Dual-Functional Biomimetic Materials: Nonfouling Poly(carboxybetaine) with Active Functional Groups for Protein Immobilization

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We introduce a dual-functional biocompatible material based on zwitterionic poly(carboxybetaine methacrylate) (polyCBMA), which not only highly resists protein adsorption/cell adhesion, but also has abundant functional groups convenient for the immobilization of biological ligands, such as proteins. The dual-functional properties are unique to carboxybetaine moieties and are not found in other nonfouling moieties such as ethylene glycol, phosphobetaine, and sulfobetaine. The unique properties are demonstrated in this work by grafting a polyCBMA polymer onto a surface or by preparing a polyCBMA-based hydrogel. PolyCBMA brushes with a thickness of 10−15 nm were grafted on a gold surface using the surface-initiated atom transfer radical polymerization method. Protein adsorption was analyzed using a surface plasmon resonance sensor. The surface grafted with polyCBMA very largely prevented the nonspecific adsorption of three test proteins, that is, fibrinogen, lysozyme, and human chorionic gonadotropin (hCG). The immobilization of anti-hCG on the surface resulted in the specific binding of hCG while maintaining a high resistance to nonspecific protein adsorption. Transparent polyCBMA-based hydrogel disks were decorated with immobilized fibronectin. Aortic endothelial cells did not bind to the polyCBMA controls, but appeared to adhere well and spread on the fibronectin-modified surface. With their dual functionality and biomimetic nature, polyCBMA-based materials are very promising for their applications in medical diagnostics, biomaterials/tissue engineering, and drug delivery.

Introduction

For biosensor or biomaterial applications, it is desirable to have a nonfouling surface or material as a background while presenting an abundance of functional groups for ligand immobilization.[1,2] Mixed carboxylic (or amino) and hydroxyl-terminated oligo(ethylene glycol) (OEG) self-assembled monolayers (SAMs)[3] or carboxymethyl dextran polymers[2,4] are currently used in biosensor applications. Copolymers containing decoratable segments and non-fouling poly(ethylene glycol) (PEG) polymers or low-fouling poly(hydroxyethyl methacrylate) segments have been prepared to control cell and tissue responses.[1] However, extra (or unreacted) functional groups can compromise the specificity of a sensor or the biocompatibility of a biomaterial.[3]

Surface resistance to protein adsorption and cell adhesion is critical for the development of biosensors with high specificity and biomaterials with superior compatibility. OEG- or PEG-based materials such as OEG SAMs[5] or poly(OEG methacrylate)[6] are the most commonly used nonfouling materials. However, it is now recognized that OEG or PEG is subject to oxidation in the presence of oxygen and transition metal ions found in most biochemically relevant solutions.[7−9] Biomimetic phosphorylcholine (PC)-based materials contain PC headgroups, which are found in the outside layer of cell membranes. Polymers or surfaces modified with PC have been shown to reduce protein adsorption.[10−13] Our recent studies demonstrate for the first time that poly(sulfobetaine methacrylate) (polySBMA)-grafted surfaces have very low protein adsorption, that is, <0.3 ng/cm^2 fibrinogen adsorption, when they are grafted from a surface via the surface-initiated atom transfer radical polymerization (ATRP) method.[14] We also detected 3 ng/cm^2 fibrinogen adsorption when copolymers containing polySBMA are physically adsorbed on a surface.[15] These results indicate that polySBMA surfaces are capable of resisting nonspecific protein adsorption to a level comparable with well-packed OEG SAMs.[16] Recent studies attribute the nonfouling properties of OEG SAMs to their strong hydration capabilities and well-packed structures.[7,9,17] While hydrophilic and neutral OEG or PEG forms a hydration layer via hydrogen bonds, zwitterions form a hydration layer via electrostatic interactions.[13] It is expected that zwitterions are capable of binding significant quantities of water and are therefore potentially excellent candidates for nonfouling materials. However, like OEG- or PEG-based polymers, PC and SBMA do not have functional groups for ligand immobilization.

