Development of Hydrogel Lenses with Surface-immobilized PEG Layers to Reduce Protein Adsorption

Jun-Pil Jee[†] and Ho-Joong Kim[‡,*]

[†]*College of Pharmacy, Chosun University, Gwangju 501-759, Korea*
[‡]*Department of Chemistry, Chosun University, Gwangju 501-759, Korea. *E-mail: hjkim@chosun.ac.kr*
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This paper describes the synthesis and characterization of a series of poly(2-hydroxyethyl methacrylate) (pHEMA)-based hydrogel lenses coated with poly(ethylene glycol) (PEG) chains. A novel tri-branched PEG-substituted hydrazide is synthesized, which imparts densely packed, covalently bound PEG layers on hydrogels, to determine whether branching provides improved coverage of the lens surface, thereby reducing protein adsorption. Surface modification of hydrogels with PEG was performed via amide-coupling reactions between PEG-substituted hydrazide and the pHEMA matrix. Protein adsorption, water content, optical transparency, and surface properties of the hydrogels were investigated. The hydrogels exhibited transmittance of >90% and improved surface hydrophilicity. Notably, the amount of lysozyme adsorbed on tri-branched PEG-coated hydrogels decreased significantly compared to the amount adsorbed onto the surface of control and linear PEG-coated hydrogels. These results provide insight into the mechanism by which PEGs reduce lysozyme adsorption and suggest that PEG coating may offer an intriguing potential for ophthalmic biomaterials as well as protein-resistant devices.

Keywords: Hydrogel, Poly(ethylene glycol), Protein adsorption, Contact lens

Introduction

The development of antifouling materials is of great interest for multiple biomedical and biotechnological applications, including medical implants, contact lenses, biosensors, drug delivery, and catheters.[1–4] Surface coating and modification methods that utilize antifouling polymers include physical adsorption, layer-by-layer (LbL) assembly, self-assembled monolayers (SAMs), surface-initiated atom transfer radical polymerization (ATRP), interpenetrating polymer network (IPN), and reaction of the specific groups of polymers with the substrate.[5–11] Polyhydrophilic polymers, including poly(ethyleneglycol) (PEG), polysaccharides, and polyamides, and polyzwitterionic polymers, such as 2-methacryloyloxylethyl phosphorylcholine (MPC) and carboxybetaine methacrylate (CBMA), have been employed in the development of bio-antifouling substrates to reduce protein adsorption and biofilm formation.[12–17] In particular, the materials used to prepare contact lenses require an elaborate design and synthesis, with a focus on their protein-resistant properties and biocompatibility, since the deposition of proteins onto lens surfaces has been the major cause of a number of adverse effects, including reduced vision, shortened lifespan of the lenses, bacterial keratitis, and increased discomfort.[18–22]

Protein-resistant coatings on contact lenses can be prepared via physical/chemical adsorption and covalent grafting, using antifouling polymers.[23–27] Recently, we have functionalized hydrogel lenses by using polysaccharides, such as hyaluronic acid (HA) and alginic acid, by constructing an IPN structure composed of cross-linked polysaccharides and a hydrogel matrix.[23] The polysaccharide modification resulted in significantly reduced protein adsorption, compared to that observed in the control hydrogel. Sheardown and coworkers have developed protein-resistant hydrogels via physical adsorption (and/or IPN construction) using HA, exhibiting reduced non-specific protein adsorption compared to that observed in unmodified controls.[24,25] Anti-biofouling contact lenses have been prepared by silylation of copolymers such as poly(MPC) with the contact lenses surface hydroxyl. The modified contact lenses showed resistance to protein adsorption and bacterial adhesion.[26] Although a considerable progress has been made in the preparation of antifouling coatings or hydrogels using protein-resistant polymers, only a few studies have reported improved reduction of protein adsorption on hydrogel lenses. This method poses a considerable number of challenges, including the comprehensive understanding of the underlying protein adhesion mechanisms and the development of more environmentally friendly and effective protein-antifouling materials for the efficient performance of contact lens.

