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PRINCIPAL INVESTIGATOR: Dr. Matthew Bernards

CONTRACTING ORGANIZATION: Regents of the University of Idaho
Moscow, ID 83844-3020

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Dr. Matthew Bernards

E-Mail: mbernards@uidaho.edu

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14. ABSTRACT

It is hypothesized that one or more of the SIBLING (small integrin binding, N-linked glycoprotein) proteins is responsible for recruiting cells for bone tissue repair and regeneration and their use in a tissue engineering scaffold will induce a natural, expedited wound healing response for segmental bone defects. To test this hypothesis, four major tasks have been proposed and during this reporting period progress has been made in all four of these areas. Major Task #1 was to evaluate the SIBLING protein family member that facilitated the best MC3T3-E1 osteoblast-like cell attachment to a multifunctional polyampholyte hydrogel. In Major Task #2, the top performing SIBLING protein was to be evaluated in primary cell proliferation, differentiation, and penetration into the base case polyampholyte hydrogel. In addition, in Major Task #4 the primary cell proliferation, differentiation, and penetration into polyampholyte hydrogels with a range of mechanical properties was to be evaluated. The creation of polyampholyte hydrogels with a range of mechanical properties was Major Task #3.

During this annual reporting period it was determined that osteopontin leads to the highest levels of cell attachment in a 2 hour adhesion assay (~120 cells/mm^2), initially completing Major Task #1. Osteopontin conjugated polyampholyte hydrogels with a range of mechanical properties were then prepared and subjected to primary cell cultures with both canine derived bone marrow cells and synoviocytes (Major Tasks #2 and #4). The results from these studies indicate that there are few to no viable cells following 15 days of primary cell culture on all of the osteopontin conjugated polyampholyte hydrogels that were evaluated. This was determined through a combination of fluorescent microscopy and total proteoglycan, alkaline phosphatase, and collagen I assays. It was concluded that the polyampholyte construct is releasing an acidic component that is causing widespread cell death. This is being addressed in on-going activities, which has led the team to revisit Major Task #1. Finally, additional efforts were completed under Major Task #3 during this reporting period, to broaden the range of demonstrated mechanical properties for polyampholyte hydrogels. The range of compressive stress at failure was nearly doubled from previous efforts (max ~900 kPa) based on the use of diethylene glycol dimethacrylate as the cross-linking species, rather than the base case triethylene glycol dimethacrylate. Major Task #3 is deemed complete at this time.

The long term application for this research is to develop an off the shelf implant technology that can be used by surgeons to improve the healing of patients and war fighters who have critical size defects in their bone tissue due to injury or disease. The results that are being obtained will be used to guide the development of a new implant technology that will be proposed for future testing in the body. Ultimately, this technology will help improve the recovery time and functionality of people with significant injuries to their bone tissues.

15. SUBJECT TERMS

Polyampholyte hydrogels; SIBLING proteins; Primary Synoviocytes; Bone marrow derived connective tissue progenitor cells.

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1. Introduction

This report follows three years of work on the project "Development of a Novel Segmental Bone Defect Construct" and it summarizes the accomplishments over the last project year (1 October 2017 – 30 September 2018). In this work a novel bone tissue engineering scaffold material is being developed to address current limitations of bone replacement scaffolds by combining a multi-functional polyampholyte polymer scaffold with a SIBLING protein biological cue. The first phase of this work was to develop polyampholyte hydrogels with a range of mechanical properties by simply changing the underlying composition of the hydrogel (project year 1). The second phase of this work has been to isolate the effects of the SIBLING proteins on the adhesion of MC3T3-E1 ostoeblast cells (project year 2-3). The third phase of this work was to determine the role of the SIBLING protein that promotes the highest cell adhesion in the second phase on the proliferation, differentiation, and biological activity of both primary synoviocytes and bone marrow derived connective tissue progenitor cells (project year 3). Significant progress was made on the project during this annual reporting period. However the project recently requested and was granted a no cost time extension because of one significant challenge, hydrogel acidity, which was identified over the last six months of effort and prevented the completion of the approved scope of work. Key accomplishments over this reporting period include expanding the range of physical properties that are achievable by varying the cross-linker species, identifying that osteopontin (OPN) promotes the highest levels of MC3T3-E1 osteoblast-like cell adhesion to the base case polyampholyte chemistry, the identification of hydrogel acidity as the source of cell death, and the development of revised hydrogel synthesis procedures to address the cytotoxicity.

2. Keywords

Polyampholyte hydrogels; SIBLING proteins; Primary Synoviocytes; Bone marrow derived connective tissue progenitor cells.

3. Accomplishments

Major Task 1 (All Subtasks): Under Major Task #1, the specific objective during this annual reporting period was to complete the screening of the adhesion of MC3T3-E1 osteoblast-like cells to the SIBLING proteins, when conjugated individually to polyampholyte hydrogels. The specific SIBLING proteins and fragments that are being used in this study are as follows: osteopontin (OPN), bone sialoprotein (BSP), dentin sialoprotein (DSP), dentin phosphoprotein (DPP), dentin matrix protein 1 N-terminal fragment (DMP1-N), and dentin matrix protein 1 C-terminal fragment (DMP1-C).

During this specific subtask, polyampholyte hydrogels were prepared from equimolar mixtures of 2-[(acryloyloxy) ethyl] trimethyl ammonium chloride (TMA) and 2-carboxy ethyl acrylate (CAA) monomers (1 mM of each). These monomers are mixed with a triethylene glycol dimethacrylate (TEGDMA) cross-linker in a 26.3:1 ratio of total monomers to cross-linker within a buffer composed of a 1.5:1:1.5 volume ratio of ethylene glycol, ethanol, and 3 M sodium hydroxide. The polymerization reaction is initiated using a 40% w/w solution of ammonium persulfate in water and a 15% w/w solution of sodium metabisulfate in water. All of the polymerization components are well-mixed and placed into a flat plate hydrogel mold composed of two microscope slides

clamped around a 1/8 inch polytetrafluoroethylene spacer. The reaction proceeded at room temperature for 24 hours. Following the reaction, the hydrogel was removed from the mold and placed into phosphate buffered saline (PBS; 150 mM, pH 7.4) where it was soaked for 24 hours. Following the PBS soak, the hydrogel was removed and punched into 5 mm circles using a biopsy punch. These disks were again soaked in PBS overnight prior to use.

