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

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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.

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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] phosphorylcholine (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.

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.

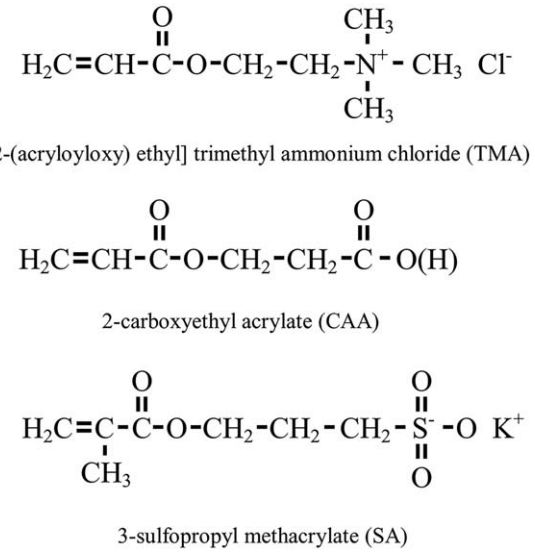

Figure 1. Structures of the three monomers used in this study.

EXPERIMENTAL

Materials

Phosphate buffered saline (PBS; 150 m*M*, 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 (≥90%) were purchased from Sigma Aldrich (St. Louis, MO). Ethylene glycol, sodium metabisulfite (SMS), urea, and hydrogen peroxide were purchased 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× 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 μg/mL HRP-conjugated anti-FBG in PBS buffer or 10 μg/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 μL/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× 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-Kα 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

