Preparation of Collagen-Immobilized Poly(ethylene glycol)/Poly(2-hydroxyethyl methacrylate) Interpenetrating Network Hydrogels for Potential Application of Artificial Cornea

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ABSTRACT: To enhance the mechanical strength of poly(ethylene glycol)(PEG) gels and to provide functional groups for surface modification, we prepared interpenetrating (IPN) hydrogels by incorporating poly(2-hydroxyethyl methacrylate)(PHEMA) inside PEG hydrogels. Formation of IPN hydrogels was confirmed by measuring the weight percent gain of the hydrogels after incorporation of PHEMA, as well as by ATR/FTIR analysis. Synthesis of IPN hydrogels with a high PHEMA content resulted in optically transparent and extensively crosslinked hydrogels with a lower water content and a 6 $\sim$ 8-fold improvement in mechanical properties than PEG hydrogels. Incorporation of less than 90 wt % PHEMA resulted in opaque hydrogels due to phase separation between water and PHEMA. To overcome the poor cell adhesion properties of the IPN hydrogels, collagen was covalently grafted to the surface of IPN hydrogels via carbamate linkages to hydroxyl groups in PHEMA. Resultant IPN hydrogels were proven to be noncytotoxic and cell adhesion study revealed that collagen immobilization resulted in a significant improvement of cell adhesion and spreading on the IPN hydrogel surfaces. The resultant IPN hydrogels were noncytotoxic, and a cell adhesion study revealed that collagen immobilization improved cell adhesion and spreading on the IPN hydrogel surfaces significantly. These results indicate that PEG/PHEMA IPN hydrogels are highly promising biomaterials that can be used in artificial corneas and a variety of other load-bearing tissue engineering applications. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 637–645, 2012

Key words: poly(ethylene glycol); poly(2-hydroxyethyl methacrylate); interpenetrating hydrogels; artificial cornea

INTRODUCTION

The cornea is the clear front window of the eye and comprises three main layers: an outermost stratified epithelium (50-µm thick), a hydrated collagen-based stroma (500-µm thick), and an innermost endothelial monolayer. The cornea serves mainly as an optical element to transmit and focus light into the eye, but also acts as a physical barrier to harmful agents, protecting the interior of the eyes.[1,2] Therefore, a healthy transparent cornea is critical to proper eye function. Approximately 10 million people worldwide are estimated to be blind because of corneal disease and the only widely acceptable treatment is corneal transplantation using human donor corneas.[3] However, a lack of donor tissue and various complications related to allograft transplantation limit the use of corneal transplantation as a solution for treatment of corneal diseases and have sparked intense interest in developing artificial corneas that possess the following properties: (1) optical transparency, (2) permeability to various nutrients, (3) good mechanical strength to withstand changes in intraocular pressure, eye rubbing, and minor trauma, (4) flexibility to minimize stress at the host-device interface and to allow for monitoring of postimplantation intraocular pressure, (5) nontoxicity to cells as well as biocompatibility, and (6) support of surface epithelialization.[4] Because most of the cornea is occupied by a hydrated matrix that contains about 80% water, the recent availability of softer and more hydrophilic materials has led to a shift in research to soft and wet materials including hydrogels and biopolymeric scaffolds.[5,6] The initial successful example of a hydrogel-based synthetic cornea is the "AlphaCor," a poly (hydroxyethyl methacrylate) (PHEMA) copolymer developed by Chirila and coworkers.[7–9] Despite some promising results, however, a key disadvantage of

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PHEMA-based materials is their low water content (<40%), which does not allow transport of nutrients from tear fluid to epithelial cells and subsequently cannot support long-term surface epithelialization. Recently, collagen-based hydrogels were developed as corneal substitutes by several groups and these hydrogels possesses outstanding physical, chemical, and biological properties.[10–12] However, synthesis of hydrogels using natural polymers, such as collagen, makes it difficult to control and manipulate the various physical properties of the resultant hydrogels.

