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Study of Polyion Complex Structure Formation
from Mixing Oppositely-Charged Block Copolypeptides

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

By

Yintao Sun

2019

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ABSTRACT OF THE DISSERTATION

Study of Polyion Complex Structure Formation
from Mixing Oppositely-Charged Block Copolypeptides

by

Yintao Sun

Doctor of Philosophy in Bioengineering

University of California, Los Angeles, 2019

Professor Timothy J. Deming, Chair

Synthetic polypeptides are a versatile class of biomaterials with many interesting properties such as biodegradability, biocompatibility and ordered secondary conformations. In particular, block copolypeptides with well-defined block composition and versatile selection of amino acid constituents allow for controlled assembly into supramolecular structures such as micelles, vesicles and hydrogels. In recent years, polyion complexation has been developed as a new strategy for supramolecular structure assembly, resulting in formation of unique polyion complex (PIC) systems that have seen growing applications in drug delivery and gene therapy. However, the usage of PIC assembly in controlling block copolypeptide supramolecular structure

formation has been largely unexplored. This dissertation will focus on the study of polyion complex (PIC) structure formation by mixing oppositely charged block copolypeptides.

Synthetic diblock copolypeptides were developed to incorporate oppositely charged ionic segments that form β -sheet structured hydrogel (DCH_{PIC}) assemblies via polyion complexation when mixed in aqueous media. The polyionic block length as well as polymer concentration can be used to tune hydrogel properties. The PIC hydrogel system has self-healing properties, microporous architecture, and stability against dilution in aqueous media. Neural stem progenitor cells were also successfully loaded into the hydrogel with good cell viability. Together, these promising attributes and unique features of the β -sheet structured PIC hydrogels highlighted their potential applications as carriers for stem cell therapy.

Diblock (DB), triblock (TB) and pentablock (PB) copolypeptide PIC hydrogels with identical overall amino acid compositions and ionic block lengths were assembled and their mechanical properties were compared. Specifically, the pentablock copolypeptides were designed to be equivalent to two connected triblock copolypeptides. As a result, PB hydrogels have demonstrated drastic improvement of mechanical properties over the DB and TB hydrogels. Furthermore, low concentrations of cationic PB components can be incorporated within the DB or TB hydrogels and act as linkers to significantly increase mechanical properties.

A dual network physically cross-linked hydrogel (DCH_{DN}) was developed that consists of two separate interpenetrating diblock copolypeptide networks based on discrete modes of assembly: polyion complexation (DCH_{PIC}) and hydrophobic association (DCH_{MO}). The PIC precursors were mixed within a preformed amphiphilic hydrogel to give hydrogels with two distinct networks. The DCH_{DN} components were shown to have synergistic effects that significantly enhanced mechanical properties of the overall system. The PIC component imparts

its stability against dilution to the DN hydrogel system while the amphiphilic component introduces hydrophobic domains within the network that potentially allow for hydrophobic cargo encapsulation. Contrary to many reported dual network hydrogels systems, DCH_{DN} retains the self-healing properties of its components, which makes this hydrogel system a potential injectable carrier for controlled release applications.

PIC diblock copolypeptides have been synthesized, assembled and characterized to form assemblies. Assembly size and structure can be tuned by varying the poly(ionic) block lengths and chirality. PIC assemblies were found to have core-shell micellar structures by electron microscopy and confocal imaging. Potential use of these assemblies for protein delivery was explored with lysozyme as the model protein. The polypeptide-protein complex formed assemblies that are stable under physiological salt and osmotic conditions.

The dissertation of Yintao Sun is approved.

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Table of Contents

Abstract.....	vi
Committee.....	v
Table of Contents	vi
List of Figures.....	viii
List of Abbreviations	x
Acknowledgements	xiii
Vita	xiv
Chapter 1: Polyion Complex Copolypeptide Materials for Biomedical Applications.....	1
1.1 Polypeptides for Biomedical Applications.....	1
1.2 Copolypeptide Synthesis via NCA polymerization.....	2
1.3 Polypeptide based Polyion Complexes.....	5
1.4 Polyion Complex polypeptide hydrogel.....	9
1.5 References	12
Chapter 2: Conformation Directed Formation of Self-healing Diblock Copolypeptide	
Hydrogels via Polyion Complexation	18
2.1 Abstract	18
2.2 Introduction	18
2.4 Conclusion.....	37
2.5 Experimental.....	37
2.5.1 Materials and Methods	37
2.5.2 Experimental Procedures.....	38
2.5.3 References	46
Chapter 3: Self-healing Multiblock Copolypeptide Hydrogels via Polyion Complexation .	49
3.1 Abstract	49
3.2 Introduction	49
3.3 Results and Discussion	53
3.4 Conclusion.....	61
3.5 Experimental	61
3.5.1 Materials and Methods	61
3.5.2. Experimental Procedures.....	62
3.6 References	74
Chapter 4: Self-healing Diblock Copolypeptide Dual Network Hydrogel	77

4.1 Abstract	77
4.2 Introduction	77
4.3 Results and Discussion	79
4.4 Conclusion.....	89
4.5 Experimental	89
4.5.1 Materials and Methods	89
4.5.2. Experimental Procedures.....	90
4.6 References	93
Chapter 5: Polyion Complex Diblock Copolypeptide Assemblies.....	93
5.1 Abstract	95
5.2 Introduction	95
5.3 Results and Discussion	97
5.4 Conclusion.....	104
5.5 Experimental	105
5.5.1 Materials and Methods	105
5.5.2. Experimental Procedures.....	106
5.6 References	115

List of Figures

Figure 1.1 Scheme for polymerization of NCAs to produce polypeptides	2
Figure 1.2 Proposed amine mechanism of NCA polymerization	3
Figure 1.3 Proposed mechanism of transition metal initiated NCA polymerization.	5
Figure 1.4 Solid and liquid polyion complex formation	6
Figure 1.5 Scheme of PIC micelle formation	9
Figure 1.6 Scheme of PIC hydrogel formation.	11
Figure 2.1 Schematic of assembly process for PIC hydrogel.	22
Figure 2.2 Circular dichroism spectra of poly(M ⁰) and poly(M ⁰ /A)C.	23
Figure 2.3 Synthesis of PIC diblock copolypeptides.	24
Figure 2.4 PIC diblock copolypeptide hydrogel mechanical propertie	27
Figure 2.5 Rheology data for PIC hydrogels.....	29
Figure 2.6 Rheology data for racemic PIC system.	31
Figure 2.7 ATR-IR spectra of lyophilized PIC hydrogel samples in the amide region.	31
Figure 2.8 ATR-IR spectra of lyophilized PIC copolypeptide samples in the amide region	32
Figure 2.9 LSCM and cryoEM imaging of PIC hydrogel.....	33
Figure 2.10 Stability of PIC hydrogels against dilution.....	35
Figure 2.11 Viability of neural stem progenitor cells (NSPCs) encapsulated in hydrogels.	36
Figure 3.1 Schematic of diblock, triblock, and pentablock copolypeptides for PIC hydrogels...	51
Figure 3.2 Chemical structures and compositions of multiblock PIC copolypeptides	
.....	53
Figure 3.3 Rheology data for multiblock PIC hydrogels.	54

Figure 3.4 Mechanical properties of PIC block copolypeptide hydrogels.....	55
Figure 3.5 ATR-IR spectra of multiblock PIC hydrogels in the amide region..	57
Figure 3.6 Mechanical properties of mismatched multiblock PIC hydrogels.....	58
Figure 3.7 Mechanical recovery of multiblock PIC hydrogels.....	60
Figure 3.8 Normalized swelling ratio measurement of pentablock hydrogel.	60
Figure 4.1 Schematic of assembly process for dual network hydrogels.....	80
Figure 4.2 Chemical structures and compositions of dual network hydrogel components	81
Figure 4.3 Rheology data for dual network hydrogels.....	83
Figure 4.4 Dual network hydrogel mechanical properties.	84
Figure 4.5 Recovery measurement for dual network hydrogels	85
Figure 4.6 Stability of dual network hydrogels against dilution.....	86
Figure 4.7 Normalized swelling ratio measurement of dual network hydrogel.....	87
Figure 4.8 Dual network hydrogel sample loss during swelling study.....	87
Figure 4.9 Laser scanning confocal images of dual network hydrogel	88
Figure 5.1 Schematic of PIC assembly system.	98
Figure 5.2 Synthesis of PIC diblock copolypeptides.	100
Figure 5.3 Dynamic Light Scattering of PIC assemblies.....	102
Figure 5.4 DIC, LCSM, EM and cryo-EM imaging of PIC assemblies.	103
Figure 5.5 Dynamic Light Scattering of PIC copolypeptide – Lysozyme complex assemblies.	104

List of Abbreviations

AF	Alexa Fluor
Bn	Benzyl
br	Broad
CSA	(1S)-(+)-Camphorsulfonic acid
d	Doublet
D	Poly(L-aspartate) chain
d-TFA	Deuterated trifluoroacetic acid
DB	Diblock
DCH	Diblock copolypeptide hydrogel
DCH _K	Poly(L-lysine)- <i>block</i> -poly(L-leucine) diblock copolypeptide hydrogel
DCH _{MO}	Poly(L-methionine sulfoxide- <i>stat</i> -L-alanine)- <i>block</i> -poly(L-leucine) diblock copolypeptide hydrogel
DCH _{PIC}	Polyion complex diblock copolypeptide hydrogel
DP	Degree of polymerization
E	Poly(L-glutamate)
Eq	Equivalents
Et ₂ O	Diethyl ether

FTIC	Fluorescein isothiocyanate
Glu	L-glutamate
GPC	Gel Permeation Chromotography
HFIP	Hexafluoroisopropanol
K	Poly(L-lysine) chain
L	Poly(L-leucine) chain
Leu	L-leucine
Lys	L-lysine
M	Poly(L-methionine) chain
MeOH	Methanol
Met	L-methionine
M ^O	Poly(L-methionine-sulfoxide) chain
MSA	Methanesulfonic acid
MWCO	Molecular weight cut-off
NCA	<i>N</i> -carboxyanhydride
PB	Pentablock
PEG	Polyethylene glycol methyl ether

PIC	Polyion Complex
ROP	Ring-opening polymerization
RT	Room temperature
s	Singlet
TB	Triblock
TBHP	Tert butyl hydrogen peroxide
TFA	Trifluoroacetic acid
THD	Tetrahydrofuran
TRITC	Tetramethylrhodamine

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Publications

1) Sun, Y.; Wollenberg, A. L.; O'Shea, T. M.; Cui, Y.; Zhou, Z. H.; Sofroniew, M. V.; Deming, T. J. Conformation-Directed Formation of Self-Healing Diblock Copolypeptide Hydrogels via Polyion Complexation. *Journal of the American Chemical Society* **2017**, 139, 15114–15121.

2) Sun, Y.; Deming, T. J. Self-healing Multiblock Copolypeptide Hydrogels via Polyion Complexation. Submitted to *ACS Macro Letters* **2019**

Chapter 1: Polyion Complex Copolypeptide Materials for Biomedical Applications

1.1 Polypeptides for Biomedical Applications

Proteins, or biological polypeptides, are fascinating products of nature because they have well controlled sequences and compositions of the constituent amino acid monomers that allow them to fold into 3D structures with specific biological and catalytic functions.¹ In recent years, synthetic polypeptides have been developed as mimics of natural proteins to make biomaterials for applications in stem cell transplantation², drug delivery³, biomineralization⁴, optics⁵ and diagnostic.⁶

Polypeptides have many advantages over conventional synthetic polymers as biomaterial candidates due to their versatility in functionalization, biodegradability, biocompatibility and unique ordered conformations. Polypeptides are prepared from polymerization of α -amino acid *N*-carboxyanhydride (NCA) monomers. Given the large library of both naturally occurring and synthetic amino acids, polypeptides can readily be prepared with various functional groups needed for specific interactions (e.g. electrostatic, dipolar and hydrophobic interactions). Furthermore, their unique ability to adopt stable and ordered secondary structures (e.g. α -helices, β -sheets, and turns) that imparts different solubility allows polypeptide to self- assemble into more defined macrostructures, such as micelles, vesicles, sheets and hydrogels.^{7,8} In contrast to most synthetic polymers, polypeptides are protein mimics and therefore can be more readily metabolized and eliminated from the body, leaving no toxic degradation product. Finally, polypeptide biocompatibility can be tuned for specific applications. For example, long poly(lysine) can be employed to increase cytotoxicity⁹ while poly (methionine sulfoxide) can act as non-charged hydrophilic groups similar to PEG.³

1.2 Copolypeptide Synthesis via NCA polymerization.

Two common ways to synthesize polypeptides are solid-phase peptide synthesis (SPPS) and ring-opening polymerization (ROP) of α -amino acid-*N*-carboxyanhydrides (NCAs). While SPPS allows for sequential control of α -amino acid content, it suffers from limitations, such as sequence defects, low molecular weight ceiling, high production costs and low overall yields.^{10,11} In late 1940s, ROP of NCAs has been developed as an economical and expedient alternative of conventional SPPS for preparing large-scale high molecular weight polypeptides (>100 residues). This method requires simpler reagents and fewer deprotection steps, which result in higher yields with no detectable racemization at the chiral centers (Figure 1.1).¹¹ With a wide library of well-developed NCAs, a variety of polypeptide can be synthesized with diverse properties.¹²

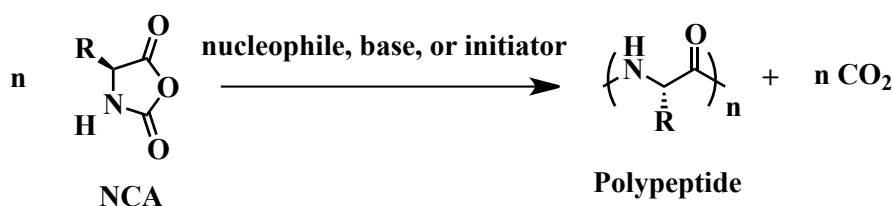


Figure 1.1 Ring-opening polymerization (ROP) of α -amino acid-*N*-carboxyanhydrides (NCAs)

ROP of NCAs follows two most likely pathways, amine and activated monomer (AM) mechanisms. While amine mechanism follows the nucleophilic ring-opening chain growth process (Figure 1.2), AM mechanism first undergoes initiation via deprotonation of an NCA.¹³ However, problem occurs when both chain growth mechanism occur at the same time during a polymerization. The side reactions can result in chain termination and transfer, which significantly limit the control over molecular weight, dispersity, block architecture and end group

functionality. There are also challenges in NCA and solvent purification due to NCA having high sensitivity towards minute trace of moisture, base and acid.¹⁴

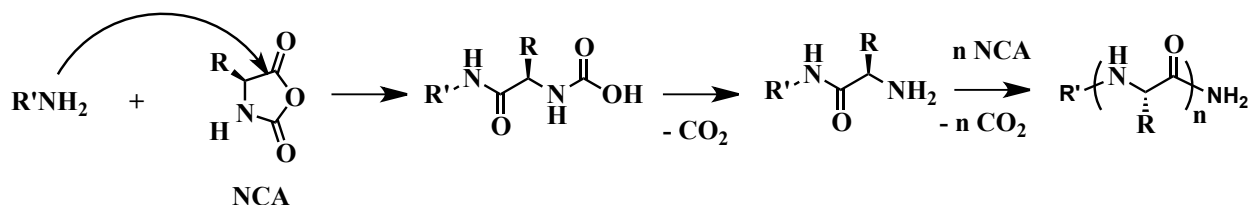


Figure 1.2 Proposed amine mechanism of NCA polymerization

In recent years, much research has been focused on improving the classical primary amine polymerization. Living polymerization has been accomplished under high vacuum conditions, which served to remove moisture and other impurities that can catalyze side reactions with monomer, polymer or solvents.¹⁵ This technique allowed for block copolypeptide synthesis with controlled molecular weight and chain distribution. Similar controlled polymerization was obtained by lowering reaction temperature to 0 °C without removal of impurities.¹⁶ When temperature is lowered, the activation barrier for chain propagation decreases more than that of the side reactions, thus reducing side reactions. Primary amine hydrochloride imitators were also developed as a way to reduce reactivity of end group amine and avoid formation of NCA anions. However, higher temperature (40 - 80 °C) is needed for controlled polymerization instead of single NCA addition reactions.¹⁷

In 2007, the Cheng group reported a new controlled NCA polymerization system using hexamethyldisilazane (HMDS) as the initiator.¹⁴ This method was demonstrated to have excellent control over molecular weight and dispersity. Interestingly, HMDS mechanism differs from the typical amine and AM chain growth mechanisms. Silylamine transfers a trimethylsilyl (TMS) group to the NCA to yield a silyl carbamate endgroup. Propagation occurs through

continuous transfer of the TMS group from the terminal TMS-carbamate to incoming NCAs. In general, organosilicon-mediated NCA polymerization is much faster (24 h) than those initiated by amines at low temperature or using amine hydrochloride initiators, but is still slower compared to those mediated by transition metal initiators (30-60 min).

More rapid living polymerization of NCAs can be achieved by using transition metal catalyst.¹⁸ Deming has developed zerovalent nickel and cobalt initiators that form metallacyclic complexes with NCA monomers via oxidative addition across the anhydride bonds of NCAs (Figure 1.3).^{19,20} Addition of a second NCA monomer results in formation of six-membered amido-alkyl metallacycle intermediates, which contracts to five-membered amido-amidate metallacycles upon further additions of NCAs.²¹ The amido-amidate metallacycle propagates through nucleophilic attack on another NCA monomers to form a large metallacycle that can contract through elimination of CO₂. Proton transfer from the free amide to the tethered amidate group further contracts the ring to reform the amido-amidate propagating species, while releasing the end of the polymer chain and becoming available for the next NCA addition. The transition metal migrates along the growing chain, while being steadily chelated at the active chain end. These transition metal initiators can produce polypeptide with narrow chain distributions (PDI <1.2) and a wide range of molecular weight (500-500,000 g/mol) in relatively short reaction times (1 h for most NCAs). In addition, this polymerization method is very effective for polymerizing most existing NCAs as either pure enantiomers (D- or L –configuration) or racemic mixtures. Finally, these transition metal initiators can readily produce block copolypeptides with well-defined sequence and compositions, which is a requirement for synthesizing biomaterials for many biomedical applications.²²

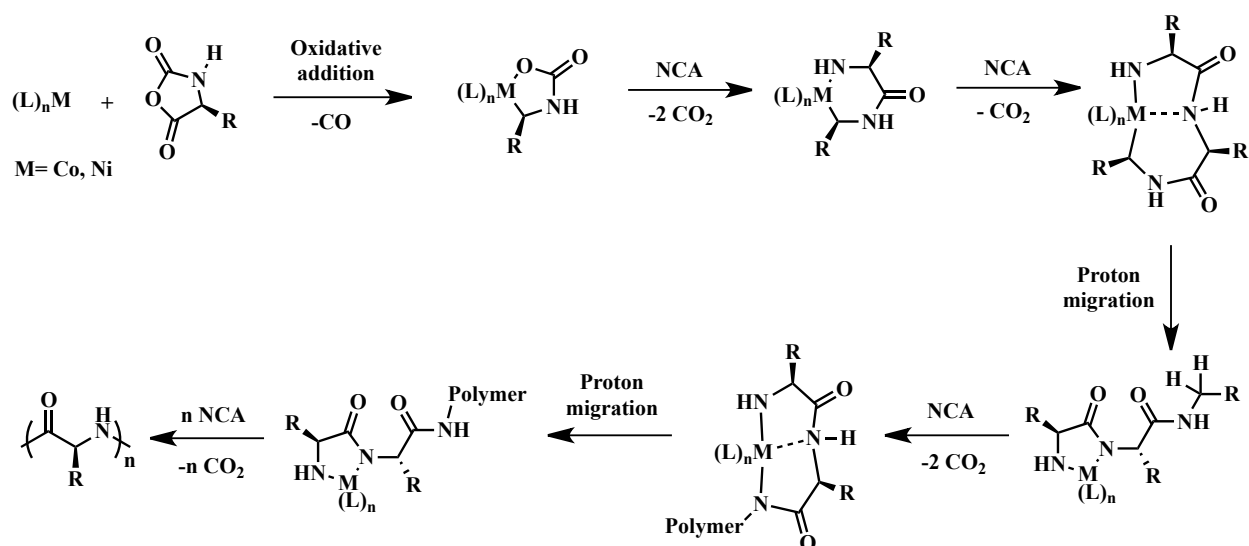


Figure 1.3 Proposed mechanism of transition metal initiated NCA polymerization.

1.3 Polypeptide based Polyion Complexes

Polyion complexes (PICs) are structures formed from mixing of oppositely charged polyelectrolytes.²³ When oppositely charged polymers associate, their counterions are released, thus driving PIC formation entropically.²⁴ PICs can form either solid precipitates with limited utility without processing²⁵ or water-rich liquid coacervates, which have seen applications in food industry²⁶, underwater adhesives²⁷ and electronic ink²⁸ (Figure 1.4). Homopolymer PIC systems can form macroscopic bulk phase separation. When block copolymers are utilized, nanoscale phase separation can be produced.^{23,24} While complex biopolymers (e.g. protein, DNA, etc) based PIC systems have been well explored in the past²⁹, polypeptide based PIC systems have gained a lot of attention in recent years. Polypeptides serve as interesting alternatives to complex biopolymers due to their inherent biocompatibility, and ability to form secondary structures, which introduces additional intermolecular forces that influence PIC stability and phase separation.³⁰

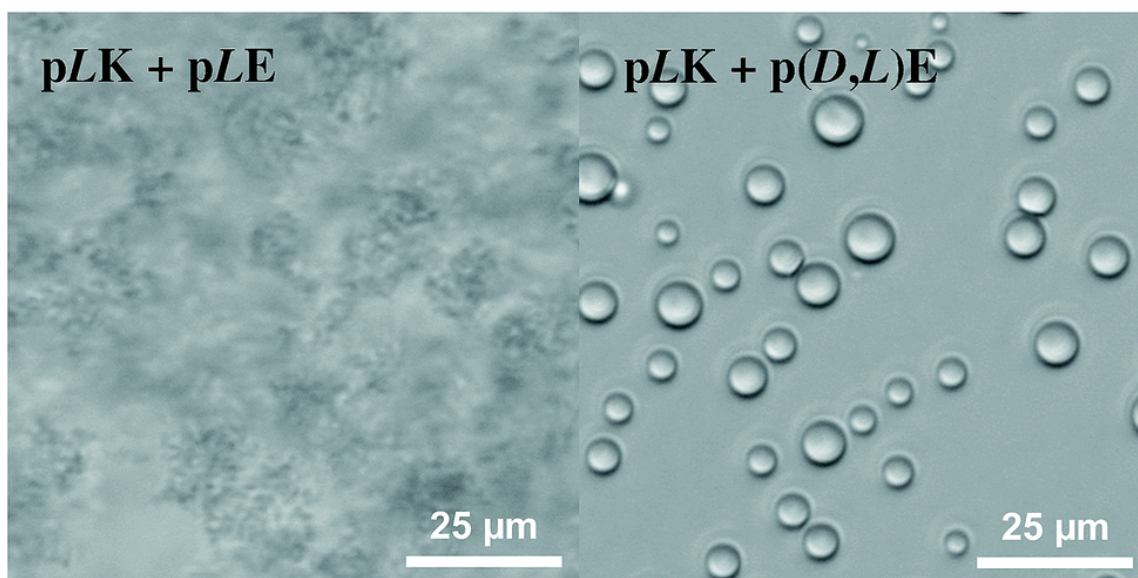


Figure 1.4 Solid and liquid polyion complexes formation using poly(lysine) and poly(glutamate). Precipitates (left image) are formed using homochiral poly(L-lysine) and poly(L-glutamate). Coacervates (right image) are observed when homochiral poly(L-lysine) was mixed with racemic poly(rac-glutamate). Scale bar 25 μm . Reprinted (adapted) with permission from Hoffmann, K. Q.; Perry, S. L.; Leon, L.; Priftis, D.; Tirrell, M.; Pablo, J. J. D. A molecular view of the role of chirality in charge-driven polypeptide complexation. *Soft Matter* 2015, 11, 1525–1538. Copyright © 2015, Royal Society of Chemistry

Homogeneous PICs are formed when two oppositely charged homo-polypeptides are mixed together. PIC systems based on poly(lysine) and poly(glutamate) were extensively investigated.^{30,31,32,33} PIC structures and properties were found to depend on parameters, such as pH, temperature, molecular weight, polyanion/polycation ratio and overall polymer concentration.³³ Secondary structure also has significant effects on PIC structures. Homochiral poly(L-lysine) (K) and poly(L-glutamate) (E) form β -sheet structures that precipitates out of solution. However, when one polyion component is racemic, β -sheet is interrupted and liquid coacervates are formed.^{30,32} However, this chirality dependency can be removed when a spacer is introduced to increase the space between the charged group and polypeptide backbone. The resulting oppositely charged homochiral polypeptides can retain their α -helicity upon mixing, preventing β -sheet and precipitate formation.³⁴

Heterogeneous PICs are formed by mixing one polyion polypeptide with an oppositely charged synthetic polymers or biopolymers. In particular, PICs have been widely developed as potential DNA, RNA and oligonucleotide delivery vehicles for gene therapy applications.³⁵ Poly(cationic) polypeptides are promising due to their versatile functionalities that can be used to overcome many challenges of gene therapy, such as (1) biocompatible components, (2) stabilization of nucleic acid, (3) reduction of non-specific interactions, (4) targeting of specific tissues and cells, (5) disruption of endosome membrane, and (6) release of cargo in cytoplasm or nucleus. Poly(lysine) had been used as a model polymer to investigate plasmid DNA complexation. The studies showed that low molecular weight poly(lysine), and stoichiometric ratio of negative charge from DNA to positive charge from polypeptide results in unstable complexes.³⁶ Poly(lysine) also suffers from high cytotoxicity, tendency to form aggregates and poor endosomal escape capability. Therefore, poly(lysine) derivatives have been developed to increase delivery efficiency. For instance, imidazole groups were introduced to facilitate endosomal escape, disulfide bonds and acid sensitive linkages provided triggered release of nucleic acids, while cross-linking and coating increased complex stability and allowed control over net surface charges.^{37, 38} Besides polypeptide- nucleic acid complexes, PIC has also seen applications in complexation with proteins³⁹ (e.g. BSA) and polysaccharides⁴⁰ (e.g. hyaluronic acid) for cargo release applications.

The aforementioned homopolymer based PIC systems have limited applications in cargo release, due to their large complex size and instability against cargo dissolution. However, adding non-charged hydrophilic blocks to the polyion components can easily solve these problems. The resulting block copolymers allow formation of more defined nanoscale stabilized structures, such as micelles and vesicles. The Kataoka group first developed PIC micelles based

on diblock copolymers: polyethylene glycol (PEG)-b-poly(lysine) (PEG-K) and polyethylene glycol (PEG)-b-poly(aspartic acid) (PEG-D). These micelles exhibited monodispersity as well as chain length recognition phenomena.^{41,42} By mixing the diblock PEG- K with homopolymer D, structures such as cylindrical micelles, connected cylindrical micelles and lamellae could also be produced.^{43,44} PIC micelle formation is also heavily dependent on secondary structure of the polyion core. For instance, homochiral PEG-K mixed with homochiral E forms micelles with β -sheet core structure. However, when racemic E is employed instead, coacervate micelles with random coiled core structure were observed. In addition, stimuli responsive PIC micelles have been developed, incorporating components with responses to redox⁴⁵, temperature⁴⁶, pH⁴⁷ and sugar presence⁴⁷ for triggered release applications. The Kataoka group also reported formation of the first semipermeable vesicles using similar block copolymers.⁴⁸ These vesicles consist of a PIC membrane surrounded by inner and outer PEG shells. Vesicle size and membrane thickness can be tuned by varying the concentration of the PIC components.⁴⁹ Stability, membrane permeability and drug release properties can also be modified by using carbodiimide crosslinker chemistry to enhance protease resistance and pH sensitivity.⁵⁰

PIC micelles containing hydrophilic PEG blocks can also increase delivery efficiency of nucleic acids⁵¹ and proteins⁵² for therapeutic applications (Figure 1.5). The PEG outer block can neutralize surface charge and prevent non-specific interactions while the cationic or anionic block complexes with the cargo to form the PIC core. Peg-K/ pDNA micelles was shown to remain soluble at stoichiometric N:P ratio, unlike the bulk K/pDNA complexes that precipitates out of solution.⁵¹ To overcome extra- and intracellular delivery barriers, poly-histidine⁵¹ and poly(N-(N-(2-aminoethyl)-2-aminoethyl)aspartamide) (poly(Asp(DET)))⁵² were employed in aiding endosomal escape via proton sponge effect. Nucleic acids were also conjugated to PEG

via an acid labile β -thiopropionate linkage that can be cleaved in the acidic endosome environment to release the active nucleic acid.⁵³ To improve the stability of nucleic acid against degradation, modification was also made to K to enable disulfide cross-linking in the micellar core.⁵⁴ In addition, cell targeting peptides such as, REKA, VHPKQHR were incorporated into the PIC micelle and shown to minimize non-specific interactions and enhance delivery specificity.⁵⁵ Anionic PEG-D was also complexed with cationic lysozyme and the resulting PIC micelles formed were evaluated as potential carriers for enzyme release applications. Results have shown that these micelles can preserve both lysozyme structure and activity within their PIC core.⁵⁶

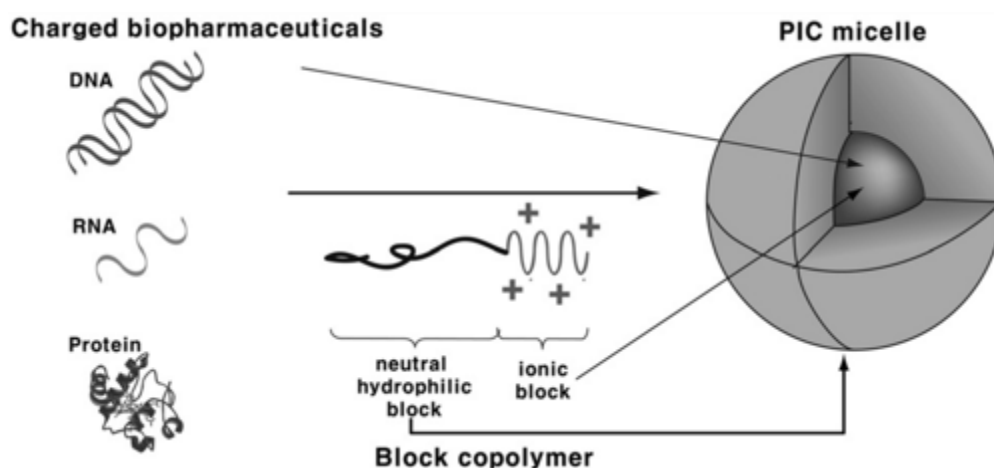


Figure 1.5 Scheme of PIC micelle formation from mixing of a diblock copolymer containing neutral hydrophilic block and ionic block with biopolymers such as nucleic acid, and protein. Reprinted (adapted) with permission from Lee, Y.; Kataoka, K. Biosignal-sensitive polyion complex micelles for the delivery of biopharmaceuticals. *Soft Matter* 2009, 5, 3810. Copyright © 2009, Royal Society of Chemistry

1.4 Polyion Complex Hydrogels

Hydrogels are three-dimensional hydrophilic polymeric networks that consists of high water content (up to >99 wt%). Hydrogels encompass a variety of chemical compositions and bulk physical prosperities and are used in a wide range of applications, such as diagnostics⁵⁷,

cellular immobilization⁵⁸, and regenerative medicine.⁵⁹ Additionally, these soft materials possess porous microstructures that resemble extracellular matrix, making them excellent candidates as cell carriers for tissue engineering.⁶⁰ There are two broad categories of hydrogels- chemical or covalently cross-linked and physical or non-covalently cross-linked. Advantages of physical hydrogels over chemical hydrogels include, (1) reversible sol-gel transition, (2) shear thinning properties that makes these materials injectable, (3) facile loading of cargoes into preformed hydrogel, (4) incorporation of responsive to stimuli such as temperature, pH, redox and ionic strength, and (5) circumvention of using permanent chemical crosslinkers for gelation.⁶¹ These unique features make physical hydrogels invaluable biomaterials for minimal invasive delivery applications.

Physical hydrogel systems consist of a major hydrophilic domain that solvates the system in aqueous environment and an associative domain that stabilizes the hydrogel through various physical interactions such as, hydrophobic, electrostatic, H-bonding, and host/guest.⁶² In particular, polyion complex (PIC) based hydrogels formed via electrostatic interactions have gained much attention in the recent years. There are also many merits to using these hydrogel materials based on polyion complexation. Electrostatic interactions are much stronger and will form more stable structures compared to those formed by hydrophobic interactions. The initial water-solubility of the unmixed PIC components bypasses the usage of organic solvent and allows homogenous mixing of the cargoes within the hydrogel. This is important for encapsulation of certain proteins, which may denature in organic solvents. Furthermore, rapid gelation of PIC hydrogel facilitates processing of the hydrogel and can benefit certain applications, such as 3D printing. Finally, mechanical properties of PIC hydrogels can be easily tuned by controlling external stimuli such as pH, salt and polymer concentration.⁶³

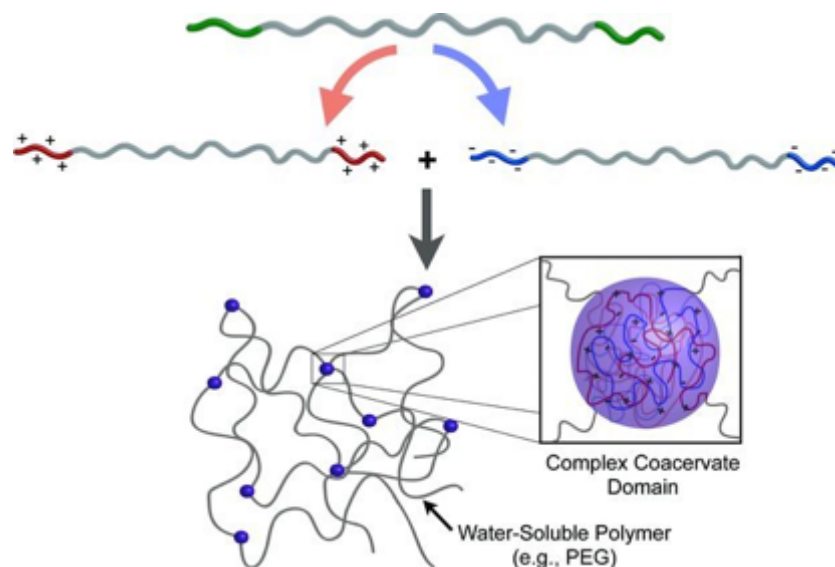


Figure 1.6 Scheme of PIC hydrogel formation from mixing of two ABA triblock copolymers with neutral hydrophilic middle block and oppositely charged ionic end blocks. Reprinted (adapted) with permission from Hunt, J. N.; Feldman, K. E.; Lynd, N. A.; Deek, J.; Campos, L. M.; Spruell, J. M.; Hernandez, B. M.; Kramer, E. J.; Hawker, C. J. *Advanced Materials* 2011, 23, 2326–2326. Copyright © 2011, John Wiley & Sons, Inc.