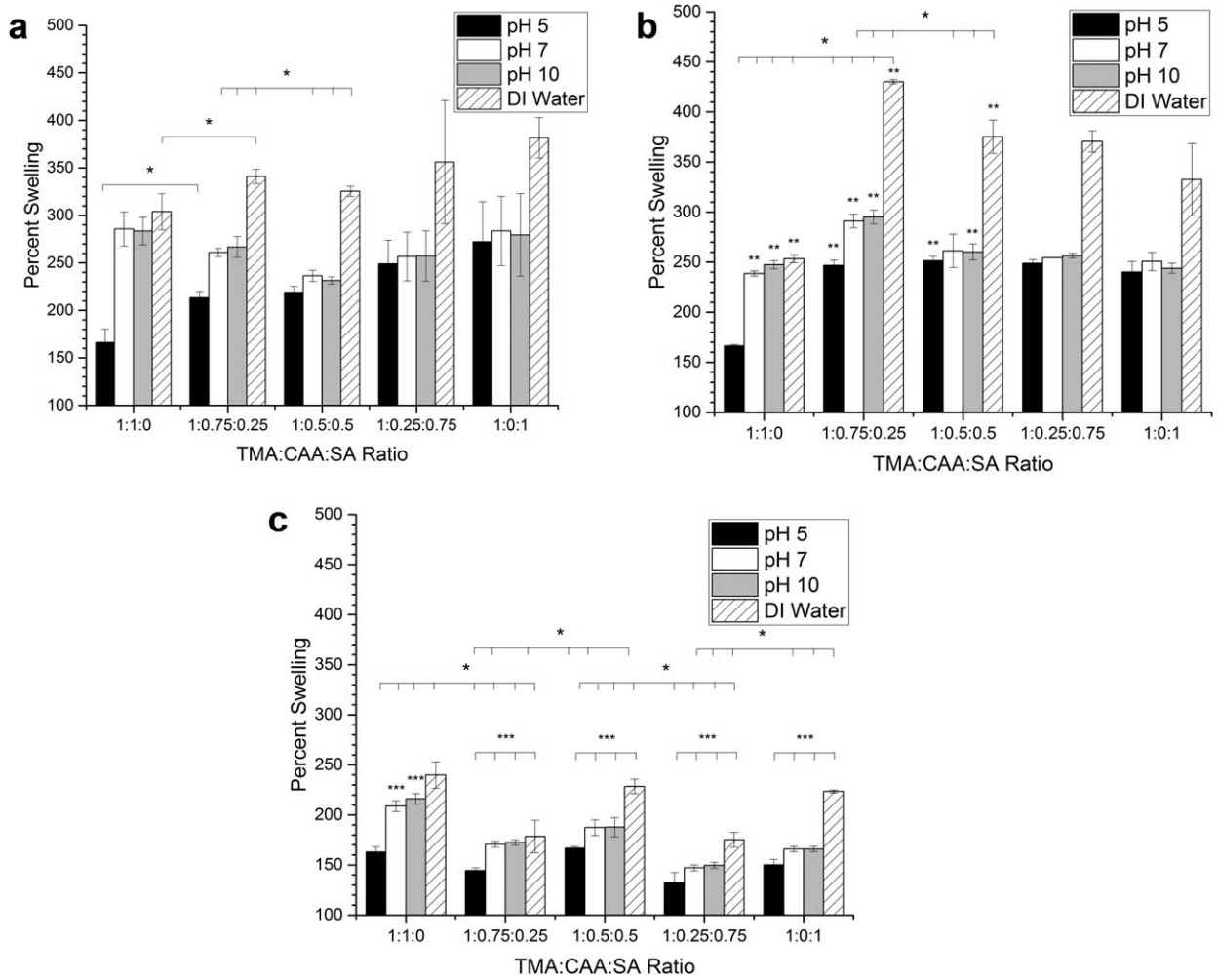

Figure 2. Mean ± standard deviation of the equilibrium swelling percentage for hydrogels with different TMA:CAA:SA ratios at cross-linker densities of (a) 1×, (b) 2×, and (c) 4× in different buffers. A * indicates a statistically significant difference between the indicated compositions at each cross-linker density, a ** indicates a statistically significant difference between the 1× and 2× cross-linker densities for the indicated composition and property, and a *** indicates a statistically significant difference between the 2× and 4× 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×), 13.2:1 (2×), and 6.6:1 (4×). 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× to 4×. 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×, 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× and 2× 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

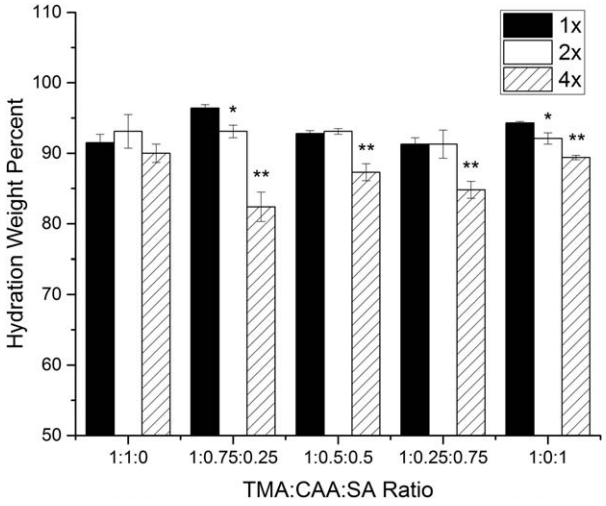

Figure 3. Average ± standard deviation of the hydration weight percentage for hydrogels with different TMA:CAA:SA ratios. A * indicates a statistically significant difference from only the 1× cross-linker density and a ** indicates a statistically significant difference from both the 1× and 2× cross-linker 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

