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Bioinspired Pseudozwitterionic Hydrogels with Bioactive Enzyme Immobilization via pH-Responsive Regulation

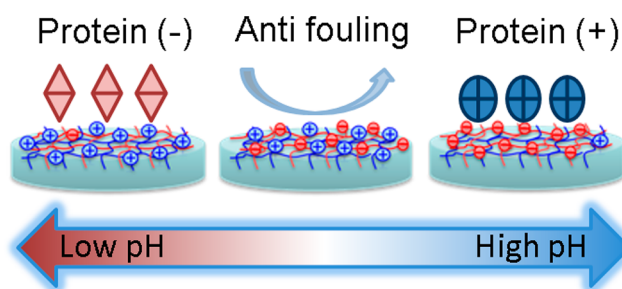
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Supporting Information

ABSTRACT: Hydrogels are hydrated networks of flexible polymers with versatile biomedical applications, and their resistance to nonspecific protein adsorption is critical. On the other hand, functionalization with other biomacromolecules would greatly enhance their biotechnological potential. The aim of this research is to prepare low fouling hydrogel polymers for selective protein immobilization. Initially, hydrogels were prepared by controlling the composition ratios of 2-carboxyethyl acrylate (CA) and 2-dimethylaminoethyl methacrylate (DMAEMA) monomers in an *N,N*-methylene-bis-acrylamide (NMBA) cross-linked free radical polymerization reaction. This series of hydrogels (C1D9 to C9D1) were then analyzed by X-ray photoelectron spectroscopy (XPS) and dynamic laser scattering to confirm the actual polymer ratios and surface charge. When the composition ratio was set at CA:6 vs DMAEMA:4 (C6D4), the hydrogel showed nearly neutral surface charge and an equivalent reaction ratio of CA vs DMAEMA in the hydrogel. Subsequent analysis showed excellent antifouling properties, low blood cell adhesion, hemocompatibility, and platelet deactivation. Moreover, this hydrogel exhibited pH responsiveness to protein adsorption and was then used to facilitate the immobilization of lipase as an indication of active protein functionalization while still maintaining a low fouling status. In summary, a mixed-charge nonfouling pseudozwitterionic hydrogel could be prepared, and its pH-responsive adsorption holds potential for designing a biocompatible tissue engineering matrix or membrane enzyme reactors.



1. INTRODUCTION

One of the key issues in the biomedical field is the development of biomimetic materials with antifouling properties and biofunctionality. An implantable biomaterial must prevent nonspecific protein adsorption upon contact with blood and other bodily fluids because it is usually the initial cascade leading to the inflammatory response followed by the encapsulation of the material or even clotting.¹ As a result, nonfouling hydrogels have long been a potential biomimetic material ever since the first generation of poly(2-hydroxyethyl methacrylate) (HEMA)-based highly hydrated polymer with a cross-linked 3D structure.² These materials are known for their good biocompatibility and have had versatile applications in biomedical research. For example, hydrogels made by HEMA are the forerunners of applications such as drug delivery,³ biosensors,⁴ and tissue-regenerating scaffolds.⁵

The second generation of antifouling hydrogels was based on poly(ethylene glycol) (PEG) polymer technology for its excellent biocompatibility.⁶ The primary mechanism of this antifouling polymer came from its water-binding capacity.⁷ PEG hydrogels were also degradable and suitable for drug release system design.⁸ However, PEG is vulnerable to oxidation and loses its antifouling status in the case of long-

term implants.⁹ Furthermore, PEG-based hydrogels were also inert for incorporating functional biomacromolecules or with unfavorable alteration chemically to affect its antifouling properties.¹⁰

Researchers then turned to zwitterionic functional groups with both positive and negative charge on the same branch. These include sulfobetaine (SB), carboxybetaine (CB), and phosphorylcholine (PC). They have recently been proposed as better biocompatible, antifouling biomaterials.¹¹ For instance, PC-based hydrogels were used to coat silica wafers with controlled ATRP (atom-transfer radical polymerization) to lessen unwanted protein adsorption when the optimal length could be modulated.¹² As for the CB-based hydrogel, Zhang and colleagues found similar performance improvements with CB-based polymer brushes functionalized onto a gold surface using a controlled ATRP protocol.¹³ Further development of

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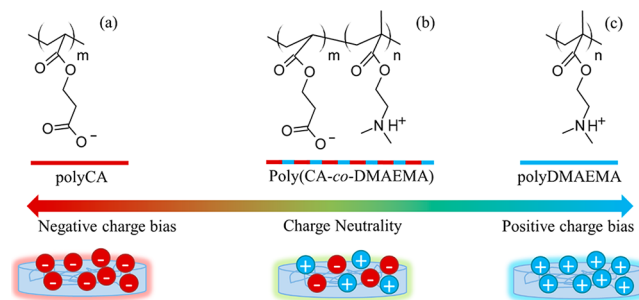
the CB-type polymer into a hydrogel format led to an antibacterial biocompatible coating.¹⁴ In addition, SBMA (2-(*N*-3-sulfopropyl-*N,N*-dimethylammonium) ethyl methacrylate) showed great versatility on multiple functionalized surfaces.¹⁵ Chang et al. developed a temperature-sensing smart hydrogel with an optimal combination of antifouling SBMA and temperature-sensitive NIPAAM (*N*-isopropylacrylamide) monomers.¹⁶ Such a strategy seems to be a valid option for hydrogel tuning. However, like the PEG-based hydrogels, the antifouling properties of most zwitterionic hydrogels may be compromised in conjugation chemistry. Interestingly, the carboxyl-group-containing CB-based materials have been shown to retain their antifouling capacity while still being open to protein immobilization through traditional EDC ((1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide)/NHS (*N*-hydroxysuccinimide) conjugation.¹⁷

On the other hand, pseudozwitterionic hydrogels are composed of positively and negatively charged monomer subunits mixed to create a neutralized water-containing surface and have been proposed to mimic antifouling zwitterionic polymers through a simplified procedure.¹⁸ Theoretically, if the charged groups from the monomers could be homogeneously distributed, then the oppositely charged functional groups could form an antifouling network of water-attracting ions through electrostatic forces.¹⁹ Subsequently, Shih et al.²⁰ have demonstrated that antifouling characteristics, the protein resistance, and the hydration capability of prepared copolymers can be effectively controlled by regulating the charge balance of the copolymers of [2-(methacryloyloxy) ethyl] trimethylammonium (TMA) and sulfopropyl methacrylate (SA) charged monomer composition. Venault et al.²¹ have also shown that pseudozwitterionic copolymer systems could be directly applied in membrane fabrication to achieve an antifouling status. On the other hand, a pseudozwitterionic hydrogel composed of 2-carboxyethyl acrylate (CA) and TMA can be designed to be pH-responsive due to the variation of the pK_a value of the charged monomers.²² Such a system was demonstrated successfully to “catch and release” bacteria upon careful situating in buffer pH. Furthermore, because the monomer contains carboxylic acid as the negatively charged side chain, the pseudozwitterionic hydrogels possess a protein conjugation capacity similar to that of the aforementioned zwitterionic CB-based materials.²³ Certain biomedical applications, such as implantable biosensors, would benefit from the combination of antifouling properties and effective enzyme conjugation onto a permeable hydrogel surface. However, effective protein immobilization seems to be contradictory to antifouling material development, particularly given the fact that the pseudozwitterionic antifouling materials are resistant to protein adsorption by nature. As a result, the pH responsiveness of the pseudozwitterionic hydrogel may serve as a new strategy for selective protein immobilization, while the effects of conjugation pH and proof of sustained antifouling in the functionalized hydrogel are still worthy of investigation. We hypothesized that pseudozwitterionic hydrogels can be formed with the ability to conditionally immobilize proteins while retaining their antifouling capacity even after thorough biofunctionalization. Such dual functionality will show wide potential for various applications.