In search of suitable matrix for an artificial cornea, poly(ethylene glycol)(PEG)-hydrogels can be one of the top candidates. PEG, one of the few synthetic polymers with FDA approval for internal consumption and injection in a variety of foods, cosmetics, personal care products, and pharmaceuticals, has been used in various biomedical applications.[13] Different molecular weight (MW) forms of PEG can be converted into acrylates, such as PEG diacrylate (PEG-DA), and polymerization of the acrylated PEGs yields a highly crosslinked hydrogel network.[14] PEG-based hydrogels possess many desirable properties that are highly attractive for tissue engineering applications.[15–17] For example, they have high water content and physical properties similar to soft tissues, including high permeability for oxygen, nutrients, and other water-soluble metabolite. 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.[18–22] However, although PEG hydrogels have several ideal properties for various applications, there are two major obstacles to their application as a material for artificial corneas. The first obstacle is their relatively poor mechanical strength because of the reduced concentration of network chains in the swollen state. The second is their resistance against cell adhesion and lack of available functional groups for covalent immobilization of cell adhesion proteins through surface modification. As mentioned earlier, for the long-term success of artificial corneas, corneal epithelial cells can adhere and proliferate on the hydrogel surfaces, which requires the immobilization of cell adhesion proteins, such as fibronectin and collagen, or cell adhesion peptides onto the hydrogel surfaces. One strategy to overcome these obstacles is to synthesize interpenetrating polymer networks (IPN). An IPN is a mixture of two or more polymers that have been synthesized or crosslinked with no significant degree of covalent bonds between them. Recently, much attention has been dedicated to the use of IPNs to obtain hydrogels with better mechanical properties and greater multifunctionality not only for artificial cornea but also for various other applications.[23–31] Especially, Myung et al. developed artificial cornea using PEG/poly(acrylic acid)(PAA) IPN hydrogel and demonstrated the possibility to overcome the problem of current artificial corneas.[32–34] However, despite promising results, using pH and salt-sensitive PAA as second network lead to produce IPN hydrogel possessing inconsistent physical properties depending on external environment. Furthermore, it required additional photochemical fixation steps to covalently immobilize collagen onto IPN hydrogels due to lack of functional groups.

In this study, we prepared PEG/Poly(2-hydroxyethyl methacrylate)(PHEMA)-based IPN hydrogels and investigated their material properties to evaluate if they can potentially be used as materials for artificial corneas. PHEMA was chosen as the second network to improve the mechanical properties of the PEG hydrogel and to provide functional groups that can be modified to promote cell adhesion. After the synthesis of an IPN of PEG and PHEMA, the physical properties of the IPN hydrogels were characterized in terms of water content, optical transparency, and tensile strength. The surfaces of the IPN hydrogel were then modified to covalently immobilize collagen, and finally, cytotoxicity and cell adhesion were investigated.

EXPERIMENTAL

Materials

Poly(ethylene glycol) (PEG)(MW 3400), 2-hydroxyethyl methacrylate (HEMA), acryloyl chloride, triethylamine, hexane, tetrahydrofuran (THF), 2-hydroxy-2-methylpropiophenone (HOMPP), triethylene glycol dimethacrylate (TEGDMA), Bradford reagent, collagen type I-FITC conjugate (collagen-FITC), collagen type I from human skin, Ham's F12, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), insulin-transferrin-selenium (ITS), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gentamycin, and trypsin/ethylenediamine-tetra-acetate (trypsin/EDTA) were purchased from ς-Aldrich (Milwaukee, WI). SV-40 transformed human corneal epithelial cells (HCECs) were obtained from the American Type Culture Collection (Manassas, VA). A Live/Dead Viability/Cytotoxicity kit (L-7013) and calcein-AM were purchased from Molecular Probes (Carlsbad, CA). PEG was converted to PEG-diacrylate (DA) according to a published protocol. Phosphate buffered saline (PBS, 0.1*M*, pH 7.4) was prepared with 1.1 m*M* potassium phosphate monobasic, 3 m*M* sodium phosphate dibasic heptahydrate, and 0.15*M* NaCl in deionized water.

