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14. ABSTRACT

This report contains the final summary of all project efforts conducted on the project "Development of a Novel Segmental Bone Defect Construct". The original project hypothesis was 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. This hypothesis was tested through the completion of four major tasks. Major Task #1 was to determine 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 evaluated in primary cell proliferation, differentiation, and penetration into the base case polyampholyte hydrogel. In Major Task #3, a family of polyampholyte hydrogels with a range of mechanical properties was developed. Finally, in Major Task #4 the primary cell proliferation, differentiation, and penetration into polyampholyte hydrogels with a range of mechanical properties was evaluated. All of the major tasks were completed during the project lifetime, but successful results were only obtained under Major Task #1 and #3.

The results from project efforts under Major Task #1 led to a revision of the polyampholyte hydrogel synthesis procedures, in order to address biocompatibility concerns. In addition, osteopontin was identified as the SIBLING family member that promoted the highest level of initial cell adhesion. The results from project efforts under Major Task #2 and #4 led to the conclusion that the original, literature-based formulation for synthesizing polyampholyte hydrogels was cytotoxic. The primary cell efforts identified acid release from the hydrogel platform as the primary source of cytotoxicity, and these efforts led to the revision of the polyampholyte hydrogel synthesis procedures resulting in Major Task #1. Unfortunately due to time and budget constraints, the revised hydrogel formulation was not tested with the primary cell lines. The results from project efforts under Major Task #4 led to the demonstration of polyampholyte hydrogels with a compressive fracture stress range of ~50-900 kPa. This range is easily obtained through control of the underlying monomer composition, the cross-linker species, and/or the cross-linker density. Overall, the project results have been disseminated through the publication of three manuscripts, with a fourth in preparation, along with three invited lectures, 4 conference oral presentations, and 4 conference posters.

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 have been 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 the completion of work on the project “Development of a Novel Segmental Bone Defect Construct” and it summarizes the accomplishments over the project lifetime. In this work a novel bone tissue engineering scaffold material was 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 was to isolate the effects of the SIBLING proteins on the adhesion of MC3T3-E1 osteoblast 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 over the lifetime of the project. However, the final project phase was not completed successfully due to an unforeseen biocompatibility concern, hydrogel acidity, that led to widespread cell death. This concern was ultimately overcome, but the project lifetime was complete, and budget was expended, thereby not allowing for repeat studies with primary synoviocytes and bone marrow derived connective tissue progenitor cells. Key accomplishments over the project lifetime include expanding the range of physical properties that are achievable with nonfouling polyampholyte hydrogels, the identification of osteopontin (OPN) as the SIBLING protein that promotes the highest levels of MC3T3-E1 osteoblast-like cell adhesion to polyampholyte hydrogels, and the development of revised hydrogel synthesis procedures to address the cytotoxicity, resulting in hydrogels capable of supporting cell adhesion and proliferation over at least 5 days.

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 primary objective was to complete the screening of the adhesion of MC3T3-E1 osteoblast-like cells to the SIBLING proteins, when conjugated individually to polyampholyte hydrogels, in order to identify the SIBLING protein that facilitated the greatest level of cell adhesion. The specific SIBLING proteins and fragments that were 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). Effort completed at Site #2, under the direction of Dr. Chunlin Qin, was completed throughout the project lifetime to isolate and purify these SIBLING proteins for all investigations discussed in this report per the proposed scope of work.

During this Major Task, 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 sodium hydroxide. The concentration of sodium hydroxide used in the buffer was varied throughout the project lifetime, beginning at a literature-based concentration of 3M¹ and ending at an experimentally determined concentration of 6.7 M as detailed in this report. The initial cell screening efforts were initiated with the literature-based formulation. 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 μ L 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 conducting 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.

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.

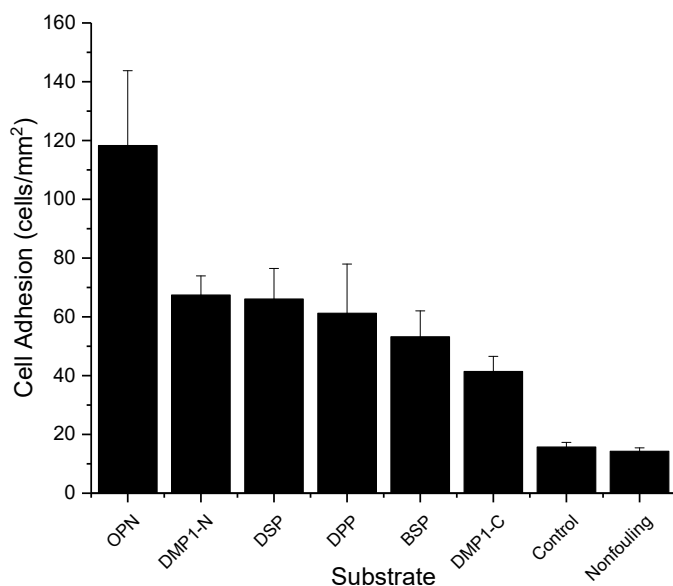


Figure 1: Average number of MC3T3-E1 cells (cell/mm²) that adhered to substrates with or without conjugated SIBLING proteins. The data are presented as the mean $\pm$ standard error of the mean from nine total samples completed over three separate occasions. A minimum of three light microscopy images were captured for each sample ($n \geq 27$).

The viability of cells adherent to polyampholyte hydrogels with conjugated SIBLING proteins was validated with the use of a live-dead stain. 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 viable cell populations 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 2, below.

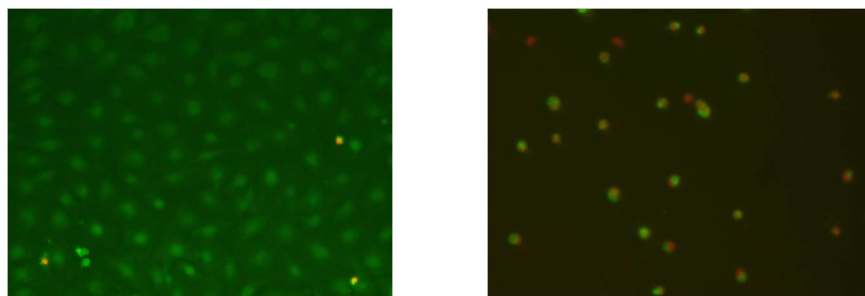


Figure 2: Representative live/dead fluorescent staining of MC3T3-E1 cells adherent on tissue culture polystyrene (left) and OPN-conjugated to a TMA:CAA polyampholyte hydrogel (right) following 24-hours of culture. Green indicates alive cells, while red indicates non-viable cells.

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 and he conducted 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 1×10^5 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 than 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 3. 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 (efforts detailed below).

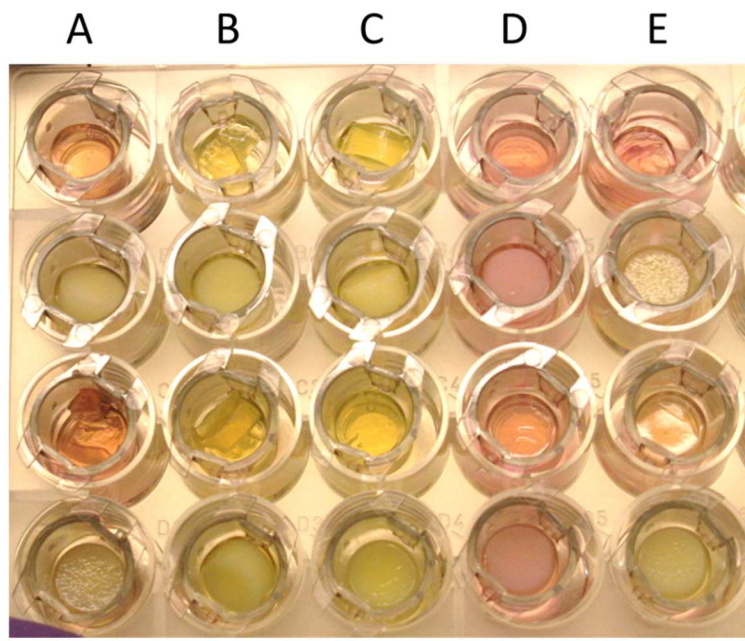


Figure 3: Constructs soaked in either (A) 1 mL of fresh fetal bovine serum, (B) 1 mL of fresh phosphate buffered saline, (C) the original phosphate buffered saline, (D) 8 mL of fresh fetal bovine serum, or (E) 8 mL of fresh phosphate buffered saline. After soaking the constructs were cultured overnight 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.

The efforts to complete Major Task #1 were initially deemed complete following the development of the revised soaking procedure discussed above. However, the results from efforts under Major Task #2 and #4, discussed below, led the project team to conclude that the polyampholyte hydrogels were releasing acidity even following the extended soaking procedures, resulting in cell death. When it was determined that additional soaking time did not eliminate the cytotoxic acidic component release, the polyampholyte hydrogel formulation was reevaluated. During synthesis the TMA, CAA, and TEGDMA monomers are diluted in a buffer composed of ethylene glycol, ethanol, and 3 M NaOH in a 1.5:1:1.5 volume ratio based on the literature.¹ NaOH was initially included to ensure that the resulting hydrogel had nonfouling properties. Polymerization is initiated through chemical means using 40% w/w ammonium persulfate (APS) and 15% w/w sodium metabisulfite (SMS). To address the acidity concern, the buffer formulation was reevaluated to include additional NaOH. As shown in Figure 4, the use of a 6.7 or 7.2 M NaOH in place of the original 3 M NaOH leads to minimal pH impacts when hydrogels are soaked in phosphate buffered saline for up to 7 days.

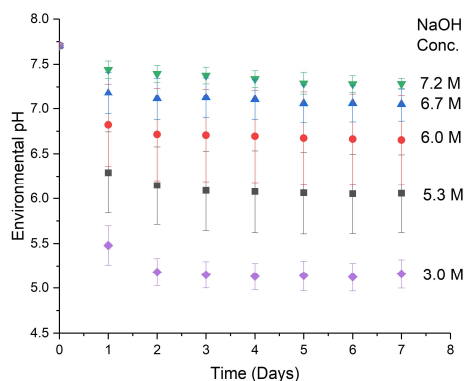


Figure 4: Additional results demonstrating the neutralization of acidic components during hydrogel synthesis through increased NaOH. The data are presented as the mean $\pm$ standard deviation from three samples completed over three separate occasions.

The revised polyampholyte formulation was evaluated for its nonfouling and protein conjugation capabilities using fluorescently labeled albumin, with no discernable impacts relative to the original formulation. Additionally, the short- and long-term cellular response to the revised formulation were evaluated using MC3T3-E1 cells and conjugated fibrinogen, a well-known cell adhesion promoting protein. A live/dead fluorescent assay was used, along with Confocal microscopy, following 2-hours, 24-hours, and 5-days in culture and the results demonstrate viable increasing cell populations at all time points on the revised polyampholyte hydrogel platform. Representative images can be seen in Figure 5.

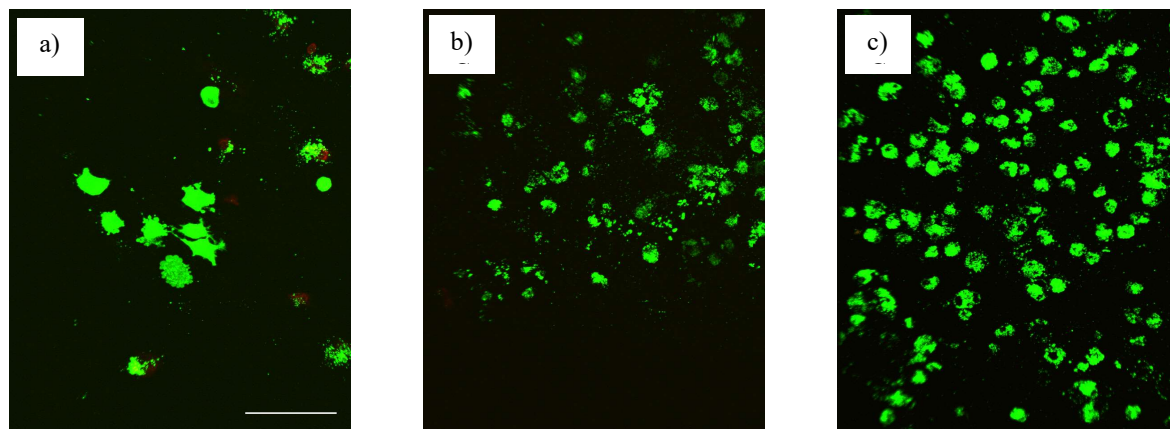


Figure 5: Representative confocal microscopy images demonstrating viable cells (green) and dead cells (red) following a) 2-hours, b) 24-hours, and c) 5-days of cell culture. The scale bar in a) is 100 μ m.

Milestones Achieved: The Statement of Work milestone for Major Task 1 was “Identify the adhesion capability of the SIBLING proteins conjugated to polyampholyte hydrogels”. It was determined that osteopontin (OPN) promoted the highest level of cell adhesion to polyampholytes. However, it was also identified that the polyampholyte hydrogel formulation required revision and the resulting hydrogel has demonstrated the ability to promote viable cell populations over 5 days with conjugated cell adhesion proteins. Major Task #1 is considered to be accomplished, although an additional cell adhesion screening with conjugated SIBLING proteins and the revised hydrogel

formulation has not been completed. This is recommended as a future stage of work. One manuscript is under preparation detailing the revised polyampholyte hydrogel synthesis procedures and the characterization of the resulting hydrogels.²

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 was not completed as discussed below.

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 literature procedures, with extended soaking, as 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 was 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, hydrogels 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 hydrogels were seeded with 100,000 cells/hydrogel. The cells were allowed seed to the hydrogels 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 hydrogels and the wells where the hydrogels were originally seeded in, to determine the viability of the cells in both wells. Cells and hydrogels were cultured for 15 days. After 15 days hydrogels were evaluated for cell metabolism using the resazurin assay, for viable cell adhesion using Calcein AM, and fluorescent microscopy (Figure 6). There were no viable cells observed on any of the hydrogels following 15 days of culture. Monolayer cells were seen in the original wells from where the hydrogels were transferred for both SYN and BMAC cultures, indicating cell viability. This indicates that the hydrogels 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 hydrogels, indicating that OPN does not allow for cell adherence to the hydrogels, or the hydrogels have a slowly released cytotoxic or acidic component.

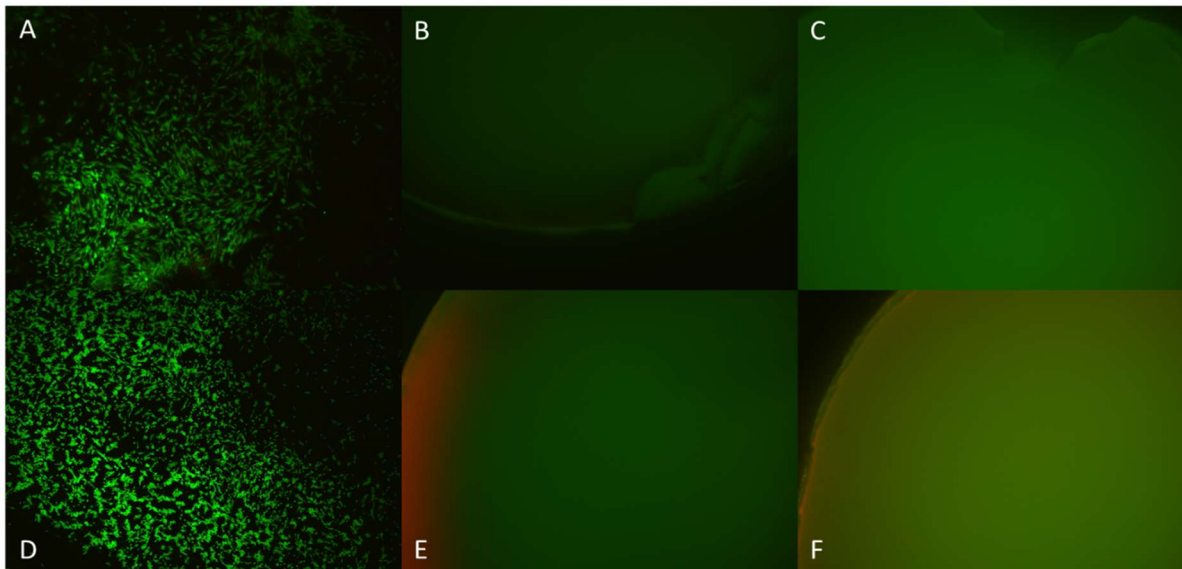


Figure 6: Representative fluorescent micrographs of cell viability staining of (A) synoviocytes, (B) 1x cross-linker density hydrogels seeded with synoviocytes, (C) 4x cross-linker density hydrogels seeded with synoviocytes, (D) bone marrow cells, (E) 1x cross-linker density hydrogels seeded with bone marrow cells, and (F) 4x cross-linker density hydrogels seeded with bone marrow cells. All images were captured 15 days after seeding.

After the 15 day cell viability assessment, half of the hydrogel 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 hydrogel cross-linker density. The other half of the hydrogel 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 7).

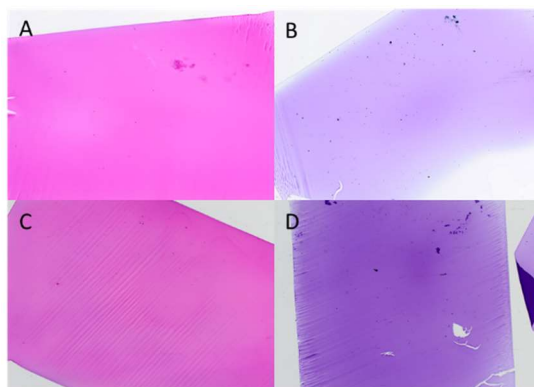


Figure 7: Hydrogels cultured for 15 days with bone marrow cells and synoviocytes were fixed in 10% formalin and then stained with either H&E or T-blue for histological evaluation.

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 8), alkaline phosphatase (Figure 9), and collagen I (Figure 10) was low in all of the samples, again regardless of cell type or hydrogel cross-linker density. No consistent significant differences indicative of stimulation of cellular matrix production or differentiation into a chondrocytic or osteogenic phenotype was seen.

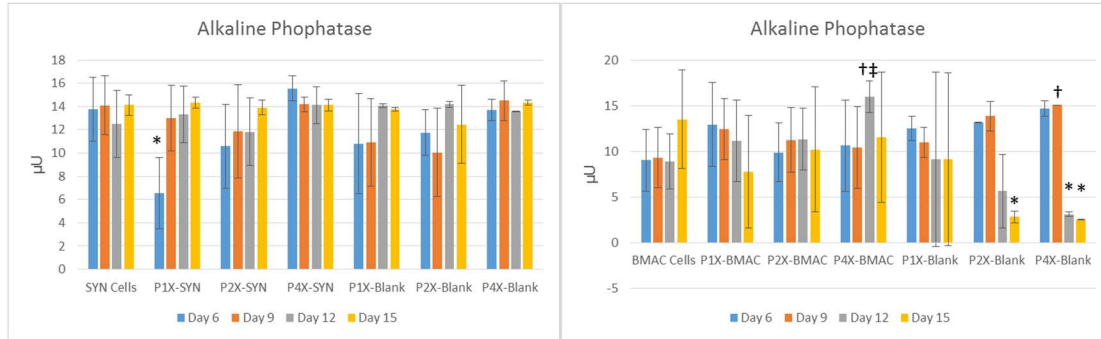


Figure 8. Media alkaline phosphatase concentration on days 6-15 from the bone marrow cell (BMAC) and synovioocyte (SYN) cultures. Hydrogels with and without (blank) bound OPN, and monolayer cell cultures (cells). A * indicates a significantly lower level than monolayer cell culture, a † indicates significantly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

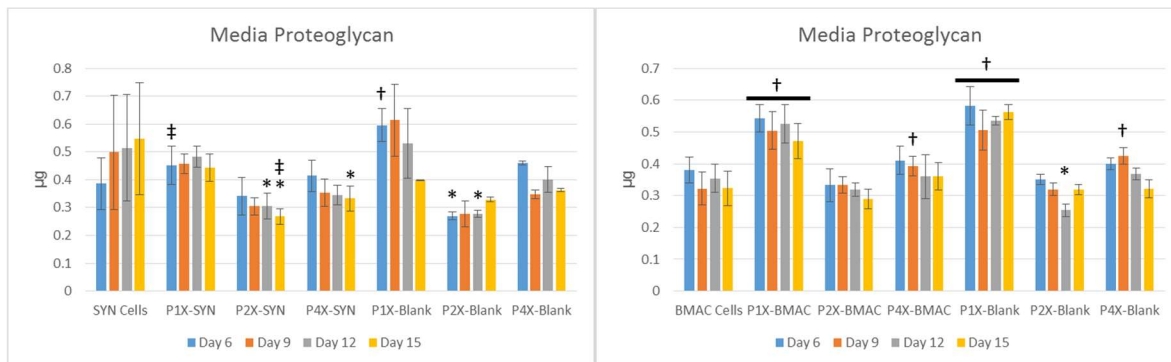


Figure 9. Media proteoglycan concentration on days 6-15 from the bone marrow cell (BMAC) and synovioocyte (SYN) cultures. Hydrogels with and without (blank) bound OPN, and monolayer cell cultures (cells). A * indicates a significantly lower level than monolayer cell culture, a † indicates significantly higher level than monolayer culture, and a ‡ indicates a significantly lower than corresponding blank culture.

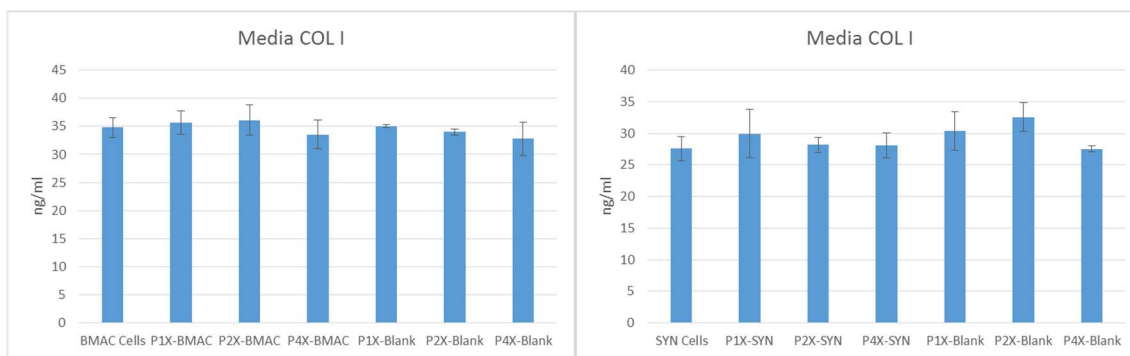


Figure 10: Media collagen I concentration on day 15 in the bone marrow (BMAC) and synoviocyte (SYN) cultures. Hydrogels with and without (blank) bound OPN, and monolayer cell cultures (cells). No significant differences were seen between groups on day 15.

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 led the project team to reevaluate the polyampholyte hydrogel synthesis procedures as discussed in Major Task #1. In addition, subtask 3 in both Major Task #2 and #4 was not evaluated at this time due to the fact that the final biocompatible hydrogel formulation was determined at the end of the project lifetime.

Milestones Achieved: The Statement of Work milestone for Major Task 2 was “Identify which SIBLING protein facilitates maximum cell proliferation and differentiation down a mineralizing lineage for each cell type”. The Statement of Work milestone for Major Task 4 was “Determination of the best polyampholyte hydrogel composition for facilitating maximum cellular proliferation and differentiation down a mineralizing lineage for each cell type”. Through the efforts described above, it was concluded that none of the SIBLING protein conjugated polyampholyte hydrogels facilitated the adhesion of viable cell cultures. While a revised hydrogel formulation was ultimately developed, time and budget constraints did not allow for verification of the performance with the two primary cell lines. This repeated evaluation is recommended as a future stage of this work. No manuscripts resulted from the efforts described in Major Task 2 and 4.

