Grafting of Poly(acrylic acid) on the Poly(ethylene glycol) Hydrogel Using Surface-initiated Photopolymerization for Covalent Immobilization of Collagen

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Abstract: The modification of the protein-repellent poly(ethylene glycol) (PEG) hydrogel surface was achieved by a two-step process using immobilization of benzophenone on the PEG hydrogel as surface initiator and subsequent surface-initiated graft polymerization of acrylic acid by UV irradiation. Formation of poly(acrylic acid) (PAA) layer on the PEG hydrogel was demonstrated by confirming the presence of carboxyl groups in the poly(acrylic acid) (PAA) with FTIR/ATR spectroscopy and measuring the height of PAA layers with alpha-step surface profiler. In the grafted region, PAA and PEG hydrogel formed an interpenetrating polymer network extending 200 µm into PEG hydrogel and homo PAA protruded 14 ∼ 17 µm above the PEG hydrogel surface. Activation of the carboxyl groups in PAA allowed covalent immobilization of collagen, a cell adhesion protein, on the PAA-grafted PEG hydrogel, which was demonstrated with FTIR/ATR spectroscopy by confirming the formation of a new amide bond. Surface-initiated graft polymerization combined with photolithography produced well-defined PAA micropatterns on the PEG hydrogels and collagen was immobilized only on the PAA region due to the lack of adhesion for proteins to PEG, producing protein micropattern on the PEG hydrogel.

Keywords: poly(ethylene glycol) hydrogel, surface-initiated graft polymerization, photolithography, benzophenone, poly(acrylic acid) micropattern

Introduction

Hydrogels are crosslinked polymers with a three-dimensional network arrangement that are able to retain a large amount of water. Hydrogels have been used as thin films, scaffolds, or nanoparticles in a wide range of biomedical and biological applications [1]. Among the various hydrogels, poly(ethylene glycol) (PEG)-based hydrogels have been widely used in biology and medicine since they are nontoxic, non-immunogenic, and approved by the US Food and Drug Administration (FDA) for various clinical uses [2-4]. PEG is nondegradable, hydrophilic polymer that has been widely employed as a biomaterial to obtain biocompatibility because of its remarkable nonadhesivity towards protein and cells [5]. Typically, PEG has been incorporated onto biomaterial surfaces via surface grafting, plasma polymerization, surface interpenetrating networks or simple adsorption of PEG-containing block copolymers [6,7]. Recent researches have primarily focused on using PEG or oligoethylene glycol (OEG) as a surface modification technique to control protein or cell adhesion. For example, soft lithography has been utilized to self assemble PEG and OEG molecules on silicon, gold and plastic in order to control protein and cell interaction with these substrates [8]. Different molecular weight of PEGs could be easily converted into acrylates such as PEG diacrylate (PEG-DA), and polymerization of acrylated PEG in the presence of light and photoinitiator yields a highly crosslinked hydrogel network [9]. The physical properties of PEG hydrogels, such as permeability, mechanical strength, and biocompatibility can be easily controlled for a particular application by varying the MW of PEG [2]. PEG hydrogels have a high equilibrium water content, which should provide rapid transport of small molecules through gels, and it was demonstrated that microporosity
of PEG hydrogels could be easily altered. The aqueous environment of PEG hydrogels is appropriate for the immobilization of various biomolecules such as proteins, nucleic acids, and even whole cells. Furthermore, PEG hydrogels have been shown to be both biocompatible and nonfouling in complex environments [10]. Because of these characteristics, PEG hydrogels have been evaluated for in vivo use such as implanted glucose sensors, drug delivery devices, tissue engineering and cell transplantation [3,4,7,11-14]. Although high protein resistance of PEG hydrogel prevents unwanted non-specific adsorption to a material and increases the long-term biocompatibility, to make PEG hydrogel a versatile template for various applications, the surface of PEG hydrogel can be modified to covalently immobilize various biomolecules such as peptides or proteins. However, the dynamic swelling behavior, soft elastic nature, and unavailable functional groups of PEG hydrogels limit the incorporation of biomolecules and only a few strategies have been devised to modify PEG hydrogel [15,16]. In this study, surfaces of the PEG hydrogels were modified with poly(acrylic acid) (PAA) by photoinduced graft polymerization and collagen, a cell adhesion protein, was covalently immobilized to the PAA-grafted PEG hydrogel surface. Furthermore, the photolithographic method was used to direct surface modification at selective regions of the PEG hydrogel with micrometer precision, producing collagen micropatterns on the PEG hydrogel.