PEG coatings among the various biocompatible polymers have been the subject of considerable interest as a means of reducing protein adsorption and cell adhesion on a variety of substrates.[28–30] The non-fouling properties of PEGs are primarily attributed to their high chain mobility, large exclusion volume, and steric hindrance effect.[31–34] In particular, dendritic PEG chains have hydrophilic repeating units, conformational flexibility, and highly branched architecture, which

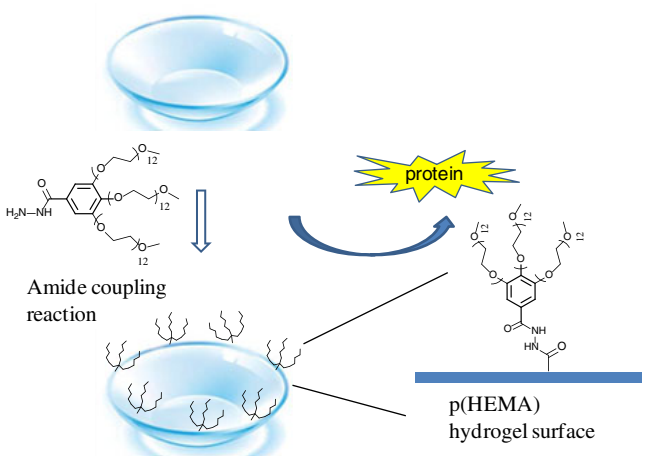

Figure 1. Schematic representation of branched PEG-functionalized hydrogel lenses exhibiting protein adsorption resistance.

correlate with steric hindrance. These features led us to develop surface coatings with dendritic PEG chains that combine all of the above-mentioned properties, which might produce hydrogel lenses with better surface wettability and bio-antifouling property.

In this paper, novel tri-branched PEG-substituted hydrazides were designed and synthesized for surface modification of poly(2-hydroxyethyl methacrylate) (pHEMA)-based hydrogels (Figure 1). PEG chains were covalently immobilized onto hydrogel surfaces by a facile one-step amide coupling reaction. The effect of PEG immobilization on the wettability, surface properties, protein adsorption, and optical transmittance of the resultant hydrogels were investigated, with a particular focus on the branching of PEGs compared to the linear PEG-coated hydrogels and unmodified control.

Experimental

Chemicals. 2-Hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MA), polyethyleneglycol monomethylether (PEG, MW 550), hydrazine hydrate, *N*-hydroxysuccinimide (NHS), 4-hydroxybenzoic acid, gallic acid, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), lysozyme (chicken egg lysozyme), dimethylformamide (DMF), tetrahydrofuran (THF), and albumin (bovine serum albumin) were purchased from Sigma Aldrich (St. Louis, MO, USA). *N*-vinylpyrrolidone (NVP) and azobisisobutyronitrile (AIBN) were purchased from Junsei (Tokyo, Japan). Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma Aldrich, Acros Organics (Geel, Belgium), TCI (Tokyo, Japan), etc.) All atmosphere-sensitive reactions were performed in the presence of nitrogen. UV light and iodine vapors were used for visualization.

Equipment. ^{1}H-NMR spectra were recorded from $CDCl_3$ solutions on a Bruker AM 250 spectrometer (Billerica, MA, USA). The purity of the products was determined via thin-layer chromatography (TLC, silica gel 60; Merck Millipore, Darmstadt, Germany). Fourier transform infrared (FT-IR) spectra of the hydrogels were recorded over the range 500–4000 cm^{-1} using a Nicolet 5700 IR spectrometer (Thermo Electron, Beverly, MA, USA). The surface roughness was investigated by atomic force microscopy (AFM) (NX10; Park Systems, Suwon, Korea). The transmittance of the hydrogel lenses was measured in duplicate over the wavelength range 200–800 nm, using a Shimadzu UV-1650PC (Shimadzu, Japan) spectrometer. Contact angles were measured using a DSA 100 (KRÜSS, Hamburg, Germany) apparatus.

Synthesis of Linear and Tri-Branched PEGs

Synthesis of Compounds 3a and 3b. Compounds **3a** and **3b** were synthesized using similar procedures. A representative example is described for compound **3a**. A mixture of 4-hydroxybenzoic acid (5.0 g, 36.2 mmol), ethanol (50 mL), and a few drops of conc. H_2SO_4 was refluxed for 5 h. Excess solvent was removed *in vacuo*; the reaction mixture was poured into cold water and extracted repeatedly using methylene chloride. Combined organic extracts were washed with 10% $NaHCO_3$, water, and brine, subsequently dried over anhydrous $MgSO_4$, and concentrated to obtain a white solid (yield, 97%).