Major Task 3: During the project lifetime, the project team has successfully completed Major Task #3 in the approved Statement of Work. Under Subtask 1, three cross-linker species were evaluated: diethylene glycol dimethacrylate (DEG), triethylene glycol dimethacrylate (TEG,) and tetra-ethylene glycol dimethacrylate (Tetra).

DEG, TEG, and Tetra cross-linker species were synthesized at 26.3:1 (1x), 13.1:1 (2x), and 6.7:1 (4x) monomer to cross-linker ratios using the procedures outlined under Major Task 1 (3 M NaOH). 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 11, indicate that the 4x DEG hydrogels present the greatest compressive mechanical properties, with a value that is almost double that seen previously (~900 kPa vs ~500 kPa). In addition, the DEG samples also presented the highest strain values at failure across all

three cross-linker species.

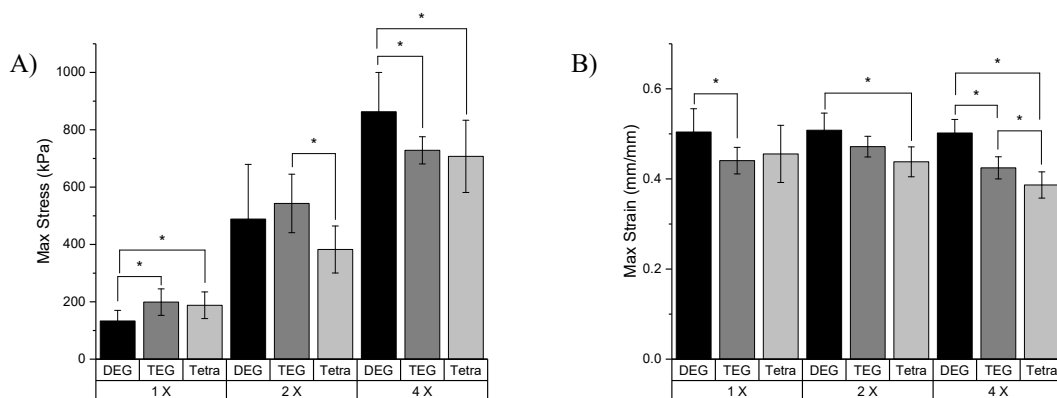


Figure 11: Compressive A) stress and B) strain at failure for TMA:CAA hydrogels with varying cross-linker species and cross-linker densities. A * indicates a statistically significant difference between the two conditions indicated.

Under Subtask 2, three different monomer subunits were mixed across five different ratios (1:1:0, 1:0.75:0.25, 1:0.5:0.5, 1:0.25:0.75, 1:0:1). The specific monomers used were the TMA and CAA monomers discussed previously, with the addition of 3-sulfopropyl methacrylate (SA). These three component hydrogels were formed with TEG cross-linker at the same three ratios of monomer to cross-linker discussed above. As seen in Figure 12, the range of fracture strengths that were achieved ranged from ~50 kPa to 500 kPa and the range of Young's modulus that were achieved was ~20 kPa to 450 kPa.

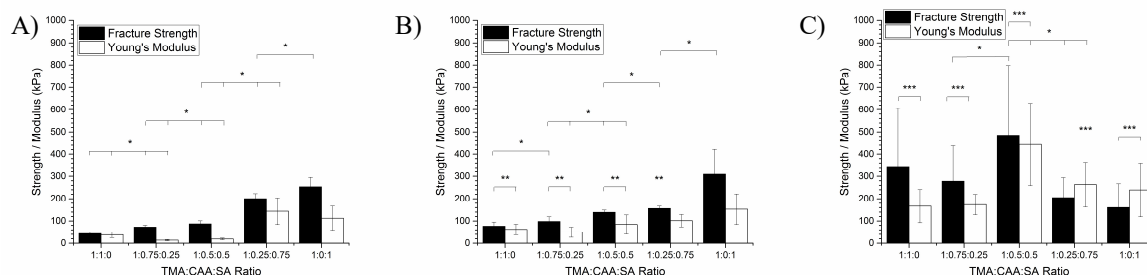


Figure 12: Average $\pm$ standard deviation of the fracture strength and Young's modulus for TMA:CAA:SA hydrogels with different compositions at cross-linker densities of A) 1x, B) 2x, and C) 4x. A * indicates a statistically significant difference between the indicated species, a ** indicates a statistically significant difference between the 1x and 2x cross-linker densities, and a *** indicates a statistically significant difference between the 2x and 4x cross-linker densities.

The cumulative results of Subtasks 1-2 led to the conclusion that the mechanical properties can be easily tuned by varying the monomer composition of the polyampholyte hydrogel, the cross-linker density, the cross-linker species, or all three in concert. However, there is a limit to the tunability as a function of the cross-linker density as there is a saturation concentration of cross-linker that masks the impact of the underlying monomer combinations.

Under Subtask 3, all of the conditions discussed above were tested to demonstrate their resistance to nonspecific protein adsorption and their ability to conjugate targeted proteins. In the work completed, fibrinogen (FBG) and lysozyme (LYZ) or fluorescently labeled albumin were used instead of the SIBLING family of proteins as a proof of concept.

Figure 13, below, provides evidence that all of the samples maintained their nonfouling characteristics confirming the hypothesis that mixed-charge polymer systems have nonfouling properties as long as the charged monomer subunits are homogeneously distributed throughout the hydrogel. In addition, the representative results in Figure 13 also demonstrate that all of the samples are capable of conjugating proteins. This was unexpected for the three component system, as it was anticipated that there would be a composition dependence to the conjugation capabilities due to the chemistry used. The conjugation takes place between a carboxylic acid group on the hydrogel and a primary amine in the protein, and the SA monomer subunit does not have a terminal carboxylic acid group. It was determined that this monomer was undergoing a saponification reaction that results in a terminal carboxylic acid group. Therefore, it is possible to have a wide range of polyampholyte polymer compositions that are both nonfouling and capable of conjugation, depending on the susceptibility of the monomers to undergo saponification.

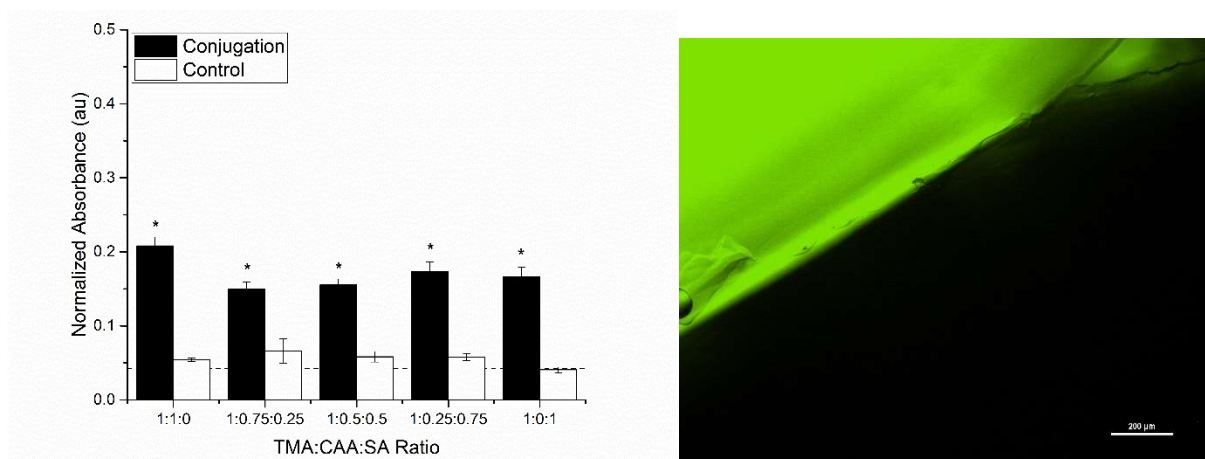


Figure 13: A) Mean $\pm$ propagated error of the anti-fibrinogen response to TMA:CAA:SA hydrogels with (Conjugation) or without (Control) EDC/NHS conjugation procedures. B) Representative fluorescent microscopy image of a DEG cross-linked TMA:CAA hydrogel with (green) or without (black) EDC/NHS conjugation procedures.

Milestones Achieved: The Statement of Work milestone for Major Task 3 was “Develop range of polyampholyte hydrogel platforms for cellular testing”. Polyampholyte hydrogels with compressive fracture strengths ranging from ~50-900 kPa have been synthesized. This range of properties were achieved through three separate approaches, varying the monomer composition, the cross-linker species, and the cross-linker density. Major Task #3 is considered fully accomplished. Additionally, two publications resulted from the work under this task.³⁻⁴

References

1. Schroeder, M. E.; Zurick, K. M.; McGrath, D. E.; Bernards, M. T., Multifunctional Polyampholyte Hydrogels with Fouling Resistance and Protein Conjugation Capacity. *Biomacromolecules* **2013**, *14* (9), 3112-3122.
2. Haag, S. L.; Bernards, M. T., Assessing the Long-Term In Vitro Biocompatibility of Polyampholyte Hydrogels. **In Preparation 2019**.
3. Cao, S.; Barcellona, M. N.; Pfeiffer, F.; Bernards, M. T., Tunable multifunctional tissue engineering scaffolds composed of three-component polyampholyte polymers. *Journal of Applied Polymer Science* **2016**, *133* (40), 10.
4. Mariner, E.; Haag, S. L.; Bernards, M. T., Impacts of Cross-Linker Length on the Physical Properties of Polyampholyte Hydrogels. *Biointerphases* **2019**, *14* (3), 031002.

4. Impact

The primary impact from the efforts expended on this project have been in the advancement of knowledge regarding polyampholyte hydrogels and their application to tissue engineering. The proposed strategies for controlling hydrogel mechanical properties has opened a new avenue for easily tuning biomaterials for their intended application. Equally important is the fact that this was demonstrated for hydrogels that retain both resistance to nonspecific protein adsorption (nonfouling) and the ability to deliver biochemical signals through common bioconjugation techniques. Finally, the revised formulation which demonstrates biocompatibility offers a platform for reevaluating the SIBLING protein influence over the long term.

5. Changes/Problems

The most significant logistical challenge during the project lifetime was the change in home institution by the PI, from the University of Missouri to the University of Idaho, in 2016. This change led to a delay in the project by approximately 1 year as the grant was transferred between Universities (no cost time extension #1).

The most significant technical challenge faced by the team was the lack of biocompatibility of the literature-based polyampholyte hydrogel formulation. This problem was ultimately overcome, but at a point when the project timeline had reached completion, even though a second no cost time extension was granted to address the issue. In addition, because investigations were completed for the two primary cell lines prior to identifying the need for a revised hydrogel formulation, there was no remaining budget to readdress Major Tasks 2 and 4 a second time. It is recommended that these tasks be revisited in a future study.

6. Products

Over the lifetime of this project, the project team has produced three published manuscripts, with one additional manuscript in preparation. The results have also been disseminated through three invited seminars, four oral presentations at conferences or technical meetings, and four poster presentations at conferences or technical meetings. All of these items acknowledged support from this grant. A bibliographic list follows:

Publications

- S.L. Haag and M.T. Bernards. “Assessing the Long-Term In Vitro Biocompatibility of Polyampholyte Hydrogels. In Preparation 2019.
- Mariner, E., Haag, S.L., and M.T. Bernards. “Impacts of Cross-Linker Length on the Physical Properties of Polyampholyte Hydrogels.” *Biointerphases*, 14(3): 031002, 2019.
- Haag, S., and M.T. Bernards. “Polyampholyte Hydrogels in Biomedical Applications.” *Gels*, 3(4): 41, 2017. Invited Review.
- Cao, S., Barcellona, M., Pfeiffer, F., and M.T. Bernards. “Tunable Multi-Functional Tissue Engineering Scaffolds Composed of Three Component Polyampholyte Polymers.” *Journal of Applied Polymer Science*, 133(40): 43985, 2016.

Invited Seminars

- “Addressing Biomedical Challenges with Polyampholyte Polymers”. Idaho IDeA Network of Biomedical Research Excellence (INBRE) State Conference. August 1, 2018.
- “Multifunctional Polyampholyte Polymers as a Novel Biomaterial Platform”. Washington State University, Materials Science and Engineering Graduate Seminar Series, September 12, 2016.
- “Pursuing Polyampholyte Polymers as a Universal Biomaterial Platform”. Brigham Young University Chemical Engineering Department Seminar Series, April 7, 2016.

Conference or Technical Meetings

- S.L. Haag, and M.T. Bernards, “Biocompatibility of Polyampholyte Polymers for Tissue Engineering Applications.” 2019 AIChE Annual Meeting, Poster Presentation, November 2019, Orlando, FL.
- Mariner, E., Haag, S.L., and M.T. Bernards, “Evaluating the Role of Electrostatic Interactions on Macroscale Properties of Polyampholyte Hydrogels.” 4th International Conference on Bioinspired and Zwitterionic Materials, Oral Presentation, June 2019, Kerkrade, Netherlands.
- S.L. Haag and M.T. Bernards, “Biocompatibility of Polyampholyte Polymers for Tissue Engineering Applications.” 4th International Conference on Bioinspired and Zwitterionic Materials, Poster Presentation, June 2019, Kerkrade, Netherlands.
- Bernards, M.T., Haag, S., and E. Mariner, “Multi-Functional Polyampholyte Hydrogels with Covalently Attached SIBLING Proteins for Bone Tissue Engineering”, 2018 Pacific Rim Symposium on Surfaces, Coatings and Interfaces, Oral Presentation, December 2018, Waikoloa, HI.
- E. Mariner and M.T. Bernards, “Characterizing the Physical Properties of Polyampholyte Hydrogels with Different Ethylene Glycol Cross-Linkers”, 2018 AIChE Annual Meeting, Oral Presentation, October 2018, Pittsburgh, PA.
- S. Haag and M.T. Bernards, “Osteoblast Adhesion and Proliferation on Multi-Functional Polyampholyte Hydrogels with Covalently Attached SIBLING Proteins”, 2018 American Institute of Chemical Engineers (AIChE) Annual Meeting, Poster Presentation, October 2018, Pittsburgh, PA. Awarded 2nd Place Poster Award by the Biomaterials Subdivision.
- Bernards, M.T., Qin, C., and S. Haag, “Bone Tissue Engineering Scaffolds based on Polyampholyte Hydrogels with Conjugated SIBLING Proteins”, 2018 Society for Biomaterials (SFB) Annual Meeting, Poster Presentation, April 2018, Atlanta, GA.
- M.T. Bernards, Barcellona, M., and S. Cao, “Tailoring the Mechanical Properties of Multi-

7. Participants & Other Collaborating Organizations

Site #1: University of Idaho, Moscow, ID

Site #2: Texas A&M University, College Station, TX

Site #3: University of Missouri, Columbia, MO

Name:	Dr. Matthew Bernards (Site 1)
Project Role:	PI
Nearest person month worked:	2
Contribution to project:	As PI, Dr. Bernards supervised all project activities and coordinated all work between sites during this project.

Name:	Dr. Ferris Pfeiffer (Site 3)
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 (Site 3)
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.

Name:	Dr. Chunlin Qin (Site 2)
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.

Name:	Dr. Hua Zhang (Site 2)
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.

Name:	Stephanie Haag (Site 1)
Project Role:	Graduate Research Assistant

Nearest person month worked:	18
Contribution to project:	Stephanie 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 all MC3T3-E1 cell adhesion studies.
Name:	Emily Mariner (Site 1)
Project Role:	Graduate Research Assistant
Nearest person month worked:	9
Contribution to project:	Emily completed all efforts to characterize the mechanical properties of TMA:CAA hydrogels with varying ethylene glycol cross-linker species.
Name:	Marcos Barcellona (Site 3)
Project Role:	Undergraduate Research Assistant
Nearest person month worked:	1
Contribution to project:	Marcos completed the synthesis of multiple polyampholyte hydrogels for mechanical testing. Marcos was supported with other funding for his work on this project.
Name:	Siyu Cao (Site 3)
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. Siyu was supported with other funding for her work on this project.
Name:	Nicole Walden (Site 3)
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.

8. Special Reporting Requirements

A final 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 wound healing response for segmental bone defects. Therefore the impact of these proteins will be individually determined using a multi-functional nonfouling polyampholyte polymer scaffold.

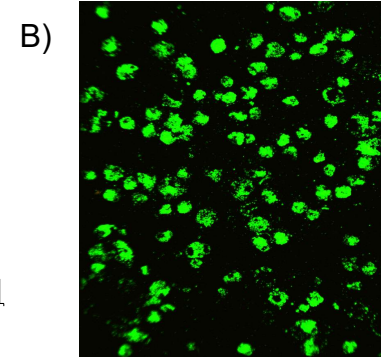
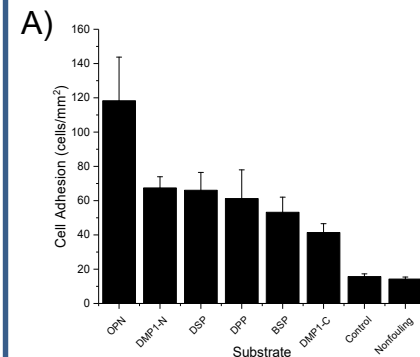


Figure: A) MC3T3-E1 adhesion to SIBLING protein conjugated scaffolds. B) Revised hydrogel formulation demonstrating viable cell populations after five days in culture (green) with limited dead cells (red).

Key Accomplishments: Influence of cross-linker species on the overall physical properties has been characterized. Screening of cell adhesion to SIBLING proteins complete. Biocompatible hydrogel formulation identified.

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/June/2019

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

☒ Track proliferation of cells following adhesion

CY18 Goal – Determine impact of hydrogel chemistry on cells

☒ Characterize differentiation of cells

☐ Characterize cell penetration into hydrogels Characterize differentiation as a function of hydrogel chemistry

☐ Characterize cell penetration as a function of hydrogel chemistry

Budget Expenditure to Date

Projected: \$284,397 Actual Expenditure:\$284,190

9. Appendices

Attached are copies of all of the published manuscripts that have resulted from this work.

Tunable multifunctional tissue engineering scaffolds composed of three-component polyampholyte polymers

Siyu Cao,¹ Marcos N. Barcellona,² Ferris Pfeiffer,^{2,3} Matthew T. Bernards^{1,2}

¹Department of Chemical Engineering, University of Missouri, Columbia, Missouri 65211

²Department of Bioengineering, University of Missouri, Columbia, Missouri 65211

³Department of Orthopaedic Surgery, University of Missouri, Columbia, Missouri 65211

Correspondence to: M. T. Bernards (E-mail: BernardsM@missouri.edu)

ABSTRACT: Resistance to nonspecific protein adsorption and the capability to provide targeted bioactive signals are essential qualities for implantable biomaterials. The development of materials that combine these multifunctional characteristics and tunable mechanical properties has been a target in the tissue engineering field over the last decade. This study is the first to demonstrate that polyampholyte hydrogels prepared with equimolar quantities of positively charged and negatively charged monomer subunits from multiple monomer compositions have great potential to address these needs. The hydrogels were synthesized with positively charged [2-(acryloyloxy)ethyl] trimethylammonium chloride and different monomer ratios of the negatively charged 2-carboxyethyl acrylate and 3-sulfopropyl methacrylate monomers. The physical and chemical properties of the hydrogels were fully characterized, including swelling, hydration, mechanical strength, and chemical composition, and the fouling resistance of the hydrogels was demonstrated using enzyme-linked immunosorbent assays. Additionally, the capability of the hydrogels to facilitate protein conjugation via EDC/NHS conjugation chemistry was assessed. The results clearly demonstrate that the polyampholyte hydrogels have a range of tunable mechanical strength based on the monomer subunits, while maintaining their excellent nonfouling properties. Additionally, high levels of conjugated protein were achieved for all of the monomer combinations investigated. Therefore, the broadly applicable multifunctional properties of polyampholyte hydrogels and their tunable mechanical properties clearly demonstrate the potential of these materials for tissue engineering. © 2016 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* **2016**, 133, 43985.

KEYWORDS: adsorption; biomaterials; properties and characterization; stimuli-sensitive polymers

Received 7 March 2016; accepted 31 May 2016

DOI: 10.1002/app.43985

INTRODUCTION

The use of biomaterials as a scaffold basis for tissue engineering is a widely used approach.^{1,2} However, the definition of an ideal biomaterial scaffold has undergone a dramatic revision over the last decade as the goal of a biomaterial has transitioned from simply being inert to one that is able to induce the integration of the adjacent tissues.^{3–5} Therefore there has been a resurgence of interest in developing novel polymeric materials that can meet the redefined needs of a biocompatible tissue engineering scaffold. These needs include a significant reduction or elimination of the foreign body response, the integration of bioactive signaling molecules to induce native tissue integration, and the ability to easily tune the physical properties like mechanical strength and degradation rate, among others. The most challenging of these features to achieve is the multifunctional ability to resist nonspecific protein adsorption, while retaining the ability to deliver bioactive signaling molecules.

There are a number of functional groups that have been demonstrated to have excellent resistance to nonspecific protein adsorption including polyethylene glycol (PEG),^{2,6} phosphorlycholine (PC),^{7–9} sulfobetaine (SB),^{10,11} carboxybetaine (CB),^{12–14} and two-component polyampholyte polymers.^{15–17} This resistance to nonspecific protein adsorption has been demonstrated in biosensing, drug delivery, and tissue engineering applications.^{18–21} Furthermore, it has been demonstrated that hydrogels formed from these chemistries can significantly reduce the foreign body response to implanted materials, thereby demonstrating that multiple nonfouling polymers can achieve the first feature of an “ideal” biomaterial for tissue engineering.^{7,22}

The drawback of many nonfouling chemistries is the fact that in order to incorporate bioactive signaling molecules, the underlying polymer functional groups must be chemically modified.²³ This leads to a reduction in the nonfouling properties as the

Additional Supporting Information may be found in the online version of this article.

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chemistry is modified, compromising the original advantages of these polymers over others. However, there are two exceptions to this, CB-based polymers and polyampholyte polymers containing carboxylic acid terminated monomers. Both of these functional chemistries have been demonstrated to be capable of covalently attaching bioactive molecules without impacting the surrounding nonfouling chemistry using standard *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) chemistry. This has been demonstrated in both highly sensitive biosensor investigations,^{24–26} as well as hydrogel based systems.^{27–29} Therefore, these two chemistries provide a distinct advantage over other nonfouling chemistries for tissue engineering applications.

The final characteristic of an “ideal” tissue engineering platform is the ability to easily tune the physical properties of the polymer scaffold. The Jiang group has demonstrated multiple approaches for controlling the physical properties of CB-based hydrogels by changing the cross-linker density or the cross-linker molecule,^{12,13,30} but ultimately the physical properties of these systems are defined by the underlying CB monomer. Our group has previously demonstrated that polyampholyte hydrogels composed of different monomer subunits have different physical properties despite similar hydrogel synthesis procedures.^{27,31} It was hypothesized that multicomponent (three or more) polyampholyte polymers represent a promising approach for creating hydrogel scaffolds with tunable physical properties, while retaining their nonfouling characteristics and protein conjugation capacity.

In this investigation this hypothesis was directly tested by synthesizing a series of polyampholyte hydrogels from different combinations of three monomer subunits. Overall neutral polyampholyte hydrogels were prepared from positively charged [2-(acryloyloxy)ethyl] trimethylammonium chloride (TMA) monomers and varying combinations of negatively charged 2-carboxyethyl acrylate (CAA) and 3-sulfopropyl methacrylate (SA) monomers. The structure of these monomers can be seen in Figure 1. The physical properties, nonfouling properties, and conjugation capability of the resulting hydrogels were all characterized and discussed relative to the underlying chemical composition. The results of this study confirmed the hypothesis that the nonfouling properties would be maintained as long as the overall hydrogel was neutrally charged and there were clear trends in the mechanical properties of the hydrogels as a function of the underlying chemistry. Surprisingly, it was also demonstrated that there was no impact on the conjugation capacity of the hydrogels as a function of composition and it is believed that this was a direct result of a saponification reaction of the SA monomer during the hydrogel synthesis process.