Experimental

Materials
PEG with a MW of 3400 g/mol, acryloyl chloride, triethylamine, hexane, tetrahydrofuran (THF), ethyl alcohol, acrylic acid, benzophenone, benzyl alcohol, sodium periodate, 1-vinyl-2-pyrrolidinone, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), collagen type I-FITC conjugate (collagen-FITC), toluidine blue, N-hydroxy succinimide (NHS) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS, 0.1 M, pH 7.4) was prepared with 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate, and 0.15 M NaCl in deionized water. PEG was converted to PEG-diacrylate (DA) according to published protocol and acrylation of PEG was confirmed by attenuated total reflectance/Fourier transform infrared (ATR/FTIR) spectroscopy (Thermo Nicolet Corp., Madison, WI, USA) [17].

Preparation of Hydrogel Substrate
PEG hydrogel substrates were prepared by UV-initiated free radical polymerization. Purified PEG-DA was dissolved in PBS to form 50 wt% PEG solution. A 300 mg/mL solution of DMPA in 1-vinyl-2-pyrrolidinone was added and mixed to the PEG solution at 5 µL/mL PEG-DA solution to initiate photopolymerization. This precursor solution was cast into a Teflon mold (1 cm diameter, 1 mm thick) and exposed to a 75 W xenon ultraviolet (UV) light source (Oriel Instruments, Mountain View, CA, USA) for 5 min after being covered with glass plates. Upon UV exposure, the precursor solution underwent free-radical induced gelation and became insoluble in common PEG solvents such as water. The resultant PEG hydrogel was washed extensively for a minimum of three days in deionized water to remove any unreacted components and to reach equilibrium swelling in deionized water.

Surface-initiated Graft Polymerization
The surface modification of PEG hydrogels was achieved by two-step process based on photoinduced graft polymerization of acrylic acid. In the first step, surfaces of the hydrogels were dabbed dry and 100 µL of a 10 wt% benzophenone solution in ethanol was drop-cast on the hydrogel and spread evenly over the surface. The solvent was then allowed to evaporate in a fume hood to ensure deposition of the benzophenone on the hydrogel. For the second step, a monomer solution composed of acrylic acid (10~30 wt% in water), benzyl alcohol (0.5 wt%) and 0.5 mM of sodium periodate was coated on the benzophenone-immobilized hydrogel surface, which was then exposed to 365 nm, 300 mW/cm² UV light (EXFO Omnicure UV spot lamp, Mississauga, Ontario, Canada). After UV exposure, the hydrogel substrates were rinsed with deionized water and immersed in ethanol to remove poly(acrylic acid) (PAA) which was not covalently attached to the hydrogel. Characterization of the modified surfaces was performed using ATR/FTIR spectroscopy. The modified PEG hydrogel was immersed in 10 wt% toluidine blue in alcohol for five minutes, and rinsed with alcohol and water. After staining process, cross-section of PAA-grafted PEG hydrogel was observed with optical microscopy to determine the depth of PAA polymerization within hydrogel.

