coupling with gelatin (as an attractive candidate for sealing internal wounds), enabling an array of catechol groups to be grafted per carbodiimide reaction (Figure 8.1a). Injectable solutions of gelatin modified with L-DOPA derivatives (GelDOPA) are then tuned in terms of their thermosensitive rheological properties by means of L-DOPA polymerization before conjugation to (i) facilitate pre-gel control when applying onto the double-curved tissue surfaces, and (ii) to enable their fast thermal gelation upon contact with the tissues (Figure 8.1b). Physical gels are oxidatively crosslinked *in situ* on-demand to provide wet stability in physiological conditions. Here, the roles of larger catechol contents in enhancing cohesion and minimizing hydrogel swelling are studied. Subsequently, bioadhesion capacity of GelDOPA hydrogels are examined through *in vitro* and *ex vivo* studies. Further, antimicrobial properties and thermal responsiveness of GelDOPA hydrogels to near-infrared light are demonstrated for potential drug delivery and photothermal therapy applications. Finally, biocompatibility of GelDOPA bioadhesives are tested *in vivo* and *in vitro* to assess their suitability for sutureless wound closure in surgical settings.

8.2 Results and Discussion

8.2.1 Synthesis of bioadhesives

As shown in Figure 8.1a, L-DOPA was first oxidized by NaIO₄ oxidizers to form poly(L-DOPA) moieties. As opposed to dopamine wherein conjugation sites (*i.e.*, primary amine groups) are consumed (to secondary amine groups) in their oxidatively polymerized polydopamine products,^{688,631} carboxyl conjugation sites of L-DOPA are preserved in each repeating unit of poly(L-DOPA). The abundant carboxyl groups present on poly(L-DOPA) moieties could favor carbodiimide coupling reactions. Polymerization kinetics of L-DOPA was studied based on variations of UV-vis spectra in response to oxidation with NaIO₄ (Figure S8.1a,b and Figure 8.1c). Pristine L-DOPA exhibited two characteristic peaks at *ca.* 250 and 290 nm, which are attributed to π - π^* and L_a - L_b transitions in phenolic catecholamines, respectively.^{689,690} Upon addition of NaIO₄, the peak at 290 nm immediately intensified and red-shifted to $\sim$ 300 nm while a broad shoulder in the visible region ($\sim$ 400 nm) appeared with the formation of dopachrome intermediates (possibly due to n - π^* transitions in the carbonyl groups of *ortho*-quinones).^{689,691} This broad peak then decayed rapidly, while the absorbance gradually increased in the 300-350 nm range, suggesting further transformation of dopachromes to indole derivatives (λ_{max} =324 nm).^{689,691} The gradual increase in absorbance at 324 nm reached a plateau after $\sim$ 2 h, based on which we determined the polymerization reaction time of L-DOPA (Figure 8.1d). This plateau was found to be higher at larger concentrations of NaIO₄, which we attribute to the formation of larger molecular weight poly(L-DOPA) products. This result is in agreement with size-exclusion chromatography (SEC) tests where the samples polymerized at higher NaIO₄

concentrations peak-shifted to higher retention times, suggesting poly(L-DOPA) with larger molecular weights. Excessive NaIO_4 content (>75 mg/10 ml), however, led to notable phase separation (Figure 8.1c), based on which we limited the maximum NaIO_4 concentration in subsequent experiments to prevent insolubility in bioadhesives. Following oxidative polymerization in 1 M HCl, poly(L-DOPA) nanoparticles of ~ 8 nm average size were formed *e.g.*, at 15 mg/10 ml NaIO_4 concentration according to the results of Figure 8.1e. These particles could grow up to *ca.* 200 nm by the end of the coupling reaction conducted in pH ~ 6 -7.

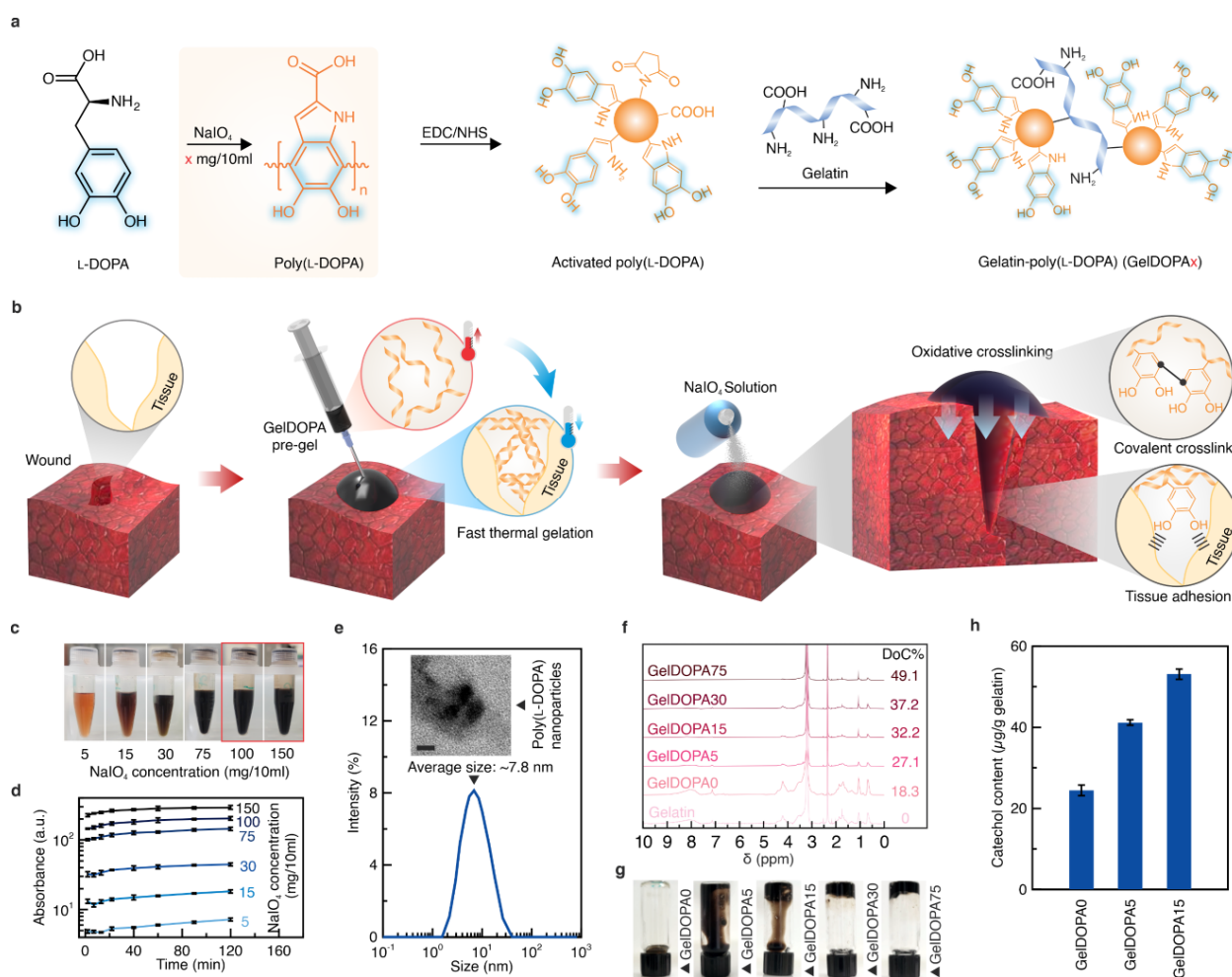


Figure 8.1. Design and characterization of injectable bioadhesives based on gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Synthesis route of GelDOPA_x

bioadhesives, where x represents the concentration of polymerizing NaIO₄ agent in mg/10 ml. Oxidation of L-DOPA prior to carbodiimide coupling (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC/*N*-hydroxysuccinimide, NHS), results in poly(L-DOPA) conjugates, which can reinforce catechol effects compared to unmodified L-DOPA conjugates when grafted to gelatin. (b) Application of GelDOPA hydrogels for surgical wound closure involves rapid thermal gelation enabled by poly(L-DOPA) conjugates, followed by topical treatment with crosslinking solutions such as NaIO₄ for diffusive crosslinking and thereby aqueous stability. (c) Solutions of L-DOPA polymerized with different amounts of NaIO₄ for 2 h. Visible phase separation occurred at NaIO₄ concentrations above 75 mg/10 ml highlighted in red. (d) Oxidative polymerization kinetics of L-DOPA in terms of absorbance at 324 nm measured by UV-vis spectroscopy. Data are shown as mean ± SD (n=3). (e) Results of dynamic light scattering (DLS) for poly(L-DOPA) nanoparticle size distribution of L-DOPA samples polymerized at 15 mg/10 ml NaIO₄. Inset shows transmission electron microscopy (TEM) images of poly(L-DOPA) nanoparticle aggregates (scale bar = 10 nm). (f) Proton nuclear magnetic resonance (¹H-NMR) results, showing that a higher degree of amine conversion (DoC) associated with GelDOPA products synthesized *via* more strongly oxidized L-DOPA. (g) Vial tilt assay for 20 %w/v solutions of GelDOPA bioadhesives dissolved at 80 °C. Lack of injectability was observed for GelDOPA solutions with poly(L-DOPA) conjugates produced at NaIO₄ concentrations of larger than 15 mg/10 ml. (h) Effect of pre-conjugation oxidation of L-DOPA on the amount of grafted catechol to gelatin for injectable GelDOPA bioadhesives. Data are shown as mean ± SD (n=3).

Covalent binding of L-DOPA derivatives to gelatin (labeled as GelDOPAx, where x represents the concentration of NaIO₄ used to polymerize L-DOPA in mg/10 ml) was studied by proton nuclear magnetic resonance (¹H-NMR), as shown in Figure 8.1f. It was found that more amine groups were consumed when L-DOPA was polymerized with higher concentrations of NaIO₄. The enhanced degree of amine conversion (DoC) is assigned to the synergistic contributions of (i) carbodiimide coupling reactions facilitated by the presence of multiple carboxyl conjugation sites in polymers of L-DOPA, (ii) Schiff base formation of oxidized quinone species in poly(L-DOPA) with gelatin, and (iii) homocoupling of gelatin.

Note that GelDOPA samples wherein L-DOPA polymerized at NaIO₄ concentrations of >15 mg/10 ml (*i.e.*, GelDOPA30 and GelDOPA75) did not dissolve in water (Figure 8.1g) and therefore, they were not studied further due to lack of injectability. Conjugation of L-DOPA derivatives was examined through UV-vis spectra of GelDOPA solutions (Figure S8.2a). The increase in absorbance of GelDOPA samples was in accord with their corresponding conjugated L-DOPA derivatives. The significantly higher absorbance of GelDOPA synthesized at higher NaIO₄ concentrations signifies larger molecular weight poly(L-DOPA) contents conjugated to the gelatin backbone. Correspondingly, catechol content more than doubled in GelDOPA15 compared to GelDOPA0 samples according to the results of Arnow assays (Figure 8.1h). This improvement was even more prominent compared to gelatin-dopamine conjugates reported in the literature (*i.e.*, >4×),⁴⁶ possibly because, as opposed to L-DOPA, dopamine is not able to homocouple during EDC/NHS activation, which would otherwise reduce its conjugation efficacy.

According to SEC test results, the molecular weight of gelatin increased with conjugation of L-DOPA-based components (Figure S8.1c) partly due to grafted masses of L-DOPA derivatives. This increase was most significant for GelDOPA15, supporting the idea that Schiff base reactions through the conjugated quinones crosslink gelatin chains to some extent. The larger molecular weight of GelDOPA0 compared to gelatin also indicates homocoupling of gelatin in the presence of EDC/NHS reagents. Positive charges can play synergistic roles with catechol groups in bioadhesion of mussel foot proteins.^{692,693} We note here that the number of primary amine groups in GelDOPA15 was found to be ~25% larger than under other conditions (see Figure S8.2b), which thereby enhances bioadhesion of GelDOPA15 *via* electrostatic interactions with negatively charged protein residues on tissue

surfaces. The hydrodynamic size of GelDOPA15 was characterized *via* dynamic light scattering (DLS) in water solution as shown in Figure S8.2c. The results suggest an average size of 124 nm, which is ~ 15 times larger than the average size of poly(L-DOPA) measured in Figure 8.1c. A slight peak around 20 nm can be attributed to the free poly(L-DOPA) present in the solution.

