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A Novel Photopolymerizable Chitosan Collagen Hydrogel for Bone Tissue Engineering

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biomedical Engineering

By

Christopher Kenji Arakawa

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ABSTRACT OF THE THESIS

A Novel Photopolymerizable Chitosan Collagen Hydrogel for Bone Tissue Engineering

By

Christopher Kenji Arakawa

Master of Science in Biomedical Engineering

University of California, Los Angeles, 2012

Professor Benjamin Wu, Chair

Photopolymerizable hydrogels derived from naturally occurring polymers have attracted significant interest in bone and cartilage tissue engineering due to their mechanical and chemical similarities to extracellular matrices, biodegradability spatial and temporal control of polymerization, and ability to be cured in situ through a minimally invasive procedure. While hydrogels are used for a number of soft tissue regeneration, few studies have investigated the use of a photopolymerizable injectable hydrogel for bone tissue engineering applications. The objective of this thesis is to synthesize a novel hybrid chitosan/collagen hybrid hydrogel characterize its mechanical and chemical properties, evaluate its biological effect and investigate the use of such hydrogels for sustained growth factor release. It was found that these chitosan collagen hybrid scaffolds exhibited extensive incorporation of collagen and proper collagen fibril formation throughout the hydrogel and that incorporation of these fibrils resulted in increased
stiffness as compared with pure chitosan hydrogels. Furthermore, incorporation of collagen did not result in observable differences in gelation time but did increase resistance to lysozyme based degradation. When utilized as a 2D substrate, the hybrid hydrogel enhanced and encouraged cellular attachment, spreading, proliferation, and osteogenic differentiation of mouse bone marrow stromal cells as there was observed an upregulation in alkaline phosphatase (ALP) activity as well as increased mineralization. Similarly when cells were encapsulated within a 3D CSCol hydrogel matrix, mBMSCs spread on the surface of the hydrogel, proliferated and exhibited greater ALP activity and larger mineral deposits. In addition to use of CSCol hybrid hydrogels as 2D and 3D systems, it was proven that CS photopolymerizable hydrogels could be used for controlled growth factor release by co-encapsulating protein-loaded poly(lactic-co-glycolic acid) (PLGA) microparticles. PLGA microparticles encapsulated within CS hydrogels exhibited controlled sustained release of a fluorescent bovine serum albumin protein conjugate.

Collectively, this thesis provides the initial understandings of a photopolymerizable hybrid CSCol hydrogel as a potential injectable bone tissue engineering system.
The thesis of Christopher Kenji Arakawa is approved

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2012
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1. **Introduction and Significance:**

Bone is the principal calcified tissue in vertebrates and is a complex organ responsible for structural support, protection, hearing, mineral storage, fat storage, pH regulation and metabolism. Although often considered static, bone is a living, growing and dynamic organ constantly being resorbed and formed through a carefully regulated process\(^1\). Bone remodeling requires the coordination of cellular, molecular, hormonal, and systemic components in response to both chemical and mechanical stimuli\(^2,3\). The ability for bone to be both remodeled and its high degree of vascularity gives rise to a medium to high regenerative capacity.

Although bone has true regenerative capacity and is repaired by generation of new and healthy bone rather than scar tissue, osseous regeneration is limited \(^4\). Bone defects that grow to critical sizes beyond the ability of natural repair result in non union and loss of function \(^5\). Such defects can arise from trauma, congenital disease, surgery, or degenerative disorders like osteoarthritis and pose a significant health risk as they impair a patient’s quality of life and mobility.

In 2003, more than 25% of Americans exhibit musculoskeletal conditions requiring attention, and the annual costs for bone and joint treatment approach $240 million USD, approximately 2.9% of the gross domestic product\(^6\). Moreover estimates project that these statistics will dramatically rise with the graying of the baby boomer generation. However despite this increasing demand for bone and cartilage replacement, current treatments are lacking. The limited performance of artificial implants, the associated costs of joint replacement, the loosening of bone screws and high costs of bone grafts further augment this existing burden.
Although the autologous bone graft remains the gold standard for healing large bone defects, the grafting procedure is complicated by donor site morbidity, increased risk of infection and poor ability to fill complex defects\textsuperscript{7} \textsuperscript{8}.
2. **Review of Literature**

A. **Injectable Hydrogels**

Due to the limited capability of artificial implants and the complications associated with bone grafts, there is an increasing interest in the orthopedic community to investigate tissue engineering solutions to bone healing. In particular, injectable hydrogels derived from natural polymers that can be cured in situ have attracted significant attention as scaffolding to deliver transplanted cells due to their mechanical and chemical similarities to extracellular matrix proteins and the ability to degrade *in vivo* into non-toxic monomers. Injectable hydrogels can be delivered through a minimally invasive manner minimizing infection risk and can fill very complex defects.

B. **Photopolymerizable Hydrogels**

More specifically, photopolymerizable injectable hydrogels can form three-dimensional hydrophilic polymer networks able to swell large amounts of water, exhibit high permeability, resemble the physical characteristics of soft tissues and extracellular matrices and cure quickly *in situ*. Furthermore, photopolymerization is by nature a modifiable process which grants both temporal and spatial control of the polymerization.

Bulk and interfacial film polymerization, the most common forms of photopolymerization are achieved by dissolving both the photopolymerizable material (as either a monomer or macromer) and the photoinitiator in an appropriate solvent. The solution is then converted to the hydrogel state via irradiation with a light source that can be absorbed by the photoinitiator. Interfacial thin hydrogels for surface coating and have
been used for many different applications. These applications include surface coatings over the islet of Langerhans to provide immunoisolation of implanted tissue, while allowing quick and rapid diffusion of nutrients and insulin\textsuperscript{10} as well as hydrogel coatings over arterial surfaces to minimize thrombosis and restenosis following balloon arthroplasty, stent deployment, and intravascular catheterization\textsuperscript{8,13}.

In addition to thin films, transdermal photopolymerization has gained significant interest for implantation of materials, tissue engineering, and drug delivery\textsuperscript{14,10}. These studies take advantage of long wavelength UW light to initiate photopolymerization. While light of these wavelengths tend to exhibit poor transmission through the skin, sufficient light penetration did convert the solution to a hydrogel state.

While typical photopolymerization techniques like those mentioned utilize high energy ultraviolet light, such techniques can cause mutagenesis, cell death, and protein denaturing in addition to poor tissue penetration\textsuperscript{15,16}. Visible light photopolymerization and use of photoinitiator that absorb within the visible and near-infrared range presents a safer alternative. Visible light polymerization is known to be non-thermogenic, exhibit fewer cytotoxic and mutagenic effects, and transmit deeper through tissue\textsuperscript{17}.

C. Free Radical Photopolymerization

Photopolymerization is defined as the increase of molecular weight of a polymer resulting from the light irradiation of a substance or solution and includes the cross-linking of macromolecules to form a polymeric network. Vinyl polymerization, perhaps the most common type of photopolymerization can be initiated by either free radicals or
ionic species. In the case of free radical photopolymerization, high energy light is absorbed by a photoinitiator, exciting the molecule to a high energy state which then generates a free radical. These free radicals subsequently attack alkene groups on adjacent molecules forming highly reactive carbons which can then join to create new carbon-carbon bonds. This irreversible reaction can be utilized to generate both linear polymer chains and or three dimensional crosslinked networks.\textsuperscript{18}

D. Chitosan as a biomaterial in bone tissue engineering

![Chemical structure of chitin and chitosan](Image)

Figure 1: The chemical structure and deacetylation process of chitin (left) to chitosan (right)\textsuperscript{19}

Chitosan, a linear polysaccharide copolymer of N-acetyl-glucosamine and N-glucosamine units is a well established polymer widely researched for applications in wound healing, drug delivery, ophthalmology, implant surface treatment and tissue engineering\textsuperscript{20,21}. Its precursor chitin is second only to cellulose as the most abundant biopolymer found in nature and can be extracted from the exoskeleton of many arthropods including crab, shrimp
and lobster. Processing of chitosan from chitin is a simple procedure. Shells are washed and crushed to remove calcium mineral and are then deacetylated by treating the shell debris with NaOH. Chitosan is defined when the ratio of glucosamine to N-acetyl glucosamine of chitin exceeds 50%. The simplicity of processing and the over abundance of chitin in nature gives rise to a low, affordable, and readily available biopolymer\textsuperscript{22}.

Chitosan has played a major role in bone tissue engineering over the past 25 years as it presents a large number of properties making it appropriate for tissue engineering. Chitosan is biodegradable, biocompatible, bioadhesive, antibacterial, and promotes wound healing. In tissue engineering a number of chitosan based scaffolds have been utilized for bone regeneration including sponges\textsuperscript{23–25}, hydrogels\textsuperscript{26,27}, as well as composite synthetic/natural polymer hybrids\textsuperscript{28}.