Protein conjugation was completed by first exposing the 5 mm circular hydrogels to a 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution for 7 minutes. Following this 30 µL of a 1 mg/mL solution of individual SIBLING proteins in PBS was placed on the top of the activated hydrogel, where it remained for 15 minutes. Following exposure to the protein solution, the hydrogels were soaked in a PBS solution containing NaCl with a pH of 8.9 for 30 minutes. Finally, these hydrogels with conjugated SIBLING proteins were soaked for 40 minutes in a PBS buffer (pH 7.4) prior to cell adhesion studies.

In the meantime, MC3T3-E1 cells (ATCC #CRL-2594) were grown on tissue culture polystyrene (TCPS) flasks in alpha-minimum essential media (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. These cells were passaged each time they reached confluency by detaching the cells from the flask using trypsin-EDTA and diluting the detached cells into new TCPS flasks. Only passages 5-10 were used for conducing cell adhesion experiments.

To complete cell adhesion experiments, confluent MC3T3-E1 cells were detached from their flask using trypsin-EDTA and they were mixed in 5 mg/mL soybean trypsin inhibitor in PBS buffer. The cells were centrifuged and the supernatant was removed and then they were rinsed two times in a solution of 1 mg/mL bovine serum albumin (BSA) in α-MEM. Finally, the cells were counted and diluted to a concentration of $1*10^5$ cells/mL in unsupplemented α-MEM.

The cell adhesion experiments were conducted by placing individual 5 mm polyampholyte hydrogels with conjugated SIBLING proteins into individual wells in a 24-well plate. The diluted cells were added to each well containing a sample (1 mL per well). In addition, TMA:CAA hydrogels without conjugated SIBLING proteins were used as a negative, non-adhesive control (labeled as Nonfouling). TMA:CAA hydrogels that were subjected to the protein conjugation procedures without exposure to SIBLING proteins were also included as a control (labeled as Control). The samples were exposed to the cell solution for 2 hours, after which the solution was removed, and the wells were rinsed with PBS buffer. The cells were fixed with 4% paraformaldehyde for 5 minutes and then the samples were imaged with light microscopy. A minimum of three random images were captured for each sample, including the controls, and three independent samples were prepared for each experiment. The experiment was replicated three times for a minimum total of 27 images per sample. The cell adhesion was determined by counting the number of cells adhered to the substrate as captured in each image.

During this annual reporting period, the above experiments were conducted for polyampholyte hydrogels with conjugated OPN, BSP, DSP, DPP, DMP1-C, and DMP1-N and the results are shown below in Figure 1. The results shown in Figure 1 demonstrate that MC3T3-E1 cells show the highest level of cell adhesion to polyampholyte hydrogels with conjugated OPN. However, while the highest adhesion levels are seen, it can be seen in Figure 2 that the cells on OPN

conjugated samples have a rounded morphology indicating they may not survive over longer times.

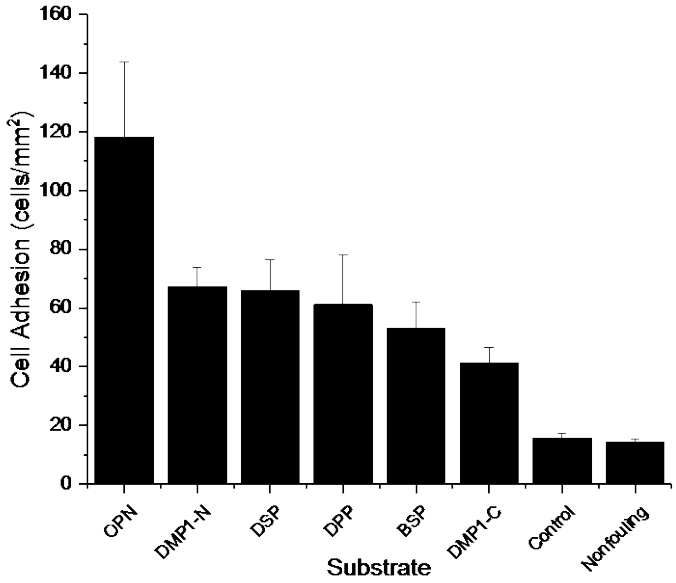


Figure 1: Average number of MC3T3-E1 cells (cell/mm^2) that adhered to substrates with or without conjugated SIBLING proteins. The data are presented as the mean ± standard error of the mean from nine total samples completed over three separate occasions. A minimum of three light microscopy images were captured for each sample ($n \geq 27$).

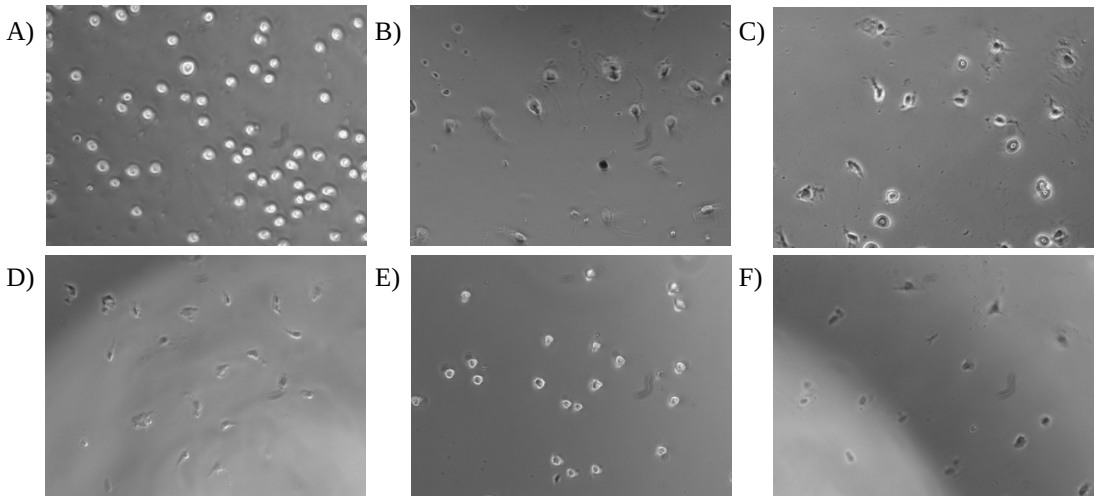


Figure 2: Representative 10x light microscopy images of MC3T3-E1 adhesion to polyampholyte hydrogels with conjugated A) OPN, B) DMP1-N, C) DSP, D) DPP, E) BSP, and F) DMP1-C.