3a: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.95 (d, 2H, Ar), 6.91 (d, 2H, Ar), 4.35 (t, 2H, COOCH_2), 1.38 (t, 3H, CH_3).

3b: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.47 (m, 2H, Ar), 4.35 (t, 2H, COOCH_2), 1.54 (t, 3H, CH_3).

Synthesis of Compounds 4a and 4b. Compounds **4a** and **4b** were synthesized using identical procedures. A representative example is described for compound **4a**. A mixture of **3a** (250 mg, 1.5 mmol), anhydrous K_2CO_3 (623 mg, 4.51 mmol), and PEG- monomethylether monotosylate (MW 550, 1.37 g, 1.95 mmol) was dissolved in DMF (20 mL) and refluxed for 12 h, in the presence of argon. The reaction mixture was cooled and distilled to remove the DMF. The resulting solution was poured into water and extracted with methylene chloride. The methylene chloride solution was washed with water, dried over anhydrous $MgSO_4$, and filtered. The crude product was purified by column chromatography to yield 955 mg (91%) of a viscous liquid.

4a: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.95 (d, 2H, Ar), 6.88 (d, 2H, Ar), 4.31 (q, 2H, COOCH_2), 4.11 (t, 2H, Ar-OCH_2), 3.67 (t, 2H, Ar-OCH$_2$CH_2), 3.47–3.63 (m, 44H, OCH_2), 3.34 (s, 3H, OCH_3) 1.32 (t, 3H, CH_3).

4b: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.26 (s, 2H, Ar), 4.32 (q, 2H, COOCH_2), 4.15 (m, 6H, Ar-OCH_2), 3.82 (m, 6H, Ar-OCH$_2$CH_2), 3.45–3.75 (m, 132H, OCH_2), 3.34 (s, 9H, OCH_3) 1.34 (t, 3H, CH_3).

Synthesis of Compounds 1 and 2. Compounds **1** and **2** were synthesized using similar procedures. A representative example is described for compound **1**. Compound **4a** (500 mg, 0.714 mmol) and hydrazine monohydrate (178 mg, 3.57 mmol) were dissolved in MeOH (10 mL) and THF (3 mL). The reaction mixture was stirred at 70 °C for 12 h, cooled to

room temperature, and the organic solvents were removed under reduced pressure. The mixture was dissolved in methylene chloride and washed with H_2O. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was evaporated to obtain the crude product, which was purified by column chromatography. The chromatography procedure utilized 8% MeOH in ethyl acetate as the eluent in order to obtain the pure product as a viscous liquid (450 mg, 92%).

1: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.92 (d, 2H, Ar), 6.92 (d, 2H, Ar), 4.15 (m, 2H, Ar-OCH_2), 3.87 (t, 2H, Ar-OCH$_2$CH_2), 3.49–3.73 (m, 44H, OCH_2), 3.36 (s, 3H, OCH_3).

2: ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.13 (s, 2H, Ar), 4.19 (m, 6H, Ar-OCH_2), 3.83 (m, 6H, Ar-OCH$_2$CH_2), 3.47–3.77 (m, 132H, OCH_2), 3.37 (s, 9H, OCH_3).

Preparation of pHEMA Hydrogels Functionalized with PEG. HEMA monomer was purified by vacuum distillation prior to polymerization. EGDMA (0.05 g), NVP (1.20 g), MA (0.10 g), and AIBN (0.04 g) were dissolved in HEMA (8.65 g). The standard monomer formulations for the three hydrogels used in this study are shown in Table 1. The monomer solution was injected into 0.08-mm-thick polypropylene contact lens molds. The molds were then placed in an oven and heated at 100 °C for 5 h. After polymerization, the samples were removed from the molds and cooled in water for 2 days to remove any unreacted monomers and initiators in the samples. The pHEMA samples were then dried in a 24-well plate at 40 °C overnight. The hydrogels were prepared by swelling each dried pHEMA sample in distilled water for 3 days at room temperature. In order to functionalize the surface of hydrogels with PEGs, they were immersed in a 75% ethanol (7.5 mL)/25% water (2.5 mL) solution containing PEG-substituted hydrazides (0.2 mmol), EDC (0.3 mmol), and NHS (0.3 mmol). The mixture solutions were shaken for 24 h at room temperature to induce amide-coupling reactions between the amino groups of hydrazides and carboxylic groups of MA. The hydrogels were washed with water and ethanol and subsequently immersed in water for 72 h to remove any unreacted PEGs, EDC, and NHS prior to characterization.