EXPERIMENTAL

Materials

Phosphate buffered saline (PBS; 150 mM, pH 7.4), TMA, CAA, SA, triethylene glycol dimethacrylate (TEGDMA), NHS, EDC, *o*-phenylenediamine (OPD), phosphate-citrate buffer, and lysozyme (LYZ) from chicken egg white ($\geq 90\%$) were purchased from Sigma Aldrich (St. Louis, MO). Ethylene glycol, sodium metabisulfite (SMS), urea, and hydrogen peroxide were pur-

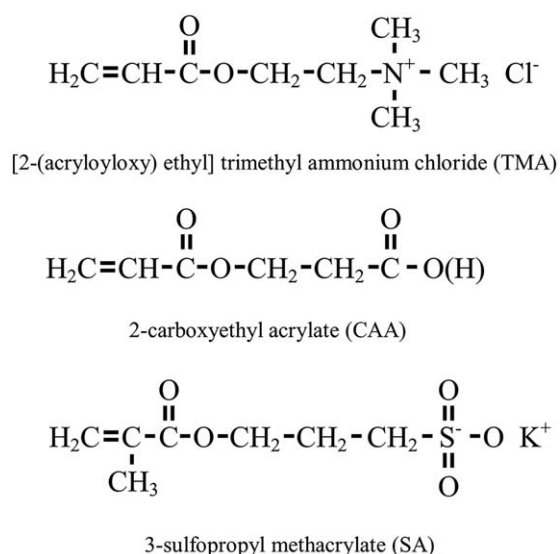


Figure 1. Structures of the three monomers used in this study.

chased from Fisher Scientific Inc. (Pittsburgh, PA). Fibrinogen (FBG) from human plasma (100%) was purchased from CalBiochem (San Diego, CA). Horseradish peroxidase (HRP) conjugated fibrinogen alpha chain antifibrinogen (human plasma) was purchased from GeneTex Inc. (Irvine, CA). HRP-conjugated polyclonal anti-LYZ (chicken egg white) was purchased from United States Biological (Swampscott, MA). *N*-(3-Sulfopropyl)-*N*-methacryloxyethyl-*N,N*-dimethylammonium betaine (SBMA) was purchased from Monomer-Polymer and Dajac Laboratories (Trevose, PA). Ammonium persulfate (APS) and sodium hydroxide were purchased from Arcos Organics (Pittsburgh, PA). Hydrochloric acid, 1.0 normal volumetric solution, was purchased from Mallinckrodt Chemicals (St. Louis, MO).

Hydrogel Synthesis

TMA:CAA:SA hydrogels were synthesized with a series of molar ratios of each monomer using procedures adapted from our previous work.²⁷ Specifically, five ratios of TMA:CAA:SA were investigated as follows: 1:1:0, 1:0.75:0.25, 1:0.5:0.5, 1:0.25:0.75, and 1:0:1. In each hydrogel, 2.0 mmol of total monomer was used, with 1.0 mmol consisting of TMA and the other 1.0 mmol being a combination of CAA and SA based on the ratio under investigation. The solvent, cross-linker conditions, and polymerization initiators were identical to the previous work.²⁷ The polymerization solution was well mixed and then inserted into a hydrogel mold and the reaction took place over 4 h, including 1 h at an elevated temperature of 60 °C. The two ratios with greater SA content relative to CAA content required sonication for 15 min in order to ensure that the solutions were well mixed. Nonfouling control hydrogels composed of SBMA monomers were created with previous procedures.^{27,31}

Swelling and Weight Percentage Hydration

All five mixtures at all three cross-linker densities were prepared as described above and then their swelling and weight percent hydration were characterized. Swelling studies were conducted in solutions of PBS buffer at pH 5, pH 7.4, pH 10, or DI water

and the hydrogel samples were measured daily and the buffer was replaced daily over a period of 6 days. The samples were monitored for a total of 6 days. The average percent swelling was determined by comparing the swollen lateral surface area to the original, due to the fact that the lateral dimensions of the hydrogel are significantly larger than the gel thickness and under the assumption that the gels swell uniformly in all three dimensions. The results are expressed as the equilibrium percent swelling from three independently prepared samples for each cross-linker density ($n = 3$).

In order to quantify the weight percentage hydration all five mixtures at all three cross-linker densities were prepared and then immersed in ultrapure water for a minimum of 48 h. After reaching equilibrium, the gel samples were removed, patted dry, and weighed. Then the samples were fully dehydrated using a desiccator containing calcium sulfate. The change in sample weights from before and after desiccation was used to calculate the average mass percent of water. These experiments were conducted for three independently prepared gels at each cross-linker density ($n = 3$).

Mechanical Characterization

All five mixtures at all three cross-linker densities were synthesized using the procedures described above with the exception that they were formed in a 15-mL centrifuge tube rather than the flat hydrogel mold using $10\times$ more material to compensate for the larger volume. The mechanical testing was completed following a 24 h soak in pH 7.4 PBS buffer using a TAHDi Texture Analyzer (Texture Technologies Corp, Scarsdale, NY) equipped with a 100 kg load cell and testing conditions identical to those used previously to test two-component polyampholyte hydrogels.^{27,31} A minimum of three sections for each of three independently prepared samples were analyzed for each composition and cross-linker density ($n = 9$).

ELISA

To test for nonspecific protein adsorption to TMA:CAA:SA across all of the test ratios, ELISA evaluations were used based on procedures from previous work.^{17,27,31} Briefly, hydrogel disks with a diameter of 5 mm were used for all investigations and the nonspecific adsorption from 1 mg/mL solutions of either FBG or LYZ were tested following 1.5 h of exposure. The adsorbed protein was detected using HRP-conjugated antibodies to the respective proteins at concentrations of 1.25 $\mu\text{g/mL}$ HRP-conjugated anti-FBG in PBS buffer or 10 $\mu\text{g/mL}$ HRP-conjugated anti-LYZ in PBS buffer following 1.5 h of antibody exposure. The relative level of bound antibody, and therefore protein, was determined following exposure to an OPD solution by monitoring the absorbance at 492 nm using a BioTek PowerWave XS2 multiwell plate reader (Winooski, VT) and Gen5 1.07 software (BioTek). The final measured absorbance in each sample well were averaged and normalized to the average tissue culture polystyrene (TCPS) value, which represents a full monolayer of adsorbed protein. Three independent trials with three hydrogel disks each, for both proteins were completed ($n = 9$). Propagation of experimental uncertainty was conducted throughout all of the trials and was used to represent the error in the experimental data.

Protein Conjugation

The ability to covalently attached protein using EDC/NHS was tested for all five ratios at all three cross-linker densities using previously published procedures.²⁷ Briefly, 5 mm hydrogel disks were activated in a solution EDC/NHS for 7 min and then they were transferred to either PBS buffer (control) or 1 mg/mL FBG in PBS buffer (conjugation) for 15 min. Then the samples were deactivated by sequentially soaking them in a PBS buffer with a pH of 9, followed by PBS buffer with a pH of 7.4. The samples were then exposed to 1.25 $\mu\text{L/mL}$ HRP-conjugated anti-FBG for 1.5 h, followed by an OPD solution. As with the ELISA procedures, the relative levels of protein were detected by measuring the absorbance at 492 nm, 30 min after the exposure to OPD. However, due to the extensive color change within the gels, the absorbance was measured in the absence of the gels as a single test point read. As before, the results for each sample were normalized to TCPS. The averages were obtained from three samples for each of three independent trials for all of the hydrogel conditions ($n = 9$). Propagation of experimental uncertainty was conducted throughout all of the trials and was used to represent the error in the experimental data.

Chemical Characterization

In order to characterize the composition of the hydrogel samples, TMA:CAA:SA hydrogels at all five ratios along with a 1:0:1 TMA:CAA:SA hydrogel with no NaOH were prepared with a $4\times$ cross-linker density as described above. Following synthesis, these samples were immersed in DI H_2O for 48 h and then they were fully dehydrated in a vacuum desiccator using calcium sulfate. The chemical composition of the samples were determined using a Kratos Axis 165 photoelectron spectrometer (XPS; Kratos Analytical, Inc.; Chestnut Ridge, NY). Spectra were collected following a 60-s sputter cleaning using a monochromatized Al-K α source with an energy of 1486.6 eV, a takeoff angle of 60° , and a spot size of approximately 700 μm . Data analysis was completed using previously described procedures.^{27,31} A minimum of two data points were analyzed for each hydrogel composition.

Statistical Analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) and results were considered to be statistically significant at a 95% confidence interval ($p < 0.05$). Statistical analysis was conducted using Origin Lab 9.20.00 (OriginLab Corporation, MA).

RESULTS AND DISCUSSION

Physical Properties

Our group has previously characterized polyampholyte hydrogels composed of only two monomer subunits and found that the hydrogels had significantly different physical properties, while maintaining excellent nonfouling characteristics as long as the monomer subunits have an equimolar and homogeneous distribution throughout the hydrogel.^{27,31} Therefore, the focus of this study is to characterize the dependence of the hydrogel properties on the composition in order to demonstrate the tunability of the final material properties through monomer selection. In addition, the most common method for controlling the mechanical properties of a hydrogel is to tune the cross-linker

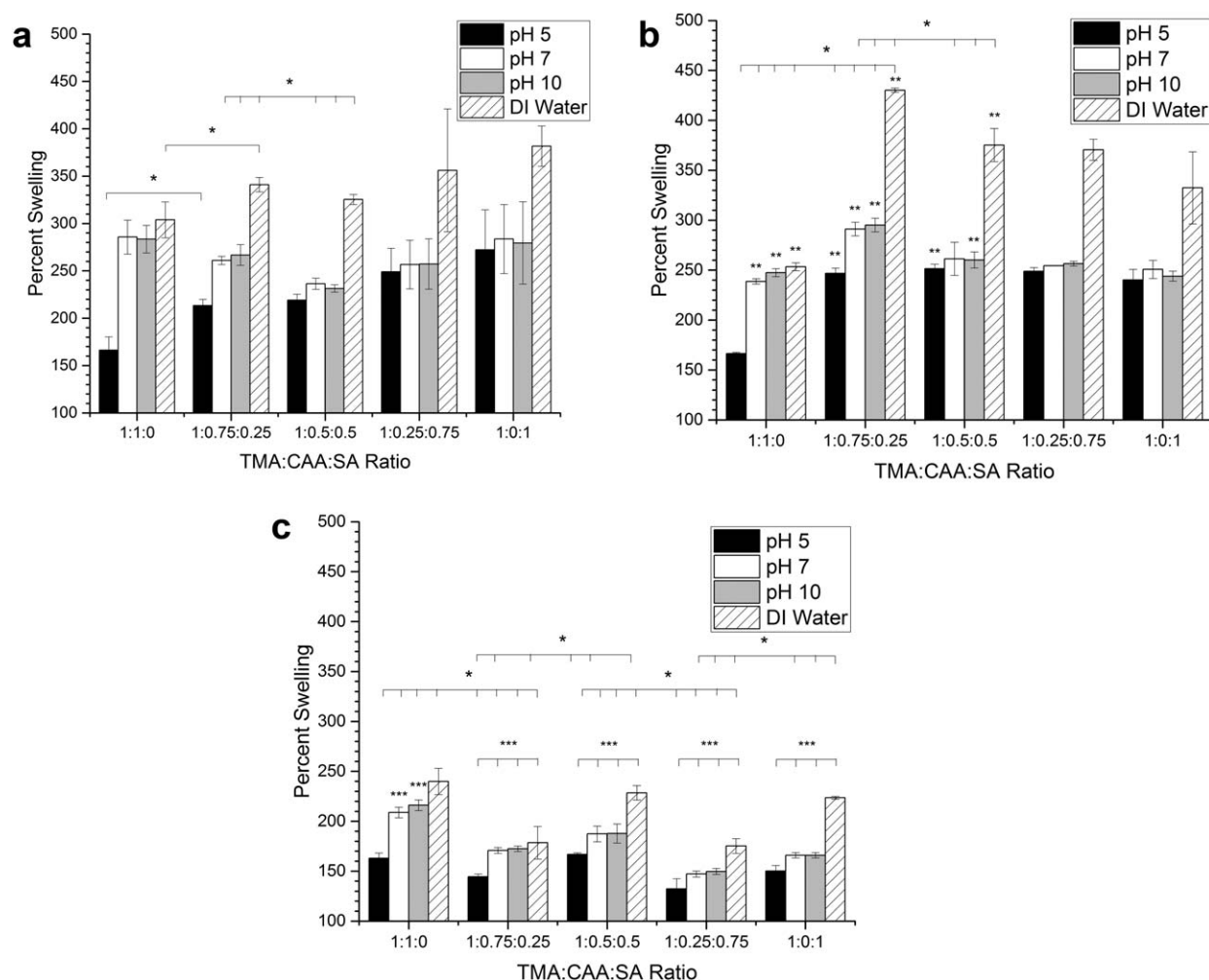


Figure 2. Mean $\pm$ standard deviation of the equilibrium swelling percentage for hydrogels with different TMA:CAA:SA ratios at cross-linker densities of (a) 1 $\times$, (b) 2 $\times$, and (c) 4 $\times$ in different buffers. A * indicates a statistically significant difference between the indicated compositions at each cross-linker density, a ** indicates a statistically significant difference between the 1 $\times$ and 2 $\times$ cross-linker densities for the indicated composition and property, and a *** indicates a statistically significant difference between the 2 $\times$ and 4 $\times$ cross-linker densities for the indicated composition and property, all at a 95% confidence interval ($p < 0.05$).

density, so the composition dependence of the hydrogel properties were also compared across three cross-linker densities. By doing this it is possible to demonstrate the full range of mechanical properties attainable with this approach. The three cross-linker densities represented total monomer to cross-linker ratios of 26.3:1 (1 $\times$), 13.2:1 (2 $\times$), and 6.6:1 (4 $\times$). The initial characterizations were of the physical properties including swelling behavior, weight percent hydration, and mechanical characteristics.

The swelling characteristic of the hydrogels are crucial for our later studies because the surface area of each hydrogel must be identical in order for us to test their protein adsorption levels without normalization due to differences in surface area. The swelling test was performed for all of the TMA:CAA:SA ratios and cross-linker densities to determine the extent of swelling as a function of the composition. This was done across multiple pH values and in DI water and the results are shown in Figure 2. The swelling percentage is defined as the ratio of the swollen

surface area to the original one before being immersed in the test solution. The swelling characterization studies confirmed prior results that the hydrogel samples completely swell within 1 day, with no quantifiable size change thereafter.

As expected, it is readily apparent that the swelling percentage decreases as the cross-linker density is increased. This is especially apparent when looking at the swelling characteristic differences that occur when the cross-linker concentration is increased from 2 $\times$ to 4 $\times$. As seen in Figure 2(c), all but two of the swelling conditions examined are statistically smaller than the results determined with a cross-linker density of 2 $\times$, but this is expected because the higher cross-linker concentration leads to the formation of a more rigid structure. In addition, when one compares the swelling results for the 1 $\times$ and 2 $\times$ cross-linker concentrations, it appears that the presence of CAA has a more significant influence on the swelling behavior of the gels as compared to the SA monomer, as there are statistically significant differences between these two cross-linker concentrations

in most of the test buffers until the SA monomer is enriched in the hydrogel (1:0.25:0.75).

In Figure 2(a), it can be seen that there are relatively few statistically significant changes in the swelling behavior as the composition is modified to increase the SA concentration relative to the CAA concentration for the 1× cross-linker density. It appears that the swelling percentage increases as the amount of SA increases in the pH 5 buffer, however, the only statistically significant change in the pH 5 swelling behavior occurs when the composition is modified from the 1:1:0 to the 1:0.75:0.25 ratio of TMA:CAA:SA. Additionally, the majority of the statistically significant differences in swelling behavior in the other test buffers primarily occur as the composition is changed from the 1:0.75:0.25 TMA:CAA:SA ratio to the 1:0.5:0.5 TMA:CAA:SA ratio.

It is also possible to compare the endpoints of this study to previous work. When the results for the TMA:CAA:SA ratio of 1:0:1 are compared to a previous investigation of [2-(methacryloyloxy)ethyl] trimethylammonium chloride (TM) and SA hydrogels (TM:SA = 1:1), it can be seen that the swelling percentage has been greatly improved in this study.³¹ The improved swelling may be a result of switching the positively charged monomer from TM to TMA. However, TMA has the same structure as TM, only missing one methyl group. It is not likely that one methyl group will drastically change the swelling characteristics, so this was the first indication that there may be changes in the chemistry of the SA monomer, as discussed in more detail later. When we compare the results for the TMA:CAA:SA ratio of 1:1:0 to a previous work with TMA:CAA hydrogels, it can be seen that the swelling percentages are a little lower, but much closer to previous results.²⁷ Furthermore, all of the swelling results demonstrate that these polyampholyte hydrogels have similar swelling characteristics to the closely related family of zwitterionic polymer hydrogels.^{12,13,32}

After characterizing the swelling percentage, we determined the hydration weight percent of the hydrogel at various TMA:CAA:SA ratios. The hydration capability is essential for biocompatibility and the nonfouling properties rely on the formation of a strong hydration layer. In Figure 3, we can easily observe that all of the hydration weight percentage reach or exceed 90%, which suggests that all of the systems are highly hydrated. As expected, the hydration weight percent is statistically lower for all but one of the 4× cross-linker densities and this is likely due to the decreased ability to swell to allow additional hydration. Again, it is relevant to compare the 1:1:0 and 1:0:1 characteristics to previous work. The results for the 1:0:1 are noticeably higher in this study as compared with Dobbins *et al.* while the 1:1:0 results are identical to Schroeder *et al.* This again suggests there are changes in the chemistry of the SA monomer as discussed below.^{27,31}

The final physical characteristics that were evaluated were the mechanical properties of the hydrogels at different compositions and cross-linker densities. The results for the fracture stress and Young's modulus as a function of composition are summarized in Figure 4. In addition, representative compressional stress-strain curves for all five ratios at all three cross-linker densities

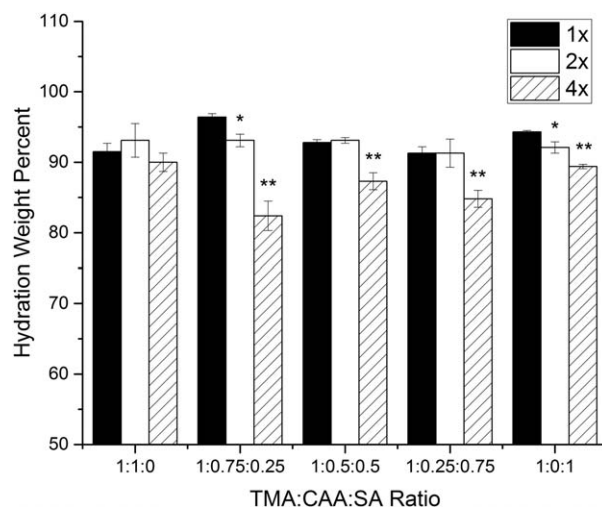


Figure 3. Average $\pm$ standard deviation of the hydration weight percentage for hydrogels with different TMA:CAA:SA ratios. A * indicates a statistically significant difference from only the 1× cross-linker density and a ** indicates a statistically significant difference from both the 1× and 2× cross-linker densities at a 95% confidence interval ($p < 0.05$).

can be found in the Supporting Information in Figure S1(a–c). It can be seen in Figure 4 that there is a steady increase in the compressive strength and Young's modulus as the concentration of the SA monomer increases across both the 1× and 2× cross-linker densities. It is important to note that only the increase in compressive strength is statistically significant for each incremental composition change for both cross-linker densities, while the same cannot be said for the Young's modulus results. These changes in mechanical properties as a function of composition are attributed to the underlying native properties of the monomer subunits. However, it can be seen in Figure 4(c) that this trend does not continue as the cross-linker density is increased further to 4×. As the cross-linker density is increased to this level it appears that the properties of the cross-linker species TEGDMA begins to overshadow the properties of the monomer subunits, thereby leveling out the mechanical properties regardless of the underlying polyampholyte chemistry. Additionally, it can be seen that there are much larger errors associated with the mechanical testing results with a 4× cross-linker density. In an effort to reduce the standard deviation across the 4× cross-linker density results, three times as many samples were tested as compared to the 1× and 2× samples. However, the increased sample size had no noticeable effect on the variability in the results. It is hypothesized that the reaction rate of the polymerization process is so quick that cross-linker inhomogeneity leads to increased variation in the mechanical properties across multiple samples. However, a detailed investigation of the polymerization rate is beyond the scope of this investigation. Finally, it is noted that the mechanical properties achieved with these hydrogels are similar to those found for other nonfouling hydrogel chemistries.^{12,13,30,33}

Nonfouling Characterization

The nonfouling properties of the hydrogels were assessed using positively charged LYZ and negatively charged FBG through ELISA techniques. The tests were performed on hydrogels of all

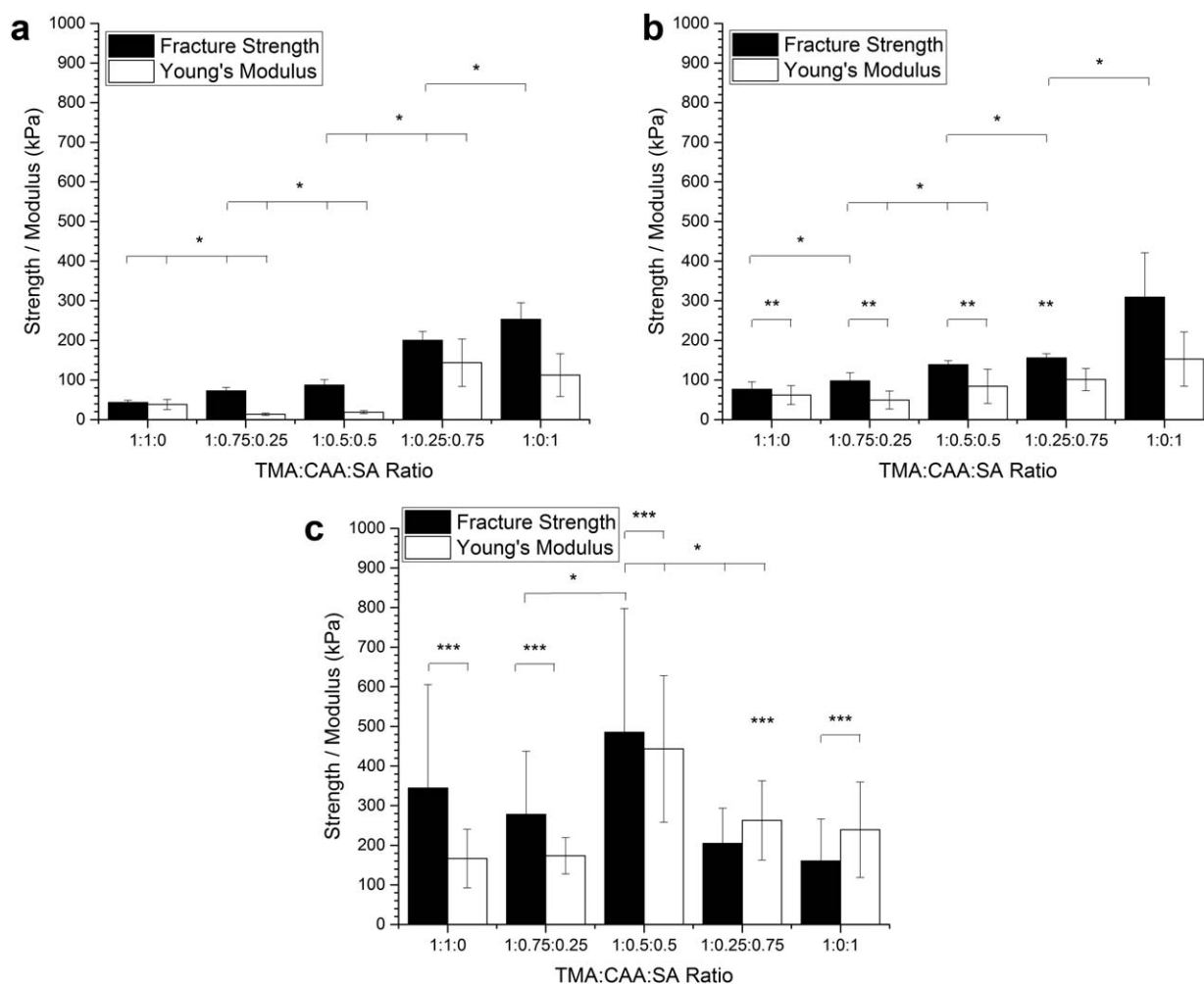


Figure 4. Average $\pm$ standard deviation of the fracture strength and Young's modulus for TMA:CAA:SA hydrogels with different compositions at cross-linker densities of (a) 1 $\times$, (b) 2 $\times$, and (c) 4 $\times$. A * indicates a statistically significant difference between the indicated compositions at each cross-linker density, a ** indicates a statistically significant difference between the 1 $\times$ and 2 $\times$ cross-linker densities for the indicated composition and property, and a *** indicates a statistically significant difference between the 2 $\times$ and 4 $\times$ cross-linker densities for the indicated composition and property, all at a 95% confidence interval ($p < 0.05$).

five different ratios and all three concentrations of cross-linker. It has previously been demonstrated that if a polyampholyte polymer chemistry is enriched with either a positively charged or negatively charged monomer relative to the other, the resulting polymer will strongly adsorb proteins with opposite charge.¹⁵ Therefore, the purpose of these ELISA investigations are to provide indirect evidence that the oppositely charged monomer subunits are homogeneously distributed in equimolar concentrations following the hydrogel synthesis. There is also a wide body of literature reviewed elsewhere,^{18,21} that support the hypothesis that neutrally balanced polyampholyte hydrogels are nonfouling regardless of chemistry. However, there are limited, if any, studies that demonstrate the nonfouling properties of three component polyampholyte polymers prior to this work. Therefore these ELISA investigations will also provide evidence that three component polyampholyte polymers also demonstrate nonfouling properties.