Preparation of PEG/Phema IPN hydrogels

IPN hydrogels were synthesized by a two-step sequential network formation technique based on

UV-initiated free radical polymerization as previously described.[32,33] The first hydrogel network was prepared from PEG-DA (MW 3400) that was synthesized using a published protocol.[35] Briefly, Purified PEG-DA was dissolved in PBS to form a 50% w/v solution and then 10 μL of HOMPP was added to 1 mL of PEG solution to initiate photopolymerization. This precursor solution was cast into a Teflon mold and exposed to 365 nm, 300 mW/cm^2 UV light (EFOS Ultracure 100ss Plus, UV spot lamp, Mississauga, Ontario) for 10 s after being covered with glass plates. Upon exposure to UV light, the precursor solution underwent free-radical induced gelation and became insoluble in common PEG solvents such as water. To incorporate the second network, the water-swollen PEG hydrogels were immersed in HEMA monomer containing 2% v/v photoinitiator solution and 1% v/v of TEGDMA as a crosslinking agent for different periods of time. After removing excess monomer solution from the surface of the PEG hydrogels, the swollen PEG hydrogels were exposed to the same UV source for 5 min and a second network, poly (-hydroxyethyl methacrylate) (PHEMA), was polymerized and crosslinked inside the first PEG hydrogel network to form an interpenetrated structure consisting of two polymer (PEG and PHEMA) networks entangling each other. The resultant IPN hydrogels were washed extensively in deionized water to remove any unreacted components and allowed to reach equilibrium with water. The formation of an IPN hydrogel was confirmed by measuring the weight percent gain of the hydrogel after incorporation of PHEMA network. The weight percent of PHEMA (W_{PHEMA}) within the IPN hydrogel was calculated as follows:

$$W_{\mathrm{Phema}} = (W_{\mathrm{IPN}} - W_{\mathrm{PEG}})/W_{\mathrm{IPN}} \times 100,$$

where W_{IPN} and W_{PEG} are the weights of the dried IPN hydrogel and PEG hydrogel, respectively.

Swelling studies

The water contents of the hydrogels were evaluated in terms of their swelling ratio. Swelling studies were performed by immersing the weighed dry hydrogel in water. The swollen gels were lifted, patted dry, and weighed at regular intervals until equilibrium was attained. The water content percentage (WC) was calculated by

$$\mathrm{WC} = (W_s - W_d)/W_s \times 100,$$

where W_s and W_d are the weights of the swollen and dry hydrogel, respectively.

Mechanical tests

The mechanical properties of the hydrogels were investigated by measuring the tensile strength of the hydrogels. Hydrogel samples were prepared according to ASTM D638-V standards and tested using an Instron 5844 testing apparatus equipped with a 10*N* load cell, BioPuls bath, and submersible pneumatic grips (Instron Corp., Norwood, MA). The crosshead speed was set at 15 mm/min. Load and extension measurements were collected automatically by a computer, and were used to calculate true stress and strain values by considering the thinning of the samples by extension. A minimum of five strips of each type of material were tested, and average values with associated standard deviations of maximum tensile strength and strain at break of water-saturated hydrogels were calculated.

Characterization

Transparency of hydrogel was investigated by measuring the percentage (%) of light transmittance through the hydrogel at 470, 520, and 630 nm using a UV-Vis Spectrometer OPTIZEN 3220UV (Mechasys Corp, Seoul, Korea) following the method described by Saito and colleagues.[36] Attenuated total reflectance/Fourier transform infrared (ATR/FTIR) spectroscopy (Thermo Nicolet Corp., Madison, WI) was used to identify the FTIR spectra of different hydrogels.

Surface modification of the IPN hydrogels

The surfaces of PEG/PHEMA hydrogels were modified as described previously.[37,38] Briefly, dried hydrogel samples were incubated in 0.01 mg/mL CDI solution in 1,4-dioxane for 2.5 h at 37°C and subsequently rinsed with 1,4-dioxane to remove unreacted CDI. The CDI-activated hydrogels were then incubated in collagen Type I solution (1 mg/mL, in 0.3% acetic acid) for 24 h at 37°C to couple reactive protein amine groups to the activated hydrogel surfaces. Finally, the hydrogels were washed extensively with PBS to remove nongrafted collagen. Immobilization of collagen was visualized with fluorescence microscopy, and the amount of collagen immobilized was quantified by measuring the initial and final amount of collagen within the incubation solution using BCA standard working agent (Pierce, Rockford, IL).

Cell culture

SV-transformed HCECs were cultured in a 1 : 1 mixture of Ham's F12 and DMEM supplemented with 0.1% v/v gentamycin, 0.01% ITS, and 5% FBS. Cells

were grown to confluence in 75 cm^2 polystyrene tissue culture flasks at 37°C in 5% CO_2 and 95% air, and confluent cells were subcultured every 2–3 days by trypsinization with trypsin/EDTA.