Conventional PIC hydrogel systems require usage of ABA triblock copolymer system, which consists of a neutral hydrophilic middle block and ionic end blocks. The Stuart group developed the first PIC hydrogel system, which was formed by complexation between anionic triblock copolymer, poly(3-sulfopropylmethacrylate)-b-PEG- poly(3-sulfopropylmethacrylate) (PSPMA-b-PEG- PSPMA), and cationic homopolymer, poly(allylamine hydrochloride)(AH). The reversible PIC hydrogel network was found to be composed of interconnected PIC micelles while hydrogel properties were shown to be dependent on polymer concentration, temperature, ionic strength, pH and charge ratio.⁶⁴ The Hawker and Kramer groups have developed a more robust PIC hydrogel by mixing two oppositely charged triblock copolymer with a central poly(ethylene glycol) (PEG) block and cationic (ammonium or guanidium) or anionic (sulfonate or carboxylate) end blocks in stoichiometric ratio (Figure 1.6).⁶³ Due to the strong and highly efficient electrostatic crosslinking by the end blocks within the coacervate domains, high

modulus hydrogels rapidly formed within seconds upon mixing at concentrations as low as 3-5 wt%. The Chen group also developed a similar polypeptide-based triblock hydrogel system with a PEG middle block and poly(L-glutamic acid) or poly(L-lysine) as the anionic or cationic end blocks. The resulting hydrogel showed tunable mechanical properties by varying concentration, pH and polymer composition. Furthermore, the polypeptide based PIC hydrogels were shown to be injectable, biocompatible and biodegradable.⁶⁵

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Chapter 2: Conformation Directed Formation of Self-healing Diblock Copolypeptide

Hydrogels via Polyion Complexation

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2.1 Abstract

Synthetic diblock copolypeptides were designed to incorporate oppositely charged ionic segments that form β -sheet structured hydrogel assemblies via polyion complexation when mixed in aqueous media. The observed chain conformation directed assembly was found to be required for efficient hydrogel formation, and provided distinct and useful properties to these hydrogels including self-healing after deformation, microporous architecture, and stability against dilution in aqueous media. While many promising self-assembled materials have been prepared using disordered or liquid coacervate polyion complex (PIC) assemblies, the use of ordered chain conformations in PIC assemblies to direct formation of new supramolecular morphologies is unprecedented. The promising attributes and unique features of the β -sheet structured PIC hydrogels described here highlight the potential of harnessing conformational order derived from PIC assembly to create new supramolecular materials.

2.2 Introduction

The concept of conformation directed assembly has been widely utilized in the design and preparation of ordered supramolecular structures.¹⁻³ In particular, assemblies containing peptides and polypeptides often take advantage of hydrophobic and H-bonding interactions between ordered chain conformations in these molecules, e.g. α -helices and β -strands, to produce

materials with advantageous properties, such as defined morphology or enhanced stability.⁴ Polypeptide containing block copolymer assemblies, such as micelles, vesicles, and hydrogels, have also been prepared using polyion complexes (PICs),^{5,6} where oppositely charged chain segments aggregate and phase separate upon mixing in aqueous media. In these systems, as well as those based on other synthetic polymers, formation of unstructured, liquid PIC coacervate domains is common and often desired. Fluidity in PIC coacervates can assist rapid complex formation and equilibration, while formation of solid β -sheet structures can lead to irregular assemblies with less desirable properties.⁵⁻⁸ In a contrarian approach, we sought to generate conformational order in polypeptides using PIC assembly to direct supramolecular assemblies into unprecedented morphologies with desirable properties. Using knowledge that certain homochiral polypeptide PICs form β -sheet aggregates,⁹⁻¹¹ we designed diblock copolypeptides incorporating such oppositely charged ionic segments that were able to form supramolecular hydrogels via PIC assembly in aqueous media. Since these hydrogels assemble via ordered chain conformations, different from other PIC hydrogels that require triblock copolymers and utilize disordered conformations,¹²⁻¹⁵ they possess distinct properties such as rapid recovery after stress and microporous architecture. Furthermore, these PIC diblock copolypeptide hydrogels (DCH_{PIC}) possess certain advantages over hydrophobically assembled DCH¹⁶ in that they are resistant to dilution in aqueous media, and are readily prepared at high concentrations for increased hydrogel stiffness.

Aqueous PIC assembly of block copolymers containing both non-ionic and oppositely charged ionic segments has been used to prepare a diverse array of micelles, vesicles, and hydrogels.^{5,6,17} Most polypeptide containing PIC assemblies utilize polyethylene glycol (PEG) chains as hydrophilic non-ionic segments, as well as ionic polypeptide segments that form

disordered or liquid coacervate immiscible phases.^{5,6,12-15,18,19} The resulting lack of internal order in the complexes tends to favor formation of spherical assemblies as found in diblock copolymer micelles and triblock copolymer hydrogels.^{5,6,12-15,18,19} Kataoka's lab has further shown that use of polypeptide segments that are long relative to PEG chain lengths results in formation of PICs some vesicular membrane assemblies.^{20,21} In the few examples where internal order has been incorporated into polypeptide PIC assemblies, via use of ionic α -helical segments⁸ or by β -sheet formation during assembly,^{7,22} only minimal perturbation of spherical micelle formation or slowed formation of micelles with increased polydispersity was observed. While there are examples of peptides and polypeptides where β -sheet structures are used to direct formation of self-assembled materials, these all rely on other components, such as hydrophobic and non-ionic residues to drive β -sheet formation.^{23,24} To the best of our knowledge, there are no reports demonstrating formation of new supramolecular morphologies that rely on assembly of polyelectrolyte segments into β -sheet rich domains.

The complexation of oppositely charged polypeptide electrolytes in aqueous media has been under study for many decades.^{5,6} Blout and Idelson were the first to report that mixture of homochiral poly(L-lysine-HCl), **K**, and poly(L-glutamate-Na), **E**, in water gave rise to phase separation of PICs rich in β -sheet content.⁹ Later studies confirmed this result and showed that while the mixture of L-lysine and L-glutamate homopolymers formed β -sheets, other combinations with L-ornithine or L-aspartate homopolymers gave only disordered PICs.¹⁰ This observation led to the hypothesis that interactions between hydrophobic side-chains, which are longer in lysine and glutamate, helped stabilize formation of the observed PIC β -sheet structure. More recently, the importance of chirality was shown by studies where replacement of one or both of the homochiral **K** and **E** components with racemic equivalents. i.e. *rac*-**K** or *rac*-**E**, led to

formation of liquid coacervates instead of solid β -sheets.^{7,22} Although most designs for polypeptide PIC assemblies favor formation of complexes with disordered internal structure,^{5,6} we sought to take advantage of the ability of homochiral **K** and **E** based PICs to form ordered β -sheet assemblies.

The design of DCH_{PIC} was developed from our understanding of hydrophobically assembled amphiphilic DCH. In these materials, the combination of long, hydrophilic segments with disordered conformations, and shorter hydrophobic segments with either α -helical or β -sheet ordered conformations, e.g. poly(L-lysine-HCl)₁₈₀-*block*-poly(L-leucine)₂₀, **K**₁₈₀**L**₂₀ or poly(L-lysine-HCl)₁₆₀-*block*-poly(L-valine)₄₀, **K**₁₆₀**V**₄₀, respectively, was found to direct their assembly in water into anisotropic structures that branch and entangle to give 3D hydrogel networks.¹⁶ The key molecular elements required for hydrogel formation are the conformation directed assembly of ordered hydrophobic segments into anisotropic structures instead of spherical micelles, and long, disordered hydrophilic chains that sterically limit packing of hydrophobic segments into tape-like assemblies as opposed to flat 2D membranes. To create DCH_{PIC}, we sought to test if the ordered hydrophobic component in amphiphilic DCH, e.g. β -sheet forming **V** segments, could be replaced with β -sheet PICs formed from mixing of **K** and **E** chains (Figure 2.1). Concurrently, it was also necessary to replace the disordered, ionic hydrophilic component of amphiphilic DCH, e.g. charged **K** segments, with a disordered, non-ionic hydrophilic component. While there are a few candidate water soluble, disordered, non-ionic polypeptides,²⁵ we chose to use segments based on water soluble poly(L-methionine sulfoxide), **M**^O, as this polymer is readily prepared, avoids the need to use racemic amino acid monomers, and is a naturally occurring residue that shows minimal toxicity.²⁶⁻²⁸

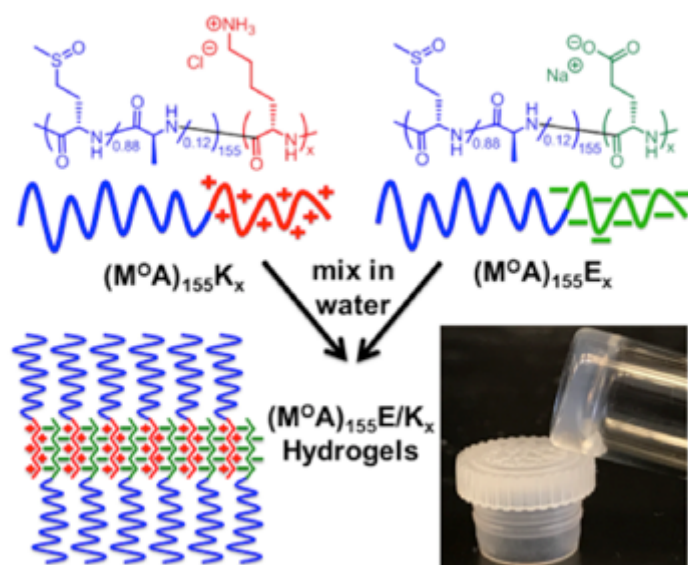


Figure 2.1 Schematic showing assembly process for preparation of polyion complex $(M^O A)_{155}E/K_x$ diblock copolyptide hydrogels.

2.3 Results and Discussion

Candidate DCH_{PIC} compositions were designed to include long, disordered hydrophilic segments as well as oppositely charged ionic segments that were able to form β -sheet complexes upon mixing (Figure 2.1). For shorter chain lengths (*ca.* 60 residues), non-ionic, hydrophilic M^O segments have been conveniently prepared from poly(L-methionine), **M**, precursors by post-polymerization oxidation.²⁸ However, at longer chain lengths, i.e. >100 residues, **M** polymers aggregate during polymerization, which limits the ability to control chain length and prepare well-defined block copolymers.²⁹ This issue was circumvented by mixing a small amount of L-alanine *N*-carboxyanhydride (NCA) monomer (*ca.* 12 mol %) with L-methionine NCA to prepare statistical copolymer segments that did not aggregate during polymerization due to disrupted side-chain packing.²⁹ The incorporation of a small amount of minimally hydrophobic alanine was found to allow efficient copolyptide synthesis without adversely affecting the

water solubility or disordered conformation of the resulting poly(L-methionine sulfoxide-*stat*-L-alanine), $\mathbf{M}^{\mathbf{O}}\mathbf{A}$, segments compared to $\mathbf{M}^{\mathbf{O}}$ homopolymer (Figure 2.2).^{26,28}

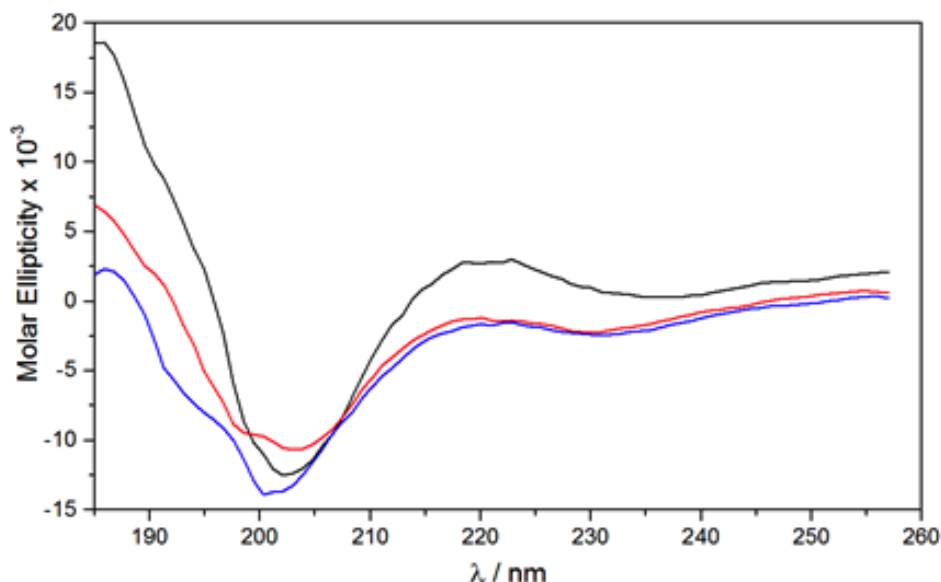


Figure 2.2 Circular dichroism spectra of $(\mathbf{M}^{\mathbf{O}})_{105}$ (black), $(\mathbf{M}^{\mathbf{O}}_{/0.90}\mathbf{A}_{/0.10})_{98}$ (blue), and $(\mathbf{M}^{\mathbf{O}}_{/0.82}\mathbf{A}_{/0.18})_{103}$ test polypeptides in DI water at 20 °C.

Using this strategy, we prepared diblock copolypeptides containing poly(L-methionine-*stat*-L-alanine), $\mathbf{MA}$, segments *ca.* 155 residues long, followed by side-chain protected $\mathbf{K}$ or $\mathbf{E}$ segments of different length (Figure 2.3).^{16,28} Subsequent oxidation of $\mathbf{M}$ residues, followed by side-chain deprotection of $\mathbf{K}$ and $\mathbf{E}$ residues and purification gave the target copolypeptides poly(L-methionine sulfoxide-*stat*-L-alanine)₁₅₅-*block*-poly(L-lysine-HCl)_x, $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{K}_x$; and poly(L-methionine sulfoxide-*stat*-L-alanine)₁₅₅-*block*-poly(L-glutamate-Na)_x, $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{E}_x$, where $x = 30, 60, 90$, and 120 (Figure 2.3). All copolymers were isolated in high yield with compositions that closely matched expected values (Table 2.1). The $\mathbf{M}^{\mathbf{O}}\mathbf{A}$ segment length was chosen based on values known, from previous studies on amphiphilic DCH,¹⁶ to be sufficiently long to promote hydrogel formation. The $\mathbf{K}$ and $\mathbf{E}$ lengths were varied in order to study the role of structured PIC domain size on hydrogel formation and properties.

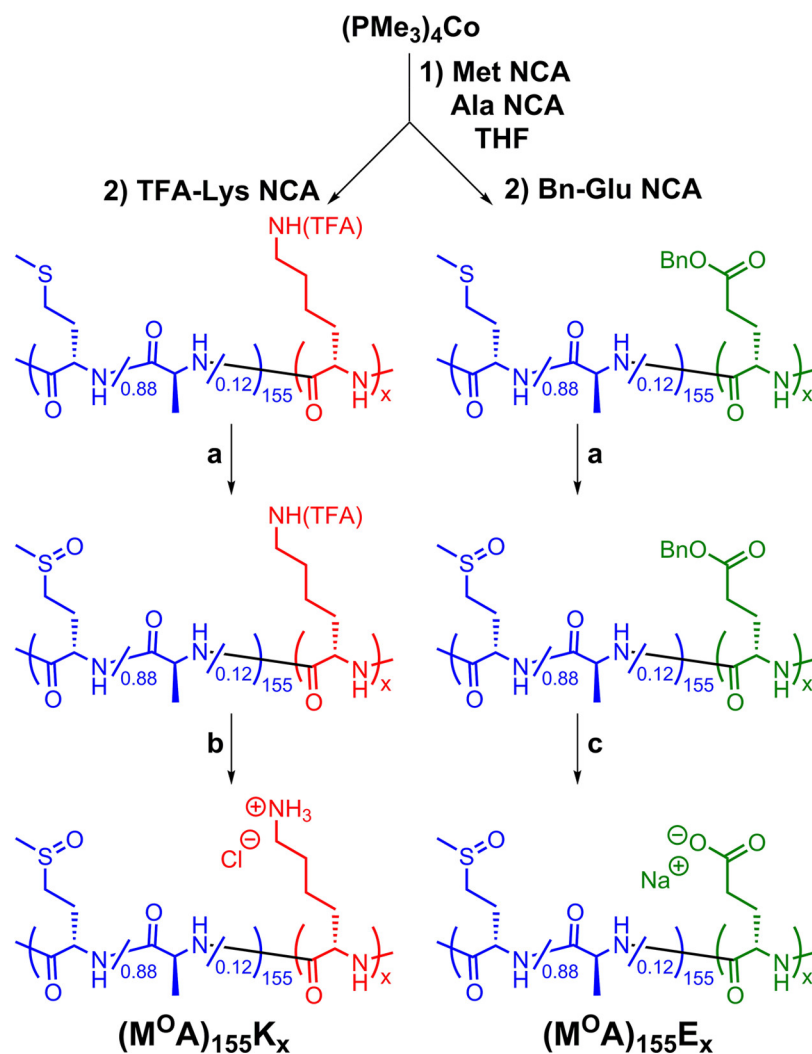


Figure 2.3 Synthesis of oppositely charged, dual hydrophilic diblock copolypeptides $(\text{M}^0\text{A})_{155}\text{K}_x$ and $(\text{M}^0\text{A})_{155}\text{E}_x$. a) TBHP, CSA, H_2O , $20\text{ }^\circ\text{C}$, 1 d. b) K_2CO_3 , $\text{MeOH}/\text{H}_2\text{O}$, $50\text{ }^\circ\text{C}$, 8 h. c) MSA, TFA, anisole, $20\text{ }^\circ\text{C}$, 1.5 h.

Table 2.1 Copolymerization data for diblock copolypeptide synthesis.

Sample	M_w/M_n ^a	Composition ^b	Yield (%) ^c
(M ^O A) ₁₅₅ E ₃₀	1.35	(M ^O A) ₁₅₆ E ₂₇	94
(M ^O A) ₁₅₅ E ₆₀	1.41	(M ^O A) ₁₅₆ E ₅₉	96
(M ^O A) ₁₅₅ E ₉₀	1.45	(M ^O A) ₁₅₆ E ₈₈	92
(M ^O A) ₁₅₅ E ₁₂₀	1.42	(M ^O A) ₁₅₆ E ₁₁₇	97
(M ^O A) ₁₅₅ (<i>rac</i> -E) ₆₀	1.45	(M ^O A) ₁₅₆ (<i>rac</i> -E) ₅₆	92
(M ^O A) ₁₅₅ K ₃₀	1.38	(M ^O A) ₁₅₆ K ₂₈	97
(M ^O A) ₁₅₅ K ₆₀	1.41	(M ^O A) ₁₅₆ K ₆₂	95
(M ^O A) ₁₅₅ K ₉₀	1.40	(M ^O A) ₁₅₆ K ₈₈	95
(M ^O A) ₁₅₅ K ₁₂₀	1.37	(M ^O A) ₁₅₆ K ₁₁₉	96

^a Dispersity of oxidized, protected block copolypeptides were determined by GPC/LS. ^b Relative amino acid compositions of oxidized, deprotected block copolypeptides were determined by ¹H NMR integrations. Degree of polymerization of initial **MA_x** segment was determined by end-group analysis using ¹H NMR. ^c Total isolated yield of purified block copolypeptides following deprotection.

For initial evaluation, matching length (M^OA)₁₅₅K_x and (M^OA)₁₅₅E_x samples were separately dissolved in aqueous 1x PBS buffer (5.0 wt% of each copolypeptide) to give clear solutions. These solutions were then combined in equal volumes at essentially stoichiometric **E** to **K** ratios (*ca.* 1.02-1.04 to 1) and agitated briefly in a vortex mixer, whereupon all samples ((M^OA)₁₅₅E/K_x, x = 30, 60, 90, and 120; 5.0 wt% total copolypeptide after mixing) were observed to form hydrogels within 15 seconds to 1 minute. These observations were confirmed by oscillatory rheology measurements where storage moduli (G') were found to dominate over loss moduli (G''), indicating elastic behavior for all samples (Figure 2.4A, Table 2.2).¹⁶

Mismatched mixtures, prepared either using non-stoichiometric **E** to **K** ratios, or by maintaining

E to **K** stoichiometry but combining samples of different length (e.g. three $(\text{M}^{\text{O}}\text{A})_{155}\text{K}_{30}$ to one $(\text{M}^{\text{O}}\text{A})_{155}\text{E}_{90}$), were found to give substantially weaker hydrogels compared to corresponding stoichiometric and length-matched samples. Stoichiometric hydrogels prepared using longer **E/K_x** segments (90 and 120) were opaque, likely due to microscopic aggregate precipitation. Hydrogels prepared using shorter **E/K_x** segments (30 and 60) were translucent, with only slight turbidity. In general, hydrogel stiffness (G') was found to increase with **E/K_x** segment length, yet aggregate precipitation with longer segments diminished this trend, as can be seen in G' for the **E/K₉₀** sample (Figure 2.4A). The minimum total copolyptide concentration required for hydrogel formation was found to be *ca.* 4.0 wt% for the $(\text{M}^{\text{O}}\text{A})_{155}\text{E/K}_{30}$ sample, and decreased with increasing **E/K_x** segment length.

Table 2.2 Properties of diblock copolyptide PIC hydrogels.

Composition(Acutal)	Concentration(wt%)	G' (Pa)	G'' (Pa)	Clarity
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{28}/\text{K}_{26}$	5.0	30	4	Clear
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{86}/\text{K}_{88}$	5.0	99	7	opaque
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{117}/\text{K}_{120}$	5.0	197	15	opaque
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{59}/\text{K}_{57}$	2.0	3	0.7	clear
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{59}/\text{K}_{57}$	3.0	29	2	clear
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{59}/\text{K}_{57}$	5.0	116	9	Clear
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{59}/\text{K}_{57}$	7.0	484	22	clear
$(\text{M}^{\text{O}}\text{A})_{156}\text{E}_{59}/\text{K}_{57}$	15	2280	181	clear

Samples were prepared in PBS buffer, 20 °C. G' = storage modulus; G'' = loss modulus. Values are averages of triplicate runs at 5 rad/s and strain amplitude of 0.05.

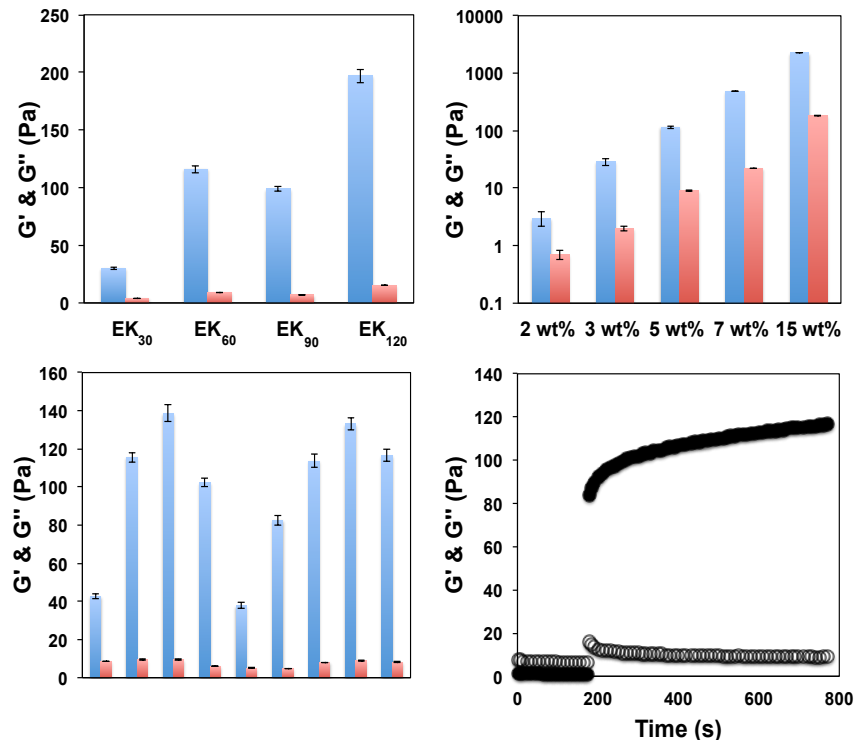


Figure 2.4 PIC diblock copolyptide hydrogel properties. (A) Storage modulus, G' (Pa, blue), and loss modulus, G'' (Pa, red), of hydrogels formed from stoichiometric $(M^0A)_{155}E/K_x$ with different ionic segment lengths ($x = 30, 60, 90$, and 120). Samples (5.0 wt % total combined cationic and anionic copolyptide) were prepared in 1x PBS buffer at 20 °C. (B) G' (Pa, blue) and G'' (Pa, red), of $(M^0A)_{155}E/K_{60}$ hydrogels at different concentrations in PBS buffer at 20 °C. (C) G' (Pa, blue) and G'' (Pa, red), of 5.0 wt % $(M^0A)_{155}E/K_{60}$ hydrogels prepared in different buffers at 20 °C. DMEM = Dulbecco's Modified Eagle Medium; FBS = fetal bovine serum. For (A, B, D), all G' and G'' values were measured at an angular frequency of 5 rad/s and strain amplitude of 0.05. (D) Recovery of 5.0 wt % in PBS $(M^0A)_{155}E/K_{60}$ hydrogel properties (G' , filled circles; G'' , open circles) over time after large amplitude oscillatory breakdown (strain amplitude of 10 at 5 rad/s for 200 s), followed by linear recovery measurement (strain amplitude of 0.05 at 5 rad/s).

Due to its desirable combination of hydrogel stiffness and minimal turbidity, $(M^0A)_{155}E/K_{60}$ was chosen as an optimized DCH_{PIC} composition for further study. Preparation of hydrogels using different concentrations of $(M^0A)_{155}E/K_{60}$ in 1x PBS was found to be a convenient means to adjust hydrogel stiffness (Figure 2.4B). All samples formed elastic hydrogels of similar clarity ($G' \gg G''$ over a range of frequency, Figure 2.5), and their stiffness was found to increase with higher copolyptide concentrations. The lack of visible aggregates

in these samples suggests that polymer chains were able to assemble into the desired structures even with fast mixing at high concentrations. Since the precursor solutions of two-component DCH_{PIC} are low viscosity liquids, it is noteworthy that these hydrogels can be readily prepared at significantly higher concentrations relative to one-component, amphiphilic DCH, where dissolution of copolypeptide at higher concentration (i.e. > 5.0 wt%) is hindered by spontaneous gel formation.¹⁶ (M^OA)₁₅₅E/K₆₀ hydrogels could also be prepared in a variety of aqueous media (Figure 2.4C). Solution ionic strengths in the range of *ca.* 100 to 300 mM were found to be superior for PIC hydrogel formation, while deionized water and higher salt concentrations (e.g. 500 mM NaCl) resulted in weaker hydrogels. For 5.0 wt % (M^OA)₁₅₅E/K₃₀ in 500 mM NaCl, a hydrogel did not form, and for 5.0 wt % (M^OA)₁₅₅E/K₉₀, hydrogels were able to form up to 1 M NaCl, but did not form in 2 M NaCl. These results suggest that lack of charge screening in pure water prevents annealing of PIC chains into ordered complexes (*vide infra*), while conversely excess charge screening in higher salt concentrations leads to weakened complexation, and is PIC length dependent.^{5,6} Finally, elevated temperature (80 °C for 1.5 h) was found to have no visible effect on a 5.0 wt% (M^OA)₁₅₅E/K₆₀ hydrogel in 1x PBS, showing that DCH_{PIC} possess good thermal stability. The self-healing properties of DCH_{PIC} after mechanical breakdown were studied by subjecting a 5.0 wt% (M^OA)₁₅₅E/K₆₀ sample in 1x PBS to high amplitude oscillatory strain, and then monitoring the recovery of elasticity over time by measuring G' at a much smaller strain amplitude (Figure 2.4D). During the initial 200 s of high strain amplitude, G' dropped by two orders of magnitude to below the level of G'', indicating the sample had become a viscous liquid. Upon switching to low strain amplitude, the sample began recovering its elastic properties, with most of the original gel stiffness regained within the brief period (*ca.* 10 s) needed to switch between strain amplitudes. Full recovery of DCH_{PIC} elasticity continued to

occur over a time scale of minutes. The rapid self-healing ability of DCH_{PIC}, which allows delivery of DCH_{PIC} via injection through small bore needles, is remarkably similar to that observed in amphiphilic DCH, such as **K**₁₈₀**L**₂₀ or **K**₁₆₀**V**₄₀, despite their substantial differences in chemical composition and mode of assembly.¹⁶ The observed similarity in physical properties may arise from the modular design of these block copolymers that likely leads to formation of similar supramolecular assembled structures.

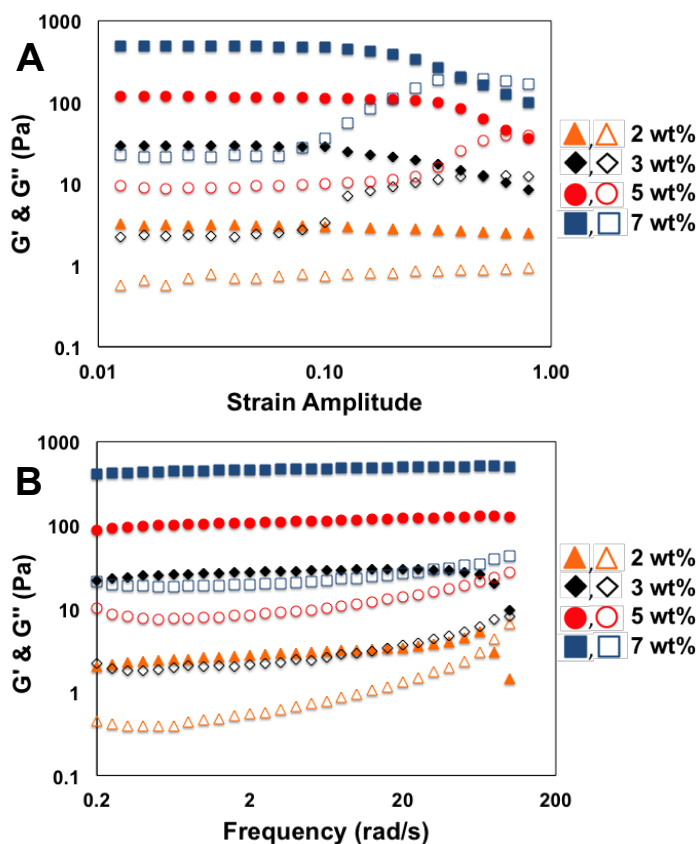


Figure 2.5 Rheology data for $(M^O A)_{155}E/K_{60}$ hydrogels at different concentrations in PBS buffer at 20 °C. (A) Storage modulus, G' (Pa, solid symbols), and loss modulus, G'' (Pa, open symbols), of $(M^O A)_{155}E/K_{60}$ hydrogels as functions of strain amplitude. (B) G' (Pa, solid symbols) and G'' (Pa, open symbols) of $(M^O A)_{155}E/K_{60}$ hydrogels as functions of angular frequency. The crossover of G' and G'' in the 2.0 and 3.0 wt% samples at high frequency is an artifact attributable to limitations of the measuring geometry (gap loading limit) and should not be considered to be a relaxation time.

To better understand the assembly of DCH_{PIC}, the influence of polyelectrolyte chirality on hydrogel formation was studied. We designed DCH_{PIC} such that the **K** and **E** segments were envisioned to form structured PICs, rich in β -sheet content, upon complexation.^{7,9,10} To test this hypothesis, a new copolypeptide component, $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}(\textit{rac-E})_{60}$, was prepared, where the *rac-E* segment was composed of racemic residues that should inhibit β -sheet formation in PICs.^{5-7,22} When equivalent amounts of $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}(\textit{rac-E})_{60}$ and $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{K}_{60}$ were mixed (total 5.0 wt% in 1x PBS), the resulting sample did not form a hydrogel and gave only a low viscosity liquid (Figure 2.6). This result confirmed the importance of chirality in formation of $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{E}/\mathbf{K}_{60}$ hydrogel structure. To directly verify the formation of β -sheet assembly in $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{E}/\mathbf{K}_x$ DCH_{PIC}, the hydrogels were also analyzed using FTIR, since different polypeptide conformations possess characteristic stretching frequencies for their Amide I and Amide II bands.³⁰ In FTIR analysis of lyophilized $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{E}/\mathbf{K}_x$ hydrogels ($x = 30, 60, 90$, and 120), all samples possessed strong 1653 cm^{-1} Amide I bands due to the disordered chain conformations of the $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}$ segments (Figure 2.2, 2.7). The samples also possessed 1630 cm^{-1} Amide I bands, characteristic of β -sheet chain conformations, which increased in intensity as $\mathbf{E}/\mathbf{K}_x$ segment length increased suggesting that this band resulted from PIC formation (Figure 2.7). The β -sheet Amide I band at 1630 cm^{-1} was only present in the homochiral $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{E}/\mathbf{K}_x$ PICs, and was absent in the individual components as well as the $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}(\textit{rac-E})/\mathbf{K}_{60}$ PIC formed with a racemic component (Figure 2.8). Together, these data confirmed that the **K** and **E** segments in $(\mathbf{M}^{\mathbf{O}}\mathbf{A})_{155}\mathbf{E}/\mathbf{K}_x$ PIC are assembling as β -sheets, and that this organized assembly is required for hydrogel formation.