Based on the results shown in Figure 2 for OPN-conjugated samples, the team added in one additional set of experiments regarding SIBLING protein conjugated polyampholyte hydrogels prior to initiating work on Major Task #2, the use of a live-dead stain to assess cell viability. In these studies, a live-dead fluorescent assay (Life Technologies, Corporation) was used for SIBLING protein conjugated polyampholyte hydrogels following a two-hour cell adhesion in

serum free media using the procedures outlined above, followed by an additional 24 hours of cell culture in unsupplemented media or in media supplemented with 1% fetal bovine serum. Following 24-hours, cells were exposed to the live/dead staining following the manufacturer's recommended protocol, resulting in alive cells being stained green and dead cells being stained red. This additional investigation demonstrated that SIBLING protein conjugated polyampholyte hydrogels did not maintain cell viability following 24 hours in culture for any of the SIBLING proteins or culture conditions. Representative results for a control tissue culture polystyrene and OPN-conjugated hydrogel can be seen in Figure 3, below.

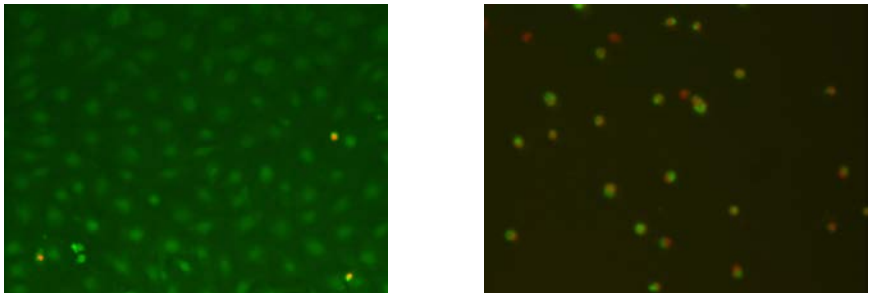


Figure 3: Representative live/dead fluorescent staining of MC3T3-E1 cells adherent on tissue culture polystyrene (left) and OPN-conjugated to a TMA:CAA polyampholyte hydrogel (right) following 24-hours of culture. Green indicates alive cells, while red indicates non-viable cells.

Following the assessment of the cell viability using the live/dead assay, procedures were developed to confirm the presence of bound SIBLING proteins to the polyampholyte hydrogels. A Stains-All stain was purchased and the manufacturer's recommended protocols were followed. This stain binds to protein and peptides, resulting in a red to blue color. While the results were not quantified, these efforts did confirm the presence of SIBLING proteins under all conjugation conditions that were tested.

Dr. Aaron Stoker (Site 3) was then consulted for additional approaches. Dr. Stoker recommended an assessment of the cytotoxicity of the TMA:CAA polyampholyte hydrogels over longer time periods given the fact that they did not demonstrate immediate cytotoxicity following the 2 hour adhesion assay. Both control and OPN-conjugated samples were prepared per the procedures above, and sent to Missouri (MU, Site 3) for cytotoxicity evaluations. MU assessed the cytotoxicity of samples under the following conditions:

- Samples remained in the PBS they were shipped in (1 mL)
- Samples were rinsed upon arrival and placed into fresh PBS (1 mL)
- Samples were rinsed upon arrival and placed into fresh FBS (1 mL)
- Samples were moved to a larger well plate and soaked in 8 mL of PBS
- Samples were moved to a larger well plate and soaked in 8 mL of fresh FBS

All of these samples were stored for 4 days at 4°C before being placed into fresh well plates. Samples were then seeded with 1 mL of $1x10^5$ cells/mL of either synoviocytes or primary bone marrow derived tissue progenitor cells, and all samples were incubated with cells overnight before analysis.

Upon evaluation it was determined that all of the samples (with and without OPN) that were left in the original PBS they were shipped in were cytotoxic, turning the cell culture media yellow, indicating a highly acidic environment. The samples that were soaked in 1 mL of fresh PBS or FBS also demonstrated cytotoxicity, but to a lesser extent that those that remained in the shipping buffer. This was indicated by a less acidic culture media, but no viable cells. Finally, those samples that were soaked in 8 mL of PBS or FBS showed negligible acidity, relative to the untreated control wells. This is shown in Figure 4. These efforts resulted in the conclusion that the TMA:CAA polyampholyte hydrogels must be soaked for longer time periods and in larger volumes of solution prior to cell seeding, to remove by-products from the reaction or initial degradation. With this conclusion, it was agreed by the project team that work would be initiated on Major Task #2 and #4.

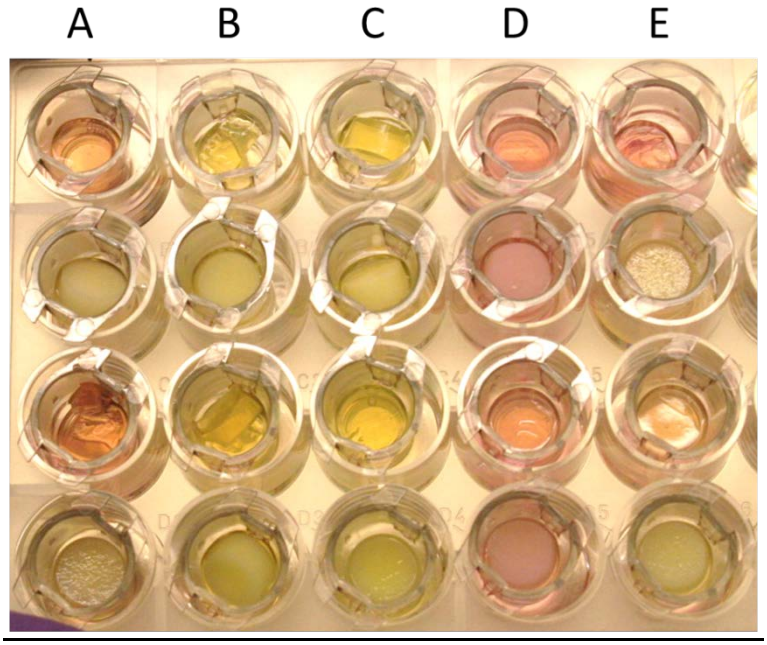


Figure 4: Constructs soaked in either (A) 1 mL of fresh fetal bovine serum, (B) 1 mL of fresh phosphate buffered saline, (C) the original phosphate buffered saline, (D) 8 mL of fresh fetal bovine serum, or (E) 8 mL of fresh phosphate buffered saline. After soaking the constructs were cultured overnight with either bone marrow derived cells or primary synoviocytes. The yellow color changes indicates acidic culture conditions. The red color indicates neutral, normal culture pH conditions.