Cytotoxicity assay

To test the cytotoxicity of the hydrogels, surface-modified IPN hydrogels were placed on top of a layer of HCECs cultured in wells of a tissue culture plate. Briefly, cells were allowed to attach in 24-well plates at a final density of 20,000 cells/well. After 24 h, culture medium was replaced with fresh medium and hydrogel samples were added to each well. As a control, cells were also cultured in the absence of hydrogel samples. After a 24-h incubation, hydrogel samples were removed from the wells and the viability of cells was investigated with a Live/Dead Viability/Cytotoxicity fluorescence assay and MTT assay as described previously.[39,40]

Cell adhesion study

HCECs were seeded at a concentration of 1 × 10^5 cells/mL onto unmodified and surface-modified PEG/PHEMA hydrogels. After 24-h incubation, the hydrogel substrates were rinsed with PBS to remove nonadherent cells. For visualization, adhered HCECs were incubated with 5 μM calcein-AM for 1 h and subsequently fixed with 4.0% glutaraldehyde at 4°C for 4 h.

Image acquisition and characterization

All fluorescence images of HCECs were obtained with a Zeiss Axiovert 200 microscope equipped with an integrated color CCD camera (Carl Zeiss, Thornwood, NY). For protein and cell-related studies, error bars represent standard deviation that was obtained from repeated experiments over five different samples.

RESULTS AND DISCUSSION

Preparation of PEG/Phema IPN hydrogel

All hydrogels were formed by photopolymerization with UV light using HOMPP as a photoinitiator. Before IPN hydrogels were prepared, single network hydrogels based on PEG and PHEMA were synthesized separately to confirm the formation of gels at a given formula and to investigate the physical properties of single networks. The formation of PEG hydrogels is based on the UV-initiated free-radical polymerization of the acrylate end group of PEG derivatives.[41] That is, when exposed to UV light in the presence of a photoinitiator, acrylate groups form reactive free radical sites that react with each other, thus resulting in the formation of polyacrylate networks that are highly crosslinked with PEG. A Raman spectroscopy study revealed that the C=C bonds of PEG-DA at 1630 cm^{-1} diminished significantly after UV exposure (data not shown), indicating that most of the terminal double bonds in PEG-DA were consumed during photopolymerization to produce crosslinked PEG-based hydrogels. As a result, polymerization and crosslinking of HEMA monomers within the preformed PEG-DA network should yield an interpenetrating structure in which the two networks are independently crosslinked with little, if any, copolymerization between the two. This is in contrast to PEG-*co*-PHEMA networks where the PEG-DA macromonomers and HEMA monomers are mixed and simultaneously photopolymerized. The synthesized PEG hydrogels were flexible and completely transparent when swollen in deionized water.

TABLE I
Characteristics of PEG/PHEMA IPN Hydrogels

Sample	Incubation time (min)[a]	Weight percent of PHEMA (%)	Water content (%)
Gel 1	10	82.89	74.40
Gel 2	20	87.95	64.88
Gel 3	30	90.22	58.37
Gel 4	40	91.52	54.62
Gel 5	50	92.52	53.39
Gel 6	60	92.58	52.91

[a] Incubation time of PEG hydrogel with HEMA monomer solution.

To incorporate PHEMA, PEG hydrogels were immersed in HEMA monomer containing a photoinitiator and crosslinker, and photopolymerization of the PHEMA network within the PEG hydrogel resulted in synthesis of IPN hydrogels. In this study, the amount of PHEMA within the IPN hydrogel was controlled by changing the amount of time the PEG hydrogel was incubated with the HEMA monomer solution. As shown in Table I, the weight percent of PHEMA increased with incubation time and became saturated after 50 min. The swelling properties of the hydrogels were characterized in terms of water content (WC). Table I also shows the equilibrium swelling values of IPN hydrogels based on PEG and different amounts of PHEMA. As expected, the WC of the hydrogels decreased after incorporation of the second network, and the magnitude of decrease was dependent on the amount of PHEMA. The degree of hydrogel swelling is an important parameter affecting mass transfer in swollen hydrogels, and it is known that diffusion coefficients through hydrogels decrease as the degree of swelling decrease.[42] Therefore, hydrogel with a higher water content are more