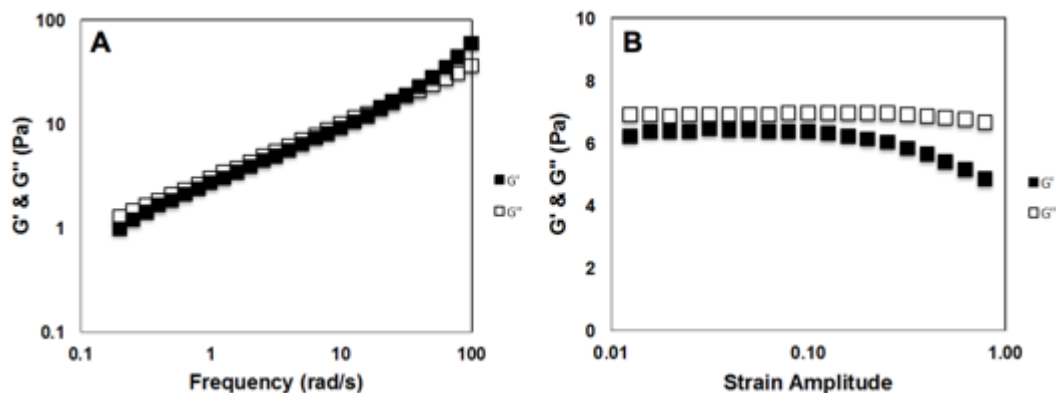


Figure 2.6 Rheology data for 5.0 wt% $(M^O A)_{155}(rac-E)/K_{60}$ in PBS buffer at 20 °C. (A) Storage modulus G' (solid symbols) and loss modulus G'' (open symbols) as a function of angular frequency (strain amplitude of 0.05). (B) Strain sweep at angular frequency of 6 rad/s.

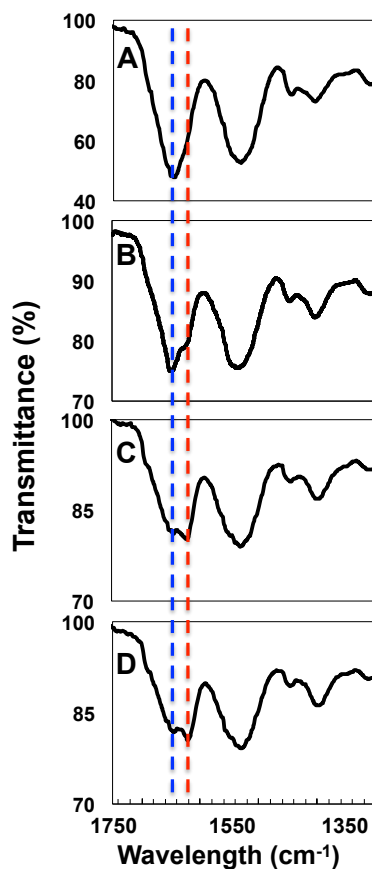


Figure 2.7 ATR-IR spectra of lyophilized $(M^O A)_{155}E/K_x$ samples in the amide region. Blue line = 1653 cm^{-1} Amide I band characteristic of α -helical and disordered chain conformations. Red line = 1630 cm^{-1} Amide I band characteristic of β -sheet chain conformations. (A) E/K_x ; $x = 30$. (B) E/K_x ; $x = 60$. (C) E/K_x ; $x = 90$. (D) E/K_x ; $x = 120$. 1630 cm^{-1} β - Amide I band increases with E/K_x content.

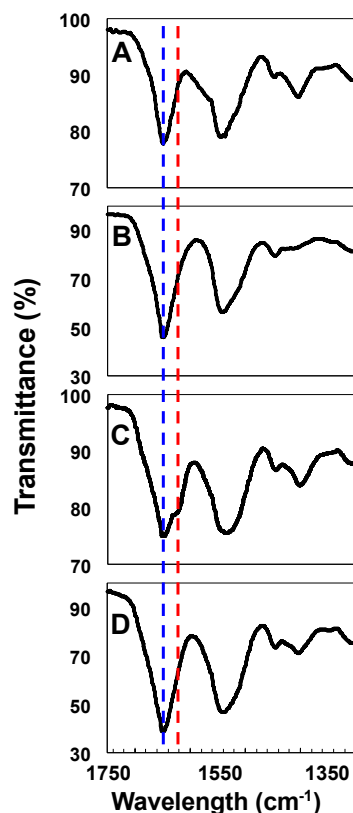


Figure 2.8 ATR-IR spectra of lyophilized samples in the amide region. Blue line = 1653 cm^{-1} Amide I band characteristic of α -helical and disordered chain conformations. Red line = 1630 cm^{-1} Amide I band characteristic of β -sheet chain conformations. (A) $(\text{M}^{\text{O}}\text{A})_{155}\text{E}_{60}$. (B) $(\text{M}^{\text{O}}\text{A})_{155}\text{K}_{60}$. (C) $(\text{M}^{\text{O}}\text{A})_{155}\text{E}/\text{K}_{60}$. (D) $(\text{M}^{\text{O}}\text{A})_{155}(\text{rac-E})/\text{K}_{60}$. Only $(\text{M}^{\text{O}}\text{A})_{155}\text{E}/\text{K}_{60}$ shows presence of β -sheet content.

The supramolecular structure of $(\text{M}^{\text{O}}\text{A})_{155}\text{E}/\text{K}_{60}$ hydrogels was analyzed at both microscale and nanoscale resolution. To visualize microscopic structure, chains of $(\text{M}^{\text{O}}\text{A})_{155}\text{E}_{60}$ and $(\text{M}^{\text{O}}\text{A})_{155}\text{K}_{60}$ were separately conjugated with different fluorescent probes (tetramethylrhodamine and fluorescein, respectively) and then mixed to form DCH_{PIC} . Laser scanning confocal microscopy (LSCM) was then used to visualize the labeled chains and the hydrogel network (Figure 2.9A,B). Both **K** labeled (TRITC, red) and **E** labeled (FITC, green) channels showed DCH_{PIC} are composed of microporous networks containing interconnected polypeptide rich domains that coexist with domains primarily composed of water, seen as dark regions in the images.³¹ An overlay of red and green channels revealed that **K** and **E** segments

are co-localized, indicating good mixing of the components within the DCH_{PIC} domains (Figure 2.9C). Cryo electron microscopy (cryoEM) imaging of a thin layer of vitrified (**M^OA**)₁₅₅**E/K**₆₀ hydrogel showed structures resembling “plumber’s nightmare” morphologies, which consist of membrane like regions interconnected with tape-like struts, and contain many defects that form a nanoporous network (Figure 2.9D). The combination of microscale and nanoscale structure in DCH_{PIC} is reminiscent of structure observed in amphiphilic DCH.¹⁶ The design of DCH_{PIC} appears to have been successful in mimicking amphiphilic DCH by replicating many of their structural features and properties even though DCH_{PIC} utilize PICs instead of hydrophobic segments for self-assembly.

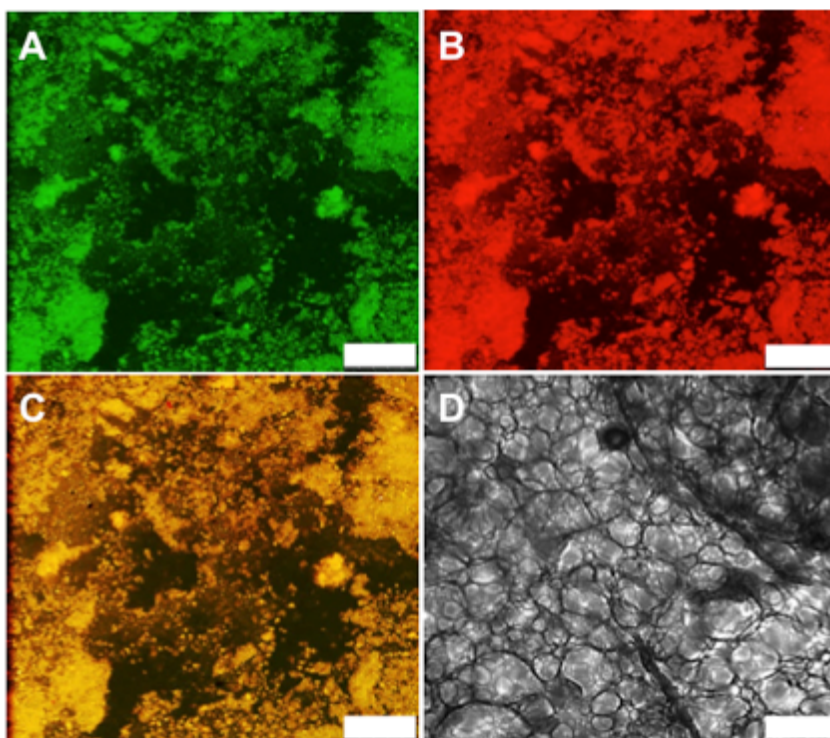


Figure 2.9 Laser scanning confocal and cryoelectron microscopy (cryoEM) images of (**M^OA**)₁₅₅**E/K**₆₀ hydrogels. (A,B,C) LSCM images (z-thickness = 0.78 μm) of TRITC labeled (**M^OA**)₁₅₅**K**₆₀ (red) and FITC labeled (**M^OA**)₁₅₅**E**₆₀ (green) hydrogel mixtures showing microporous structure (3.0 wt% in PBS). (A) FITC channel; (B) TRITC channel; (C) Merge of A and B. (D) CryoEM image of (**M^OA**)₁₅₅**E/K**₆₀ hydrogel showing nanoporous structure (2.0 wt% in PBS). Scale bars: A,B,C = 25 μm; D = 200 nm.

Compared to amphiphilic DCH, DCH_{PIC} were found to possess a significant difference in hydrogel stability against dilution in aqueous media. Amphiphilic DCH are formed by direct dissolution of solid copolypeptide in aqueous media, where samples, e.g. **K₁₈₀L₂₀**, swell to fill the volume and spontaneously form hydrogels. If additional media is then added, the copolypeptides will continue to disperse to give diluted samples that fill the larger volume.¹⁶ While this property allows facile preparation of DCH formulations for a number of applications,³² it does limit the ability to suspend hydrogels in excess media, as could be desired for *in vitro* cell culture.³³ To study the stability of DCH_{PIC} against dilution in aqueous media, a 5.0 wt% (**M^OA**)₁₅₅**E/K₆₀** hydrogel in PBS was prepared, and then an equal volume of DMEM cell culture media was added on top of the hydrogel (Figure 2.10A). For comparison, a similar experiment was performed using a 2.0 wt% **K₁₈₀L₂₀** hydrogel in PBS (Figure 2.10B), where its concentration was chosen to match the stiffness of the (**M^OA**)₁₅₅**E/K₆₀** hydrogel. Initially, the DMEM solutions formed clear layers above both hydrogels. After 3 days, the **K₁₈₀L₂₀** sample had fully mixed with the DMEM layer and the diluted sample was a viscous liquid. With (**M^OA**)₁₅₅**E/K₆₀**, although the DMEM solutes were able to diffuse into the sample over 3 days, the hydrogel was able to retain its shape and stiffness (Figure 2.10C,D). Compared to the hydrophobic interactions of amphiphilic DCH, the combination of H-bonding and electrostatic interactions present in the assemblies of DCH_{PIC} was found to impart superior resistance against dissolution in aqueous media.

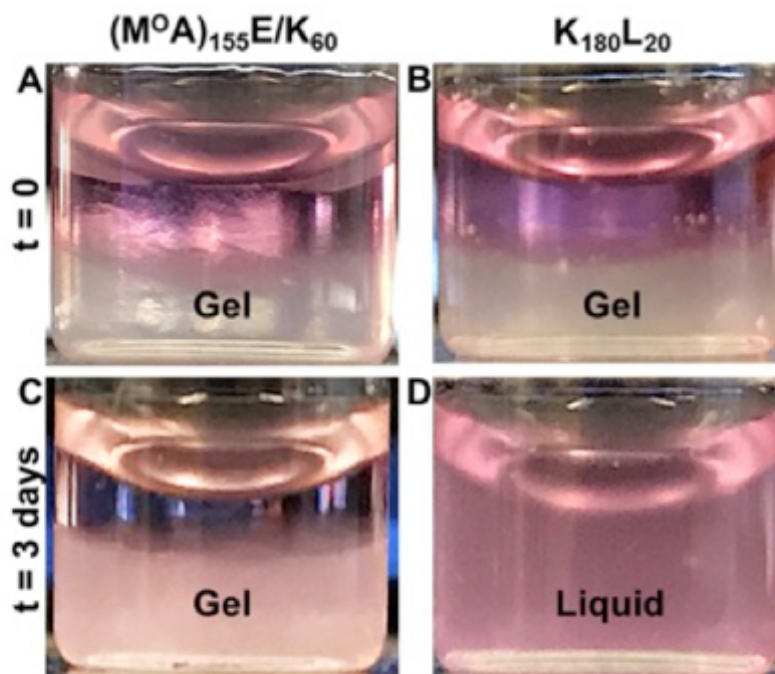


Figure 2.10 Stability of diblock copolyptide hydrogels against dilution. Polyion complex $(M^O A)_{155}E/K_{55}$ (A and C, 5.0 wt%) and hydrophobic assembled $K_{180}L_{20}$ (B and D, 2.0 wt%) hydrogels of equivalent stiffness ($G' \approx 120$ Pa) in 1x PBS were each diluted with an equal volume of DMEM cell culture media. (A,B) A layer of cell media formed over both gels at the beginning of the experiment (time = 0). (C,D) After 3 days, the $(M^O A)_{155}E/K_{60}$ hydrogel remained intact, while $K_{180}L_{20}$ had dispersed into the full volume of media and was a liquid.

The ability of DCH_{PIC} to resist dissolution or swelling once formed provides a means to cast hydrogel shapes from precursor solutions, and then use these stable hydrogels for various applications in aqueous media. To showcase their potential utility, we encapsulated primary neural stem progenitor cells (NSPCs)³³ in a $(M^O A)_{155}E/K_{60}$ hydrogel (Figure 2.11). The NSPCs were encapsulated by mixing a suspension of cells in media with an equal volume of $(M^O A)_{155}E_{60}$ solution in media, which was then combined with an equal volume of $(M^O A)_{155}K_{60}$ solution in media to rapidly form the cell containing hydrogel. This sample, as well as cell only and cell in $K_{180}L_{20}$ hydrogel controls in media, was incubated for 1 day, and then cell viability was quantified using a Live/Dead assay (Figure 2.11).³⁴ The cationic $K_{180}L_{20}$ hydrogel was

found to be highly cytotoxic, as expected from the literature,³³ and served as a good negative control. The $(M^O A)_{155}E/K_{60}$ hydrogel provided good cell viability, similar to the cells in media only control, which suggests that DCH_{PIC} may be promising for use as a cell carrier. Although DCH_{PIC} contain long, charged polypeptide segments, these are effectively sequestered by PIC formation and steric shielding from the uncharged $M^O A$ hydrophilic segments, resulting in hydrogels that are effectively non-ionic.³⁵ Although cells were exposed to the non-complexed, charged components of DCH_{PIC} during the mixing process, this brief exposure, regardless of mixing order, was found to have minimal adverse effects on cell viability. More detailed studies on cell suspension in DCH_{PIC} will be the subject of future investigations.

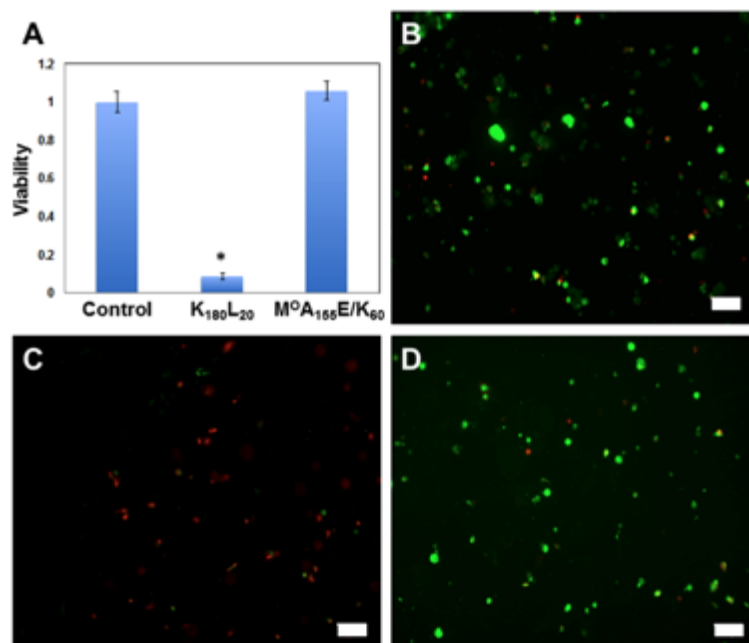


Figure 2.11 Viability of neural stem progenitor cells (NSPCs) encapsulated in hydrogels. (A) Plot of NSPC viability after 1 day incubation in different conditions: cells in media only control, in media plus 2.0 wt% $K_{180}L_{20}$ hydrogel, or in media plus 5.0 wt% $(M^O A)_{155}E/K_{60}$ hydrogel. (B-D) Fluorescence microscopy images of NSPC cells after 1 day incubation under different conditions and then stained using the Live/Dead® viability/cytotoxicity assay where green is due to calcein (live cells) and red is due to EthD-1 (dead cells). (B) cells in media only control. (C) cells in media plus 2.0 wt% $K_{180}L_{20}$ hydrogel. (D) cells in media plus 5.0 wt% $(M^O A)_{155}E/K_{60}$ hydrogel. Scale bars = 100 μm . * $p < 0.0001$ (Unpaired student's t-test for $K_{180}L_{20}$ with either cell control or $(M^O A)_{155}E/K_{60}$).

2.4 Conclusion

A new class of cell-compatible copolypeptide hydrogels, DCH_{PIC}, that possess chain conformation directed PIC supramolecular architectures was reported. The use of polypeptide components that form β -sheets upon polyion complexation, in combination with non-ionic disordered segments, led to anisotropic supramolecular assembly into anisotropic structures that form extended hydrogel networks capable of rapid self-healing after deformation. Unlike amphiphilic DCH, the use of PIC assembly in DCH_{PIC} was found to impart these materials with stability against dilution in aqueous media, a valuable feature for downstream applications. While there are many examples of useful materials based on liquid coacervate or disordered PIC assemblies, the promising attributes and unique features of DCH_{PIC} highlight the potential for use of conformational order in PIC assembly to create new supramolecular materials.

2.5 Experimental

2.5.1 Materials and Methods

Tetrahydrofuran (THF) and hexanes were dried by purging with nitrogen and passage through activated alumina columns prior to use. Co(PMe₃)₄ and amino acid *N*-carboxyanhydride (NCA) monomers were prepared according to literature procedures.³⁶ All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Selecto silica gel 60 (particle size 0.032– 0.063 mm) was used for flash column chromatography. Fourier Transform Infrared (FTIR) measurements were taken on a Perkin Elmer RX1 FTIR spectrophotometer calibrated using polystyrene film, and attenuated total reflectance (ATR-IR) data were collected using a PerkinElmer Spectrum 100 FTIR spectrometer equipped with a universal ATR sample accessory. ¹H NMR spectra were acquired on a Bruker

ARX 400 spectrometer. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed at 25 °C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 mm columns at 30 °C with 0.5% (w/w) KTFA in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) as eluent and sample concentrations of 10 mg/ml. Pyrogen free deionized water (DI) was obtained from a Millipore Milli-Q Biocel A10 purification unit. Circular Dichroism spectra were recorded in quartz cuvettes of 0.1 cm path length with samples prepared at concentrations between 0.10 to 0.17 mg/mL using Millipore deionized water. The spectra are reported in units of molar ellipticity $[\theta]$ ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$), using the formula, $[\theta] = (\theta \times 100 \times M_w)/(c \times l)$, where θ is the measured ellipticity in millidegrees, M_w , is the average residue molecular mass in g/mol, c is the peptide concentration in mg/mL; and l is the cuvette path length in cm.

2.5.2 Experimental Procedure

All polymerization reactions were performed in an N₂ filled glove box using anhydrous solvents. To a solution of L-methionine NCA (Met NCA) and L-alanine NCA (Ala NCA) in THF (50 mg/ml), a solution of Co(PMe₃)₄ in THF (20 mg/ml) was added.¹ The reaction was let to stir at ambient temperature (*ca.* 22 °C) for 60 min. Complete consumption of NCA was confirmed by FTIR spectroscopy, and then the desired amount of γ -benzyl-L-glutamate NCA (Bn-Glu NCA) or ϵ -TFA-L-lysine NCA (TFA-Lys NCA) in THF (50 mg/ml) was added to the reaction mixture, which was let to stir for an additional 60 min. FTIR was used to confirm complete consumption of all NCAs. Outside the glove box, the block copolypeptide solutions were precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl (2x 20 ml)

to remove residual cobalt ions. The white precipitates were then washed with DI water (3x 20 ml) and freeze-dried.³⁶

Example synthesis of poly(L-methionine_{0.88}-stat-L-alanine_{0.12})₁₅₅-block-poly(ε-trifluoroacetyl-L-lysine)₆₀, (MA)₁₅₅(TFA-K)₅₅ and poly(L-methionine_{0.88}-stat-L-alanine_{0.12})₁₅₅-block-poly(γ-benzyl-L-glutamate)₆₀, (MA)₁₅₅(Bn-E)₆₀

Met NCA (120 mg, 0.71 mmol) and Ala NCA (11 mg, 0.097 mmol) were dissolved together in THF (2.7 ml) and placed in a 20 ml scintillation vial containing a stir bar. To the vial, (PMe₃)₄Co initiator solution (260 μl of a 20 mg/ml solution in THF) was added via syringe. The vial was sealed and allowed to stir in the glove box for 1 h. An aliquot (20 μl) was removed and analyzed by FTIR to confirm that all the NCA was consumed. In the glove box, α-methoxy-ω-isocyanoethyl-poly(ethylene glycol)₄₅ (mPEG₂₃-NCO)¹ (20 mg) was dissolved in THF (1 ml) in a 20 ml scintillation vial. An aliquot (350 μl) of the polymerization solution containing active chain ends was removed and added to the solution of mPEG₂₃-NCO. The PEG end-capped sample (MA_x-mPEG₂₃) was sealed, allowed to stir for 24 h, and then used for chain length determination (*vide infra*). Separately, aliquots of the polymerization solution containing active chains (1.2 ml each) were added to vials containing either Bn-Glu NCA (32 mg, 0.12 mmol) or TFA-Lys NCA (33 mg, 0.12 mmol) dissolved in THF (64 μl or 65 μl, respectively). The vials were sealed and allowed to stir in the glove box for 1 h to give the diblock copolypeptides, (MA)₁₅₅(TFA-K)₆₀ and (MA)₁₅₅(Bn-E)₆₀. FTIR was used to confirm complete consumption of NCAs in both reactions. Outside the glove box, the block copolypeptide solutions were precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl (2 x 20 ml) to remove residual cobalt ions. The white precipitates were then washed with DI water (3 x 20 ml) and freeze-dried (average yield = 98%).

Analytical data: (MA)₁₅₅(Bn-E)₆₀

¹H NMR (400 MHz, d-TFA, 25 °C): δ 7.38 (br m, 2.3H), 5.24 (br m, 0.93H), 4.97 (br s, 1H), 4.81 (br m, 0.54H), 2.81 (br m, 2H), 2.6 (br m, 1.06 H), 2.40 - 2.05 (br m, 6.37H), 1.61 (br s, 0.42H). FTIR (THF, 25 °C): 1738 cm⁻¹ (benzyl ester), 1652 cm⁻¹ (amide I), 1550 cm⁻¹ (amide II).

Analytical data: (MA)₁₅₅(TFA-K)₆₀

¹H NMR (400 MHz, d-TFA, 25 °C): δ 4.86 (br s, 0.94 H), 4.60 (br m, 0.54H), 3.46 (br m, 1.23 H), 2.69 (br m, 2H), 2.17 (br m, 5H), 1.9 (br m, 1.42 H), 1.69 (br m, 1.34 H), 1.50 (br m, 1.32 H), 1.31 (br m, 0.68 H). FTIR (THF, 25 °C): 1726 cm⁻¹ (TFA amide), 1652 cm⁻¹ (amide I), 1550 cm⁻¹ (amide II).

Sample procedure for MA_x chain length determination using end-group analysis

Outside of the glove box, the PEG end-capped sample (MA_x-mPEG₂₃) from above was washed with 10 mM aqueous HCl (2x). After stirring for 1 h, MA_x-mPEG₂₃ was collected by centrifugation and washed with DI water (3 x 20 ml) to remove all non-conjugated mPEG₂₃-NCO. The remaining MA_x-mPEG₂₃ was then freeze-dried to remove residual H₂O. To determine MA_x molecular weights (M_n), ¹H NMR spectra were obtained. Since it has been shown that end-capping is quantitative for (PMe₃)₄Co initiated NCA polymerizations when excess isocyanate is used,³⁷ integrations of methionine (δ 2.70) and alanine (δ 1.52) resonances versus the polyethylene glycol resonance at δ 3.92 could be used to obtain both **M** to **A** ratios and MA_x lengths (found: x = 156, designated as MA₁₅₅). ¹H NMR (400 MHz, d-TFA, 25 °C): 4.87 (br s, 1H), 4.68 (br s, 0.167H), 3.92 (br m, 0.71H), 2.70 (br m, 2.03 H), 2.30 - 2.05 (br m, 5.16H), 1.52 (br s, 0.43H).

Preparation of poly(L-methionine sulfoxide_{0.88}-stat-L-alanine_{0.12})₁₅₅-block-poly(ε-trifluoroacetyl-L-lysine)₆₀, (M^OA)₁₅₅(TFA-K)₆₀, and poly(L-methionine sulfoxide_{0.88}-stat-L-alanine_{0.12})₁₅₅-block-poly(γ-benzyl-L-glutamate)₆₀, (M^OA)₁₅₅(Bn-E)₆₀

In separate scintillation vials (5 ml) containing stir bars, (MA)₁₅₅(TFA-K)₆₀ and (MA)₁₅₅(Bn-E)₆₀ were suspended in 80% *tert*-butyl hydroperoxide (TBHP) in water (16 eq TBHP per methionine residue). Camphorsulfonic acid (0.2 eq per methionine residue) was then added to each vial, and DI water was added to give final copolymer concentrations of *ca.* 20 mg/ml. These reactions were stirred for 16 h at ambient temperature (*ca.* 22 °C). Saturated sodium thiosulfate (0.5 ml) was then added dropwise to each vial in order to quench the reactions, and the samples were transferred to 2000 MWCO dialysis tubes and then dialyzed against DI water for 2 d with frequent water changes. The resulting solutions were freeze-dried to yield white fluffy solids (average yield =97%).³⁸

Analytical Data: (M^OA)₁₅₅(Bn-E)₆₀

¹H NMR (400 MHz, d-TFA, 25 °C): δ 7.24 (br m, 2.2H), 5.10 (br m, 0.91H), 4.85 (br s, 1H), 4.69 (br m, 0.55H), 3.45 - 3.10 (br m, 2.06H), 2.90 (br m, 3 H), 2.62 (br m, 1.04 H), 2.47 (br m, 1.86 H), 2.18 (br m, 0.45H), 1.97 (br m, 0.45), 1.49 (br s, 0.40 H).

Analytical Data: (M^OA)₁₅₅(TFA-K)₆₀

¹H NMR (400 MHz, d-TFA, 25 °C): δ 4.91 (br s, 1H), 4.64 (br m, 0.52H), 3.52-3.10 (br m, 2.96 H), 2.96 (br m, 3.03H), 2.67 (br m, 1.04 H), 2.46 (br m, 1 H), 1.96 (br m, 0.86 H), 1.73 (br m, 0.88 H), 1.54 (br m, 1.27 H).

Preparation of poly(L-methionine sulfoxide_{0.88}-*stat*-L-alanine_{0.12})₁₅₅-*block*-poly(L-lysine)₆₀, (M^OA)₁₅₅K₆₀

A sample of (M^OA)₁₅₅(TFA-K)₆₀ was dispersed in a 9:1 methanol:water mixture (20 mg/ml) and K₂CO₃ (2 eq per lysine residue) was added. The reaction was stirred for 8 h at 50 °C, and the majority of the methanol was then removed under vacuum. The resulting solution (*ca.* 10% of original volume) was then diluted with water (3 times the remaining volume), transferred to a 2000 MWCO dialysis bag, and then dialyzed against 0.10 M aqueous NaCl at pH 3 (HCl) for 24 h, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give a white solid (yield = 93%).¹ ¹H NMR (400 MHz, D₂O, 25 °C): δ 4.52 (br s, 1H), 4.37 (br m, 0.52H), 3.2-2.8 (br m, 3.18 H), 2.75 (br m, 3.1 H), 2.40 - 2.20 (br m, 2.2 H), 1.73 (br m, 1.62H), 1.44 (br m, 1.32H). ATR-IR (25 °C): 1653 cm⁻¹ (amide I), 1546 cm⁻¹ (amide II).

Preparation of poly(L-methionine sulfoxide_{0.88}-*stat*-L-alanine_{0.12})₁₅₅-*block*-poly(L-glutamate)₆₀, (M^OA)₁₅₅E₆₀

A sample of (M^OA)₁₅₅(Bn-E)₆₀ was dissolved in trifluoroacetic acid (TFA, 30 eq per benzyl glutamate residue) in an ice bath. Methanesulfonic acid (MSA, 35 eq) and anisole (5 eq) were then added under vigorous stirring. The reaction mixture was stirred for 20 min in the ice bath, and then for an additional 90 min at ambient temperature. Next, the copolymer was precipitated using Et₂O (20 ml) and collected by centrifugation. The pellet was dissolved in 10% aqueous NaHCO₃ (3 ml), extensively dialyzed (2000 MWCO) against DI water for 2 d, and then freeze-dried to give a white solid (yield = 95%).³⁹ ¹H NMR (400 MHz, D₂O, 25 °C): δ 4.50 (br s, 1H), 4.40 (br m, 0.57H), 3.00 (br m, 2.03H), 2.75 (br m, 2.95 H), 2.40 - 2.10 (br m, 3 H), 2.10 -

1.80 (br m, 1H), 1.44 (br s, 0.4 H). ATR-IR (25 °C): 1653 cm^{-1} (amide I), 1546 cm^{-1} (amide II).

Preparation of $(\text{M}^{\text{O}}\text{A})_{155}\text{E}/\text{K}_x$ PIC hydrogels

Samples of $(\text{M}^{\text{O}}\text{A})_{155}\text{E}_x$ and $(\text{M}^{\text{O}}\text{A})_{155}\text{K}_x$ were separately dissolved in a desired aqueous medium (e.g. DI water, 1x PBS, etc.) at a desired concentration (e.g. 1, 1.5, or 2.5 wt%). Once each copolymer was fully dissolved, the copolymer solutions were combined in a scintillation vial (2 ml) and vortexed rigorously for 15 s using a Fisher Vortex Genie 2. The concentration of PIC hydrogel was same as the concentrations of the two components. The duration of time before gelation occurred (i.e. gelation time) was found to vary from seconds to minutes depending on sample concentration, the ionic strength, and copolymer composition. A “5 second inversion test” was used to initially confirm gel formation.⁴⁰

Rheology measurements on $(\text{M}^{\text{O}}\text{A})_{155}\text{E}/\text{K}_x$ PIC hydrogels

A TA Instruments AR 2000 rheometer with a 20 mm parallel plate geometry and solvent trap was used for all measurements. Frequency sweeps were measured at a constant strain amplitude of 0.05. Strain sweeps were measured at a constant frequency of 5 rad/s. All measurements were repeated 3 times for each hydrogel sample and the results were averaged and plotted. In general, the standard errors for frequency sweeps were less than 3.5%, while the standard errors for strain sweeps were less than 2.5%.

Fluorescent probe conjugation to $(\text{M}^{\text{O}}\text{A})_{155}\text{E}_{60}$ and $(\text{M}^{\text{O}}\text{A})_{155}\text{K}_{60}$ copolypeptides

Tetramethylrhodamine isothiocyanate (TRITC) was conjugated to amine groups of lysine side chains. $(\text{M}^{\text{O}}\text{A})_{155}\text{K}_{60}$ (10 mg) was dissolved in pH 10 $\text{H}_2\text{O}/\text{NaOH}$ (1 ml) in a scintillation vial (20 ml). TRITC was dissolved in DMSO (1 mg/ml) and added to the 1 % (w/v) copolypeptide solution at a 5:1 molar ratio of copolypeptide chains to fluorescent probes. The

reaction was allowed to proceed for 24 h at ambient temperature. After TRITC modification, the resulting solution was dialyzed (2000 MWCO) against DI water for 2 d, and then freeze-dried to yield the product as an orange solid. Fluorescein isothiocyanate (FITC) was conjugated onto the N-terminal amine of $(\text{M}^{\text{O}}\text{A})_{155}\text{E}_{60}$ using a similar procedure.

Laser scanning confocal microscopy (LSCM) of fluorescently labeled hydrogels

LSCM images of hydrogels (3.0 wt% in PBS) were taken on a Leica TCS-SP1 MP-Inverted Confocal and Multiphoton Microscope equipped with an argon laser (476 and 488 nm blue lines), a diode (DPSS) laser (561 nm yellow-green line), and a helium-neon laser (633 nm far red line). Fluorescently labeled hydrogel samples were visualized on glass slides with a spacer between the slide and the cover slip (double-sided tape) allowing the self-assembled structures to be minimally disturbed during focusing. A Z-slice thickness of 0.78 μm was used. Sample imaging was performed at the Advanced Light Microscopy/Spectroscopy Center (ALMS) at the UCLA California NanoSystems Institute (CNSI).