8.2.2 Rheological analyses of pre-gel solutions

Rheological properties of gelatin-based bioadhesive solutions were optimized for facile injectability and applicability on wound surfaces. To this end, dynamic interactions introduced by L-DOPA-based motifs (shown in Figure 8.2a) enabled tuning of gelatin's viscosity and intrinsic thermosensitivity. Aqueous solutions of gelatin at 20 %w/v concentration in the sol state (at 37 °C) are low-viscous liquids (Figure 8.2b); they are thereby difficult to control on double-curved and complex-shaped tissue surfaces (due to dripping under gravity). Likewise, GelDOPA0 and GelDOPA5 showed similar orders of viscosity to gelatin. Further polymerization of L-DOPA in GelDOPA15, however, led to a significant rise in viscosity, which is desired for more controllable flow of the pre-gel solution. Moreover, the decreasing trend of viscosity with shear rate in GelDOPA15 demonstrates shear-thinning behavior, rendering its pre-gel solutions injectable.

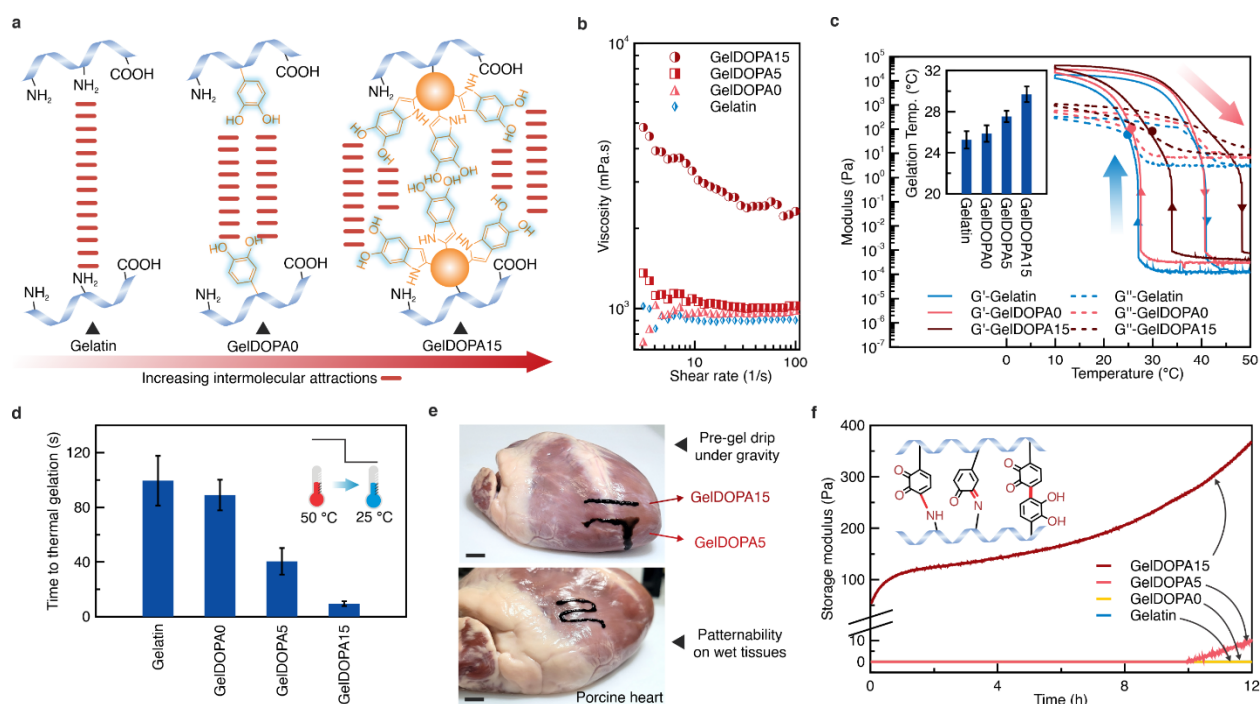


Figure 8.2. Rheological properties of 20 %w/v solutions of gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Schematic of increase in intermolecular interactions with pre-conjugation oxidative polymerization of L-DOPA in GelDOPAx samples where x is concentration of NaIO_4 agent in mg/10 ml used to polymerize L-DOPA. (b) Viscosity-shear rate characteristics of GelDOPA bioadhesives. The greater extent of L-DOPA polymerization was associated with more viscous bioadhesive solutions. Data are shown as mean $\pm$ SD (n=3). (c) Reversible thermal sol-gel transition of GelDOPA bioadhesives studied across 10-50 °C. The inset shows the measured gelation temperature of GelDOPA solutions during cooling. (d) Gelation time of GelDOPA solutions in response to the temperature step from 50 °C to 25 °C. Rapid physical gelation was obtained for GelDOPA15 upon exposure to room temperature. (e) Demonstration of better pre-gel controllability and patternability of GelDOPA15 solutions on porcine heart tissues (synthesized at high L-DOPA oxidation) compared to GelDOPA5 (synthesized at low L-DOPA oxidation). Scale bar = 1 cm. (f) Self-gelation of GelDOPA solutions at 37 °C in post-operation timescales due to the covalent interactions triggered by oxidized catecholic moieties through mechanisms shown in the inset.

Thermosensitive physical gelation of gelatin was tuned by polymerization of L-DOPA prior to conjugation, to enable fast thermal gelation *in situ* after application on tissue. As shown in Figure 8.2c, phase transition temperatures during cooling and heating increased

with the polymerization of L-DOPA conjugates. This effect is primarily attributed to: (i) higher density of junction zones due to hydrogen bonds introduced *via* catechol compounds and (ii) increased molecular weight of the gelatin backbone (Figure S8.2d). The stronger interchain associations *via* higher molecular weight poly(L-DOPA) conjugates are further supported by the higher modulus of GelDOPA15 compared to gelatin solution at 37 °C (see Figure S8.3a). We studied the time it takes to form a physical hydrogel in response to a sharp temperature change from 50 °C to room temperature, *i.e.*, 25 °C. According to the results shown in Figure S8.3b,c and Figure 8.2d, the physical gelation time of ~100 s for gelatin was reduced to ~10 s for GelDOPA15. The accelerated physical gelation along with enhanced viscosity of GelDOPA15 facilitated its application on non-flat tissue surfaces, as demonstrated in Figure 8.2e. While GelDOPA5 dripped under gravity, GelDOPA15 solution formed a physical gel rapidly after exposure to room temperature; hence, the pre-gel solution could be manually patterned on the inferior wall of porcine myocardial tissue in a controllable manner. Rapid gelation of bioadhesives is also critical when it comes to bleeding wounds;² however, a sufficient period is nonetheless required before polymer solidification to allow their injection onto tissue. In this case, the ~10 s gelation time provided by GelDOPA15 is sufficient to enable bioadhesive applications in surgical settings.

We monitored the rheological evolution of bioadhesive solutions over a longer period, 12 h, at 37 °C under dry conditions (Figure 8.2f). As opposed to gelatin and GelDOPA0, which remained in the sol state, GelDOPA5 approached a gelation point at the end of the test period. Stronger self-association was observed in GelDOPA15 samples according to the continuous increases in storage modulus. We attribute the spontaneous crosslinking of GelDOPA to the Schiff base and Michael addition reactions *via* quinone

moieties in oxidatively polymerized L-DOPA conjugates. Self-gelation of hydrogels enhances their cohesion under physiological conditions on relevant time scales after surgical implantation. However, we note that self-gelation was only seen in concentrated hydrogels (tested under dry conditions). Thermal hydrogels of GelDOPA could still dissociate when maintained in wet media at 37 °C overnight. Hence, a crosslinking step was necessary to prevent hydrogels from dilution and for wet stability *in vivo*.

8.2.3 Crosslinking of bioadhesives

As highlighted above, covalent crosslinking was required following *in situ* thermal gelation to provide wet stability and retention after surgical implantation. For this purpose, we performed diffusive crosslinking by treating physical hydrogels with crosslinking solutions (*i.e.*, Fe^{3+} , BO_4^- , IO_4^-) for 5 min. Crosslinked hydrogels are labeled as GelDOPAx-y, where y denotes the concentration of crosslinking solution in mM. Diffusive crosslinking enabled on-demand covalent bonding of a topical layer on hydrogel surfaces, preventing their dissociation in aqueous media. This method is preferred over direct mixing because (i) uniform mixture is a challenge in viscous solutions of GelDOPA and requires pre-gel dilution, (ii) catechol groups at the tissue interface are non-selectively consumed for crosslinking, limiting their tissue adhesion capacity, and (iii) crosslinking is time sensitive, leading to injectability issues such as clogging. Crosslinking of GelDOPA hydrogels was assessed in terms of hydrogel stability in wet conditions and release at 37 °C for different crosslinking solutions of IO_4^- , BO_4^- , and Fe^{3+} at concentrations between 2 and 100 mM (Figure 8.3a,b). All GelDOPA0 hydrogels dissociated in aqueous media, whereas gelatin-poly(L-DOPA) conjugates (treated with NaIO_4 solutions at concentrations > 10 mM) remained integral. We attribute this difference to the larger amounts of catechol groups in GelDOPA15 and

GelDOPA5 compared to GelDOPA0, which agrees with the results of the Arrow assay (Figure 8.1h). The data also demonstrated the strongest crosslinking provided by IO_4^- , compared to BO_4^- and Fe^{3+} , according to the low release (higher stability) in hydrogels crosslinked with IO_4^- .