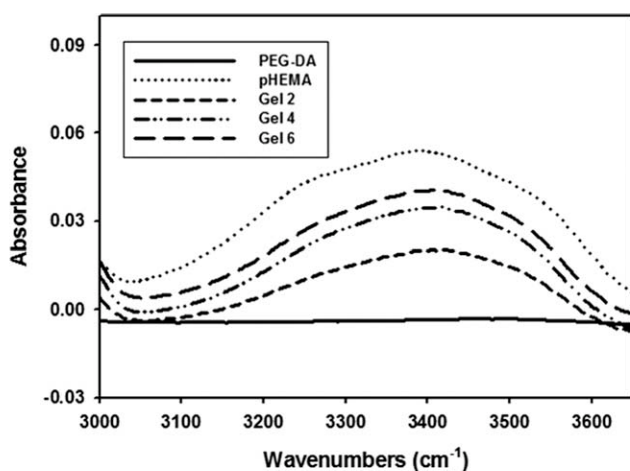

Figure 1 FTIR spectra of PEG and PEG/PHEMA IPN hydrogels around 3000 (3600 cm^{-1}).

permeable to nutrients. Although the incorporation of the PHEMA network into the PEG hydrogel caused a decrease in equilibrium swelling compared with PEG hydrogels (92.8%) and the human cornea (about 81.0%),[12] the IPN hydrogel retained a higher level of hydration than the PHEMA hydrogel (38.2%). Incorporation of a PHEMA network inside a PEG hydrogel was also characterized by ATR/FTIR. Figure 1 shows that compared with the PEG hydrogels, PEG/PHEMA IPN hydrogels possessed new peaks around 3000 (3600 cm^{-1}), which are characteristic of O-H stretching originating from the hydroxyl groups in PHEMA. Additionally, we observed that more OH groups could be introduced in the IPN hydrogels by increasing the incubation time, which would enhance the ability of hydrogels to covalently immobilize proteins.

Optical and mechanical properties of hydrogel

When PHEMA hydrogels are prepared from a water solution containing HEMA monomer, initiator, and crosslinker, the transparency of the resultant hydrogels is dependent on the ratio of water to the HEMA monomer.[43] If the concentration of water in a monomer mixture is lower than a critical level, transparent homogeneous hydrogels are formed. On the other hand, if the concentration of water is above a critical concentration, the water induces phase separation at the onset of polymerization, leading to heterogeneous hydrogels that are opaque. In this study, the HEMA monomer mixture diffused into PEG hydrogels that were swollen with water, producing an environment in which the HEMA monomer mixed with water within the PEG hydrogels. Therefore, the transparency of the PEG/PHEMA IPN hydrogels was dependent on the amount of time the PEG hydrogel was incubated with the HEMA monomer mixture solution, as this determined the amount of HEMA that diffused into PEG hydrogel and consequently the weight percentage of PHEMA in the IPN hydrogel. As shown in Figure 2(a), individual PEG and PHEMA hydrogel were transparent, whereas IPN hydrogels with a smaller amount of PHEMA were opaque (Gel 1 and Gel 2) and a higher level of transparency was obtained by incorporating more PHEMA in the PEG hydrogel. To provide a quantitative measurement of hydrogel transparency light transmission of three different wavelengths of