Because ELISA does not yield a specific adsorbed amount, the results were normalized using a fouling control material, TCPS. TCPS is assumed to represent a complete monolayer of adsorbed protein

under the conditions used here. In addition to TCPS, ELISA was also performed on SBMA. SBMA was selected as the negative control because of its well-acknowledged nonfouling properties.^{10,11} If the results of protein adsorption on the TMA:CAA:SA samples are not statistically different from the SBMA control, it suggests the TMA:CAA:SA hydrogel is nonfouling and charge balanced. The results of the ELISA studies are summarized in Figure 5. As can be seen in Figure 5, none of the TMA:CAA:SA ratios at any cross-linker density have nonspecific protein adsorption levels that are significantly different from the SBMA control for both probe proteins. This was expected and it supports the hypothesis that all polyampholyte chemistries that have a homogeneously dispersed, net neutral mixture of charged subunits have nonfouling properties, especially when it is combined with the existing body of literature regarding polyampholyte polymers.^{18,21}

Protein Conjugation

To further investigate the potential to use polyampholyte hydrogels for tissue engineering applications, the ability to covalently attach proteins to the hydrogels was characterized next. In order to activate the TMA:CAA:SA hydrogels, the samples are placed

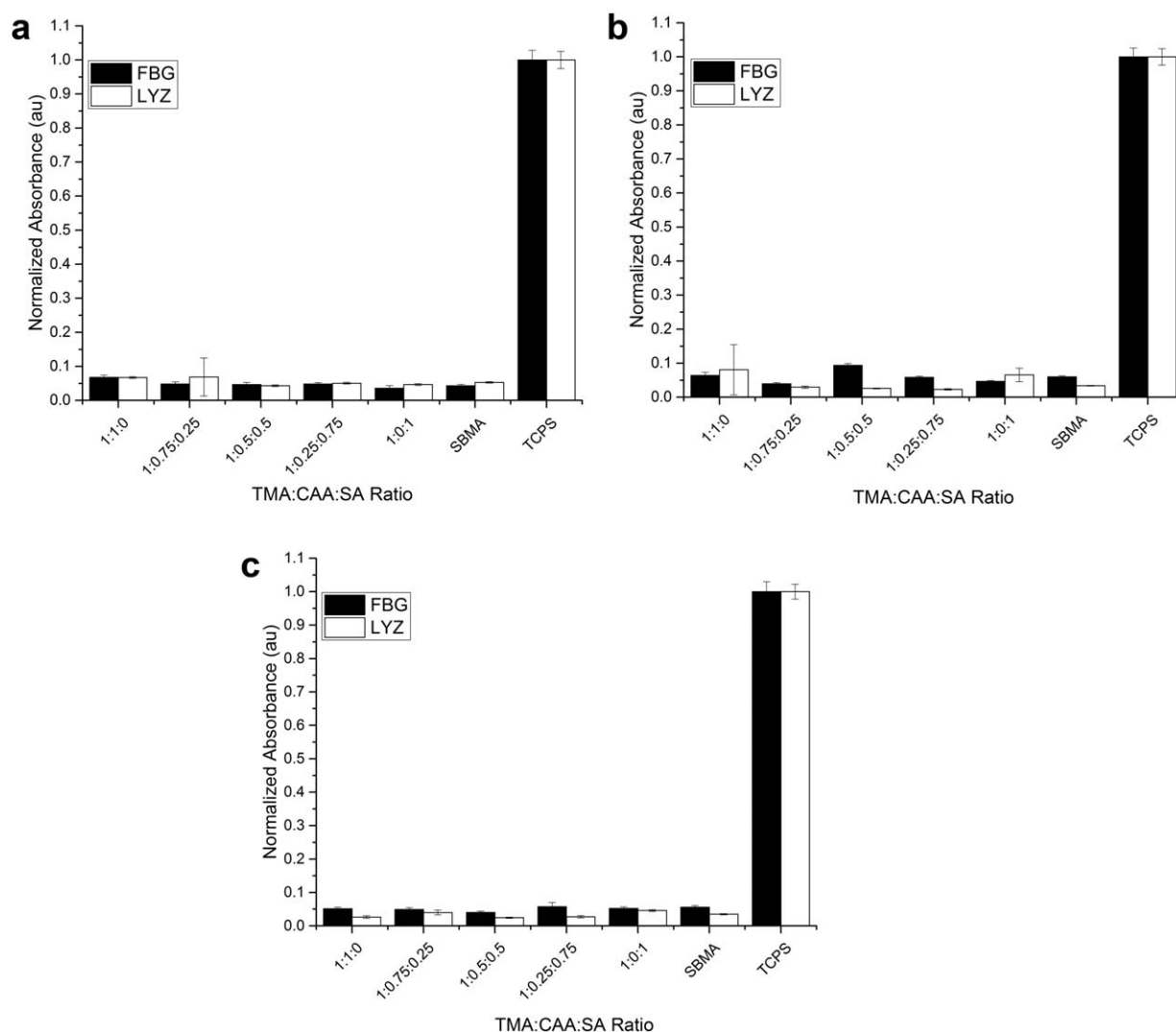


Figure 5. Mean $\pm$ propagated error of the nonspecific protein adsorption to hydrogels with different TMA:CAA:SA ratios at cross-linker densities of (a) 1 $\times$, (b) 2 $\times$, and (c) 4 $\times$.

into an acidic solution of EDC/NHS, during which the negatively charged carboxylic acid groups of CAA become protonated. The advantage of this approach is the fact that unreacted carboxyl group can be returned to their negatively charged form with a slightly basic pH buffer solution rinse, enabling the hydrogel to maintain its nonfouling property.²⁷

The conjugation experiment was conducted for all five ratios at all three cross-linker densities using FBG. The protein conjugation level is then compared to the control group and the nonfouling results discussed above. The control group hydrogels are activated and deactivated, with the exception that PBS buffer is used in place of FBG. The results are summarized in Figure 6, where it can be clearly seen that all of the TMA:CAA:SA hydrogel ratios at all three cross-linker densities have statistically higher protein conjugation levels as compared to the control group and previous SBMA nonfouling control results. This result is somewhat surprising given the fact that the native SA monomer is not capable of undergoing the EDC/NHS conjugation reaction. The fact that the 1:0:1 sample had protein conju-

gation levels that were identical to the 1:1:0 samples at all three cross-linker densities confirmed the earlier results that suggested there is a change in the chemistry of the SA monomer. It was hypothesized that due to the presence of NaOH in the hydrogel synthesis buffer, the SA monomer was undergoing a saponification reaction. This reaction dissociates the ester group, leaving a carboxyl group just like the end group of CAA and a schematic of this reaction shown in Figure 7.

To test this hypothesis, XPS analysis was completed to investigate the underlying chemistry of all of the TMA:CAA:SA hydrogels. Additionally, the 1:0:1 ratio was also evaluated with a modified hydrogel synthesis buffer where the NaOH was replaced with DI water, to eliminate the catalyst for the saponification reaction. Representative broad survey spectra for all five ratios of TMA:CAA:SA and the 1:0:1 TMA:CAA:SA without NaOH along with average atomic compositions for all six samples can be found in the Supporting Information. Due to the similarity in the composition of the monomer subunits, the theoretical changes in atomic composition are not large enough to

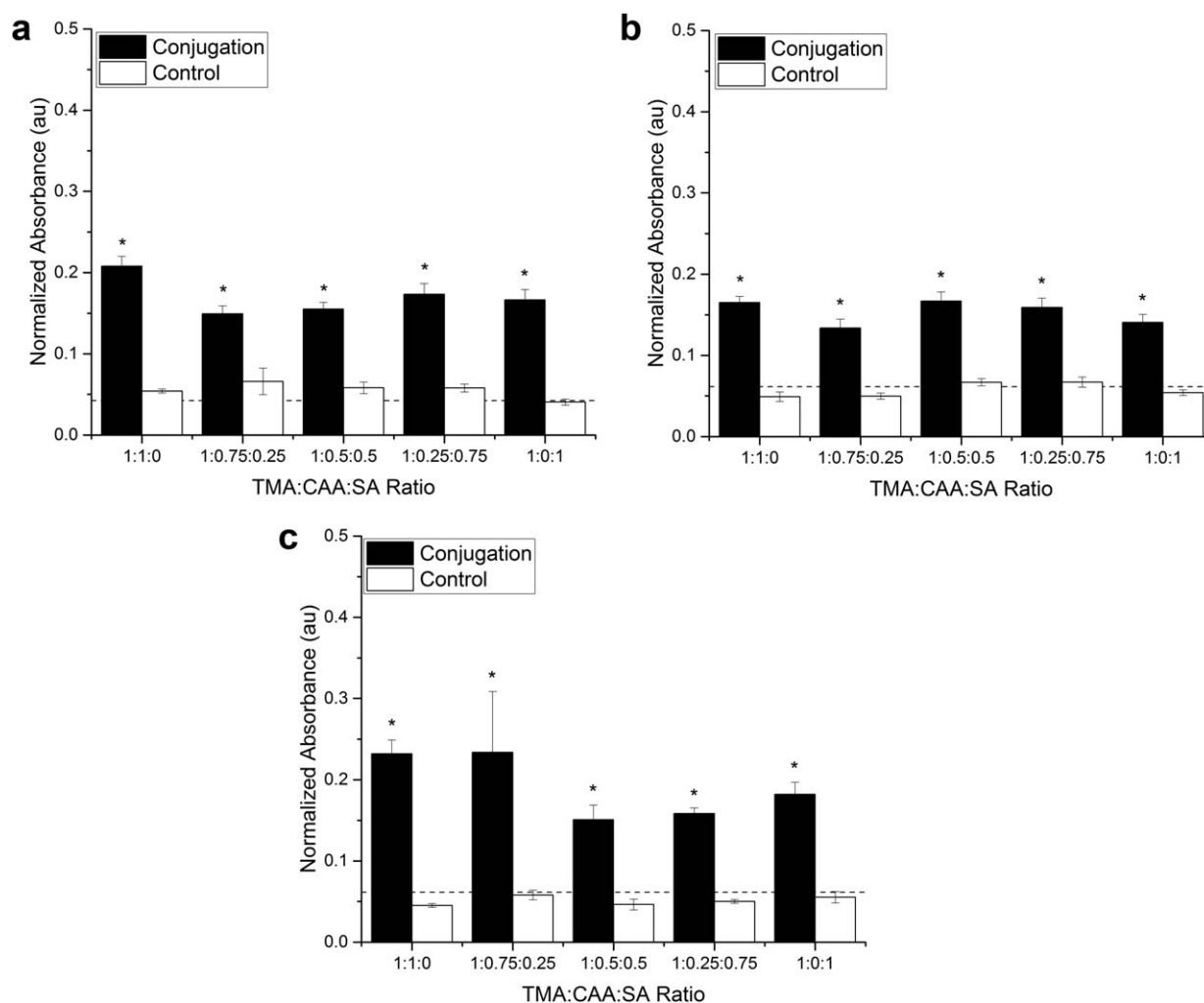


Figure 6. Mean $\pm$ propagated error of the anti-FBG response following EDC/NHS mediated conjugation of FBG to hydrogels with different TMA:CAA:SA ratios and cross-linker densities of (a) 1 $\times$, (b) 2 $\times$, and (c) 4 $\times$. The dashed line in each panel represents the anti-FBG response for the associated SBMA nonfouling control from Figure 5. A * represents a statistically significant difference from both the EDC/NHS control and the nonfouling controls at a 95% confidence interval ($p < 0.05$).

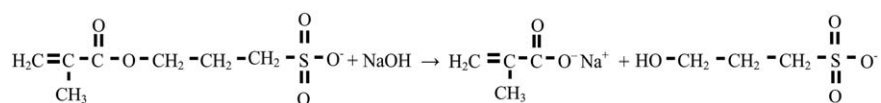


Figure 7. Proposed saponification reaction of the SA monomer.

be distinguishable using XPS combined with curve fitting (atomic composition changes of $<1\%$). However, the theoretical ratio of sulfur to nitrogen should increase from 0 to 1 in 0.25 increments as the CAA monomer is replaced with SA. Table I shows the S/N ratio that was determined for each of the TMA:

CAA:SA hydrogels where it can clearly be seen that there are no clear changes in the S/N ratio as a function of the TMA:CAA:SA ratio. The only significant differences are seen between the 1:1:0 to 1:0.75:0.25 ratios as the SA monomer is introduced to the sample and the 1:0:1 ratio in the presence or absence of NaOH

Table I. Sulfur to Nitrogen Ratios for TMA:CAA:SA Hydrogels

TMA:CAA:SA	1:1:0	1:0.75:0.25	1:0.5:0.5	1:0.25:0.75	1:0:1	1:0:1 No NaOH
Theoretical S/N^a	0.00	0.25	0.50	0.75	1.00	1.00
XPS $S/N \pm$ propagated error	0.00 ± 0.00	0.49 ± 0.12	2.35 ± 0.99	0.90 ± 1.35	1.71 ± 0.33	8.11 ± 1.83

^a Assuming that the saponification reaction does not take place.

in the hydrogel synthesis buffer. When the results for these two synthesis conditions are compared it is clear that there is more sulfur present in the final hydrogel when the NaOH has been removed from the system. It should also be noted that all of the *S/N* ratios are greater than the theoretical ratio due to the presence of APS and SMS initiator that have not been fully removed from the hydrogel during the soaking process. This has been seen previously in related studies.^{18,21}

To better understand the state of the sulfur species that remain in the 1:0:1 TMA:CAA:SA hydrogel following the two synthesis conditions, high resolution XPS spectra were collected for the S 2p peak and representative spectra can be seen in Figure 8. When these two spectra are compared side-by-side it is quite clear that there are differences in the type of sulfur present. In Figure 8(a), the strongest sulfur peak is based around the region for mercaptan (C—S) species, with a smaller peak based in the region for sulfite species (SO₃). These are representative of trace amounts of the unreacted SA monomer, the cleaved sulfur group following saponification, or the polymerization initiator species. In addition, high resolution sulfur spectra for all of the other SA monomer containing ratios had a similar appearance.

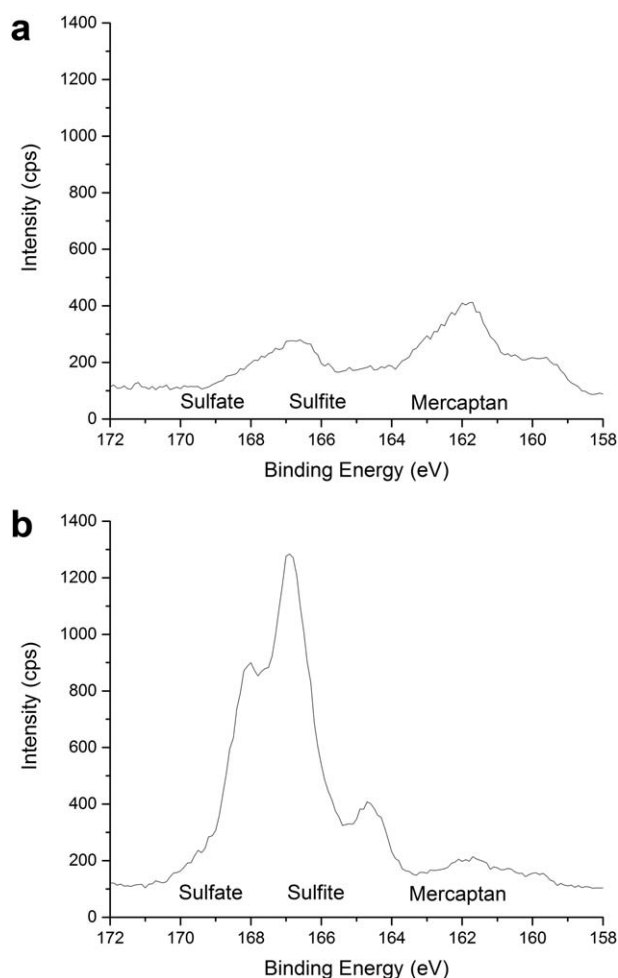


Figure 8. Representative high resolution XPS sulfur spectrum for 1:0:1 TMA:CAA:SA hydrogels with a 4× cross-linker density formed in the (a) presence or (b) absence of NaOH in the hydrogel synthesis solvent.

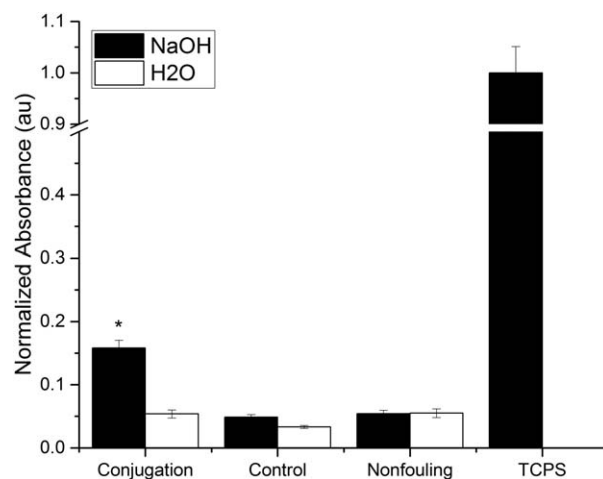


Figure 9. Mean $\pm$ propagated error of the anti-FBG response to 1:0:1 TMA:CAA:SA hydrogels with a 4× cross-linker density formed in the presence or absence of NaOH in the hydrogel synthesis solvent. A * represents a statistically significant difference in the results compared with the sample formed with water and the nonfouling controls at a 95% confidence interval ($p < 0.05$).

In contrast, Figure 8(b) shows a much stronger sulfite associated peak, along with a sulfate (SO₄) associated peak. These two peaks greatly overshadow the peak associated with the mercaptan species, suggesting that the sulfonic acid group of the SA monomer dominates the sulfur related signal. The drastic differences between the XPS sulfur analysis also provide direct evidence that the saponification reaction has occurred over a large percentage of the SA monomer species when NaOH is present in the hydrogel synthesis buffer.

In addition, to further confirm these results, we conducted another set of experiments to compare the protein conjugation capabilities of the 1:0:1 ratio formed with NaOH to one formed with DI water substituted in place of the NaOH and the results are shown in Figure 9. It can be seen in Figure 9 that the hydrogels made in a solvent containing NaOH have a much higher conjugation level compared to those made in DI water containing solvent. This further supports the conclusion that the SA monomer has undergone saponification. This result also suggests that multifunctional polyampholyte hydrogels can be formed from multiple chemistries. This opens up an even wider range of potential monomers that can be used to form multifunctional polyampholyte hydrogels, which should result in a wider range of tunable physical properties based on monomer selection. Our group is actively pursuing the demonstration of this concept.

CONCLUSIONS

In this work polyampholyte hydrogels were formed from mixtures of positively charged TMA and negatively charged CAA and SA monomer subunits. They were fully characterized as a function of cross-linker density and monomer ratio. These hydrogels were demonstrated to have a high swelling capacity and hydration level, and hydrogels with higher SA ratios or higher cross-linker densities were found to have greater

mechanical properties. All of the hydrogels were also shown to have nonfouling properties for both FBG and LYZ. Furthermore, they were demonstrated to have pH-dependent protein conjugation capability, which enables them to be bioactive for cell adhesion. This was true even in hydrogel samples without CAA due to the occurrence of a saponification reaction with the SA monomer. The results of this work demonstrate an effective and unique path for creating mechanically tunable, multifunctional polyampholyte hydrogels simply by modifying the monomers used in the synthesis.

ACKNOWLEDGMENTS

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Supporting Information for:

**Tunable Multi-Functional Tissue Engineering Scaffolds Composed of Three Component
Polyampholyte Polymers**

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Mechanical Characterizations

As discussed in Section 3.1 of the manuscript, the mechanical properties for all five monomer ratios at all three cross-linker densities were characterized by compressing the hydrogels to failure. The results of these studies are summarized in Figures 4 a-c. Figures S.1 a-c show representative stress-strain curves for all of the hydrogels that were tested.

Chemical Composition Characterizations

As discussed in Section 3.3 of the manuscript, the composition of all five monomer ratios at a 4× cross-linker density were characterized using XPS analysis. The primary evaluation of the XPS data was based on analyzing the sulfur to nitrogen ratio and high resolution sulfur spectra as discussed in the manuscript. Figures S.2 a-f show representative broad survey spectra for all of the hydrogels that were analyzed. The average composition for each of the hydrogel samples following analysis of these spectra are summarized in Table S.1.