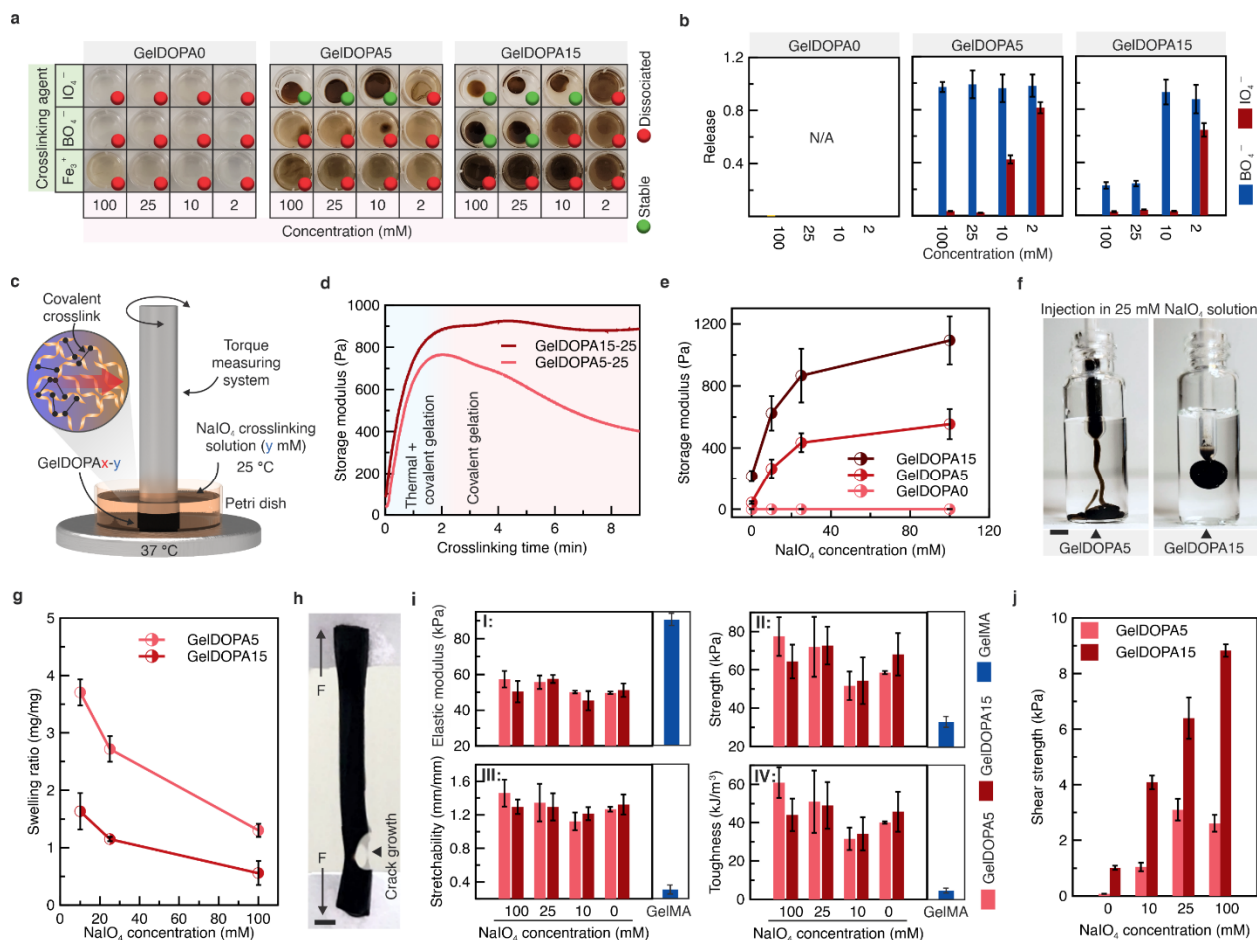


Figure 8.3. Crosslinking, physical and mechanical properties of gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Crosslinking assessment of different crosslinking agents and concentrations in terms of aqueous stability in GelDOPAx hydrogels (x indicates the concentration of NaIO $_4$ used to polymerize L-DOPA in mg/10 ml) after 24 h incubation at 37 °C. (b) Release of GelDOPA components from their crosslinked hydrogels. Data are shown as mean $\pm$ SD (n=3). (c) Rheological experimental setup used for evaluation of crosslinking kinetics in GelDOPAx-y samples (y indicates the mM concentration of NaIO $_4$ solution used to crosslink GelDOPAx). (d) Crosslinking profiles of GelDOPA hydrogels upon exposure to 25 mM NaIO $_4$ crosslinking solution. (e) Storage modulus of GelDOPA hydrogels after 5 min peripheral diffusive

crosslinking using different concentrations of NaIO_4 . Data are shown as mean $\pm$ SD (n=3). (f) Demonstration of rapid covalent crosslinking of GeldOPA15 solution when injection in 25 mM NaIO_4 solution compared to its GeldOPA5 counterpart with less-oxidized L-DOPA conjugates (scale bar = 2 mm). (g) Swelling ratio of GeldOPA hydrogels crosslinked at different concentrations of NaIO_4 solution after 8 days incubation in human blood serum at 37 °C. Data are shown as mean $\pm$ SD (n=3). (h) Failure mechanism and crack propagation in tensile tests for GeldOPA15-25 samples (scale bar = 3 mm). (i) Tensile mechanical properties of GeldOPA hydrogels involving I: elastic modulus, II: strength, III: stretchability and IV: toughness, in comparison with gelatin methacryloyl (GelMA) controls. Data are shown as mean $\pm$ SD (n=3). (j) Maximum shear strength of GeldOPA hydrogels at 37 °C measured *via* rheological strain sweep tests. Data are shown as mean $\pm$ SD (n=3).

The crosslinking kinetics of wet-stable hydrogels (*i.e.*, GeldOPA5 and GeldOPA15 crosslinked using NaIO_4 solutions at concentrations > 10 mM) were further studied *via* rheological responses of pre-gel solutions to peripheral diffusion of crosslinking solutions at 37 °C (Figure 8.3c,d). We attribute initial increases in storage modulus upon the addition of NaIO_4 to thermal gelatin (cooling because of contact with crosslinking solutions) and oxidative crosslinking of GeldOPA hydrogels. The increasing trend of storage modulus was reversed for GeldOPA5 samples by increasing the temperature back to 37 °C, whereas GeldOPA15 either reached a plateau (25 and 10 mM NaIO_4) or increasingly grew (100 mM NaIO_4), suggesting stronger covalent crosslinking in GeldOPA15 samples. Accordingly, greater values of storage modulus were attained with further polymerization of L-DOPA conjugates and higher concentrations of NaIO_4 (Figure 8.3e). Rapid crosslinking of samples with highly oxidized L-DOPA conjugates was shown in Figure 8.3f *via* injection in NaIO_4 crosslinking solutions. As opposed to GeldOPA5, which precipitated after injection, rapid crosslinking occurred when GeldOPA15 was injected in a 25 mM NaIO_4 solution.

Post-implantation swelling of hydrogels is a challenge associated with injectable

bioadhesives, which can degrade mechanical strength, adhesion, and cause pressure on surrounding tissues. Swelling is also another measure of crosslinking density in hydrogels and is characterized for GelDOPA in Figure S8.4. Here, GelDOPA15 reached a steady swelling level after ~ 16 h, whereas no plateau was seen for GelDOPA5 after 4 days. The samples crosslinked using NaIO_4 solution lower than 25 mM never reached a plateau, as well. As shown in Figure 8.3g, swelling of GelDOPA15 hydrogels crosslinked at *e.g.*, 25 mM NaIO_4 was approximately half that of GelDOPA5 after 8 days due to stronger intermolecular associations.

8.2.4 Mechanical properties of crosslinked bioadhesives

Tensile properties of catechol-modified gelatin (GelDOPA hydrogels) are compared to methacryloyl-modified gelatin (GelMA hydrogels) Figure 8.3h,i. Lower elastic modulus of GelDOPA hydrogels relative to GelMA suggests enhanced compliance (Figure 8.3iI). Softer bioadhesives can deform more easily and their deformation can absorb more mechanical energy from dynamic tissue substrates, such as lungs. Hence, less energy is left to be dissipated by interfacial forces. Further, tensile strength and stretchability of GelDOPA hydrogels (*e.g.*, GelDOPA15 crosslinked at 25 mM NaIO_4 concentration), were improved by $\sim 2\times$ and $\sim 5\times$ compared to GelMA, respectively as shown in Figure 8.3iII,III. These improvements led to significant increases in their toughness (Figure 8.3iIV) compared to GelMA, due to the synergistic contributions of intermolecular dynamic interactions (*i.e.*, hydrogen bonding and hydrophobic interactions) and covalent bonding in GelDOPA hydrogels. We did not find any significant differences between the tensile properties of GelDOPA5 *versus* GelDOPA15, as well as between different NaIO_4 concentrations, most likely due to physical gelation of hydrogels under our testing conditions at room temperature.

Thus, oscillatory strain sweep tests were performed on crosslinked hydrogels at the controlled temperature of 37 °C to understand the effects of modification with L-DOPA-based moieties on mechanical properties in physiological conditions (Figure S8.5). As seen in Figure 8.3j, the maximum shear strength where a network rupture occurs is significantly larger for GelDOPA15 (~2×) compared to GelDOPA5. In both groups, increasing NaIO₄ crosslinking from 25 to 100 mM had negligible impact on shear responses; however, a significant drop in shear stress was observed for 10 mM NaIO₄.

8.2.5 Bioadhesion performance

Different adhesion tests were conducted underwater to evaluate bioadhesion of GelDOPA hydrogels. A tack test at the controlled temperature of 37 °C demonstrated ~3× larger adhesion strength in GelDOPA15 compared to GelDOPA5 (see Figure 8.4a) due to the lack of sufficient cohesion, as shown in strain sweep test results of Figure 8.3j. As shown in Figure 8.4b, the results of *in vitro* burst pressure tests suggested considerably larger (~3-5×) adhesion strength in GelDOPA hydrogels compared to commercial bioadhesives. We attribute the drops in burst pressure for the group of hydrogels crosslinked at 10 mM (compared to 25 and 100 mM) to their poor cohesion, as discussed for Figure S8.5. In addition, a cyclic tack test on the physical hydrogels at 37 °C (representative of the hydrogel state at the interface with tissue) suggested little adhesion for all samples, because of their complete phase transition to the sol state, whereas GelDOPA15 showed durable adhesion forces across 50 cycles (Figure S8.6). Hence, we infer that the enhanced adhesion of GelDOPA15 is primarily due to better cohesion at 37 °C *via* strengthened internal association, and because of the large amounts of adhesive catechol groups in GelDOPA15. Note that adhesion properties of samples crosslinked with 100 mM NaIO₄ solution shown in Figure

8.4b did not show any significant improvement relative to 25 mM NaIO₄ in both burst pressure and tack tests. We conclude that GelDOPA15 represents the most favorable condition due to its robust bioadhesion (Figure 8.4b) and mechanical properties (Figure 8.3j), minimal swelling (Figure 8.3g), fast thermal gelation (Figure 8.2d), and the minimized concentration of NaIO₄ solution that enables its wet-stability *in vivo*, i.e., 25 mM (Figure 8.3b). Hence, subsequent characterization focused on this condition for further assessment of hydrogel functionality.

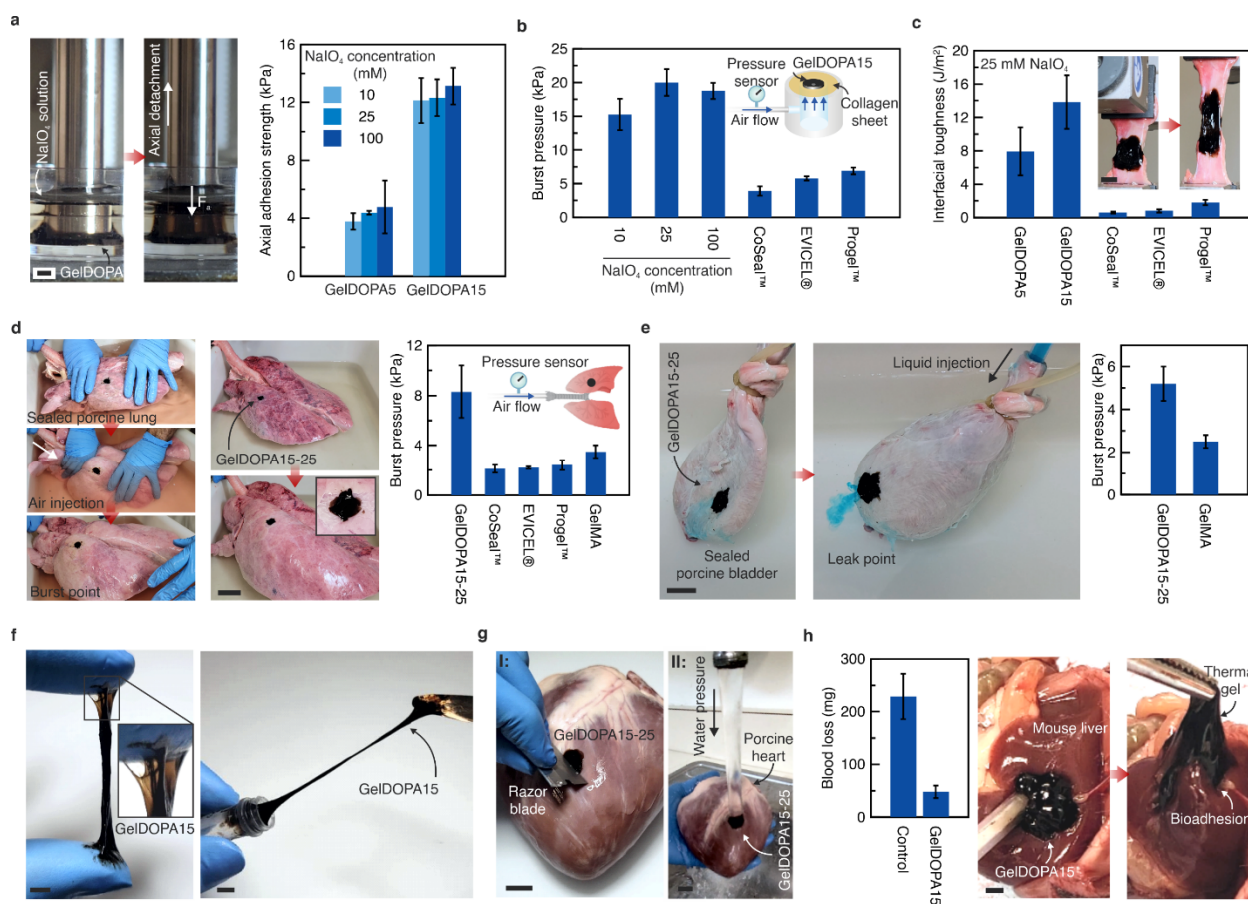


Figure 8.4. Bioadhesive properties of gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Adhesion of GelDOPAx (x shows mg/10 ml concentration of NaIO₄ used to polymerize L-DOPA during synthesis of GelDOPA) in axial mode (F_a) measured *via* tack test (scale bar = 2 mm). Data are shown as mean $\pm$ SD (n=3). (b) *In vitro* burst pressure test showing adhesion strength to collagen sheet substrates. Data are shown as mean $\pm$ SD (n=3). (c) Wound closure tests