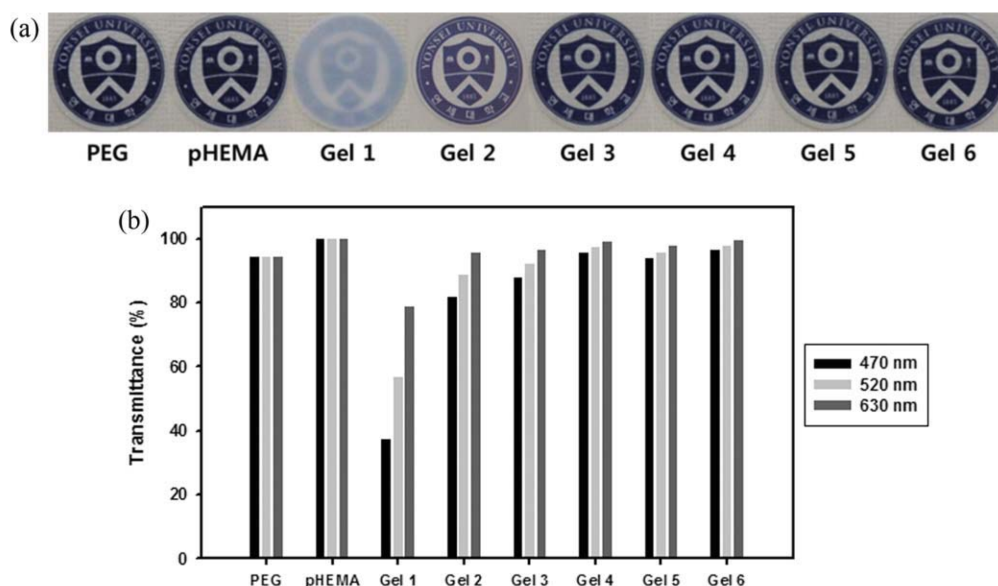

Figure 2 Optical transparency of hydrogels. (a) Photographs of different hydrogels, (b) Light transmittance through the hydrogel samples at different wavelength. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

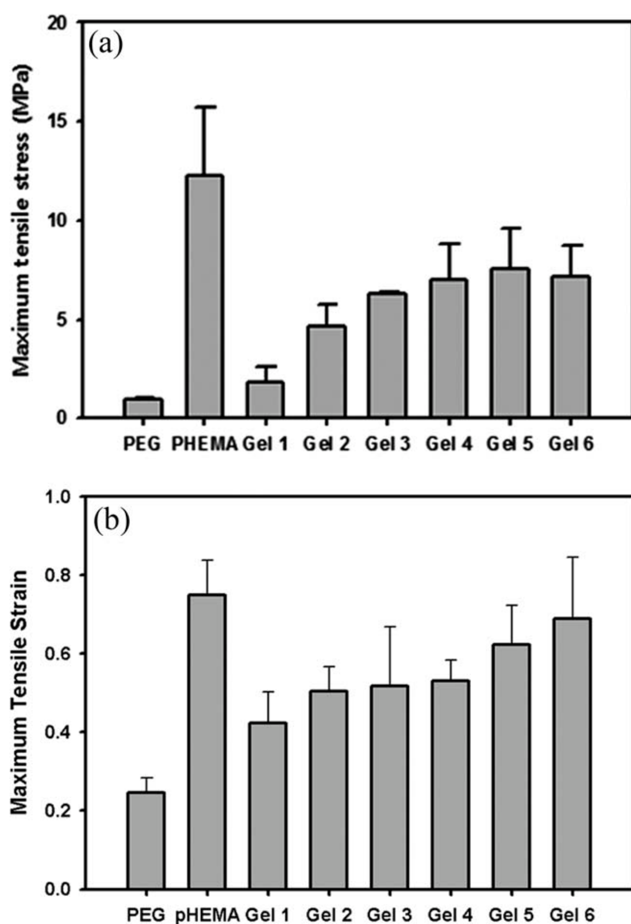

Figure 3 Mechanical properties of hydrogels. (a) Maximum tensile stress, (b) Maximum tensile strain.

light (470 nm for blue, 520 nm for green, and 630 nm for red) through the different hydrogel samples was measured, and is summarized in Figure 2(b). Considering that the percent light transmission of the human cornea is about 87%,[44] the transparency of the IPN hydrogels that contained more than 90% PHEMA (Gel 3, 4, 5, 6) is comparable to or even superior to that of the human cornea.