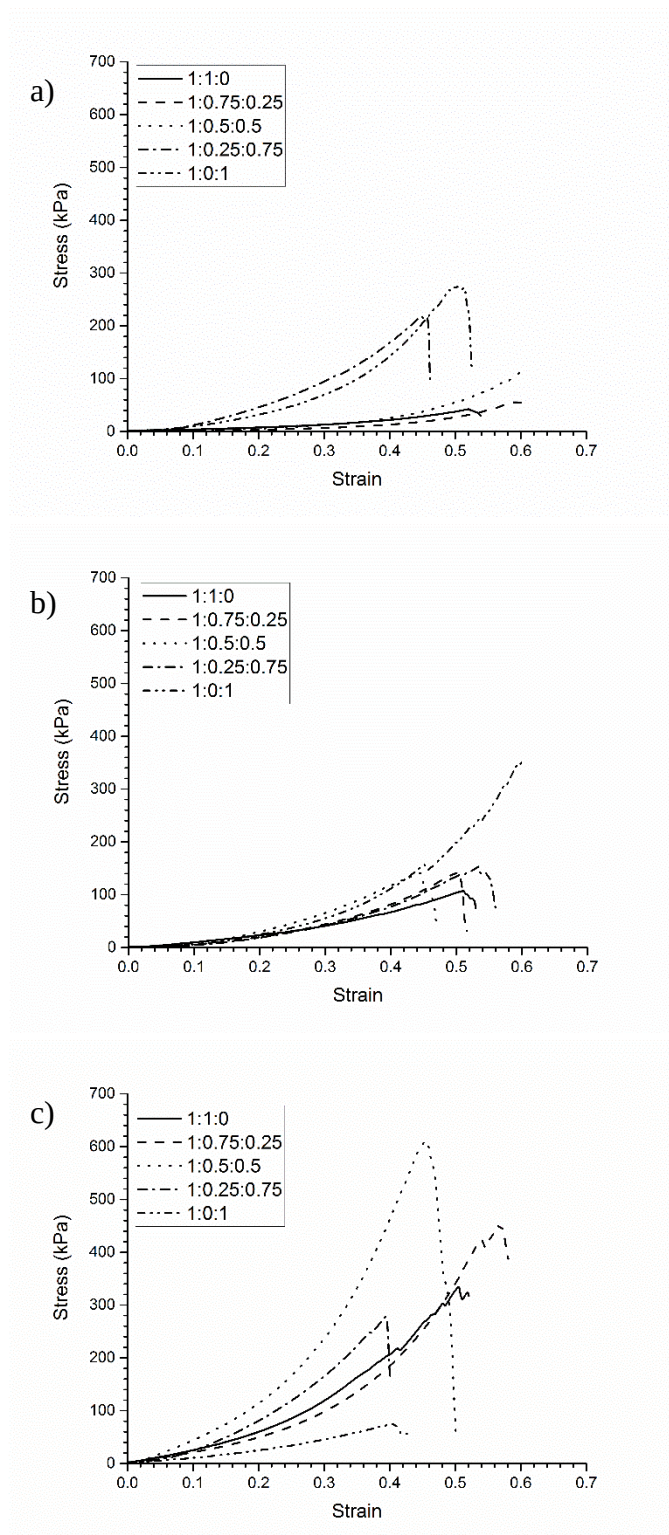


Figure S.1: Representative compressional stress-strain curves for hydrogels with different TMA:CAA:SA ratios at cross-linker densities of a) 1x, b) 2x, and c) 4x.

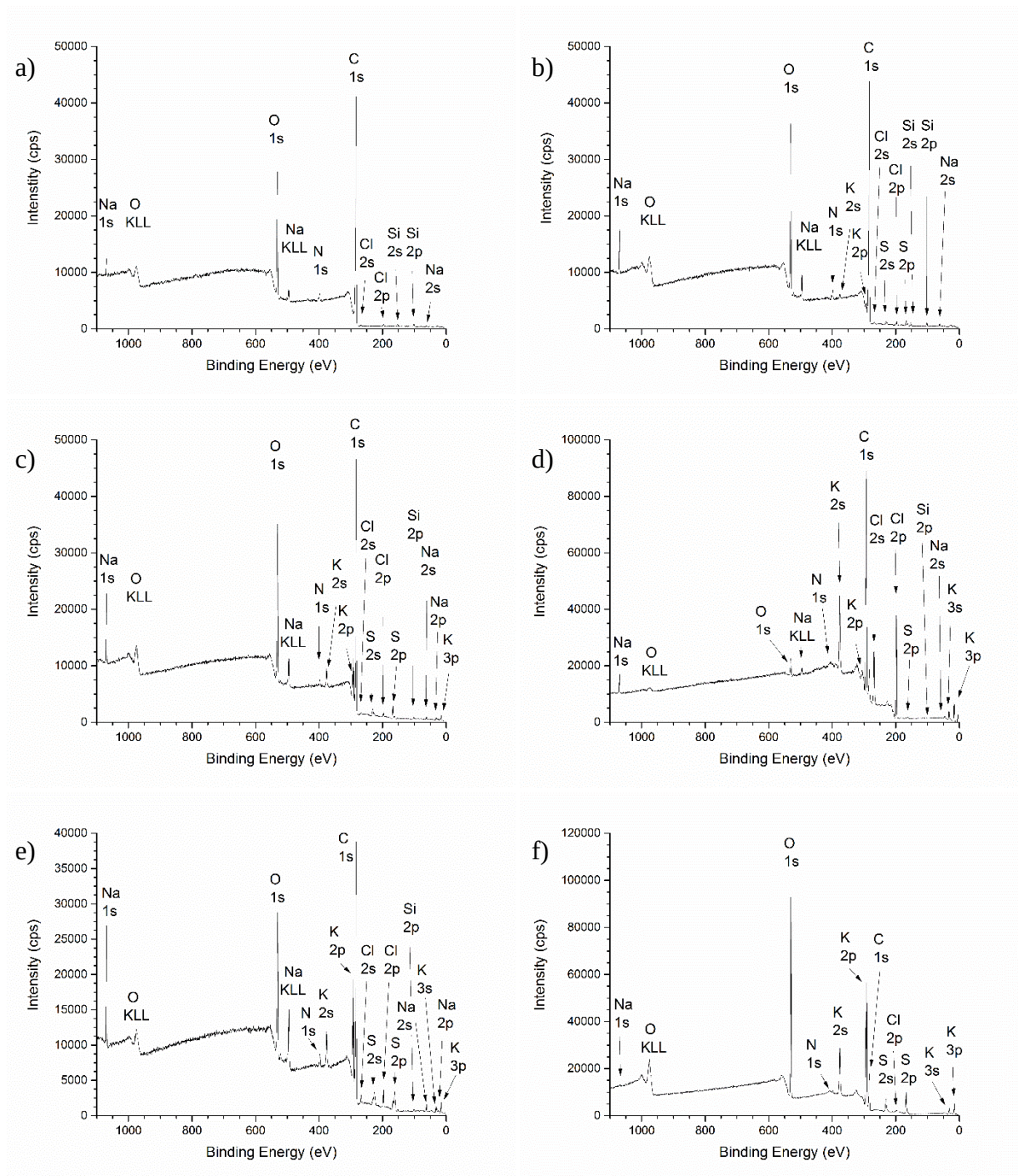


Figure S.2: Representative broad survey XPS spectra for TMA:CAA:SA hydrogels with ratios of a) 1:1:0, b) 1:0.75:0.25, c) 1:0.5:0.5, d) 1:0.25:0.75, and e-f) 1:0:1 formed in the presence (e) or absence (f) of NaOH in the hydrogel synthesis buffer.

Table S.1: Average $\pm$ Standard Deviation of the Atomic Composition for TMA:CAA:SA

Hydrogels as a Function of Composition

	1:1:0	1:0.75:0.2 5	1:0.5:0.5	1:0.25:0.7 5	1:0:1	1:0:1 No NaOH
C	83.38 $\pm$ 4.36	75.35 $\pm$ 3.10	65.97 $\pm$ 6.96	40.50 $\pm$ 25.13	68.37 $\pm$ 3.68	25.94 $\pm$ 4.44
O	13.21 $\pm$ 4.22	16.59 $\pm$ 4.80	20.58 $\pm$ 2.21	7.68 $\pm$ 2.72	15.38 $\pm$ 1.38	42.60 $\pm$ 4.42
N	1.61 $\pm$ 0.17	2.48 $\pm$ 0.42	1.44 $\pm$ 0.31	0.97 $\pm$ 1.37	2.26 $\pm$ 0.31	1.01 $\pm$ 0.23
S	0.00 $\pm$ 0.00	1.23 $\pm$ 0.22	3.38 $\pm$ 1.22	1.73 $\pm$ 0.85	3.88 $\pm$ 0.53	8.19 $\pm$ 0.23
Na	0.81 $\pm$ 0.03	1.98 $\pm$ 0.09	3.41 $\pm$ 0.76	1.92 $\pm$ 0.06	3.14 $\pm$ 0.82	0.16 $\pm$ 0.01
K	0.00 $\pm$ 0.00	0.61 $\pm$ 0.32	4.27 $\pm$ 1.65	27.08 $\pm$ 17.52	5.14 $\pm$ 0.59	21.66 $\pm$ 0.15
Cl	0.26 $\pm$ 0.04	0.68 $\pm$ 0.50	0.61 $\pm$ 0.30	19.78 $\pm$ 12.62	1.48 $\pm$ 0.04	0.45 $\pm$ 0.13
Si	0.75 $\pm$ 0.04	1.11 $\pm$ 0.16	0.36 $\pm$ 0.51	0.36 $\pm$ 0.14	0.37 $\pm$ 0.06	0.00 $\pm$ 0.00

Polyampholyte Hydrogels in Biomedical Applications

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Abstract: Polyampholytes are a class of polymers made up of positively and negatively charged monomer subunits. Polyampholytes offer a unique tunable set of properties driven by the interactions between the charged monomer subunits. Some tunable properties of polyampholytes include mechanical properties, nonfouling characteristics, swelling due to changes in pH or salt concentration, and drug delivery capability. These characteristics lend themselves to multiple biomedical applications, and this review paper will summarize applications of polyampholyte polymers demonstrated over the last five years in tissue engineering, cryopreservation and drug delivery.

Keywords: polyampholyte hydrogels; nonfouling; multi-functional

1. Introduction

A significant amount of research is being done with polyampholyte polymers in the biomedical community. Polyampholytes are polymeric systems comprised of both positively and negatively charged monomer subunits. Through the selection of monomers, one can build a polyampholyte with desired properties, tuned to specific biomedical applications. Our previous work evaluated much of the relevant literature prior to 2013 [1,2], so this paper is focused on advances over the past five years. We will first give a brief review of general polyampholyte characteristics with references to more thorough summaries, a discussion of the tunability of these systems, and an evaluation of recent findings using polyampholytes in tissue engineering, cryopreservation applications, and drug delivery.

2. General Polyampholyte Characteristics

A detailed explanation of the synthesis and properties of polyampholytes is beyond the scope of this paper and will not be provided here [3–6]. However, we will give a brief overview of the general characteristics that make polyampholytes attractive for biomedical applications. As mentioned above, polyampholytes contain both anionic and cationic functional groups. The strengths of these functional groups are often divided into four categories, that include both weak anionic and cationic groups, weak anionic and strong cationic groups, strong anionic and weak cationic groups, and lastly, both strong anionic and cationic groups. Table 1 shows the most commonly used monomers based on a survey of the recent literature. It should be noted that Table 1 is focused on summarizing synthetic organic monomer subunits. There is also a range of literature focused on naturally occurring materials that have been modified to include charged functional groups, like chitosan [7,8].

Table 1. Common Monomers Used in Polyampholyte Hydrogels.

Chemical Name	Acronym	Monomer Formula	Strength of Functional Group
Acrylamide	AM	$\text{CH}_2=\text{CHCONH}_2$	Weak cation
N-[3-(Dimethylamino)propyl] acrylamide	DMAAA	$\text{CH}_2=\text{CHCONH}(\text{CH}_2)_3\text{N}(\text{CH}_3)_2$	Weak cation
2-(Dimethylamino)ethyl methacrylate	DMAEM	$\text{CH}_2=\text{C}(\text{CH}_3)\text{COOCH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$	Weak cation
2-(Diethylamino)ethyl methacrylate	DEAEM	$\text{CH}_2=\text{C}(\text{CH}_3)\text{CO}_2\text{CH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2$	Weak cation
[2-(Methacryloyloxy)ethyl] trimethylammonium chloride	TM	$\text{CH}_2=\text{C}(\text{CH}_3)\text{CO}_2\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3\text{Cl}$	Strong cation
2-(Acryloyloxy ethyl)trimethyl ammonium chloride	TMA	$\text{CH}_2=\text{CHCO}_2\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3\text{Cl}$	Strong cation
[3-(Methacryloylamino)propyl] trimethylammonium chloride	MAPTAC	$\text{CH}_2=\text{C}(\text{CH}_3)\text{CONH}(\text{CH}_2)_3\text{N}(\text{CH}_3)_3\text{Cl}$	Strong cation
2-Carboxyethyl acrylate	CAA	$\text{CH}_2=\text{CHCO}_2(\text{CH}_2)_2\text{CO}_2\text{H}$	Weak anion
Methacrylic acid	MAA	$\text{CH}_2=\text{C}(\text{CH}_3)\text{COOH}$	Weak anion
Acrylic acid	AA	$\text{CH}_2=\text{CHCOOH}$	Weak anion
Carboxylated poly-L-lysine	COOH-PLL	$\text{NH}_2(\text{CH}_2)_4\text{CHNH}_2\text{COOH}$	Weak anion
3-Sulfopropyl methacrylate potassium salt	SA	$\text{H}_2\text{C}=\text{C}(\text{CH}_3)\text{CO}_2(\text{CH}_2)_3\text{SO}_3\text{K}$	Strong anion
2-Sulfoethyl methacrylate	SE	$\text{H}_2\text{C}=\text{C}(\text{CH}_3)\text{CO}_2(\text{CH}_2)_2\text{SO}_3\text{H}$	Strong anion

Based on the selection of the underlying functional groups, polyampholytes have a tunable isoelectric point (IEP). The IEP occurs at the pH level when a polyampholyte is overall neutrally charged. The IEP is also the state at which a polyampholyte will have the most compact conformation, due to electrostatic attractions between the balanced, oppositely charged functional groups. As pH increases or decreases from the IEP, the overall charge of the polyampholyte will move further from neutral, causing electrostatic repulsive forces between like-charged regions, to increase and expand the polyampholyte. Similarly, when salt ions are present, the ions disrupt the electrostatic interactions between oppositely charged regions of the subunits. This also causes the polyampholyte to swell, as depicted schematically in Figure 1 [1]. The extent of swelling from pH or salt is ultimately dependent on the composition and architecture of the polymer [1,6]. However, manipulation of these unique electrostatic interactions and system responses has spurred investigation into using these materials in biomedical applications, as detailed throughout the rest of this review.

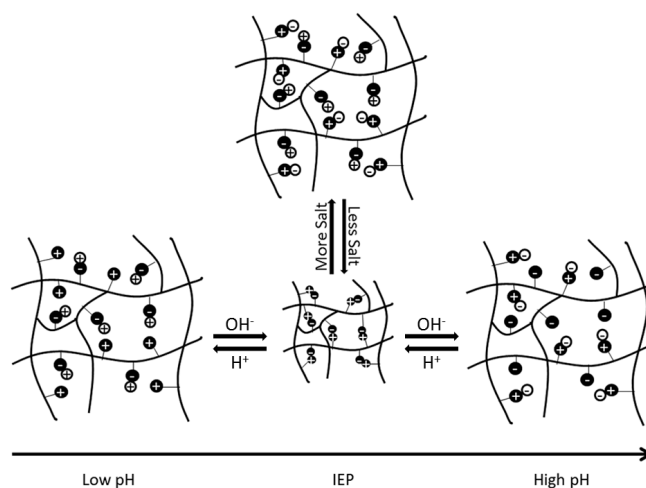


Figure 1. Schematic representation of the impact that changes in pH and salt concentrations have on electrostatic interactions within a polyampholyte hydrogel. This figure is reprinted from Ref. [1] with permission. Copyright 2013, Wiley Periodicals, Inc.

Another important general feature of overall charge neutral polyampholyte polymers is their natural nonfouling properties. It has been widely demonstrated [9–12] and reviewed previously [1,2] that this native resistance to nonspecific protein adsorption is the result of the formation of a strong hydration layer due to interactions between the naturally occurring dipole distribution in water and the charged regions of the underlying polyampholyte substrate. This is important because it is

believed that this nonfouling property will lead to a reduced foreign body response in the *in vivo* environment, as seen with related zwitterionic systems. For example, in a previously reviewed paper, it was shown that polymer brushes composed of equimolar mixtures of 2-(acryl-oyloxy)ethyl trimethylammonium chloride (TMA) and 2-carboxyethyl acrylate (CAA) had only 4.3 ± 1.7 ng/cm² of nonspecific protein adsorption from 100% fetal bovine serum [13]. Furthermore, as demonstrated throughout the remainder of this review, pH changes can be used to modify the net neutral charge of polyampholyte systems, adding in a responsive component to the utilization of these polymers in biomedical applications.

3. Mechanical Properties

The composition dependent tunability of polyampholyte systems also provides a unique approach for easily controlling the mechanical properties of the biomaterial. To facilitate better tissue regeneration and integration, it is important for an implanted biomaterial to mimic the native properties of the tissues it is supplanting [14,15]. There is, of course, great variability in the mechanical properties of tissues, as properties range from soft and flexible (skin) to strong, with the ability to absorb impact forces (bone). In addition, biomaterials must also have a high water content, to maintain their biocompatibility, and the ability for cells to penetrate into the material.

Our group demonstrated the easy tunability of polyampholyte hydrogels utilizing various ratios of monomers in three component hydrogels consisting of positively charged 2-(Acryl-oyloxy)ethyl trimethylammonium chloride (TMA), and varying mixtures of negatively charged 2-carboxyethyl acrylate (CAA) and 3-sulfopropyl methacrylate (SA) monomers [16]. Furthermore, the crosslinker density was also used as a mechanism for further tuning the mechanical properties. It was demonstrated that both the density of the crosslinker, as well as the ratio of monomers in the hydrogel, altered the fracture strength and Young's Modulus. At crosslinker densities of $1\times$ and $2\times$ (1:0.076 and 1:0.152 monomer/crosslinker ratios), the mechanical properties were dependent upon the exact combination of monomer subunits, while at a $4\times$ crosslinker density, the crosslinker became the controlling factor. However, this study clearly demonstrated the easily tuned mechanical properties of polyampholyte systems with low crosslinker densities. In a similar fashion, Jian and Matsumura were able to controllably tune the mechanical properties of their nanocomposite hydrogel designed with carboxylated poly-L-lysine (COOH-PLL), and synthetic clay laponite XLG, by changing the laponite concentration (composition dependence) or the density of the polyethylene glycol with *N*-hydroxy succinimide ester (PEG-NHS) crosslinker [17]. Changing the crosslinker density or monomer concentration are also common tuning mechanisms for mechanical properties [18–22].

A great deal of both theoretical and experimental study has been conducted to better understand the fracture mechanisms of polyampholyte gels, for use in guiding the design of stronger or more tunable systems [23]. Above a critical loading stress, moderately chemically crosslinked hydrogels resisted creep flow, while physically crosslinked and lightly chemically crosslinked hydrogels experience creep rupture. However, at large stresses, creep behavior indicated that both physically and chemically crosslinked hydrogels undergo bond breaking mechanisms. These results confirm that chemical bonds are stronger than physical bonds, therefore, chemically crosslinked systems show an improvement over systems with only ionic bonds [24]. However, the incorporation of physical crosslinks has positively influenced fracture behavior of viscoelastic hydrogels through reduced deformation rate [25] and crack blunting [26].

Due to the beneficial features of both chemical and physical crosslinks, recent studies have approached the development of mechanically strong hydrogels by combining the two mechanisms in an approach referred to as the sacrificial bond principle [14,20,27–32]. The sacrificial bond principle is based on the formation of a highly stretchable base matrix, with a high density of brittle sacrificial bonds that are weaker than the base matrix. During stress, the brittle bonds break before the stretchable base matrix, leading to improved mechanical performance. Figure 2 shows a schematic of possible fracture processes with and without sacrificial bonds present [27].

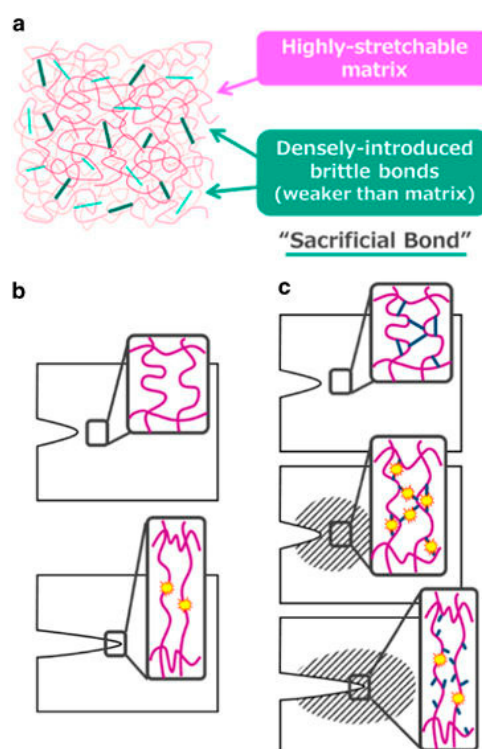


Figure 2. (a) General structure of a tough gel based on the sacrificial bond principle consisting of a highly stretchable matrix with a high density of brittle bonds; (b) Possible fracture processes of a single network gel; (c) Possible fracture processes of a sacrificial bond gel. The brittle bonds are widely ruptured prior to the macroscopic crack propagation around the crack tip (shadowed zone). This figure is reprinted from Ref. [27] with permission. Copyright 2017, the Society of Polymer Science, Japan.

These sacrificial bonds can be covalent bonds, hydrogen bonds, ionic bonds, or hydrophobic interactions, depending on the polymer matrix. They can also be incorporated into the base matrix with multiple approaches, including double network gels, ionically linked gels, metal ion chelation, and composite gels [27,33]. The resulting hydrogels from all of these approaches show great mechanical strength, energy dissipation, and force dispersion, to slow down fracture and crack propagation [20]. In just one representative example, the use of a double network hydrogel composed of poly(2-acrylamido-2-methylpropanesulfonic acid) and poly(acrylamide) was shown to improve the compressive fracture stress from 0.4–0.8 to 17.2 MPa [34].

With polyampholyte double network hydrogels showing irreversible deformation, however, efforts started on the use of other types of bonds that could be reversible and self-healing. Some work has been done using electrostatic interactions and hydrophobic interactions. The mechanical properties are extremely dependent on pH, as the interactions that hold the structure together can be weak or strong, depending on the charged state of the monomers. Furthermore, the material will also swell and collapse with changes in pH [28,29]. One study added partially quaternized poly(4-vinylpyridine) into an elastic hydrogel, thereby introducing electrostatic, hydrophobic, and hydrogen bonding interactions to better dissipate energy. This resulted in an increase in fracture energy from 44 to 1000 J/m² [30].

In polyampholytes, it is common to take advantage of the electrostatic interactions as a secondary sacrificial bond, to toughen materials via the presence of oppositely charged functional groups distributed throughout the system. Strong electrostatic interactions act as permanent crosslinks, and weaker interactions reversibly break and reform, which dissipates energy and toughens the gels [14,32]. These bonds can also occur via both inter- and intra-chain interactions. Polyampholytes and polyion complex hydrogels (PIC) both contain oppositely charged functional groups and have potential as tough, self-healing gels. PICs are formed from electrostatic interactions between oppositely

charged polyelectrolyte polymers upon mixing. Polyampholytes form the toughest hydrogels around zero net charge, where PIC systems can form tough gels at weakly off-balanced charge compositions. PICs are typically tougher than polyampholytes when they have the same monomer compositions, due to the fact that PIC hydrogels form at lower concentrations than polyampholytes [31].