Throughout all of the efforts under Major Task #1, the subtasks to be accomplished at Site #2 under the direction of Dr. Chunlin Qin were completed as needed, and SIBLING proteins were isolated and purified for use at Site #1 whenever they were required for completion of project tasks. Site #2 has provided OPN, BSP, DPP, DSP, DMP1-N, and DMP1-C to Site #1. Overall, efforts to complete Major Task #1 were initially deemed complete following the development of revised soaking procedures discussed above. However, efforts under subtask 2 are being redone at this time, to directly address the negative results that were obtained in Major Task #2 and #4, as discussed below. This remaining subtask will be completed by the end of the revised timeline.

Major Task 2 (All Subtasks) and Major Task 4 (All Subtasks): The discussion of progress made on Major Task #2 (primary cell line proliferation and differentiation on base case hydrogel) and #4 (primary cell line proliferation and differentiation on polyampholyte hydrogels with different mechanical characteristics) will be completed in parallel, as both tasks were completed simultaneously through work at Site #1 and Site #3. Under both Major Tasks, efforts were expended to complete Subtask 1 and 2, which were to evaluate the primary cell proliferation and differentiation, respectively. Subtask 3 (cell penetration) under both Major Task #2 and #4 has not been initiated and will be addressed during the no cost time extension for this project.

To initiate these efforts, the PI and Site #1 synthesized 16 TMA:CAA polyampholyte hydrogels using a TEGDMA cross-linker at a 26.3:1 (1x) monomer to cross-linker density using the procedures outlined above. Additionally, 16 TMA:CAA hydrogels with monomer to cross-linker ratios of 13.1:1 (2x) and 6.7:1 (4x) were synthesized with the same procedures by increasing the concentration of TEGDMA. Twelve hydrogels for each cross-linker density were subjected to OPN conjugation using the procedures outlined above. The remaining four hydrogels for each cross-linker density were used as negative control gels. These hydrogels were shipped from Site #1 to Site #3 for use in primary cell culture to complete subtasks 1-2 for both Major Task #2 and #4. The original intention was to complete subtask 3 as well. The two primary cell lines include canine bone marrow derived cells (BMAC) and primary synoviocytes (SYN). The breakdown of samples analyzed at Site #3 is as follows:

- 6 gels; 1x; OPN; BMAC
- 6 gels; 1x; OPN; SYN
- 6 gels; 2x; OPN; BMAC
- 6 gels; 2x; OPN; SYN
- 6 gels; 4x; OPN; BMAC
- 6 gels; 4x; OPN; SYN
- 2 gels of each cross-linker density (1x, 2x and 4x); No protein; BMAC
- 2 gels of each cross-linker density (1x, 2x and 4x); No protein; SYN

Upon arrival at Site #3, constructs were soaked at 4 °C in 1 mL of FBS, and every 24 hours the FBS was removed and replaced with fresh FBS for 5 days per the procedures determined during the cytotoxicity evaluation. On the fifth day the FBS was removed, and the constructs were seeded with 100,000 cells/constructs. The cells were allowed seed to the constructs overnight at 37 °C, 5% CO2, and 95% humidity. The next day the media was removed and the constructs were also moved to a new well. Fresh media without cells was added to the constructs and the wells where the constructs were originally seeded in, to determine the viability of the cells in both wells. Cells and constructs were cultured for 15 days. After 15 days constructs were evaluated for cell metabolism using the resazurin assay, for viable cell adhesion using Calcein AM, and fluorescent microscopy (Figure 5). There were no viable cells observed adhered to any of the constructs following 15 days of culture. Monolayer cells were seen in the original wells from where the constructs were transferred for both SYN and BMAC cultures, indicting cell viability. This indicates that the constructs were not immediately toxic to the cells, and that the cells were still viable in culture for 15 days. However, the cells either did not bind to the constructs, indicating that OPN does not allow for adherence cells to the constructs, or the constructs have a slowly released cytotoxic or acidic component.

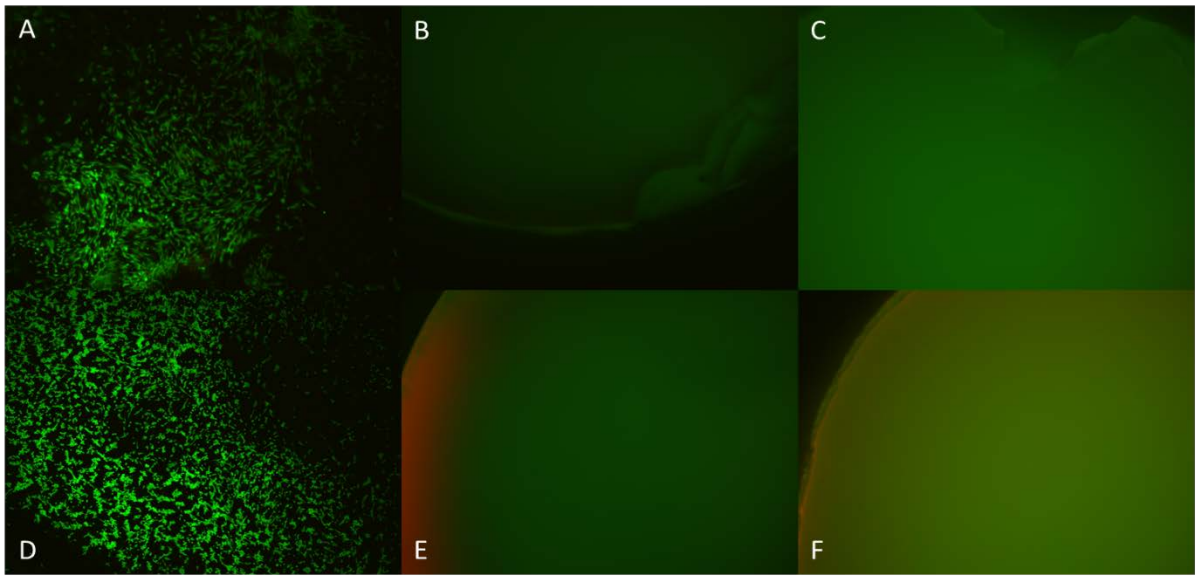


Figure 5: Representative fluorescent micrographs of cell viability staining of (A) synoviocytes, (B) 1x cross-linker density constructs seeded with synoviocytes, (C) 4x cross-linker density constructs seeded with synoviocytes, (D) bone marrow cells, (E) 1x cross-linker density constructs seeded with bone marrow cells, and (F) 4x cross-linker density constructs seeded with bone marrow cells. All images were captured 15 days after seeding.

After the 15 day cell viability assessment, half of the construct was digested and analyzed for collagen and proteoglycan content. There was no detectable collagen or proteoglycan content in any of the tissue digests, regardless of cell type or construct cross-linker density. The other half of the construct was used for histological evaluation using H&E and T-blue stains. There were no cells visible by histological evaluation, or staining for proteoglycan or collagen content (Figure 6).