To develop a new pseudozwitterionic hydrogel system as an antifouling pH-responsive material and test the hypothesis that the immobilization efficiency could be improved by adjusting the pH value during conjugation, pseudozwitterionic hydrogels

were prepared systematically from several mixtures of positively charged [(2-dimethyl amino)ethyl methacrylate] (DMAEMA) and negatively charged 2-carboxyethyl acrylate (CA) monomers with *N,N*-methylene-bis-acrylamide (NMBA) cross-linker. The molecular structures for all three of these monomers can be seen in Scheme 1. The physical and

Scheme 1. Chemical Structures of Hydrogel Polymers Used in This Work



chemical properties of the CA/DMAEMA hydrogels were analyzed, and the antifouling properties were also confirmed using BSA, fibrinogen, and lysozyme (LYZ) as model proteins. The serum biocompatibilities, hemolysis characteristics, and cell/bacteria adhesion were also compared through different hydrogels. Then the potential of antifouling CA/DMAEMA hydrogels as protein immobilization substrates was investigated through a series of protein conjugation studies at different conjugation pH values, activity tests, and antifouling properties after immobilization. The results clearly demonstrated that CA/DMAEMA hydrogels could be optimized for antifouling and adaptable to various protein immobilizations through the pH-responsive capability of the hydrogel.

2. MATERIALS AND METHODS

CA (2-carboxyethyl acrylate) and SBMA (2-(*N*-3-sulfopropyl-*N,N*-dimethylammonium)ethyl methacrylate) monomers were purchased from Sigma-Aldrich (U.S.A.). DMAEMA (2-dimethyl amino)ethyl methacrylate) was purchased from Acros Co. Ammonium persulfate (APS), NMBA (*N,N*-methylene-bis-acrylamide, cxa96%), *N,N,N',N'*-tetraethylmethylenediamine (TEMED), and diiodomethane were purchased from Alfa Aesar Co. In this experiment, deionized water (DI water) was purified with a Millipore water purification system with a minimum resistivity of 18.0 M Ω -m. Proteins (BSA, porcine pancreatic lipase (PPL), lysozyme, fibrinogen, and IgG) used in this experiment were purchased from Sigma-Aldrich Co.

Preparation of Poly(DMAEMA-co-CA) Hydrogels. Different mass ratios of CA and DMAEMA (Table 1) were mixed in an aqueous solution of 3.5 wt % NMBA and stirred consistently for 10 min at 25 °C. The polymerization of poly(DMAEMA-co-CA) networks was initiated using a final composition of 0.2 wt % APS and cross-linked using a 1 wt % TEMED catalyst. The reaction was carried out between a pair of glass slides with a spacer of thickness 0.2 mm at 25 °C for 1 h. After polymerization, the gel was cut into small disks with 10 mm diameter and 2 mm height. The disks were stored in DI water at 4 °C before use.

(a) The theoretical compositions of the poly(CA-co-DMAEMA) hydrogels were calculated on the basis of reaction ratios and molecular weight of comonomers.

(b) The mole fraction of polyCA (x_{CA}) and polyDMAEMA in the cross-linked poly(DMAEMA-co-CA) hydrogels was determined by XPS in the dry state from the spectral integrated area ratio of the atomic percentages based on N 1s of the polyDMAEMA isopropyl groups and C 1s of the polyCA side groups at approximately 399 and 298 eV, respectively.

Table 1. Chemical Analysis of CA/DMAEMA Hydrogels with Various Monomer Compositions

| sample ID | composition of monomer (mol %) | | reacted ratio by XPS (mol %) | |
|-----------|--------------------------------|--------|------------------------------|--------|
| | CA | DMAEMA | CA | DMAEMA |
| DMAEMA | 0 | 100 | 0 | 100 |
| C1D9 | 10 | 90 | 5.61 | 94.39 |
| C2D8 | 20 | 80 | 11.92 | 88.08 |
| C3D7 | 30 | 70 | 18.64 | 81.36 |
| C4D6 | 40 | 60 | 27.5 | 72.5 |
| C5D5 | 50 | 50 | 41.28 | 58.72 |
| C6D4 | 60 | 40 | 45.54 | 55.46 |
| C7D3 | 70 | 30 | 69.55 | 30.45 |
| C8D2 | 80 | 20 | 73.09 | 26.91 |
| C9D1 | 90 | 10 | 90.76 | 9.24 |
| CA | 100 | 0 | 100 | 0 |

Characterization of Poly(DMAEMA-co-CA) Hydrogels. The chemical structure of poly(DMAEMA-co-CA) hydrogels was characterized using X-ray photoelectron spectroscopy (XPS). XPS analysis was conducted with a Thermal Scientific K-Alpha spectrometer with a monochromated Al K X-ray source (1486.6 eV photons). The emitted electrons were measured for their energy level with a hemispherical analyzer at pass energies ranging from 50 to 150 eV. Data were collected at a takeoff angle of 45° with respect to the dried sample surface. The binding energy (BE) scale was referenced to the peak maximum in the C 1s spectrum at 284.6 eV. The high-resolution C 1s spectrum was convoluted using Shirley background subtraction. The quantified mole fraction of polyCA (x_{CA}) in the cross-linked poly(DMAEMA-co-CA) hydrogels was determined by the integrated area ratio of the atomic percentages based on N 1s of the polyDMAEMA isopropyl groups and C 1s of the polyCA side groups at binding energies of approximately 399 and 298 eV, respectively. The results are presented in Table 1. The swelling ratio of poly(DMAEMA-co-CA) hydrogels was determined using a gravimetric method. The stored hydrogels were equilibrated in DI water or PBS7.4 at 25 °C for 24 h. Immersed hydrogel disks were weighed (W_w) before the gel disks were dried under vacuum at 40 °C for 24 h. The disks were weighed (W_D) after drying. The average weights from three measurements were compiled to determine the swelling ratio by the following equation:

$$\text{swelling ratio} = \frac{W_w - W_D}{W_D}$$

The hydrophilicity of prepared hydrogels was measured with an oil-water angle meter (Kyowa Interface Science Co., Ltd., Japan) at 25 °C. The diiodomethane was dropped on the pre-equilibrated sample surface at three different sites and averaged for data analysis.

Plasma Protein Adsorption. Several different buffers (details in the Supporting Information) at different pH values for protein adsorption assessment were prepared using a universal ionic strength of 0.167 M. BSA, fibrinogen, or lysozymes (1 mg/mL) were added to buffers at pH = 3, 5, 7.4 (PBS), 9, and 11 for the adsorption assay. The hydrogel disks were first rinsed thoroughly with PBS and equilibrated with the corresponding buffer for 3 h before the test. The disks were transferred to protein solutions and allowed to adsorb protein for 2 h. The protein solution were withdrawn from the wells and analyzed with a standard UV-280 nm absorbance test or a Bradford assay to determine the residual concentration of proteins in the solution. The difference between the initial concentration of the protein and the concentration after adsorption was calculated to prove the antifouling properties. Furthermore, the adsorption of common human plasma proteins including human serum albumin (HSA), fibrinogen, and γ -globulin on the hydrogel disks was analyzed using a standard enzyme-linked immunosorbent assay (ELISA) method according to the standard protocol in previous publications.

Blood Platelet Adhesion and Hemolysis Tests. Human blood samples were obtained from volunteer donation from a local hospital under standard ethical guidance. Samples were first centrifuged for 1200 rpm to separate upper clarified platelet-rich plasma (PRP) and lower red blood cells (RBCs). They were stored at -20 and 4 °C, respectively. White blood cells were extracted using a diluted Ficoll method followed by mild centrifugation. The hydrogel disks were equilibrated in individual wells of a 24-well tissue culture plate filled with PBS for 12 h at 25 °C. Then the temperature was increased to 37 °C for 1 h. A total of 1000 μ L of the PRPs, RBCs, or WBCs was placed on the hydrogel surface and incubated for 2 h at 37 °C. After the adsorption, hydrogel disks were rinsed twice with 1000 μ L of PBS, and adhesion cells were fixed in 2.5% glutaraldehyde in PBS for 48 h at 4 °C. For RBC and WBC experiments, the disks were observed directly under a confocal laser microscope (CLSM). For PRPs, the disks were rinsed two more times with DI water and vacuum-dried using a freeze-drying machine. Afterward, the samples were plasma-coated with gold prior to observation under a JEOL JSM-5410 SEM. The number of adhering platelets on the hydrogels was counted from SEM.