Cryogenic transmission electron microscopy (cryoEM) of hydrogels

25 μl of a 2.0 wt% $(\text{M}^{\text{O}}\text{A})_{155}\text{E}/\text{K}_{60}$ hydrogel in PBS buffer was applied on a glass coverslip to form a flat surface onto which an EM grid with lacey carbon was gently placed against with a pair of tweezers to acquire a thin layer of sample. The EM grid was then plunged into liquid nitrogen-cooled ethane to make a cryoEM grid. The frozen sample was examined in an FEI TF20 cryo electron microscope at liquid nitrogen temperature. Low dose cryoEM images were recorded on CCD camera at 4.4 $\text{\AA}/\text{pixel}$ on the specimen level and a defocus value of about -5 μm . Sample preparation and imaging was performed at the Electron Imaging Center for Nanomachines (EICN) at the UCLA California NanoSystems Institute (CNSI).

Viability of neural stem progenitor cells (NSPCs) encapsulated in hydrogels

NSPCs were harvested from the brain cortex of postnatal day 2 (P2) mice using procedures described in detail previously³³. Tissues around the ventricles were excised, diced with a razor blade and placed in Accumax solution (Innovative Cell Technologies, San Diego, CA) for 1 hour to digest brain tissue extracellular matrix. Cells were dissociated and titrated to obtain a single cell suspension and were cultured in suspension as neurospheres within neural basal media supplemented with B27 (Thermo Fisher Scientific, Waltham, MA) and 20 ng/ml basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ). Growth media was replaced every two days and neurospheres were passaged every four days or as needed. Cell encapsulation within hydrogels was performed by adding an equal volume of dissociated NSPC suspension in cell media (30,000 cells/ μ l) to a 10 wt% ($\text{M}^{\text{O}}\text{A}$)₁₅₅**E**₆₀ solution in cell media to give a resulting copolymer concentration of 5.0 wt %. This mixture was rapidly combined with a 5.0 wt% ($\text{M}^{\text{O}}\text{A}$)₁₅₅**K**₆₀ solution in cell media to yield an overall 5.0 wt% cell containing ($\text{M}^{\text{O}}\text{A}$)₁₅₅**E/K**₆₀ hydrogel. In a similar manner, a 4.0 wt% **K**₁₈₀**L**₂₀ hydrogel control sample in cell media was diluted with cell suspensions to yield final hydrogel concentrations of 2.0 wt%. A cell suspension alone (15,000 cells/ μ l) without any hydrogel was also used as a control and baseline. For each of these samples, a 20 μ l aliquot was deposited on top of 1.0 wt% agarose gel in media within an Eppendorf tube. The samples were stored in an incubator (37 °C, 5% CO₂) and were removed after 1 day for analysis. The samples were diluted 50 fold with PBS, and the cells were pelleted using a microfuge. The Live/Dead® viability/cytotoxicity assay ((Thermo Fisher Scientific, Waltham, MA)) was employed to quantify the percentages of NSPCs both alive and dead after hydrogel encapsulation.³⁴ Samples were incubated with Live/Dead stain (2 μ M calcein AM and 4 μ M EthD-1 in PBS) for 30 min at

room temperature. The samples were examined under a Zeiss fluorescence microscope (Carl Zeiss Inc) with filters separating light emission from calcein (live, green) and EthD-1 (dead, red). Finally, all the live/dead cells were counted using imageJ. The resulting counts were averaged (6 samples of $(\text{M}^{\text{O}}\text{A})_{155}\text{K}_{60}$ and 5 samples for both cell control and $\text{K}_{180}\text{L}_{20}$) and normalized against the cell control.

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Chapter 3: Self-healing Multiblock Copolypeptide Hydrogels via Polyion Complexation

Adapted from: Sun, Y. Deming, T.J. Submitted to *ACS Macro Letters*

3.1 Abstract

Diblock, triblock and pentablock copolypeptides were designed and prepared for formation of polyion complex hydrogels in aqueous media. Increasing the number of block segments was found to allow formation of hydrogels with substantially enhanced stiffness at equivalent concentrations. Use of similar length ionic segments also allowed mixing of different block architectures to fine tune hydrogel properties. The pentablock hydrogels possess a promising combination of high stiffness, rapid self-healing properties, and cell compatible surface chemistry that makes them promising candidates for applications requiring injectable or printable hydrogel scaffolds.

3.2 Introduction

Polyion complex (PIC) assembly of dual hydrophilic block copolymers containing non-ionic and oppositely charged ionic segments has been developed as a facile method to prepare a diverse array of micelles, vesicles, and hydrogels in aqueous media.¹⁻³ Due to high water solubility of precursors, PIC formation allows preparation of supramolecular assemblies at high concentrations via simple mixing, and does not require use of either heating or co-solvents. These assemblies are experiencing extensive development in applications, including as carriers for therapeutic molecules and as scaffolds for cell culture and tissue repair.¹⁻⁵ We recently reported the design of PIC diblock copolypeptide hydrogels (DCH_{PIC}) that utilize formation of β -sheet structured complexes and were shown to be cell-compatible, self-healing, and resistant to dilution.⁶ In order to improve efficiency of hydrogel formation and to enhance mechanical properties, we report here studies on aqueous assembly of triblock and pentablock

copolypeptides designed to form PIC hydrogels. These copolymers possess block architectures that have not previously been explored for PIC assembly, and were found to significantly enhance network formation and stiffness compared to diblock architectures at equivalent concentrations.

Most block copolymer PIC hydrogels have been prepared using one or two triblock copolymer components containing ionic end-blocks flanking a non-ionic, hydrophilic center block.^{1-3,5,7-10} These hydrogels rely on formation of PIC phase-separated spherical domains that act as physical crosslinks in the networks, where the charged segments are placed as end-blocks to facilitate bridging of spherical domains by the non-ionic segments.^{5,7-11} Hydrogels have also been prepared via assembly of similar amphiphilic triblock copolymers in water, and these systems have recently incorporated more complex multiblock architectures to enhance and tune mechanical properties.¹²⁻¹⁵ We and others have also found that use of multiblock and star copolymer architectures can enhance hydrogel properties in amphiphilic polypeptide systems.¹⁶⁻¹⁹ However, these amphiphilic materials can be difficult to formulate, especially at higher concentrations, and the reported systems are not cell compatible.^{18,20} Here, we sought to develop PIC multiblock copolypeptide hydrogels that could overcome these issues and allow preparation of hydrogels with a broad range of tunable properties. Since the assembly of DCH_{PIC} in water promotes formation of PIC fibrils as opposed to spherical domains,⁶ the design of multiblock copolypeptide architectures needs to be fundamentally different from that of conventional block copolymers used to form PIC hydrogels.

The design of PIC multiblock copolypeptide hydrogels was based on triblock and pentablock amphiphilic block copolypeptides we had previously developed,¹⁶⁻¹⁸ where cationic and hydrophobic segments were now replaced with non-ionic and ionic segments,

respectively. The compositions of the non-ionic and ionic segments were based on those utilized previously in diblock (DB) DCH_{PIC},⁶ namely poly(L-methionine sulfoxide-*stat*-L-alanine)₁₀₀-*block*-poly(L-lysine-HCl)₃₀, (M^OA)₁₀₀K₃₀, and poly(L-methionine sulfoxide-*stat*-L-alanine)₁₀₀-*block*-poly(L-glutamate-Na)₃₀, (M^OA)₁₀₀E₃₀ (Figure 3.1 and 3.2). Notably, the triblock (TB) and pentablock (PB) copolypeptides were designed with non-associating (i.e. non-ionic) segments as end-blocks (Figure 3.1),¹⁶⁻¹⁸ distinct from conventional PIC hydrogels that have associating ionic segments as end-blocks.^{5,7-10} This change is due to the different mechanism of assembly in DCH_{PIC}, where the β -sheet structured PICs require substantial solvation to prevent further aggregation and precipitation.⁶

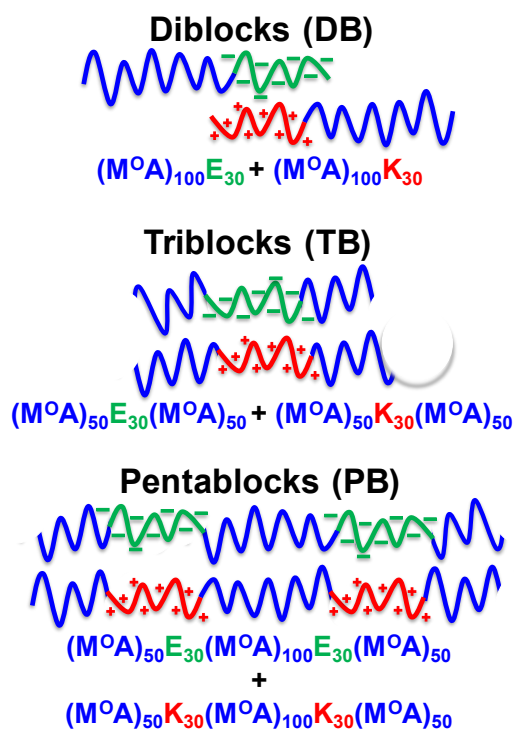


Figure 3.1 Schematic showing representations of diblock (DB), triblock (TB), and pentablock (PB) copolypeptides containing alternating non-ionic (M^OA) and ionic (K or E) segments. Oppositely charged K and E domains in copolypeptides can assemble via PIC formation in aqueous media to give hydrogels.

Table 3.1. Characterization data for multiblock copolypeptides.

Architecture	Sample	M_w/M_n^a	Composition ^b	Yield (%) ^c
Diblock	(M ^O A) ₁₀₀ E ₃₀	1.28	(M ^O A) ₉₁ E ₂₆	95
Diblock	(M ^O A) ₁₀₀ K ₃₀	1.31	(M ^O A) ₉₁ K ₂₈	93
Triblock	(M ^O A) ₅₀ E ₃₀ (M ^O A) ₅₀	1.28	(M ^O A) ₄₆ E ₂₇ (M ^O A) ₅₂	95
Triblock	(M ^O A) ₅₀ K ₃₀ (M ^O A) ₅₀	1.26	(M ^O A) ₄₆ K ₂₉ (M ^O A) ₄₉	92
Pentablock	(M ^O A) ₅₀ E ₃₀ (M ^O A) ₁₀₀ E ₃₀ (M ^O A) ₅₀	1.36	(M ^O A) ₄₆ E ₂₈ (M ^O A) ₈₉ E ₃₁ (M ^O A) ₄₈	91
Pentablock	(M ^O A) ₅₀ K ₃₀ (M ^O A) ₁₀₀ K ₃₀ (M ^O A) ₅₀	1.32	(M ^O A) ₄₆ K ₂₉ (M ^O A) ₉₅ K ₃₁ (M ^O A) ₄₆	93

^a Dispersity of oxidized, protected block copolypeptides were determined by GPC/LS. ^b Actual amino acid compositions of oxidized, deprotected block copolypeptides were determined by ¹H NMR integrations. Degree of polymerization of initial (MA)_x segments was determined by end-group analysis using ¹H NMR. ^c Total isolated yield of deprotected, purified block copolypeptides.

The TB and PB compositions were designed so that (i) their mole fractions of non-ionic and ionic residues would be identical to the DB samples, and (ii) ionic segments would be the same average length in all samples (Figure 3.2). Matching of ionic and non-ionic copolypeptide content allows for meaningful and quantitative comparison of hydrogel properties since equivalent sample concentrations (wt %) will possess the same molar concentrations of amino acid components. Maintaining similar ionic segment lengths is also important since their variation has been shown to strongly affect hydrogel mechanical properties.^{6,21} The copolypeptides in Figure 3.2 were prepared by stepwise addition of desired NCA monomers to

growing chains initiated using $\text{Co}(\text{PMe}_3)_4$,²² and gave samples with segment lengths and compositions that agreed well with predicted values (Table 3.1). Subsequent oxidation of methionine residues resulted in their conversion to methionine sulfoxides,^{23,24} and removal of protecting groups gave the final water soluble co-polypeptides in high overall yields after purification (Table 3.1).

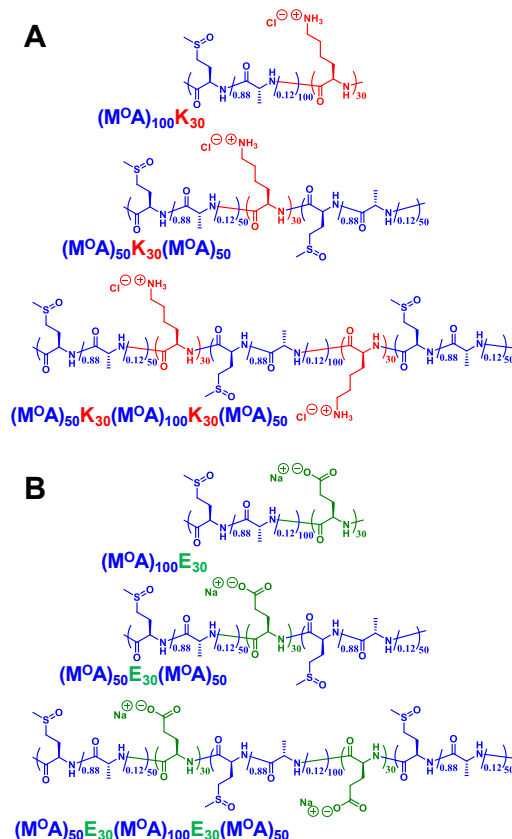


Figure 3.2 Chemical structures and compositions of DB, TB, and PB copolypeptides used in this study. (A) cationic copolypeptides. (B) anionic copolypeptides. Characterization data are given in Table 3.1

3.3 Results and Discussion

PIC assemblies were prepared by mixing aqueous solutions of matched DB, TB, or PB copolypeptides at different concentrations in 1 x PBS. Stoichiometric mixtures of oppositely charged copolypeptides resulted in formation of transparent hydrogels within seconds to minutes depending on concentration and block architecture, with PB samples forming hydrogels *ca.* 6

times faster than TB and DB samples. These samples were evaluated using oscillatory rheology to quantify their mechanical properties, and all were found to display elastic behavior ($G' \gg G''$) over a range of frequency (Figure 3.3). The hydrogels were also found to break down at high strain, as expected for physical hydrogels.⁶ While hydrogel stiffness (G') was found to increase with sample concentration for all samples, at equivalent concentrations hydrogel stiffness of PB samples was always greater than TB samples, and both were greater than DB samples (Figure 3.4, Table 2). Hence, multiblock architectures allow preparation of significantly stiffer hydrogels at equivalent amino acid contents and concentrations.

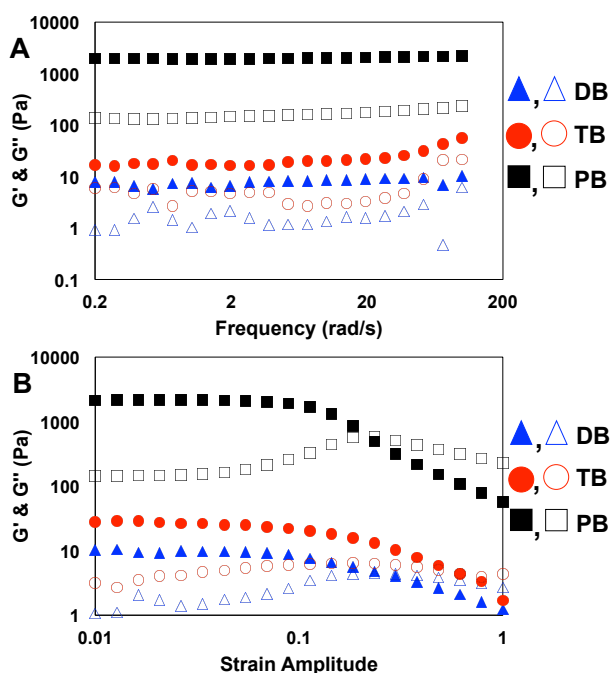


Figure 3.3 Rheology data for diblock (DB), triblock (TB) and pentablock (PB) PIC hydrogels at 7 wt% in PBS buffer at 20 °C. (A) G' (Pa, solid symbols) and G'' (Pa, open symbols) of multiblock PIC hydrogels as functions of angular frequency at constant strain amplitude of 0.01. (B) Storage modulus, G' (Pa, solid symbols), and loss modulus, G'' (Pa, open symbols), of multiblock PIC hydrogels as functions of strain amplitude at a constant frequency of 5 rad/s.

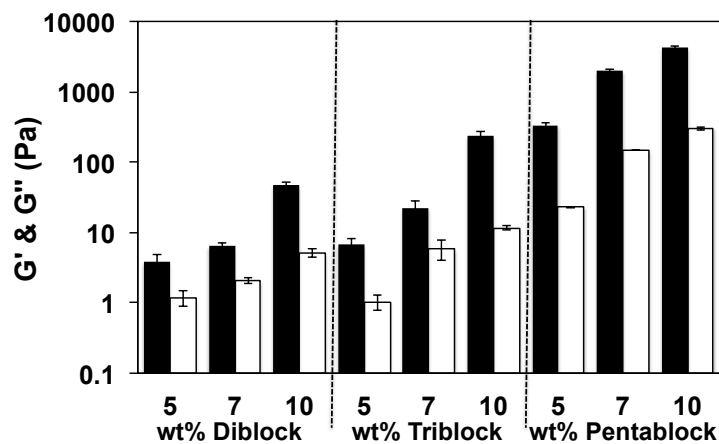


Figure 3.4 Mechanical properties of PIC block copolypeptide hydrogels. Storage modulus (G' , Pa, black) and loss modulus (G'' , Pa, white) of DB, TB, and PB hydrogels were measured at varying sample concentrations in 1×PBS buffer at 25 °C. All G' and G'' values were measured at an angular frequency of 5 rad/s and a strain amplitude of 0.01.

Table 3.2. Rheology data summary for multiblock copolypeptides.

Sample	wt%	G' (Pa)	G'' (Pa)
Diblock	5.0	3.93	1.19
Diblock	7.0	6.52	2.10
Diblock	10.0	47.1	5.12
Triblock	5.0	6.78	1.03
Triblock	7.0	22.5	5.84
Triblock	10.0	237	11.7
Pentablock	5.0	335	22.8
Pentablock	7.0	1990	147
Pentablock	10.0	4200	299

Data for 5 rad/s and strain amplitude = 0.01.

When comparing DB and TB samples, both are composed of equal length chains. Here, the replacement of single long non-ionic segments in DB samples with two shorter non-ionic

segments in TB samples is responsible for the observed modest increases in hydrogel stiffness. Although it has been found that longer non-associating (i.e. non-ionic) segment lengths in DCH enhance hydrogel formation, their effect on stiffness is modest.²¹ Consequently, it is thought that the increased density of non-ionic segments per associating segment found in the TB samples increases non-ionic chain repulsion at the block junctions upon PIC formation, resulting in formation of more extended, stiffer fibril assemblies.¹⁶⁻¹⁸

The PB samples incorporate the features of TB samples, but also double the chain length and allow for interfibril bridging or fibril re-entry due to the long central non-ionic tether segments.¹⁸ This difference resulted in the stiffness of PB hydrogels being up to *ca.* 100 times greater than equivalent concentration DB samples. We had previously found that hydrogel formation in DB samples was driven by assembly of β -sheet structures in the PIC domains, which can be monitored by examination of polypeptide Amide I bands using FTIR spectroscopy. Analysis of lyophilized DB, TB, and PB samples revealed strong Amide I bands at 1653 cm^{-1} due to the disordered chain conformations of the (M^{O} A) segments, and Amide I shoulders at 1630 cm^{-1} that are characteristic of β -sheet chain conformations (Figure 3.5).⁶ The intensity of the band at 1630 cm^{-1} was found to increase in samples from DB to TB to PB, consistent with improved β -sheet formation in the PB samples. Thus, it appears that PB samples allow for better chain ordering within PIC domains, which results in substantial enhancement of hydrogel stiffness, comparable or superior at similar concentration to the stiffest block copolymer PIC hydrogels that have been reported.^{5,7-10}

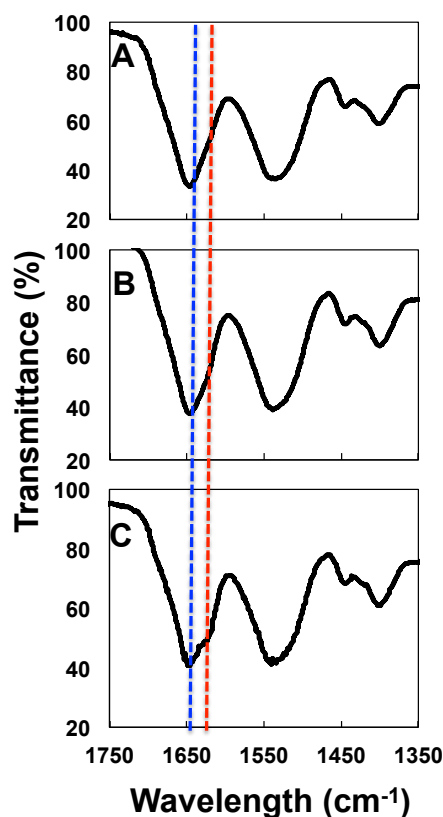


Figure 3.5 ATR-IR spectra showing the amide region for lyophilized (A) diblock, (B) triblock and (C) pentablock PIC hydrogel samples. Blue line = 1651 cm^{-1} Amide I band characteristic of α -helical and disordered chain conformations. Red line = 1621 cm^{-1} Amide I band characteristic of β -sheet chain conformations. Amide I band at 1621 cm^{-1} is more pronounced in pentablock sample (C), suggesting increased β -sheet content compared to corresponding diblock and triblock samples.

To evaluate interactions between different block architectures, we prepared charge balanced mixtures of cationic PB chains (i.e. PK) with anionic DB (i.e. DE) or TB (i.e. TE) chains. At 7.0 wt% in 1 x PBS media, these ‘mismatched’ samples were found to form hydrogels with stiffness (G') intermediate between the matched DB, TB and PB samples (Figure 3.6 A). In both cases, enhancement of stiffness was up to *ca.* 10 times greater compared to matched DB and TB samples. These results indicate that different block copolypeptide architectures containing associating ionic segments of similar length can efficiently co-assemble into hydrogel networks. This behavior is similar to results obtained from mixing amphiphilic copolypeptide hydrogels

where samples with similar hydrophobic segment lengths could be mixed to tune mechanical properties.¹⁶ Such behavior is beneficial for fine tuning of mechanical properties without having to prepare many different compositions, and potentially allows for significant enhancement of hydrogel stiffness using small amounts of multiblock copolypeptides. To test this concept, 7.0 wt% TB hydrogels were prepared using increasing amounts (wt%) of PK copolypeptides, where the amount of cationic TB (i.e. TK) chains was simultaneously decreased in the formulations to maintain charge balance. The data in Figure 3.6 B show that hydrogel stiffness can be enhanced as the fraction of cationic PK chains is increased, with a significant increase in stiffness when the fraction of PK is above 50 wt%.

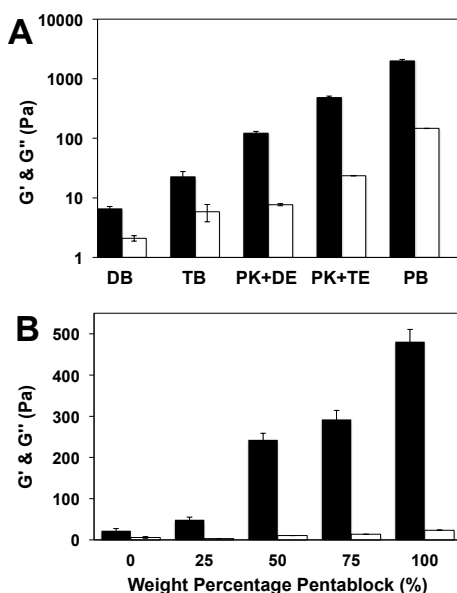


Figure 3.6 Mechanical properties of PIC block copolypeptide hydrogels prepared using matched and mismatched copolypeptides. (A) G' (Pa, black) and G'' (Pa, white) of 7 wt% PIC hydrogels measured in 1×PBS buffer at 25 °C. In matched samples, DB, TB and PB hydrogels were prepared by mixing cationic and anionic copolypeptides of the same block architecture. In mismatched samples, cationic PB (PK) is mixed with either anionic DB (DE) or anionic TB (TE), where DE = $(M^O A)_{100}E_{30}$; TE = $(M^O A)_{50}E_{30}(M^O A)_{100}$; and PK = $(M^O A)_{50}K_{30}(M^O A)_{100}K_{30}(M^O A)_{50}$. (B) G' (Pa, black) and G'' (Pa, white) of 7 wt% PIC hydrogels (PK+TB), with varying weight % incorporation of cationic PK component into TB mixtures, were measured in 1×PBS buffer at 25 °C. All samples were prepared with stoichiometric E to K ratios. All G' and G'' values were measured at an angular frequency of 5 rad/s and a strain amplitude of 0.01.

We previously noted that DCH_{PIC} were able to rapidly self-heal after mechanical breakdown after being subjected to high amplitude oscillatory strain.⁶ Rapid self-healing behavior is a desirable property in hydrogels as it allows deposition of hydrogel via injection through small bore needles, which has utility in biological applications as well as in additive manufacturing. DB, TB and PB hydrogel samples (10 wt%) were evaluated to determine if self-healing properties were affected by block architecture. Each sample was subjected to high amplitude oscillatory strain, followed immediately by monitoring the recovery of elasticity over time by measuring G' at a much smaller strain amplitude (Figure 3.7). During the initial 100 s of high strain amplitude, G' for all samples dropped substantially to below the level of G'' , indicating they all became viscous liquids. Upon switching to low strain amplitude, all samples began recovering their elastic properties over time. Remarkably, the PB sample, which possessed the greatest stiffness, was the fastest (less than *ca.* 10 s) to fully recover its mechanical properties. For the DB and TB samples, recovery of elasticity continued to occur over a time scale of minutes. The rapid self-healing properties of PB hydrogels combined with the ability to prepare samples of high stiffness by varying concentration provide a promising combination of attributes for development of injectable or printable hydrogel scaffolds. Furthermore, PB hydrogels were also found to be resistant to dissolution in media as had been previously found for DCH_{PIC} (Figure 3.8).⁶

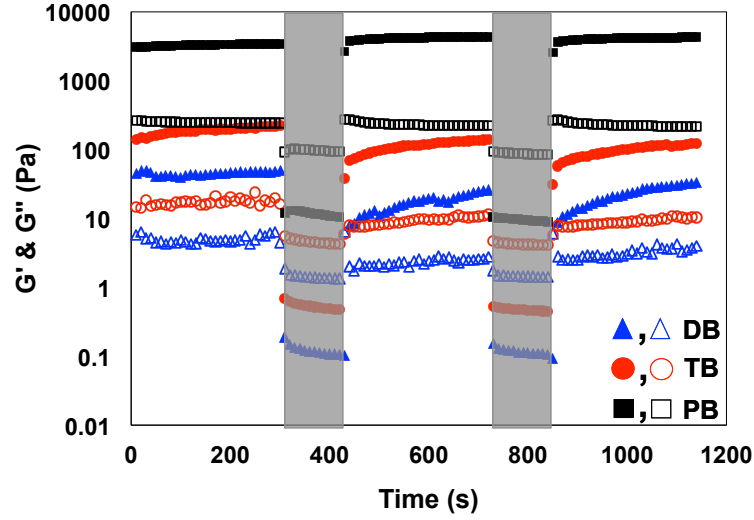


Figure 3.7 Mechanical recovery of DB, TB and PB hydrogels. Samples prepared at 10 wt% in 1×PBS buffer at 25 °C (G' = solid symbols; G'' = open symbols) after application of stepwise large-amplitude oscillatory breakdown (gray regions = strain amplitude of 10 at 10 rad/s for 120s) followed by low-amplitude linear recovery (white regions = strain amplitude of 0.01 at 5 rad/s for 300s).

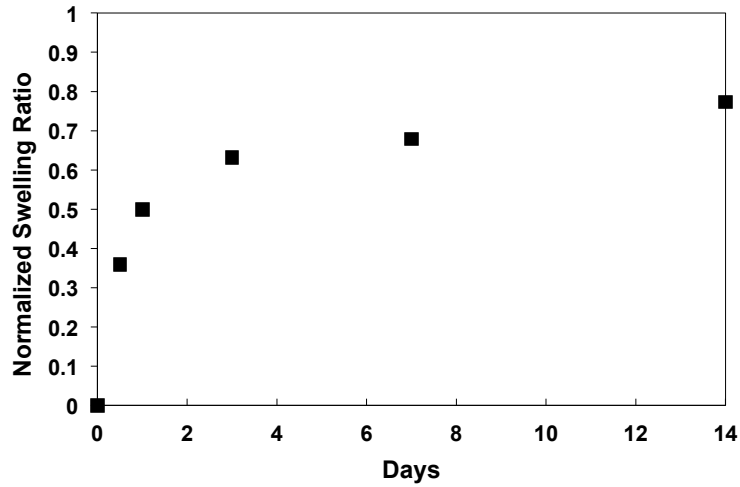


Figure 3.8 Normalized swelling ratio measurement. A sample of pentablock PIC hydrogel prepared at 10 wt% in 1×PBS was diluted with an equal volume of DMEM cell culture media. Hydrogel swelling was monitored by removal of all supernatant liquid above the hydrogel at different time points. Normalized swelling ratio was calculated as: (weight of sample after swelling - weight of initial sample) / weight of initial sample.

3.4 Conclusion

In summary, new triblock and pentablock copolypeptides capable of forming PIC hydrogels in aqueous media were reported. With a design based on previously reported diblock copolypeptides, the PB hydrogels were found to possess substantially enhanced stiffness compared to DB and TB samples at equivalent concentrations. In addition to adjusting concentration, hydrogel properties could also be tuned by mixing ‘mismatched’ block architectures in different ratios. All hydrogels were capable of rapid self-healing after deformation, with PB samples showing the fastest complete recovery. The use of multiblock architectures in PIC copolypeptide hydrogels was found to impart these physical assemblies with significantly enhanced mechanical properties, while retaining self-healing ability and stability against dilution in aqueous media. It is expected that the multiblock hydrogels will also possess good cell compatibility, similar to that shown for the diblock DCH_{PIC}.⁶

3.5 Experimental

3.5.1 Materials and Methods

Tetrahydrofuran (THF), hexanes, and methylene chloride were dried by purging with nitrogen and passage through activated alumina columns prior to use. Co(PMe₃)₄ and amino acid *N*-carboxyanhydride (NCA) monomers were prepared according to literature procedures.²⁵ All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Selecto silica gel 60 (particle size 0.032– 0.063 mm) was used for flash column chromatography. Fourier transform infrared (FTIR) spectra were acquired on a Perkin Elmer RX1 FTIR spectrophotometer calibrated using polystyrene film, and attenuated total reflectance infrared (ATR-IR) data were collected using a Perkin Elmer Spectrum 100 FTIR

spectrometer equipped with a universal ATR sample accessory. ^1H NMR spectra were acquired on a Bruker ARX 400 spectrometer. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 μm columns at 30 °C with 0.5% (w/w) KTFA in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) as eluent and sample concentrations of 10 mg/ml. Pyrogen free deionized (DI) water was obtained from a Millipore Milli-Q Biocel A10 purification unit.