In addition to its cost chitosan also exhibits few biocompatibility issues, antimicrobial effects, and similarity to osseo and chondral glycoproteins\textsuperscript{19}. Furthermore, investigation of the chemical structure of chitosan reveals a large number of amine groups which can be utilized as nucleophiles in bioconjugation techniques, making chitosan readily modifiable. Protonation of these amine groups is required to render chitosan sufficiently hydrophilic to dissolve in water. Unfortunately protonation of these groups necessitates an aqueous solution well below physiological pH ranges (~pH 5).
E. Photopolymerizable chitosan

Figure 2: The chemical structure of methacrylated glycol chitosan with glycol and methacrylate groups highlighted

While unmodified chitosan is only moderately water-soluble and can only be dissolved in acidic aqueous solutions, synthesis of a water-soluble glycol chitosan can be achieved by glycosylation of the hydroxyl group bound to carbon 6 of the chitosan backbone. Using this water-soluble glycol chitosan (GCS), and taking advantage of the large number of modifiable amine groups, one can chemically altered GCS via graft methacrylation to become photopolymerizable.29

When treated with glycidyl methacrylate, an unstable and chemically reactive molecule, the amine groups on the GCS nucleophillically attack the glycidyl methacrylate generating a methacrylated glycol chitosan. The newly conjugated terminal alkene groups on the methacrylate branches are then available to free radical attack and polymerization. The degree of methacrylation can be modified by altering the incubation time of glycidyl
methacrylate with GCS. Due to the hydrophobic nature of the methacrylate group, the degree of methacrylation may alter the hydrophilicity of the methacrylated glycol chitosan (MeGCS).

**F. Photoinitiator Screening**

Previous work by the Lee lab identified three potential photoinitiators which can be utilized to generate the necessary free radicals for polymerization when irradiated with visible blue light: riboflavin (RF), camphoquinone (CQ), and fluorescein (FR). Post-polymerization hydrogels were assessed and characterized by SEM, gelation time, elastic modulus, degradation time, and cellular viability and proliferation of primary harvest chondrocytes\textsuperscript{27}.

SEM analysis of hydrogels cured using each of the three photoinitiators did not reveal any significant differences in physical morphology. All hydrogels synthesized despite photoinitiator used exhibited pore sizes ranging from 200-300\textmu m.

While there was no observable difference in morphology, the mechanical properties of such hydrogels varied dramatically with type of photoinitiator, concentration of photoinitiator and irradiation time. Hydrogels polymerized using either CQ or FR exhibited significantly lower moduli as compared with those hydrogels polymerized using RF as a photoinitiator.

Swelling ratio and degradation rates, both functions of cross-linking density, permeability and stiffness were significantly different between samples initiated by
different photoinitiators. Due to the lower cross-linking density arising from photopolymerization initiated by CQ and FR increased degradation rates as well as increased

While blue light alone has a very low incidence of causing mutagenesis or cell death, free radicals generated by a photoinitiator upon irradiation can both disrupt the cell membrane and elicit apoptosis. In our system, it was observed that chondrocyte viability was significantly decreased in CQ and FR hydrogels as compared with RF and observed cell death was viability exhibited a dose-dependent response to both irradiation time and photoinitiator concentration. Due to the high viability, increased mechanical properties and longer but modifiable degradation rate, RF was determined to be the optimal photoinitiator for our system.

The photopolymerizable chitosan was furthermore shown to be able to fill complex osteochondral defects, and remain at the site of implantation when submerged in 1xPBS for up to 14 days.

G. Type-1 Collagen in bone

Type -1 Collagen is the most abundant protein in bone extracellular matrix, accounting for nearly 30 % of the dry nonmineralized matrix. Collagen is a heteropolymer composed of two identical α subunit chains and one β subunit chain twisted and bound together to form a super structural triple helix. Each has a chain has
the primary structure (Gly-X-Y)$_n$ where X and Y are most often proline or hydroxyproline$^{31}$. Stabilized by hydrogen-bonding, these super helices associate together to for super-super-coils also known as collagen fibrils. The degree of order and interaction with adjacent fibrils suggest that helices within collagen fibrils exhibit semi crystalline-like qualities$^{31,32}$.

During bone formation, Type I collagen forms the backbone for the deposition of mineralized tissue. Bone mineral crystals become aligned with their long axis parallel to the collagen axis$^{30}$. Proper collagen formation is furthermore known to be necessary for proper mineralization, as diseases which alter proper collagen helix formation are known to give rise to osteogenesis imperfect. Bones of these patients exhibit smaller mineral crystals and overall brittle structure$^{33}$.

Moreover, the two types of bone (woven and lamellar), can be identified via SEM according to the arrangement of collagen within the osteoid matrix. In woven bone, Type I collagen is arranged in an haphazard manner and is mechanically weak. While in lamellar bone collagen is arranged into regular and parallel-arranged sheets and is mechanically strong.

**H. Collagen as a Biomaterial**

Collagen is furthermore a common and widely used biomaterial due to its ease of extraction from murine, rat, and bovine sources, abundance and role in healthy tissue, and ability to enhance spreading, attachment and proliferation$^{34,35}$. While it is important to note that collagen itself does not facilitate mineral formation (ie: the nucleation process
by which the initial mineral is deposited) collagen and its many derivatives: tropocollagen, procollagen, and gelatin contain a unique three amino acid recognition sequence containing arginine, glycine, and aspartic acid which binds \( \alpha 1 \beta 1 \), \( \alpha 2 \beta 1 \), \( \alpha 10 \beta 1 \) and \( \alpha 11 \beta 1 \)-integrins allowing cells to attach to collagen coated surfaces\(^{36}\). More importantly this RGD sequence upregulates both focal adhesion kinase based cascades and ERK expression (a factor in the MAPK pathway)\(^{37,38,39}\). Both FAK dependent signaling cascades and ERK are associated with osteogenesis of preosteoblast and mesenchymal stem cells.
3. **Hypothesis, Thesis Objective and Specific Aims**

Photopolymerizable methacrylated glycol chitosan has been well characterized as a viable biomaterial which can be cured in situ. Furthermore, chondrocytes cultured in MeGCS exhibit high viability and ability to undergo chondrogenesis. However, hybrid MeGCS hydrogels tuned to enhance osteogenesis have not yet been investigated. The motivation of this thesis is to synthesize a practical photopolymerizable hydrogel system that promotes osteogenesis.

The core hypothesis of this thesis:

Hybrid chitosan/collagen hydrogels utilized as both as 2D substrates as well as 3D encapsulating scaffolding mediates and enhances osteoblastic differentiation *in vitro*.

The objective of this thesis is to synthesize and characterize the mechanical and chemical properties of a hybrid chitosan/collagen hydrogel, investigate its ability to mediate cellular attachment, spreading and proliferation, and determine its osteoinductive properties for both 2D and 3D culture. The 2D model system comprised of a hydrogel surface has been designed to understand the interaction of host endogenous cells with the hydrogel surface upon implantation while the 3D microenvironment is utilized for the investigation of collagen-promoted osteogenic differentiation. To further modify and enhance the osteogenic capabilities and modifiability of injected hydrogels a controlled protein release system was examined.

The following aims are designed to assist organization of the results:
**Aim 1:** To characterize and understand the chemical and mechanical properties of the hybrid CSCol hydrogel

**Aim 2:** To evaluate the *in vitro* osteoblastic response on a 2D CSCol hydrogel substrate

**Aim 3:** To evaluate the *in vitro* osteoblastic response of cells encapsulated within a 3D CSCol hydrogel

**Aim 4:** To generate and investigate a system to control the release of protein from CS hydrogels
4. **Aim 1: To characterize and understand the chemical and mechanical properties of the hybrid CSCol hydrogel**

**A. Introduction**

Polymeric hydrogels made from either synthetic or naturally derived origins have been utilized in tissue engineering to act as space fillers, drug delivery vehicles and 3D matrices to deliver transplanted cells to regenerate damaged tissue. Much of the success of the hydrogel in its particular role hinges on their ability to find an appropriate material that can address critical physical and chemical variables inherent to its application.

In tissue engineering it is important to consider the tissue of interest and its inherent mechanical properties and rate of regeneration. The mechanical properties of natural tissues vary, and due to mechanoreceptors which interact with the tissue ECM, it has been determined that the stiffness of surrounding tissue and associated strain observed by a cell can dramatically alter cellular phenotype and behavior. Likewise, the mechanical properties of a hydrogel can also alter viability, proliferation, and differentiation of stem cells cultured within a 3D matrix\textsuperscript{40,41}.