To test the hemocompatibility of the blood cells, the hydrogels were placed in a 15 mL centrifugation tube and soaked with 3.3 mL of PBS. Whole blood (200 mL) was added to the tube, and the temperature was kept constant at 37 °C for 1 h. The tubes were then centrifuged at 1200 rpm to extract 200 μ L of the upper clarified solution. The absorbance values of different samples at 542 nm were determined to evaluate the release of hemoglobin from cell breakage. On the other hand, clotting times were determined by adding 2.5 mM calcium solution and PRPs to a 96-well plate. The absorbance at 660 nm was measured dynamically to evaluate the turbidity of the solution caused by clotting formation.

Fluorescence Bacterial Adhesion Assay. Recombinant bacteria *Escherichia coli* with a plasmid expression enhanced GFP were used to investigate the adhesion behavior on the surface of poly(DMAEMA-co-CA) hydrogels. *E. coli* were cultured in LB medium supplemented with inducer IPTG after 3 h of start-up culture. The cells were then incubated in a shaker for another 12 h and rinsed for adhesion studies. The *E. coli* concentration was normalized to 10⁶ cells/mL with a 600 nm absorbance assay. The hydrogel disks were equilibrated with PBS for 1 h at 37 °C and washed with PBS three times in a 24-well plate. A total of 1 mL of a bacterial suspension was added to each well. The bacteria were then incubated with the samples for 3 h at 37 °C. The bacterial solution was removed, and each sample was then washed again with PBS three times to remove the loosely attached bacteria. Strong adhesion to the surface was fixed with 0.8 mL of 2.5% glutaraldehyde and observed under CLSM.

Protein Immobilization and Activity Assay. The hydrogel disks were equilibrated with 50 mM MES buffer at pH = 4.5, 6, or 7 for 50 min at 25 °C in 12-well plates. The disks were removed and rinsed with MES buffer and soaked in EDC solution (12 mg/mL) with shaking at 20 °C for 15 min. The modification solution was then removed and replaced with 5 mg/mL porcine pancreatic lipase solution at pH = 4.5, 6, or 7. The immobilized hydrogels were then rinsed and soaked twice for 35 min with the corresponding buffer.

Lipase activity was tested by the hydrolyzation of *para*-nitrophenyl palmitate (*p*-NPP) solution at 1.65 mM in Tris-HCl buffer at pH = 8.0 for 2 h. The aqueous solution may be turbid and was further extracted with hexane after full-speed centrifugation. This clarified solution (200 μ L) was added to 40 μ L of 0.1 N NaOH placed in 96-well plates to stop the reaction. The hydrolyzed *para*-nitrophenol group was then detected by absorbance at 410 nm and used to calculate the relative activity compared to that of free suspended lipase without immobilization. Lysozyme activity tests were conducted with *E. coli* strain DHS α with the same protocol mentioned earlier in the literature.²⁴ The optical density of the suspension was adjusted to 0.6 at 600 nm wavelength to normalize the concentration of the bacteria. This suspension was then mixed with EDTA solution at pH = 8 to chelate the metal ion affecting lysozyme activity first and then added to hydrogel disks for 1 h at 37 °C. The reaction was completed, and the OD of the remaining solution was

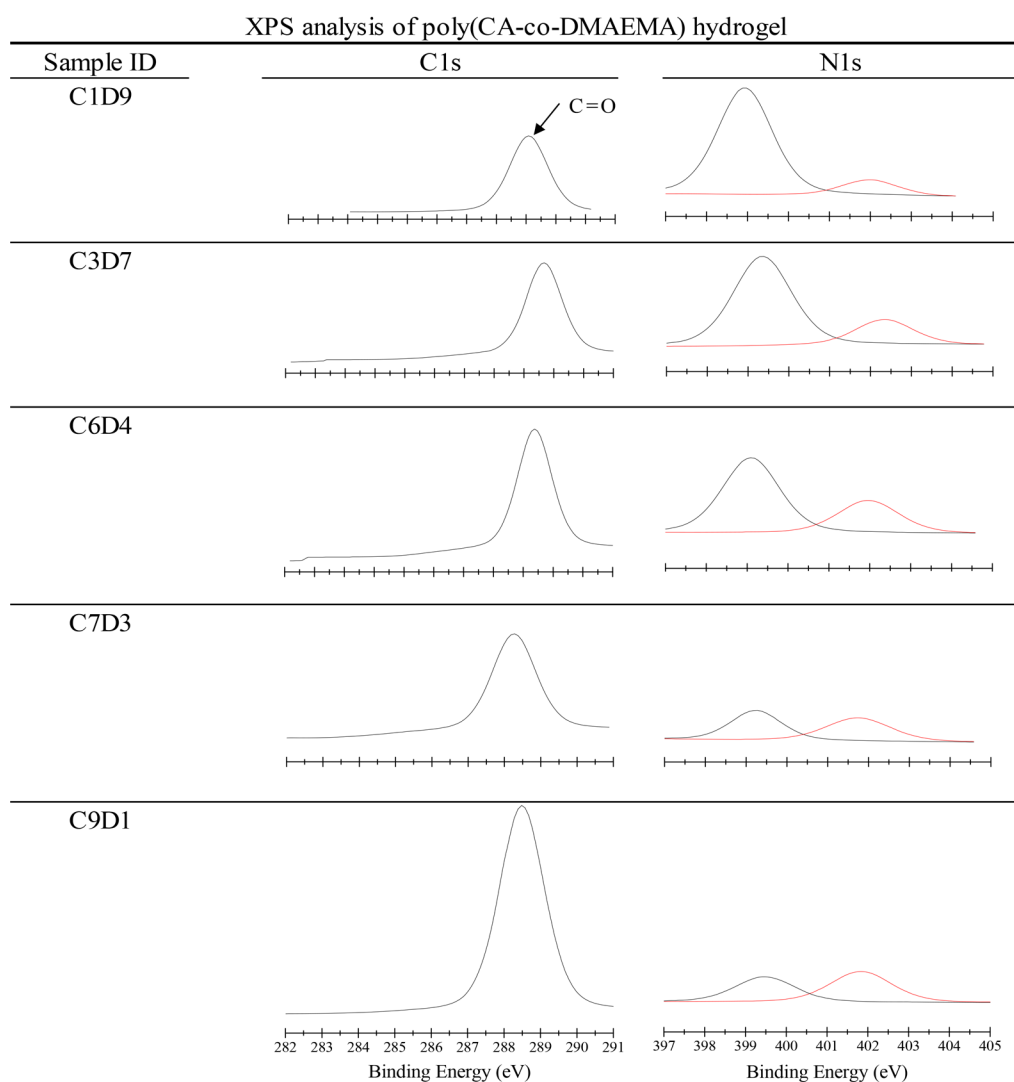


Figure 1. Analysis of the carbon–nitrogen ratio of mixed-charge hydrogels from XPS

recorded along with control groups with no hydrogel present to calculate the ΔOD value reflecting the lysozyme activity of bacterial lysis. The disks with immobilized enzymes were then subjected to the protein adsorption assay as mentioned earlier to evaluate their antifouling capability.