Quantitation of Protein Deposition onto Hydrogel Lenses. An artificial tear solution, containing 3.88 g/L of bovine serum albumin and 1.20 g/L of chicken egg-white lysozyme dissolved in phosphate buffered saline (PBS, pH 7.4), was prepared as previously reported.[35] It should be noted that human and chicken lysozymes share a high degree of similarity in their primary and tertiary structures.[36] Six lenses of each lens type were incubated for 12 h. The lenses were placed in 5 mL of the artificial tear solution and were incubated at a temperature of 37 °C under constant rotation for 12 h. After the first incubation period, the lenses were rinsed with PBS to remove any unbound protein on the lens surface. The lenses were then placed in an extraction solvent containing acetonitrile/0.2 wt % trifluoroacetic acid and were incubated in the dark at room temperature for 24 h. The concentration of proteins extracted from the hydrogels was quantified by reverse-phase high-performance liquid chromatography (RP-HPLC). The HPLC system (Shimadzu, kyoto, Japan) consisted of an LC-10AVP pump, an LC-20AD autosampler, and an SPD-10A UV detector. One hundred microliters of the extracted lysozyme solution or 10 μL of the extracted albumin was mixed with 900 or 990 μL of the mobile phase solution, respectively; 20 μL of this mixture was injected into a C18 column (LUNA-C18, 4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA, USA). The proteins were eluted with an isocratic mixture of 50% acetonitrile containing 0.1% of trifluoroacetic acid and 50% water. The run time and flow rate were set to 4.5 min and 1.0 mL/min, respectively, and the samples were detected at 220 nm. Data are given as the mean ± SD of three measurements.

Results and Discussion

Synthesis and Characterization. Two series of PEG-substituted benzohydrazides **1** and **2** were prepared as per the synthetic strategies detailed in Figure 2. PEG synthesis was initiated with ethyl-4-hydroxy benzoate (**3a**) and ethyl 3,4,5-trihydroxy benzoate (**3b**), which underwent Williamson ether reactions with the appropriate PEG-monomethylether monotosylate (MW 550) by using anhydrous potassium carbonate, to form PEG-substituted benzoates **4a** and **4b**. The subsequent amide-coupling reactions of the benzoates with hydrazine monohydrate produced linear and tri-branched PEG-substituted hydrazides, respectively. The unmodified hydrogel (H) contains 1 wt % MA, which is conjugated with PEGs through the amide-coupling reactions occurring between PEG-substituted hydrazides and carboxylic acids of MA units. The hydrogels were functionalized with linear PEG **1** and tri-branched PEG **2**, yielding P1 and P2, respectively. The hydrogels modified with PEGs were investigated by FT-IR spectroscopy (Figure 3). A strong peak, assigned to the C=O stretching band from the HEMA ester group, was observed at 1710 cm^{-1} for all hydrogels. The

Table 1. Characteristics of hydrogels functionalized with PEG.

Hydrogels	Composition ratio of monomers (wt %)				Coated PEG	Transmittance (%)	Water content (%)	Water contact angle (°)	Root mean square roughness (nm)
	HEMA	NVP	MA	EDGMA					
H	86.5	12	1	0.5	—	91	44	65.2	3.56
P1	86.5	12	1	0.5	1	90	46	53.4	6.21
P2	86.5	12	1	0.5	2	90	46	43.0	7.13