Here, we introduce a biomimetic material based on poly(carboxybetaine methacrylate) (polyCBMA), with the dual role of preventing nonspecific protein adsorption leading to fouling while permitting covalent decoration with bioactive proteins. The zwitterionic structure of CBMA is similar to that of glycine betaine, which is one of the solutes vital to the osmotic regulation of living organisms.[18] Its biomimetic nature makes it attractive for many biomedical applications. Recent studies have shown that polyCBMA copolymers reduced platelet adhesion and protein adsorption.[19,20] However, its strong resistance to protein adsorption and dual functionality have not been well recognized and explored. In this work, we demonstrate the dual-functional properties of polyCBMA materials by either grafting polyCBMA polymers outward from a surface or by preparing a polyCBMA-based hydrogel. Both polyCBMA-grafted surfaces and hydrogels are shown to be highly resistant to nonspecific protein adsorption or cell adhesion before and

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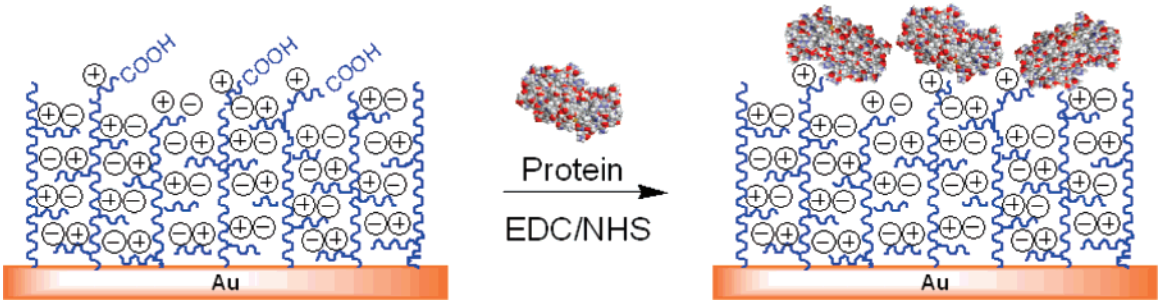
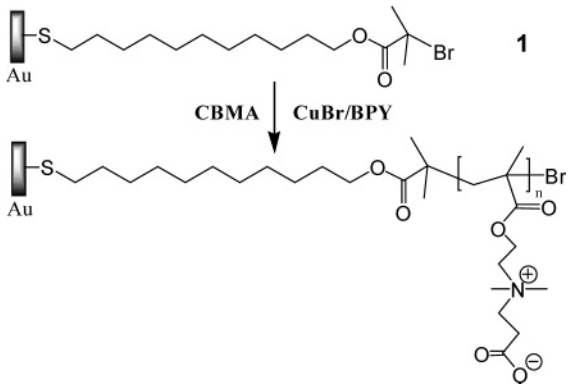


Figure 1. Schematic of a polyCBMA-grafted surface (left) with immobilized proteins (right).

Scheme 1



after ligand immobilization. At the same time, the abundant carboxylic groups of polyCBMA make it possible to covalently bind amino groups of, for example, proteins, using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysuccinimide (EDC/NHS) chemistry (Figure 1). This material is very promising for a wide range of applications in medical diagnostics, biomaterials/tissue engineering, and drug delivery.

Experimental Section

Materials. Human plasma fibrinogen and chicken egg white lysozyme were purchased from Sigma-Aldrich (Milwaukee, WI). Human plasma fibronectin was purchased from Chemicon International (Temecula, CA). Human chorionic gonadotropin (hCG) and its monoclonal mouse antibody (mAb; isotype IgG1) were purchased from Scripps Laboratories (San Diego, CA). 2-(*N*,*N*′-dimethylamino)ethyl methacrylate (DMAEM, 98%), *β*-propiolactone (95%), copper (I) bromide (99.999%), bromoisobutyryl bromide (98%), 11-mercapto-1-undecanol (97%), 2,2′-bipyridine (BPY 99%), tetrahydrofuran (THF, HPLC grade), NHS, and EDC, were purchased from Sigma-Aldrich (Milwaukee, WI). Phosphate-buffered saline (PBS: 0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4) was purchased from Sigma Chemical Co. Ethanol (absolute 200 proof) was purchased from AAPER Alcohol and Chemical Co. Water used in experiments was purified using a Millipore water purification system with a minimum resistivity of 18.0 MΩ·cm. THF for reactions and washings was dried by sodium before use.