3. RESULTS AND DISCUSSION

Hydrogel Chemical Property Analysis. For the CA/DMAEMA hydrogels to achieve antifouling status, the oppositely charged DMAEMA and CA monomer subunits should be distributed with near equality onto the synthesized polymer chain, as indicated in the previous study.²⁵ The imbalance in the “reacted ratio” would result in a net surface charge facilitating the adsorption of oppositely charged proteins. The polymerized product was examined in detail using XPS, and the results were compared to the mixed monomer ratio in the polymerization solution. Representative spectra from the XPS analysis are listed in the [Supporting Information](#), and the results of the elemental analysis based on the area below the carboxyl (C=O) peak at 288–289 eV for CA and the tertiary amine signal at 398–399 eV for DMAEMA are presented in [Table 1](#). The spectra used for quantification are listed in [Figure 1](#) for comparison. The amounts of CA and

DMAEMA presented in the hydrogels were correlated to their composition of comonomer and named after this ratio. However, DMAEMA had a higher reaction ratio in most of the copolymers, which led to a nearly equal CA/DMAEMA ratio in sample C6D4. The data suggested that this sample preparation recipe would generate a nearly neutralized surface at physiological pH of 7.4.

In order to confirm that the reaction ratios were correlated to the charge status of hydrogels in PBS (pH = 7.4), surface zeta potential measurements were conducted on CA/DMAEMA hydrogels with all 10 comonomer compositions, and the results are also shown in [Table 1](#) and [Figure 2](#). As a control, hydrogel polymers synthesized using pure CA and pure DMAEMA were analyzed first. Pure CA and DMAEMA hydrogels have surface charge zeta potentials of -33.76 and 19.94 mV, respectively, as expected. As can be seen in [Table 1](#), the proportion of the CA reaction increased while the surface potential from the C6D4 to C9D1 hydrogel decreased. Among them, especially C5D5 to C6D4, it has been observed that the potential dropped sharply from 12.6 to -11.2 mV in the PBS environment, which was also combined with the fact that the reaction ratio was closest to 1:1 in this case. Typically, a neutral interface could be defined as the zeta potential between -10

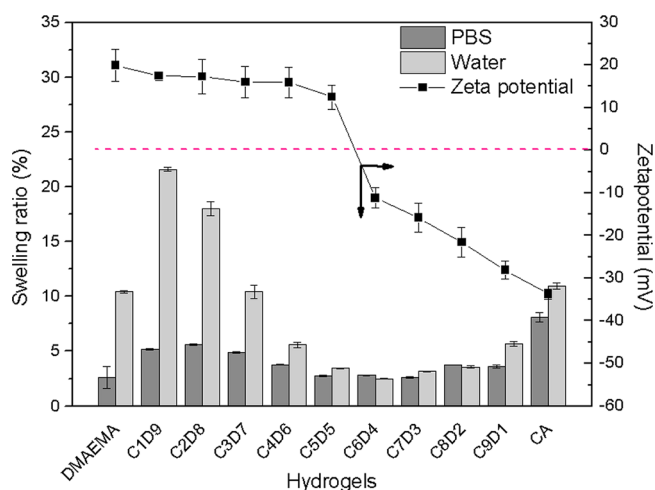


Figure 2. Physical properties of hydrogels including representative hydration ratios with varying compositions for CA/DMAEMA hydrogels and an analysis of the surface charge of mixed-charge hydrogels from DLS.

and +10 mV, and C6D4 nearly achieved this status. Venault et al.²⁶ have compared several different ratios of mixed-charge hydrogels based on negatively charged 3-sulfopropyl methacrylate (SA), 2-(methacryloyloxy)ethyl-trimethylammonium (TMA), and DMAEMA as used in this work. It was found that the SA-DMAEMA hydrogel would achieve neutrality at a slightly higher DMAEMA ratio (roughly [SA 4]/[DMAEMA 6]), but the initial monomer composition and reacted ratio showed no change. This was explained by the fact that SA has a higher charge density than DMAEMA. On the other hand, TMA with a high charge density would pair with SA to show a similar discrepancy in the initial monomer ratio and reaction ratio while neutrality could be achieved with a composition similar to that of this C6D4 hydrogel. As a result, the relative charge density of the monomer functional group may affect the formation and surface charge of mixed hydrogels.

Hydrogel Physical Characterization. The surface hydrophilic properties of the different hydrogels can be evaluated through the measurement of the diiodomethane contact angles and the calculation of the swelling ratio through the bulk mass balance. The oil-in-water diiodomethane contact angles of the hydrogels were found to be in the range of 130–145° (Supporting Information), showing overall superhydrophilicity without a clear correlation to the CA/DMAEMA ratios; this was also observed in a similar study.²⁶ The next characterization was to determine the swelling characteristics of the CA/DMAEMA hydrogels in both DI water and PBS buffer. The results were also shown in Figure 2. First, swelling occurred when the hydrogels were soaked in DI water rather than in PBS, indicating that the ionic strength tested would not trigger the so-called antipolyelectrolyte effect.^{27,28} Interestingly, the CA/DMAEMA hydrogel with a CA/DMAEMA ratio of 6:4, which possesses as nearly neutral surface as indicated in this figure, also had the lowest swelling ratio. This may result from denser cross-linking facilitated by electrostatic attraction between oppositely charged side chains. Although it is believed that a strongly hydrated surface is better for a hydrogel to be antifouling, a lower swelling ratio may actually present a smooth surface with less chance to trap larger particles. In PBS, it was clear that the ionic strength in the solution phase caused the shielding of the charged groups and reduced the swelling

ratio compared to that of the water-equilibrated hydrogels. This may indicate that the cross-linking extent was higher in C6D4 from the initial polymerization reaction. Nevertheless, the low swelling ratio suggests that the CA/DMAEMA hydrogels may serve as possible antifouling material.

Protein Adsorption Characterization. In general, the ability of a surface to resist plasma protein adsorption, especially for fibrinogen, is a direct parameter with which to evaluate the antifouling capability. To evaluate the antifouling status of this series of hydrogels, the adsorption of BSA and lysozyme on hydrogel surfaces was first evaluated using a traditional absorbance assay, and the results are presented in Figure 3. It was demonstrated that C5D5 and C6D4 did show

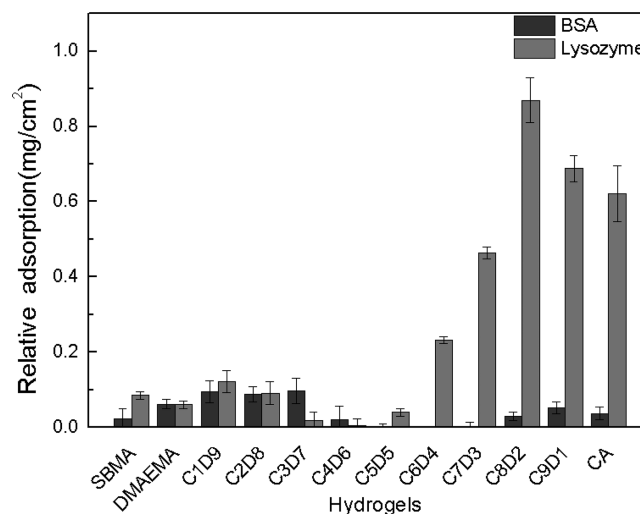


Figure 3. Evaluation of BSA and lysozyme adsorption onto hydrogels.

a comparative antifouling capability with respect to the positive control nonfouling SBMA hydrogel. Among the protein tested, BSA and fibrinogen are negatively charged in the physiological environment. As a result, better repulsion of these two proteins by the C6D4 hydrogels is expected since the zeta potential is slightly negative (−11.2 mV). However, lysozyme adsorption seems to be higher for C6D4 than for C5D5, possibly due to the slightly positive nature of the later hydrogel. To further verify the effectiveness of these two hydrogels, the adsorption of plasma proteins on surfaces including HSA and fibrinogen can be tested by a more precise ELISA method. The results are shown in Figure 4. It was found that C6D4 was also comparable with the SBMA hydrogel in a more sensitive adsorption evaluation method. Both of these types of data suggested that the C6D4 hydrogel fulfilled the antifouling requirement.