However generation of tissue and *in vitro* differentiation are only one concern. Chemical modifications in the polymer and differences in polymerization can alter gelation time, degradation time, mesh size, and physical morphology. For a biodegradable injectable hydrogel, it is ideal to also minimize gelation time to aid enhance cellular retention and minimize operation time while controlling the rate of degradation to match in growth of surrounding tissue and regeneration of healthy tissue within the hydrogel. For this reason, the CSCol hybrid hydrogel’s physical and chemical properties were characterized.
B. Materials and Methods:

i. Methacrylation of Glycol Chitosan

Glycidyl Methacrylate was added to a 2w/v% aqueous GCS solution make a 1:1 molar ratio of glycidyl methacrylate to amino groups in chitosan. The pH was adjusted to 9.0 using 1N HCl and the solution was allowed to conjugate for 36 hours. The pH of the solution was then readjusted to 7.0 and dialyzed with a molecular weight cutoff of 1kDa dialysis tubing against water. Both water and tubing was replaced after 4 hours and allowed to dialyze for an additional 24 hours. Samples were frozen, lyophilized and stored at -20 °C.

ii. Photopolymerization of Methacrylated Glycol Chitosan

Figure 3: A schematic diagram of the CS/Col polymerization process. A solution of 2% MeGCS, .08% PureCol and 6uM RF was combined and irradiated for 2 mins. Polymerized
samples were then incubated at 37 degrees to allow collagen to gelate forming a hybrid CSCol hydrogel.

Hybrid scaffolds were created by first neutralizing type-1 collagen solution (3mg/ml) with .1N NaOH and 10x PBS in an 8:1:1 v/v ratio. This neutralized collagen solution was combined with 3% MeGCS in a 1:2 ratio to achieve a final concentration of 2% MeGCS and 0.08% Type-1 collagen, thus generating a chitosan/collagen hybrid hydrogel (CSCol). Pure chitosan (CS) hydrogels were created by diluting 3% MeGCS with 1xPBS to a 2% solution. The photoinitiator RF was added to make a final concentration of 6uM and all gels were irradiated for 40s, 1min, or 2 mins. For all 2D experiments 300uL or 500uL hydrogels were polymerized for 2mins at the bottom of untreated 24-multi well tissue culture polystyrene plates.

**iii. Intron and Elastic Modulus**

The Young’s moduli of the hydrogels were determined via indentation measurements performed using an Intron electromechanical testing machine (Instron, Model 5564, Norwood, MA) with a 1.6 mm diameter indenter using a Poisson ratio of .25. 400 mL 2% hydrogels were gelated in 48-multi well tissue culture plates using 6uM RF and .08% Type-1 Collagen and measured at 1, 2, 3, and 4 hours of incubation at 37 C. Three measurements were made per hydrogel on three different hydrogels. The average of the three indentations was taken as a single data point.
iv. Picrosirius Red Staining

2% MeGCS hydrogels were irradiated for 2 mins with 6uM RF. Samples were then allowed to incubate in a 2% picrosirius red staining solution for 1 hour. Samples were removed, washed and incubated in 1xPBS for 24 hours. Samples were then viewed under bright field microscopy and images were captured.

v. Scanning Electron Microscopy

After being crosslinked, hydrogels were allowed to incubate in ddH2O for 1 hour at room temperature to swell. After swelling, water was aspirated and samples were frozen at -80 °C. Samples were lyophilized for a minimum of 24 hours. Dried samples were then carefully handled and cross-sectioned using a sharp razor blade. Cross sectioned samples were mounted on carbon tape and observed using a FEI Nova SEM230 at low vacuum with a low vacuum detector.

vi. Fourier Transform Infrared Spectrometry

Samples analyzed using FT-IR were freeze dried in a similar manner to those used in SEM. Freeze dried samples were then directly placed onto the FTIR, clamped, and analyzed. Ambient blank measurements were taken without a sample were used as background and subtracted from the sample reading.
vii. Gelation time

Gelation time was defined by the sol to gel transition (when the solution of MeGCS, RF, and PBS no longer exhibited viscous flow). Sol to gel transition was determined by using the vial tilt method. 400uL MeGCS solutions were transferred into 5uL glass vials and irradiated in 1-2s bursts. Samples were then tilted after each irradiation and deemed either solution or gel. The onset of sol to gel was recorded and plotted versus concentration of RF.

viii. Degradation

200uL of a 2%CS or CSCol solution were irradiated for 2min in the cover of a 48-multi well tissue culture plate. 3 200uL hydrogels were transferred into 1 scintillation vial and 3 sets of samples were created for each different condition. The mass of scintillation vial was determined before and after adding samples. Samples were then incubated in 3mL of either 1xPBS, 3mg/mL lysozyme in 1xPBS, or 5mg/mL lysozyme in 1xPBS. Supernatant was removed every 3-7 days and the wet sample mass was determined and recorded. Samples were observed over a 21 day period.
C. RESULTS AND DISCUSSION

i. Instron and Mechanical Properties

Figure 4: Young’s modulus of CS or CSCol hydrogels were compared after being incubated at 37 °C for 0, 1, 2, 3, or 4 hours, where 0 is immediately after polymerization and wash with 1xPBS.

Figure 4 shows the influence of incorporation of .08%Col to a 2%CS hydrogel and the changes in Young’s modulus over a 4 hour period. As one can see from the figure, the pure CS hydrogel was measured to have a Young’s modulus of approximately 2kPa.
while the CS/Col hybrid hydrogel increased in stiffness from a Young’s modulus of 2.5kPa to nearly 4kPa over the course of 4 hours. These results indicate that for culture of cells within or on the surface of a hybrid CS/Col hydrogel will not reach steady state until after a 4 hour incubation period.

This observation also supports the hypothesis that Col incorporated into the CS hydrogel undergoes a second physical cross-linking. Collagen fibrils are water-soluble when maintained in a low pH environment, however when neutralized to physiological pH (ie pH 6.5-8.5) and brought to physiological temperatures (ie 20-37 °C) collagen polymers undergo a two phase polymerization process. This process begins with an initial nucleation phase followed by rapid growth. This secondary formation of a crosslinked collagen network we believe strengthens and stiffens the hybrid hydrogel, contributing to the increase in modulus.
ii. Scanning Electron Micrographs

Figure 5: SEM micrographs of cross-sections of free-dried CS or CSCol hydrogels were obtained using a low vacuum mode and low vacuum detector on a FEI Nova SEM230.

The observed increase in modulus and incorporation of collagen is confirmed by SEM micrographs taken of cross sectioned lyophilized CS and CSCol hydrogels. Pure CS
hydrogels exhibit distinct 100-200um pores within a honeycomb like structure and smooth CS struts. CSCol hybrid hydrogels however exhibit CS struts with interconnecting characteristic collagen fibrils within the CS pores.

While SEM micrographs only portray the hydrogel morphology post-lyophilization, clear collagen fibrins can be observed incorporated within the CS struts as well within voids.

iii. Picrosirius Red

![CS and CSCol hydrogels stained with picrosirius red](image)

Figure 6: CS and CSCol hydrogels were stained using a picrosirius red stain specific for collagen. Samples were imaged by bright field microscopy.

While SEM and Instron provide visual and morphological indication that collagen fibrils are present, no chemical confirmation can be established. Here two 100uL
hydrogels were stained with picrosirius red and viewed using bright field microscopy. Yellow picric acid is a small anionic partially hydrophobic molecule while Sirius Red is a red acid dye that is large and hydrophilic. In bright field microscopy collagen is stained dark red against a pale yellow background.

In Figure 6 one sees that the pure CS hydrogel stained yellow indicating little positive stain while the CSCol hydrogel was stained dark red, indicative of collagen. Positive picrosirius red staining of collagen in, further confirms that collagen is present in the CSCol hybrid hydrogel.
### iv. Gelation Time

![Gelation Time Graph]

**Figure 7**: Gelation time was determined by the sol to gel transition and recorded as a measure of both RF concentration for gels consisting of 2%CS and .004, .008, .016, or .08% Col.

To facilitate the implantation process and to enhance the ability of an injectable gel to remain within a defect site, it is advantageous for such a gel to cure quickly. Gelation time was determined by the vial tilting method and the sol-gel transition was recorded for 2% CS solutions containing varying concentrations of the photoinitiator RF and Col. As observed in figure 7, gelation time was significantly different with increasing concentrations of RF (from 24s to 6s) however over the range of Col concentrations
tested there was no observable difference between pure CS and samples with increasing concentrations of Col.

This indicates that while collagen has been successfully incorporated into the CS network, collagen polymers within the CS solution did not interfere with the photopolymerization of CS to any observable degree.

Furthermore it is important to note that the gelation time even at the lowest concentration of RF occurs within 25s suggesting that our photopolymerizable chitosan system forms an gel solution very quickly. Other injectable systems, crosslinked by thermogelation can take minutes or even hours to cure.
v. Fourier Transform Infrared Spectrometry

Figure 8: FTIR spectra were obtained for freeze dried CS or CSCol hydrogels.

FTIR-IR spectra obtained from CS and CSCol hydrogels are shown in figure 8. For the CS spectra characteristic peaks were observed at 3500, 2900, 1650, 1560, 1380, and 1150 indicative of hydroxyl groups, free amine groups, C=O stretching, -NH$_2$
bending, C-O stretching of the primary alcohols and 1150 C-O-C stretching of the glycosidic linkages.