Despite the excellent biocompatibility and high water content of PEG hydrogels, their application as a substitute for natural tissue has been limited because of their weak mechanical properties in the wet state. To be used as a cornea substitute, hydrogels have to possess a certain amount of mechanical strength, not only to withstand internal and external pressures but also to facilitate handling and suturing during clinical implantation. A number of strategies have been used to improve the strength of hydrogels, and using IPN hydrogel has been a focus of interest since Gong et al. first reported that the mechanical properties of hydrogels could be dramatically improved without compromising their other material properties by using double network hydrogels, which is a class of IPN hydrogel.[24,26,27,33] Therefore, in this study, the incorporation of a PHEMA network into a PEG hydrogel was expected to improve the mechanical properties of the hydrogels in the swollen state. Figure 3(a,b) show the maximum tensile stress and strain of water-saturated hydrogels obtained from stress–strain data, respectively, demonstrating that incorporation of more PHEMA into the PEG hydrogel improved both the maximum tensile strength and the extensibility of the resultant hydrogels. The tensile strengths of most of the IPN hydrogels were greater than that of the human cornea (about 3.81 MPa).[45] The mechanical strength of a swollen hydrogel network is influenced by two independent factors: crosslinking density and polymer volume fraction, which are related to the structure of the hydrogel network and water content, respectively. As discussed in our previous study, IPN hydrogels have a higher crosslinking density with a smaller mesh size than PEG hydrogels due to physical entanglement of the second network with the PEG hydrogel network,[46] Furthermore, the lower water content of the IPN hydrogel indicates a higher polymer volume faction. These results indicate that the higher mechanical strength of the IPN hydrogels can be attributed to their higher crosslinking density and polymer volume fraction than PEG hydrogels. It should also be noted that there was huge difference in stress/strain values between Gel 1 and Gel 6 in spite of small difference in PHEMA contents (less than 10%). We hypothesize that additional possible source of strength enhancement is the hydrogen bonding formation between the ether groups on PEG-DA and the hydroxyl groups on PHEMA. However, when the amount of incorporated PHEMA was not enough like Gel 1, phase separation occurred, which might interrupt interpolymer hydrogen bonds, producing relatively weaker IPN hydrogels than completely miscible hydrogels like Gel 6.

Surface modification and collagen immobilization

Because of their nonadhesiveness toward proteins and cells, PEG hydrogels cannot support the adhesion and growth of corneal epithelial cells, which is an important requirement for the successful development of an artificial cornea. Furthermore, incorporation of adhesion-promoting molecules into PEG hydrogel was very limited due to the dynamic swelling behavior, soft elastic nature, and lack of available functional groups in PEG hydrogels. One of the advantages of using PHEMA as second network is that PHEMA possesses hydroxyl groups that can be easily modified to serve as sites for immobilization of various proteins. The surfaces of the IPN hydrogels were modified to enable covalent linkages with proteins and to facilitate cell adhesion by grafting

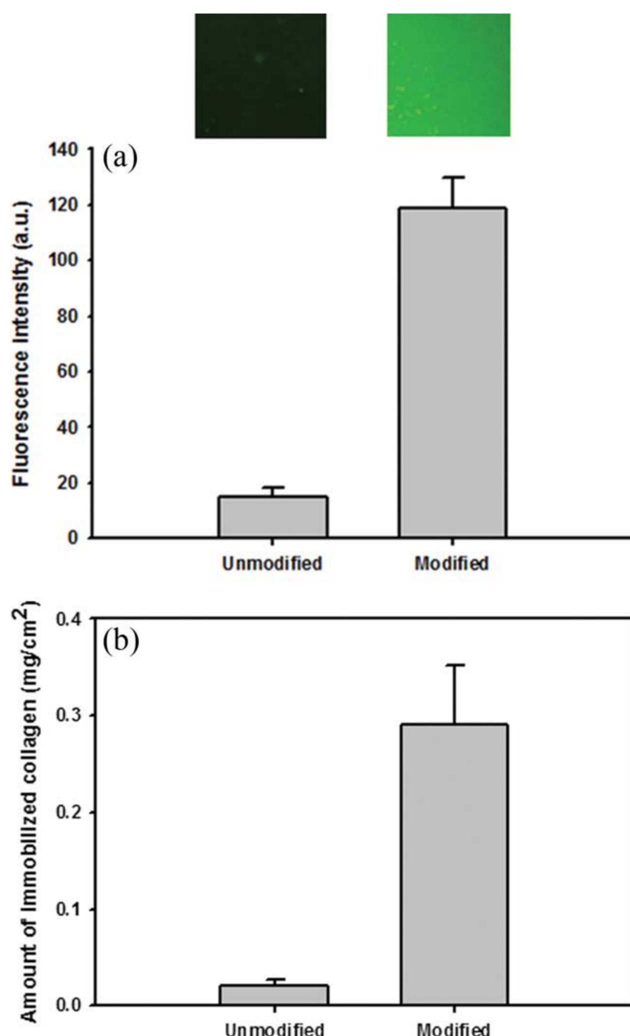

Figure 4 Immobilization of collagen onto PEG/PHEMA IPN hydrogels. (a) Fluorescence images and intensities of hydrogels that were incubated with collagen-FITC, (b) Actual amount of collagen immobilized onto hydrogel surfaces.