Compared to other mixed-charge systems, the zeta potential within ± 10 mV can be considered to be antifouling.^{23,29} With a zeta potential at ~ -10 mV, the closest example would be SAS/DMAEMAS as mentioned in the investigation conducted by Venault et al.²⁶

Hemolysis and Clotting Time Assay. In order to apply the hydrogel materials in biomedical engineering, it is necessary to test the biocomparability of hydrogels against blood cells because inflammatory responses may follow hemolysis and clotting caused by inappropriate chemistry on the material surface. First, the hemolysis ratios of various hydrogels were evaluated in Figure 5. Overall, a material can be considered to be nonhemolytic if the hemolysis can be

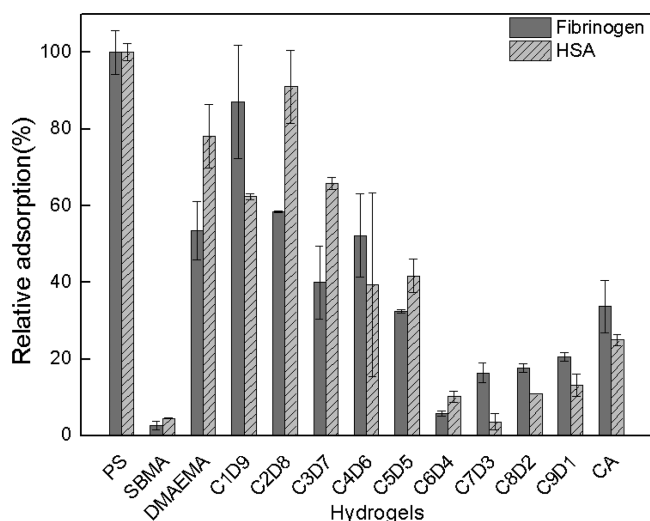


Figure 4. ELISA-based adsorption of HSA and lysozyme onto hydrogels.

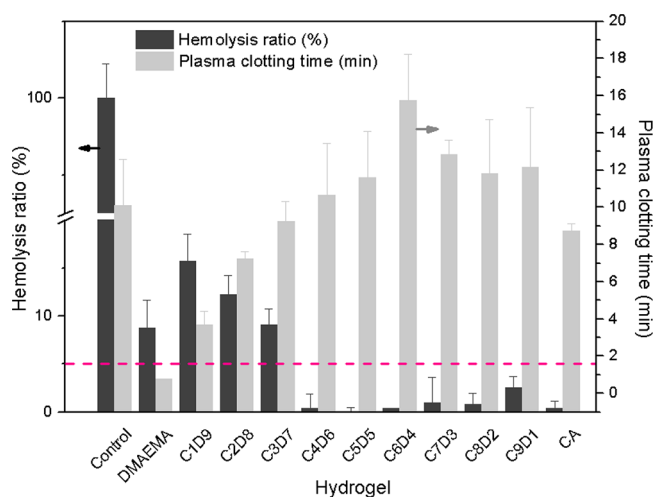


Figure 5. Evaluation of hemolysis ratio and plasma clotting time of different hydrogels

maintained below 5%, which was indicated in the figure as the thin red line.³⁰ Apparently, hydrogels with a higher DMAEMA ratio (from pure DMAEMA to C3D7) and a positive zeta potential, as demonstrated in Figure 5, were over this threshold. Fernandes et al.³¹ have proposed that the higher positive charge would attract the negatively charged membrane of erythrocytes and lead to subsequent breakage. In accordance, this was also observed in our red blood cell attachment test (Supporting Information). On the other hand, hemolysis was soon almost nonexistent for other hydrogels, especially C6D4, which is essential for even more stressful environments in practical applications.

Upon contacting blood, biomaterials may induce clotting resulting from a cascade of platelet adhesion, activation, and procoagulation.³² These combined effects usually induce thrombin formation and shorten the plasma clotting time. Because the mechanism behind these hydrogels was designed to prevent platelet adhesion to improve hemocompatibility, platelet-rich plasma (PRP) was used to challenge disks of hydrogels in a 96-well plate at 37 °C to evaluate their recalcified plasma clotting time. The results were presented in

Figure 5 with the clotting time on the right axis. As indicated, the average clotting time in blank wells was 10 min and served as a control standard, which generally should be the lower threshold for hemocompatible materials. Compared to the zeta potential results in Figure 2, when the positively charged hydrogels (DMAEMA to C5D5) were placed in the recalcified solution, the clotting time decreased to below the standard time. In the literature, positively charged surfaces can activate plasma clotting through factor VII activating protease pathway.³³ Among all of the hydrogels tested, C6D4 exhibited 60% more prolonged clotting time with statistical significance. This value slightly exceeded that of other pseudozwitterionic materials using similar testing protocols.³⁴ In general, material interfaces can be considered to be blood-inert if they present a high surface and bulk hydrophilicity along with the neural zeta potential close to 10 to -10 mV. Consequently, C6D4 showed optimal performance in these tests, with both a low hemolysis percentage compared to blank polystyrene surface and the longest clotting time among all hydrogels. These results showed that C6D4 can be used as a potential biocompatible material.

Blood Cell Attachment Assays. For a long-term implant to be stably functional in the vascular system, the biomaterial must exhibit a low fouling ability against both proteins and larger blood cells. There are three types of blood cells interacting with materials (RBCs, WBCs, and PRPs) with quite diverse interaction schemes toward different materials. Here, all three types of blood cells were tested against hydrogels with 10 different mixed ratios to compare their efficacy. Because of the limiting paragraph, only results obtained from ImageJ-quantitative CLSM data from an area of 0.645 mm × 0.645 mm = 0.416 mm² is presented in Figure 6. The original images

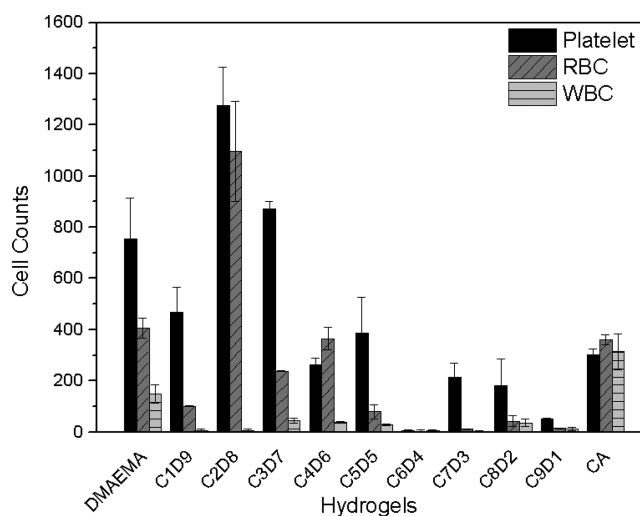


Figure 6. Quantitative results of blood cells adhesions onto CA:DMAEMA hydrogels including platelets, erythrocytes (RBC), and leukocytes (WBC).

for analysis can be found in the Supporting Information. It can be found that C6D4 is almost immune to fibrinogen adhesion, which is usually the most difficult to repel because of its fibrous nature and potential danger in activating clotting formation. As to other types of blood cells, the intermediate monomer ratio ranging from C5D5 to C7D3 exhibited nonattachment results.