3.5.2. Experimental Procedure

General procedure for copolypeptide synthesis

All polymerization reactions were performed in an N_2 filled glove box using anhydrous solvents. To a solution of L-methionine NCA (Met NCA) and L-alanine NCA (Ala NCA) in THF (50 mg/ml) was added a solution of $\text{Co}(\text{PMe}_3)_4$ in THF (20 mg/ml).²⁵ The reactions were let to stir at ambient temperature (*ca.* 22 °C) for 60 min. Complete consumption of NCA was confirmed by FTIR spectroscopy, and then the desired amount of γ -benzyl-L-glutamate NCA (Bn-Glu NCA) or ϵ -trifluoroacetyl-L-lysine NCA (TFA-Lys NCA) in THF (50 mg/ml) was added to the reaction mixtures, which were let to stir for an additional 60 min. FTIR was used to confirm complete consumption of all NCAs. Monomer additions were repeated as necessary. Once polymerizations were completed the block copolypeptide solutions were removed from the glove box, precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl (2 x 20 ml) to remove residual cobalt ions. The white precipitates were then washed with DI water (3 x 20 ml) and freeze-dried to give products as white solids.¹ Subsequent oxidation of samples,

followed by deprotection of Bn-Glu or TFA-Lys groups were performed as previously described.⁶

Poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₁₀₀-*block*-poly(L-lysine)₃₀, (M^OA)₁₀₀K₃₀ and poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₁₀₀-*block*-poly(L-glutamate)₃₀, (M^OA)₁₀₀E₃₀

These samples were prepared as previously described.⁶

Poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀-*block*-poly(L-lysine)₃₀-*block*-poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀, (M^OA)₅₀K₃₀(M^OA)₅₀ and poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀-*block*-poly(L-glutamate)₃₀-*block*-poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀, (M^OA)₅₀E₃₀(M^OA)₅₀

In the glove box, a stock solution of Met NCA (110 mg, 0.62 mmol) mixed with Ala NCA (9.9 mg, 0.085 mmol) was prepared using THF (2.2 ml) and placed in a 20 ml scintillation vial. **First block synthesis:** The desired amount of Met/Ala NCA stock solution (1.2 ml) was added to a 20 ml scintillation vial containing a stir bar. To the vial, (PMe₃)₄Co initiator solution (500 µl of a 20 mg/ml solution in THF) was added via syringe. The vial was sealed and allowed to stir in the glove box for 1 h. An aliquot (20 µl) was removed and analyzed by FTIR to confirm that all the NCA was consumed. In the glove box, α-methoxy-ω-isocyanoethyl-poly(ethylene glycol)₄₅ (mPEG₂₃-NCO) (20 mg) was dissolved in THF (1 ml) in a 20 ml scintillation vial. An aliquot (550 µl) of the polymerization solution containing active chain ends was removed and added to the solution of mPEG₂₃-NCO. The PEG end-capped sample (MA₅₀-mPEG₂₃) was sealed, allowed to stir for 24 h, and then used for chain length determination (*vide infra*). **Second**

block synthesis: Separately, aliquots of the polymerization solution containing active chains (0.4 ml each) were added to vials containing either Bn-Glu NCA (21 mg, 0.078 mmol) or TFA-Lys NCA (21 mg, 0.078 mmol) dissolved in THF (410 μ l or 420 μ l, respectively). The vials were sealed and allowed to stir in the glove box for 1 h to give the diblock copolypeptides, (MA)₅₀(TFA-K)₃₀ and (MA)₅₀(Bn-E)₃₀. FTIR was used to confirm complete consumption of NCAs in both reactions. Aliquots (400 μ l) of each polymerization solution were removed for ¹H NMR analysis to determine the second block lengths. **Third block synthesis:** 470 μ l of the Met/Ala NCA stock solution was added to each of the polymerization solutions to prepare the triblock copolypeptides, (MA)₅₀(TFA-K)₃₀(MA)₅₀ and (MA)₅₀(Bn-E)₃₀(MA)₅₀. The solutions were allowed to stir for 1 hr and were checked by FTIR to ensure completed NCA consumption. Outside the glove box, the triblock copolypeptide solutions were precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl (2 x 20 ml) to remove residual cobalt ions. The white precipitates were then washed with DI water (3 x 20 ml) and freeze-dried (94% average yield).⁶ Subsequent oxidation of samples, followed by deprotection of Bn-Glu or TFA-Lys groups were performed as previously described.⁶

Poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀-block-poly(L-lysine)₃₀-block-poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₁₀₀-block-poly(L-lysine)₃₀-block-poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀, (M^OA)₅₀K₃₀(M^OA)₁₀₀K₃₀(M^OA)₅₀ and poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀-block-poly(L-glutamate)₃₀-block-poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₁₀₀-block-poly(L-glutamate)₃₀-block-poly(L-methionine sulfoxide_{0.88-stat-L-alanine_{0.12}})₅₀, (M^OA)₅₀E₃₀(M^OA)₁₀₀E₃₀(M^OA)₅₀

A stock solution of Met NCA (240 mg, 1.4 mmol) mixed with Ala NCA (22 mg, 0.19 mmol) was prepared using THF (4.8 ml) and placed in a 20 ml scintillation vial. 50 mg/ml stock

solutions of Bn-Glu NCA (45 mg, 0.17 mmol) and TFA-Lys NCA (45 mg, 0.17 mmol) were also prepared using THF in 20 ml scintillation vials. **First block synthesis:** The desired amount of Met/Ala NCA stock solution (1.2 ml) was added to a 20 ml scintillation vial containing a stir bar. To the vial, $(\text{PMe}_3)_4\text{Co}$ initiator solution (500 μl of a 20 mg/ml solution in THF) was added via syringe. The vial was sealed and allowed to stir in the glove box for 1 h. An aliquot (20 μl) was removed and analyzed by FTIR to confirm that all the NCA was consumed. In the glove box, α -methoxy- ω -isocynoethyl-poly(ethylene glycol)₄₅ (mPEG₂₃-NCO) (20 mg) was dissolved in THF (1 ml) in a 20 ml scintillation vial. An aliquot (550 μl) of the polymerization solution containing active chain ends was removed and added to the solution of mPEG₂₃-NCO. The PEG end-capped sample (MA₅₀-mPEG₂₃) was sealed, allowed to stir for 24 h, and then used for chain length determination (vide infra). **Second block synthesis:** Separately, aliquots of the polymerization solution containing active chains (0.4 ml each) were added to vials containing either Bn-Glu NCA (410 μl of stock) or TFA-Lys NCA (420 μl of stock). The vials were sealed and allowed to stir in the glove box for 1 h to give the diblock copolypeptides, (MA)₅₀(TFA-K)₃₀ and (MA)₅₀(Bn-E)₃₀. FTIR was used to confirm complete consumption of NCAs in both reactions. Aliquots (400 μl) of each polymerization solution were removed for NMR analysis to determine the second block lengths. **Third block synthesis:** 470 μl of the Met/Ala NCA stock solution was added to each of the polymerization solutions to give the triblock copolypeptides, (MA)₅₀(TFA-K)₃₀(MA)₅₀ and (MA)₅₀(Bn-E)₃₀(MA)₅₀. The solutions were allowed to stir for 1 hr and were checked by FTIR to ensure completed NCA consumption. Aliquots (400 μl) of each polymerization solution were removed for ¹H NMR analysis to determine the third block lengths. **Fourth block synthesis:** 180 μl of each Bn-Glu NCA and TFA-Lys NCA stock solution was added to the corresponding polymerization solution to give the tetrablock copolypeptides,

(MA)₅₀(TFA-K)₃₀(MA)₅₀(TFA-K)₃₀ and (MA)₅₀(Bn-E)₃₀(MA)₅₀(Bn-E)₃₀. The solutions were allowed to stir for 1 hr and were checked by FTIR to ensure completed NCA consumption. Aliquots (400 µl) of each polymerization solution were removed for ¹H NMR analysis to determine the fourth block lengths. **Fifth block synthesis:** 110 µl of the Met/Ala NCA stock solution was added to each of the polymerization solutions to give the final pentablock copolypeptides, (MA)₅₀(TFA-K)₃₀(MA)₅₀(TFA-K)₃₀(MA)₅₀ and (MA)₅₀(Bn-E)₃₀(MA)₅₀(Bn-E)₃₀(MA)₅₀. The solutions were allowed to stir for 24 hr and were checked by FTIR to ensure completed NCA consumption. Outside the glove box, the pentablock copolypeptide solutions were precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl (2 x 20 ml) to remove residual cobalt ions. The white precipitates were then washed with DI water (3 x 20 ml) and freeze-dried (97% average yield). ⁶Subsequent oxidation of samples, followed by deprotection of Bn-Glu or TFA-Lys groups were performed as previously described.⁶

Sample procedure for MA_x chain length determination using end-group analysis

Outside of the glove box, a PEG end-capped sample (MA_x-mPEG₂₃) from above was washed with 10 mM aqueous HCl (2x). After stirring for 1 h, MA_x-mPEG₂₃ was collected by centrifugation and washed with DI water (3 x 20 ml) to remove all non-conjugated mPEG₂₃-NCO. The remaining MA_x-mPEG₂₃ was then freeze-dried to remove residual H₂O. To determine MA_x molecular weights (M_n), ¹H NMR spectra were obtained. Since it has been shown that end-capping is quantitative for (PMe₃)₄Co initiated NCA polymerizations when excess isocyanate is used,²⁶ integrations of methionine (δ 2.70) and alanine (δ 1.52) resonances versus the polyethylene glycol resonance at δ 3.92 could be used to obtain both M to A ratios and MA_x lengths.

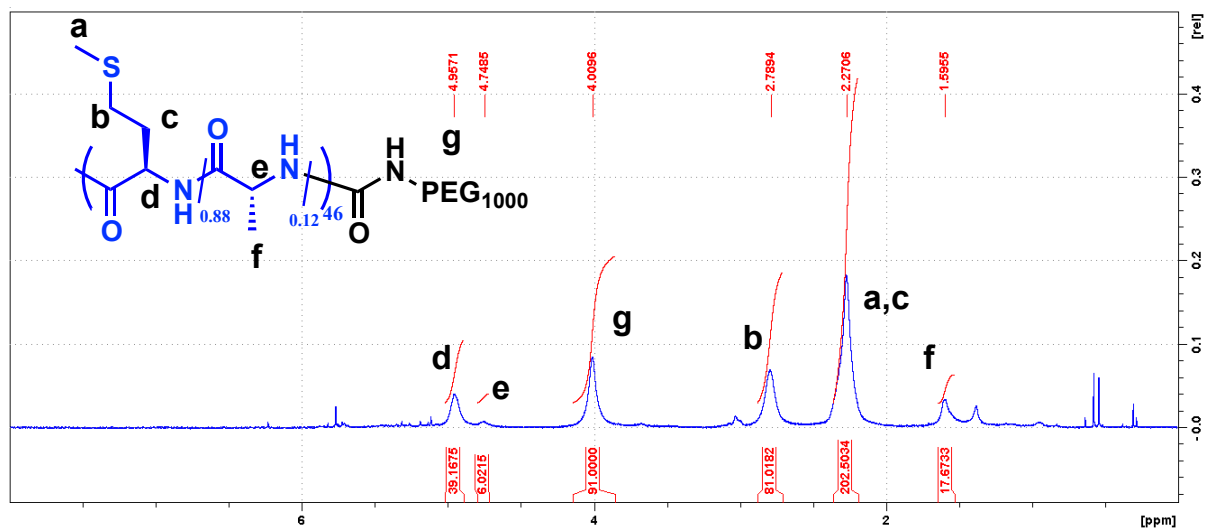
Rheology measurements on multiblock copolypeptide hydrogels

An Anton Paar Instruments MCR 302 rheometer with a 25 mm diameter and 1° cone plate geometry and solvent trap was used for all measurements. Frequency sweeps were measured at constant strain amplitude of 0.01. Strain sweeps were measured at a constant frequency of 5 rad/s. All measurements were repeated 3 times for each hydrogel sample and the results were averaged and plotted. To evaluate shear thinning and recovery behavior of DCH, the strain amplitude was stepped from 0.01 to 10, maintained at 10 for 2 min and then returned to 0.01 to evaluate the recovery of mechanical properties at a fixed frequency of 5 rad/s.

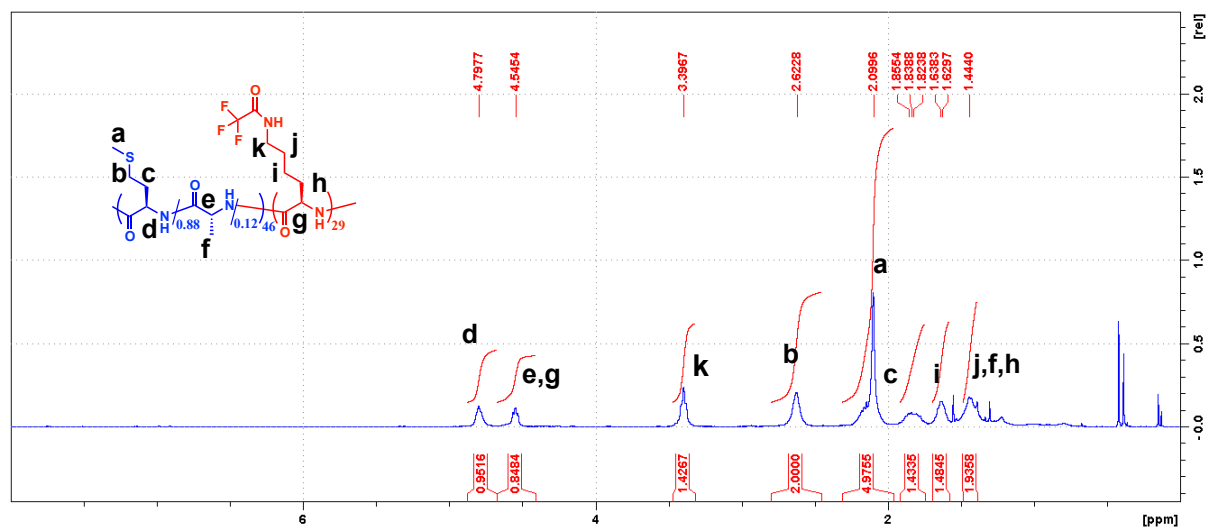
Hydrogel swelling measurements

10 wt% hydrogels of $(M^O A)_{100}E/K_{30}$, $(M^O A)_{50}K_{30}(M^O A)_{50}$ and $(M^O A)_{50}K_{30}(M^O A)_{100}K_{30}(M^O A)_{50}$ were prepared in 2 ml scintillation vials and allowed to stand for 1 hr. DMEM cell culture media was then placed on top of each hydrogel sample and all were stored in a refrigerator (0 °C) for different periods of time. At each time point, the supernatant liquid was pipetted out of each sample without disturbing the gel at the bottom. The supernatant volumes were subtracted from the original media volume to determine swelling ratios. The hydrogel samples were also subjected to inversion tests to verify hydrogel integrity. Finally, the supernatant liquid was replaced on top of each hydrogel and incubation of samples allowed to continue.

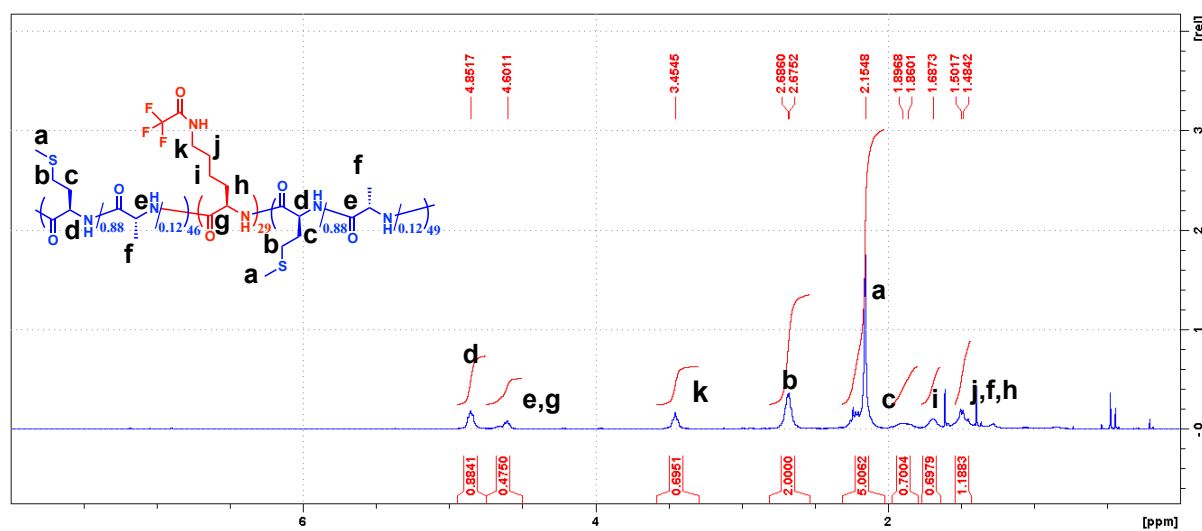
¹H NMR Spectra of polypeptide samples and intermediates



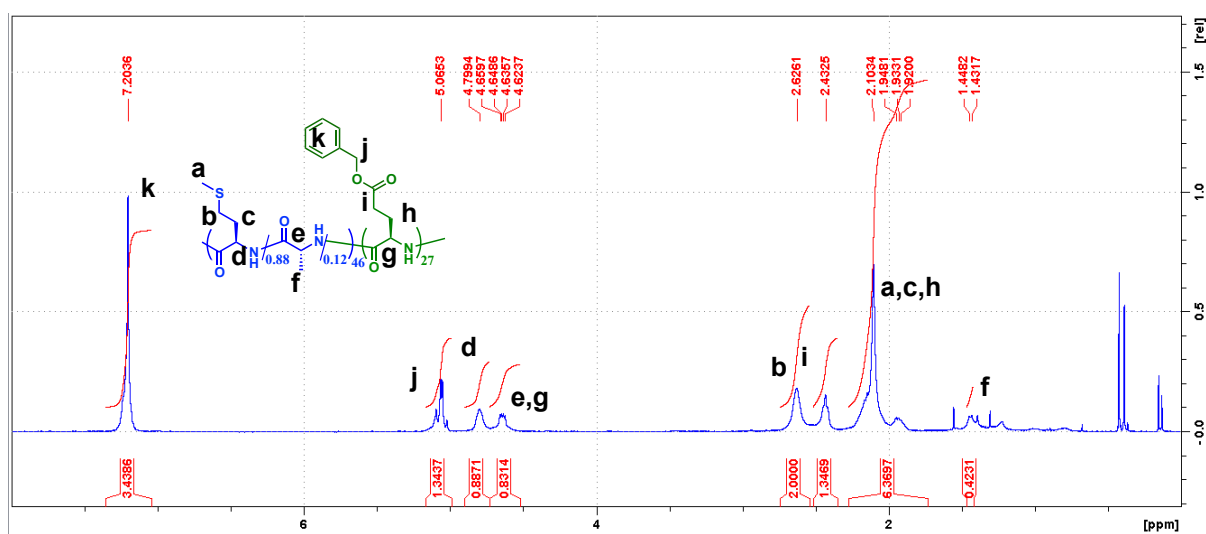
(MA)₄₆-PEG₁₀₀₀ in d-TFA.



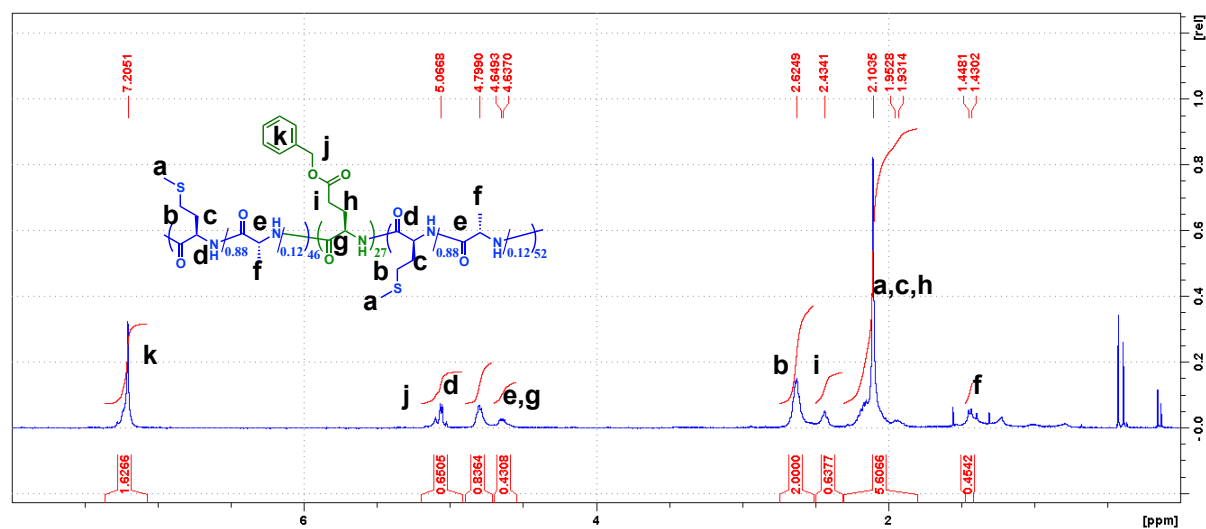
2nd block addition for triblock, (MA)₄₆(TFA-K)₂₉, in d-TFA.



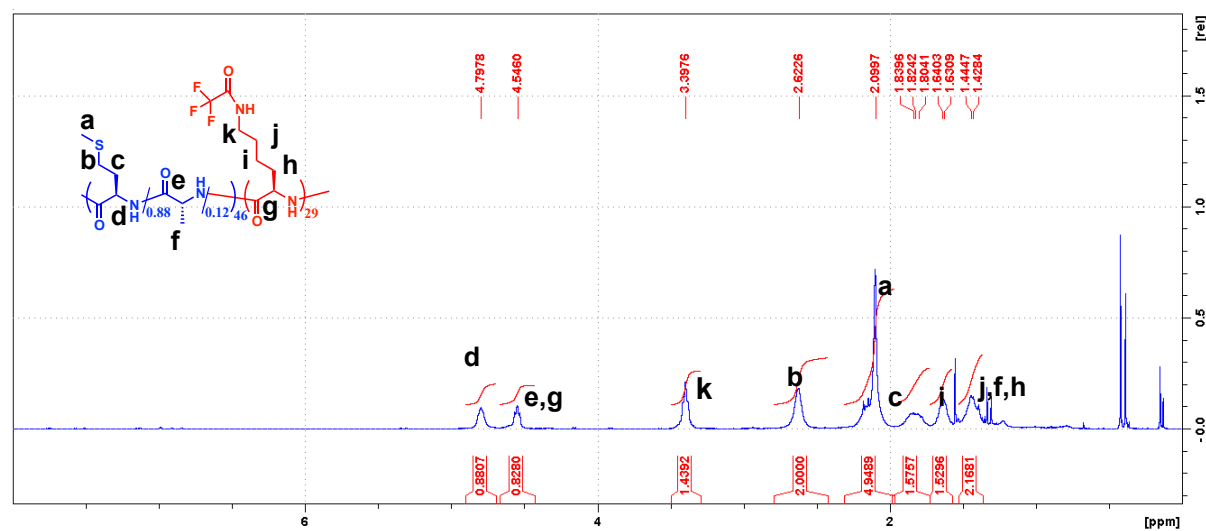
3rd block addition for triblock, (MA)₄₆(TFA-K)₂₉(MA)₄₉, in d-TFA.



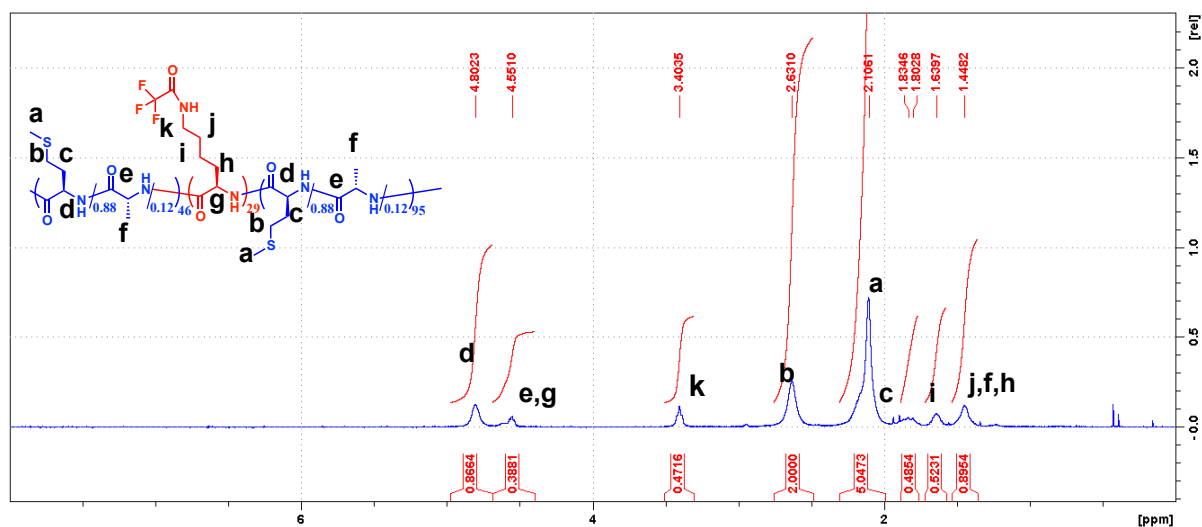
2nd block addition for triblock, (MA)₄₆(Bn-E)₂₇, in d-TFA.



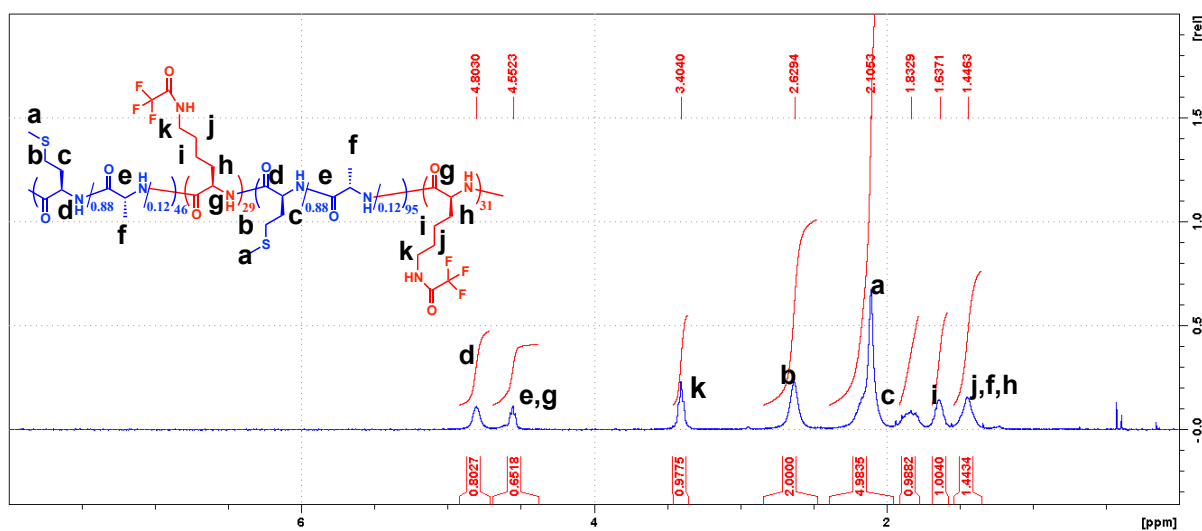
3rd block addition for triblock, $(MA)_{46}(Bn-E)_{27}(MA)_{52}$, in d-TFA.



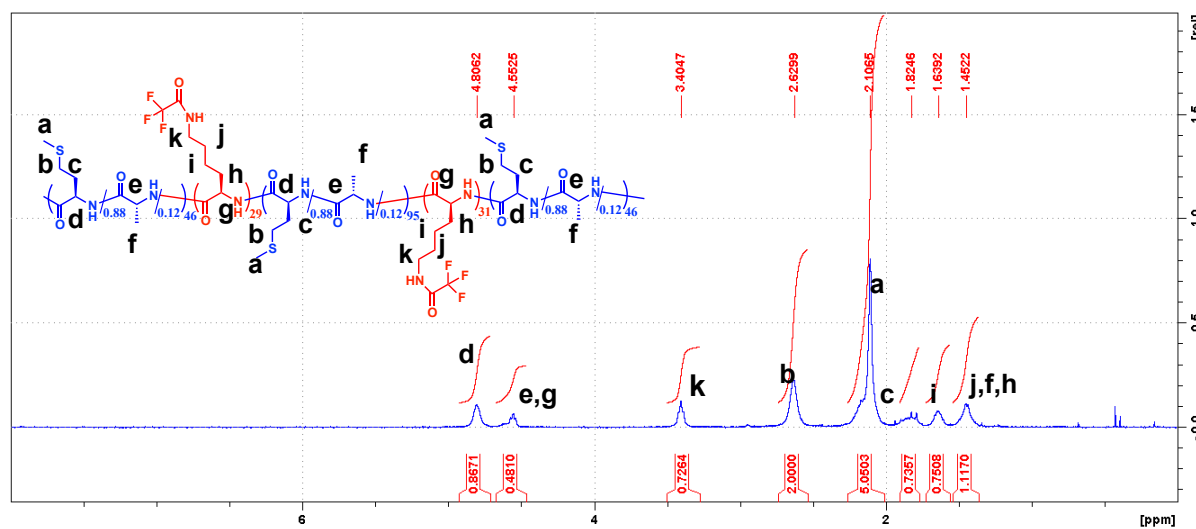
2nd block addition for pentablock, $(MA)_{46}(TFA-K)_{29}$, in d-TFA.



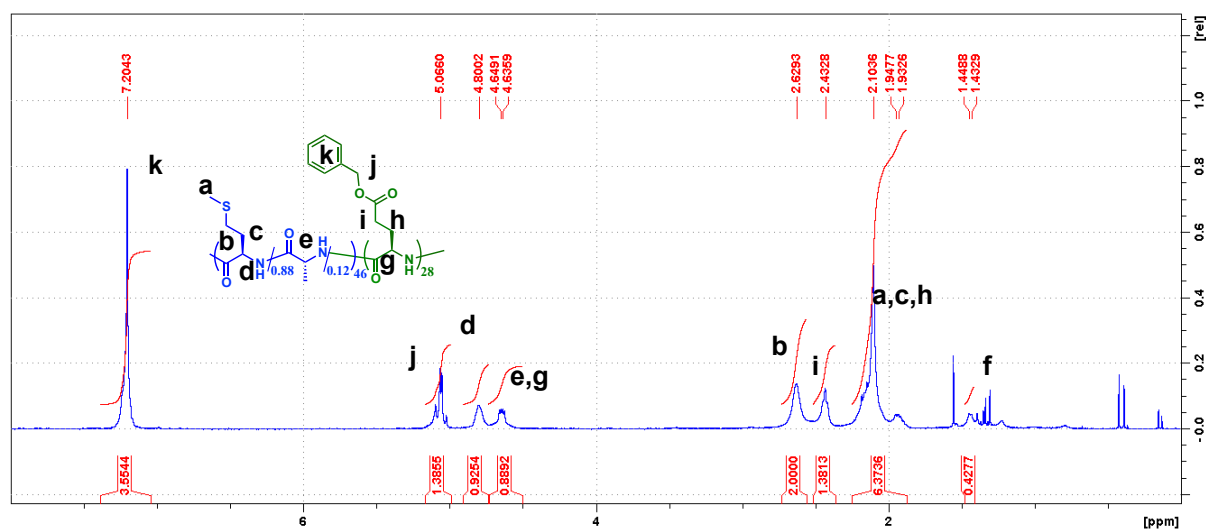
3rd block addition for pentablock, $(MA)_{46}(TFA-K)_{29}(MA)_{95}$, in $d-TFA$



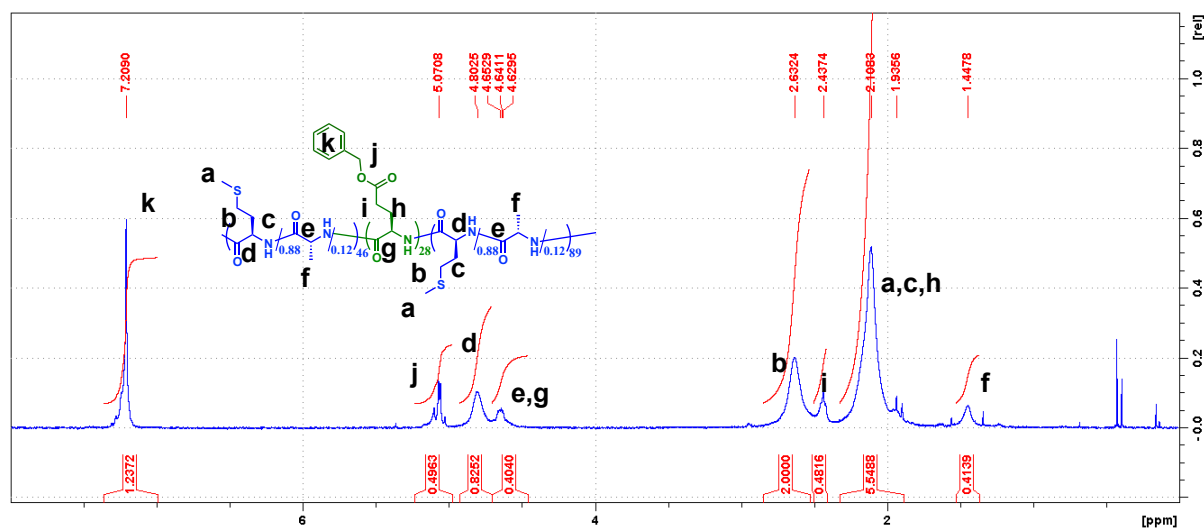
4th block addition for pentablock, $(MA)_{46}(TFA-K)_{29}(MA)_{95}(TFA-K)_{31}$, in $d-TFA$.



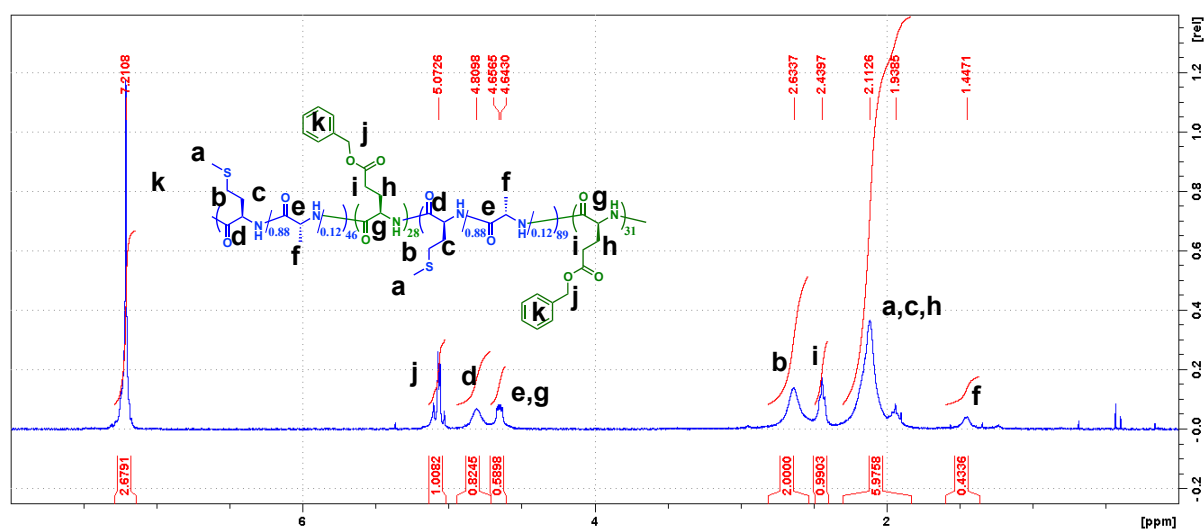
5th block addition for pentablock, (MA)₄₆(TFA-K)₂₉(MA)₉₅(TFA-K)₃₁(MA)₄₆, in d-TFA.