Synthesized CSCol hydrogels exhibited very similar FTIR spectra as pure CS and no observable differences could be determined. This indicates the hybrid scaffold exhibits greater overall similarities in bonding and structure to CS.

vi. Degradation time

![Graph showing degradation time](image)

Figure 9: *In vitro* degradation of CS and CSCol hydrogels irradiated for 2 mins was determined after incubation in 1xPBS, 3mg/mL or 5mg/mL lysozyme solutions for 3 weeks at 37 °C.
One requirement of modern successful biomaterials used for scaffolding is that they are fully degradable and completely replaced with new healthy tissue. As such it is imperative that a material degrade as transplanted cells proliferate, differentiate and build new tissue.

Chitosan is enzymatically degraded in vivo by lysozyme, which acts by bonding to and attacking chitosan’s six sugar chains backbone. Here we incubate 200uL scaffolds in solutions containing 0, 3, or 5mg/mL of lysozyme and observe the degradation of our hydrogel. As seen in Figure 9, the CSCol hybrid hydrogel degraded at a slower rate than pure CS at nearly every time point. Also, degradation of either CS or CSCol was dependent on lysozyme concentration. More concentrated solutions of lysozyme resulted in faster degradation rates for all samples. CS and CSCol when incubated in only PBS maintained their initial unswollen mass and exhibited very little swelling.

These results indicate that although the concentration of Col within the CS hydrogel is small in comparison to the CS composition, the polymerization of Col within the CS hydrogel and the resulting collagen based network provides resistance to lysozyme based degradation. More importantly both CS and CSCol hydrogels are expected to persist throughout the bone differentiation and mineral deposition period.
D. Conclusions

The existence and incorporation of collagen throughout the hydrogel is verified by both picrosirius red staining as well as SEM. Numerous thin collagen fibrils can be observed incorporated into the chitosan struts, as well as in the void space between chitosan indicating extensive collagen integration. These microscopic observations give rise to a CSCol hybrid hydrogel that exhibits greater mechanical properties and higher stiffness. We account for this change in mechanical properties over a 4 hour time span as a result of the second polymerization of collagen in addition to the original photopolymerization of MeGCS.

Despite these observations however, the hydrogel itself appears to be mostly chitosan as there were no observable differences in FTIR spectra between the CS and CSCol hydrogels. Furthermore, it can be established by the gelation time analysis that incorporation of Type-1 Collagen did not alter the sol to gel transition time.

CSCol degradation however was slower than CS degradation when incubated in lysozyme solutions. Lysozyme which is specific for polysaccharides and sugar residues on glycosaminoglycans does not interact nor degrade collagen. CSCol hydrogels that had been incubated in lysozyme solutions therefore appeared to maintain their hemispherical initial shape, while CS hydrogels quickly became flattened and deformed. A 3-5 week time frame for degradation is estimated to be relatively good time frame for the hydrogel to degrade as mineralization usually occurs between 3-4 weeks.

In vivo however we might expect that both lysozyme and collagen would be present at the defect site, as both exist endogenously within bone. Conditions however
are difficult to generate \textit{in vitro} as it has been reported that lysozyme may inhibit collagenase activity\textsuperscript{43}.

The CSCol hydrogel thus exhibited greater mechanical properties and was observed to be more robust than the pure CS hydrogels despite being mostly CS in bonding nature.
5. Aim 2: To evaluate the *in vitro* osteoblastic response on a 2D CSCol hydrogel substrate

A. Introduction

![Figure 10: A schematic of the 2D culture system. mBMSCs were cultured atop 300 or 500uL CS or CSCol hydrogel substrates and cultured in 500uL of media.](image)

While more recent studies using hydrogels examine cell behavior of encapsulated cells, it is important to note that implanted systems interact with both transplanted cells as well as host endogenous cells which interact with the hydrogel surface. As such, 2D cell studies were pursued to model and assess how host cells might interact with the hydrogel post implantation and post-polymerization. We sought to prove that endogenous progenitor or stem cells could attach to the surface of our hybrid CSCol hydrogel implants, and would exhibit sufficient attachment, proper cell spreading and morphology, enhanced proliferation, and osteogenic differentiation.

2D cell growth and proliferation findings on the surface of CS and CSCol however are not limited to only understanding endogenous cell/hydrogel interactions. One can image that studies might also encourage the investigation of such CSCol photopolymerizable polymers for use as surface coatings.
B. Materials and Methods

i. Cell Culture

mBMSC cells were cultured in normal growth media conditions using 4.5mg/mL glucose supplemented DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. Media was replaced every 3 days.

ii. Quant-iT™ PicoGreen® assay

The Quant-iT™ PicoGreen® assay [Invitrogen] utilizes an asymmetric cyanine dye that binds dsDNA and can quantifiably detect as little as 250pg/mL of DNA. The picogreen assay was performed by combining 10uL of cell lysis solution with 90uL of a 1:200 dilution of picogreen in 7.4pH tris buffer solution. Samples were then analyzed by plate reader analysis. Samples were excited at 480nm and fluorescent intensity was measured at 520 nm.

iii. Attachment Efficiency

To determine the attachment efficiency of cells to the surface of the hydrogels, mBMSCs were seeded at a density of 250 cells/mm² atop 300uL CS or CSCol hydrogels. Attachment efficiency was determined by Picogreen DNA assay.
iv. Attachment and Spreading

To observe the changes in morphology of seeded cells, mBMSCs were seeded at a density that was adjusted to ensure that upon attachment approximately 250cell/mm\(^2\) would attach. Prior to imaging using the Live/Dead staining procedure, media was aspirated and cell monolayers were carefully washed once using 1xPBS. Stained samples were observed by fluorescent microscopy.

v. 2D Proliferation

CS and CSCol hydrogels were seeded with at 250cells/mm\(^2\). At 1, 4, 7, 10, 14 and 21 days media was aspirated and samples were collected. Each sample was then placed in 300uL of lysis buffer consisting of .02% Tween-20 diluted in 1xPBS and sonicated for 5-10s. Cell lysate was allowed to incubate for 1 hour before centrifugation at 16,000 RPM for 3 minutes. 10uL of supernatant was then analyzed using the Picogreen assay.

vi. 2D Cell Morphology and Live/Dead

Cells were seeded atop 500uL CS or CSCol hydrogels at 250cells/mm\(^2\). At 1, 4, 7, 10, 14, and 21 days media was aspirated, samples were washed with 1xPBS and media was replaced with L/D reagent diluted in growth media. Samples were then observed by fluorescent microscopy.
vii. 2D ALP Quantification

Cells seeded atop 300uL CS or CSCol hydrogels at 250cells/mm² were grown in either CM or OM in triplicates. Samples at 1, 7, 14, and 21 days were washed with 1xPBS, and collected. Samples were incubated in a lysis buffer consisting of .02% Tween-20 diluted in 1xPBS. Samples were sonicated for 5s and centrifuged at 16,000RPM for 3 mins. 15 uL of sample was then transferred to a 96-well plate and combined with 200uL of phosphatase substrate (1 phosphatase substrate capsule in alkaline buffer solution) and allowed to incubate for 10 mins. Samples were read with a plate reader measuring at 405nm.

viii. 2D Quantitative Calcium Assay

Samples collected for 2D ALP Quantification were then digested in .1N HCl by the addition of 20uL of 1N HCl. Digested samples were allowed to incubate at 4 degree C for 24 hours. Samples were centrifuged again at 16,000 RPM for 3 minutes and 10uL of sample supernatant was combined with 90uL of a reaction buffer created by combining a .024% pthalein purple/ .25% 8-hydroxyquiolone solution with a 500mM 2-amino-2methyl-1,3-propanedial solution.

ix. 2D Histology Preparation

Cells were seeded on 500uL CS or CSCol hydrogels, cultured in typical culture conditions using either CM or OM and collected at days 7, 14, 21, and 28. Samples were
prepared for paraffin embedding and cross-sectioning by first fixing in 10% formalin for 12 hours. Samples were then embedded in approximately 1mL Histogel (Richard-Allan Scientific). Each sample was then placed in a tissue cassette and placed in 70% EtOH. Samples were delivered to the UCLA Pathology core facility where they were further prepared, embedded in paraffin, cross sectioned and H&E stained.

x. **2D Histology H&E Samples**

Samples returned from the histology lab were then deparaffinized by serial washes in xylene and ethanol. Samples were then dried and stained using a 2% Alizarin Red solution for 5 minutes. Stained samples were then briefly washed in water twice, dried, and then viewed under bright field microscopy.
C. Results and Discussion

i. 2D Attachment

![Images of cell attachment at different time points](image)

Figure 11: mBMSCs were seeded atop CS or CSCol hydrogels and stained with L/D stain at 6, 12, and 24 hours. Samples were viewed by fluorescent microscopy to observed cell spreading, morphology and viability.

Attachment of cells to a substrate is necessary for both anchoring to prevent cells from being washed away from the implantation site and to encourage attachment and growth of host cells to the implant. More importantly, enhanced binding and spreading of cells to surfaces coated with collagen or collagen based hydrogels via integrin receptors is known to upregulated proliferation and differentiation.