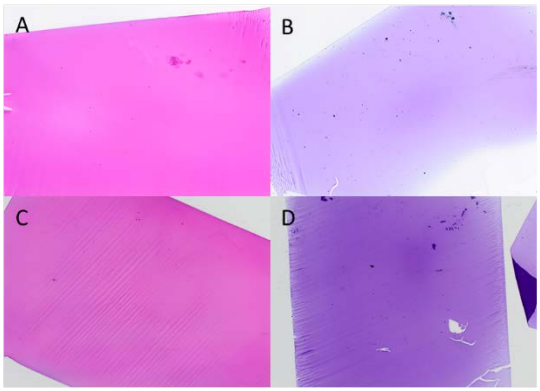


Figure 6: Constructs cultured for 15 days with bone marrow cells and synoviocytes were fixed in 10% formalin and then stained with either H&E or T-blue for histological evaluation.

Finally, the media from each well from days 6-15 in culture was evaluated for proteoglycan content and alkaline phosphatase content, and the media from day 15 was evaluated for collagen I. The media concentration of proteoglycans (Figure 7), alkaline phosphatase (Figure 8), and collagen I (Figure 9) was low in all of the samples, again regardless of cell type or cross-linker density. No

consistent significant differences indicative of stimulation of cellular matrix production or differentiation into a chondrocytic or osteogenic phenotype was seen.

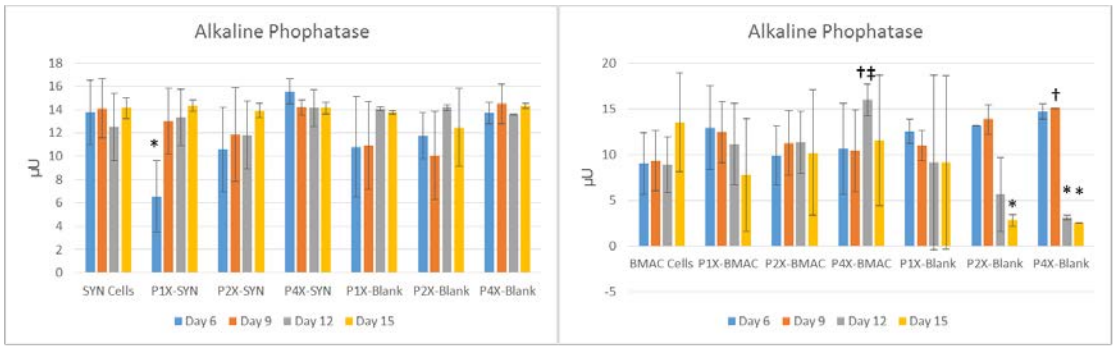


Figure 7. Media alkaline phosphatase concentration on days 6-15 from the bone marrow cell (BMAC) and synoviocyte (SYN) cultures. Constructs with and without (blank) bound OPN, and monolayer cell cultures (cells). A * indicates a significantly lower level than monolayer cell culture, a † indicates significantly higher level than monolayer culture, and a ‡ indicates a significantly higher than corresponding blank culture.

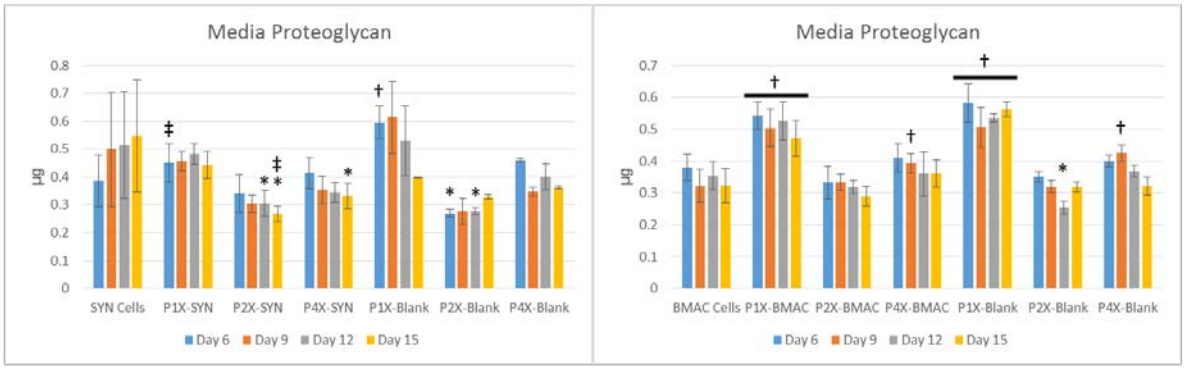


Figure 8. Media proteoglycan concentration on days 6-15 from the bone marrow cell (BMAC) and synoviocyte (SYN) cultures. Constructs with and without (blank) bound OPN, and monolayer cell cultures (cells). A * indicates a significantly lower level than monolayer cell culture, a † indicates significantly higher level than monolayer culture, and a ‡ indicates a significantly lower than corresponding blank culture.

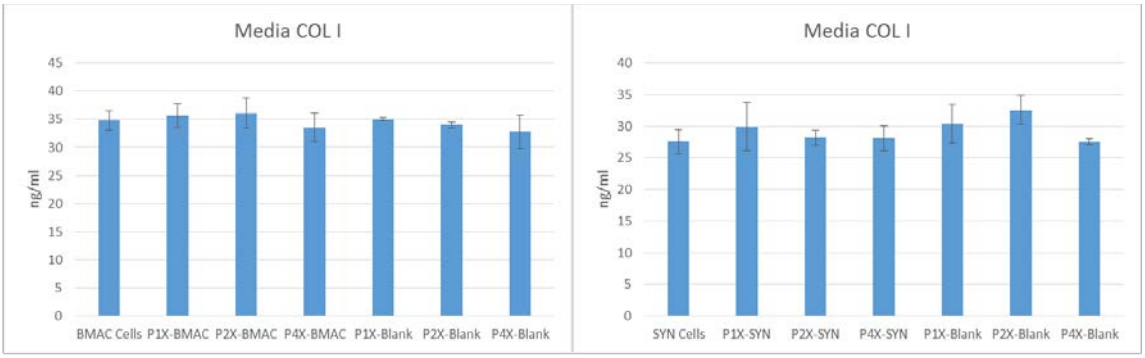


Figure 9: Media collagen I concentration on day 15 in the bone marrow (BMAC) and synoviocyte (SYN) cultures. Constructs with and without (blank) bound OPN, and monolayer cell cultures (cells). No significant differences were seen between groups on day 15.