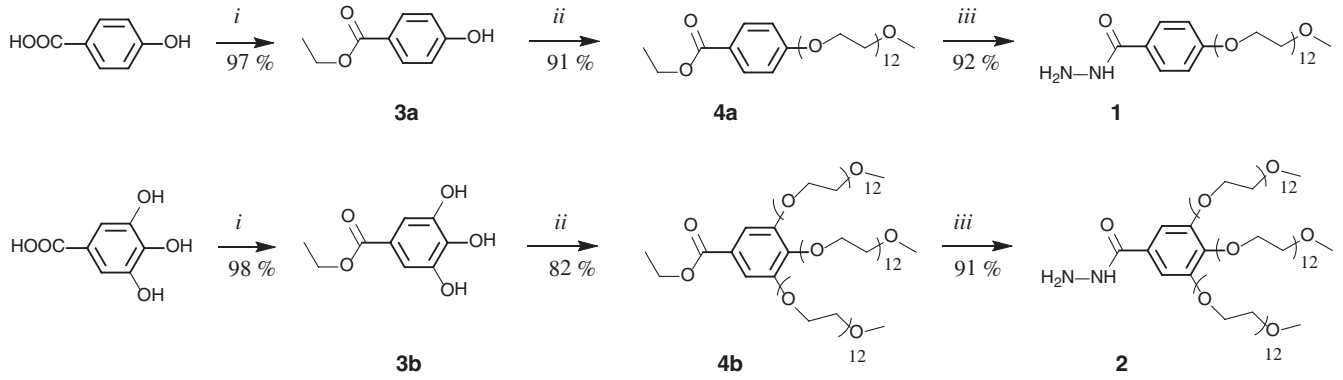

Figure 2. Synthetic routes for amine-functionalized PEG **1** and **2**. Reagents and conditions: (i) ethanol, H_2SO_4, reflux; (ii) poly(ethylene glycol) monomethylether monotosylate, K_2CO_3, DMF, 80 °C; (iii) hydrazine hydrate, MeOH/THF, 70 °C 0.5 wt % of AIBN (initiator) were added to the total monomers and crosslinking mixtures prior to polymerization.

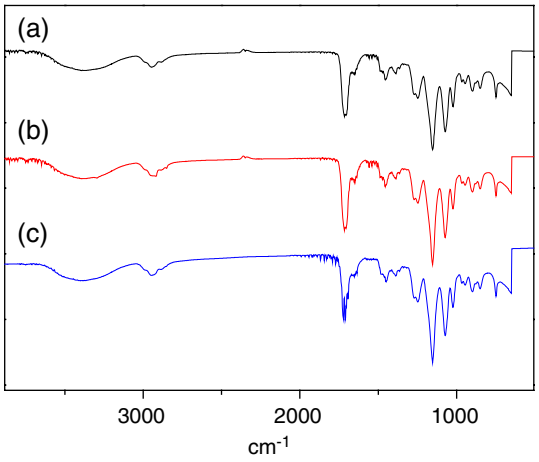

Figure 3. FT-IR spectra of (a) H, (b) P1, and (c) P2.

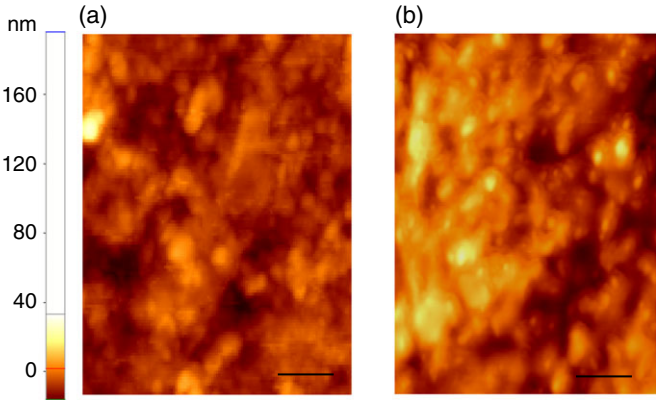

Figure 4. AFM images of dried (a) H and (b) P2. Scale bar is 1 μm.

functionalization of the hydrogels with PEGs did not show the expected absorption peaks of aliphatic ether (C—O—C) stretching at 1120 cm^{-1} in the PEGs and an amide band I peak at 1650 cm^{-1} because of their overlap with the absorption peaks of ester and amine units from HEMA and NVP.