Additional approaches have also been used to improve the mechanical properties of hydrogels based on ionic bonding. In one example, the removal of co-ions prior to gelation was shown to facilitate improved ionic bond formation [35]. In another study, Cui et al. developed a method referred to as pre-stretching, where hydrogels are prepared and then stretched. This stretching helps align the chains parallel to each other, as opposed to the original random alignment. When the chains are parallel, stronger ionic bonds form, which in turn strengthens the overall polyampholyte hydrogel [36]. Fang et al. explored a similar approach to attain a tough and stretchable hydrogel by altering the structure of the material [37]. Starting with a protein-based hydrogel, they forced the unfolding of the globular domains. The subsequent collapse and aggregation of the unfolded material allows for physical intertwining and linking through electrostatic interactions. The resulting hydrogels have the unusual properties of a negative swelling ratio, high stretchability, and toughness.

Byette et al. took inspiration from the mechanisms used by mussels to attach to wet surfaces as an approach to toughen polyampholyte materials [38]. Mussels use byssus, a protein-based material, to secure themselves to solid surfaces. Byssus shows a self-healing ability combined with strength, partially due to metal ions forming sacrificial bonds with the amino acid subunits. Byette et al. created a hydrogel from byssus protein hydrolysate, and treated it with Ca^{2+} or Fe^{3+} . The films with Fe^{3+} showed the greatest increase in strength and toughness. A similar approach was used by Huang et al., who made a semi-interpenetrating polymer network composed of carboxymethyl chitosan (CMCH), acrylamide, and maleic acid with carboxylic- Fe^{3+} interactions serving as ionic sacrificial bonds [39]. By changing the ratio of maleic acid and the concentration of Fe^{3+} , the best hydrogels showed a tensile stress of 1.44 MPa. Additionally, the CMCH provided the gels with antibacterial characteristics against *Staphylococcus aureus* and Gram negative *Escherichia coli*.

4. Tissue Engineering Applications

Polyampholyte hydrogels are an attractive option for tissue engineering, due to the general characteristics described above. In addition to their tunable, responsive, and nonfouling properties, they also have a high moisture holding capacity, which is generally associated with biocompatibility. Our group has demonstrated multifunctional polyampholyte hydrogels for tissue engineering using TMA and CAA monomer subunits [40,41]. These gels show excellent resistance to nonspecific protein adsorption, including negatively charged fibrinogen (FBG) and positively charged lysozyme (LYZ), and they prevent the short-term adhesion of MC3T3-E1 cells [41]. The elimination of nonspecific cell adhesion is intended to reduce the occurrence of the foreign body response in the in vivo environment, but it is not desirable for facilitating tissue regeneration through the implanted scaffold. However, the multifunctional capabilities of the polyampholyte hydrogel platform demonstrated in this work provides an easy mechanism for incorporating cell adhesive biological cues. The pH responsive nature of the CAA monomer can be taken advantage of with the use of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) bioconjugation chemistry to covalently attach bioactive signaling molecules. This was used to attach FBG, which subsequently facilitated MC3T3-E1 cell adhesion to the hydrogel, as demonstrated in Figure 3 [41]. Furthermore, the background hydrogel (locations without conjugated FBG) was tested, and verified that it retained the native nonfouling properties away from the conjugated proteins, upon return to neutral pH [41]. It is believed that the incorporation of tissue specific biological cues will facilitate targeted cell adhesion and interrogation interactions. This multifunctional capability is not limited to just TMA/CAA polyampholyte hydrogels, either. Three component polymers, using equimolar combinations of positively charged TMA and varying combinations of negatively charged CAA and SA monomers, have also shown the same nonfouling

properties and pH dependent protein conjugation capabilities, regardless of the underlying charge balanced composition [16]. This combination of nonfouling properties, protein conjugation capability, and tunable cell adhesion suggests polyampholyte hydrogels have excellent potential for applications as tissue engineering scaffolds.

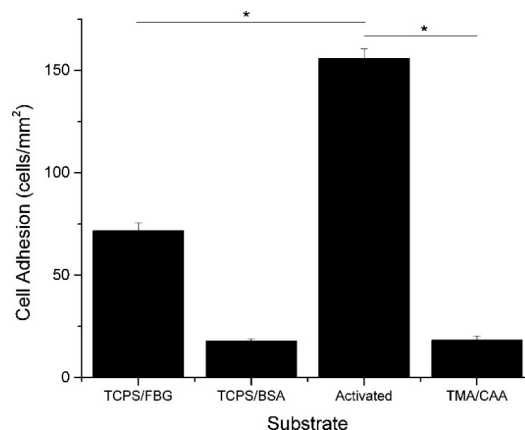


Figure 3. Average number of MC3T3-E1 cells (cells/mm²) that adhered to tissue culture polystyrene (TCPS) and TMA/CAA hydrogels with or without adsorbed or conjugated proteins. * Represents a statistically significant difference between the surfaces being compared ($p < 0.05$). This figure is reprinted from Ref. [41] with permission. Copyright 2013, American Chemical Society.

Advances in the application of polyampholyte hydrogels for tissue engineering are not limited to our efforts. For example, Jian and Matsumura developed a nanocomposite hydrogel using COOH-PLL and synthetic clay laponite XLG, that showed promise as a tissue engineering scaffold due to its controlled release profiles, good mechanical properties, and cell adhesion capability [17]. These gels were cytocompatible, and had adjustable degradation properties. Furthermore, cell adhesion was tunable by controlling the hydrogel formulation. When the polymer chains were covalently cross-linked with PEG-NHS, it hid some of the laponite surface and reduced cell adhesion. Alternatively, when the hydrogels were only physically crosslinked (no PEG-NHS), there was more exposed laponite surface area, leading to enhanced cell attachment.

5. Cryopreservation Applications

Another important aspect of tissue engineering is the preservation of cells over long-term scenarios. This is most generally done using cryopreservation in a liquid nitrogen cell freezer. In order to prevent cell death, a cryoprotective agent (CPA) is typically added to the cell solution prior to freezing. One of the most commonly used CPAs is dimethyl sulfoxide (DMSO), but it shows high cytotoxicity and needs to be removed quickly after thawing. DMSO has also been seen to influence the differentiation of many cell types. The need for a new and more effective CPA has driven research into the use of polyampholytes for cryopreservation.

Matsumura et al. demonstrated the use of COOH-PLL as a new polyampholyte CPA for human bone marrow derived mesenchymal stem cells (hBMSCs) [42]. They found that the polyampholyte CPA did not penetrate the cell wall, but instead provided protection by attaching to the membrane. When the ratio of carboxylation was within the range of 0.5–0.8, there was >90% cell viability upon seeding after being frozen for 24 months, with no significant differences compared to cells frozen in the presence of DMSO. The hBMSCs also showed better retention of their properties inherent before freezing, such as differentiation potential, as compared to samples with DMSO as the CPA. COOH-PLL was further tested as a CPA during fast and slow vitrification of two-dimensional cell constructs. Figure 4a,b below, show the cell viability directly after warming, and after one day of culture. It can be clearly seen that there are no significant differences in the cell viability immediately after thawing

in the presence of COOH-PLL (denoted as P-VS), DMSO (denoted as DAP213), or no CPA (denoted as VS). After both one day of culture and over longer time periods, the proliferation curves (Figure 4c) show a distinct improvement when the cells were frozen with the polyampholyte CPA, as compared to either DMSO or no CPA. Through these studies, it was concluded that the use of a polyampholyte CPA significantly improved the viability of hBMSCs while maintaining differentiation capacity, making it promising for the long-term storage of tissue engineered constructs [42,43].

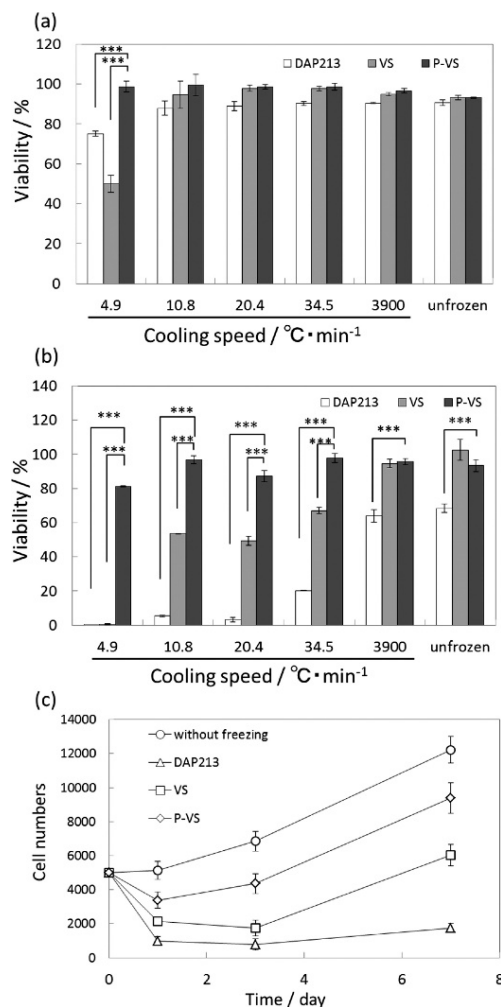


Figure 4. Quantitative viability results of mesenchymal stem cells (MSCs) after slow and fast vitrification with various VSs and different cooling speeds (a) immediately after warming and (b) after 1 day of culture. (c) Cell proliferation curves after slow vitrification at a cooling rate of 10.8 °C/min with various VSs (***p* < 0.001). This figure reprinted from Ref. [43] with permission. Copyright 2016, American Chemical Society.

Based on the positive results seen with COOH-PLL, other polyampholytes have also been investigated as CPAs. These studies were to both expand the formulation range of CPAs, as well as to better understand how polyampholytes protect the cell membrane during freezing. In one example, 2-(dimethylamino) ethyl methacrylate (DMAEMA) and methacrylic acid (MAA) were copolymerized in various ratios [44]. In addition, hydrophobic groups in the form of *N*-butyl methacrylate (Bu-MA) and *N*-octyl methacrylate (Oc-MA) were introduced into the polymer backbone at 2–10% mole percent of the total monomer amount. This range of polyampholyte chemistries were tested, and at an overall solution polymer concentration of 10%, with 5% consisting of Bu-MA or Oc-MA, significantly increased cell viability was seen following freezing. By testing

this range of polyampholyte compositions, it was determined that the cryoprotective properties are strongly correlated with hydrophobicity. This approach has also been adapted to the closely related zwitterionic polymers 3-((3-acrylamidopropyl)dimethylammonio)-propane-1-sulfonate and 2-((2-methacryloyloxy)ethyl)-dimethylamino acetate [45]. The cryoprotective capabilities of the zwitterionic species were compared to poly(MAA-DMAEMA), and they did not show comparable cell viability, providing further insight into the mechanism of preservation. Through these studies, it was concluded that the cryoprotective property results from strong interactions between the polyampholyte CPA and the cell membrane, which are greatly aided by limited hydrophobic interactions [44,45].

Cell sheets and constructs have added complexity for successful cryopreservation. A dextran-based polyampholyte hydrogel was developed to encapsulate cell constructs prior to cryopreservation, and it has shown promise for tissue engineering applications in preliminary studies [46]. Another variation on the use of COOH-PLL CPAs was explored by Jian and Matsumura. Cells were cryopreserved with 7.5–20% COOH-PLL solutions. After thawing, nanosilicates were injected, turning the solution into a thixotropic hydrogel. Cell viability was excellent, remaining >90% for all tested polyampholyte concentrations. This unique gel system was proposed for direct cell injection for site specific cell delivery and tissue repair, without the need to wash out the cryoprotective agent [47]. Furthermore, the thermoresponsiveness of this class of polyampholyte materials, and their demonstrated biocompatibility, make them promising for other biomaterial and drug delivery applications [48].

6. Drug Delivery Applications

Due to the naturally occurring responsive nature of polyampholyte polymers addressed earlier, they have gained increasing interest for drug delivery applications. The cryoprotective properties of some polyampholyte formulations, discussed above, have been taken a step further by Ahmed et al. as a novel approach to deliver proteins into cells [49]. Proteins were adsorbed on/into nanoparticles made from hydrophobically modified polyampholytes synthesized by the succinylation of ϵ -poly-L-lysine with dodecyl succinic anhydride and succinic anhydride. L929 cells were then frozen with the protein loaded nanoparticles as a CPA. The high affinity between the cell membrane and the hydrophobic subunits of the nanoparticles caused the protein-loaded nanoparticles to condense on the peripheral cell membrane during freezing. The adsorbed protein and nanoparticles were found to be internalized after thawing via endocytosis during culture, thereby delivering the protein payload. However, there was a critical concentration above which these nanoparticle delivery systems became cytotoxic. The Matsumura group also adapted this approach to polyampholyte-modified liposomes in additional protein delivery studies, demonstrating its adaptability for protein delivery in immunotherapy applications [50].

At the same time, much of the recent work in polyampholyte mediated drug delivery takes advantage of the pH responsive behavior of polyampholyte systems. For example, chitosan based polyampholytes have recently been shown to have potential in protein delivery applications, as they have exhibited the ability to adsorb and desorb bovine serum albumin (BSA) in a pH dependent manner [7,8]. However, a combination of design characteristics is required to optimize drug delivery that include biocompatibility, multifunctionality, and responsiveness to the microenvironment. Nanogels have been investigated for use as delivery systems, and have shown tremendous promise due to the ability to control drug release, provide the drug protection from degradation, and target specific tissues. Some of the loading and drug release methods include covalent conjugation, passive/diffusion based, or through environmental stimuli, such as pH [51].

Our group previously investigated the fundamental release characteristics of polyampholyte hydrogels composed of equimolar ratios of TMA and CAA using neutral caffeine, positively charged methylene blue, and negatively charged metanil yellow [52]. These species were selected as methylene blue and metanil yellow have nearly identical molecular weights, thereby eliminating this variable when comparing the release kinetics, while caffeine is approximately one half the size of the other

species, to allow for a characterization of the influence of size. Hydrogels were synthesized in the presence of the drug analogues, and then the release characteristics were monitored as a function of crosslinker density, pH, and ionic concentration. The release of the smaller, neutral caffeine molecule was shown to be mediated by diffusion alone, although this release was tunable, based on environmental stimuli induced swelling of the polyampholyte hydrogels. Conversely, the release of the charged molecules was strongly dependent on electrostatic interactions throughout the system, which could be modified through the environmental cues of pH and ionic strength. Figure 5 shows a schematic of the relative drug release levels from the TMA/CAA hydrogel. Importantly, it was also demonstrated that following the release of the various drug molecules, it was verified that the TMA/CAA platforms retained their native nonfouling characteristics. Therefore, this platform shows great potential for long-term biomolecule delivery.

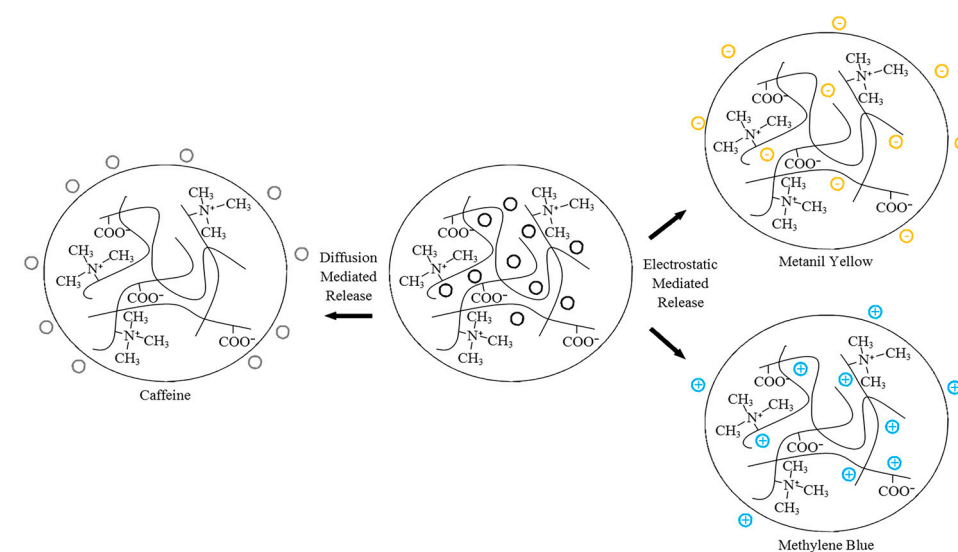


Figure 5. Schematic depicting the release of caffeine, metanil yellow and methylene blue from TMA/CAA gels. This figure is reprinted from Ref. [52] with permission. Copyright 2015, American Chemical Society.

Kudaibergenov et al. also used a variety of guest molecules to characterize the adsorption and release from a macroporous amphoteric cryogel composed of *N,N*-dimethylaminoethyl methacrylate and methacrylic acid with a *N,N'*-methylenebisacrylamide crosslinker [53]. The guest species tested included methylene blue, methyl orange, sodium dodecylbenzene sulfonate (SDBS), and lysozyme. Lysozyme and methylene blue were adsorbed at pH 9.5, and SDBS and methyl orange were adsorbed at pH 7.5. Similar to the work by Barcellona et al., the binding interactions between the cryogel and the guest molecules was driven by electrostatic forces. However, at the IEP of pH 7.1, the amphoteric cryogel allowed for the release of 93–98% of the adsorbed species. The conclusions drawn by both Barcellona et al. and Kudaibergenov et al. are also supported by simulation based studies that concluded that electrostatic interactions play the most significant role in mediating drug release from polyampholyte systems [54].

A variety of specific drug species have also been used to test drug delivery from assorted polyampholyte mediums. Mishra et al. used poly 3-[(methacryloylamino)propyl trimethylammonium chloride-*co*-methacrylic acid] (PMAPTACMAAc) copolymers with various concentrations of monomers and loaded indomethacin (IND) [55]. IND is a nonsteroidal anti-inflammatory drug that is used for the treatment of rheumatoid arthritis, ankylosing spondylitis, and osteoarthritis, to name a few. The hydrogel composition played a large role in the sustained release of IND, and PMAPTACMAAc-5 led to the highest percentage of IND release. This formulation released 75% of the entrapped IND within 8 h and 82% after 12 h. Other hydrogel formulations showed release percentages ranging

from ~44% to 77% after 12 h. The release was primarily diffusion based, and it followed non-Fickian release kinetics. Although diffusion is often effective for drug delivery, a controlled release response can provide a more targeted delivery. Salicylic acid was used as a model drug in a polyampholyte composed of casein and poly(*N*-isopropylacrylamide), and the release was affected by temperature, pH, and crosslinker density [56]. This led Cao et al. to conclude this delivery vehicle was appropriate for orally administered drug delivery. Finally, Sankar et al. demonstrated the pH sensitive release of promethazine hydrochloride from polyampholyte hydrogels containing carbon nanotubes [57]. These nanotubes were incorporated into the hydrogel as an approach to reinforce the mechanical properties of this delivery system, without impacting the drug delivery capabilities.

Investigators have also begun incorporating polyampholytes into multicomponent systems to enhance performance or offer additional benefits. For example, Wang et al. examined a polyampholyte hydrogel release system based on pyromellitic diester diacid chloride (PDDC) combined with combinations of diethylenetriamine (DETA) and triazine [58]. This polyampholyte system showed a pH dependent release capability that overcame previous issues seen with related encapsulants formed with terephthaloyl chloride (TC) in place of PDDC. This new microcapsule formulation showed high loading capacity, and steady, controlled release at pH 7.4. It also demonstrated accelerated release at both pH 5 and pH 10, as shown in Figure 6. The release characteristics were also tunable by varying the ratio of DETA to triazine, indicating the ability to refine this microcapsule formulation for tunable release rate applications.

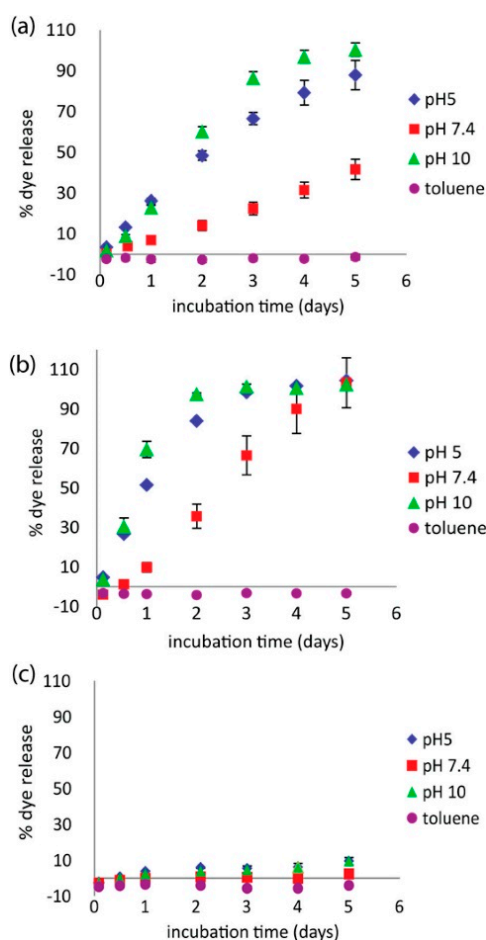


Figure 6. Release profiles of coumarin 1 dye under different solvent conditions for PDDC capsules with (a) 3:1 triazine/DETA and (b) 1:1 triazine/DETA. (c) Control experiments: 1:1 triazine/DETA with TC. This figure is reprinted from Ref. [58] with permission. Copyright 2017, American Chemical Society.

Others have also incorporated polyampholyte polymers into their drug delivery vehicles to add pH responsive release characteristics. For example, Schulze et al. saw potential in lamellar liquid crystalline systems, but the structure did not react to environmental stimuli such as pH. When the polyampholyte poly(*N,N'*-diallyl-*N,N'*-dimethyl-*α*-maleamic carboxylate) (PalH) was integrated into a lamellar liquid crystalline system of sodium dodecyl sulfate, decanol, and water, it was found that release from the new structure could be tuned by varying the pH or temperature. This suggests it has promise as a new structural material for drug delivery systems [59]. In another example, papacetamol, an analgesic drug, was released from a polyampholyte hydrogel matrix composed of laponite, polyacrylamide and poly(3-acrylamidopropyl) trimethylammonium chloride. Drug release was tested as a function of environmental changes in pH and ionic strength, and in the presence of an electric field. Without an electric field, papacetamol was only released at pH 1.1, but with the application of an electric field, sustained drug release occurred at other pH values [60]. Finally, Ali et al. created a novel polymer containing residues of alendronic acid, that showed pH sensitive responses that were proposed to be used as a drug delivery system [61].

Asayama et al. also incorporated a polyampholyte polymer, carboxymethyl poly(1-vinylimidazole) (CM-PVIm), into an existing system. CM-PVIm was used to coat poly(ethylenimine)/DNA (PEI/DNA) complexes, to reduce nonspecific protein adsorption to this delivery platform. The results demonstrated this coating did not significantly reduce gene transfection or cell viability. Therefore, the authors concluded that CM-PVIm is an effective coating for improved circulation of gene therapy agents [62].

Another application of adding polyampholytes to drug delivery vehicles is based on their strong water holding capacity. Polyampholyte acrylic latexes were incorporated into drug tablet coatings to minimize the amount of water removed from the drugs during the tablet drying step [63]. During the optimization of this approach, Ladika et al. focused on finding a polymer solution with similar viscosity to the industry standard, that contained a much higher concentration of solids. Typical tablet coatings on the market today range from 4–10 wt % solids and the new polyampholyte acrylic latexes showed a range of 37–39 wt % solids. Three types of latexes were explored: weak acid/strong base latexes, strong acid/weak base latexes, and combinations of anionic and cationic latexes. Latex formulations for all three combinations were determined that had viscosities similar to current coating solutions, had higher solids composition, and were pH-tunable, to enable targeted delivery of active pharmaceutical ingredients.

7. Future Directions

Throughout this review, many exciting advancements applying polyampholyte hydrogels to biomedical applications were highlighted. However, despite this progress and the clearly demonstrated capabilities of polyampholytes, these materials have not yet been investigated in the *in vivo* environment in depth. This is the critical next step in the continued development of these materials, and our group is pursuing these efforts in the application of polyampholyte hydrogels for bone tissue engineering. Additionally, while the tunability and responsive properties of polyampholytes have been widely demonstrated, the ease of tuning polyampholyte materials for targeted applications of these capabilities must also be further pursued.