Micropatterning of Hydrogel Surface
Surface micropatterning was performed using photolithography. As previously described, a monomer solution was coated on the benzophenone-immobilized hydrogel substrates and exposed to UV light through a photomask to direct surface modification at the selected areas. The photomask for photolithography contained
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Immobilization of Collagen on the Hydrogel Substrates
Collagen was covalently immobilized on the PEG hydrogel surface via a EDC/NHS-mediated reaction. Specifically, PAA-grafted hydrogels were immersed into a solution containing 2 mM EDC and 5 mM NHS for three hours to convert the carboxyl groups in PAA into reactive intermediates which are susceptible to be attacked by amine groups from proteins. The activated hydrogel substrates were then washed with PBS and immediately reacted with collagen-FITC solutions for three hours at 36°C, which resulted in the covalent attachment of the proteins to the hydrogel surface by amide formation. Immobilization of collagen onto PAA-grafted hydrogel was characterized with ATR/FTIR. Fluorescence microscopy was used to confirm the selective attachment of collagen to the micropatterned hydrogels.

Image Acquisition and Analysis
A Zeiss Axiovert 200 microscope equipped with an integrated color CCD camera (Carl Zeiss Inc., Thornwood, NY, USA) was used to obtain optical and fluorescence images. Image analyses were performed using commercially available image analysis software (KS 300, Carl Zeiss Inc.).

Results and Discussion

Grafting of PAA on the PEG Hydrogel
Photopolymerization of precursor solution containing PEG-DA and photoinitiator produced crosslinked PEG hydrogels which were 2 mm thick, flexible and transparent when swollen in deionized water (water content of hydrogel was 83.4 ± 5.5%). Formation of PAA layers from the different concentration of acrylic acid on the PEG hydrogel substrates was demonstrated by ATR/FTIR spectroscopy. Figure 1 shows FTIR spectra of unmodified PEG hydrogel and PAA-grafted PEG hydrogel. Although all the spectra have a common absorption band at around 1730 cm⁻¹ which is attributed to carbonyl bonds (C = O), spectra of PAA-grafted PEG hydrogels shows higher intensity of this band than that of unmodified PEG hydrogel and the intensity increased with the concentration of acrylic acid. The concentration of carbonyl groups in the PEG hydrogel is relatively low, as they exist in acrylate groups at the termini of the large MW of PEG (MW 3400). On the other hand, for PAA, carbonyl bonds in the carboxyl groups appear on every acrylic acid monomer unit (MW 72). Therefore, PAA-grafted PEG hydrogels possess more carbonyl group than PEG hydrogel, resulting in an increase of peak intensity at around 1730 cm⁻¹ as shown Figure 1. As a control, same experiment was carried out without a benzophenone-immobilizing step. No evidence of surface modification was detected, corroborating the role of benzophenone as a surface initiator.

Figure 2 shows the thickness of PAA layers prepared from different concentration of acrylic acid on the PEG hydrogel, which was measured with alpha-step surface profiler (KLA-Tencor Co., San Jose, CA, USA). As indicated in this Figure, polymerization with high concentration of monomer resulted in the thicker PAA layer on
Penetration of PAA into PEG Hydrogel

Since the benzophenone could diffuse into the PEG hydrogel, it was possible that graft polymerization occurred within the PEG hydrogel itself as well as on the surface. To determine the location of the PAA, PAA-grafted PEG hydrogels were stained with toluidine blue, which has positively charged amine groups and can adsorb to the negatively charged carboxyl groups of PAA, but not PEG hydrogel. As shown in Figure 3, majority of the dye was observed within the PEG hydrogel and penetration depth of dye was about 200 µm deep. Considering the thickness of PAA layer from the PEG hydrogel surface was less than 20 µm, PAA formation occurred primarily within the PEG hydrogel. This result proposes that a PAA/PDMS interpenetrating polymer network was formed during grafting polymerization. In the UV exposed regions, decomposition of surface-implanted benzophenone generates free radicals within the PEG hydrogel. Free radicals directly initiated polymerization of acrylic acid, which has diffused into the PEG hydrogel. In addition, the free radicals reacted with the hydrogen atoms of PEG, creating free radicals on the PEG chains. The free radicals on the PEG chains also initiate graft polymerization of acrylic acid within the hydrogel [18-21]. As a result, PAA and PEG hydrogel formed an interpenetrating polymer network to a depth of 200 µm and homo PAA protruded about 17 µm above the PEG hydrogel surface.