were performed on porcine lung tissue substrates (scale bar = 5 mm). Data are shown as mean $\pm$ SD (n=4). *Ex vivo* burst pressure tests were performed on (d) porcine lung (scale bar = 5 cm) and (e) bladder tissues (scale bar = 2 cm) by injection of oxygen gas and liquid dye, respectively. Here, y in GelDOPAx-y is the mM concentration of NaIO₄ solution used to crosslink GelDOPAx. The optimized condition of GelDOPA15-25 showed significantly improved bioadhesion performance *in vitro* and *ex vivo* compared to commercial sealants. Data are shown as mean $\pm$ SD (n=3). (f) Demonstration of GelDOPA15 tackiness to gloves and metal surface of spatula (scale bar = 5 mm). (g) I: The GelDOPA bioadhesives did not detach when rubbed by a razor blade (scale bar = 2 cm). II: Resistance of GelDOPA15-25 hydrogels crosslinked on porcine heart tissue *in situ* to harsh tap water pressure (~37 °C) without failure for more than 1 min. (h) *In vivo* mouse liver bleeding model showing the capacity of GelDOPA bioadhesives to minimize bleeding due to the rapid thermal gelation upon cooling at the application conditions and strong adhesion to tissue substrates (scale bar = 3 mm). Data are shown as mean $\pm$ SD (n=3).

Bioadhesion of GelDOPA hydrogels was evaluated on different porcine tissues. A wound closure test performed on porcine lung tissues (Figure 8.4c) suggested enhanced interfacial toughness of GelDOPA hydrogels compared to commercial counterparts. Likewise, *ex vivo* burst pressure tests on porcine lung demonstrated approximately 2-4 $\times$ improvements relative to commercial bioadhesives (Figure 8.4d). Burst pressures of GelDOPA hydrogels exceeded those of GelMA samples, as GelMA failed to accommodate the underlying tissue expansion; whereas GelDOPA hydrogels stretch more easily, and thereby conformed better to expanding tissues (as discussed above for the results of elastic modulus presented in Figure 8.3II). In addition to this effect, the high content of catechol groups promoted adhesion further in GelDOPA hydrogels. The same explanation is responsible for the higher burst pressure of GelDOPA compared to GelMA in the porcine bladder, as shown in Figure 8.4e.

We demonstrated tackiness of pre-gel GelDOPA15 solutions in Figure 8.4f, with

stickiness to the gloves as well as to a spatula. The GelDOPA bioadhesives crosslinked on porcine heart tissue exhibited resistance against scratching using blades (see Figure 8.4gI). Furthermore, durability of hydrogels under harsh tap water pressure at 37 °C was demonstrated in Figure 8.4gII, where no detachment was observed under water flow for more than 2 min. Finally, we assessed the ability of hydrogels to seal bleeding wounds physically in a mouse liver bleeding model (Figure 8.4h). As compared to blank controls, the amount of bleeding was reduced up to ~75% due to rapid gelation of GelDOPA15 solutions upon application to the tissue surface.

Bioadhesion of GelDOPA hydrogels is attributed primarily to the interactions between polycatecholic moieties, *i.e.*, poly(L-DOPA) with tissue substrates. Those interactions include non-covalent bonds such as hydrogen bonding and π - π interactions.⁹ Stronger covalent interactions between oxidized quinone species on the grafted poly(L-DOPA) chains and nucleophilic groups on tissue surfaces including Michael addition and Schiff base formation are also another contributing factor to adhesion performance.^{9,24}

8.2.6 Antibacterial activity

Antibacterial properties in bioadhesives are essential for treatment of bacterial wounds and to prevent surgical site infection. Antibacterial activity of GelDOPA hydrogels were examined *in vitro* towards *E. coli* and *S. aureus* bacterial strains (Figure 8.5a,b). As shown in Figure 8.5b, a bacteriostasis ring appeared around the NaIO₄-crosslinked hydrogels (*i.e.*, GelDOPA15-25) against both bacterial strains whereas no antiseptic effect was seen in all other non-crosslinked pre-gel solutions (*i.e.*, gelatin, GelDOPA5, and GelDOPA15). These results suggest that release of the infused NaIO₄ and its crosslinking reaction byproducts (*i.e.*, iodate ions) back to the media can lead to bacterial cell disruption likely due to oxidation of

amino acid and nucleic acids on bacterial cell membranes.⁶⁹⁴ In addition, as shown in Figure 8.5c, a stronger antibacterial effect was obtained towards *S. aureus* compared to *E. coli*.

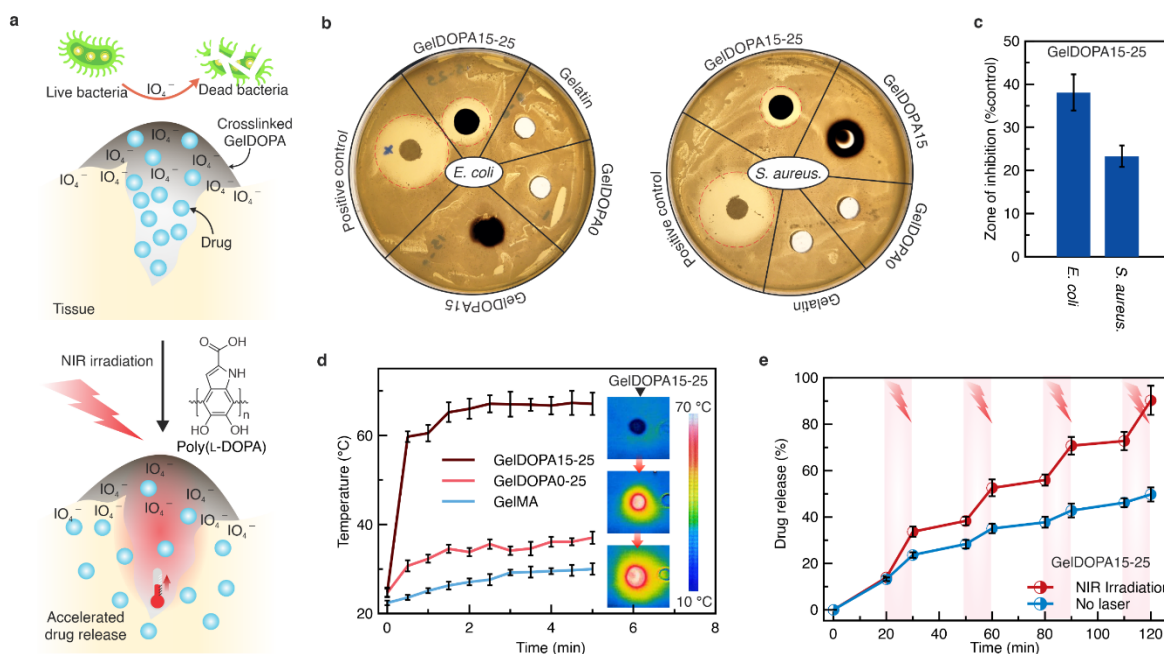


Figure 8.5. Antibacterial and near-infrared (NIR) responsiveness of gelatin-based bioadhesives modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Mechanism of antibacterial and NIR-responsiveness. The release of IO_4^- ions diffused when crosslinking the hydrogels provides antibacterial functionality. Furthermore, photothermal activity of poly(L-DOPA) moieties enables NIR-responsive temperature and thereby drug release control in GelDOPA hydrogels. (b) Antibacterial zone of inhibition for uncrosslinked (GelDOPAx, where x shows mg/10 ml concentration of NaIO_4 used to polymerize L-DOPA during synthesis of GelDOPA) and crosslinked (GelDOPAx-y, where y is the mM concentration of NaIO_4 solution used to crosslink GelDOPAx) samples. (c) Relative zone of inhibition in GelDOPA15-25 compared to positive control against *E. coli* and *S. aureus* strains. Data are shown as mean $\pm$ SD (n=3). (d) Temperature response of GelDOPA hydrogels in wet media to NIR irradiation. Insets show thermal camera photos taken from GelDOPA15-25 under NIR light. Data are shown as mean $\pm$ SD (n=3). (e) On-off drug release profile of GelDOPA15-25 under increments of NIR irradiation (Rhodamine B was used as a drug model). GelMA, gelatin methacryloyl. Data are shown as mean $\pm$ SD (n=3).

8.2.7 Photothermal responsiveness to near-infrared light

Photothermally responsive hydrogels can leverage on-demand functions for applications in antibiotic drug delivery and cancer thermo-chemotherapy (Figure 8.5a).⁶⁹⁵⁻⁶⁹⁸ Here, we evaluated the photothermal response of GelDOPA hydrogels in terms of temperature changes under near-infrared (NIR) light irradiation. As shown in Figure 8.5d, the temperature of GelDOPA15-25 samples in wet conditions rose rapidly to ~ 65 °C (after NIR irradiation for ~ 60 -90 s), exceeding the temperatures required for cancer cell death, *i.e.*, ~ 50 °C^{696-697,699-701} without damaging the healthy tissues.⁷⁰² The final temperatures of hydrogels were tunable by NIR light intensity, as shown in Figure S8.7a. Moreover, higher temperatures were reached when the hydrogels were irradiated with NIR in dry conditions (see Figure S8.7b), suggesting applicability for topical use. Note that we did not find any differences between photothermal activities of crosslinked GelDOPA15 and GelDOPA5 hydrogels. Although, significantly lower steady temperatures were measured for GelDOPA0 and gelatin/GelMA samples, indicating the role of pre-conjugation polymerization of L-DOPA in photothermal activity of hydrogels. With the aid of this effect, drug release capacity of the hydrogels could be controlled in an on-demand “off-on” manner, as demonstrated in Figure 8.5e. Here, using a drug model, (*i.e.*, Rhodamine B) drug release from GelDOPA15-25 hydrogels accelerated in time increments with NIR irradiation, whereas slower kinetics corresponded to drug release in the absence of NIR light. The NIR-triggered heating also enabled self-healing properties due to thermosensitivity of GelDOPA hydrogels. As seen in Figure S8.7c,d, the crosslinked hydrogels cut in the middle were merged after NIR irradiation for ~ 30 s and recovered their tensile mechanical properties.

Note that photothermal hydrogels have also been shown to be effective in wound-

healing applications.⁷⁰³ Local heating of wound beds can accelerate blood flow and thereby stimulate fibroblast proliferation and inhibit inflammation.⁷⁰⁴⁻⁷⁰⁶ Antibacterial effects due to local heating has also been reported, which can have synergistic effects for enhancing wound healing.^{14,707}

8.2.8 Biocompatibility

In vitro biocompatibility studies were performed to evaluate cytotoxicity of the crosslinked hydrogels. According to the results of live/dead assays (Figure 8.6a,b), viability of C2C12 cells co-cultured with the crosslinked GelDOPA15-25 hydrogels did not result in significant changes compared to the blank and GelMA (as a most widely used biomaterial in tissue engineering applications due to its superior biocompatibility)⁵⁹⁷ during the 5 day culture period. Cell viability remained above ~85% for all tested samples. In addition, according to results of PrestoBlue™ assay (Figure 8.6c), cell proliferation increased over 5 days of culture, where no significant differences in metabolic activity were observed between the groups of GelDOPA and GelMA hydrogels. Biocompatibility of GelDOPA hydrogels were studied subcutaneously *in vivo*, as shown in Figure 8.6d. The staining images suggest no obvious systemic cytotoxicity to the organs of the mice implanted with crosslinked GelDOPA hydrogels. The hydrogels after 1 week of implantation were harvested as shown in Figure S8.8. An accumulation of cells around and their infiltration within the degraded structure of hydrogels was dominant with no observable capsule formation. Both crosslinked (GelDOPA15-25) and non-crosslinked (GelDOPA15) samples were degraded where the degradation rate was more significant for the non-crosslinked hydrogels. The observed residues of GelDOPA15 hydrogels can be due to self-gelation of GelDOPA pre-gel solutions without NaIO₄ oxidative crosslinking as discussed for Figure 8.2f. This result

indicates that for applications where faster degradation rates are required, GelDOPA bioadhesives can be applied without the need for NaIO_4 treatment. Elimination of NaIO_4 crosslinking in these cases can further reduce the inflammatory response as per the lower accumulation of cells in GelDOPA15 compared to GelDOPA15-25.