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Impacts of cross-linker chain length on the physical properties of polyampholyte hydrogels

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Impacts of cross-linker chain length on the physical properties of polyampholyte hydrogels

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Polymeric tissue engineering scaffolds have shown promise to aid in regeneration and repair of damaged tissue. In particular, nonfouling polymers have been proposed for eliminating biomaterial-induced concerns such as infection, scarring, and rejection by the immune system. Polyampholyte polymers are one class of nonfouling polymers that are composed of an equimolar mixture of positively and negatively charged monomer subunits. They possess nonfouling properties, bioactive molecule conjugation capabilities, and tunable mechanical properties. In this study, the influence of the cross-linker species on the degradation behavior, mechanical strength, and nonfouling properties of polyampholytes composed of a 1:1 molar ratio of [2-(acryloyloxy)ethyl] trimethylammonium chloride (positively charged) and 2-carboxyethyl acrylate (negatively charged) monomers was investigated. Specifically, the impact of ethylene glycol repeat units on the overall material performance was evaluated by synthesizing and characterizing hydrogels containing di-, tri-, and tetra-ethylene glycol dimethacrylate cross-linker species. The degradation studies were conducted for over 100 days in Sorenson's buffer with pH values of 4.5, 7.4, and 9.0 by tracking the swelling behavior and weight change over time. The mechanical properties were assessed using compression and tensile testing to failure. The retention of the nonfouling and protein conjugation capabilities was demonstrated using fluorescently labeled bovine serum albumin. The results demonstrate the tunability of both degradation behavior and mechanical properties through the cross-linker selection, without impacting the underlying nonfouling and biomolecule delivery capabilities. Therefore, it is concluded that polyampholyte hydrogels represent a promising platform for tissue engineering. *Published by the AVS.*

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I. INTRODUCTION

In the biomedical field, there is a need for engineered scaffold materials that can improve and facilitate wound healing in severely damaged tissues. These scaffolds must have several important properties to ensure successful tissue recovery. First, the biomaterial must be nonfouling or resistant to nonspecific protein adsorption. It is hypothesized that this feature will minimize any foreign body response, preventing connective tissue from becoming inflamed and scarred.^{1,2} Inflammation and scarring lead to the encapsulation of the implanted biomaterial, preventing the biomaterial from fully integrating and maintaining functionality.³⁻⁷ Second, the biomaterial must also facilitate targeted cell adhesion, migration, and remodeling to promote tissue regeneration.^{7,8} To do this, the material must present biochemical cues specific to the target tissue. Third, the scaffold material should be biodegradable and not break down into components that are toxic or harmful to the surrounding cells and tissues.^{9,10} Ideally, the material will break down at the same rate at which tissue is reforming to prevent defects in the developing tissue.^{1,11} Finally, an implant must have mechanical properties similar to the tissue it will be integrated with,

to allow for faster return to functionality.^{9,10,12} Mechanical properties also impact the differentiation of seeded or recruited stem cells which is important for the regeneration of the intended tissue.^{13,14}

There have been many approaches to develop engineered biomaterials to fulfill these criteria, but it has been difficult to design something that incorporates all of these properties. Hydrogels are the standard for tissue engineering because of their high water content and general biocompatibility.^{1,15,16} They also have tunable properties and the ability to be synthesized on a larger scale.^{1,8} Some common chemistries that have been explored for tissue engineering include poly(lactic acid) (PLA), poly(hydroxyethyl methacrylate) (PHEMA), and poly(caprolactone) (PCL).^{1,17} PLA is biologically inert and has high degradability.^{16,18} However, this material still invokes an inflammatory response as it is not nonfouling.^{18,19} Additionally, its degradation rate is relatively slow.¹⁹ PHEMA has been used in tissue engineering for decades because it is biocompatible and has some ability to reduce nonspecific protein adsorption.¹⁷ Since this material is only low fouling, it still causes inflammation when implanted. While PCL is biodegradable and inexpensive, it is hydrophobic and has poor cytocompatibility.^{20,21}

Another common material is polyethylene glycol (PEG), which is resistant to nonspecific protein adsorption, leading to its widespread use in biomedical systems. However, PEG based materials are known to oxidize *in vivo*.²² This ultimately

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leads to protein adsorption and cell attachment as it breaks down into acids and aldehydes.²² It has also been reported that humans have developed antibodies to PEG, suggesting that there is a need to find alternative biomaterials.^{23,24}

Zwitterionic materials have recently become of interest for biomedical applications because they have shown great antifouling properties when compared to other nonfouling materials such as PEG.^{5,25,26} These neutral systems are composed of mixed charged functional groups throughout the polymer. As a result, they are extremely hydrophilic due to the large number of charged regions.²³ The most widely studied zwitterionic materials are polycarboxybetaine (pCB), polysulfobetaine, and polyphosphorylcholine.^{23,25} pCB-based materials, in particular, have gained a lot of attention because of their superior antifouling properties, biocompatibility, and functionalization capabilities.^{6,7,23,25,27} Functionalization allows for the covalent attachment of biomolecules, and pCB is highly functionalizable due to its many carboxyl groups.^{27,28} However, one limitation of zwitterionic polymers is that their physical properties are dependent on the underlying monomer, making their physical properties less tunable than other materials.^{5,29}

One family of materials that presents many of the desired properties for tissue engineering are polyampholyte hydrogels, which are a subclass of zwitterionic polymers. They are being considered for tissue engineering and drug delivery applications due to their unique characteristics including nonfouling properties, ability to deliver biomolecules to the body, and tunable mechanical properties.^{30–32} Polyampholyte hydrogels are composed of equimolar mixtures of positively and negatively charged monomer subunits, resulting in an overall neutral charge.³³ Their nonfouling properties are likely derived from the formation of a strong hydration layer on the

surface of these hydrogels due to electrostatic interactions with the charged terminal groups of the monomers.^{31,32,34,35} Polyampholyte hydrogels also have tunable mechanical properties depending on both the underlying monomer composition and the cross-linker density.^{31,32} Finally, salt concentration can have an effect on the physical properties due to the salt ions interacting with the charged groups and disrupting the electrostatic interactions between the monomer subunits.^{30,33,36}

While several beneficial features of polyampholytes have been demonstrated, their degradation behaviors are relatively unknown. Understanding the degradation behavior will provide insight into the *in vivo* behavior of these materials, allowing for targeted applications in tissue engineering. It was hypothesized that different ethylene glycol chain lengths will lead to variation in the physical and mechanical properties, without impacting the multifunctional nonfouling capability. Therefore, the goal of this investigation is to characterize the degradation and physical properties of polyampholytes with different ethylene glycol cross-linkers. The polyampholytes used in this study contain equimolar amounts of cationic [2-(acryloyloxy)ethyl] trimethylammonium chloride (TMA) and anionic 2-carboxyethyl acrylate (CAA) monomers, which are shown in Fig. 1. Hydrogels were synthesized with one of three chemical cross-linkers, di-, tri-, or tetra-ethylene glycol dimethacrylate (DEGDMA, TEGDMA, and Tetra-EGDMA), also shown in Fig. 1, to study how the cross-linker chain length impacts the properties of polyampholyte hydrogels. The multifunctionality was validated using fluorescently labeled proteins and microscopy, the compressive and tensile properties were quantified, and the degradation behavior as a function of pH was determined.

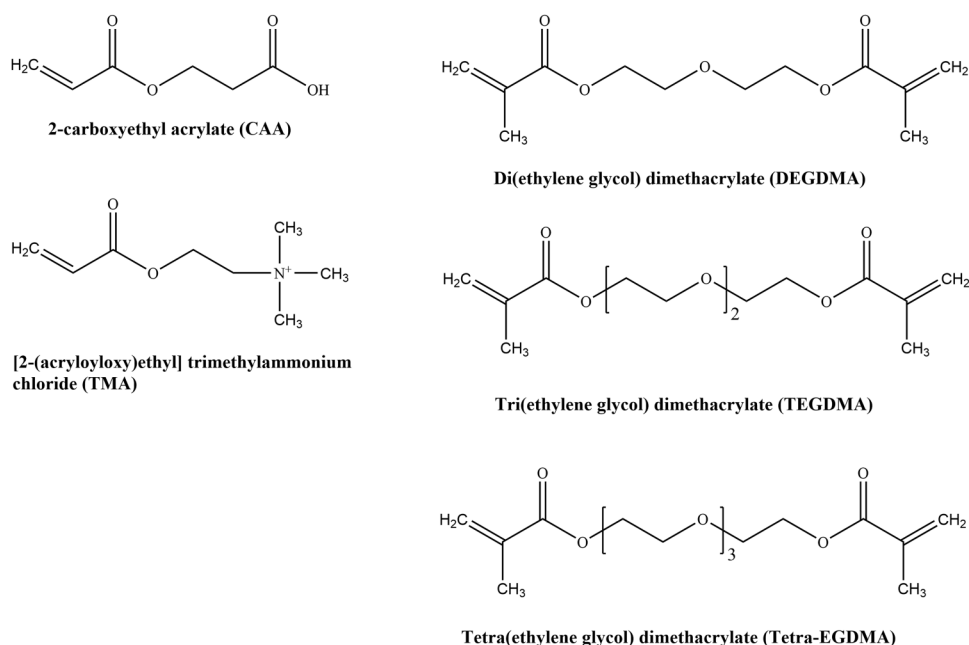


FIG. 1. Structures of the monomers and cross-linkers used in this study.

II. METHODS

A. Materials

Ethylene glycol, phosphate-buffered saline (PBS, pH 7.4), fluorescein isothiocyanate bovine serum albumin (FITC-BSA), TMA, CAA, TEGDMA, DEGDMA, Tetra-EGDMA, sodium hydroxide, ammonium persulfate (APS), sodium metabisulfate (SMS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Potassium phosphate monobasic and sodium phosphate dibasic, anhydrous were purchased from VWR. Ethanol was purchased from Greenfield Global. All chemicals were used as received.

B. Hydrogel synthesis

Hydrogels were synthesized using equimolar concentrations of TMA and CAA using procedures described previously.³¹ In short, 2 ml of a buffer solution containing 3 M NaOH, ethanol, and ethylene glycol in a 1.5:1:1.5 volume ratio was mixed with 4 mmol of TMA and CAA each and mixed thoroughly. One of the three cross-linkers was added in with monomer to cross-linker ratios of 26.3:1 (1X), 13.2:1 (2X), or 6.6:1 (4X). The reaction was initiated by adding 32 μ l of 40% w/w APS and 32 μ l of 15% w/w SMS and the solution was then transferred into a polyfluoroethylene (PTFE) mold. The hydrogels polymerized at room temperature for 24 h. With the exception of degradation, all hydrogels were then soaked for 24 h in PBS and used in subsequent studies.

C. Nonfouling and protein conjugation

For nonfouling and conjugation testing, hydrogels were synthesized in a flat plate mold formed from two microscope slides clamped around a 1/8 in. PTFE spacer. Following synthesis, the hydrogels were soaked in PBS for 24 h and then they were punched with a 5 mm biopsy punch.³¹ Conjugation and control samples were first soaked in a solution of 0.05 M NHS and 0.2 M EDC for 7 min, while the nonfouling samples were soaked in neutral PBS. The nonfouling and conjugation samples were then exposed to 10 μ l of 1 mg/ml FITC-BSA in PBS for 15 min, while the control samples were soaked in neutral PBS. Finally, all samples were soaked in 1 ml of NaCl-PBS with a pH of 9.0 for 30 min, followed by 1 ml neutral PBS for 40 min. Nonfouling and conjugation were evaluated by comparing the fluorescence of these samples to the control sample using an inverted fluorescent Nikon microscope with a 4X objective. Images were captured using NIS Elements imaging software.

D. Compression testing

For compression testing, hydrogels were synthesized in a cylindrical PTFE mold with a 5/8 in. inner diameter. Compression samples were made by tripling the above procedures. After the samples soaked for 24 h in PBS, they were cut using a razor and mold to obtain 10 mm tall hydrogel cylinders. Compression tests were conducted using an Instron equipped with a 100 kN load cell. Samples were

compressed to failure at a rate of 0.106 mm/s. The maximum stress and strain were obtained from the force displacement curve and Young's modulus was taken from the linear region (75%–85% of the maximum strain) of the stress–strain curve.

E. Tensile testing

For tensile testing, hydrogels were synthesized in a PTFE dog-bone shaped mold. Tensile testing was conducted using a custom tensile load frame equipped with a 150 g load cell.³⁷ Samples were stretched at a rate of 0.1 mm/s until fractured. The maximum stress and strain were obtained from the force displacement curve and Young's modulus was taken from both the linear region (50%–100% of the maximum strain) and the toe region (0–0.1 strain values) of the stress–strain curve.

F. Degradation studies

For degradation testing, hydrogels were synthesized in the flat plate mold described above. After polymerizing for 24 h, samples were immediately cut into 1 cm squares and placed in individual 50 ml centrifuge tubes containing Sorenson's buffer. Sorenson's buffer with pH values 4.5, 7.4, and 9.0 was used to induce degradation under different conditions. Degradations studies were conducted over a period of 113 days with 1X cross-linker density hydrogels. At each measurement time point, the samples were removed from the buffer and characterized before being placed in fresh buffer. Wet weight was measured using a scale, and swelling measurements were taken using calipers.

G. Data analysis

All results are presented as the average $\pm$ standard deviation from the evaluation of three samples replicated three independent times ($n = 9$). Statistical significance was evaluated using a one-way analysis of variance at a 95% confidence interval ($p < 0.05$) using OriginPro 2017 (OriginLab Corporation, MA).

III. RESULTS

A. Nonfouling and protein conjugation

Polyampholyte hydrogels are of interest for tissue engineering applications because of their demonstrated multifunctionality.³¹ Therefore, it was important to verify that their previously demonstrated nonfouling and conjugation capabilities were not impacted by the additional cross-linker species investigated in this study. Nonspecific adsorption of FITC-BSA was used to verify the nonfouling properties using fluorescent microscopy. Figures 2(a)–2(c) show representative images of TMA:CAA hydrogels following exposure to FITC-BSA and control hydrogels that have not been exposed to any protein. There are minimal to no differences in the fluorescence between these samples. Therefore, the results clearly show that the nonfouling properties are not impacted by the different cross-linker species. The protein

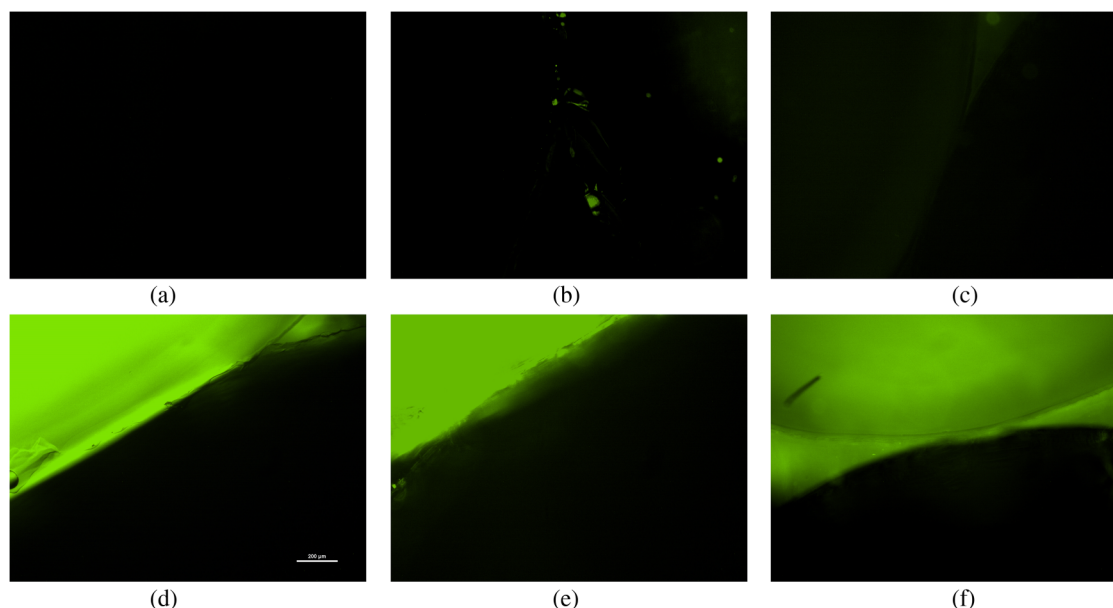


FIG. 2. Representative fluorescent microscopy images of FITC-BSA: (a)–(c) adsorption and (d)–(f) conjugation to TMA:CAA hydrogels with 1X cross-linker densities of [(a) and (d)] DEGDMA, [(b) and (e)] TEGDMA, and [(c) and (f)] Tetra-EGDMA cross-linkers. Control samples are included in the bottom right of each image and they were not exposed to FITC-BSA.

conjugation capabilities were also verified with FITC-BSA and fluorescent microscopy. The results can be seen in Figs. 2(d)–2(f). These images show hydrogels with conjugated FITC-BSA adjacent to control samples. FITC-BSA conjugated samples showed bright fluorescence indicating that the FITC-BSA is successfully conjugated to the hydrogels, again demonstrating that the known conjugation capabilities are not impacted by the variation in cross-linker species.

B. Compressive strength

The physical properties under compression were evaluated next, to identify impacts of the cross-linker length and density. Figure 3(a) shows representative stress–strain curves for 4X cross-linker density hydrogels with each cross-linker species and representative stress–strain curves for the 1X and 2X cross-linker densities can be seen in Figs. S1(a) and S1(b).⁴¹ It can be seen that decreasing the cross-linker chain length increases both stress and strain at failure. The quantitative analysis of multiple samples, in Fig. 3(b), shows that increased cross-linker density improves the stress at failure for all the cross-linker species, as expected.³¹ The maximum stress at failure value across all samples was found for the 4X cross-linker density DEGDMA hydrogels with an average stress of 860 ± 140 kPa. This value was statistically greater than the max stress at failure for both 4X cross-linker density TEGDMA and Tetra-EGDMA samples. At the 1X cross-linker density, DEGDMA samples had the statistically lowest fracture stress. DEGDMA samples also demonstrated the largest impact as cross-linker density was increased. The only statistically significant difference in the 2X cross-linker density samples was seen between the TEGDMA and Tetra-EGDMA results, with TEGDMA samples having a

higher stress at failure. The maximum strain at failure results is shown in Fig. 3(c). Only the Tetra-EGDMA samples showed significant variation in the max strain at failure as a function of cross-linker density, with a statistically significant drop between the 1X and 4X cross-linker density samples. Across all cross-linker densities, DEGDMA samples presented the highest max strain at failure, with the greatest average strain of 0.51 ± 0.04 for the 2X cross-linker density, but this value was not statistically significant from the 1X and 4X DEGDMA density results. At the 4X cross-linker density, there were statistically significant differences between all of the cross-linker species. The results indicate that increasing cross-linker chain length decreases the compressive strain at failure. Finally, the elastic moduli of the various samples were evaluated, and these results are shown in Fig. 3(d). For the most part, the cross-linker species did not impact the elastic modulus under compression. The modulus increased uniformly across the cross-linker species with increasing cross-linker density and the maximum elastic modulus was seen for 4X density DEGDMA samples with a value of 3300 ± 825 kPa.

C. Tensile strength

The physical properties under tension were evaluated, again to identify the influence of the cross-linker species and density. The tensile properties of this family of materials have not been previously tested. Figure 4(a) shows representative stress–strain curves for 4X cross-linker density hydrogels in tension, and representative curves for the 1X and 2X cross-linker densities can be seen in Figs. S2(a) and S2(b).⁴¹ Each curve presents a different profile with a low strain inflection point seen with the Tetra-EGDMA

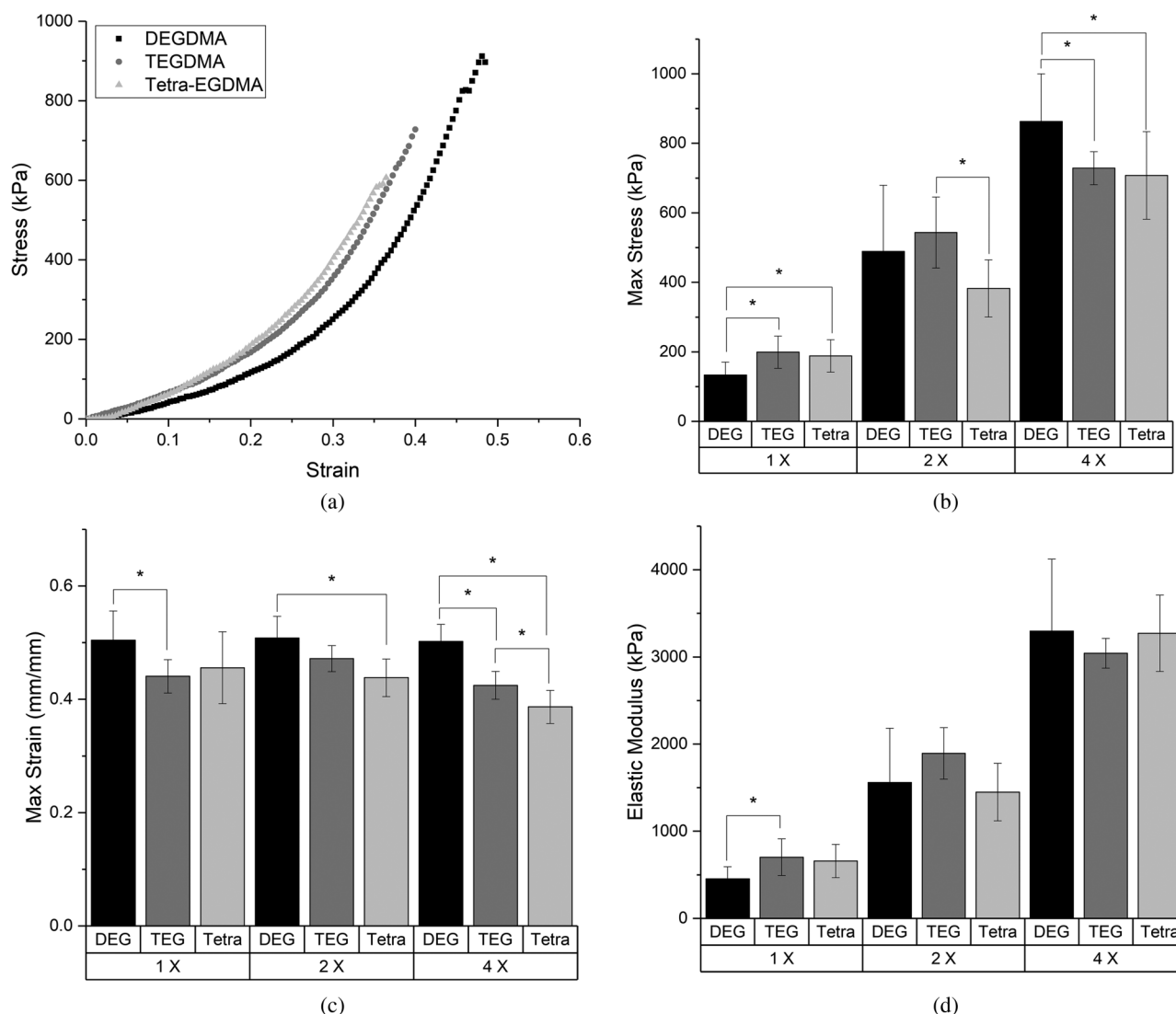


Fig. 3. Compressive properties of hydrogels with different cross-linker species and densities. (a) Representative stress-strain curves for 4X cross-linker density hydrogels, (b) maximum stress at failure, (c) maximum strain at failure, and (d) elastic modulus taken from the linear region. A * indicates a statistically significant difference at a 95% confidence interval ($p < 0.05$) between the samples identified.

hydrogel curves (~18% of total strain), no inflection point with the TEGDMA hydrogels, and a mid strain inflection point in the DEGDMA hydrogel curves (~56% of the total strain). The TEGDMA hydrogel curves have a nearly linear stress-strain curve at a 4X cross-linker density, while the 1X and 2X cross-linker density curves have profiles similar to DEGDMA. The slope of Tetra-EGDMA stress-strain curve is similar to that of TEGDMA after the inflection point, while the slope of the DEGDMA stress-strain curve is similar to TEGDMA before its inflection point. The results in Fig. 4(b) indicate that the fracture stress at failure increases with increasing cross-linker density, again as expected. However, in contrast to the compressive fracture behaviors, Tetra-EGDMA demonstrates the highest values, reaching a maximum tensile fracture stress of 58 ± 14 kPa for the 4X cross-linker density hydrogels. Tetra-EGDMA also has the largest change as the cross-linker density is

increased. Similar to the compression study, the fracture strain decreased with increased cross-linker density. However, a much more significant change was seen for all three cross-linkers species as the cross-linker concentration increased. As before, there was little significance between the strain values as a function of cross-linker species, at a given cross-linker density, though DEGDMA had a greater strain value under all conditions. Finally, Fig. 4(d) shows the average elastic modulus for these samples under tension in both the toe region (solid) and linear region (patterned). The elastic modulus was greater in the toe region than it was in the linear region and the overall elastic modulus increases with increasing cross-linker density. As with the maximum stress under tension data, the Tetra-EGDMA samples have the highest elastic modulus values and show the largest dependence on cross-linker density.