Any integrin of the αV type or two of integrins of the β1 type binds to the RGD amino acid sequence on collagen upregulating expression and activation of the MAPK
pathway as well as FAK dependent signaling cascades associated with proliferation and osteogenesis.

In Figure 11 mBMSCs were allowed to attach to either CS or CS/Col 2D hydrogels for a 12 hour period. Cells were then stained with Live/Dead reagent (live: green, dead: red). Cells attaching to the surface of the CS hydrogel remained round in shape and do not exhibit any spreading over the course of 24 hours. Rather cells aggregate together, preferring to bind and attach to each other rather than to the CS substrate.

Cells seeded on the hybrid CS/Col hydrogels however begin to exhibit proper spread spindle-like morphology beginning at 12 hours and to an even greater extent at 24 hours.

Dead cells were very rare and viability remained very high over the course of the 24 hour incubation time.
ii. 2D Proliferation

Figure 12: Live/Dead fluorescent staining of mBMSCs cultured atop CS or CSCol hydrogels after 1, 4, 7, 10, or 14 days of culture in normal growth media.

Figure 13: DNA quantification of mBMSCs cultured on CS or CSCol substrates. Fluorescent intensity was recorded at each time point.

To minimize the number of necessary cells implanted and to generate a more healthy and robust tissue, successful scaffolding material must not only prove to be non cytotoxic, but must should also enhance cellular proliferation. As in the natural healing process, a proliferative phase is necessary to generate a sufficient number of cells to regenerate the tissue defect. Cellular proliferation in a hallmark of successful healing and can be indicative of healthy cell growth.
In previous studies by our lab, we have shown that chondrocytes encapsulated within photopolymerizable chitosan remain viable but exhibit moderate proliferation; however no studies have investigated long term cellular growth on the surface of MeGCS hydrogels.

In order to evaluate the viability, proliferation and morphology of cells cultured on the surface of our photopolymerizable hydrogels bMSCs were seeded atop 500uL CS or CSCol hydrogels in growth media and observed by both Live/Dead staining as well as quantitative DNA quantification.

After being stained with Live/Dead, it was observed that over a 14 day period, on the surface of pure CS hydrogels, mBMSCs remained round in shape. Cells aggregates appeared to grow slightly in size, forming larger clusters, however no spreading was observed. When DNA was quantified cells cultured on CS hydrogels proliferated to a very small degree in the first 7 days but reached a plateau and did not continue to proliferate.

mBMSCs cultured on the CSCol hydrogels however exhibited extensive spreading by 1d and continued to grow and proliferate over the surface of the hydrogel. Cells exhibited spindle-like shape and seemed to initially grow along collagen fibrils forming a web-like structure. By day 7 cells had completely covered the surface of the hydrogel and continued to increase in density up to 14d. Observations by fluorescent microscopy were mirrored by DNA quantification by the Picogreen assay. Cells exhibited the greatest proliferation between day 1 and 4 before exhibiting a linear like growth rate to 14d.
iii. 2D ALP Quantification

To heal bone defects, requires that implanted devices and materials not only enhance cellular attachment and proliferation but also promote osteogenic differentiation of those implanted cells. While it is well established that growth factors derived from the TGF superfamily, like bone morphogenic proteins, can greatly enhance osseous regeneration, such growth factors are very expensive. An ideal biomaterial for bone tissue engineering would encourage osteogenic differentiation and mineral deposition.

Osteogenic differentiation is a complex process that most often occurs over a three week period. The course of differentiation from a progenitor or stem cell to the differentiated osteoblast is marked by a number of changes in gene expression as well as protein production.

One such observed change is an upregulation of production of alkaline phosphatase (ALP). ALP is a hydrolase enzyme responsible for dephosphorylization of organic molecules including DNA, proteins and alkaloids. In bone, cleaving of the phosphate from phosphate sources provides maturing osteoblasts inorganic phosphate ions which can be utilized for mineralization. ALP as the name suggests is most active in alkaline environments. While ALP is often associated with osteogenesis, its expression is not limited to bone. ALP is known to be expressed by embryonic stem cells, hepatocytes, and parenchymal cells. For this reason, it is well established as an osteogenic marker but not considered definitive.
In bone ALP expression has been reported to be expressed as early as 4-7 days and normally peaks in expression by or before 14 days of culture. ALP is furthermore transient and is not normally expressed in as high of concentrations by mature osteoblasts.

Figure 14: ALP activity normalized by DNA content of mBMSCs cultured on the surface of CS or CSCol hydrogels cultured over a 2 week period.

In this experiment, bMSCs were seeded atop either CS or CSCol hydrogels and cultured for 21 days in either growth media or osteogenic media. Samples were then collected, lysed, centrifuged, and analyzed for ALP expression by combining cell lysate with an ALP substrate in an alkaline buffer solution. The kinetic enzymatic
dephosphorilation of the ALP substrate was observed as the substrate was converted from the colorless p-nitrophenol to a yellow colored derivative upon dephosphorylation using a plate reader. Measurements at 405nm taken after 4 minutes of incubation were compared. Quantified ALP was then normalized by DNA analysis.

As seen in Figure 14, ALP expression was upregulated by cells cultured on CS and CSCol substrates at 7 days, but continued to increase on CSCol substrates to 14 days by 21 days a down regulation in ALP expression was observed.

ALP being an early osteogenic marker is known to be upregulated early during osteogenesis and then later expression is down regulated during terminal differentiation. Here we observe that ALP expression reaches a greater maximum by cells cultured in CSCol hydrogels but there is little marked difference in early expression at day 7.
iv. 2D Quantification of Calcium Deposition

Figure 15: Ca content was measured for mBMSCs cultured on CS or CSCol hydrogels for 1, 7, and 14 days. The y-axis represents the Ca content as normalized by DNA.

While ALP is a well-established early marker of osteogenesis, as mentioned, ALP expression does not definitively mark that a cell will differentiate into an osteoblast lineage. Full functional differentiation of a progenitor cell into a osteoblast is confirmed upon the deposition of mineral and the formation of calcium nodules. Mineralization is a hallmark of bone tissue engineering and is the final step in process of differentiation.

In this particular experiment, samples analyzed for ALP were digested in .1N HCl and then amount of calcium was determined via a quantitative calcium assay. mBMSCs cultured in osteogenic media in pure CS and CSCol hydrogels exhibited a significantly different degree of mineralization. mBMSCs in the pure CS hydrogel did not produce
sizable amounts of calcium deposits, while mBMSCs cultured in the hybrid CSCol hydrogels generated extensive mineral by day 14 and continued to mineralize within the hydrogel to day 21.

v. 2D Bright field Images of Hydrogels

Figure 16: 40x Bright field image of mBMSCs encapsulated within CS or CSCol hydrogels cultured in osteogenic media for 7, 14, and 21 days

Extensive mineralization within hydrogel matrices could be viewed by bright field microscopy. Both CS and CSCol hydrogels upon being loaded with cells appeared transparent, however due to the extent of mineral formed, hydrogels became opaque. In
agreement with findings observed by the quantitative calcium assay, nodule formation began at day 14 on the CSCol hybrid hydrogel and continued to grow in size to day 21. Cells seeded on the pure CS hydrogel did not exhibit mineralization until day 21 and only in the immediate vicinity of the cell aggregate.

vi. 2D Histology and H&E Staining

Figure: 17: 100x Bright field image. Haematoxylin and Eosin staining of cross sections of cells seeded atop CS or CSCol hydrogels over a 3 week culture in osteogenic media. Samples were encapsulated within Histogel just prior to paraffin embedding to preserve the cell/hydrogel interface.
Cross-sections of the hydrogels were generated by first embedding hydrogels and cells into Histogel to maintain and secure the monolayer of cells onto the surface of the gel. Samples were then embedded in paraffin and cross sectioned. Hemalum, a complex formed from aluminum ions and oxidized haematoxylin, stains nuclei blue, while a counter stain of eosin Y an acidic dye stains basic molecules (largely cytosolic proteins) pink, red, or orange. As expected, the chitosan hydrogel is stained lightly with eosin generating a light pink color, while the CSCol hydrogel is slightly darker due to the incorporation of collagen.

Cell aggregates atop pure CS hydrogels vary from single cells to bundles of 5-10 cells at day 1 but grow in size over the 21 day period. Cell aggregates however reach a critical mass by day 14 and no longer proliferate. Cells seeded atop the hybrid CSCol hydrogel however begin as singles cells but quickly by day 7 and to a larger extent, day 14, form a complete monolayer across the surface of the gel. By day 21, the mBMSC cells extent being to form multilayered cultures.

Also it was observed that on the pure CS cells did not exhibit any surface remodifications of the hydrogel, however cellular monolayers atop the CSCol hydrogel appear rough and remodeled. While it remains unclear the mechanism by which the cells have modified the surface, mostly likely the cells both enzymatically degraded small portion of the gels and due to growth and proliferation exhibited contractile stress over the surface, pulling on the hydrogel causing it to buckle.
vii. 2D Mineralization and Alizarin Red Staining

Figure 18: Cross sections of cells cultured on either CS or CSCol hydrogels in osteogenic media were stained with the calcium specific Alizarin red dye and imaged using bright field microscopy at 100x.