Platelets adhered to biomaterials may also be activated by surface chemistry with a proper activator such as calcium ions

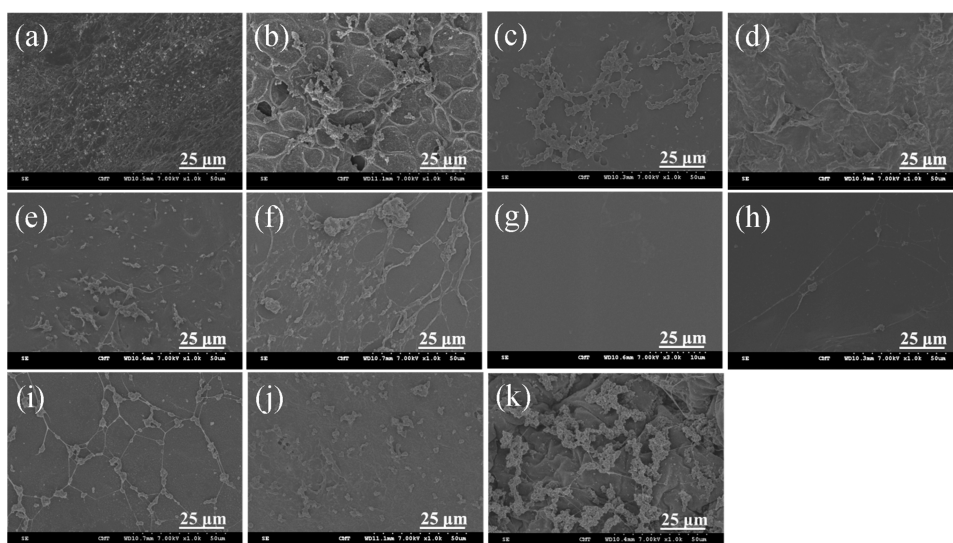


Figure 7. Evaluation of activated platelet adhesion on the CA/DMAEMA hydrogel with SEM images: (a) DMAEMA, (b) C1D9, (c) C2D8, (d) C3D7, (e) C4D6, (f) C5D5, (g) C6D4, (h) C7D3, (i) C8D2, (j) C9D1, and (k) CA.

and provide a site for further clotting cascades and serious fouling.³⁵ To assess the activated platelet adhesion, hydrogels disks were soaked in a calcium-induced platelet test solution, washed, and lyophilized to prepare the sample for imaging. SEM pictures were taken and compared in Figure 7. Surpassing the expectation, the surface of C6D4 hydrogels had a smooth texture without the presence of any activated fibers from platelet adhesion. The performance of the C6D4 hydrogel was thus on par with the more expensive SBMA with respect to blood compatibility. Kwak et al.³⁶ showed that the adhesion and activation of platelets from the blood-derived fluid could be correlated with the adsorption of fibrinogen on surfaces, which is examined in Figure 4 with respect to this work. The two data sets agreed with each other. In the literature, it is generally agreed that the charge neutrality achieved by the proper mixing of amine with the carboxyl group would allow low platelet adhesion and activation.³⁷

Zeta Potential Change for pH Variation and Protein Adhesion Analysis. Because the C6D4 hydrogel could now be considered to be a good antifouling candidate, it is crucial to evaluate the pH responsiveness of surface charge against charged biased proteins such as negatively charged BSA. As a result, it is essential to measure the zeta potential change with respect to the pH value for both hydrogels and proteins. This was done by soaking proteins and ground hydrogels in different pH buffers with the same ionic strength and analyzing using a DLS device. The result is illustrated in Figure 8. It is demonstrated that at physiological pH, C6D4 carries a slightly negative charge, and this might be the reason that lysozyme, which is a positively charged protein with a pI value of 11, could be attractive to C6D4 hydrogels. As a result, we proposed that the immobilization of negatively charged protein could be favored at lower pH while positively charged protein could be used at higher physiological pH. As indicated in Figure 9, the adsorption of BSA was higher at lower pH while the adsorption of lysozyme was higher at higher pH, corresponding to the zeta potential change.

Protein Immobilization and Activity Tests. To take advantage of the surface charge responsiveness of this hydrogel material, a reasonable application is to exploit protein immobilization under different pH values and test its activity.

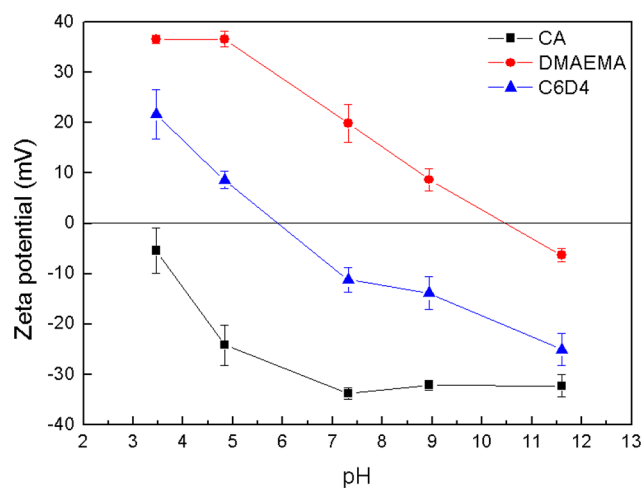


Figure 8. pH responsiveness of a C6D4 hydrogel during a zeta potential measurement.

For comparison biofunctionalization of the hydrogel for protein recognition and biocatalysis applications, it is necessary to develop a viable protocol to covalently bind active protein. As a result, a biochemical assay showing that the protein still possesses its catalytic function may be direct evidence of structural integrity. Here we selected lipase as a model enzyme for its comprehensive immobilization data in the literature³⁸ and various application in the biotechnology and fine chemicals industries.³⁹ Lipases are biphasic proteins operating optimally at the oil–water interface, and hydrogel could be a proper substrate to provide an aqueous environment. The model enzyme is a negatively charged protein and has an isoelectric point ($pI = 5.18$)⁴⁰ that is close to that of BSA. The protein could be conjugated onto hydrogel using EDC chemistry targeting the carboxyl group on the hydrogel surface. In Figure 10, the activity test results are illustrated using different pH values with different immobilization methods. The hydrogels were processed in three distinctive ways. In the “Modify (no enzyme)” group, the background levels of substrate hydrolyzation in C6D4 hydrogels treated with EDC, but without enzyme loading, were tested for control

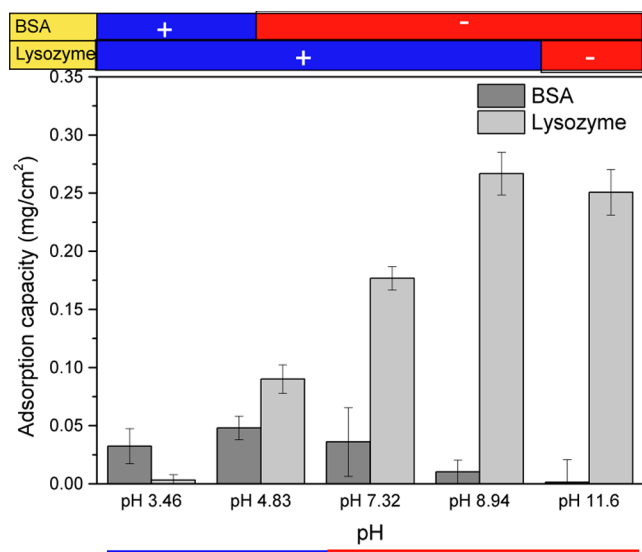


Figure 9. BSA and lysozyme adsorption onto a C6D4 hydrogel at various pH values.