PAA Micropattern on the PEG Hydrogels

To examine whether surface graft polymerization can provide micro-order precision, a micropatterning process was performed using a photomask with different designs. Figure 4 shows the optical images of the PEG hydrogel substrate micropatterned with PAA. The combination of photolithography and surface initiated graft polymerization successfully created the clearly defined microstructures that corresponded to the pattern of the photomask. Only the UV-exposed region determined by the photomask, was susceptible to photoinduced graft polymerization, resulting in no residual polymer on the substrate after washing. PAA micropatterns were firmly anchored to the hydrogel substrates and did not delaminate during washing or prolonged exposure to an aqueous environment.

Collagen Immobilization onto the PAA-grafted PEG Hydrogel

PAA possesses carboxyl groups that could serve as sites for immobilization of various proteins. Using the EDC/NHS-mediated reaction, carboxyl groups in PAA...
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Figure 5. Comparison of FTIR spectra between PAA-grafted PEG hydrogels with and without immobilized collagen.

Figure 6. Fluorescence image of the micropatterned PEG hydrogel after collagen-FITC was attached to the activated PAA area.

are converted to N-hydroxysuccinimide ester, which can react with amine groups in proteins to form a stable amide linkage. Collagen was incubated with the PAA-modified PEG hydrogel to confirm the ability of grafted PAA to react and form the covalent bond with proteins. Immobilization of collagen on the PEG hydrogels was demonstrated with ATR/FTIR. Comparing with PAA-grafted PEG hydrogel, the FTIR, spectra of collagen-immobilized PEG hydrogel possessed new peaks at 1653 and 1540 cm\(^{-1}\), which were characteristic of the C = O stretching and the N-H bending of the amide bond (Figure 5). These results suggest that collagen was successfully incorporated to the PEG hydrogel surface through surface modification.

Because of nonadhesivity of PEG hydrogel toward proteins, micropatterning of the PEG hydrogel created a clear contrast between the adhesion promoting PAA micropattern and adhesion resisting PEG hydrogel surface. Feasibility of selectively immobilizing proteins on the micropatterned PAA was investigated by incubating collagen-FITC with the micropatterned PEG hydrogel substrate to visualize the localization and patterning of the protein. The result is shown in Figure 6 where fluorescent and dark regions correspond to the PAA micropatterns with immobilized collagen and unmodified PEG region, respectively. As indicated by these results, proteins were immobilized only onto the PAA micropattern with PEG hydrogel serving as effective barrier to protein adsorption, demonstrating the spatial control of protein immobilization was possible on the PEG hydrogel substrates with a micrometer scale. Because PEG hydrogel was used as a substrate, passivation step which is necessary for most of protein patterning techniques could be skipped.

Conclusion

In this study, a protein-repellent PEG hydrogel surface was modified by photoinduced graft polymerization using acrylic acid as a monomer and benzophenone as a surface initiator. Due to the carboxyl groups in PAA, collagen, cell adhesion protein, could be covalently immobilized on to the PEG hydrogel. Surface-initiated graft polymerization combined with the photolithographic method produced well-defined PAA micropatterns on the PEG hydrogels, which did not delaminate during washing or prolonged exposure to an aqueous environment. Proteins were selectively immobilized on the PAA micropattern, while the bare PEG region effectively prevented non-specific protein adsorption. The techniques described in this study provided the new method to modified PEG hydrogel surface for the protein immobilization and can be used for tissue engineering or biochip-based analytical system where the control of protein immobilization is crucial for the successful results.

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References