The overall conclusions reached following the primary cell culture work involving both BMAC and SYN cell types is that the constructs do not support viable cell adhesion, proliferation, and differentiation under the conditions evaluated. It is uncertain if this is due to a lack of cell adhesion in the initial 24 hour seeding time frame or the extended release of a cytotoxic component. In either case, these results have led the project team to reevaluate the polyampholyte hydrogel synthesis procedures developed and used during Major Task #1. In addition subtask 3 in both Major Task #2 and #4 was not evaluated at this time.

It is hypothesized that the primary cytotoxicity concerns result from acidity in the culture media as shown in the results discussed above. Therefore, an evaluation was completed to determine the pH of each component added to the polyampholyte hydrogel synthesis (ethanol, ethylene glycol, 3 M NaOH, TMA, CAA, TEGDMA, SMS initiator, and APS initiator). It was determined that all of the monomers and initiators in the hydrogel system have an acidic pH. The concentration of the NaOH was determined to be insufficient to balance out these components, resulting in an overall acidic hydrogel synthesis solution. It is noted that the NaOH was originally included in the hydrogel buffer not for pH control, but to ensure the deprotonation of the CAA monomer.[1] Therefore, in the remaining project time the team will be reformulating the hydrogel synthesis buffer of ethanol, ethylene glycol, and NaOH to better counter-balance the acidic monomers and initiators. This work will be completed during the remaining project time and the successful reformulation will be evaluated using MC3T3-E1 cells at Site #1. These activities fall under the original scope of Major Task #1.

Major Task 3: In addition, during this annual reporting period the PI and Site #1 initiated additional research activities that fall under Major Task #3, without impacting the primary scope of work and remaining timeline. Major Task #3 is to "Develop polyampholyte hydrogels with a range of mechanical properties." The primary subtasks under this Major Task were previously deemed complete, but the overall range of mechanical properties that were achieved was not a wide as originally hypothesized (40 – 500 kPa fracture strength). In this additional research effort, polyampholyte hydrogels were prepared using TMA and CAA, with identical procedures to those described above. The one variable that was changed was the cross-linker species. Two additional cross-linker species were tested and the resulting mechanical properties were compared to those

1 Schroeder, *et al. Biomacromolecules*, 14(9), 3112-3122, 2013.

achieved with the original TEGDMA cross-linker. Diethylene glycol dimethacrylate (DEG) and tetraethylene glycol dimethacrylate (Tetra) cross-linker species were tested at 26.3:1 (1x), 13.1:1 (2x), and 6.7:1 (4x) monomer to cross-linker ratios. All of the hydrogel species were compressed to failure using an Instron equipped with a 100 kN load cell. The samples were compressed to failure at a compression rate of 2 mm/s and the fracture stress and strain were determined as the highest values obtained prior to failure. The results, shown in Figure 10, indicate that the 4x DEG hydrogels present the greatest compressive mechanical properties, with a value that is almost double that seen in the previous project efforts (~900 kPa vs ~500 kPa). In addition, the DEG samples also presented the highest strain values at failure across all three cross-linker species.

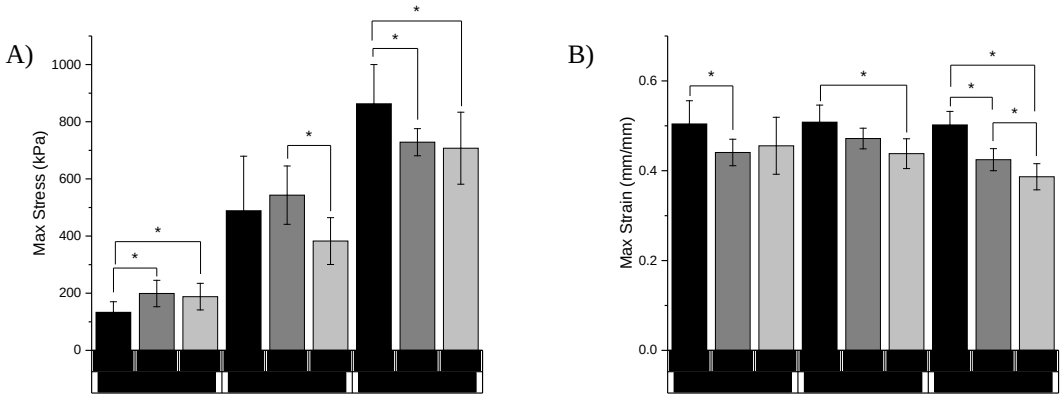


Figure 10: Compressive A) stress and B) strain at failure for TMA:CAA hydrogels with varying cross-linker species and cross-linker densities. A * indicates a statistically significant difference between the two conditions indicated.

Milestones Achieved: The Statement of Work milestone for Major Task 3 was "Develop range of polyampholyte hydrogel platforms for cellular testing" and polyampholyte hydrogels with compressive fracture strengths ranging from ~50-900 kPa have been synthesized. The additional efforts undertaken during this annual reporting period have led to polyampholyte hydrogels with a mechanical property range that is almost twice that of the previously documented project results, indicating that the additional efforts were worthwhile. At this stage, Major Task #3 is considered to be accomplished and no additional efforts are planned. These new results will also be included in a manuscript that is currently in preparation and will be published by the end of the revised project timeline.

4. Impact

During this reporting period, the project team gave one poster presentation at the Society for Biomaterials Annual Meeting, which was held in April 2018 in Atlanta, GA. This poster was entitled "Bone Tissue Engineering Scaffolds based on Polyampholyte Hydrogels with Conjugated SIBLING Proteins". The project team also presented this work at an invited talk at the regional IDEA Network of Biomedical Research conference which was held in August 2018 in Moscow, ID.

5. Changes/Problems

The project timeline has just recently been modified again. The team requested a no cost time extension to more thoroughly address the cytotoxicity issues that were discovered during primary cell culture activities with the polyampholyte hydrogels. This extension was granted the week that this report was prepared and the project timeline has been extended to March 2019. During this extension the project team will reinvestigate the adhesion of MC3T3-E1 cells to the SIBLING proteins following conjugation to the reformulated polyampholyte hydrogels. Penetration of cells into the hydrogels will also be investigated to complete the original scope of work during this extension.

6. Products

The products obtained during this reporting period are one poster presentation at the 2018 Society for Biomaterials Annual Meeting which took place in Atlanta, GA. In addition, the PI was invited to give a presentation at the regional IDEA Network of Biomedical Research (INBRE) conference in August 2018, in Moscow, ID, where the progress on this project was presented. Finally, the project team has submitted two abstracts for consideration at the 2018 American Institute of Chemical Engineers Annual Meeting, both of which have been accepted for presentations (1 oral and 1 poster). This meeting will be held in October/November 2018 in Pittsburgh, PA.