Mercaptoundecyl bromoisobutyrate was synthesized through the reaction of bromoisobutyryl bromide and 11-mercapto-1-undecanol using a method published previously.[21] 1H NMR (300 MHz, $CDCl_3$): 4.15 (t, J = 6.9 Hz, 2H, OCH_2), 2.51(q, J = 7.5 Hz, 2H, SCH_2), 1.92 (s, 6H, CH_3), 1.57−1.72(m, 4H, CH_2), and 1.24−1.40 (m, 16H, CH_2). Surface characterization of mercaptoundecyl bromoisobutyrate SAMs (**1**, Scheme 1) on gold surfaces with X-ray photoelectron spectroscopy (XPS) and atomic force microscopy was reported in our previous paper.[14]

Synthesis of a CBMA Monomer. A CBMA monomer, 2-carboxy-*N*,*N*-dimethyl-*N*-(2′- methacryloyloxyethyl) ethanaminium inner salt, was synthesized by the reaction of DMAEM with *β*-propiolactone. *β*-Propiolactone (0.87 g, 12 mmol) in 10 mL of dried acetone was added dropwise to a solution of (1.57 g, 10 mmol) DMAEM dissolved in 50 mL of anhydrous acetone. The reaction mixture was stirred under nitrogen protection at 15 °C for ∼5 h. The white precipitate was washed with 50 mL of anhydrous acetone and 100 mL of anhydrous ether. The product was dried under reduced pressure to obtain the final CBMA monomer product. The monomer was kept at 2−8 °C before polymerization. Yield: 91%. NMR was recorded on a Bruker spectrometer using deuterated water as a solvent:[22] 1H NMR (300 MHz): 6.06 (s, 1H, ═CH), 5.68(s, 1H, ═CH), 4.55 (t, 2H, OCH_2), 3.70 (t, 2H, CH_2N), 3.59 (t, 2H, NCH_2), 3.10 (s, 6H, NCH_3), 2.64(t, 2H, CH_2COO), 1.84 (s, 3H, ═CCH_3). 13C NMR (500 MHz): 180.27(COO^-), 172.43 (COO), 139.16, 131.77, 66.38, 66.31, 62.44, 55.35, 34.76, 21.30.

Surface-Initiated Polymerization on Surface Plasmon Resonance (SPR) Sensors. Electron beam evaporation under vacuum was used to coat glass chips with a layer of chromium (2 nm thick) to promote the adhesion of a subsequent surface plasmon active layer of gold layer (48 nm thick). Before SAM preparation, the substrates were washed with pure ethanol, cleaned with a UV−ozone cleaner (Jelight, model 42) for 20 min, and washed with water and pure ethanol. The initiator SAMs were formed by soaking gold-coated substrates in a pure ethanol solution containing 1 mM *ω*-mercaptoundecyl bromoisobutyrate at room temperature for 24 h. Before the polymerization, the substrates were rinsed with pure ethanol, followed by THF, and dried with a stream of nitrogen.

CuBr and substrates with immobilized initiators were placed in a reaction tube in a dry box under nitrogen protection and sealed with rubber septum stoppers before removal from the dry box. A degassed solution (pure water and methanol in a 1:1 volume ratio) with CBMA and BPY was then transferred to the tube using a syringe under nitrogen protection. After the reaction, the substrate was removed and rinsed with ethanol and water, and the samples were kept in water overnight. The substrate was rinsed with PBS buffer to remove unbound polymers before testing. For a typical polymerization, the substrate was reacted with 7.5 mmol CBMA, 2 mmol BPY, and 1 mmol CuBr in 25 mL of CH_3OH/H_2O (1:1 volume ratio) for 1 h under nitrogen protection.

SPR Analysis and Protein Adsorption. Protein adsorption was measured with a custom-built SPR sensor, which is based on wavelength interrogation. An SPR chip (32 × 18 × 2 mm) was attached to the base of the prism, and optical contact was established using refractive index matching fluid (Cargille). A four-channel flow cell with four independent parallel flow channels (contact area: 17 × 3 mm each) was used to contain liquid samples during experiments. A peristaltic pump (Ismatec) was utilized to deliver liquid samples to the four channels of the flow cell. A fibrinogen solution of 1.0 mg/mL in PBS was flowed over the sensor surface at a flow rate of 0.05 mL/min. An SPR detector was used to monitor protein−surface interactions in real time. In this study, wavelength shift was used to measure the change in surface concentration (or mass per unit area). The smallest change in the signal that can be measured by the SPR sensor was 0.02 nm or lower (i.e., 10 times the standard deviation of baseline noise, which is 0.002 nm for the custom-built SPR sensor used). For normal molecules, at an operating wavelength of around 800 nm, a 1 nm SPR wavelength

shift corresponds to an adsorption of ~15 ng/cm^2 on the surface.[23,24] For each surface, at least three samples were measured for protein adsorption.