Samples collected over a 3 week period were stained with Alizarin red or 1, 2-dihydroxyanthraquinone, a small organic compound that binds calcium ions, calcium precipitates, and calcium phosphate crystals. Staining and subsequent imaging under bright field microscopy revealed extensive calcium nodule formation by cells cultured on the CSCol hybrid hydrogel surface at day 14 and day 21, but only moderate mineralization by cells cultured on the pure CS hydrogel at day 21. Depth of mineral formation according to the stain penetrated approximately 10um into the surface of the
hydrogel by day 14 in the CSCol hydrogels and approximately 20um into the surface by day 21. Mineral formation by cells on the hybrid scaffold was clearly denser and more extensive across the surface of the CSCol hydrogel as compared with the pure CS hydrogel.
mBMSCs cultured on pure CS hydrogel surfaces attached to the surface of the gel but were unable to spread as a result of a lack of binding epitopes. Cellular attachment is most likely a result of a minimal amount of adsorbed serum proteins from the growth media and subsequent adherence to those proteins. Over a 21 day period, the mBMSCs, a cell line with a doubling time of nearly 12 hours, exhibited very little cellular proliferation but did not display any significant signs of apoptosis or cell death. Furthermore these cells, while did express a large amount of ALP was marginally less than cells seeded on CSCol hydrogels. Despite this large expression of ALP however cells cultured on the pure CS did form mineral but only to a small degree. Alizarin red staining of the surface of the hydrogel as well as H&E staining revealed little morphological changes to the cells or their substrate.

mBMSCs cultured on the CSCol hybrid hydrogel however exhibited greater spreading, proliferation, ALP expression, and mineralization capabilities. Complete coverage of the substrate surface was observed by day 7 and proliferation of cells continued over a 14 day period, forming single and even multilayered structures on the surface of the gel. Furthermore, these cells after growth and proliferation remodeled the surface of the gel forming ridged rough surfaces. By day 14 calcium nodules could be detected by observation under by bright-field microscopy, quantitative calcium assay, and cross sectioned samples stained by Alizarin red indicative of osteogenic differentiation.

This study confirms the ability of our hybrid CSCol hydrogels to enhance cell spreading, proliferation and promote osteogenic differentiation.
6. **Aim 3: To evaluate the *in vitro* osteoblastic response of cells encapsulated within a 3D CSCol hydrogel**

   **A. Introduction**

   ![Diagram](image)

   Figure 19: A schematic of the 3D culture system. mBMSCs were encapsulated within 100uL CS or CSCol hydrogels and cultured within 1mL of media.

   Our injectable hydrogel system is designed not only to interact with endogenous cells but must deliver autologous or allogenic cells to the implantation site within the hydrogel matrix and be encapsulated at the bone defect. For this reason, it is favorable that the hydrogel enhance cellular proliferation, appropriate morphology, and most importantly differentiation. A successful cell-hydrogel implant used to fill the void should produce preliminary mineralized tissue that can be remodeled into mature healthy bone.

   While 2D cell culture is well established and popular for its ease of use, cells in their natural environment rarely exist in a 2D microenvironment. Osteoblasts and precursor cells more specifically exist within a 3D environment in which they are fully encapsulate within...
an ECM matrix which they form and mineralize. Furthermore, critical sized defects to be filled are by definition 3D voids which exceed the body’s natural ability to fill and replace. For this reason it is crucial that one understand how transplanted cells behave within a 3D matrix.

It is also well established that culture in 2D and in 3D are very different. Cells within a 3D matrix it is well established the cell growth and differentiation is very different when grown and cultured in
B. Materials and Methods

i. Cell Culture

mBMSC cells were cultured in normal growth media conditions using 4.5mg/mL glucose supplemented DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. Media was replaced every 3 days.

ii. 3D Cell Encapsulation

In all 3D experiments, cells were encapsulated at concentrations of 200,000 cells/hydrogel within 100uL 2%CS or 2%CS/.08%Col hydrogels. To create each sample, 100uL of cell/hydrogel mixture was delivered atop 96-multiwell tissue culture polystyrene plate covers and irradiated for 2 mins. Samples were then removed using a spatula and transferred to a 24-multiwell plate and incubated in 1mL of media. Due to the migration of cells out of the gel and their subsequent proliferation within the tissue culture plate, samples were transferred to a new plate every 7 days.

iii. 3D Proliferation

To determine the proliferation of cells within our hydrogels, cells were encapsulated in the typical 3D manner. Encapsulated cell hydrogel complexes were grown in normal growth media. Triplicates were made per time point and at 0, 7, 14; 21 days samples were collected, digested in 300uL of .02% Tween-20 lysis buffer and
sonicated for 5s. Samples were centrifuged at 16,000 RPM for 3 mins and 10uL of supernatant was analyzed using the Picogreen assay.

iv. 3D Cell Morphology

Encapsulated cells were prepared in the typical 3D manner, cultured in normal growth media and were observed over a 21 day time course using the Live Dead Assay. At 0, 7, 14, and 21 days samples were collected and incubated in 1mL of Live Dead working reagent for 20mins. Samples were then observed by fluorescent microscopy.

v. 3D ALP Quantification

Triplicates per time point were generated in the typical 3D manner and were cultured in either CM or OM for a 21 day period. At 0, 7, 14, and 21 days samples were collected, digested in 300uL of .2% Tween-20 in 1xPBS and sonicated for 5s. The heterogeneous cell mixture was then centrifuged at 16,000 RPM for 3 mins. 15 uL of sample was analyzed by combining lysate with 200uL of phosphatase substrate (1 phosphatase substrate capsule in alkaline buffer solution) and allowed to incubate for 10 mins. Samples were read with a plate reader measuring at 405nm. Samples were normalized to 10uL analyzed using the Picogreen assay.
vi. **3D Quantitative Calcium Assay**

Samples collected and tested for ALP were digested in .1N HCl by addition 30uL of 1N HCl to each vial. Samples were then vortexed and allowed to incubate for 24hrs at 4 °C. After digestion, vials were centrifuged at 16,000 RPM for 3 mins. 10uL of sample supernatant was combined with 90uL of a reaction buffer created by combining a .024% pthalein purple/.25% 8-hydroxyquiolone solution with a 500mM 2-amino-2methyl-1,3-propanedial solution.

vii. **3D Histology**

Cells were encapsulated in the typical 3D manner in either CS or CSCoI hydrogels and cultured in either CM or OM. Samples were collected at 7, 14, and 21 days. Each hydrogel was transferred to a new24-well plate and fixed in 10% formalin for 12 hours. Samples were then washed with PBS, collected in tissue cassettes and delivered to the UCLA Core Pathology Lab to be embedded in paraffin, cross sectioned and stained for H&E.

viii. **3D Mineralization Stain**

Cross sectioned slides were deparrafinized by serial washes in xylene and ethanol, dried, and stained with a 2% Alizarin Red stain for 5 mins. Samples were then washed by dipping in water 10 times, dried and viewed under bright microscopy.
C. **Results and Discussion**

i. **3D Proliferation**

Cells cultured in a true 3D scaffolding material are entrapped in all directions within the hydrogel matrix and therefore interactions between the cell and its surroundings become increasingly important. In addition to simple adhesion and spreading, considerations within a hydrogel shift to mass transport of nutrients in and out of the scaffold.

![Figure 20: Live/Dead fluorescent microscopy of mBMSCs encapsulated within CS or CSCol hydrogels for 3 days of culture in regular growth media.](image)
Figure 21: DNA quantification as measured in fluorescent units by the Picogreen assay. mBMSCs were cultured within either a CS or CSCol hydrogel for 1, 7, 14, or 21 days.

Here bMSCs were encapsulated within either a CS or CSCol hydrogel and observed over a 21 day period and analyzed for DNA content using the Picogreen assay. Initially, within the first day in both CS and CSCol hydrogels, scaffolds exhibited a 30-50% loss of DNA followed by a dramatic increase in DNA content by day 14. It was observed that cells cultured in the CSCol hydrogel exhibited the greatest growth, nearly tripling in number over the 3 week period, while cells seeded within the pure CS hydrogel exhibited an initial drop in DNA content before returning to initial loading concentrations.

Despite DNA measurements that reported a large decease in cells, decrease in cell number did not appear to be a result of cell death, as cells, when observed using the live dead stain, appeared to reflect a nearly 90+%viability. This level of viability remained very high over the 3 week period. While cells remained viable in both types of hydrogel, cellular morphology was quite different in as well as on the surface of the hydrogels.

Cells cultured in the pure CS hydrogel, exhibited no real change in morphology, nor were any significant proliferation observed within or on the surface of the gel. Cells remained static and neither grew nor died but instead appeared to remain in stasis. Cells cultured in and near the surface of the hybrid CSCol hydrogels however appeared to begin to spread within the first day, and exhibited extensive spreading and proliferation between days 7, 14, and 21. This dramatic growth rate near the surface of the hydrogel
accounts for the increase in DNA content. Cells towards the center of the hydrogel however did not exhibit extensive changes in morphology and like the pure CS hydrogel appeared static.