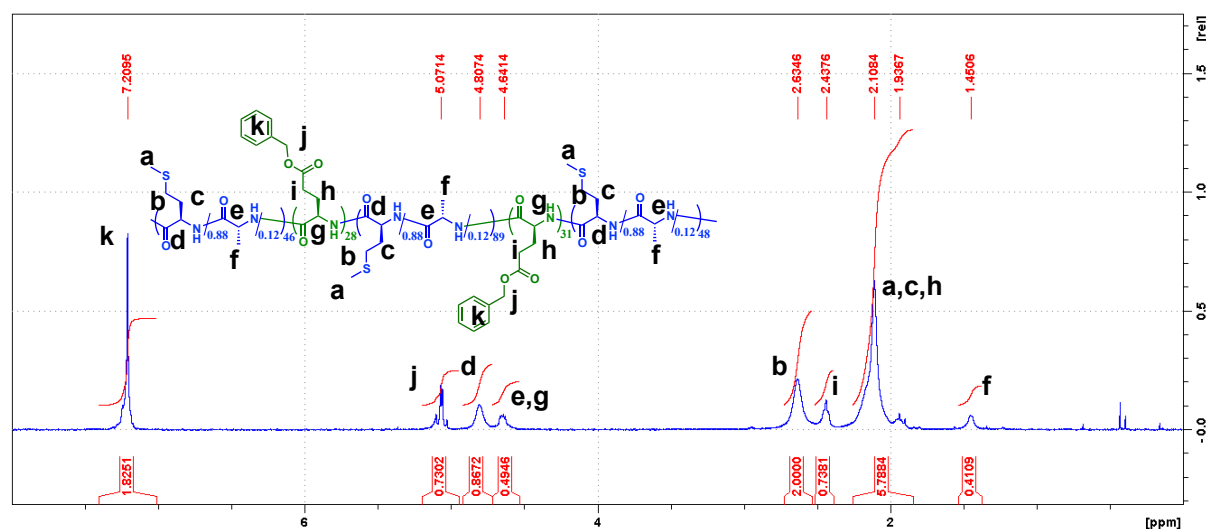
2nd block addition for pentablock, (MA)₄₆(Bn-E)₂₈, in d-TFA.



3rd block addition for pentablock, (MA)₄₆(Bn-E)₂₈(MA)₈₉, in d-TFA.



4th block addition for pentablock, (MA)₄₆(Bn-E)₂₈(MA)₈₉(Bn-E)₃₁, in d-TFA.



5th block addition for pentablock, (MA)₄₆(Bn-E)₂₈(MA)₈₉(Bn-E)₃₁(MA)₄₈, in d-TFA.

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Chapter 4: Self-healing Physically-Crosslinked Diblock Copolypeptide Dual Network

Hydrogels

Adapted from: Sun, Y. Deming, T.J. *manuscript in preparation*

4.1 Abstract

A new type of physically cross-linked dual network hydrogel system was designed and prepared by combining two cell compatible copolypeptide hydrogel components: polyion complex diblock hydrogel (DCH_{PIC}) and nonionic amphiphilic diblock hydrogel (DCH_{MO}). The dual network hydrogel (DCH_{DN}) consists of two distinct hydrogel networks that interpenetrate at a microscopic level, resulting in enhanced stiffness. DCH_{DN} also inherits stability against aqueous dilution and rapid self-healing properties from its hydrogel components, which makes it a promising candidate for use as an injectable or printable cell scaffold for tissue engineering applications.

4.2 Introduction

Dual network (DN) hydrogels are composed of two interpenetrating networks that are separately cross-linked by covalent or noncovalent bonds, which cooperatively enhance mechanical properties of the overall system.¹ Most common DN hydrogels are covalently cross-linked and consist of two components: 1) a minor fraction of a stiff and brittle highly cross-linked network, and 2) a major fraction of a soft and ductile loosely cross-linked network.² Upon deformation, the sacrificial bonds in the minor network break to dissipate energy, allowing the major network to retain its integrity.³ To synthesize these DN hydrogels, the minor network is typically pre-formed and then swollen in the polymerization solution of the major network. Then, polymerization of the major component forms an interpenetrating network with the minor component, resulting in formation of the DN

hydrogel.⁴ However, one major disadvantage for covalently cross-linked DN hydrogels is their lack of ability to self-heal due irreversible bond breaking.⁵

In recent years, a new generation of physically cross-linked DN hydrogels has been developed to replace sacrificial covalent bonds with dynamic and reversible bonds (e.g. hydrogen bonding, hydrophobic, ionic interactions). The physically cross-linked bonds can reversibly break and dissipate energy upon deformation.⁶ After stress is removed, the physical bonds can reform, allowing the networks to self-heal.⁷ The Zheng group has designed a physically cross-linked DN hydrogel based on hydrogen bond associated agar networks, and hydrophobically associated polyacrylamide or ionically crosslinked polyacrylic acid networks.^{5,8} Poly(vinyl alcohol) (PVA) network based DN hydrogels have also been heavily explored, incorporating chitosan, agar and poly(acrylamide-co-acrylic acid) as the second network.^{7,9,10} However, while these agar and PVA based DN hydrogels exhibit high mechanical strength and self-healing properties, their harsh gelation temperature requirement (i.e. -10 and 85 °C for PVA and agar respectively) limits their application as scaffolds for tissue engineering and stem cell therapy. Therefore, there is a strong demand for a DN hydrogel system that 1) allows for facile *in situ* gelation under mild conditions, 2) enhanced mechanical properties and 3) rapid self-recovery.

The Deming lab has developed two synthetic biomimetic diblock copolypeptide systems that form hydrogels via different conformation directed assembly mechanisms. The polyion complex diblock hydrogels (DCH_{PIC}) assemble via formation of PIC β -sheets while the nonionic amphiphilic diblock hydrogels (DCH_{MO}) assemble via association of hydrophobic α -helices.^{11,12} These hydrogel materials are promising due to their cell compatibility, versatile chemical functionality, response to biological stimuli, self-healing properties, and potential for

encapsulation of hydrophobic and charged cargoes.¹³ In particular, DCH_{PIC} allows for rapid gelation *in situ* and exhibits stability against dilution in aqueous solution that amphiphilic assembled hydrogels lack. However, like most existing physically cross-linked hydrogel systems, these DCH systems also suffer from lower hydrogel stiffness that limits their use for certain tissue engineering applications. Herein we sought to enhance hydrogel properties by developing physically cross-linked diblock copolypeptide dual network hydrogels (DCH_{DN}) composed of DCH_{PIC}, based on poly(L-methionine sulfoxide-*stat*-L-alanine)₁₅₅-*block*-poly(L-lysine-HCl)₅₅, (M^OA)₁₅₅K₅₅ and poly(L-methionine sulfoxide-*stat*-L-alanine)₁₅₅-*block*-poly(L-glutamate-Na)₅₅, (M^OA)₁₅₅E₅₅, and DCH_{MO}, based on poly(L-methionine sulfoxide-*stat*-L-alanine)₂₀₀-*block*-poly(L-leucine)₃₀, (M^OA)₂₀₀L₃₀ (Figure 4.1 and Figure 4.2).^{11,12} By facile mixing of these copolypeptide components, DCH_{DN} with enhanced stiffness and self-healing properties can be readily and rapidly formed, making this hydrogel system potentially useful for development of injectable cell carriers.

4.3 Results and Discussion

The block copolypeptides were synthesized by ring opening polymerization of appropriate NCA monomers with Co(PMe₃) initiator.¹⁴ The resulting copolypeptides had block lengths and compositions that agreed well with predicted values (Table 4.1). Subsequent oxidation of methionine residues and deprotection of ionic blocks yielded the final diblock copolypeptides in high overall yields (Table 4.1).

The dual network hydrogels were prepared by first dissolving both PIC components in 1 x PBS. Then, the cationic PIC component, M^OA₁₅₅K₅₅, was mixed with the nonionic DCH_{MO}, M^OA₂₀₀L₃₀, to form a viscous solution. Finally, the anionic PIC component, M^OA₁₅₅E₅₅, was

added to the cationic viscous solution, mixed and vortexed for 20 seconds to give DCH_{DN} within 5-30 seconds depending on the polypeptide concentration and composition (Figure 4.1).

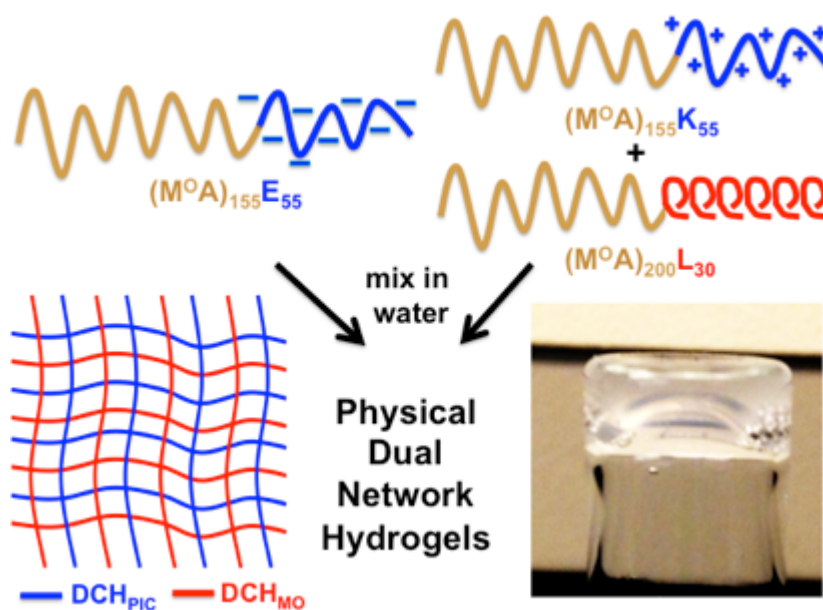


Figure 4.1 Schematic showing the assembly process for preparation of dual network hydrogel DCH_{DN} , consisting of polyion complex $(\text{M}^{\text{O}}\text{A})_{155}\text{E}/\text{K}_{55}$ (PIC) and nonionic $(\text{M}^{\text{O}}\text{A})_{200}\text{L}_{30}$ (MO) diblock copolypeptide hydrogels.

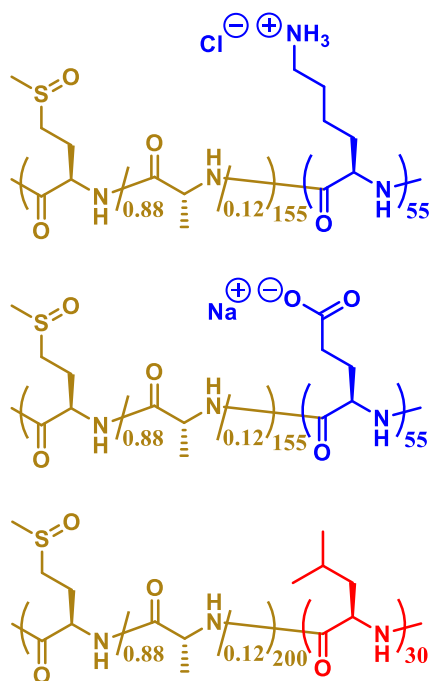


Figure 4.2 Chemical structures and compositions of $(M^O A)_{155}K_{55}$, $(M^O A)_{155}E_{55}$, and $(M^O A)_{200}L_{30}$

Table 4.1. Copolymerization data for diblock copolypeptide synthesis.

Sample	Mw/Mn ^a	Composition ^b	Yield (%) ^c
$(M^O A)_{155}E_{55}$	1.372	$(M^O A)_{152}E_{52}$	92
$(M^O A)_{155}K_{55}$	1.378	$(M^O A)_{152}K_{54}$	94
$(M^O A)_{200}L_{30}$	1.402	$(M^O A)_{215}L_{31}$	91

^a Dispersity of oxidized, protected block copolypeptides were determined by GPC/LS. ^b Relative amino acid compositions of oxidized, deprotected block copolypeptides were determined by ¹H NMR integrations. Degree of polymerization of initial MA_x segment was determined by end-group analysis using ¹H NMR. ^c Total isolated yield of purified block copolypeptides following deprotection.

Rheological data were collected for the individual DCH_{MO} and DCH_{PIC} components at different concentrations in PBS for reference (Figure 4.3, Figure 4.4A). A series of dual network

hydrogels were also been analyzed, either with varying DCH_{MO} and constant DCH_{PIC} concentrations (Figure 4.4B), or varying DCH_{PIC} and constant DCH_{MO} concentrations (Figure 4.4C). As shown in Figure 4.4B&C, all DCH_{DN} samples had greater G' (*ca.* 5 times) compared to the sum of their respective DCH_{MO} and DCH_{PIC} components, suggesting that there is a synergistic effect that enhances mechanical properties. With increasing DCH_{MO} concentration, G' for the dual network hydrogels increased and reached its highest value at 3 wt% DCH_{MO} , after which the G' decreased (Figure 4.4B). These results suggest that there is a narrow threshold of DCH_{MO} concentration that results in optimized dual network hydrogel network formation. As DCH_{MO} concentration was increased past 3 wt%, the dual network hydrogel stiffness decreased. This is likely due to increased viscosity during component mixing that prevented optimized PIC formation and annealing. By holding DCH_{MO} concentration constant, we also demonstrated that mechanical properties of DCH_{DN} can be easily tuned by varying concentration of DCH_{PIC} (Figure 4.4C).

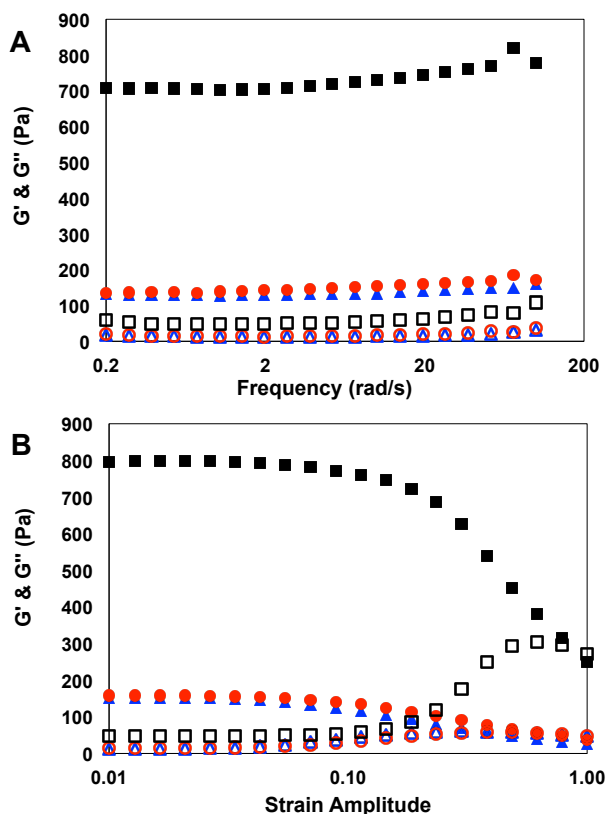


Figure 4.3 Rheology data for 5 wt% DCH_{PIC} (triangle), 5 wt% DCH_{MO} (circle), DCH_{DN} (3 wt% DCH_{MO} + 5 wt% DCH_{PIC}) (square) in PBS buffer at 25 °C. (A) G' (Pa, solid symbols) and G'' (Pa, open symbols) of hydrogel samples as functions of angular frequency. (B) Storage modulus, G' (Pa, solid symbols), and loss modulus, G'' (Pa, open symbols), of hydrogel samples as functions of strain amplitude.

We previously observed that both DCH_{PIC} and DCH_{MO} were able to rapidly self-heal after mechanical breakdown, which allows them to be injected through small bore needles.^{11,12} DCH_{DN} (3 wt% DCH_{MO} + 5 wt% DCH_{PIC}) was evaluated to determine if self-healing properties were inherited from its components. The DCH_{DN} sample was subjected to high amplitude oscillatory strain, followed immediately by monitoring the recovery of elasticity over time by measuring G' at a much smaller strain amplitude (Figure 4.5). During the initial 100 s of high strain amplitude, G' became less than G'' , indicating that the hydrogel has become a viscous liquid. Upon removal of the high strain, DCH_{DN} rapidly recovered its elastic properties similar to DCH_{MO} and DCH_{PIC}.

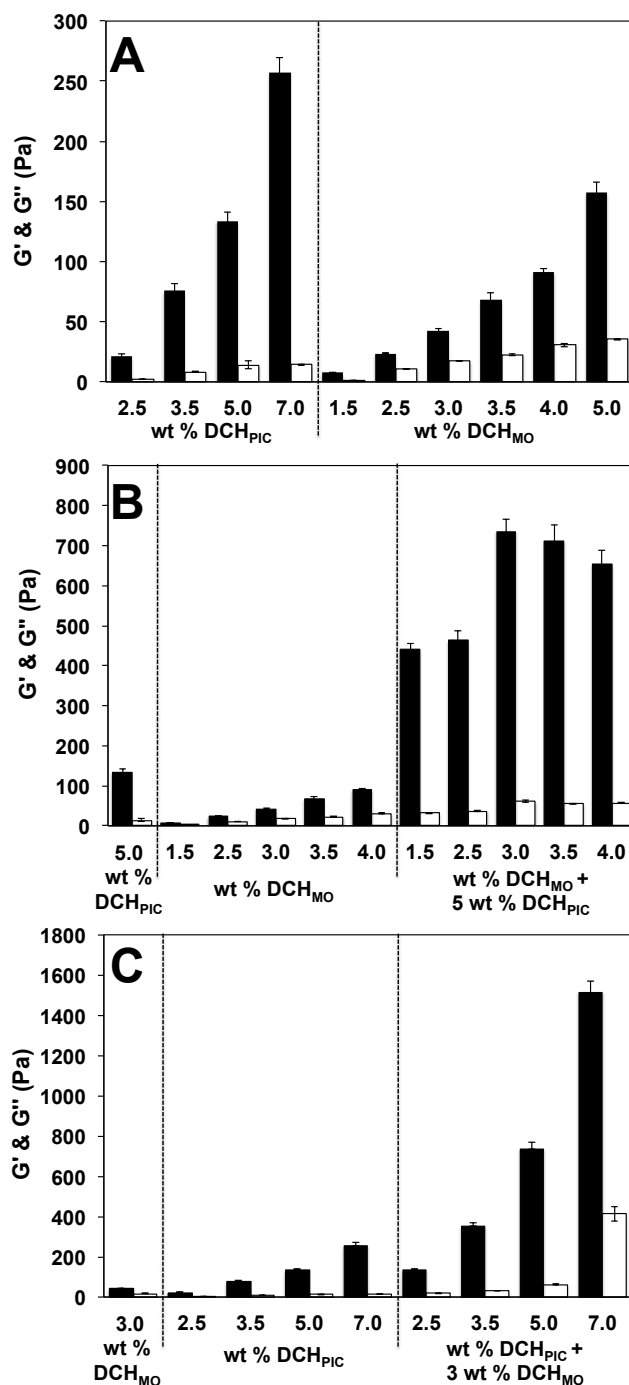


Figure 4.4 Dual network diblock copolyptide hydrogel properties. (A) Storage modulus, G' (Pa, black), and loss modulus, G'' (Pa, white), of DCH_{PIC} and DCH_{MO} at varying concentrations in $1\times$ PBS buffer at 25 °C. (B) G' (Pa, black) and G'' (Pa, white) of DCH_{DN} made from mixing 5 wt% DCH_{PIC} with varying concentrations of DCH_{MO} in PBS buffer at 25 °C. (C) G' (Pa, black), and loss modulus, G'' (Pa, white), of DCH_{DN} made from mixing 3 wt% DCH_{MO} with varying concentrations of DCH_{PIC} in PBS buffer at 25 °C. All G' and G'' values were measured at an angular frequency of 5 rad/s and a strain amplitude of 0.01.

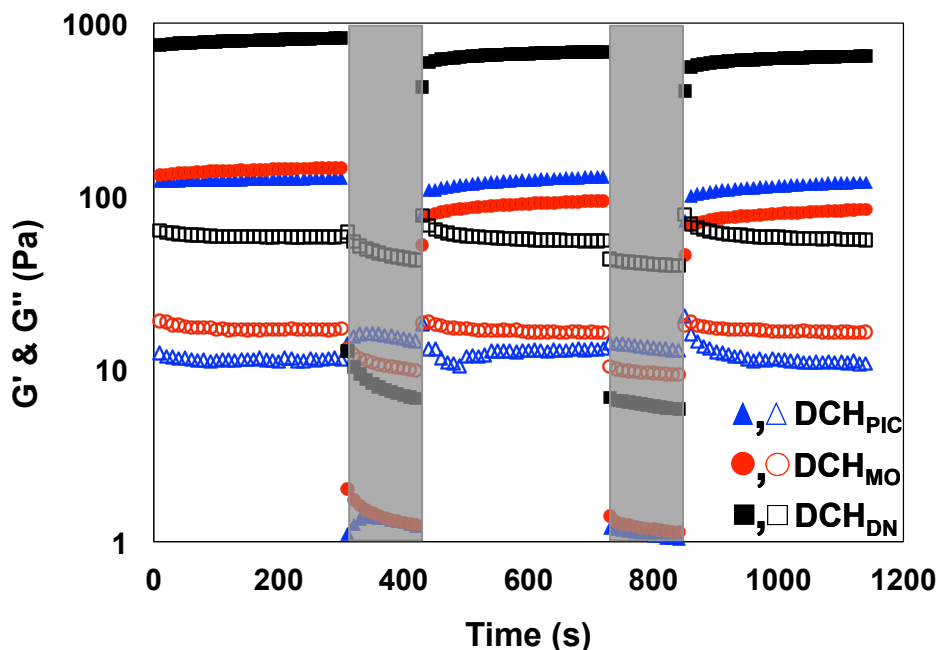


Figure 4.5 Recovery for 5 wt% DCH_{PIC}, 5 wt% DCH_{MO}, DCH_{DN} (3 wt% DCH_{MO} + 5 wt% DCH_{PIC}) in PBS buffer at 25 °C over time (G' , solid symbols and G'' , open symbols) after application of stepwise large-amplitude oscillatory breakdown (gray area, percent strain of 1000 at 10 rad/s for 120 s) followed by low-amplitude linear recovery (white area, percent of strain of 1 at 5 rad/s for 300s)

Previously, DCH_{PIC} was reported to possess significantly enhanced resistance against dilution in aqueous media compared to amphiphilic DCH (i.e. DCH_{MO}), which was observed to directly dissolve into aqueous media.¹¹ To study the stability of the DN hydrogel against dilution in aqueous media, a DCH_{DN} (3 wt% DCH_{MO} + 5 wt% DCH_{PIC}) sample was prepared in PBS, and then an equal volume of DMEM cell culture media was added on top of the hydrogel (Figure 4.6C). For comparison, 5 wt% samples of DCH_{MO} and DCH_{PIC} in PBS were prepared in the same manner (Figure 4.6A & B). Initially, the DMEM solutions formed clear layers above all hydrogel samples. By day 4, the DCH_{MO} sample had fully dissolved into the DMEM layer (Figure 4.6D). However, both DCH_{PIC} and DCH_{DN} were able to retain their hydrogel shape even after 7 days (Figure 4H and I). The swelling ratio was calculated for all three hydrogel samples

and DCH_{DN} was observed to have higher degree of swelling than DCH_{PIC} over the duration of 7 days (Figure 4.7). This phenomenon can be explained by partial dissolution of DCH_{MO} domains within DCH_{DN} that results in additional swelling. To verify that the DCH_{MO} network within DCH_{DN} can be stabilized by the DCH_{PIC} network upon dilution, we measured the weight loss from DCH_{DN} by diffusion into the supernatant. We found that DCH_{DN} retains 81% of its original total mass after 7 days of dilution in media, which is only slightly less than the 88% mass retention of the DCH_{PIC} control (Figure 4.8). The rapid self-healing properties of DCH_{DN} combined with the stability against aqueous dilution makes it a promising material for potential development as an injectable hydrogel scaffold.

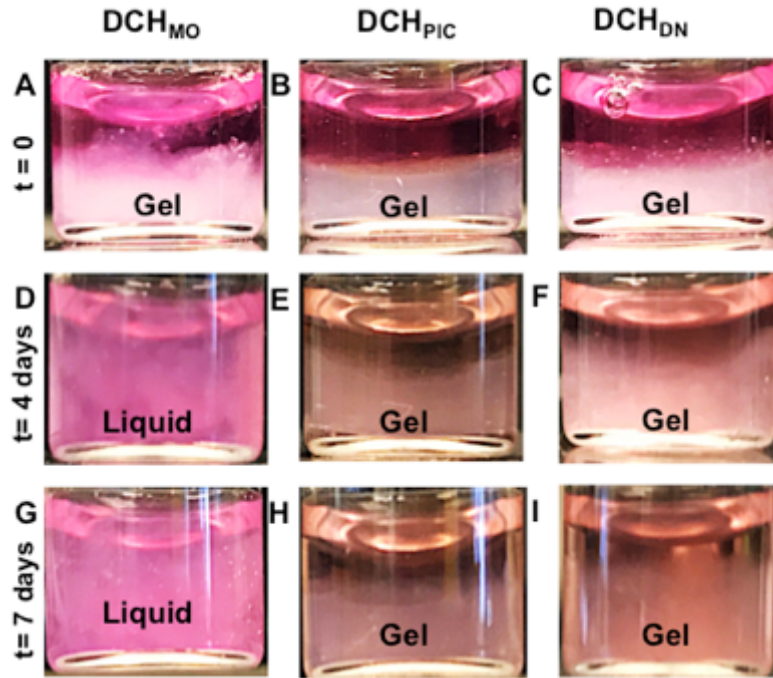


Figure 4.6 Stability of diblock copolyptide hydrogels against dilution. Nonionic amphiphilic DCH_{MO} (A, D, G; 5.0 wt %), polyion complex DCH_{PIC} (B, E, H; 5.0 wt %), and dual network DCH_{DN} (C, F, I; 3 wt % DCH_{MO} and 5 wt% DCH_{PIC}) in 1× PBS were each diluted with an equal volume of DMEM cell culture media. (A, B, C) Layer of cell media formed over all gels at the beginning of the experiment (time 0). (D, E, F) After 4 days, the DCH_{PIC} and DCH_{DN} remained intact while DCH_{MO} had dispersed into the full volume of media and was a liquid. (G, H, I) After 7 days, the DCH_{PIC} and DCH_{DN} still remained intact as gels underneath the media.

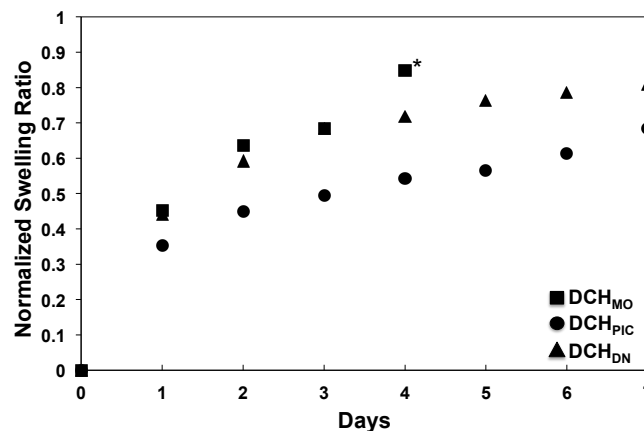


Figure 4.7 Normalized swelling ratio measurements. Hydrophobic assembled DCH_{MO} (5.0 wt %), polyion complex DCH_{PIC} (5.0 wt %), and dual network DCH_{DN} (3 wt % DCH_{MO} and 5 wt% DCH_{PIC}) in 1× PBS were each diluted with an equal volume of DMEM cell culture media. Swelling was monitored by removal of supernatant above the hydrogels at each time point. Normalized swelling ratio was calculated as: (weight of sample after swelling - weight of initial sample) / weight of initial sample. *DCH_{MO} no longer remains as a hydrogel by day 4.

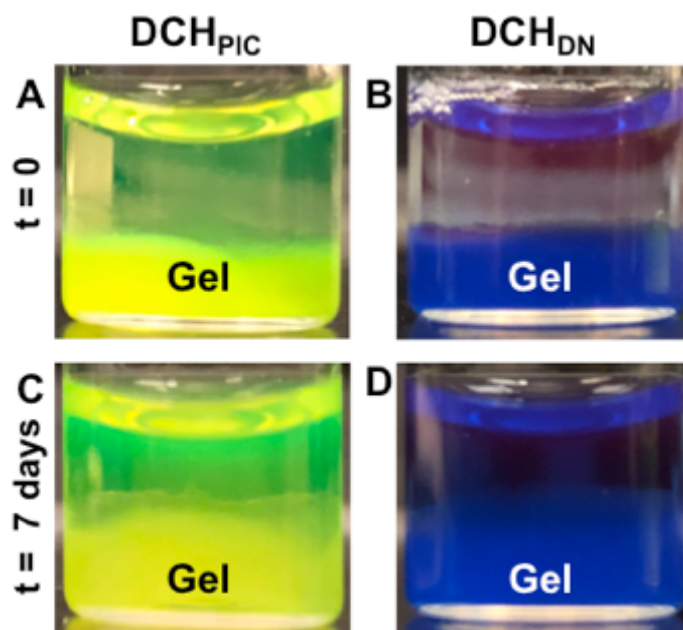


Figure 4.8 Sample loss during swelling study. DCH_{PIC} [A, C; 5.0 wt %; Alexa Fluor 488 - labeled (M⁰A)₁₅₅E₅₅ (yellow)], and dual network DCH_{DN} [B, D; 3 wt % DCH_{MO} and 5 wt% DCH_{PIC}; Alexa Fluor 633 -labeled (M⁰A)₂₀₀L₃₀ (blue)] in 1× PBS were each diluted with an equal volume of PBS. At day 7, supernatants were removed and lyophilized. Dried polymer from supernatants was weighed accounting for salt from PBS. It was found that 88 % and 81 % of the components were retained in DCH_{PIC} and DCH_{DN}, respectively.

The supramolecular structure of DCH_{DN} was characterized at microscale resolution via laser scanning confocal microscopy (LSCM). To visualize the microscopic structures, chains of (M^OA)₁₅₅E₆₀ and (M^OA)₂₀₀L₃₀ were conjugated with fluorescent probes (Alexa Fluor NHS esters 488 and 633 respectively) and then assembled into DCH_{DN}. Both the DCH_{PIC} (Alexa Fluor 488, green) and the DCH_{MO} (Alexa Fluor 633, red) channels were found to contain microporous networks with interconnected polypeptide-rich domains (Figure 4.9A&B). An overlay of the red and green channels revealed that the two networks had minimal overlap and were interpenetrating (Figure 4.9C). Additional 3D rendering of the z-stack series shows greater detail of the interpenetrating DCH_{MO} and DCH_{PIC} networks at a microscopic level (Figure 4.9D, E &F).

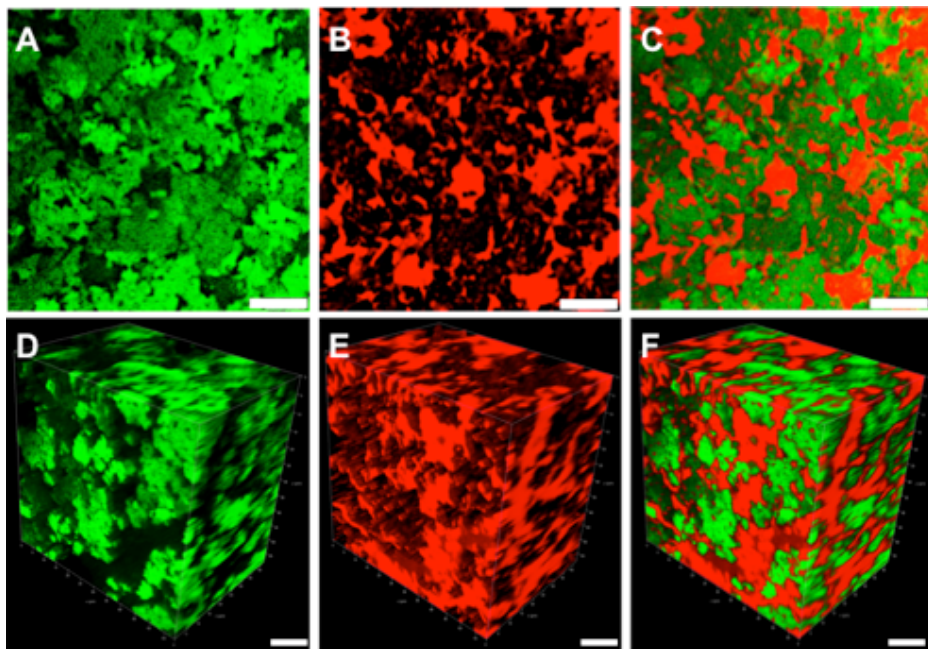


Figure 4.9 Laser scanning confocal images of DCH_{DN} (3 wt% DCH_{MO} + 5 wt% DCH_{PIC}). (A–C) LSCM images (z-thickness 0.896 μm) of DCH_{DN} consisting of Alexa Fluor 488-labeled (M^OA)₁₅₅E₅₅ (green) and Alexa Fluor 633-labeled (M^OA)₂₀₀L₃₀ (red) showing interpenetrating DCH_{MO} and DCH_{PIC} networks. (A) Alexa Fluor 488 channel, (B) Alexa Fluor 633 channel, (C) merge of (A) and (B). (D–F) 3D renderings of DCH_{DN} z-stacks showing interpenetrating DCH_{MO} and DCH_{PIC} networks. (D) Alexa Fluor 488 channel, (E) Alexa Fluor 633 channel, (F) merge of (D) and (E). Scale bars: (A–F) 10 μm

4.4 Conclusion

In summary, a new diblock copolypeptide based dual network hydrogel (DCH_{DN}) system has been developed. DCH_{DN} are composed of two interpenetrating networks based on previously reported polyion complex hydrogels (DCH_{PIC}) and nonionic amphiphilic hydrogels (DCH_{MO}). DCH_{DN} was found to possess substantially enhanced stiffness compared to both of its hydrogel components. Dual hydrogel stiffness was sensitive to concentration of the DCH_{MO} component, which affects viscosity of the pre-hydrogel mixture, and thus PIC assembly. By varying the concentration of DCH_{PIC} , mechanical properties of DCH_{DN} can be easily tuned. Finally, DCH_{DN} was shown to inherit rapid self-healing and stability against aqueous dilution properties from the DCH_{PIC} component. It is also expected that DCH_{DN} will inherit good cell compatibility from both DCH_{PIC} and DCH_{MO} components.^{11,12} A combination of these properties makes DCH_{DN} viable candidates for applications as injectable or printable scaffold for tissue engineering and stem cell therapy.