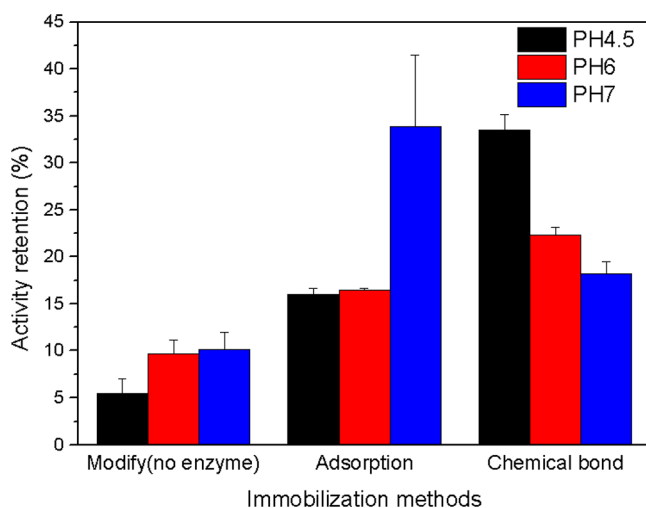


Figure 10. Activity tests among immobilized hydrogels at various pH values.

and comparison. Next, enzymes were loaded and washed in hydrogels without EDC conjugation in the “Adsorption” group, while the hydrogels were treated with EDC and loaded with enzyme solution for covalent immobilization in the “Chemical bond” group. Among all of the parameters tested, the highest lipase activity was observed with immobilization carried out at pH = 4.5, followed by simple physical adsorption at pH = 7. The latter could be considered to be a control for protein activity at neutral pH. Despite the low pH, it was indicated that the zeta potential difference was favorable for lipase to be adhered and covalently bonded to the surface as shown by the XPS analysis presented in the [Supporting Information](#). The data suggested that lipase was not denatured at lower pH and the facilitated conjugation by EDC only without NHS would result in a higher retention of enzymatic activity as the literature suggested.⁴¹ From the swelling ratio measurements with pH values from 4.5 to 9.0 ([Supporting Information](#)), it was clear that the poly(CA-co-DMAEMA) hydrogel displayed a relatively stable swelling ratio. This indicates that the protein conjugation at low pH might not be

due to the variation in the physical structure of the hydrogel upon buffer switching.²⁸ On the other hand, the direct conjugation capability by EDC-only chemistry seems to favor lower pH as reported previously. These results suggested the electrostatic interaction at the protein–poly(CA-co-DMAEMA) interface and might be the major contributors to the significant increase in protein conjugation at lower pH.

Protein Adsorption Characterization on Immobilized Hydrogel Disks. One key issue that is often neglected in protein immobilization studies is the relapse of protein fouling due to the immobilized protein surface. As a result, it is interesting to determine if such functionalization would result in serious fouling after immobilization. The BSA adsorption onto lipase-immobilized hydrogel is illustrated in [Figure 11](#). To

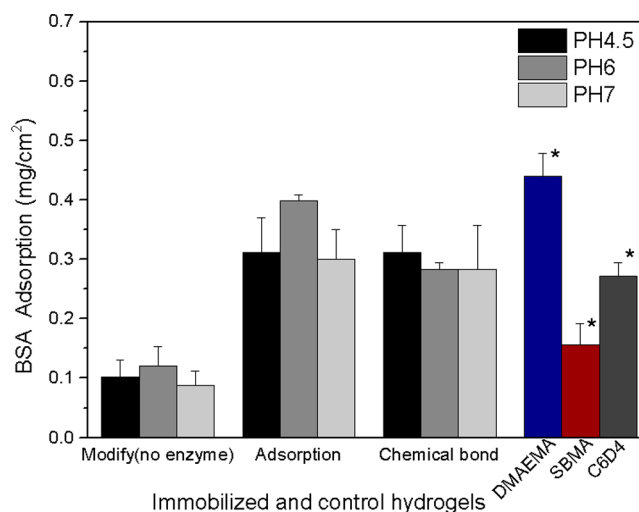


Figure 11. Protein adsorption on C6D4 hydrogels with immobilized lipase.

our surprise, C6D4 modified with only EDC showed improved anti-BSA adhesion while lipase covalently bound to hydrogels had a similar extent of adhesion compared to blank C6D4, but both physically adsorbed and covalent-immobilized groups were slightly inferior to low-fouling-standard SBMA. On the other hand, the immobilization pH did not cause measurable differences among various groups. This may indicate that the EDC may serve as an extra cross-linking initiator without the presence of enzymes. On the other hand, lipase may share similar charge properties compared to C6D4, resulting in overall similar protein adhesion behavior.

CONCLUSIONS

To summarize this work, multifunctional pseudozwitterionic hydrogels were synthesized from mixtures of positively charged DMAEMA and negatively charged CA monomers, and their physical and chemical properties were characterized. These hydrogels were demonstrated to resist nonspecific protein adsorption from both BSA and fibrinogen. Subsequent studies using blood cells and bacteria showed that the C6D4 hydrogel could perform well with high biocompatibility and excellent antifouling status. Furthermore, the CA/DMAEMA hydrogels were shown to have different absorption profiles toward proteins with charge bias, while retaining both their biochemical activity and fouling-resistant properties away from immobilized protein/hydrogel complexes. This research article presents a novel approach to the development of

biofunctionalized materials with pseudozwitterionic materials that may provide low fouling properties while exhibiting favorable bioactivity upon choice.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.langmuir.8b02483](https://doi.org/10.1021/acs.langmuir.8b02483).

Complete XPS spectrum for mixed charged hydrogels; XPS analysis for a reacted ratio of monomers in hydrogels; oil-in-water contact angle of mixed charged hydrogels; image of platelet adhesion observed by confocal microscopy; image of erythrocyte adhesion observed by confocal microscope; image of leukocytes adhesion observed by confocal microscope; image of fluorescent *E. coli* adhesion observed by confocal microscope (PDF)