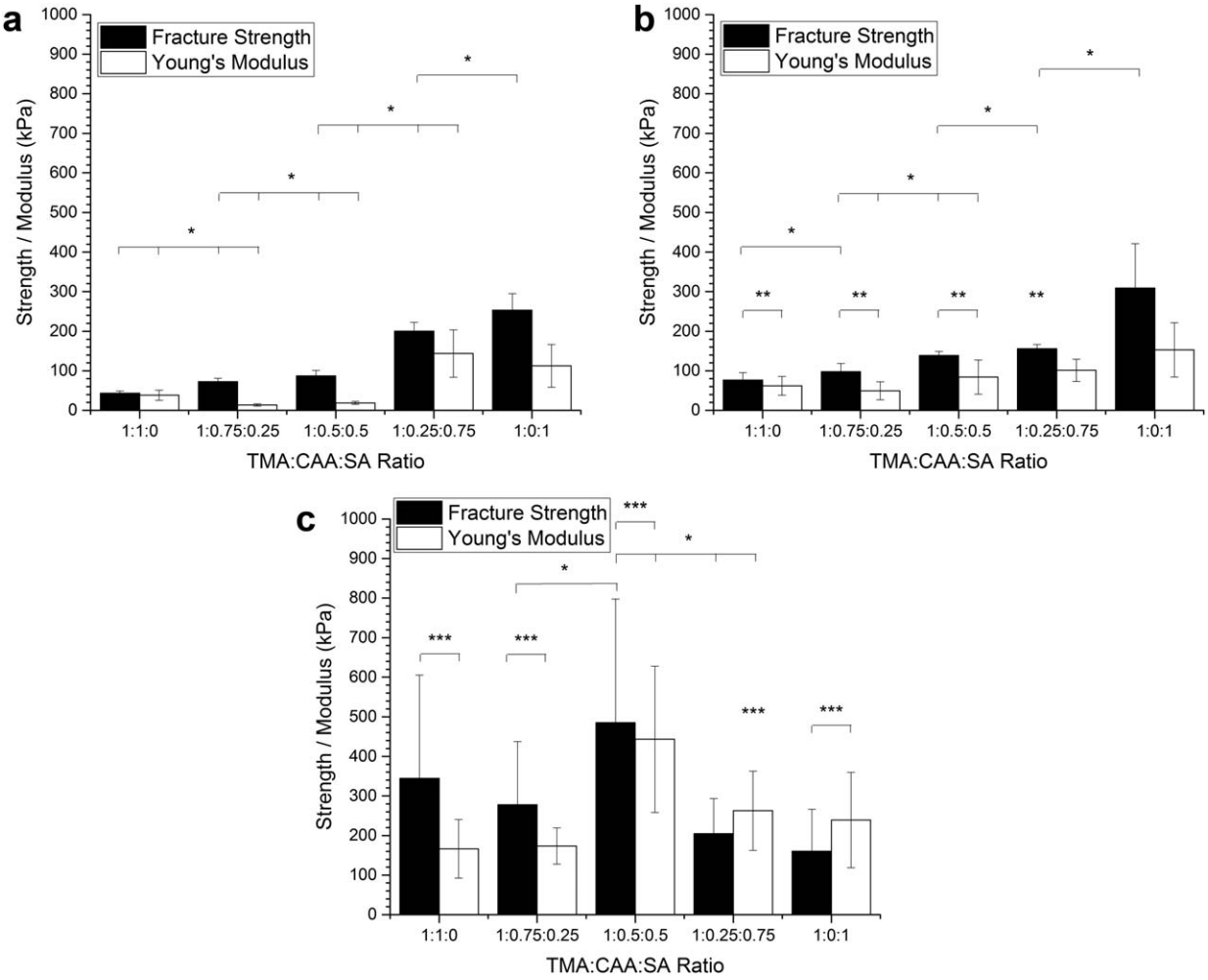

Figure 4. Average ± standard deviation of the fracture strength and Young's modulus for TMA:CAA:SA hydrogels with different compositions at cross-linker densities of (a) 1×, (b) 2×, and (c) 4×. A * indicates a statistically significant difference between the indicated compositions at each cross-linker density, a ** indicates a statistically significant difference between the 1× and 2× cross-linker densities for the indicated composition and property, and a *** indicates a statistically significant difference between the 2× and 4× 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