The above data suggest that GelDOPA bioadhesives are biocompatible candidates to seal open wounds in surgical settings. The biocompatibility of GelDOPA hydrogels was favored by the minimal concentration of NaIO_4 agents required for crosslinking due to the amplified catechol content. The accelerated crosslinking kinetics enabled a diffusive treatment with NaIO_4 to provide adequate wet stability without the need for physical mixing. Such a mechanism will provide the flexibility for washing the residue crosslinking solution post-crosslinking while the diffused periodate ions that drove the di-catechol coupling reactions are decomposed into less harmful⁷⁰⁸ iodate ions⁷⁰⁹ within the hydrogel matrix.

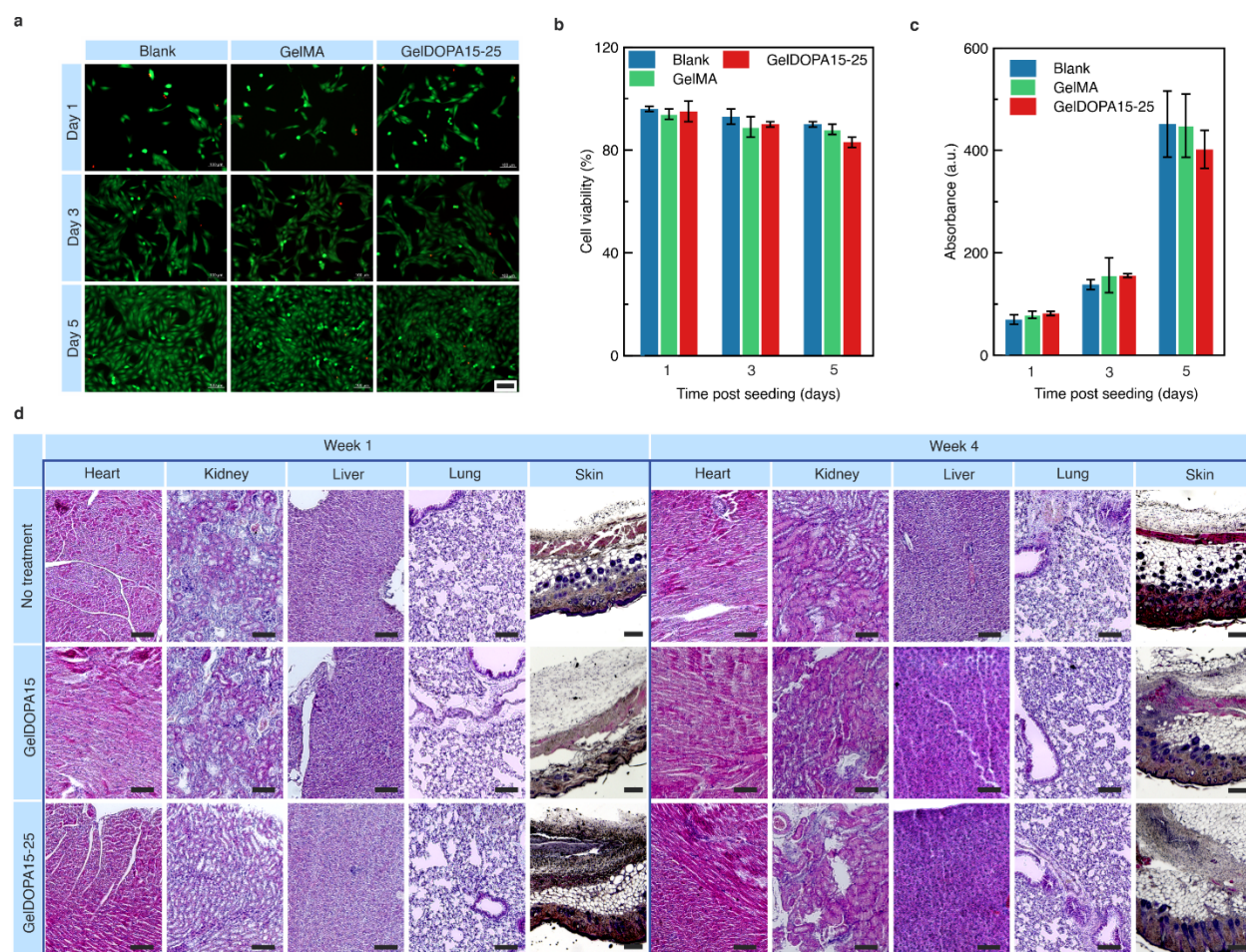


Figure 8.6. Biocompatibility analysis of gelatin-based bioadhesives modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Live/dead images of C2C12 mouse myoblasts cultured in presence of crosslinked GelDOPA hydrogels (GelDOPAx-y, where x and y are the concentration of NaIO₄ used for polymerization of L-DOPA during synthesis in mg/10 ml and for crosslinking of GelDOPA in mM, respectively) compared to gelatin methacryloyl (GelMA) hydrogels (scale bar = 100 μ m). (b) Cell viability of cells calculated from live/dead assays. Data are shown as mean $\pm$ SD (n=3). (c) Metabolic activity of cells in exposure to GelDOPA samples measured via Prestoblue™ assay. Data are shown as mean $\pm$ SD (n=3). (d) Hematoxylin and eosin (H&E) staining of organs extracted from mice subcutaneously implanted with GelDOPA hydrogels to evaluate biocompatibility *in vivo*.

8.3 Conclusions and Prospects

Biodegradable and injectable mussel-inspired bioadhesives are leveraged by catechol

modification of naturally derived biomacromolecules, such as gelatin proteins. One popular approach for catechol-functionalization of gelatin is carbodiimide coupling with catechol-carrying small molecules. However, this approach is limited by low conjugation efficacy, leading to weak catechol function. Here, we showed that oxidative polymerization of catechol-carrying molecules (*i.e.*, L-DOPA) prior to conjugation reaction is an effective solution to boost catechol content in gelatin-based bioadhesives. By means of strong intermolecular associations due to augmented content of catecholic moieties, pre-gel solutions of injectable bioadhesives were strengthened for better control, patternability, and applicability on curved tissue surfaces. In addition, the spontaneous thermal gelation of gelatin is accelerated to enable rapid sealing of wounds (gelation within ~ 10 s) upon exposure of the pre-gel to tissue surface temperature. The proposed process favors oxidative crosslinking kinetics, leading to higher crosslinking densities using lower concentrations of NaIO_4 solution. As a result, better cohesion with minimized swelling in wet conditions is possible in hydrogels crosslinked only with on-demand topical treatment with NaIO_4 (diffusive crosslinking), eliminating the need for complete mixing with NaIO_4 . Treatment with NaIO_4 also drives antibacterial properties *via* the release of crosslinking reaction byproducts. By virtue of enhanced cohesion, stronger adhesion is attained when catecholic moieties are polymerized prior to the coupling reaction, exceeding those of commercially available bioadhesives. In addition to favorable bioadhesive properties, polymerized L-DOPA components endow gelatin backbones with photothermal NIR-responsiveness for applications in controlled drug release and photothermal cancer therapy. Future studies on implementing the presented approach on other natural biomacromolecules, their tissue regenerative capacity, more in-depth pathological analysis, and long-term stability in

various storage conditions will explore the potential of pre-conjugation polymerization of catecholic moieties in bioadhesive materials design.

8.4 Experimental Methods

8.4.1 Materials

Gelatin from porcine skin type A, 3,4-dihydroxy-L-phenylalanine (L-DOPA), sodium periodate (NaIO_4), sodium nitrite (NaNO_2), sodium molybdate (Na_2MoO_4), sodium dodecyl sulfate (SDS), 2,4,6-trinitrobenzene sulfonic acid (TNBSA) solution 5 w/v% in DI water, hydrochloric acid (HCl) solution 1 M, and methacrylic anhydride (MA) were supplied by Sigma Aldrich, USA. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from TCI Chemicals, USA. Porcine bladder and lung tissues were provided by Sierra for Medical Science, USA. Sodium bicarbonate (NaHCO_3) was supplied by Fisher Scientific, USA. Human blood-derived plasma was purchased from Innovative Research, USA.

8.4.2 Synthesis

Gelatin was modified with polymerized L-DOPA to obtain L-DOPA modified gelatin (GelDOPA) bioadhesives. First, 340 mg L-DOPA was dissolved in 10 ml 1 M HCl solution in de-ionized (DI) water. Different batches of poly(L-DOPA) were synthesized by adding various amounts of NaIO_4 (*i.e.*, 0, 5, 15, 30, 75 mg) to the L-DOPA solution, and the reaction proceeded for 2 h under vigorous shaking. Then, the pH was increased to ~ 6 by adding NaOH. In each batch, 15 ml solution containing 160 mg EDC and 100 mg NHS was added to initiate activation of L-DOPA. After 2 h stirring, a solution of 1 g gelatin dissolved in 75 ml

was added to each group and coupling reaction proceeded for 24 h at room temperature. Subsequently, the reaction solutions were dialyzed against DI water for 3 days and the water was swapped three times a day. Final products were freeze-dried for 4 days to obtain dry GelDOPA. Samples were labeled as GelDOPAx where x indicates the mg NaIO_4 used for polymerizing L-DOPA.

Modification of gelatin with methacryloyl groups was performed as follows: 1 g gelatin was dissolved in 100 ml phosphate-buffered saline (PBS) solution (pH $\sim$ 7.4), followed by dropwise addition of 8 ml MA. The reaction was maintained at 60 °C for 3 h, and the reaction solution was transferred to dialysis membranes. The dialysis and freeze-drying processes were performed as described for synthesis of GelDOPA.

8.4.3 Hydrogel preparation

Pre-gel solutions of GelDOPA bioadhesives were prepared by dissolving dry GelDOPA in DI water at 20 w/v%. For dissolution, GelDOPA was gradually added to the pre-heated DI water stepwise in an Eppendorf tube involving cycles of vortex followed by mini-centrifugation and heating in 80 °C oven to remove bubbles until complete dissolution. The GelDOPA solutions were pipetted using a 100 μm positive-displacement pipette (Pos-D™, USA) and left at room temperature for 45-60 s for thermal gelation. To form a topical covalent crosslink layer, the physical gels were treated with a crosslinking solution of 25 mM NaIO_4 for 5 min, unless otherwise noted, followed by washing with DI water.

8.4.4 Ultraviolet-visible absorption spectroscopy

Ultraviolet-visible absorption (UV-vis) spectroscopy was performed using a NanoDrop OneC Microvolume spectrophotometer (2 μl solution) to study polymerization of

L-DOPA, and its conjugation to gelatin. For the former, different solutions of NaIO₄ (*i.e.*, 0, 2.5, 7.5, 15, 25, 37.5, 50, 75, 100 mg/ml) in 1 M HCl was prepared. Then, 103 µl of the NaIO₄ solution was mixed with 397 µl of 42.8 mg/ml L-DOPA solution in 1 M HCl to start the L-DOPA polymerization reaction at similar final concentrations as those of synthesis concentrations. The absorbance at 324 nm was measured relative to 1 M HCl solution. For the latter, the absorbance spectra of GelDOPA solutions in DI water were measured at 2 w/v%.