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Notes

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■ REFERENCES

- (1) Anderson, J. M.; Rodriguez, A.; Chang, D. T. Foreign body reaction to biomaterials. *Semin. Immunol.* **2008**, *20*, 86–100.
- (2) Hoffman, A. S. Hydrogels for biomedical applications. *Adv. Drug Delivery Rev.* **2002**, *54*, 3–12.
- (3) Alarcon, C. D. H.; Pennadam, S.; Alexander, C. Stimuli responsive polymers for biomedical applications. *Chem. Soc. Rev.* **2005**, *34*, 276–285.
- (4) Brahim, S. I.; Maharajh, D.; Narinesingh, D.; Guiseppi-Elie, A. Design and characterization of a galactose biosensor using a novel polypyrrole-hydrogel composite membrane. *Anal. Lett.* **2002**, *35*, 797–812.
- (5) Liu, L.; Chen, G.; Chao, T.; Ratner, B. D.; Sage, E. H.; Jiang, S. Reduced foreign body reaction to implanted biomaterials by surface treatment with oriented osteopontin. *J. Biomater. Sci., Polym. Ed.* **2008**, *19*, 821–835.
- (6) Chang, Y.; Ko, C.-Y.; Shih, Y.-J.; Quemener, D.; Deratani, A.; Wei, T.-C.; Wang, D.-M.; Lai, J.-Y. Surface grafting control of PEGylated poly(vinylidene fluoride) antifouling membrane via surface-initiated radical graft copolymerization. *J. Membr. Sci.* **2009**, *345*, 160–169.
- (7) Ostuni, E.; Chapman, R. G.; Holmlin, R. E.; Takayama, S.; Whitesides, G. M. A Survey of Structure–Property Relationships of Surfaces that Resist the Adsorption of Protein. *Langmuir* **2001**, *17*, 5605–5620.
- (8) Zustiak, S. P.; Leach, J. B. Hydrolytically Degradable Poly(Ethylene Glycol) Hydrogel Scaffolds with Tunable Degradation and Mechanical Properties. *Biomacromolecules* **2010**, *11*, 1348–1357.
- (9) Han, S.; Kim, C.; Kwon, D. Thermal/oxidative degradation and stabilization of polyethylene glycol. *Polymer* **1997**, *38*, 317–323.
- (10) Michel, R.; Pasche, S.; Textor, M.; Castner, D. G. Influence of PEG Architecture on Protein Adsorption and Conformation. *Langmuir* **2005**, *21*, 12327–12332.
- (11) Chen, S.; Li, L.; Zhao, C.; Zheng, J. Surface hydration: Principles and applications toward low-fouling/nonfouling biomaterials. *Polymer* **2010**, *51*, 5283–5293.
- (12) Chen, S. F.; Zheng, J.; Li, L. Y.; Jiang, S. Y. Strong resistance of phosphorylcholine self-assembled monolayers to protein adsorption: Insights into nonfouling properties of zwitterionic materials. *J. Am. Chem. Soc.* **2005**, *127*, 14473–14478.
- (13) Zhang, Z.; Chao, T.; Chen, S.; Jiang, S. Superlow fouling sulfobetaine and carboxybetaine polymers on glass slides. *Langmuir* **2006**, *22*, 10072–10077.
- (14) Cheng, G.; Xue, H.; Li, G.; Jiang, S. Integrated Antimicrobial and Nonfouling Hydrogels to Inhibit the Growth of Planktonic Bacterial Cells and Keep the Surface Clean. *Langmuir* **2010**, *26* (13), 10425–10428.
- (15) Chang, Y.; Chen, S. F.; Zhang, Z.; Jiang, S. Y. Highly protein-resistant coatings from well-defined diblock copolymers containing sulfobetaines. *Langmuir* **2006**, *22* (5), 2222–2226.
- (16) Chang, Y.; Chen, W.-Y.; Yandi, W.; Shih, Y.-J.; Chu, W.-L.; Liu, Y.-L.; Chu, C.-W.; Ruaan, R.-C.; Higuchi, A. Dual-Thermoresponsive Phase Behavior of Blood Compatible Zwitterionic Copolymers Containing Nonionic Poly(N-isopropyl acrylamide). *Biomacromolecules* **2009**, *10*, 2092–2100.
- (17) Vaisocherova, H.; Yang, W.; Zhang, Z.; Cao, Z.; Cheng, G.; Piliarik, M.; Homola, J.; Jiang, S. Ultralow fouling and functionalizable surface chemistry based on a zwitterionic polymer enabling sensitive and specific protein detection in undiluted blood plasma. *Anal. Chem.* **2008**, *80*, 7894–7901.
- (18) Chen, S.; Jiang, S. A new avenue to nonfouling materials. *Adv. Mater.* **2008**, *20*, 335.
- (19) Chang, Y.; Shu, S. H.; Shih, Y. J.; Chu, C. W.; Ruaan, R. C.; Chen, W. Y. Hemocompatible Mixed-Charge Copolymer Brushes of Pseudozwitterionic Surfaces Resistant to Nonspecific Plasma Protein Fouling. *Langmuir* **2010**, *26*, 3522–3530.
- (20) Shih, Y. J.; Chang, Y.; Quemener, D.; Yang, H. S.; Jhong, J. F.; Ho, F. M.; Higuchi, A.; Chang, Y. Hemocompatibility of Polyampholyte Copolymers with Well-Defined Charge Bias in Human Blood. *Langmuir* **2014**, *30*, 6489–6496.
- (21) Venault, A.; Wei, T. C.; Shih, H. L.; Yeh, C. C.; Chinnathambi, A.; Alharbi, S. A.; Carretier, S.; Aimar, P.; Lai, J. Y.; Chang, Y. Antifouling pseudo-zwitterionic poly(vinylidene fluoride) membranes with efficient mixed-charge surface grafting via glow dielectric barrier discharge plasma-induced copolymerization. *J. Membr. Sci.* **2016**, *516*, 13–25.
- (22) Mi, L.; Bernards, M. T.; Cheng, G.; Yu, Q.; Jiang, S. pH responsive properties of non-fouling mixed-charge polymer brushes based on quaternary amine and carboxylic acid monomers. *Biomaterials* **2010**, *31*, 2919–2925.
- (23) 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*, 3112–3122.
- (24) Muriel-Galet, V.; Talbert, J. N.; Hernandez-Munoz, P.; Gavara, R.; Goddard, J. M. Covalent Immobilization of Lysozyme on Ethylene Vinyl Alcohol Films for Nonmigrating Antimicrobial Packaging Applications. *J. Agric. Food Chem.* **2013**, *61*, 6720–6727.
- (25) Zhang, Z.; Chen, S. F.; Chang, Y.; Jiang, S. Y. Surface grafted sulfobetaine polymers via atom transfer radical polymerization as superlow fouling coatings. *J. Phys. Chem. B* **2006**, *110*, 10799–10804.
- (26) Venault, A.; Hsu, K. J.; Yeh, L. C.; Chinnathambi, A.; Ho, H. T.; Chang, Y. Surface charge-bias impact of amine-contained

pseudozwitterionic biointerfaces on the human blood compatibility. *Colloids Surf, B* **2017**, *151*, 372–383.

(27) van der Linden, H. J.; Herber, S.; Olthuis, W.; Bergveld, P. Stimulus-sensitive hydrogels and their applications in chemical (micro)analysis. *Analyst* **2003**, *128*, 325–331.

(28) Qiu, Y.; Park, K. Environment-sensitive hydrogels for drug delivery. *Adv. Drug Delivery Rev.* **2012**, *64*, 49–60.

(29) Dobbins, S. C.; McGrath, D. E.; Bernards, M. T. Nonfouling Hydrogels Formed from Charged Monomer Subunits. *J. Phys. Chem. B* **2012**, *116* (49), 14346–14352.

(30) Singhal, J. P.; Ray, A. R. Synthesis of blood compatible polyamide block copolymers. *Biomaterials* **2002**, *23*, 1139–1145.

(31) Fernandes, H. P.; Cesar, C. L.; Barjas-Castro, M. d. L. Electrical properties of the red blood cell membrane and immunohematological investigation. *Revista brasileira de hematologia e hemoterapia* **2011**, *33*, 297–301.

(32) Chang, Y.; Shih, Y. J.; Ko, C. Y.; Jhong, J. F.; Liu, Y. L.; Wei, T. C. Hemocompatibility of Poly(vinylidene fluoride) Membrane Grafted with Network-Like and Brush-Like Antifouling Layer Controlled via Plasma-Induced Surface PEGylation. *Langmuir* **2011**, *27*, 5445–5455.

(33) Sperling, C.; Maitz, M. F.; Grasso, S.; Werner, C.; Kanse, S. M. A Positively Charged Surface Triggers Coagulation Activation Through Factor VII Activating Protease (FSAP). *ACS Appl. Mater. Interfaces* **2017**, *9*, 40107–40116.

(34) Zhang, Z.; Zhang, M.; Chen, S.; Horbetta, T. A.; Ratner, B. D.; Jiang, S. Blood compatibility of surfaces with superlow protein adsorption. *Biomaterials* **2008**, *29*, 4285–4291.

(35) Sivaraman, B.; Latour, R. A. The relationship between platelet adhesion on surfaces and the structure versus the amount of adsorbed fibrinogen. *Biomaterials* **2010**, *31*, 832–839.

(36) Kwak, D.; Wu, Y. G.; Horbett, T. A. Fibrinogen and von Willebrand's factor adsorption are both required for platelet adhesion from sheared suspensions to polyethylene preadsorbed with blood plasma. *J. Biomed. Mater. Res., Part A* **2005**, *74*, 69–83.

(37) Chuang, W. H.; Lin, J. C. Surface characterization and platelet adhesion studies for the mixed self-assembled monolayers with amine and carboxylic acid terminated functionalities. *J. Biomed. Mater. Res., Part A* **2007**, *82*, 820–830.

(38) Ye, P.; Wan, R. B.; Wang, X. P. Quantitative enzyme immobilization: Control of the carboxyl group density on support surface. *J. Mol. Catal. B: Enzym.* **2009**, *61*, 296–302.

(39) Stergiou, P. Y.; Foukis, A.; Filippou, M.; Koukouritaki, M.; Parapouli, M.; Theodorou, L. G.; Hatziloukas, E.; Afendra, A.; Pandey, A.; Papamichael, E. M. Advances in lipase-catalyzed esterification reactions. *Biotechnol. Adv.* **2013**, *31*, 1846–1859.

(40) Donner, J. Preparation of porcine pancreatic lipase free of co-lipase activity. *Acta Chemica Scandinavica Series B-Organic Chemistry and Biochemistry* **1976**, *30*, 430–434.

(41) Rodrigues, A. R.; Cabral, J. M. S.; Taipa, M. A. Immobilization of *Chromobacterium viscosum* lipase on Eudragit S-100: coupling, characterization and kinetic application in organic and biphasic media. *Enzyme Microb. Technol.* **2002**, *31*, 133–141.