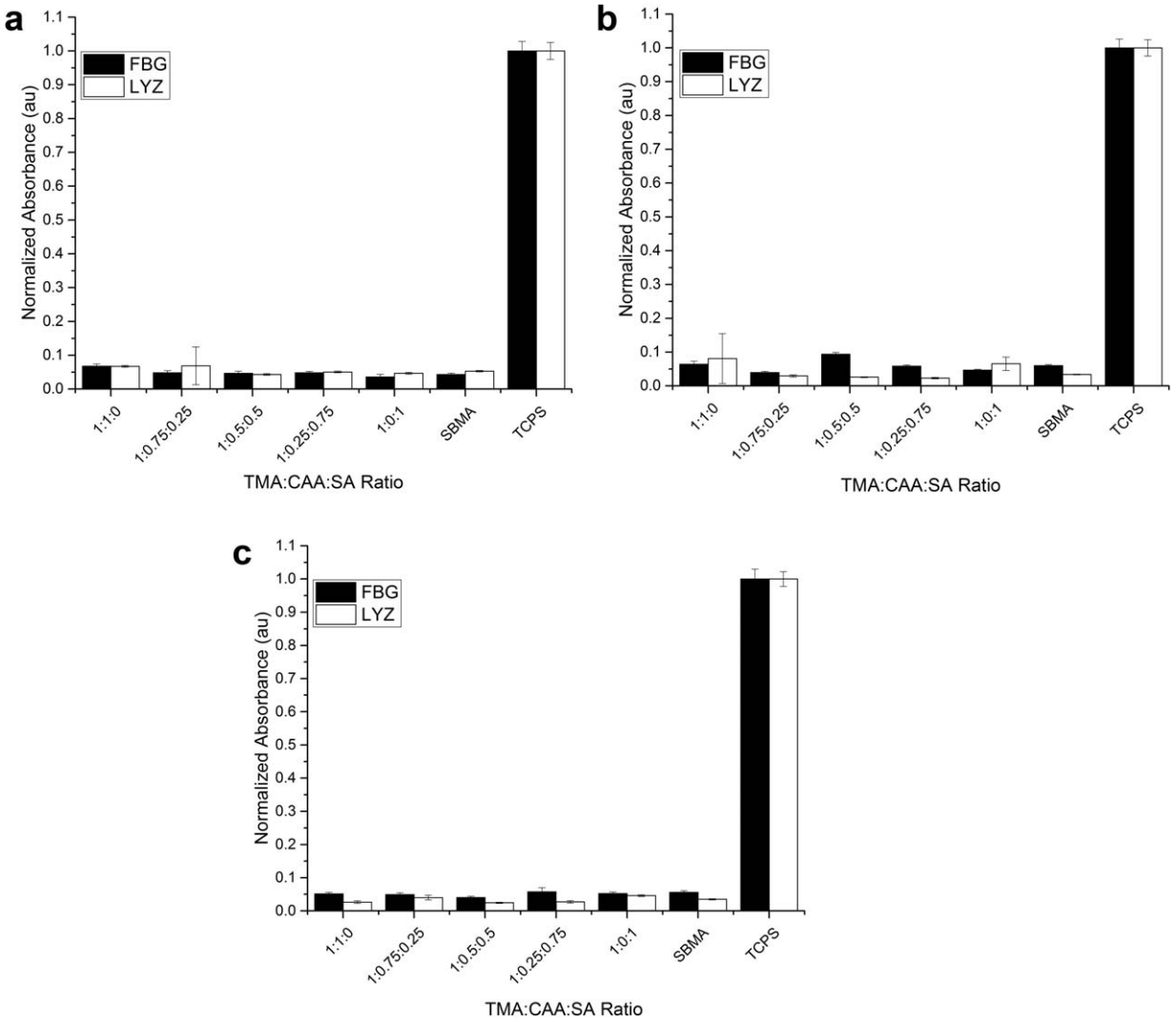

Figure 5. Mean ± propagated error of the nonspecific protein adsorption to hydrogels with different TMA:CAA:SA ratios at cross-linker densities of (a) 1×, (b) 2×, and (c) 4×.