8.4.5 Nuclear magnetic resonance

To study consumption of amine groups as a result of conjugation reaction, proton nuclear magnetic resonance (¹H NMR) spectroscopy was performed using a 400 MHz Bruker AV400 spectrometer. The synthesized GelDOPA was dissolved in dimethyl sulfoxide (DMSO)-d₆ at 10 mg/ml concentration for ¹H NMR experiments, and degree of amine consumption was calculated based on the procedures reported elsewhere.⁶⁴³

8.4.6 Size-exclusion chromatography

Measurements of molecular weight were conducted *via* size-exclusion chromatography (SEC). Briefly, the samples were diluted to 0.5 mg/ml in DI water and filtered using a filter paper with ~200 nm pore size. The standard samples with molecular weights 1000 Da to 600 kDa were purchased from GE Healthcare (NY, USA), diluted 10×, and run-on SEC. For poly(L-DOPA) samples, the polymerized solutions (prepared following the procedure explained for synthesis) were diluted 100× before the SEC run. Samples were inserted into an Agilent 1200 high-performance liquid chromatography (HPLC) equipped with a UV detector at 270 nm. The mobile phase was PBS (pH~7.4) with a flow rate of 0.8 ml/min. A Yarra™ 3 µm SEC-3000 (Phenomenex, Torrance, CA, USA) column was used at a

running time of 30 min.

8.4.7 Dynamic light scattering

Dynamic light scattering (DLS) tests were carried out using a Malvern Panalytical Zetasizer Nano ZS to characterize the size distributions of the poly(L-DOPA) nanoparticles. The poly(L-DOPA) solutions were prepared through the same process as described for their synthesis, followed by 10× dilution before loading into DTS1070 disposable folded capillary cuvettes. Refractive indices for dispersant and poly(L-DOPA) were defined as 1.33 and 1.59, respectively.

8.4.8 Arnow catechol content assay

To measure conjugated catechol content, the Arnow assay was carried out according to the following procedure, reported elsewhere.⁴⁶ Briefly, the Arnow reagent was prepared by dissolving 10 w/v% sodium molybdate and 10 w/v% sodium nitrite in 0.5 M HCl. Equal volumes of a 1.5 mg/ml GelDOPA solution already dissolved in 0.5 M HCl were mixed with the Arnow reagent, and the final solutions were shaken for 5 min. Subsequently, the solutions were diluted 2.5× and transferred to a 96 well plate (volumes of 200 µl). The absorbance at 520 nm was registered using a plate reader (BioTek Synergy 2, USA) and the catechol content was based on to the standard curves obtained from dopamine solutions at different concentrations.

8.4.9 Primary amine content assay

To quantify the concentrations of the primary amine groups, GelDOPA was dissolved at 0.4 mg/ml in 0.1 M sodium bicarbonate. The GelDOPA solutions were diluted to concentrations of 200-20 µg/ml in a 96-well plate with a final volume of 100 µl per well.

Then, 50 μ l of 500 $\times$ diluted TNBSA solution was added to each well, followed by incubation at 37 $^{\circ}$ C for 2 h. To stop the reaction, 50 μ l 10 w/v% SDS and 25 μ l 1 M HCl solution were added to the wells, and the absorbance at 335 nm was measured by a plate reader (BioTek Synergy 2, USA). Primary amine contents were calculated based on calibration curves obtained from glycine controls tested at concentrations between 20-2 μ g/ml.

8.4.10 Transmission electron microscopy

Nanoscale structural features of poly(L-DOPA) components were characterized using transmission electron microscopy (TEM). As synthesized poly(L-DOPA) dispersions were diluted 10 $\times$ and a volume of 2.5 μ l was pipetted on a membrane-supported copper mesh grid. Then, TEM imaging was performed using a FEI Tecnai T12 TEM microscope at 200 kV acceleration voltage.

8.4.11 Rheological tests

The rheological experiments were carried out by an MCR 302, Anton Paar rheometer using an 8 mm parallel-plate torque measuring system. For each experiment, 80 μ L of samples were tested at 1 mm gap size, 10% strain, and 1 Hz frequency, unless otherwise noted.

For thermosensitivity tests, GelDOPA pre-gels were maintained for 5 min at 50 $^{\circ}$ C for thermal equilibrium. Then, a linear thermal cycle of cooling-heating was applied between 10 to 50 $^{\circ}$ C at a rate of 1 $^{\circ}$ C/min with 3 min isothermal period defined before the onset of heating. To measure time to thermal gelation, a temperature step was defined from 50 to 25 $^{\circ}$ C at the maximum rate of cooling (20 $^{\circ}$ C/min). Viscosity-shear rate characteristics were evaluated at 37 $^{\circ}$ C across shear rates from 1 to 100 s $^{-1}$ with 0.75 mm gap size. Time sweep

tests were performed at a constant temperature of 37 °C over 12 h to study self-gelation of pre-gel solutions.

To study diffusive crosslinking of GelDOPA hydrogels, pre-gel solutions were maintained at 37 °C for 5 min to reach thermal stabilization. Then, they were exposed to different crosslinking solutions so that the pre-gels were fully submerged for peripheral diffusion while recording variations of modulus in time sweep tests. Strain sweep tests were performed on hydrogels crosslinked *ex situ* in 8 mm diameter circular polydimethylsiloxane (PDMS) molds filled with 80 µl pre-gel solution. These tests were started following thermal stabilization at 37 °C for 5 min at a 1.2 mm gap size.

8.4.12 Mechanical tensile tests

To evaluate tensile mechanical properties, 200 µl GelDOPA solutions were pipetted in rectangular PDMS molds of 25 mm × 4 mm. The samples were crosslinked by soaking them in crosslinking solutions for 5 min and then glued to plain paper using Krazy glue (OH, USA) for better gripping. The samples were loaded onto an Instron 5943 (MA, USA) universal testing equipment. Tensile tests were performed at a cross-head displacement rate of 8 mm/min, and the force-displacement data were recorded using a 100 N load cell.

8.4.13 Swelling tests

Fluid absorption capacity of the hydrogels was evaluated in GelDOPA hydrogels fabricated by molding 100 µl GelDOPA solution in 8 mm circular PDMS mold. After crosslinking, the hydrogels were incubated at 37 °C in 12 well plates containing 2 ml human blood-derived plasma per well. The weight of hydrogels was registered at different time points, and the swelling ratio was calculated based on Equation (5):

$$\text{swelling ratio} = \frac{w_s - w_0}{w_0} \quad (6)$$

where w_0 is weight at $t = 0$, and w_s represents the weight of swollen hydrogel at different time points.

8.4.14 Axial adhesion tests

Adhesion performance of hydrogels along the axial direction was measured using a rheometer (MCR 302, Anton Paar). Pre-gel solutions were crosslinked *in situ* by peripheral diffusion of crosslinking solutions on a 37 °C substrate. Then, axial adhesion force was measured at a vertical displacement rate of 0.5 mm/s. All measurements were performed using an 8 mm parallel-plate torque measuring system at a gap size of 1 mm.

Cyclic tack tests were also performed to assess the durability of interfacial adhesion with substrates at body temperature. Hence, physical hydrogels of GelDOPA (prepared to be 8 mm diameter and 1 mm height) were tested. The hydrogels were initially cooled to 10 °C, and a pre-load of 2 N was applied to the hydrogel surface. Then, cyclic adhesion tests were started after 10 min of thermal stabilization at 37 °C.

8.4.15 *In vitro* burst pressure tests

Adhesion strength of hydrogels was assessed under air pressure using a burst pressure setup. For this purpose, 25 mm wet collagen sheets were punctured using a 3 mm puncher. Collagen sheets were fixed onto an air chamber connected to a digital pressure sensor and a 60 ml syringe. The volumes of 80 µl GelDOPA solutions were applied to seal the punctured collagen sheets, followed by a crosslinking process. Then, the crosslinked hydrogels were soaked in 37 °C water before the air was injected at a rate of 20 ml/min while the chamber pressure was recorded real time. The maximum recorded pressure was

reported as burst pressure.

8.4.16 Wound closure tests

The capacity of hydrogels to prevent wound edges from opening transversally was evaluated. Two pieces of 10 mm × 20 mm tissues extracted from the porcine lung surface were placed head-to-head, and the interfacial edge was covered by GelDOPA pre-gel solution over a 10 mm × 10 mm area. The GelDOPA solutions underwent thermal gelation followed by oxidative crosslinking through the topical surface. The samples were incubated at 37 °C for 15 min. Then, an axial tensile displacement was applied to the two ends of tissue strips at a rate of 25 mm/min, while the force-displacement data was recorded. Adhesion toughness was calculated as the area under force-displacement curve normalized to the total surface area under pre-gel solution.

8.4.17 *Ex vivo* burst pressure tests

Burst pressure tests on porcine tissues (*i.e.*, lung and bladder) were performed to assess the sealing capability of bioadhesives. For experiments on lungs, an oxygen tank was connected to a pressure sensor and porcine lung through a T-joint. A 3 mm diameter puncture was sealed by GelDOPA bioadhesives and the entire tissue was submerged in 37 °C water. Pressure variations were monitored while oxygen gas was injected into the lungs until adhesion failure was observed. For burst tests on the bladder, food dye diluted in water was injected into bladder tissue with a 3 mm puncture sealed with GelDOPA similar to lung tests. The tests were performed underwater at 37 °C, and pressure was recorded at the point where leaking was seen.

8.4.18 Near-infrared responsiveness tests

Thermal responses of GelDOPA hydrogels to exposure to NIR irradiation (808 nm) was evaluated by real-time monitoring of hydrogel temperature *via* a thermocouple-assisted (type-K) thermal camera (Teledyne FLIR TG267). Irradiation of NIR was performed using a light source (Pilot® therapeutic diode laser system, CAO Group Inc., UT, USA) at different power levels (between 1 and 9 W, which was shined over an area of ~2 cm diameter). For this purpose, crosslinked hydrogel disks of 8 mm diameter and 2 mm height were placed at 25 mm from the light guide tip. To test hydrogels in wet conditions, the samples were placed in 12-well plates containing 1 ml DI Water.

Drug release studies were performed for crosslinked GelDOPA samples containing 2 mg/ml Rhodamine B. The hydrogels were immersed in 1 ml PBS and the hydrogel absorbance at 556 nm was measured at different time intervals with and without NIR irradiation. For NIR irradiation, each 10 min NIR irradiation (6 W) was followed by an “off” interval for 20 min. The amount of Rhodamine B released from the hydrogels were calculated based on calibration curves obtained from Rhodamine B solutions.

8.4.19 Antibacterial test

The antimicrobial activity of GelDOPA hydrogels was assessed *via* agar well diffusion. The microbial strains (*i.e.*, *E. coli* and *S. aureus*) were incubated in nutrient broth where optical density (OD) at 600 nm for *E. coli* and *S. aureus* were measured 1.24 and 0.48, respectively using a UV-vis spectrometer. The prepared inoculum was spread over the agar plates punched with 8 mm biopsy punches. The pre-gel solutions were pipetted (100 µl) in the punctured wells while the crosslinked hydrogels were placed on the agar surface. The

plates were incubated at 37 °C for 24 h to evaluate the zone of inhibition. For positive control, filter papers disks of 8 mm were infiltrated with silver sulfadiazine (at 15 µg/disk) and placed on the plates.

8.4.20 *In vitro* biocompatibility tests

Biocompatibility of GelDOPA bioadhesives was studied *via* two-dimensional (2D) cell culture using C2C12 murine myoblasts cell lines (ATCC). The cells were incubated at 37 °C and 5% CO₂ in an incubator and seeded at the density of 10⁴ cells per well in a 6 well plate for 1 d using 2 ml Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10 w/v% fetal bovine serum (FBS) and 1 w/v% penicillin–streptomycin. The GelDOPA hydrogels (molded to 8 mm diameter disks with 1.5 mm height) were crosslinked by soaking in NaIO₄ solutions for 5 min. The crosslinked hydrogel disks were sterilized under UV light for 1 h (each side) in PBS and transferred to the cell culture plates. Live/dead assay (Invitrogen, USA) at different time points was performed by adding 8 µL calcein and 12 µL ethidium homodimer-1 to each well, followed by incubation at 37 °C for 15 min. The cells were washed a few times before treatment with the live/dead solution. To image the cells, fluorescence imaging was carried out using an Axio Observer 5, Zeiss fluorescence microscope at the excitation/emission wavelengths of 494/515 nm for calcein (green) and 528/617 nm for ethidium homodimer-1 (red). Live and dead cells were counted using ImageJ software, based on which cell viability was calculated as the ratio of live cells to the total number of cells. The metabolic activity of cells was quantified *via* PrestoBlue™ assay (Thermo Fisher Scientific, USA). Cells were washed with PBS, and treated with the PrestoBlue™ reagent diluted 10× in DMEM media for 2 h. The fluorescence intensity of the media was measured at the excitation/emission wavelengths of 560/590 nm using a plate

reader (BioTek Synergy 2, USA).