Over the lifetime of this project, there have been a total of two submitted/accepted/published manuscripts, one oral presentation at a professional conference, one poster presentation at a professional conference, and one invited talk at a regional conference.

7. Participants & Other Collaborating Organizations

Name:	Dr. Matthew Bernards
Project Role:	PI
Nearest person month worked:	2
Contribution to project:	As PI, Dr. Bernards has supervised all project activities and coordinated all work between sites during this reporting period.

Name:	Dr. Ferris Pfeiffer
Project Role:	Co-I
Nearest person month worked:	1
Contribution to project:	Dr. Pfeiffer supervised the completion of the hydrogel mechanical compression testing in the first year of work on this project.

Name:	Dr. Aaron Stoker
Project Role:	Co-I
Nearest person month worked:	2
Contribution to project:	Dr. Stoker completed work to isolate primary synoviocytes and primary bone marrow derived connective tissue

progenitor cells from canines and to evaluate the adhesion, proliferation, and differentiation of these two cell lines on hydrogels with covalently attached SIBLING proteins with a range of mechanical properties. This work was completed during this reporting period.

Name:	Dr. Chunlin Qin
Project Role:	Co-I
Nearest person month worked:	1
Contribution to project:	Dr. Qin supervised efforts to isolate SIBLING proteins from rat incisors and long bones. This work has been successful and is on-going in support of the project needs.

Name:	Dr. Hua Zhang
Project Role:	Postdoctoral Research Associate
Nearest person month worked:	1
Contribution to project:	Hua worked under the guidance of Dr. Qin to isolate and purify SIBLING proteins. This work has been successful and is on-going in support of the project needs.

Name:	Stephanie Haag
Project Role:	Graduate Research Assistant
Nearest person month worked:	12
Contribution to project:	Stephanie has completed synthesis and characterization of TMA:CAA hydrogels as described under Major Task #1 and varied the hydrogel synthesis procedures to address cytotoxicity. Stephanie also initiated and completed MC3T3-E1 cell adhesion studies over this project year and continues to evaluate cell response to the reformulated hydrogels.

Name:	Emily Mariner
Project Role:	Graduate Research Assistant
Nearest person month worked:	3
Contribution to project:	Emily completed all efforts to characterize the mechanical properties of TMA:CAA hydrogels with varying ethylene glycol cross-linker species. This work falls under Major Task #3.

Name:	Marcos Barcellona
Project Role:	Undergraduate Research Assistant
Nearest person month worked:	1
Contribution to project:	Marcos completed the synthesis of multiple polyampholyte hydrogels for mechanical testing in the first year of work on this project. Marcos was supported with other funding for his work on this project.

Name:	Siyu Cao
Project Role:	Graduate Research Assistant
Nearest person month worked:	1
Contribution to project:	Siyu completed the nonfouling and protein conjugation measurements for multiple polyampholyte hydrogels in the first year of work on this project. Siyu was supported with other funding for her work on this project.

Name:	Nicole Walden
Project Role:	Undergraduate Research Assistant
Nearest person month worked:	1
Contribution to project:	Nicole worked under the supervision of Dr. Stoker on the isolation of cells for use in this project during the first year of work on this project.

8. Special Reporting Requirements

An updated project Quad Chart can be seen on the next page.

Development of Novel Segmental Bone Defect Construct

W81XWH-15-1-0664

PI: Dr. Matthew Bernards **Org:** University of Idaho / University of Missouri **Award Amount: $**284,397

Study/Product Aim(s)

- Elucidate the role of the SIBLING proteins on the adhesion, proliferation, and differentiation of both primary synoviocytes and bone marrow derived connective tissue progenitor cells.
- Determine the influence of the underlying polyampholyte polymer on the cellular adhesion, proliferation, and differentiation of both primary synoviocytes and bone marrow derived connective tissue progenitor cells.

Approach

It is hypothesized that one or more of the SIBLING proteins is responsible for recruiting cells for bone tissue repair and regeneration and their use in a tissue engineering scaffold will induce a natural, expedited would healing response for segmental bone defects. Therefore the impact of these proteins will be individually determined using a multi-functional nonfouling polyampholyte polymer scaffold.

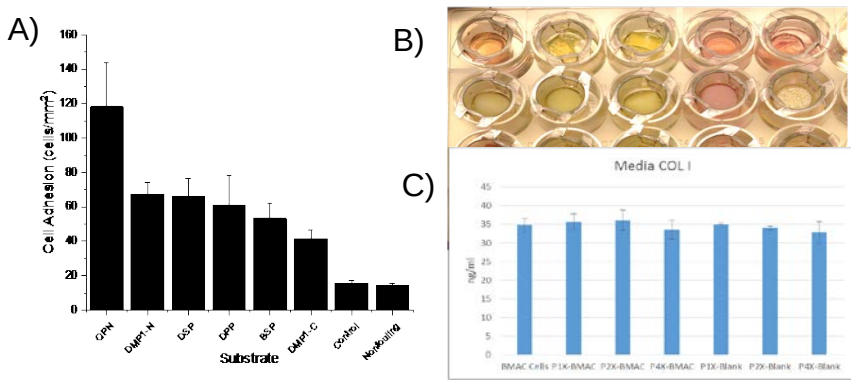


Figure: A) MC3T3-E1 adhesion to SIBLING protein conjugated scaffolds. B) Representative cytotoxicity assay where yellow indicates acidic conditions. C) Collagen content in culture media following 15 days of primary cell culture.

Annual Accomplishments: Influence of cross-linker species on the overall physical properties has been characterized. Primary cell studies indicate that the gels still lead to cell death, so the hydrogel formulation is being modified.

Timeline and Cost

Activities	CY 15	17	18
Attach SIBLING Proteins			
Determine SIBLING Roles			
Modify Hydrogel Characteristics			
Determine Hydrogel Roles			
Estimated Budget ($K)	**$46.1**	**$92.6**	**$145.6**

There was a project break between CY15 and CY17 to transfer the project from the University of Missouri to the University of Idaho.