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 conjugation 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

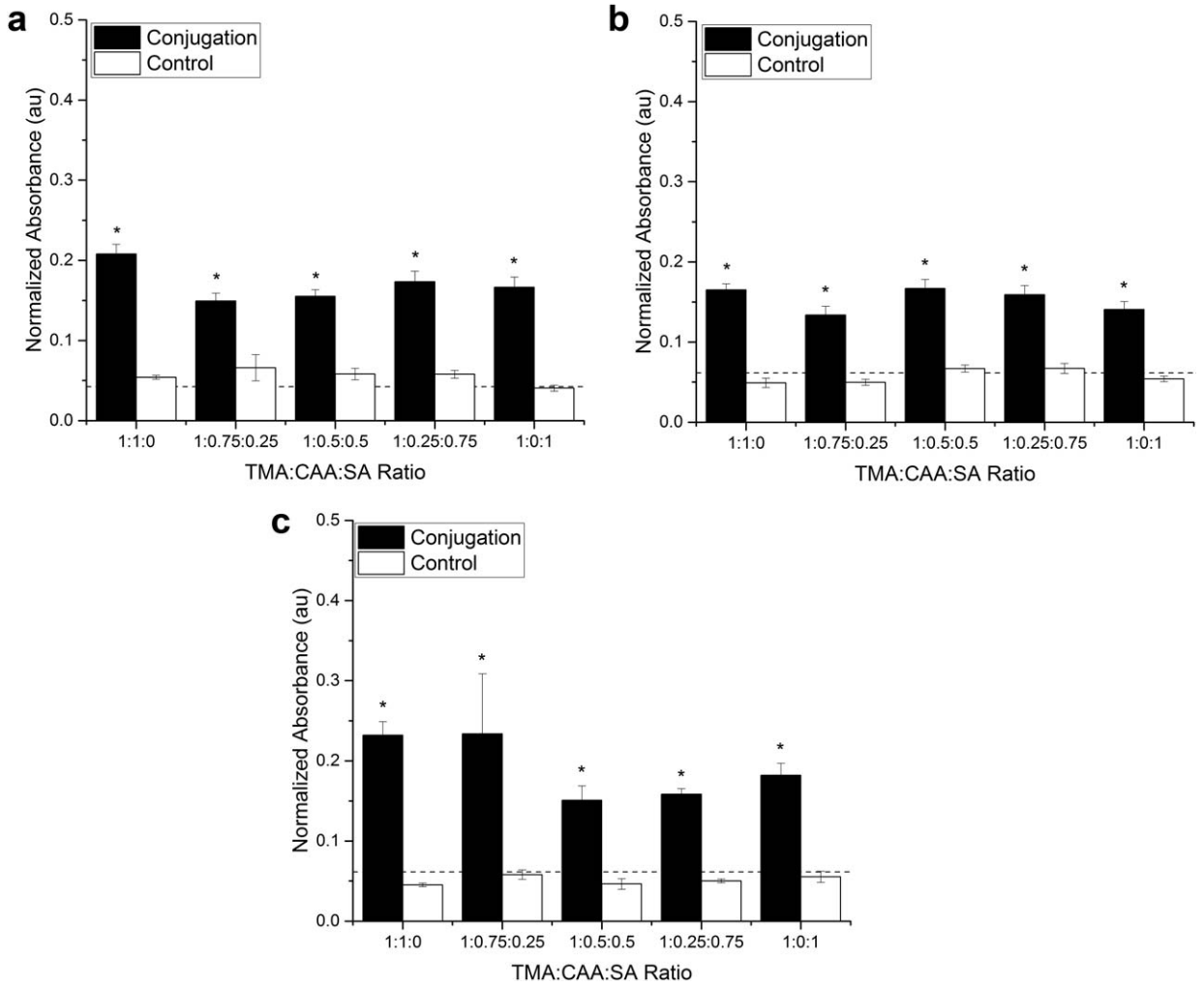

Figure 6. Mean ± propagated error of the anti-FBG response following EDC/NHS mediated conjugation of FBG to hydrogels with different TMA:CAA:SA ratios and cross-linker densities of (a) 1×, (b) 2×, and (c) 4×. The dashed line in each panel represents the anti-FBG response for the associated SBMA nonfouling 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 ($p < 0.05$).