Ellipsometry. Ellipsometry was performed using a spectroscopic ellipsometer (Sentech SE-850, GmbH).[14] Gold-coated silicon chips were used for ellipsometry analysis. The procedure for SAM preparation was the same as that for the SPR sensors. Five separate spots were measured at three different angles of incidence (50, 60, and 70°) in the visible region. The same batch of gold-coated chips was cleaned with a UV−ozone cleaner for 20 min, washed with ethanol and Millipore water, and dried with nitrogen. The bare gold-coated chips were used as a reference. The thicknesses of the films studied were determined using the Cauchy layer model with an assumed refractive index of 1.45.

Anti-hCG Immobilization on PolyCBMA-Grafted SPR Sensors. The polyCBMA-grafted SPR sensor surface was activated by incubating the SPR chip in a freshly prepared solution containing 2 mg/mL NHS and 2 mg/mL EDC in a mixed solvent of dioxane/water (v/v 14:1) for 1 h at room temperature. The anti-hCG mAb was linked to the activated surface by putting a 10 μL drop of 2 mg/mL anti-hCG in PBS onto the surface, covering the surface with a glass cover slip, and then incubating the mAb with the activated surface for approximately 24 h at 4 °C in a humid environment. The substrate was then treated with 1 M ethanolamine (pH 8.5) for 10 min to remove any unreacted NHS.

Fibronectin Immobilization/Cell Culture on PolyCBMA Hydrogels. The transparent hydrogel was prepared by adding 2.7 M CBMA monomer into tetraethylene glycol dimethacrylate (5.9 mol %) and through free radical polymerization initiated by sodium metabisulfite (1.2 mol %) and ammonium persulfate(2.6 mol %) in a mixed solution (ethylene glycol/ethanol/H_2O = 3:1:1 volume ratio). The reaction was carried out between a pair of glass substrates, separated with a PTFE spacer of 0.4 mm at 37 °C for 12 h. After the polymerization, the gel was immersed in a large amount of deionized water for 3 days, and the water was changed every day to remove residual chemicals. The gel was then equilibrated in sterilized PBS solution, which was changed every day for another week. Hydrogels were punched into disks with a diameter of 5 mm and stored in sterilized buffer solution before use.

The hydrogel disks were immersed into a dioxane/water (v/v 14:1) mixture containing 2 mg/mL NHS dioxane and 2 mg/mL EDC for 1 h at room temperature. The hydrogel disks shrank during the dioxane/water solution soak. The disks were removed from the solution, soaked in Millipore water to swell them back to their original size, rinsed with Millipore water, and soaked in PBS buffer for another 30 min. The samples were immersed in a 100 μg/mL fibronectin solution at 4 °C for 24 h.

Bovine aortic endothelial cells (BAECs) were maintained in continuous growth in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% nonessential amino acids, and 2% penicillin streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2 on tissue culture polystyrene flasks. BAECs were removed from the flask surfaces by washing twice with 10 mL of PBS followed by incubation in 2 mL of trypsin/ethylenediaminetetraacetic acid (0.05%/0.53 mM) for detachment. After cells were detached, cells were then resuspended in 8 mL of DMEM, and the suspension was centrifuged at 1000 rpm for 5 min. The supernatant was removed and the cells were diluted in DMEM with 5% or 10% FBS at a final concentration of 10^5 cells/mL. The hydrogel disks were washed by PBS in a 24-well plate and 2 mL cell suspension was added to each well. The cells were then incubated with the samples for 3 days at 37 °C in a humidified atmosphere of 5% CO_2. The morphology and proliferation of the cells were observed using a Nikon TE200 phase contrast microscope equipped with a digital camera using a 10× objective. Cell seeding density was determined using a hemocytometer. Cell culture medium and reagents were obtained from Gibco (Gaithersburg, MD).