8.4.21 *In vivo* biocompatibility

Hydrogel biocompatibility was evaluated *in vivo* by subcutaneous implantation of crosslinked GelDOPA hydrogels in mice under an approved protocol at The Lindquist Institute (#22747-01). Black C57B6 mice (purchased from The Jackson Laboratory, USA) were maintained under standard laboratory conditions (*i.e.*, fed by laboratory pellets and purified water and kept at 25 °C in pathogen-free (SPF) facilities). The samples of ¼ circular disks with 8 mm diameter and 2 mm height were prepared as described above. The samples were implanted subcutaneously using ~15 mm incisions. All surgeries were used under anesthesia using 1.5 %v/v isoflurane in oxygen gas adjuvant to carprofen injection. Then, at different time points (weeks 1 and 4) the tissues from the mice were collected after they were euthanized (by carbon dioxide inhalation). Subsequently, the samples were fixed by soaking in a 10 %v/v formalin solution. Cell morphology was evaluated by hematoxylin and eosin (H&E) staining. To this end, the fixed organs and tissues were embedded in paraffin and sliced into 5 µm layers.

8.4.22 Statistical analyses

The experiments were performed at least in triplicates. The reported quantities represent means ± standard deviations (SD). Statistical analyses were conducted using Graphpad Prism 8 software *via* one-way analysis of variance (ANOVA) where $P < 0.05$ was considered as statistically significant. Experimental measurements were performed in triplicates (n=3) unless noted otherwise.

8.5 Supporting Information

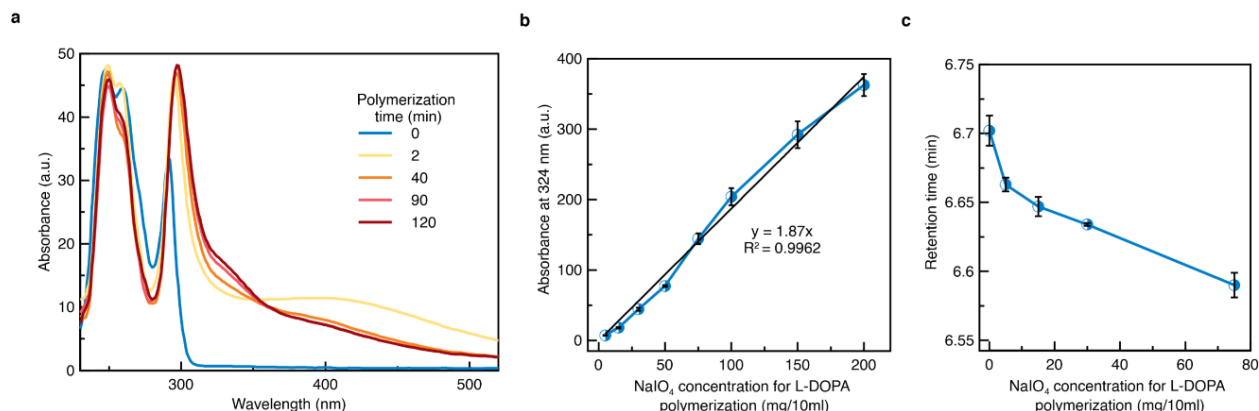


Figure S8.1. Characterization of 3,4-dihydroxy-L-phenylalanine (L-DOPA) polymerization. (a) Real-time evolution of UV-vis spectra for L-DOPA solutions in 1 M HCl with the addition of 15 mg/10 ml NaIO₄. (b) Absorbance at 324 nm after 120 min polymerization of L-DOPA at different concentrations of NaIO₄. Data are shown as mean $\pm$ SD ($n=3$). (c) Retention time in size-exclusion chromatography (SEC) tests performed on poly(L-DOPA) samples synthesized at different concentrations of NaIO₄ following *N*-hydroxysuccinimide (NHS) activation. Data are shown as mean $\pm$ SD ($n=3$).

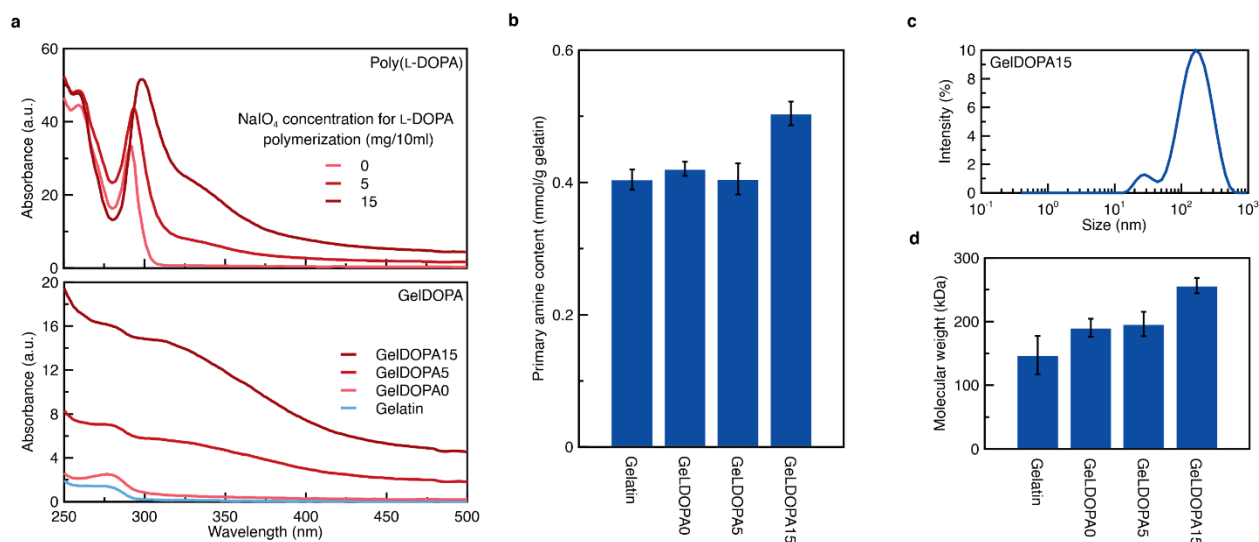


Figure S8.2. Chemical characterization of gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Ultraviolet-visible spectra of GelDOPAx solutions (where x is concentration of NaIO₄ agent in mg/10 ml to polymerize L-DOPA) and their corresponding poly(L-

DOPA) conjugates. (b) Variations in the primary amine content in the gelatin backbone as a result of modification with L-DOPA derivatives. Data are shown as mean $\pm$ SD (n=3). (c) Hydrodynamic size distribution of GelDOPA15 solutions in water. (d) Molecular weights of GelDOPA products after modification reaction measured *via* size-exclusion chromatography (SEC). Data are shown as mean $\pm$ SD (n=3).

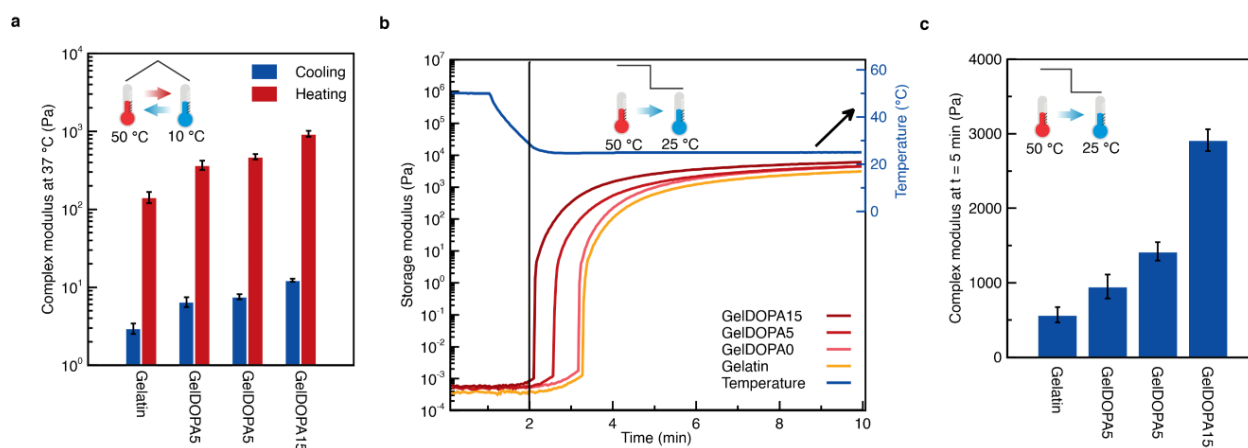


Figure S8.3. Thermosensitivity of gelatin-based solutions modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). (a) Complex modulus of GelDOPAx solutions (where x is the concentration of the NaIO₄ agent in mg/10 ml to polymerize L-DOPA) during heating and cooling cycles at 37 °C in rheological studies of the sol-gel transition temperature. Data are shown as mean $\pm$ SD (n=3). (b) Thermal gelation of GelDOPA hydrogels in response to a step temperature profile from 50 to 25 °C, and (c) values of storage modulus 5 min after the beginning of the test. Data are shown as mean $\pm$ SD (n=3).

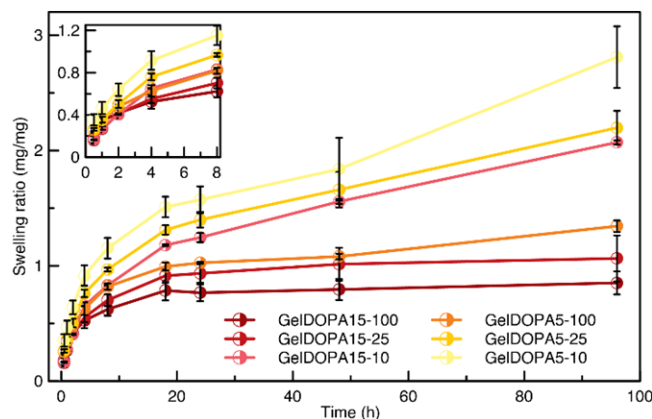


Figure S8.4. Swelling of crosslinked bioadhesives of gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). In GelDOPAx-y, x and y are the concentration of NaIO_4 used for polymerization of L-DOPA during synthesis in mg/10 ml and for crosslinking of GelDOPA in mM, respectively. Data are shown as mean $\pm$ SD (n=3).

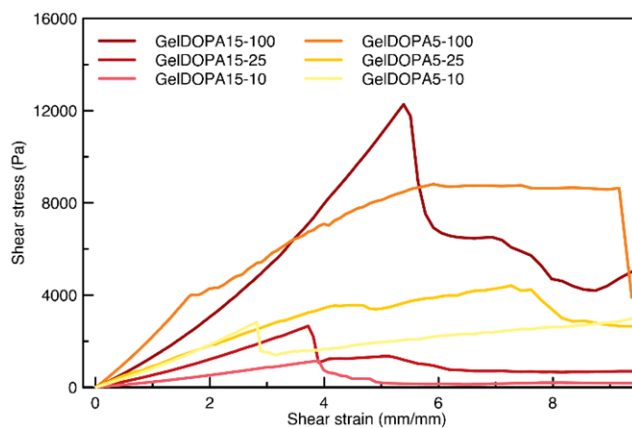


Figure S8.5. Shear stress *versus* shear strain measured in rheological strain sweep tests at 37 °C for bioadhesives based on gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA). The values of x and y in GelDOPAx-y denote the concentrations of NaIO_4 used for polymerization of L-DOPA during synthesis in mg/10 ml and for crosslinking of GelDOPA in mM, respectively.