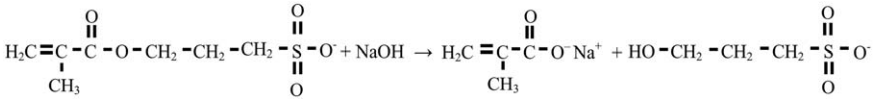

Figure 7. Proposed saponification reaction of the SA monomer.

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 ± 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 (SO_3). 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.

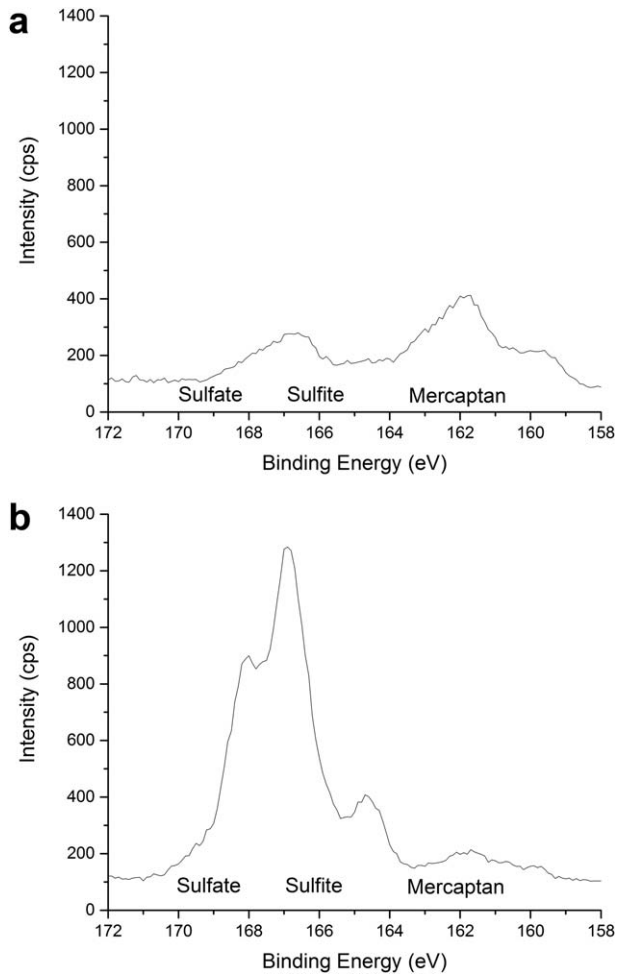

Figure 8. Representative high resolution XPS sulfur spectrum for 1:0:1 TMA:CAA:SA hydrogels with a 4× cross-linker density formed in the (a) presence or (b) absence of NaOH in the hydrogel synthesis solvent.

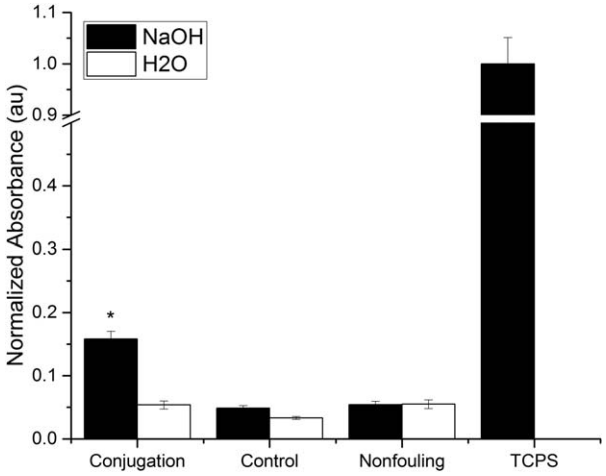

Figure 9. Mean ± propagated error of the anti-FBG response to 1:0:1 TMA:CAA:SA hydrogels with a 4× cross-linker density formed in the presence or absence of NaOH in the hydrogel synthesis solvent. A * represents a statistically significant difference in the results compared with the sample formed with 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 (SO_4) 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.

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