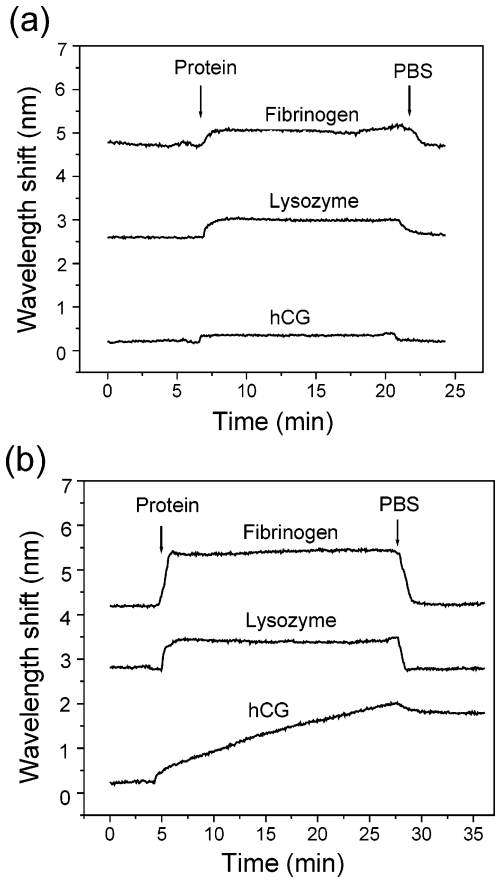


Figure 2. Adsorption of 1 mg/mL fibrinogen, 1 mg/mL lysozyme and 20 μg/mL hCG from PBS (150 mM and pH 7.4) on (a) polyCBMA-grafted surfaces and (b) polyCBMA surfaces with immobilized anti-hCG from SPR measurements.

Results and Discussion

In this work, a highly protein-resistant surface was prepared by grafting polyCBMA onto a gold surface covered with initiators via the surface-initiated ATRP method (Scheme 1). After a typical ATRP polymerization, homogeneous carboxybetaine polymer brushes were grafted outward from the surface. XPS and attenuated total reflection Fourier transform infrared (ATR-FTIR) analysis showed that the surfaces were covered with polyCBMA (see Supporting Information). The thickness of the polymer layer was around 10−15 nm, as measured by ellipsometry. Protein adsorption was measured with a custom-built SPR sensor based on wavelength interrogation.[22] Protein adsorption is defined as the wavelength shift before protein injection and after buffer wash. The wavelength shift after protein injection is mainly due to the change in the bulk refractive index. The adsorption of three proteins with different sizes and isoelectric points (pI)—human fibrinogen (340 kD, pI = 5.5), lysozyme (14 kD, pI = 12), and hCG (37 kD, pI = 4.5)—on polyCBMA-grafted surfaces was shown to decrease to <0.3 ng/cm^2 (or a wavelength shift of <0.02 nm, the detection limit of the SPR sensor), as shown in Figure 2a. This level of adsorption is the same as that found for well-packed OEG SAMs[16] or surfaces covered with polySBMA via surface-initiated ATRP.[14] Thus, polyCBMA-grafted surfaces are highly resistant to nonspecific protein adsorption.

The monoclonal mAb to hCG was covalently immobilized onto the surface covered with polyCBMA brushes by reacting the amines of the mAb to the carboxyl groups of the polyCBMA via EDC/NHS coupling chemistry (Figure 1). After the im-