Updated: 29/October/2018

Goals/Milestones

Completed Goals

☑ Modify hydrogel chemistry and cross-linker density to tune mechanical properties

☑ Verify nonfouling and protein conjugation capacity of hydrogels

CY17 Goals – Attach SIBLING proteins to hydrogels and determine key SIBLING protein roles and influence of polyampholyte chemistry

☑ Quantify conjugation to polyampholyte hydrogels

☑ Test adhesion of cells to SIBLING proteins **(revisiting)**

☑ Track proliferation of cells following adhesion **(revisiting)**

CY18 Goal – Determine impact of hydrogel chemistry on cells

☑ Characterize differentiation of cells

☐ Characterize cell penetration into hydrogels **(in progress)**

☑ Characterize differentiation as a function of hydrogel chemistry

☐ Characterize cell penetration as a function of hydrogel chemistry **(in progress)**

Budget Expenditure to Date

Projected: $284,397 Actual Expenditure:$266,625

9. Appendices

Attached is a copy of the poster that was presented on this project in April 2018 at the Society for Biomaterials Annual Meeting in Atlanta, GA.

Bone Tissue Engineering Scaffolds based on Polyampholyte Hydrogels with Conjugated SIBLING Proteins

Stephanie Haag,[1] Chunlin Qin,[2] and Matthew Bernards[1]
[1]*Chemical & Materials Engineering, University of Idaho, Moscow, ID*
[2]*Biomedical Sciences, Texas A&M University College of Dentistry, Dallas, TX*

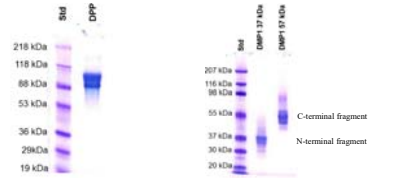
Introduction

Recently, there has been an increasing interest in polyampholyte hydrogels for use in tissue engineering applications. Previous studies have shown polyampholytes to be resistant to non-specific protein adsorption, capable of covalently binding proteins, and mechanically tunable. The combination of polyampholyte hydrogels with members of the SIBLING (small integrin binding, N-linked glycoprotein) family of proteins is hypothesized to enhance cell attachment for a bone tissue engineered scaffold. In this project, MC3T3-E1 cell adhesion to polyampholyte hydrogels with conjugated SIBLING proteins is investigated.

SIBLING Family of Proteins

The SIBLING family is made up of five family members, two of which are naturally cleaved *in vivo*. All contain a cell adhesive RGD sequence, a collagen binding domain, and a hydroxyapatite binding domain.

Osteopontin (OPN) Bone Sialoprotein (BSP) Dentin Sialoprotein (DSP)

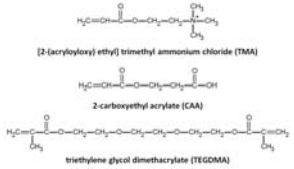


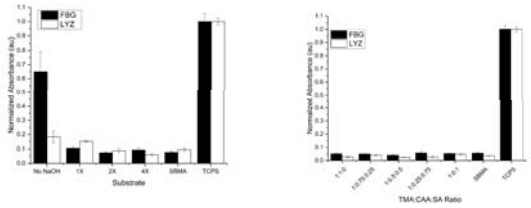
Dentin Phosphoprotein (DPP) Dentin Matrix Protein 1 (DMP1)

- Highly pure proteins isolated from rate bone or dentin.
- Matrix extracellular phosphoglycoprotein is not included due its trace concentrations *in vivo*.

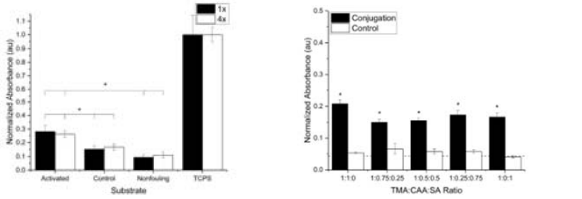
Background

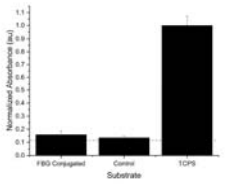
- TMA and CAA monomers mixed in a 1:1 ratio.
- TEGDMA cross-linker mixed in a ~1:26 ratio relative to the monomers.



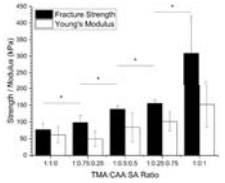


- Polyampholyte hydrogels show excellent nonfouling capabilities.
- Nonfouling is observed for multiple charge neutral compositions.



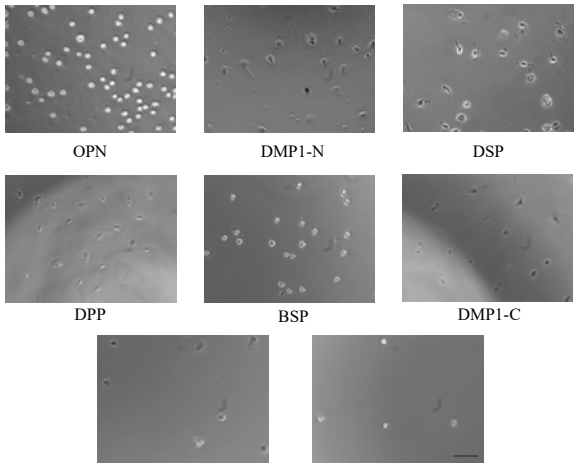


- Hydrogels capable of protein conjugation via EDC/NHS chemistry.
- Conjugation capability is independent of the underlying composition.
- Nonfouling property is retained following protein conjugation.

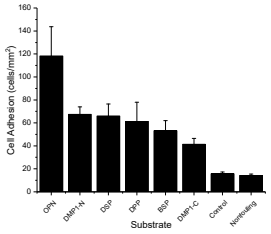


- Mechanical properties are tunable via monomer composition and cross-linker species / density.

Cell Adhesion



Cell Adhesion Quantification



- Hydrogels resist cell adhesion in the absence of SIBLING proteins.
- OPN showed highest cell adhesion with ~120 cells/mm^2, but rounded morphology.
- Intermediate adhesion, but spread morphology seen for DMP1-N, DSP, and DPP.

Conclusions/Future Work

- Hydrogels provide a "blank slate" tissue engineering platform.
- Conjugated SIBLING proteins facilitate cell adhesion.
- OPN promoted the highest cell adhesion, but unhappy morphology.
- MC3T3-E1 7-day proliferation studies in the presence of these SIBLING proteins are on-going.
- Primary synoviocyte and primary bone marrow derived connective tissue progenitor cell studies are on-going.

References

•Qin *et al. Crit Rev Oral Biol Med 15*(3), 126-136, 2004.
•Schroeder *et al. Biomacromolecules, 14*(9), 3112-3122, 2013.
•Cao *et al. Journal of Applied Polymer Science, 133*(40), 10, 2016.

University of Idaho

Acknowledgements

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