The optical analysis revealed a transparency >90% for both the control and PEG-coated hydrogels, indicating that surface immobilization with PEGs had no effect on the transparency of hydrogels (Table 1). The PEG layers coated on the hydrogels were characterized by tapping-mode atomic force microscopy (AFM) to determine whether they resulted in changes to the surface roughness. Figure 4 shows that the surfaces of PEG-modified hydrogels are rougher than those of the unmodified hydrogels, as indicated by the root mean square (RMS) roughness values presented in Table 1. While the AFM images of the unmodified surfaces of H appeared fairly smooth, with small and equally distributed narrow features, the PEG-coated hydrogels P1 and P2 showed unevenly distributed broader features. This may be attributed to heterogeneous PEG coverage, resulting in the formation of a rougher surface.

Water Content and Hydrophilicity of Hydrogels. The PEG coating did not increase the water content of hydrogels compared to the unmodified control, as the amount of attached PEGs was too small. The surface contact angle by water is an important factor for evaluating surface hydrophilicity. As shown in Table 1 and Figure 5, the presence of hydrated PEG chains decreased the contact angle of the hydrogels, implying increased surface wettability. The contact angles of P1 and P2 were observed to be 53.4° and 43.0°, respectively, which were lower than the observed value of 65.2° for H. Notably, the contact angle of P2 decreased by ~20% compared to that of P1, suggesting that tri-branched PEGs provide higher surface hydrophilicity compared to the linear PEGs. This result was attributed to surface enrichment with branched PEGs, which covered the hydrogel surfaces with greater efficiency, compared to that observed with the linear PEGs. High-water-content lens materials are commonly obtained by the copolymerization of moderately hydrophilic HEMA with highly hydrophilic ionic monomers such as methacrylic acid (MA). Because of its ionic functionality, MA is used to increase the water content in the hydrogel. However, the use of ionic monomers increases the protein adsorption of hydrogels, while the branched PEG chains increase

surface hydrophilicity without the use of ionic units, demonstrating the potential applicability of these hydrogels in the contact lens industry.

Protein Adsorption to Hydrogel Lenses. Lysozyme adsorption onto the synthesized hydrogels is shown in Figure 6. The presence of PEG layers on the hydrogel surface was highly effective in preventing the adsorption of the lysozyme. The amounts of lysozyme adsorbed onto the PEG-modified hydrogels P1 and P2 were observed to be 171.74 and 83.13 μg/lens, respectively, indicative of 36 and 69% reduction, compared to 267.52 μg/lens observed for unmodified hydrogel H (Figure 6). The amount of adsorbed lysozyme on P2 exhibited 52% reduction approximately, compared to that of P1, indicating that the protein resistance of the branched PEG is much better than that of the linear PEG. This result can be rationalized by considering the surface charge of the lysozyme and the steric hindrance effect of PEG. The lysozyme with an isoelectric point (pI) of 10.7 displayed a positively charged surface at physiological pH; therefore, its adsorption onto negatively charged substrates is electrostatically favorable. The ionic functionality of the MA unit in an aqueous environment increases the water content and surface hydrophilicity of the hydrogel. Lysozyme may be electrostatically attracted to the carboxylate anions of the MA units, which are highly hydrophilic ionic monomers that are used often in the manufacture of soft contact lenses, such as etafilcon A and vifilcon A.[37] According to some previously reported studies, lysozyme is extensively taken up by the hydrogel containing MA, implying that the H containing 1% MA may have attractive interactions with lysozyme.[38,39] It appears that the PEG immobilization between the carboxylic acids of H and the amine groups of PEG-substituted hydrazides facilitates the formation of amide bonds, which may result in a nearly neutral hydrogel surface. In addition, the presence of PEG layers reduces lysozyme adsorption, since their neutral hydrophilic characteristics repel the negatively charged lysozymes and prevent interactions with the hydrophobic domain that can cause lysozyme denaturation and subsequent surface accumulation. Notably, tri-branched PEG modification showed a better ability to repel lysozyme than the linear PEG treatment. It has been reported that the antifouling ability of PEG chains depends on diverse parameters, including the PEG grafting density, chain length, polymer architecture, and chain mobility. A comparison of the structural differences between the linear and branched PEGs revealed the importance of the effect of the PEG surface density, since a low surface density may not provide significant steric exclusion, while a high surface density may not allow enough mobility to the PEG chains. The surface hydration and steric repulsion associated with hydrogel surface coverage of branched PEG work together to impart greater efficiency to the lysozyme-repelling property of hydrogel P2 prepared from tri-branched PEG, compared to the linear PEG hydrogel.