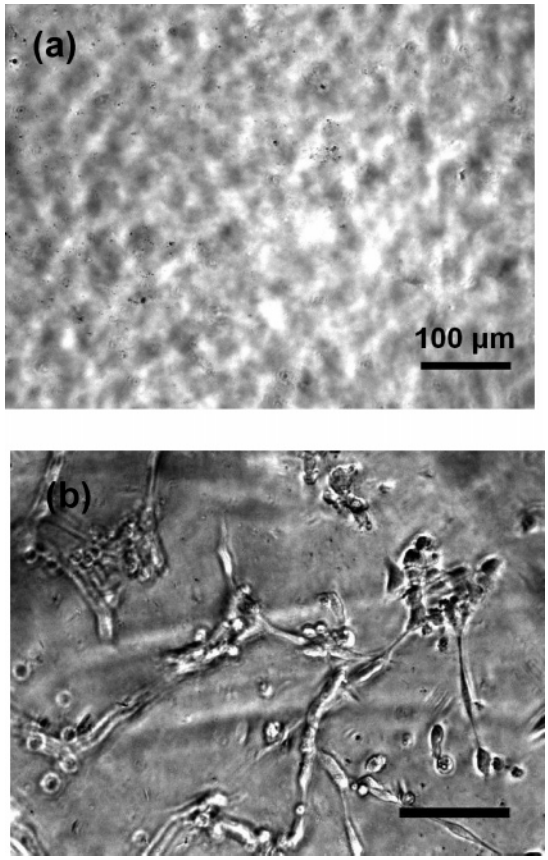


Figure 3. Microscopic images of BAEC adhesion after 24 h on (a) inactivated and (b) EDC/NHS-activated polyCBMA hydrogels. Both samples were incubated in a fibronectin solution for 24 h before the cell adhesion experiments.

mobilization, the unreacted NHS was removed with ethanolamine. The SPR response after an injection of 20 μg/mL hCG for 22 min shows that the adsorbed amount of protein is much greater on the polyCBMA-grafted surface with immobilized anti-hCG (Figure 2b) than on the control polyCBMA-grafted surfaces without the immobilized mAb. The wavelength shift of the SPR spectra is 1.6 nm, corresponding to hCG adsorption of $\sim$24 ng/cm^2. At the same time, the adsorption of both lysozyme and fibrinogen remains the same on both surfaces (less than 0.3 ng/cm^2), indicating that the surface is still nonfouling after anti-hCG immobilization. Thus, the polyCBMA-grafted surface with an immobilized anti-hCG represents a dual-functional surface with both resistance to nonspecific protein adsorption and binding to a specific protein.

We also prepared a cross-linked polyCBMA hydrogel and evaluated its dual functionality. The hydrogel was found to be noncytotoxic and contained less than 0.06 units (EU)/mL of endotoxin using a Limulus amebocyte lysate (LAL) endotoxin assay kit (Cambrex Bioscience, Walkersville, MD). The hydrogel disks that were activated with EDC/NHS were immersed in a solution containing 100 μg/mL fibronectin used to promote cell adhesion. The disks without EDC/NHS activation were also incubated in the fibronectin solution. Following protein adsorption, BAECs were cultured on both activated and inactivated disks. Cell morphology was observed between 2 h and 3 days of cultivation. While there were no adhered cells on the inactivated polyCBMA hydrogel disks, BAECs were observed on the surfaces that were treated with EDC/NHS before the fibronectin adsorption (Figure 3). These results show that the polyCBMA hydrogel itself is highly resistant to cell adhesion and can be readily immobilized with proteins or other ligands.

Because of the dual-functional properties of polyCBMA, it is very convenient to not only create nonfouling surfaces or materials, but also immobilize proteins onto the nonfouling background, which is desirable for applications in biosensors and biomaterials. Furthermore, the biomimetic nature of polyCBMA makes it very attractive for biomedical applications. In addition, as a zwitterionic polymer, polyCBMA is responsive to the changes in pH and ionic strength[25] and can be tailored for applications in drug delivery as well.

Conclusions

We introduce a biomimetic material based on polyCBMA, which has dual roles of both protein resistance and protein immobilization. This material is not only highly resistant to protein adsorption/cell adhesion, but it also has abundant functional groups that could be conjugated with biological ligands. More importantly, polyCBMA-based materials and surfaces covered with polyCBMA still maintain their excellent nonfouling properties after ligand immobilization. In this work, gold surfaces grafted with polyCBMA brushes showed protein adsorption less than 0.3 ng/cm^2. The hCG antibody was chemically immobilized on the surfaces using the EDC/NHS method. The modified surface bound specifically with hCG and highly resisted the nonspecific adsorption of other nontargeted proteins. PolyCBMA-based hydrogels were prepared and modified by EDC/NHS activation and fibronectin conjugation. BAECs attached and spread on the surface with conjugated fibronectin, while no cells attached on the hydrogel without EDC/NHS activation. With the dual functionality and biomimetic nature of polyCBMA materials, they are very promising for biomedical applications where both a specific binding and a nonfouling background are often simultaneously required.

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Supporting Information Available. XPS and ATR-FTIR spectra of polyCBMA-grafted gold surfaces. This material is available free of charge via the Internet at http://pubs.acs.org.

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