4.5 Experimental

4.5.1 Materials and Methods

Tetrahydrofuran (THF), hexanes, and methylene chloride were dried by purging with nitrogen and passage through activated alumina columns prior to use. $\text{Co}(\text{PMe}_3)_4$ and amino acid *N*-carboxyanhydride (NCA) monomers were prepared according to literature procedures.¹⁵ All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Selecto silica gel 60 (particle size 0.032–0.063 mm) was used for flash column chromatography. Fourier Transform Infrared (FTIR) measurements were taken on a Perkin Elmer RX1 FTIR spectrophotometer calibrated using polystyrene film, and attenuated

total reflectance (ATR-IR) data were collected using a PerkinElmer Spectrum 100 FTIR spectrometer equipped with a universal ATR sample accessory. ^1H NMR spectra were acquired on a Bruker ARX 400 spectrometer. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed at 25 °C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 µm columns at 30 °C with 0.5% (w/w) KTFA in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) as eluent and sample concentrations of 10 mg/ml. Pyrogen free deionized water (DI) was obtained from a Millipore Milli-Q Biocel A10 purification unit.

4.5.2. Experimental Procedure

General procedure for copolypeptide preparation

All polymerization reactions were performed in an N_2 filled glove box using anhydrous solvents. To a solution of L-methionine NCA (Met NCA) and L-alanine NCA (Ala NCA) in THF (50 mg/ml), a solution of $\text{Co}(\text{PMe}_3)_4$ in THF (20 mg/ml) was added.¹⁵ The reaction was let to stir at ambient temperature (ca. 22 °C) for 60 min. Complete consumption of NCA was confirmed by FTIR spectroscopy, and then the desired amount of γ -benzyl-L-glutamate NCA (Bn-Glu NCA), ϵ -TFA-L-lysine NCA (TFA-Lys NCA) or L-leucine NCA in THF (50 mg/ml) was added to the reaction mixture, which was let to stir for an additional 60 min. FTIR was used to confirm complete consumption of all NCAs. Outside the glove box, the block copolypeptide solutions were precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl

(2x 20 ml) to remove residual cobalt ions. The white precipitates were then washed with DI water (3x 20 ml) and freeze-dried.¹⁵

Synthesis of poly(L-methionine sulfoxide_{0.88}-*stat*-L-alanine_{0.12})₁₅₅-*block*-poly(L-lysine)₅₅, (M^OA)₁₅₅K₅₅ and poly(L-methionine sulfoxide_{0.88}-*stat*-L-alanine_{0.12})₁₅₅-*block*-poly(L-glutamate)₅₅, (M^OA)₁₅₅E₅₅

Synthesis procedure was reported in literature.¹¹

Synthesis of poly(L-methionine sulfoxide_{0.88}-*stat*-L-alanine_{0.12})₂₀₀-*block*-poly(L-Leucine)₃₀

Synthesis procedure was reported in literature.¹²

Preparation of (M^OA)₁₅₅E/K₅₅ + (M^OA)₂₀₀L₃₀ Dual Network (DCH_{DN}) hydrogels

Both anionic and cationic PIC components, (M^OA)₁₅₅E₅₀ and (M^OA)₁₅₅K₅₀ were dissolved in PBS at desired concentrations. The cationic solution was then used to dissolve M^OA₂₀₀L₃₀ to form a viscous solution. Next, the solution of anionic (M^OA)₁₅₅E₅₀ component was added to the cationic viscous solution, and the resulting sample mixed and vortexed for 20 seconds to form a hydrogel within 5-30 seconds depending on the copolyptide concentrations and compositions.

Rheology measurements on hydrogels

An Anton Paar Instruments MCR 302 rheometer with a 25 mm diameter, 1° cone plate geometry and solvent trap was used for all measurements. Frequency sweeps were measured at a constant strain amplitude of 0.01. Strain sweeps were measured at a constant frequency of 5 rad/s.

All measurements were repeated 3 times for each hydrogel sample and the results were averaged and plotted. To evaluate shear thinning and recovery behavior of DCH samples the strain amplitude parameters were stepped from 1% to 1000%, maintained at 1000% for 2 min and then returned to 1% to evaluate the recovery of the mechanical properties at a fixed frequency of 5 rad/s.

Hydrogel swelling measurement

5 wt% **DCH_{PIC}**, 5 wt% **DCH_{MO}** and **DCH_{DN} (3 wt% DCH_{MO} + 5 wt% DCH_{PIC})** hydrogels were formed in 2 ml scintillation vials and let stand for 1 hr. Cell media were then placed on top of the hydrogel samples and stored in the refrigerator (0 °C) for desired period of time. At each time point, the supernatants were pipetted out of the scintillation vial without disturbing the gel at the bottom. The supernatant volumes were subtracted from the original media volume to determine swelling ratio. The gel samples were subjected to an inversion test to assess gel integrity. Finally, the supernatant media volume was replaced on top of the hydrogel in the scintillation vial and the sample was returned to the refrigerator (0 °C).

Hydrogel loss measurement during swelling

5 wt% **DCH_{PIC}** labeled with Alexa fluor 488 and **DCH_{DN} (3 wt% DCH_{MO} + 5 wt% DCH_{PIC})** labeled with Alexa fluor 633 in 1× PBS were each diluted with an equal volume of PBS. At day 7, supernatants were removed and lyophilized. Dried polymer from supernatants was weighed accounting for salt from PBS. It was found that 88 % and 81 % of the components were retained in the **DCH_{PIC}** and **DCH_{DN}**, respectively.

Fluorescent probe conjugation to (M^OA)₁₅₅E₅₅ and (M^OA)₂₀₀L₃₀ copolypeptides

Alexa Fluor 488 NHS Ester (AF 488 NHS) was conjugated to the N-terminal amine of (M^OA)₁₅₅E₅₅. (M^OA)₁₅₅E₅₅ (10 mg) was dissolved in DI water (pH=7) (1 ml) in a scintillation vial (20 ml). AF 488 NHS was dissolved in DI water (pH=7) (1 mg/ml) and added to the 1 % (w/v) copolypeptide solution at a 1:1.25 molar ratio of copolypeptide chains to fluorescent probes. The reaction was allowed to proceed for 24 h at ambient temperature. After AF 488 NHS modification, the resulting solution was dialyzed (2000 MWCO) against DI water for 2 d, and then freeze-dried to yield the product as a yellow solid. Alexa Fluor 633 NHS Ester (AF 633 NHS) was conjugated onto the N-terminal amine of (M^OA)₂₀₀L₃₀ using a similar procedure.

Laser scanning confocal microscopy (LSCM) of fluorescently labeled hydrogels

LSCM images of hydrogels were taken on a Leica TCS-SP8 MP-Inverted Confocal and Multiphoton Microscope equipped with an argon laser (476 and 488 nm blue lines), a diode (DPSS) laser (561 nm yellow-green line), and a helium-neon laser (633 nm far red line). Fluorescently labeled hydrogel samples were visualized on glass slides with a spacer between the slide and the cover slip (double-sided tape) allowing the self-assembled structures to be minimally disturbed during focusing. An optical section of 0.896 μm was used. Leica LAS-X software was used for 3D rendering. The resulting 3D model was processed via histogram equalization. Sample imaging was performed at the Advanced Light Microscopy/Spectroscopy Center (ALMS) at the UCLA California NanoSystems Institute (CNSI).

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Chapter 5: Polyion Complex Diblock Copolypeptide Assembly

5.1 Abstract

New particle forming polyion complex diblock copolypeptides have been synthesized, assembled in aqueous media and the resulting structures characterized. The size and structure of these assemblies were shown to depend on the poly(ionic) block lengths and chirality. Various imaging techniques, including confocal microscopy, EM and cryo-EM were used to verify that the assemblies formed were core-shell structured micellar aggregates. The poly(anionic) copolypeptide component of this system was also complexed with lysozyme (a cationic protein) in aqueous media, and was found to form assemblies that are stable under physiological salt and osmotic conditions.

5.2 Introduction

Polyion complexes (PIC) are precipitates and coacervates that form primarily with a charge neutral stoichiometry when a pair of oppositely charged polyelectrolytes are mixed in aqueous media. However, when non-charged hydrophilic blocks are added to the polyelectrolyte chains, more defined structures can be formed.¹ For example, polyion complex micelles can form by association via electrostatic interactions. Kataoka *et al* first reported formation of monodisperse PIC micelles by mixing together a pair of oppositely charged, dual hydrophilic block copolymers, PEG-poly(L-lysine) and PEG-Poly(α,β -aspartic acid).^{2,3} The PIC micelles showed great potential as delivery carriers for oligonucleotides and plasmid DNA due to their high stability, reduced immune response, and prolonged blood circulation lifetime.

Kataoka *et al* also reported formation of the first semipermeable PIC vesicles using related block polymers.⁴ Similar to core-forming polypeptide based copolymer micelles, chain length recognition of oppositely charged block copolymers produced membranes of matched

polymer chains, which were shielded with a layer of PEG segments in aqueous solution.⁵ These PIC vesicles do not require organic solvents for self-assembly and can provide effective stabilization and delivery of cargoes, such as myoglobin. In addition, these vesicles have pH-sensitive properties that allow tunable permeability of the membrane and intracellular release of therapeutic agents.⁶

There are many advantages of using block copolypeptides to form the various fore-mentioned PIC structures. These materials are attractive because of their biodegradability, diverse side chain functionalities, and ability to respond to external stimuli. Our lab has shown that poly(L-methionine) can be oxidized into non-ionic, water soluble poly(L-methionine sulfoxide), which can serve as a biodegradable replacement for the PEG segments typically used in PIC assemblies. Most importantly, the Deming lab has also developed transition metal mediated living polymerization techniques that allow facile preparation of block copolypeptides with well-defined block lengths, which we sought to use to form PIC self-assembled structures for biomedical applications.^{7,8,9}

There are also many merits to using PIC systems for assembling structures. Self-assembled structures can be formed directly in water and do not require organic solvents. This is important for encapsulation of protein cargoes, which may denature in organic solvents. PIC copolypeptide system also may allow for pH responsive properties or charges conversion properties that may enhance *in vivo* blood circulation, cellular uptake and intracellular cargo release.¹⁰

The goal of this study was to 1) synthesize and characterize a pair of oppositely charged, dual hydrophilic diblock copolypeptides, and to 2) characterize the nano and microstructures formed upon mixing of the copolypeptides in aqueous media. Diblock copolypeptides, poly(L-

methionine sulfoxide)_n-b-poly(L-lysine)_m, M^O_n(K)_m, and poly(L-methionine sulfoxide)_n-b-poly(L-glutamate)_m, M^O_n(E)_m were used for this purpose. Poly(L-methionine sulfoxide) acts as the nonionic hydrophilic segment similar to PEG while poly(L-lysine) and poly(L-glutamate) are the poly(cation) and poly(anion) blocks that are involved in PIC formation (Figure 2).

Based on PIC literature, PIC assembly structure should be primarily determined by diblock copolypeptide composition as well as conformation and chirality of the charged blocks. A non-ionic segment to charged segment ratio greater than 3:1 will tend to form spherical micelles.² In contrast, vesicle formation requires greater charged segment lengths and thus smaller non-ionic to charged ratios of ca. 1:1.⁴ Also, close matching of the oppositely charged segment lengths has been found to be important in enabling formation of stable micelle and vesicle structures.

In addition to compositional requirements, conformations of the charged blocks has also been found to be important for forming regular assemblies. Homochiral charged homopolypeptides are known to aggregate into irregular precipitates due to formation of β -sheets. Meanwhile, when at least one charged chain is racemic, coacervates are formed instead of β -sheets.¹¹ Also, a charged block that can retain its α -helicity during PIC formation can also prevent β -sheet formation.¹² Therefore, it is possible to create ordered micelle and vesicle assemblies by controlling the optical purity and α -helicity of the charged segments.

5.3 Results and Discussion

We prepared diblock copolypeptides containing poly(L-methionine), **M**, segments *ca.* 60 residues long, followed by side-chain protected **K** or **E** segments of different length (Figure 5.1).^{8,13} Subsequent oxidation of **M** residues, followed by side-chain deprotection of **K** and **E**

residues and purification gave the target copolypeptides poly(L-methionine sulfoxide)₆₀-*block*-poly(L-lysine-HCl)_x, (**M**^O)₆₀**K**_x; and poly(L-methionine sulfoxide)₆₀-*block*-poly(L-glutamate-Na)_x, (**M**^O)₆₀**E**_x, where x = 10, 20, 30, 40 and 60 (Figure 5.2). All copolymers were isolated in high yield with compositions that closely matched expected values (Table 5.1). The **M**^O segment length was chosen based on previous studies on amphiphilic copolypeptide assemblies,^{8,14} where it was found to be sufficiently long to promote assembly formation. The **K** and **E** lengths were varied in order to study the role of structured PIC domain size on assembly formation and properties

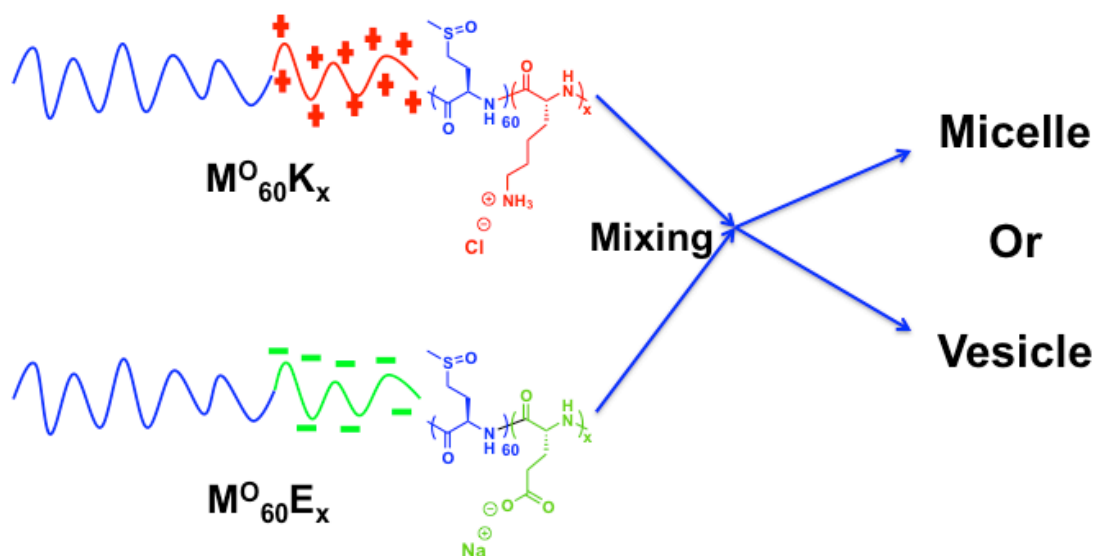


Figure 5.1 Schematic of diblock copolypeptide PIC assembly system.

The oppositely charged diblock copolypeptides were mixed in aqueous media at different polymer concentrations and salt concentrations to determine the optimal assembly parameters. Two mixing methods were explored. Method 1: separately dissolved copolypeptides in PBS were mixed directly followed by vortexing for two minutes. Method 2: copolypeptides in 1 M NaCl were mixed and then dialyzed against DI water to remove excess salt. Using method 1, the clear solutions quickly turned cloudy upon mixing, indicating formation of aggregates. It was

observed by DLS that when salt concentration reached 1 M NaCl, no assembly greater than 200 nm in diameter was formed based on number average. Therefore, method 2 was used to achieve a slower self-assembly process that might provide better annealed assemblies.. The high salt concentration in method 2 was used to initially screen the electrostatic interactions between the poly(L-lysine) and poly(L-glutamate). The resulting mixtures were then dialyzed against a decreasing gradient of NaCl solution to slowly remove excess salt and to promote assembly of more well defined structures. While the decreasing salt gradient resulted in fewer large aggregates by DIC microscopy, both methods resulted in similar particle size distribution by DLS. Therefore, due to its simplicity and short time required, method 1 was employed for rest of the study.

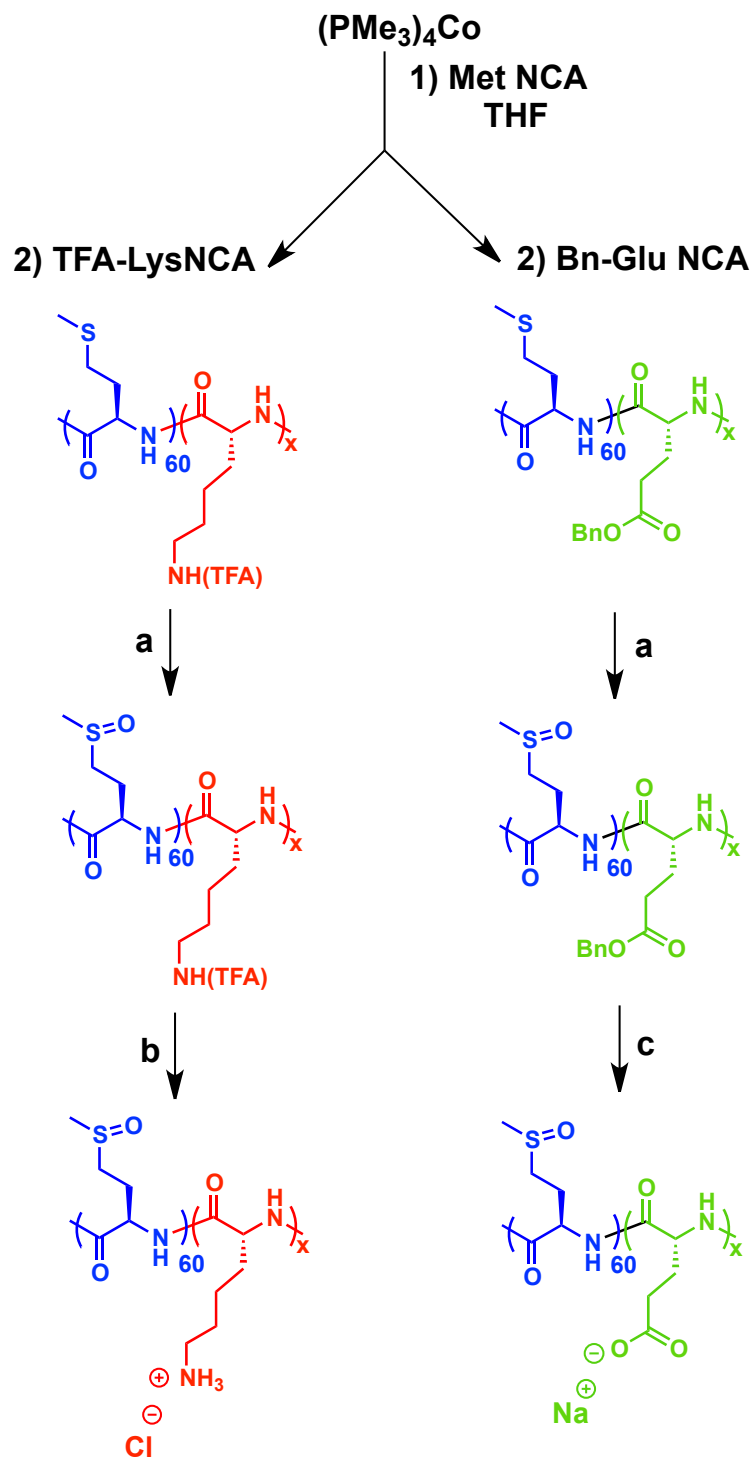


Figure 5. 2 Synthesis of oppositely charged, dual hydrophilic diblock copolypeptides $\text{M}_{60}^{\text{O}}\text{K}_x$ and $\text{M}_{60}^{\text{O}}\text{E}_x$. a) TBHP, CSA, H_2O , 20 °C, 1 d. b) K_2CO_3 , $\text{MeOH}/\text{H}_2\text{O}$, 50 °C, 8 h. c) MSA, TFA, anisole, 20 °C.

Table 5.1 Copolymerization data for diblock copolypeptide synthesis.

Sample	Composition ^a	Yield (%) ^b	PIC Formulation	Structure Observed	Average Size (nm) ^c
$M_{60}^O E_{10}$	$M_{61}^O E_9$	89	$M_{60}^O E/K_{10}$	Micelles/ Micelle Clusters	220
$M_{60}^O K_{10}$	$M_{61}^O K_{10}$	90			
$M_{60}^O E_{20}$	$M_{58}^O E_{17}$	91	$M_{60}^O E/K_{20}$	Micelles/ Aggregates	38
$M_{60}^O K_{20}$	$M_{58}^O K_{18}$	93			
$M_{60}^O E_{30}$	$M_{58}^O E_{32}$	93	$M_{60}^O E/K_{30}$	Micelles/ Aggregates	79
$M_{60}^O K_{30}$	$M_{58}^O K_{33}$	90			
$M_{60}^O E_{40}$	$M_{63}^O E_{42}$	93	$M_{60}^O E/K_{40}$	Micelles/ Aggregates	122
$M_{60}^O K_{40}$	$M_{63}^O K_{43}$	91			
$M_{60}^O E_{60}$	$M_{63}^O E_{68}$	95	$M_{60}^O E/K_{60}$	Micelles/ Aggregates	135
$M_{60}^O K_{60}$	$M_{63}^O K_{65}$	92			
$M_{60}^O E_{(rac)40}$	$M_{58}^O E_{36}$	87	$M_{60}^O E_{(rac)}/K_{40}$	Micelles	33
$M_{60}^O K_{40}$	$M_{63}^O K_{43}$	91			

^aRelative amino acid compositions of oxidized, deprotected block copolypeptides were determined by ¹H NMR integrations. Degree of polymerization of initial M_x segment was determined by end-group analysis using ¹H NMR. ^bTotal isolated yield of purified block copolypeptides following deprotection. ^cAverage particle sizes were measured by dynamic light scattering

DLS measurements showed that both PIC components $M_{60}^O E_x$ and $M_{60}^O K_x$ do not form aggregates in aqueous media prior to mixing. Upon mixing, $M_{60}^O E/K_x$ formed particles with diameters ranging from 20 nm to 400 nm by number distribution (Figure 5.3, Table 5.1). With increasing ionic segment lengths, particle sizes were observed to increase. The exception was $M_{60}^O E/K_{10}$, which also formed larger particles. The smaller ionic charged segments allowed formation of small micelles that were observed to further associate to become larger micelle clusters. The nature of the larger aggregates formed in $M_{60}^O E/K_{40}$ were studied with Differential

Interference Contrast (DIC) and confocal microscopy. DIC images (Figure 5.4A) showed formation of round assemblies greater than 1 μm in diameter. FITC labeled assemblies, $\text{M}_{60}^{\text{O}}\text{E}_{(\text{FITC})}\text{K}_{40}$, were imaged using confocal microscopy. These particles appeared to resemble aggregates of micelles with core-shell morphologies instead of vesicles with hollow sphere morphologies (Figure 5.4B). Micelle formation was further confirmed using negative stain Electron Microscopy (EM). EM images demonstrated the existence of micelles approximately 100 nm in diameter, which agrees with the DLS measurement (Figure 5.4C).

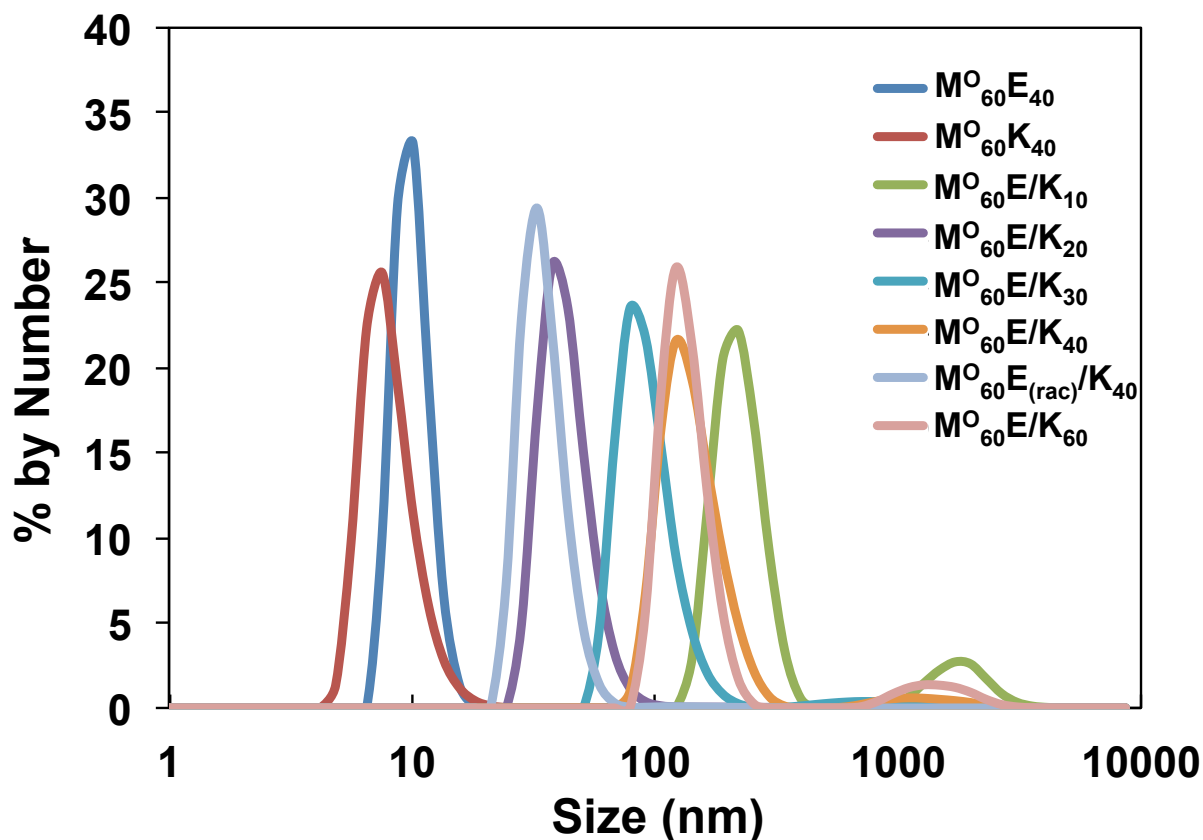


Figure 5.3 Dynamic Light Scattering data for PIC assemblies. All samples were prepared in PBS at 1 mg/ml.

It is known that when at least one of the poly(lysine) or poly(glutamate) components is racemic, PIC formation leads to coacervation instead of solid β -sheet precipitates.¹¹ Therefore, to

inhibit formation of β -sheets, a racemic glutamate segment was used in the $M_{60}^O E_{(rac)}/K_{40}$ system. DLS measurements (Figure 5.3, Table 1) showed smaller particle population sizes of 33 nm, consistent with micellar assemblies. Cryo-EM studies confirmed that these structures were forming micelles (Figure 5.2D).

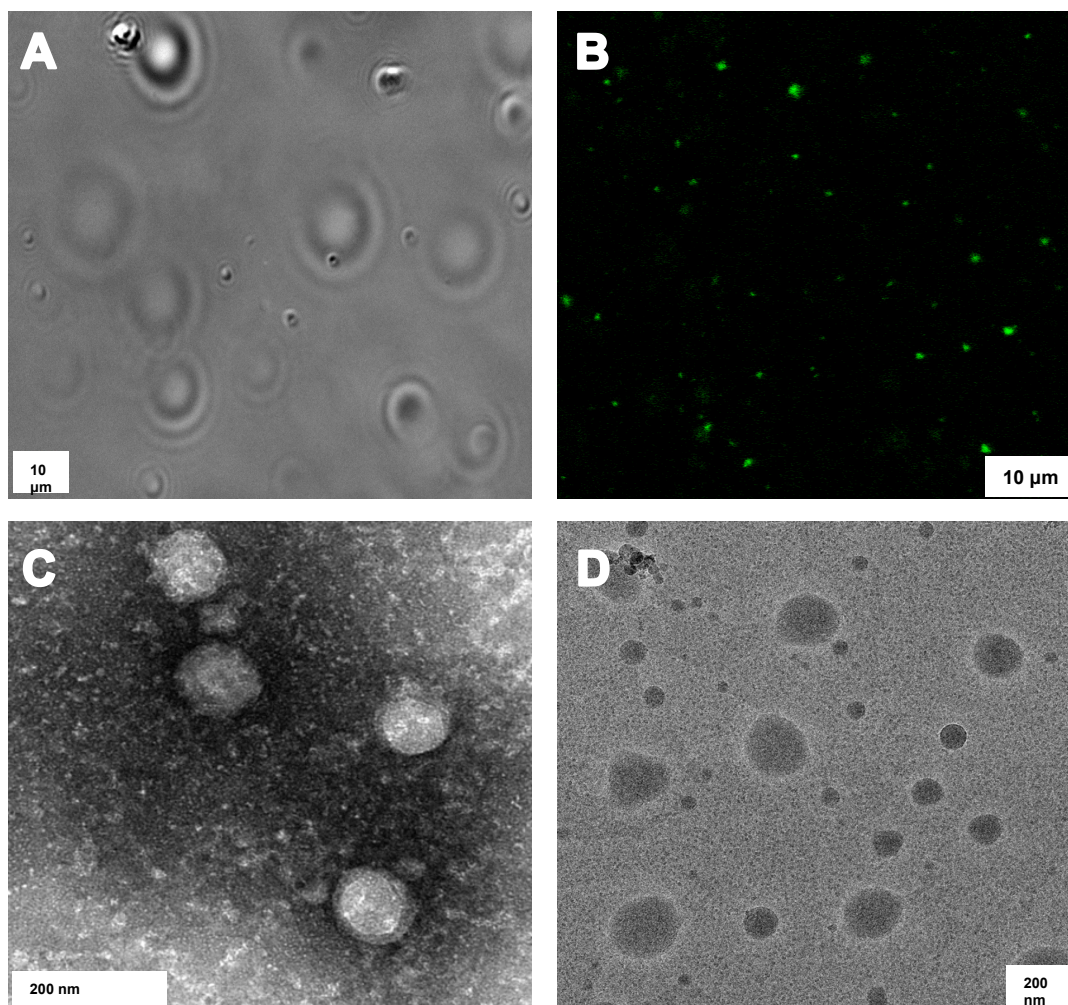


Figure 5.4 (A) DIC image of $M_{60}^O E/K_{40}$ particles. (B) Confocal microscopy image of $M_{60}^O E_{(FITC)}/K_{40}$ (C) Negative staining TEM image of $M_{60}^O E/K_{40}$ (D) cryo-EM image of $M_{60}^O E_{(rac)}/K_{40}$. (A,B,D) All samples were prepared in PBS at 1 mg/ml.

To explore the potential of PIC diblock copolypeptides for protein delivery applications, $M_{60}^O E_{20}$ was complexed with the cationic protein lysozyme. The polypeptide-protein mixture was prepared at stoichiometric charge balance in 10 mM phosphate buffer. Immediately upon

mixing, the solution turned turbid, indicating formation of aggregates. DLS measurements showed formation of assemblies with average diameter of 79 nm. To study stability, the particles were prepared in aqueous media with increased salt concentration (150 mM NaCl) or osmotic pressure (150 mM glucose). The particle size distribution was shown to remain the same under increased osmotic pressure. At physiological salt concentration, the complex particles remained stable with an increased average particle size of 220 nm (Figure 5.5). This increase in size is likely due to charge screening effects by higher salt concentration that allows the polyion components to better anneal and assemble into larger assemblies.

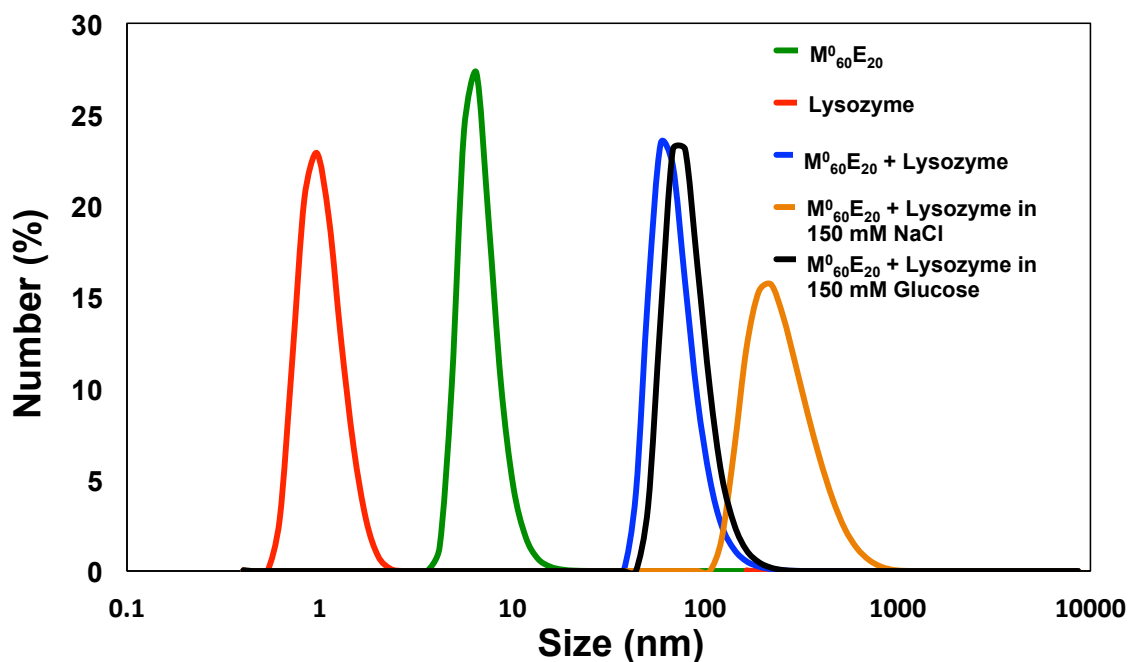


Figure 5.5 Dynamic Light Scattering of $M_{60}E_{20}$ – lysozyme complex assembly. All samples were prepared in 10 mM phosphate buffer at 2.5 mg/ml.

5.4 Conclusions

A series of polyion complex diblock copolypeptide based assembly, $M_{60}E/K_x$, has been developed and evaluated. Using dynamic light scattering, it was found that the assembly sizes

tend to increase with polyionic block lengths. However, if the ionic block lengths are too short ($x < 10$), larger compound micelles were also observed to form. Confocal microscopy and electron microscopy were used to verify that the assemblies have core-shell micelle structures. Chirality was also used to tune PIC assembly properties. PIC particles with racemic polyionic blocks were found to form smaller coacervate micelles by cryo-EM. Finally, the PIC system was demonstrated to have potential for protein delivery applications. Initial experiments have shown that the poly(anionic) copolypeptide component was able to form stable assemblies when complexed with lysozyme. The resulting complex particles remained stable under both physiological salt concentration and osmotic pressure.