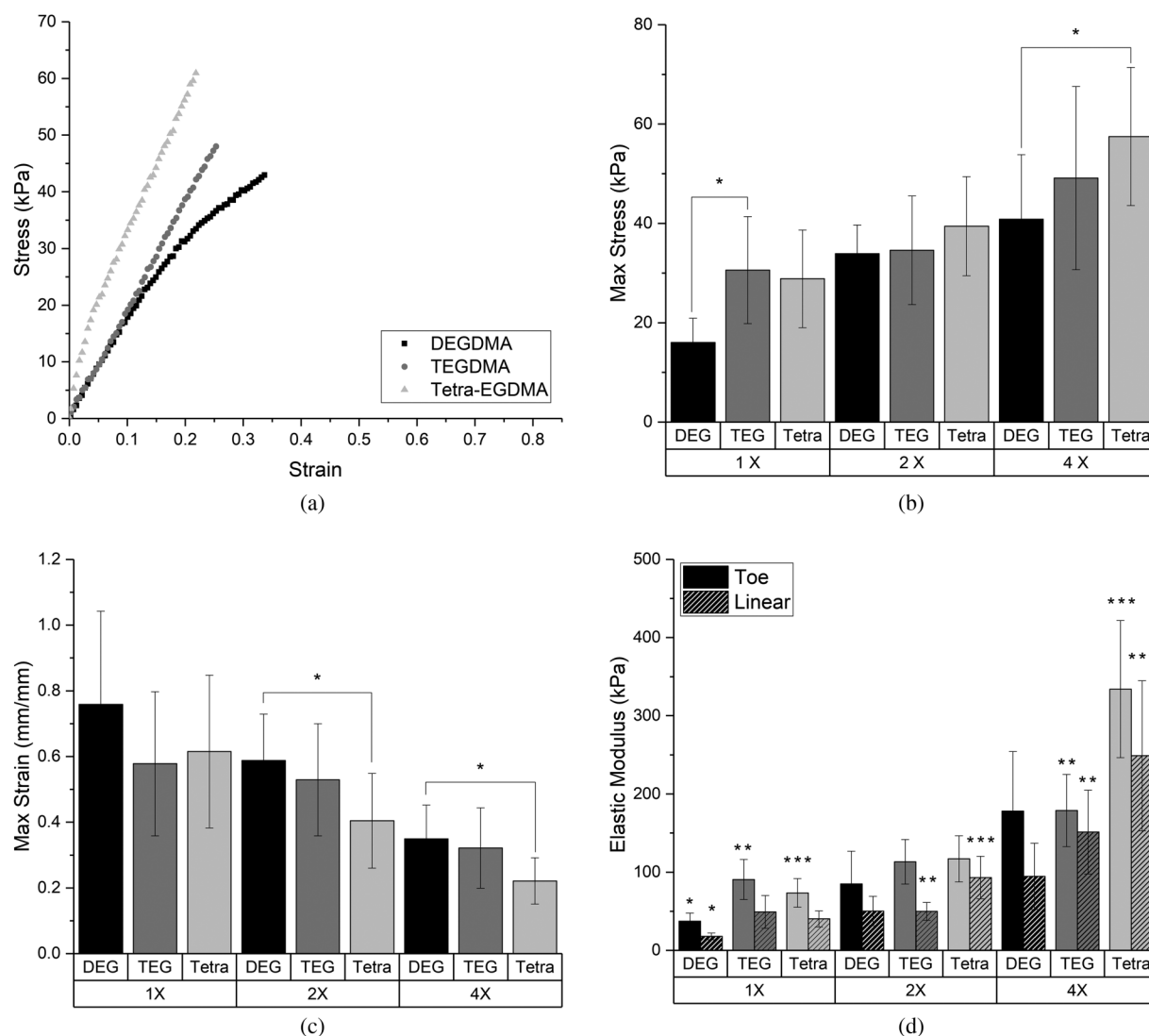


FIG. 4. Tensile properties of hydrogels with different cross-linker species and densities. (a) Representative stress-strain curves for 4X cross-linker density hydrogels, (b) maximum stress at failure, (c) maximum strain at failure, and (d) elastic modulus taken from the toe (solid) and linear (patterned) regions. A * indicates a statistically significant difference between the samples identified at a 95% confidence interval ($p < 0.05$). For the modulus, *, **, and *** indicate a statistically significant difference for the results between the DEGDMA and TEGDMA samples, the TEGDMA and Tetra-EGDMA samples, and the DEGDMA and Tetra-EGDMA samples, respectively, all at a 95% confidence interval ($p < 0.05$).

D. Degradation studies

The degradation behaviors of polyampholyte hydrogels have not been characterized previously. For this degradation study, 1X cross-linker density hydrogels with the three different cross-linkers species were monitored for 113 days in Sorenson's buffer with acidic (4.5), neutral (7.4), and basic (9.0) pH conditions. The degradation was monitored by tracking the sample swelling and weight changes over time and the results are summarized in Fig. 5. There were insignificant changes in the swelling and weight change behavior for all three cross-linker species under the neutral and basic conditions, over the time period evaluated here. Degradation occurred in the acidic environment for all three cross-linker species, ultimately leading to full sample breakdown after ~110 days for the Tetra-EGDMA cross-linked samples. The

Tetra-EGDMA hydrogels increased in both weight and swelling over time. Full breakdown was determined when the samples completely dissipated into the degradation medium. This time point was used as the endpoint for all of the degradation studies even though the full sample degradation only occurred with Tetra-EGDMA samples in acidic media. Conversely, the DEGDMA and TEGDMA cross-linked hydrogels decreased in both size and weight. They also did not reach full degradation like the Tetra-EGDMA hydrogels over the time period evaluated, although surface flaking was visible during the degradation study. These paired results suggest that the Tetra-EGDMA samples degraded via bulk degradation pathways, while the DEGDMA and TEGDMA degraded via surface degradation pathways, as discussed in more detail below.

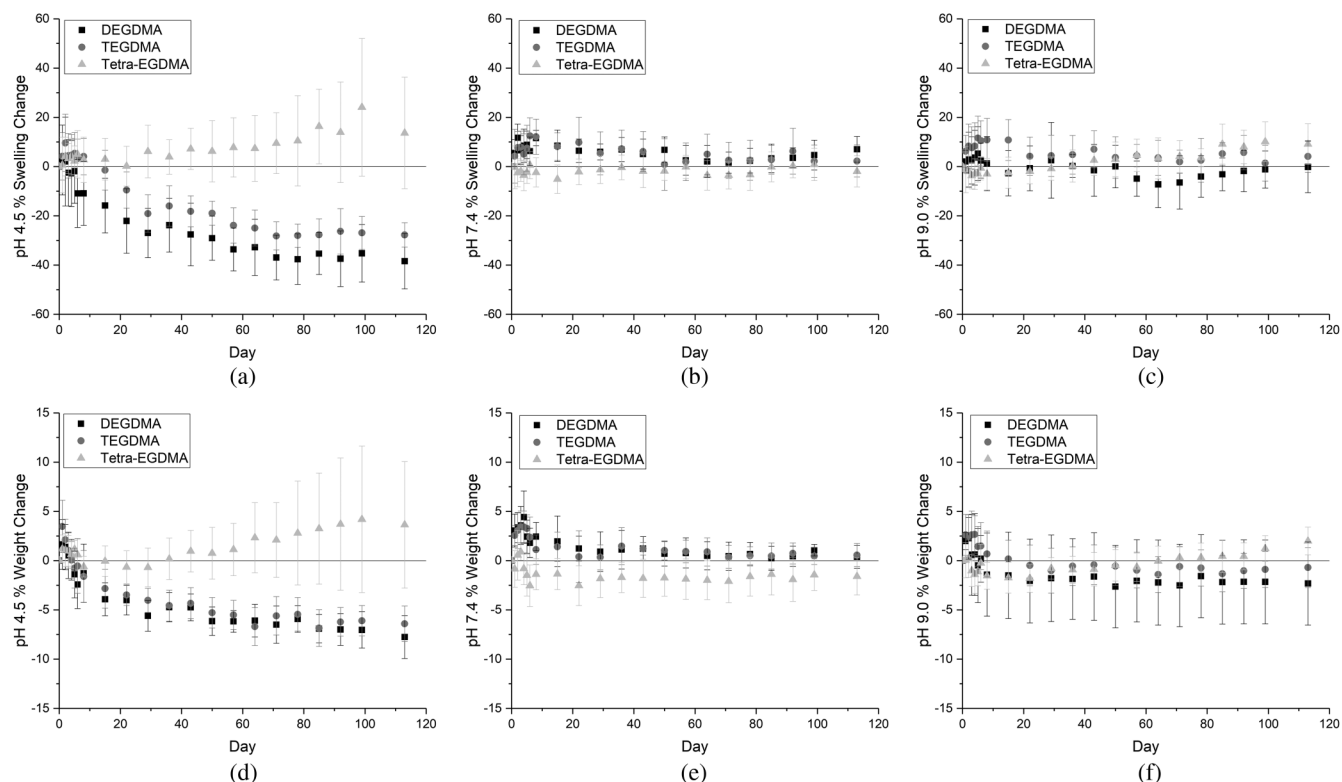


Fig. 5. Mean $\pm$ standard deviation of the percent change in [(a)–(c)] swelling and [(d)–(f)] weight for 1X cross-linker density hydrogels in [(a) and (d)] acidic, [(b) and (e)] neutral, and [(c) and (f)] basic pH conditions.

IV. DISCUSSION

The compression samples show that increasing the cross-linker density increases the stress at failure and elastic modulus, which has been seen in previous studies.^{32,38} Specifically, the physical properties under compression for TMA/CAA hydrogels with a TEGDMA cross-linker have been tested previously and the trends between the previous results and this study are similar.³¹ The results in this study show that the available range of mechanical properties can be expanded even further by varying the length of the cross-linker species, suggesting a wider range of tunability. A similar range is also demonstrated under tension in this study as well.

Polyampholyte hydrogels cross-linked with DEGDMA had the lowest stress at fracture compared to TEGDMA and Tetra-EGDMA with a 1X cross-linker density, but presented the greatest stress at fracture with a 4X cross-linker density. It is hypothesized that this is due to differences in the side-chain packing density in these various systems. The DEGDMA cross-linker has the shortest chain length and this results in the most side-chain crowding and overlap between the pendant groups of polymer chains linked by this cross-linker. The overlap is eliminated as the cross-linker chain length is increased to TEGDMA and there is the least amount of crowding between pendant groups when the length is increased to the Tetra-EGDMA cross-linker. This is shown schematically in Fig. 6. While Fig. 6 presents an example of the most tightly packed pendant group alignment,

it is readily acknowledged that the pendant groups will rotate around the polymer backbone in three-dimensions to reduce the overlap and resulting steric hindrance. However, as the cross-linker density is increased, it will reduce the degrees of freedom available for this pendant group rotation. This results in more overlap between pendant groups in hydrogels cross-linked with DEGDMA, which will not be seen in the TEGDMA and Tetra-EGDMA cross-linked systems. Further complicating the discussion is the fact that these hydrogels have been shown to have ~ 90 wt. % water in their fully hydrated state.³¹ The fact that the maximum stress at failure for the DEGDMA samples shows the greatest change from the 1X to 4X cross-linker density, while going from the lowest to highest value, is attributed to the packing density, increased interactions between the pendant side chains, and hydrating water.

As mentioned previously, increasing the cross-linker chain length decreased the compressive strain at failure, specifically at high cross-linker densities. Additionally, there were no significant differences in strain for each cross-linker species with changes in the cross-linker density. This indicates that the compressive strain is not dependent on the amount of cross-linker in the hydrogel. The high weight percentage of water ($\sim 90\%$ (Ref. 31)) leads to bulk incompressible behavior, based on the incompressible nature of water. Therefore, there is no change in strain as a function of cross-linker density. The electrostatic interactions between the pendant side chains, which are influenced by the cross-linker length, could also play a role in the strain behavior. As

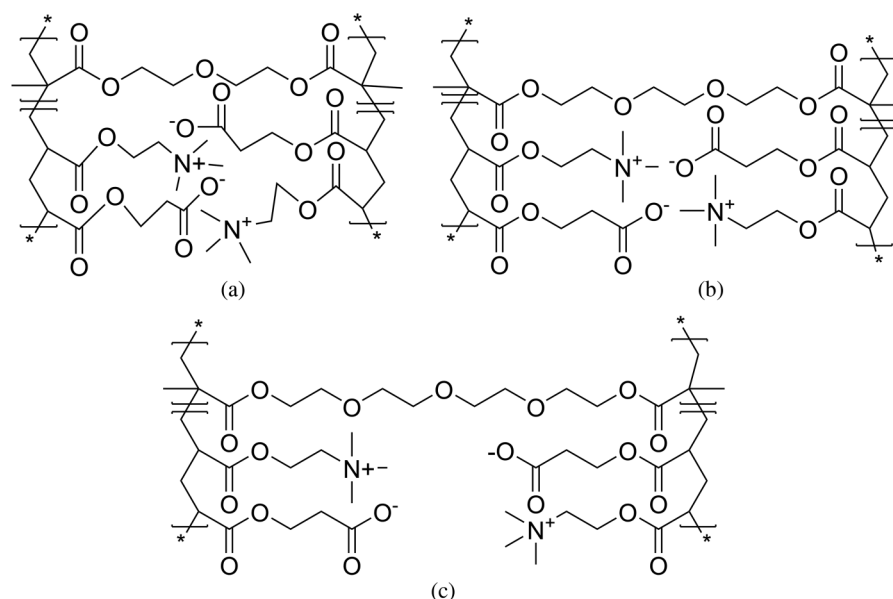


FIG. 6. Schematic showing the packing density of the pendent side chains with (a) DEGDMA, (b) TEGDMA, and (c) Tetra-EGDMA cross-linkers.

discussed above, there is potential overlap between the pendant groups in hydrogels formed with the DEGDMA cross-linker. As the system is compressed, the pendant groups can more easily slide past each other as they are potentially already overlapping. This contrasts with the pendant group interactions in TEGDMA and Tetra-EGDMA cross-linked systems. The electrostatic interactions between intrachain pendant groups in the TEGDMA and Tetra-EGDMA systems are hypothesized to lead to more tightly bound side chains, resulting in failure when the interchain groups are compressed together.

This packing density model can also be used to explain the trends seen in the tensile testing results. When the 4X cross-linker density stress-strain curves are examined [Fig. 4(a)], it is evident that all three cross-linker species have a portion of the curve that has nearly identical slope with each other. This occurs before the inflection point in the DEGDMA cross-linked samples, across the entirety of the profile for the TEGDMA cross-linked samples, and after the inflection point in the Tetra-EGDMA cross-linked samples. It is hypothesized that this slope is indicative of the simultaneous breakage of the cross-linker species and the electrostatic interactions between pendant groups of adjacent polymer chains, along with disruptions to the significant hydrating water-functional group interactions that occur throughout the hydrogel. In Fig. 6, it can be seen that the TEGDMA samples provide the best alignment of pendant side chains from adjacent polymer chains. Therefore, there is limited rearrangement prior to breakage of the electrostatic interactions, cross-linker species, and water interactions. The DEGDMA cross-linked samples initially have the same structure disruptions occurring under tension. However, the overlap of the pendant side chains from adjacent polymer chains leads to an extended domain where only the weaker

electrostatic interactions and hydrating water-functional group interactions are being broken. This occurs after the inflection point in the stress-strain curve, where there is a significantly lower elastic modulus (slope). The opposite is true in Tetra-EGDMA cross-linked samples. Prior to the inflection point, the slope of the stress-strain curve is much more significant than after, and this is attributed to the breakage of the Tetra-EGDMA cross-linker. After the inflection point, the system has very similar behavior to the TEGDMA samples. While similar trends exist in the 1X and 2X cross-linker density stress-strain curves [Figs. S2(a) and S2(b)],⁴¹ they are not as prevalent because the pendant group packing densities are lower.

The packing density model is also supported by the degradation study results. The Tetra-EGDMA cross-linked hydrogels present a different degradation behavior than the DEGDMA and TEGDMA cross-linked hydrogels under acidic conditions, as observed directly with the samples and in the quantitative analysis of the weight changes and swelling behaviors. All of the hydrogels degrade via acid hydrolysis of the ester groups present in both the cross-linkers and monomers.^{1,9} The results indicate that the DEGDMA and TEGDMA cross-linked hydrogels degrade via surface degradation pathways (shrinkage and weight loss; visible surface flaking), while the Tetra-EGDMA hydrogels degrade via bulk degradation pathways (swelling and weight gain; full sample dissipation).^{39,40} The strong electrostatic interactions that occur between adjacent polymer chains cross-linked with either DEGDMA or TEGDMA cross-linkers provide steric hindrance that limits accessibility to the ester groups of the cross-linker where overall degradation occurs (Fig. 6). Therefore, degradation only occurs at the surface and outmost layers of the hydrogels. In contrast, the pendant groups of adjacent polymer chains in hydrogels cross-linked with Tetra-EGDMA

have wider spacing, facilitating easier penetration into the hydrogel and bulk degradation. Furthermore, full degradation was only observed with the Tetra-EGDMA cross-linked samples over the ~110 day degradation time frame, at which point the samples fully dissipated into solution.

V. CONCLUSIONS

In this work, polyampholyte hydrogels were formed from equimolar mixtures of TMA and CAA charged monomer subunits with three different ethylene glycol based cross-linkers (di-, tri-, and tetra-ethylene glycol). Fluorescent microscopy evaluations confirmed that the multifunctional nonfouling and protein conjugation capabilities of TMA/CAA hydrogels were not impacted by the cross-linker species. The compression and tensile properties of this family of hydrogels were evaluated across a range of cross-linker densities and the results suggest that the cross-linker length influences the pendant side-chain packing density, which in turn impacts the macroscale physical characteristics. The cross-linker length and subsequent packing density also impact the acid hydrolysis degradation pathway for this family of hydrogels. Hydrogels cross-linked with DEGDMA and TEGDMA degraded via surface degradation pathways, while those cross-linked with Tetra-EGDMA degraded via bulk degradation pathways. Molecular dynamics investigations are ongoing, and they will provide further mechanistic insight into this proposed packing density model due to the complex interplay between side-chain density, electrostatic interactions, and water-functional group interactions. However, the results from this study clearly demonstrate the broad tunability of polyampholyte hydrogels for tissue engineering applications based on controlling the spacing and interactions between the underlying charged pendant groups.

ACKNOWLEDGMENTS

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- ⁴¹See supplementary material at <https://doi.org/10.1116/1.5097412> for representative stress-strain curves for hydrogels formed with a 1X and 2X cross-linker density for all three cross-linker species. This material is available free of charge via the internet.

Supporting Information For:

Impacts of Cross-Linker Chain Length on the Physical Properties of Polyampholyte Hydrogels

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Stress-Strain Curves

As discussed in the results section of the manuscript, compressive and tensile properties were evaluated for each cross-linker species (di-, tri-, and tetra-ethylene glycol dimethacrylate; DEGDMA, TEGDMA, Tetra-EGDMA) at 1X, 2X, and 4X cross-linker densities. Representative compressive and tensile stress-strain curves for 1X and 2X cross-linker density hydrogels are shown in Figures S1 a-b and S2 a-b, respectively.

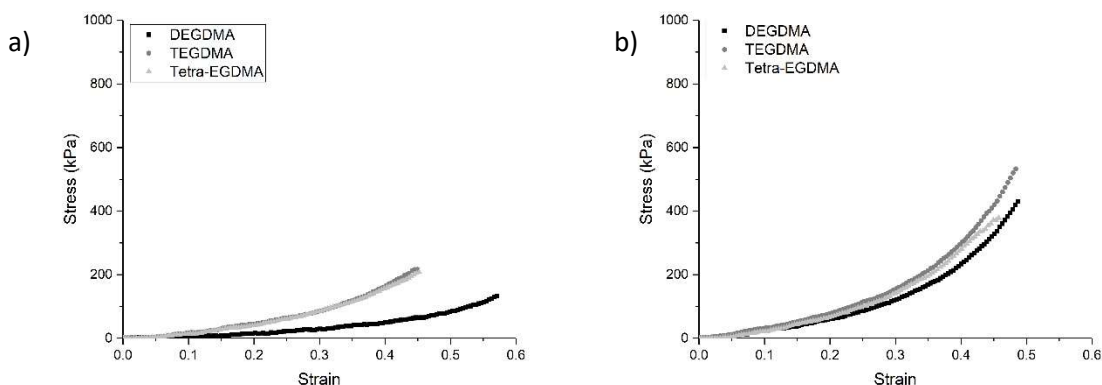


Figure S1. Representative stress-strain curves in compression for a) 1X cross-linker density hydrogels and b) 2X cross-linker density hydrogels.

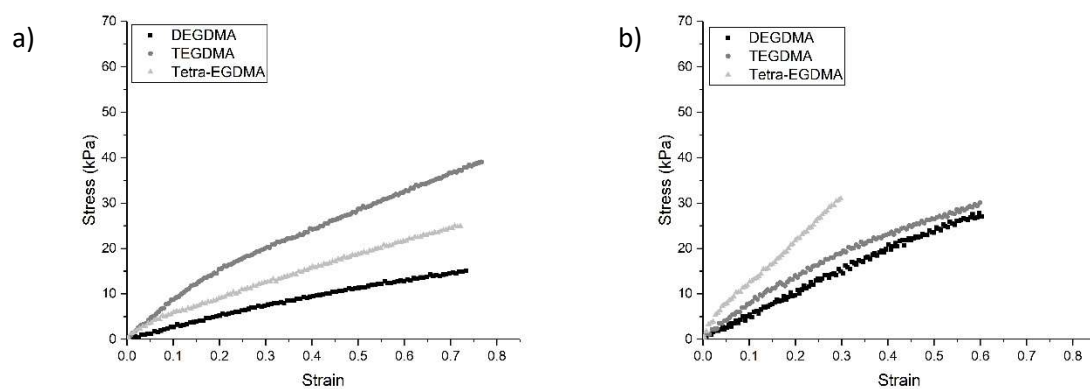


Figure S2. Representative stress-strain curves in tension for a) 1X cross-linker density hydrogels and b) 2X cross-linker density hydrogels.