The initial loss of cells was observed repeatedly over the course of many trials as well as cell types. By observation during culture, it became apparent that within the first day of culture, the initial swelling of the hydrogel after polymerization, resulted in the migration and or forcing of unbound cells out of the hydrogel matrix. Most notably, those cells towards the surface of the hydrogel became dislodged and lost from the sample. While some cells were observed to be ejected, quantitative measurement losses estimated a staggering 30-50% of DNA from the initial encapsulation was no longer accounted for by day 1. We hypothesize that this discrepancy between qualitative analysis and quantification of DNA may be a result of down regulation as a result to shock of encapsulation. In other words, in addition to cell migration out of the gel, cells remain quiescent during the first week of culture, until they have become adjusted to the new microenvironment. This initial loss of cells however is not a unique phenomenon. In fact, cell loss following encapsulation is observed in a number of natural based as well as synthetic hydrogels and often reported as cell migration out of the gel.

Although moderate increases in DNA content were observed over the three week period, proliferation rate within the 3D hydrogel and on the surface of the 2D hydrogels was much slower. Cells that remained in the center of either the CS or CSCol hydrogels likely did not proliferate.
While literature references often suggest that elasticity and stiffness of a gel may contribute to a cells inability to spread within a hydrogel matrix, both CS and CSCol hybrid hydrogels exhibit very low moduli as compared with other types of hydrogels reported in literature. We hypothesize that this decreased rate of growth is due to the fact that cells were unable to modify their surroundings to any significant degree and a result of a small mesh size. This problem may be resolved by the incorporation of more readily cleavable linkages between the chitosan macromers. Viable cleavable linkages might include MMP cleavable moieties.

ii. 3D ALP Quantification

![ALP quantification normalized by DNA for mBMSCs encapsulated in CS or CSCol hydrogels and cultured in osteogenic media for 1, 7, or 14 days.](image)

Figure 22: ALP quantification normalized by DNA for mBMSCs encapsulated in CS or CSCol hydrogels and cultured in osteogenic media for 1, 7, or 14 days.
As mentioned earlier, ALP is an early marker for osteogenesis and is normally upregulated between 7-14 days when in 2D culture. Here, ALP is quantified for samples cultured within 3D CS or CSCol hydrogels in osteogenic media over the course of 21 days. ALP activity was normalized to DNA concentration. As seen in figure 22 mBMSCs encapsulated within the CSCol Hydrogels exhibited a significant increase in ALP activity as compared to both day 1 activity as well as with cells encapsulated within the pure CS hydrogels at every time point. ALP activity peaked at day 14 and by day 21 slightly decreased.
iii. 3D Calcium Quantification

Figure 23: Calcium quantification normalized by DNA for mBMSCs encapsulated within CS or CSCol hydrogels. Samples were cultured in osteogenic media and collected at 1, 7, and 14 day time points.

Calcium deposits by cells within a pure CS or hybrid CSCol hydrogel were observed over a 21 day period by digestion in a .1NHCl solution. It was observed that samples cultured in CS hydrogels first began to osteogenically differentiate and produce mineral around day 21. However mBMSCs cultured in CSCol hydrogels began to produce mineral by day 14 and continued to produce mineral through day 21.
iv. 3D Bright-field Microscopy of hydrogels in culture

Cells cultured in osteogenic media within CS hydrogels remained relatively transparent until day 21. At day 21 white areas within the hydrogels developed making the hydrogel opaque. This was further confirmed by images taken by bright field microscopy. Cells cultured in CSCol hydrogels by day 14 appeared dark around their immediate vicinity and by day 21 the entire hydrogel appeared white by naked eye and opaque by bright field microscopy.
v. 3D Histology and H&E

Figure 25: H&E stained histological samples of mBMSCs encapsulated within CS or CSCol hydrogels cultured in osteogenic media for 7, 14, and 21 days.

In osteogenic media, cells within the 3D matrix did not exhibit as significant spreading as was observed in hydrogels constructed for proliferation studies that were cultured in normal growth media. However, by H&E staining we can observe that cells did prefer to grow in clusters on the cell surface in both CS and CSCol hydrogels. Furthermore, void spaces were observed which enlarged over the 3 week period. This is likely a result of both enlargement and expansion of cells creating void spaces and possibly a result of degrading the surrounding hydrogel.

Light white spots within the hydrogel could be observed in the 21 day CSCol sample which may correlate to mineralized tissue.
vi. 3D Calcium Staining

Figure 26: Cross sections of bMSCs cultures within CS or CSCol hydrogels cultured in osteogenic media for 7, 14, or 21 days were stained with a 2% Alizarin red dye and viewed by bright field microscopy.

Alizarin red staining of cross-sectioned samples confirmed that the opacity observed in bright field images were indeed calcium deposits. Mineralization by mBMSCs encapsulated within pure CS hydrogels cultured in osteogenic media was not observed until day 21 and only to a very small degree just adjacent to the cell. Higher magnification of individual cells revealed that not all cells formed mineral. Furthermore as confirmed by H&E staining, growth and expansion of individual cells formed voids in the hydrogel, indicating that cells had either expanded or begun to degrade their surrounding hydrogel.
Cells cultured in the CSCol hybrid scaffold however exhibited mineralization by day 14 comparable to the amount of mineral formed by cells in pure CS hydrogels at day 21. By day 21, cells had not only deposited mineral directly adjacent to themselves but also throughout the scaffold as alizarin red stain stained the entire gel.

Interestingly, although cells did not appear to exhibit proper spindle-shaped morphology as normally observed in 2D culture, mBMSCs cultured within the CSCol hydrogel exhibited increased mineral production as well as ALP expression. We believe that this change in cellular behavior is largely a result of integrin binding to the collagen fibrils and intracellular upregulation of FAK and MAPK associated signaling pathways. While many groups associate proper spreading with differentiation, a number of published works report that cell spreading, especially within a 3D matrix may not be necessary in enhance cellular differentiation. Rottmar et al displayed that human bone marrow stromal cells could be confined in single square-shaped wells allowing them to attach to fibronectin coated PDMS walls but unable to spread. Nevertheless, cells cultured in osteogenic media within the square shaped wells stained positive for the early bone marker ALP at 7 days.
D. **Conclusions**

Findings indicate that mBMSCs encapsulated and cultured within 3D hybrid CSCol hydrogels exhibited enhanced cellular proliferation, spreading across and near the surface of the hydrogel but to a smaller degree within the hydrogel matrix, and significant osteogenic potential as compared with mBMSCs cultured within a pure CS hydrogel. While cells within the hybrid hydrogel did not spread within the hydrogel nor exhibit spindle-shaped morphology, they were still able to form mineral throughout the entire hydrogel over the course of 21 days, while mBMSCs cultured in the pure CS hydrogel did not form any noticeable amount of mineral until day 21.

These results suggest that hybrid photopolymerizable CSCol hydrogels not only maintain cell viability but promote both proliferation of encapsulated mBMSCs and enhance their osteogenic potential. Observations encourage the further investigation of this photopolymerizable material and exhibit its potential as a means of delivering and healing large bone defects.
7. **Aim 4: To generate and investigate a system to control the release of protein from CS hydrogels**

A. **Introduction**

While incorporation of collagen with MeGCS can generate a hybrid hydrogel capable of encouraging cellular proliferation, attachment, spreading, and osteogenesis, our system is highly modifiable and can be further enhanced by the incorporation of a controlled release growth factor drug delivery system. The goal of this aim is to develop a controlled release growth factor system which can deliver an osteoinductive growth factor without significant loss of function.

Osteoinductive proteins like growth factors of the bone morphogenic protein family have attracted much attention in the field of bone tissue engineering because of their ability to promote both in growth, use as a chemoattractant, and ability to enhance bone formation.