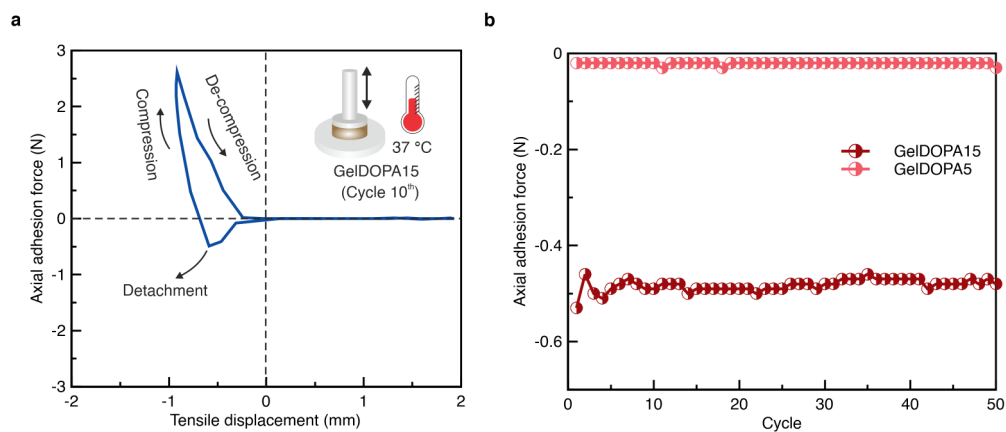


Figure S8.6. Cyclic adhesion performance of gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GelDOPA) in axial mode. (a) Force-displacement curves at the 10th cycle and (b) axial detachment force measured over 50 cycles for GelDOPAx hydrogels (x is concentration of NaIO₄ agent in mg/10 ml to polymerize L-DOPA).

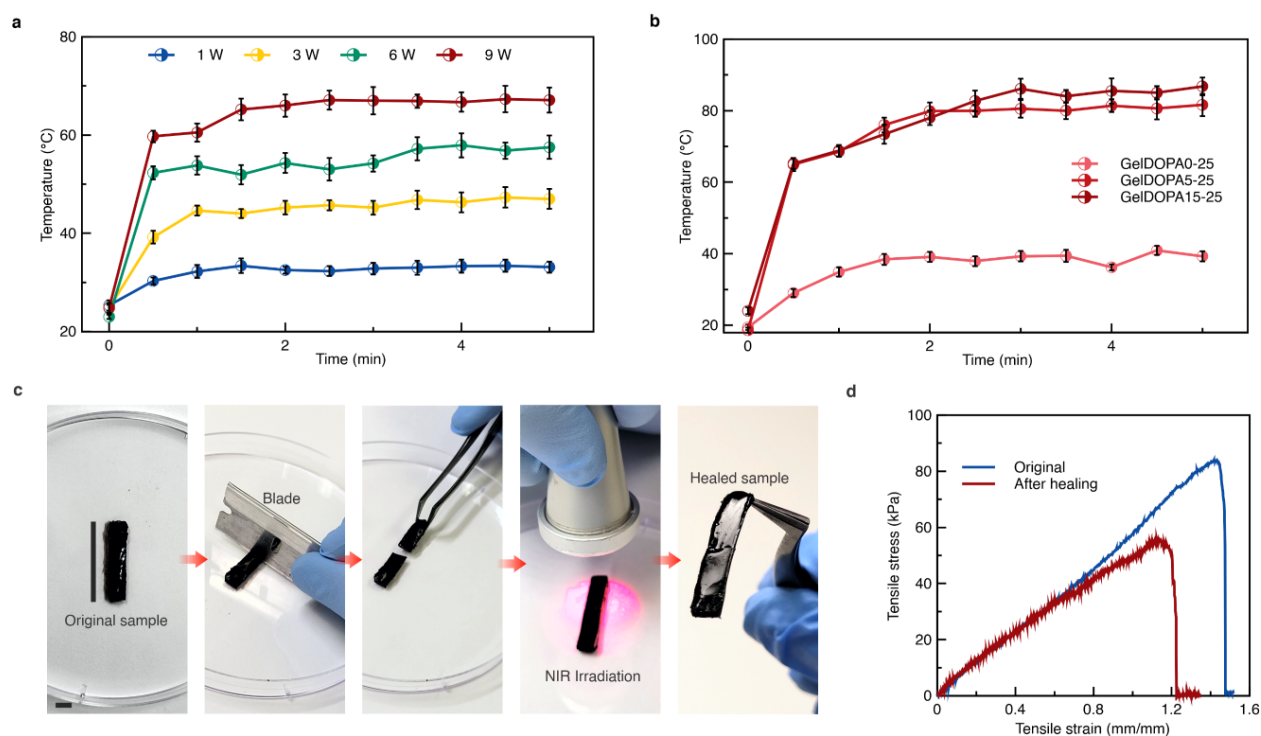


Figure S8.7. Near-infrared (NIR) responsiveness of gelatin modified with 3,4-dihydroxy-L-phenylalanine (L-DOPA) derivatives (GeldOPA). (a) Temperature response of crosslinked GeldOPAx-y hydrogels (x=15 mg/10 ml is concentration of NaIO₄ agent to polymerize L-DOPA during synthesis procedure, and y=25 mM is concentration of NaIO₄ for crosslinking GeldOPA hydrogels) at different NIR irradiation powers for control on steady temperature. Data are shown as mean $\pm$ SD (n=3). (b) Temperature profile of GeldOPAx-y samples measured in dry conditions. Data are shown as mean $\pm$ SD (n=3). (c) The process of NIR exposure enabled self-healing of GeldOPA hydrogels (scale bar = 5 mm). (d) Tensile mechanical properties of GeldOPA15-25 hydrogels before (original state) and after self-healing process.

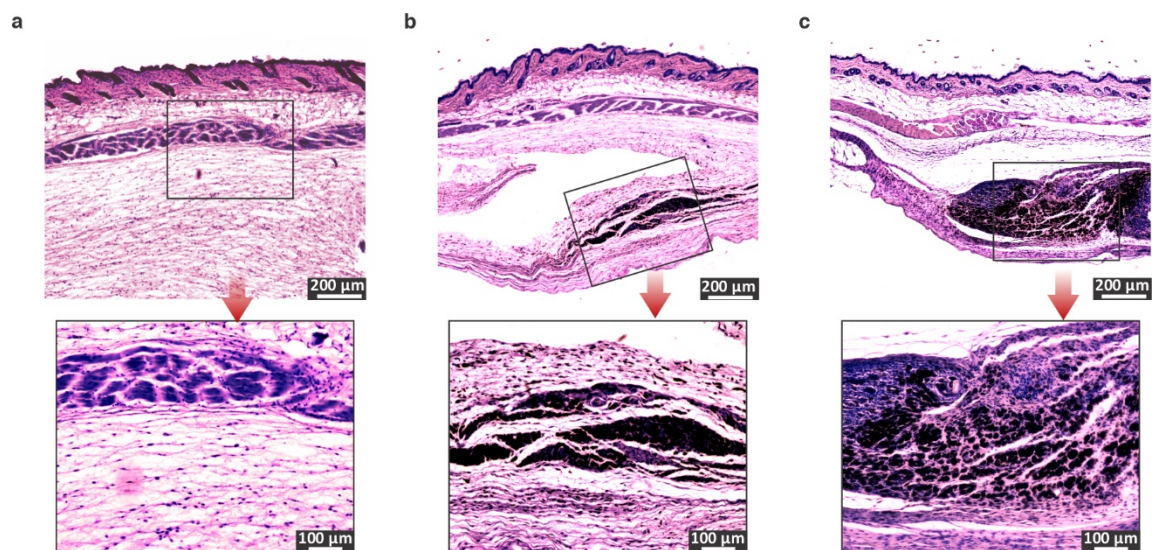


Figure S8.8. Hematoxylin and eosin (H&E) staining of subcutaneously implanted gelatin-3,4-dihydroxy-L-phenylalanine (L-DOPA) conjugates (GelDOPA) after 1 week for (a) no treatment control, (b) GelDOPA15, and (c) GelDOPA15-25 samples. The dark spots magnified in b and c show the GelDOPA hydrogels.

CHAPTER 9: CONCLUSIONS AND PROSPECTS

Gelatin proteins, due to their bioactivity and biodegradability, are attractive building blocks for tissue bioadhesion through modification with phenolic compounds. Gelatin biomaterials are economical candidates with excellent biocompatibility and tissue-mimetic properties, which can be chemically functionalized to impart bioadhesion for wound care applications. Phenolic structures are recognized as the main elements for adhesion in natural organisms, inspiring recent developments in tissue bioadhesives. Polyphenolic derivatives are produced *via* simple chemical processes and provide more robust bioadhesion compared to small-molecule phenolic structures in gelatin-based biomaterials. In addition to bioadhesion, polyphenolic compounds have various biological functions. Their antioxidant and antibacterial properties make them strong candidates for wound-healing applications. Furthermore, polyphenols impart photothermal responsiveness for use as drug carriers in cancer treatment applications. Polyphenols are therefore receiving increasing attention as a versatile and diverse toolbox for the development of gelatin-based tissue sealants and hydrogel bioadhesive patches for tissue engineering, drug delivery, wound healing, and bioelectronic devices.

Despite the exciting opportunities brought to the field, there remain many unresolved challenges to be addressed more rigorously for clinical translation of polyphenolic gelatin hydrogels. Physical incorporation of polyphenols into gelatin-based hydrogels at high concentrations (as the simplest phenolic integration method) increases toxicity²¹ despite strengthening adhesive interactions. Furthermore, polyphenol structures are not compatible with free-radical-based crosslinking methods, *e.g.*, photocrosslinking due to radical scavenging effects, leading to loosened crosslinking networks, poor cohesion, and

thereby increased toxicity triggered by polymer burst release. Oxidative crosslinking of covalently polyphenol-functionalized gelatin proteins is an effective alternative strategy for robust bioadhesion, rapid gelation, and strong cohesion due to the large amounts of grafted phenolic moieties (as compared to phenol-functionalized gelatin proteins). Although, such crosslinking approaches raise concerns on potential toxicity. Nevertheless, the majority of the current literature focuses on phenolic (mostly catechol) functionalization for the development of bioadhesives using small molecules. Reports on polyphenolic bioadhesives are predominantly based on physical mixing with hydrogel precursors, which has many limitations, such as uncontrolled release. Therefore, future research should explore the potential of gelatin biomaterials modified chemically with high molecular weight polyphenolic molecules. The state-of-art polyphenolic hydrogels rely heavily on polydopamine, specifically ignoring the large library of oxidatively polymerizable phenolic species, *e.g.*, serotonin³⁰⁶ and rosmarinic acid.

Oxidative crosslinking of phenolic biomaterials uses harsh oxidizers, raising biosafety concerns. While the concentrations of oxidizing agents required for crosslinking are reduced significantly with polyphenolic functionalization, to circumvent cytotoxicity issues, the development of safer but compatible crosslinking procedures is necessary. In this regard, using mechanisms such as physical association through electrostatic interactions and hydrogen bonding to associate proteins is beneficial. Although hydrogel patches can be designed in multiple layers to prevent unwanted adhesion to adjacent tissues, selective tissue adhesion (*i.e.*, adhesion at tissue interface and anti-adhesion on the hydrogel backing) is not straightforward to achieve in injectable bioadhesives. This property has been enabled by diffusive-oxidative crosslinking of polyphenolic gelatin proteins. However, diffusive

crosslinking is not possible for most low-viscosity pre-gel solutions. Therefore, more versatile materials design with adhesion selectivity is needed. Another major challenge with phenolic biomaterials is their long-term stability, due to autoxidation of phenolic groups in air, which requires attention in characterization with respect to storage conditions. We note that despite the plethora of existing studies on wet adhesion, there is little work on biomaterials for adhesion on oily skin surfaces and fatty tissues.

Next-generation bioadhesives introduce smart functionality such as stimuli-controllable adhesion. On-demand deactivation of bioadhesion in tough bioadhesives for their detachment from fragile patient surfaces, *e.g.*, diabetic and burn wounds and infant skin, is essential. Exploring chemistries that enable disruption of polyphenol-tissue interactions should be pursued. Introducing other functions, such as electrical conductivity, to polyphenolic adhesives will expand their applications in the reconstitution of conduction pathways after cardiac infarction and repair of damaged nerve tissues. Finally, multifunctional adhesive polyphenolic patches can serve as interfaces of bioelectronic devices and sensors with wet tissues and organs *in vivo*.

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