The amounts of albumin adsorbed onto the H, P1, and P2 surfaces were approximately 19.78, 17.83, and 16.78 μg/lens, indicating that PEG treatment reduces albumin adsorption (Figure 7). Considering the pI of albumin (4.7), the hydrogel H, containing carboxylate anions, may repel albumin with a negatively charged surface at pH 7.4. The PEG-coated surfaces of hydrogels P1 and P2 may be sterically inaccessible to albumin. Specifically, the branched PEG layer of P2 is more effective in preventing the adsorption of albumin. Therefore, the albumin-resisting ability is related to the PEG modification of hydrogels containing MA units.

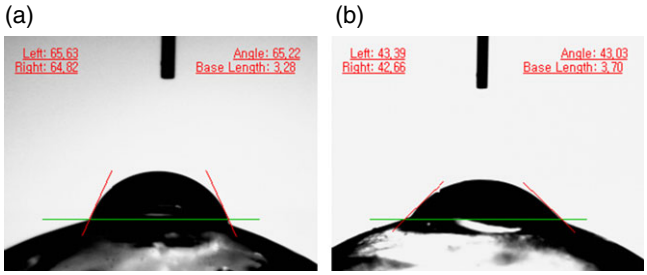

Figure 5. Photographs of contact angles with water. (a) H and (b) P2.

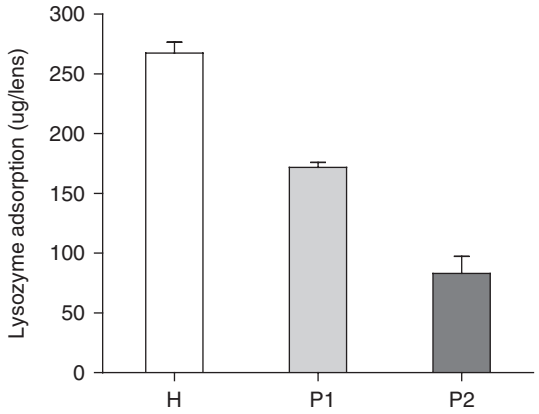

Figure 6. Graph depicting the amount of adsorbed lysozyme on the pHEMA lens and PEG-functionalized hydrogel lenses. Each value is expressed as mean ± standard deviation, $n = 3$.

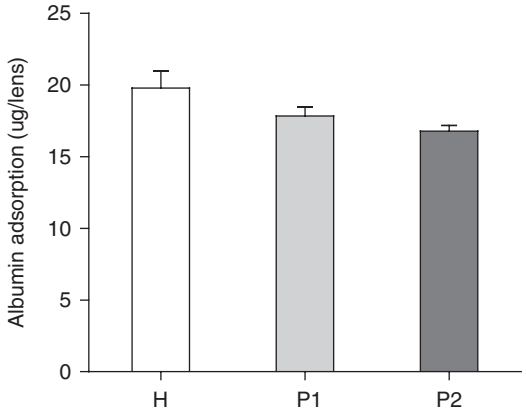

Figure 7. Graph depicting the amount of adsorbed albumin on the pHEMA lens and PEG-functionalized hydrogel lenses (μg/lens). Each value is expressed as mean ± standard deviation, $n = 3$.

Conclusion

Novel tri-branched PEG-substituted benzohydrazides were synthesized and used as building blocks for the preparation of protein-resistant hydrogels via surface modifications. The PEG-modified hydrogels exhibited high transparency and improved surface hydrophilicity compared to the untreated hydrogels. The PEG coating was proven to successfully reduce the adsorption of lysozyme onto the hydrogel surfaces because of their bioinert and highly hydrophilic nature. Notably, the tri-branched PEG treatment reduced the lysozyme adsorption with greater efficiency compared to linear PEGs. This result is attributed to the characteristic structural features of branched PEGs, which combine a highly flexible polyether with hydrophilic surface groups and a highly branched architecture, resulting in a strong steric hindrance, which repels the approaching lysozyme efficiently. The preparation of hydrogels with a PEG-immobilized surface may be a new method for the development of ophthalmic biomaterials as well as protein-resistant devices.

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