Developments in recombinant protein technology has enabled the production of human BMPs which are now used both in the laboratory and in the clinic to enhance osteogenesis and encourage healing of critical sized bone defects and spinal fusion. While BMP is a potent osteogenic factor, control over the amount of BMP delivered can greatly alter its efficacy. Zara et al showed that over exposure within a femoral defect can result in hollow cist-like bone formation.\(^{44}\)

For this reason, BMP-2 release must be carefully monitored. To control this release, a number of different types of biodegradable carriers have been developed and
utilized within bone defects including bone sponges, demineralized bone matrix and calcium phosphates. Here we present a novel combination of PLGA microparticles delivered via our photoencapsulated hydrogel system.
B. Material and Methods

i. Release from Hydrogels

100uL 2% CS hydrogels were loaded with 10ug of Rhodamine-BSA conjugates (Sigma) and irradiated for 2 mins in the covers of 96-multiwell tissue culture plates. Samples were then transferred to 24-multiwell plates and incubated in 1xPBS for 1, 4, 7, 10, 14, and 21 days. Supernatant was removed and replaced every 3 days. BSA release from the hydrogel was determined using a plate reader with excitation 550nm emission at 560nm.

ii. Microsphere Fabrication

10ug of rhodamine-BSA conjugate was combined with 500uL of .5% (w/v) 85:15 PLGA in chloroform and sonicated for 1 min at 20% amplitude in pulse mode (1s on/off) on ice. The PLGA/Protein emulsion was added to 2mL of 2% (w/v) Poly vinyl acid in ddH2O and sonicated for the aforementioned conditions for 1 min. The PLGA/BSA/PVA emulsion was then added drop wise to a 40mL 0.05% PVA/Poly vinyl propylene solution and allowed to stir overnight using a magnetic stir bar (600RPM). Samples were then collected via centrifugation at 5,000 RPM for 30 mins. Supernatant was collected and the pellet was washed twice by resuspending the particles in 1mL ddH2O followed by centrifugation at 5,000RPM for 3 mins.
iii. Microsphere encapsulation efficiency

Supernatant collected following stirring in .05%PVA/PVP and subsequent washing steps were analyzed for rhodamine-BSA by fluorescent quantification of 100uL at 560nm using a plate reader. Supernatant samples were compared to serial dilutions of rho-BSA in water and .05%PVA/PVP.

iv. Release from Microspheres

After determining encapsulation efficiency, particles containing 10ug were distributed to 3 1.5mL sample vials and resuspended in 1xPBS. At 1, 4, 7, 10, 14, and 21 days samples were centrifuged at 5,000 RPM for 30mins and supernatant was collected and replaced. 100uL of supernatant was analyzed using a plate reader and compared to a standard curve of rho-BSA in ddH2O.

v. Release from Encapsulated Microspheres

Synthesized microspheres were concentrated and encapsulated within 100uL 2%CS hydrogels such that each hydrogel contained a total of 10ug of rho-BSA. Hydrogels were irradiated for 2 mins in the covers of 96-multiwell tissue culture plates. Each hydrogel was then transferred to a 24-multiwell plate and resuspended in 1mL of 1xPBS. Supernatant was collected and replaced at 1, 4, 7, 10, 14 and 21 days. 100uL of supernatant was analyzed for rho-BSA using a plate reader and compared to serial dilutions of known amounts of rho-BSA in ddH2O.
vi. Fluorescent Images

To ensure that microparticles encapsulated in CS hydrogels remain loaded with rho-BSA fluorescent images of the encapsulated particles were gathered at 0d and 14d.
C. Results and Discussion

i. SEM of Particles

Figure 27: Fabricated PLGA particles were dried atop silicon wafers and imaged by SEM at 50,000x

PLGA particles were synthesized, concentrated via centrifugation and resuspended in water. After trying on a silicon wafer SEM micrographs were obtained. As observed in Figure 27 particles were approximately 200-600nm in size with the majority within the 200-300 range. Particles as expected appear to be solid with no observable porous structure. Observed aggregation is likely an artifact of the drying process.
ii. **Encapsulation Efficiency**

Encapsulation efficiency after synthesis was consistent varying between 40-60%.

iii. **Controlled Release**

![Graph showing controlled release](image)

Figure 28: Rhodamine conjugated BSA was encapsulated within a CS hydrogel, PLGA particles or PLGA particles encapsulated within a CS hydrogel. Samples were all incubated in 1xPBS and supernatant was collected and measured by fluorescence at 560nm.

To control release of growth factor, a diffusion/degradation based system was utilized. Protein was encapsulated within either 2min irradiated CS hydrogels, solid PLGA particles formed via a double emulsion process or PLGA particles encapsulated within a CS hydrogel. Due to the large cost associated with BMP, rhodamine conjugated
BSA was used as a model protein to observe and determine release kinetics. The Rhodamine BSA complex is about 3 times as large as rh-BMP2. BSA has a relative size of approximately 66kDa as compared with BMP-2 which has a relative weight of 13kDa.

As seen in Figure 24, protein encapsulated directly within the CS hydrogel exhibited a large burst release within the first 1 hour of incubation and by day 1 nearly 60% of the total encapsulated protein had been released. After the initial burst release, hydrogels exhibited a 1% release/day for the subsequent 13 days. Protein encapsulated within PLGA particles suspended in PBS exhibited a smaller burst release accounting for approximately 38% of total protein by day 1 before slowly releasing contents in a steady state .2%/day manner. PLGA particles encapsulated within a CS hydrogel however exhibited no burst release but rather a sustained controlled release of approximately .2%/day over the entire 14d period.
iv. Fluorescent Images

Figure 29: Fluorescent images of rhodamine BSA loaded PLGA particles encapsulated within a CS hydrogel at day 1 and day 14.

To ensure that protein was not lost and that protein remained within the hydrogel after 14 days, images of the particles encapsulated within the CS hydrogel were taken just after encapsulation and again at 14 days using the sample exposure time and microscope settings. There were no observable differences between samples at day 1 and day 14, indicating that protein remained encapsulated within the hydrogel and no sizable burst release occurred.
D. Conclusions

In addition to delivery of cells and encouraging cellular proliferation, attachment, and spreading by generating a hybrid CSCol hydrogel, our hydrogel material is capable of creating a sustained release of growth factor by co encapsulating protein loaded PLGA particles.

We believe that the release kinetics is dictated by two processes. Protein loaded within the PLGA particles and then encapsulated within the CS hydrogel must first be released from the PLGA particle via either surface or bulk degradation and then diffuse through the hydrogel matrix.


8. **Conclusion and Shortcomings**

Here we have exhibited that mBMSC cells cultured in 2D culture on the surface of our hybrid CSCol hydrogel as well as in 3D culture within our CSCol hydrogel exhibit appropriate attachment and spreading, enhanced cellular proliferation, earlier expression of bone markers like ALP, and greater levels of mineralization over a 3 week time period. Furthermore we have proved that our system may also be used to release of growth factor sized macromolecules in controlled manner by encapsulation of protein loaded PLGA microspheres. CSCol hydrogels exhibited significant advantages over the pure CS hydrogel as both 2D and 3D substrates and was proven to be a feasible means of encouraging proliferation and osteogenic differentiation *in vitro*.

While mBMSCs cultured in and on the surface of CSCol hydrogels were promoted to undergo osteogenic differentiation, cell lines less osteogenic in nature did not exhibit the same degree of mineralization. Cells derived from the mouse calvarial MC3T3 cell line as well as primary harvested mouse adipose derived stem cells extracted from the inguinal fat pads did display markedly increased proliferation and spreading on CSCol hydrogels as compared with pure CS hydrogels but were less responsive to osteogenic differentiation. Little or no differences in mineralization or ALP expression between CS and CSCol hydrogels could be observed.

For such types of cells Col incorporation at the concentrations used in this study were perhaps too small to observe noticeable differences in differentiation and could be improved by increasing Col content. Alternatively growth factors may need to be incorporated to assist in the differentiation process.
Although not shown here it was also discovered that modulus of the hydrogel as controlled by irradiation time, resulted in significant MC3T3 cell spreading of encapsulated cells within CSCol hydrogels. This decrease in modulus resulted in significant cellular contraction and shrinking of hydrogels over a 1-2 week culture period. For this reason, modulus of the hydrogel material may play role in the proliferation and differentiation of cells within our photopolymerizable chitosan hydrogel.
9. **Future Directions**

While our CSCol hydrogels exhibit promise as osteogenic cell carriers which can be delivered in an injectable fashion, there remain a number of avenues of interest that should be explored.

First and foremost, all findings reported in this thesis are *in vitro* and further *in vivo* studies are required to ensure that CSCol hydrogels enhance healing of critical-sized defects. While results are very promising in culture, crossing the chasm to an *in vivo* model can be very difficult and might require alterations in either the concentration of Col or the co delivery of growth factors. Furthermore, while the hydrogels have been proven to fill complex osteochondral defects, no long term studies have been performed with either pure CS or CSCol hydrogels in a defect site.

Furthermore, while the incorporation of PLGA microparticles suggests that growth factors can be incorporated into the CS hydrogel and used for sustained release of a growth factor, sustained release from such microparticles to encapsulated cells has not yet been proven to enhance osteogenic differentiation of cells encapsulated within the hydrogel matrix nor during implantation. This work encourages further investigation to determine if growth factors encapsulated within the CS or CSCol hydrogel remain bioactive after processing, and both *in vitro* and *in vivo* testing.

Finally, findings suggest that incorporation of Col enhances attachment, proliferation, and osteogenic differentiation; however the two step polymerization process requires that Col is added just prior to culture or implantation. To increase shelf-life and minimize the need for a 4 hour incubation, it may be of interest to conjugate
RGD peptides to the MeGCS backbone rather than incorporate Col. The RGD as mentioned earlier is identified as an important epitope for integrin receptors and essential for cell attachment, spreading and differentiation. Conjugation directly to the MeGCS would then generate a single polymer solution which can be directly irradiated with light in a one step manner.
10. Bibliography