5.5 Experimental

5.5.1 Materials and Methods

Tetrahydrofuran (THF), and hexanes were dried by purging with nitrogen and passage through activated alumina columns prior to use. $\text{Co}(\text{PMe}_3)_4$ and amino acid *N*-carboxyanhydride (NCA) monomers were prepared according to literature procedures.⁸ All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Selecto silica gel 60 (particle size 0.032–0.063 mm) was used for flash column chromatography. Fourier Transform Infrared (FTIR) measurements were taken on a Perkin Elmer RX1 FTIR spectrophotometer calibrated using polystyrene film, and attenuated total reflectance (ATR-IR) data were collected using a PerkinElmer Spectrum 100 FTIR spectrometer equipped with a universal ATR sample accessory. ^1H NMR spectra were acquired on a Bruker ARX 400 spectrometer. Pyrogen free deionized water (DI) was obtained from a Millipore Milli-

Q Biocel A10 purification unit. Lysozyme from chicken egg white was purchased from Sigma-Aldrich.

5.5.2. Experimental Procedures

General procedure for copolypeptide preparation

All polymerization reactions were performed in an N₂ filled glove box using anhydrous solvents. To a solution of L-methionine NCA (Met NCA) in THF (50 mg/ml), a solution of Co(PMe₃)₄ in THF (20 mg/ml) was added.⁸ The reaction was let to stir at ambient temperature (*ca.* 22 °C) for 60 min. Complete consumption of NCA was confirmed by FTIR spectroscopy, and then the desired amount of g-benzyl-L-glutamate NCA (Bn-Glu NCA) or e-TFA-L-lysine NCA (TFA-Lys NCA) in THF (50 mg/ml) was added to the reaction mixture, which was let to stir for an additional 60 min. FTIR was used to confirm complete consumption of all NCAs. Outside the glove box, the block copolypeptide solutions were precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl (2x 20 ml) to remove residual cobalt ions. The white precipitates were then washed with DI water (3x 20 ml) and freeze-dried.⁸

Example synthesis of poly(L-methionine)₆₀-*block*-poly(e-trifluoroacetyl-L-lysine)₄₀, M₆₀(TFA-K)₄₀ and poly(L-methionine)₆₀-*block*-poly(g-benzyl-L-glutamate)₄₀, M₆₀(Bn-E)₄₀

Met NCA (120 mg, 0.71 mmol) was dissolved in THF (2.4 ml) and placed in a 20 ml scintillation vial containing a stir bar. To the vial, (PMe₃)₄Co initiator solution (496 µl of a 20 mg/ml solution in THF) was added via syringe. The vial was sealed and allowed to stir in the glove box for 1 h. An aliquot (20 µl) was removed and analyzed by FTIR to confirm that all the NCA was consumed. In the glove box, α-methoxy-ω-isocyanoethyl-poly(ethylene glycol)₄₅

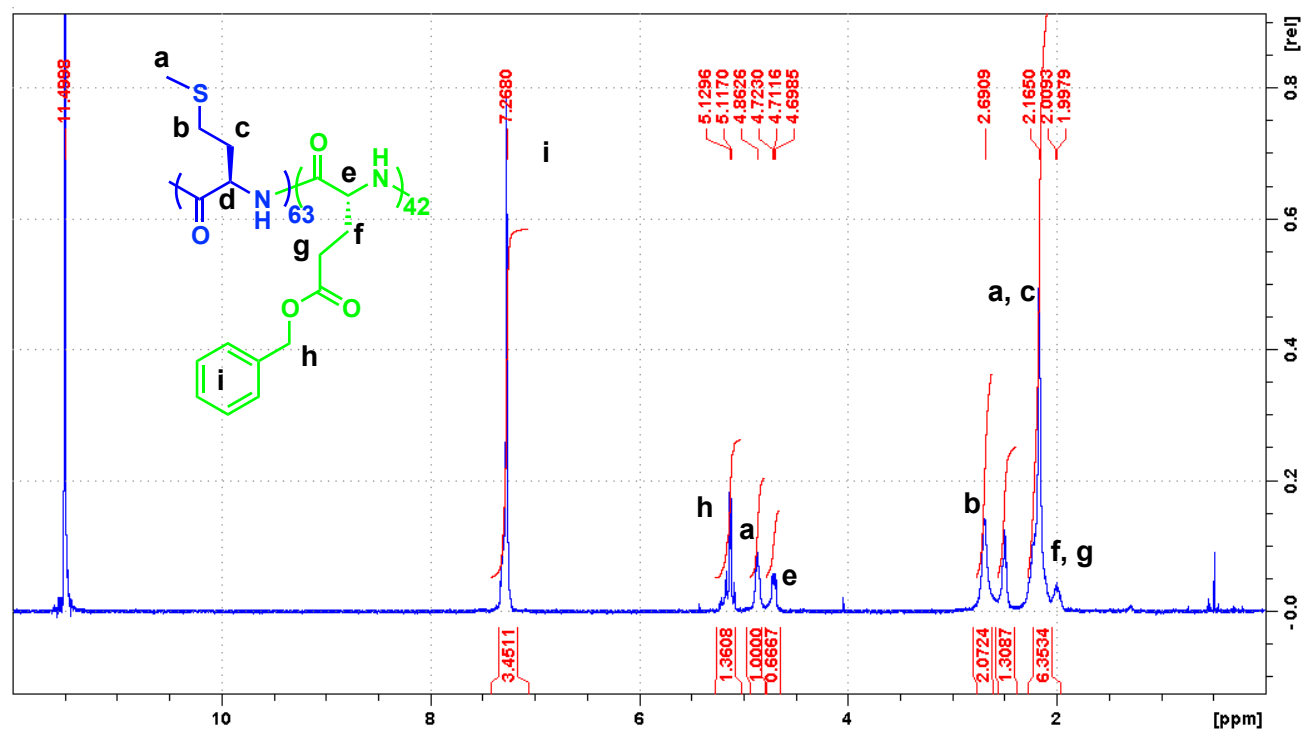
(mPEG₂₃-NCO)¹ (20 mg) was dissolved in THF (1 ml) in a 20 ml scintillation vial. An aliquot (350 μ l) of the polymerization solution containing active chain ends was removed and added to the solution of mPEG₂₃-NCO. The PEG end-capped sample (**M_x-mPEG₂₃**) was sealed, allowed to stir for 24 h, and then used for chain length determination (*vide infra*). Separately, aliquots of the polymerization solution containing active chains (1 ml each) were added to vials containing either Bn-Glu NCA (52 mg, 0.195 mmol) or TFA-Lys NCA (53 mg, 0.199 mmol) dissolved in THF (1040 μ l or 1060 μ l, respectively). The vials were sealed and allowed to stir in the glove box for 1 h to give the diblock copolypeptides, **M₆₀(TFA-K)₄₀** and **M₆₀(Bn-E)₆₀**. FTIR was used to confirm complete consumption of NCAs in both reactions. Outside the glove box, the block copolypeptide solutions were precipitated into 10 mM HCl (20 ml), and then washed with 10 mM aqueous HCl (2 x 20 ml) to remove residual cobalt ions. The white precipitates were then washed with DI water (3 x 20 ml) and freeze-dried (average yield = 93%).

Analytical data: M₆₀(Bn-E)₄₀

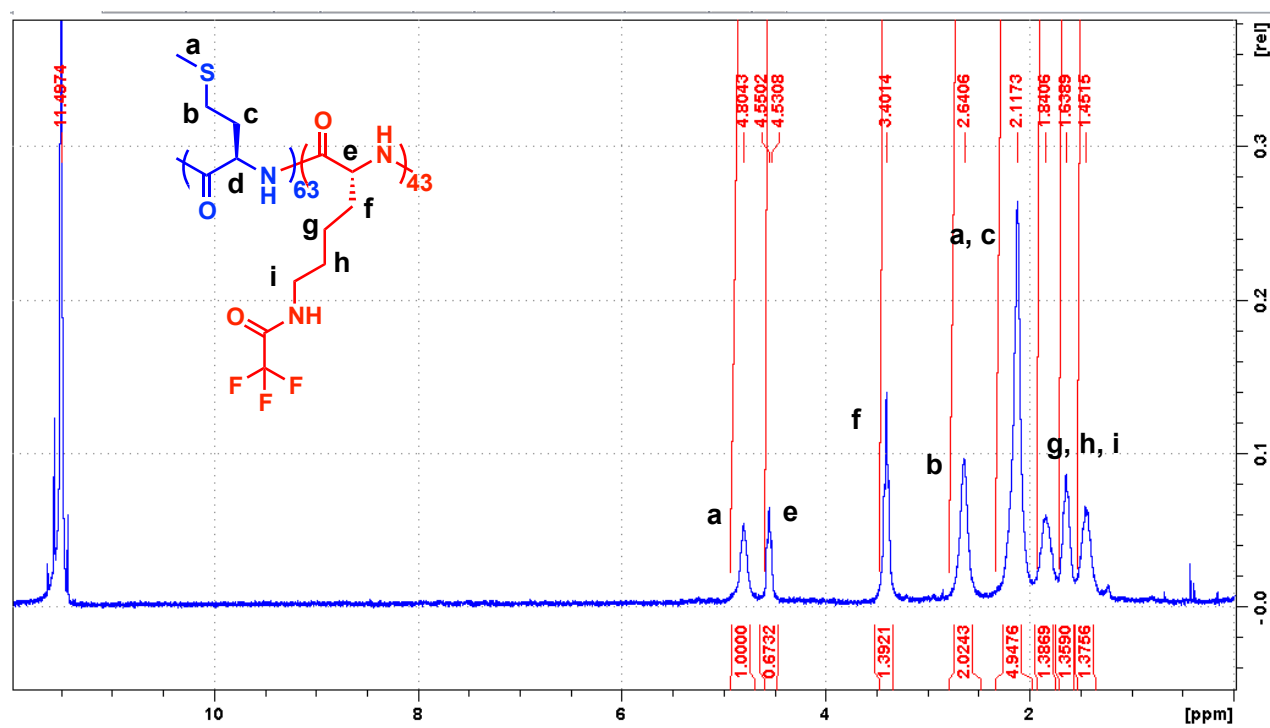
¹H NMR (400 MHz, d-TFA, 25 °C): δ 7.27 (br m, 3.45H), 5.12 (br m, 1.36H), 4.86 (br s, 1H), 4.7 (br m, 0.67H), 2.7 (br m, 2.07H), 2.49 (br m, 1.31H), 2.28 – 1.94 (br m, 6.35H), FTIR (THF, 25 °C): 1738 cm⁻¹ (benzyl ester), 1652 cm⁻¹ (amide I), 1550 cm⁻¹ (amide II).

Analytical data: M₆₀(TFA-K)₄₀

¹H NMR (400 MHz, d-TFA, 25 °C): δ 4.8 (br s, 1 H), 4.54 (br m, 0.67H), 3.4 (br m, 1.39H), 2.64 (br m, 2.02H), 2.12 (br m, 4.95H), 1.85 (br m, 1.39H), 1.64 (br m, 1.36H), 1.45 (br m, 1.38H), FTIR (THF, 25 °C): 1726 cm⁻¹ (TFA amide), 1652 cm⁻¹ (amide I), 1550 cm⁻¹ (amide II).



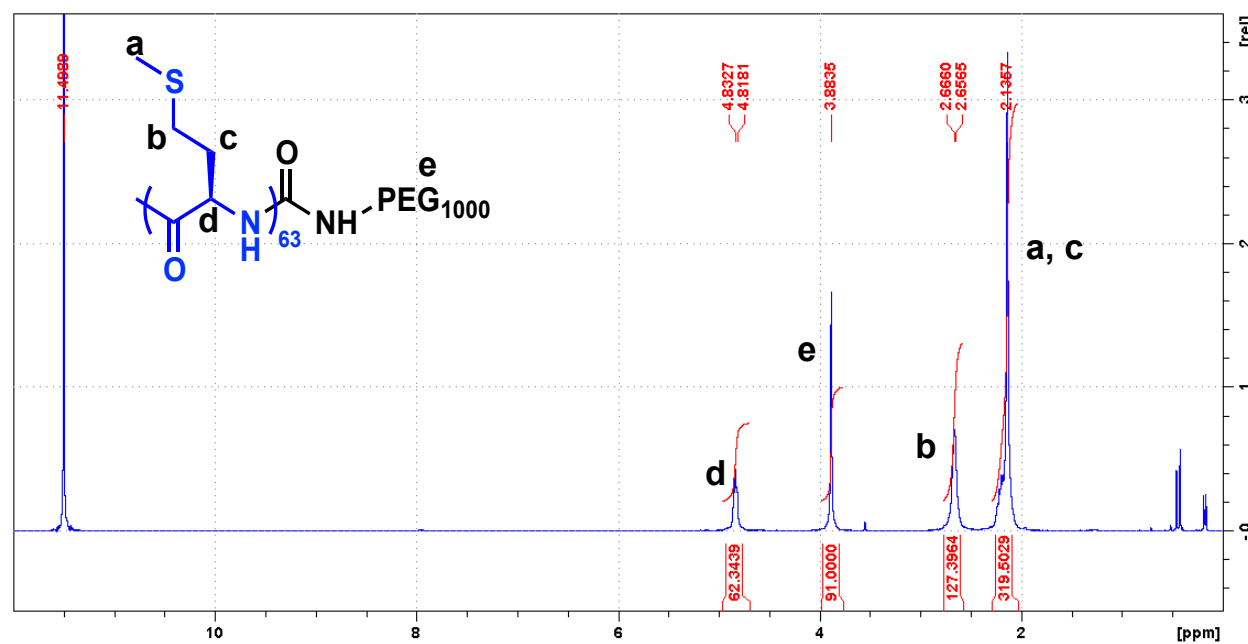
¹H NMR spectrum of $M_{60}(\text{Bn-E})_{40}$ in d-TFA.



¹H NMR spectrum of $M_{60}(\text{TFA-K})_{40}$ in d-TFA

Sample procedure for M_x chain length determination using end-group analysis

Outside of the glove box, the PEG end-capped sample (M_x -mPEG₂₃) from above was washed with 10 mM aqueous HCl (2x). After stirring for 1 h, M_x -mPEG₂₃ was collected by centrifugation and washed with DI water (3 x 20 ml) to remove all non-conjugated mPEG₂₃-NCO. The remaining M_x -mPEG₂₃ was then freeze-dried to remove residual H₂O. To determine M_x molecular weights (M_n), ¹H NMR spectra were obtained. Since it has been shown that end-capping is quantitative for (PMe₃)₄Co initiated NCA polymerizations when excess isocyanate is used,¹⁶ integrations of methionine (δ 2.66) resonances versus the polyethylene glycol resonance at δ 3.88 could be used to obtain M_x lengths (found: x = 62, designated as M_{60}). ¹H NMR (400 MHz, d-TFA, 25 °C): 4.82 (br s, 1H), 3.88 (br m, 1.46H), 2.66 (br m, 2.03 H), 2.26 - 2.0 (br m, 5.13H)



¹H NMR spectrum of M_{60} -mPEG₂₃ (1000 Da) in d-TFA.

Preparation of poly(L-methionine sulfoxide)₆₀-block-poly(L-lysine·HCl)₄₀, $M_{60}^O(K)_{40}$, and poly(L-methionine sulfoxide)₆₀-block-poly(L-glutamate·Na)₄₀, $M_{60}^O(E)_{40}$

Oxidation and deprotection procedures were reported in literature.¹⁷

Analytical Data: $M_{60}^O(\text{Bn-E})_{40}$

^1H NMR (400 MHz, d-TFA, 25 °C): δ 7.26 (br m, 3.66H), 5.12 (br m, 01.33H), 4.87 (br s, 1H), 4.71 (br m, 0.68H), 3.47 - 3.12 (br m, 2.04H), 2.92 (br m, 2.98H), 2.64 (br m, 1.05H), 2.49 (br m, 2.33H), 2.2 (br m, 0.68H), 1.99 (br m, 0.67H),

Analytical Data: $M_{60}^O(\text{TFA-K})_{40}$

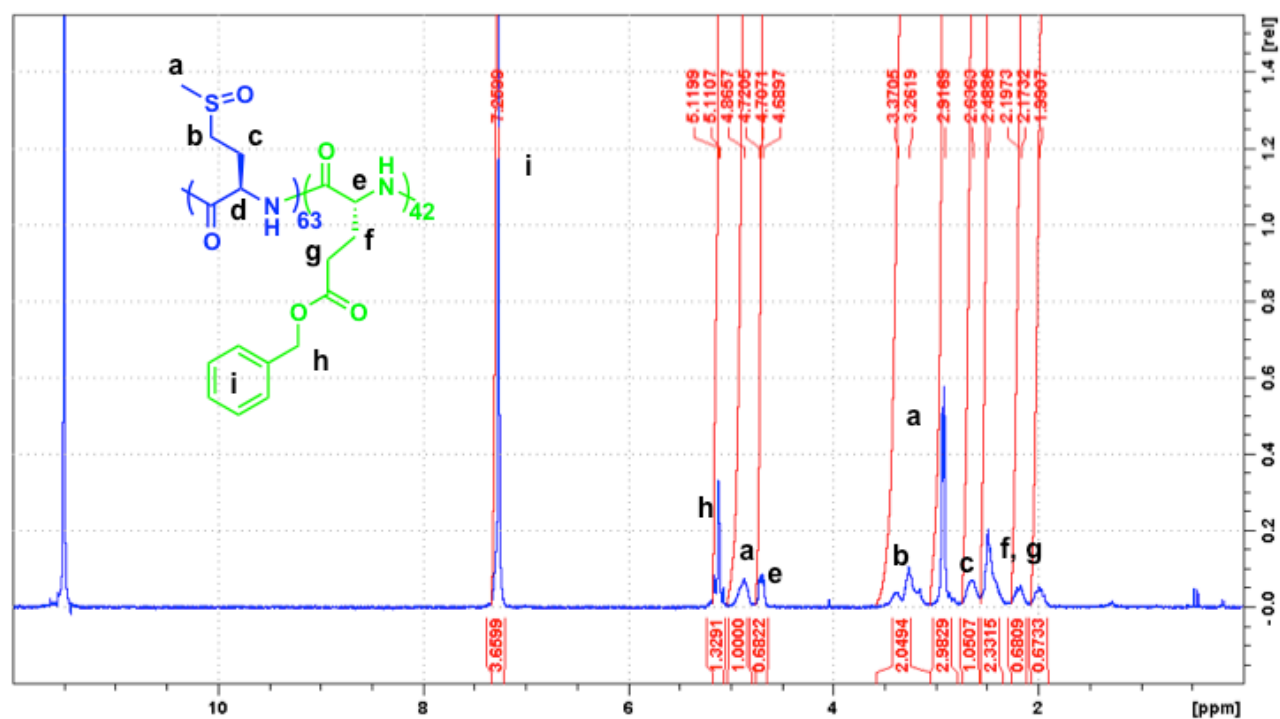
^1H NMR (400 MHz, d-TFA, 25 °C): δ 4.88 (br s, 1H), 4.61 (br m, 0.714H), 3.49-3.07 (br m, 3.43 H), 2.93 (br m, 3.06H), 2.65 (br m, 1.04 H), 2.44 (br m, 1.03 H), 1.94 (br m, 1.46 H), 1.71 (br m, 1.43 H), 1.52 (br m, 1.44 H).

Analytical Data: $M_{60}^O\text{E}_{40}$

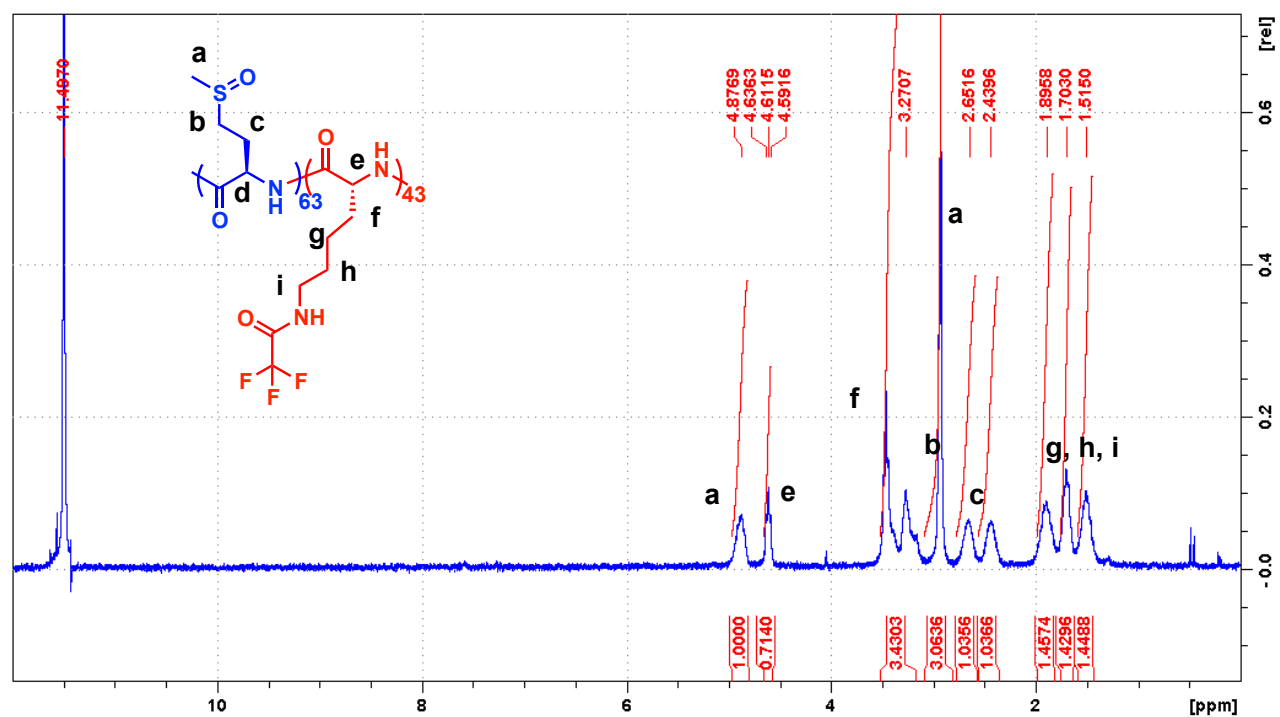
^1H NMR (400 MHz, D_2O , 25 °C): δ 4.55 (br s, 1H), 4.43 (br m, 0.69H), 3.09 (br m, 2.03H), 2.81 (br m, 3.02H), 2.45 - 2.15 (br m, 3.47H), 2.10 - 1.85 (br m, 1.45H). ATR-IR (25 °C): 1653 cm^{-1} (amide I), 1546 cm^{-1} (amide II).

Analytical Data: $M_{60}^O\text{K}_{40}$

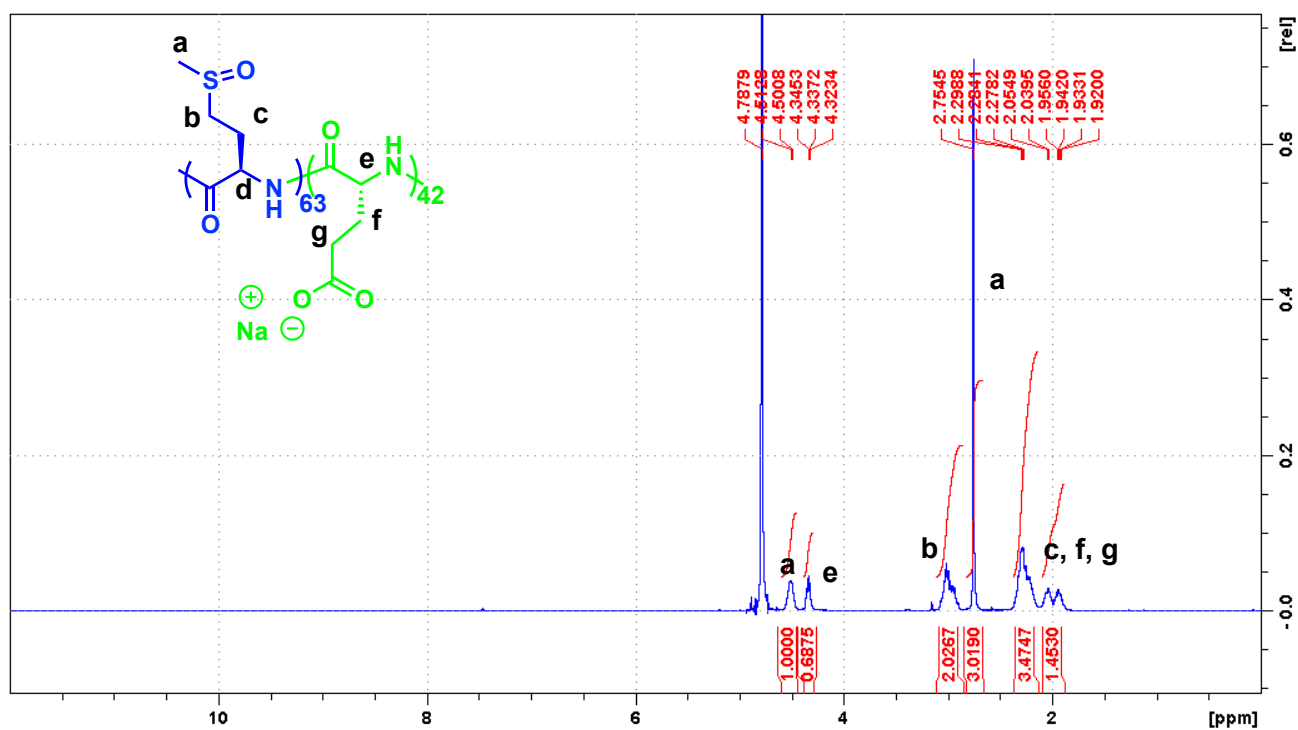
^1H NMR (400 MHz, D_2O , 25 °C): δ 4.58 (br s, 1H), 4.43 (br m, 0.68H), 3.26-2.86 (br m, 3.43H), 2.81 (br m, 3.07H), 2.46 - 2.26 (br m, 2.05H), 1.8 (br m, 2.69H), 1.5 (br m, 1.39H). ATR-IR (25 °C): 1653 cm^{-1} (amide I), 1546 cm^{-1} (amide II).



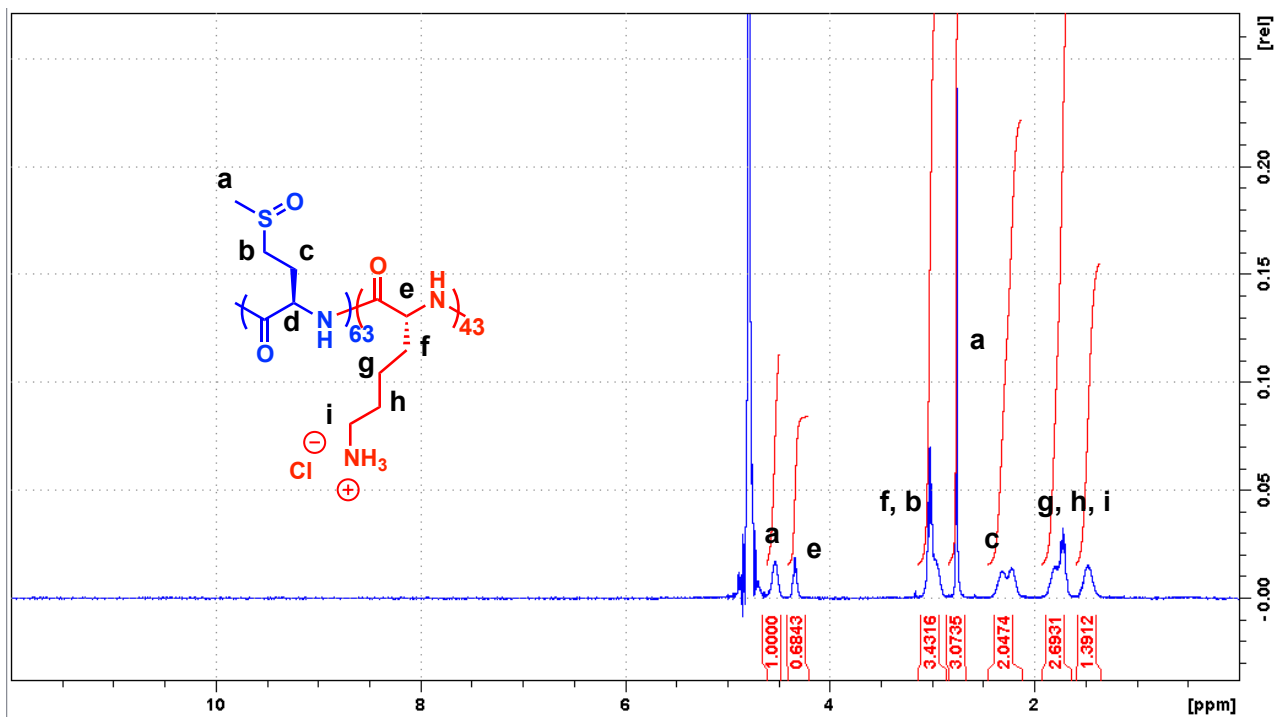
^1H NMR spectrum of $M_{60}(\text{Bn-E})_{40}$ in $d\text{-TFA}$.



^1H NMR spectrum of $M_{60}(\text{TFA-K})_{40}$ in $d\text{-TFA}$.



1H NMR spectrum of $M_{60}^O E_{40}$ in D_2O .



1H NMR spectrum of $M_{60}^O K_{40}$ in D_2O .

PIC Particle Preparation

$M_{60}^O E_x$ and $M_{60}^O K_x$ were individually dissolved in desired solvents (i.e. DI water, PBS, etc.) at the desired concentration (e.g. 1 mg/ml). Once the polymers were fully dissolved, the polymer solutions were combined in a 2 ml scintillation vial and vortexed rigorously for 2 minutes.

$M_{60}^O E_{20}$ -Lysozyme Complex Preparation

$M_{60}^O E_{20}$ was dissolved in 10 mM phosphate buffer at 2 mg/ml. A lysozyme stock solution (3.2 mg/ml) was made in 10 mM phosphate buffer. An aliquot of the lysozyme solution was added to the $M_{60}^O E_{20}$ solution to reach charge stoichiometric balance. The polypeptide-protein mixture was vortexed for 5 seconds. Cloudiness was observed immediately after mixing.

Fluorescent probe conjugation to Poly(L-methionine sulfoxide)₆₀-block-poly(L-lysine)₄₀,

$M_{60}^O K_{40}$ FITC was conjugated to the amine group of lysine side chains. $M_{60}^O K_{40}$ (10 mg) was dissolved in pH 10 water (1 mL) in a 20 mL scintillation vial. FITC was dissolved in DMSO (1 mg/mL) and added to a 1 % (w/v) copolypeptide solution at 5:1 molar ratio of copolypeptide chains to probe. The acylation was allowed to proceed for 24 h at room temperature. After FITC modification, the solution was dialyzed in DI water for 2 days and freeze-dried to yield an orange solid.

Laser scanning confocal microscopy (LSCM) of fluorescently labeled PIC particles

LSCM images of PIC particles were taken on a Leica Inverted TCS-SP1 MP-Inverted Confocal and Multiphoton microscope equipped with an argon laser (476 and 488 nm blue lines), a diode (DPSS) laser (561 nm yellow-green line), and a helium-neon laser (633 nm far red line). The fluorescently labeled PIC particles were visualized on glass slides with a spacer between the

slide and the cover slip (double-sided tape) allowing the self-assembled structures to be minimally disturbed during focusing. Z-slice thickness of 0.78 μm was used. Sample imaging was performed at the Advanced Light Microscopy/Spectroscopy Center (ALMS) at the UCLA California NanoSystems Institute (CNSI).

Dynamic light scattering (DLS) analysis on PIC particles

PIC particle suspensions were placed in disposable cuvettes (ZEN0118) and analyzed using a Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc, Westborough, MA). A total scattering intensity of approximately 1×10^5 cps was targeted.

Zeta potential analysis on complexed PIC lysozyme particles

1 % (w/v) aqueous suspension of $\text{M}_{60}\text{E}_{20}$ complexed with lysozyme was prepared in 10 mM phosphate buffer (pH=7). The suspension was placed in a disposable capillary cell (DTS1070). Zeta potentials were measured with the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc, Westborough, MA).

Differential interference contrast microscopy (DIC).

Suspensions of PIC particles were visualized on glass slides with a spacer between the slide and the coverslip (double-sided tape) allowing the self-assembled structures to be minimally disturbed during focusing. The samples were imaged using a Zeiss Axiovert 200 DIC/Fluorescence Inverted Optical Microscope.

Negative Staining transmission electron microscopy (TEM) of PIC particles

Samples (4 μL) were placed on 300 mesh Formvar/carbon coated copper grids (Ted Pella) and allowed to remain on the grid for 150 seconds. Filter paper was used to remove residual fluid. One drop of 2 % (w/v) uranyl acetate (negative stain) was then placed on each grid

for 40 seconds, and subsequently removed by washing with drops of Millipore water and removing the excess liquid with filter paper. Sample containing grids were allowed to dry in air before imaging with a JEM 1200-EX (JEOL) transmission electron microscope at 80 kV.

Cryogenic transmission electron microscopy (cryo-EM) of PIC particles

10 μ l of 2 % (w/v) $M_{60}^O E_{(rac)}/K_{40}$ PIC particles in PBS buffer were deposited onto a 300 mesh copper grid containing a Quantifoil holey carbon film (SPI Supplies, West Chester, PA) and let stand for 2 min. The sample was blotted dry with filter paper and the grid was briefly washed with DI water twice. Without delay, the copper grid was then placed in a cryogenic bath of liquid ethane and then stored under liquid nitrogen. The sample was placed, using a cold stage, into a TF20 (FEI Tecnai G2) electron microscope and imaged with an accelerating voltage of 200 kV. Sample preparation and imaging was performed at the Electron Imaging Center for Nanomachines (EICN) at the UCLA California NanoSystems Institute (CNSI).

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