collagen through a carbamate linkage between free hydroxyl groups and carbonyldiimidazole (CDI) molecules.[38] Immobilization of collagen onto the hydrogel surfaces was first monitored with fluorescence microscopy after incubating hydrogels with collagen-FITC. In contrast to unmodified IPN hydrogels, CDI-modified hydrogel surfaces emitted stronger fluorescence after incubation with collagen-FITC [Fig. 4(a)]. For further confirmation of covalent immobilization of collagen on the hydrogel via CDI modification, the amount of collagen on the hydrogel was quantified using BCA reagents, and it was found that about 0.02 mg/cm^2 of collagen existed on the surfaces of unmodified hydrogels, while about 0.29 mg/cm^2 of collagen on the surfaces of CDI-modified hydrogels [Fig. 4(b)]. These results indicate that almost no protein could physically adsorb on the PEG/PHEMA IPN hydrogel, but could be immobilized to the hydrogel only via covalent bonding by surface modification.

Cytocompatibility and cell adhesion

In the field of tissue engineering, one of the first steps in the development of a novel scaffold is the evaluation of its cytotoxicity. Among several existing standard cytotoxicity assays (ISO 10993-5: Biological evaluation of medical devices-Part 5: Tests for *in vitro* cytotoxicity), direct contact assay was used to investigate cytocompatibilty of the surface-modified PEG/PHEMA hydrogels. First, the viability of cells that were incubated with surface-modified IPN hydrogels was examined using a Live/Dead Viability/Cytotoxicity fluorescence assay that stains live cells green and dead cells red. As shown in the fluorescence images presented in Figure 5(a), there are

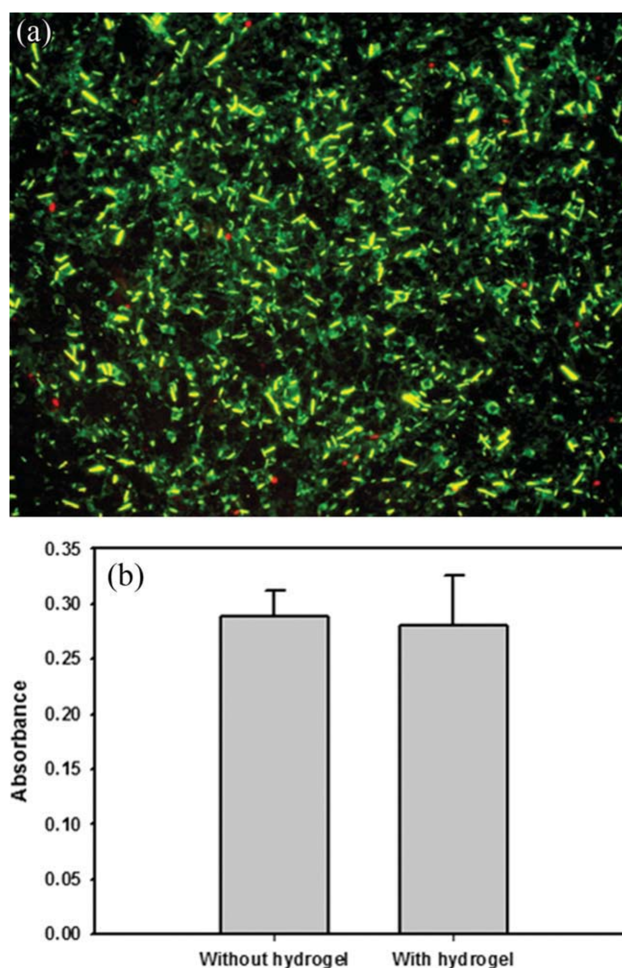

Figure 5 Cytotoxicity assays with PEG/PHEMA IPN hydrogels (Experiments were done with Gel 6). (a) Fluorescence image obtained from Live/Dead fluorescence viability assay, (b) Result of MTT assays on corneal epithelial cells incubated with and without IPN hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

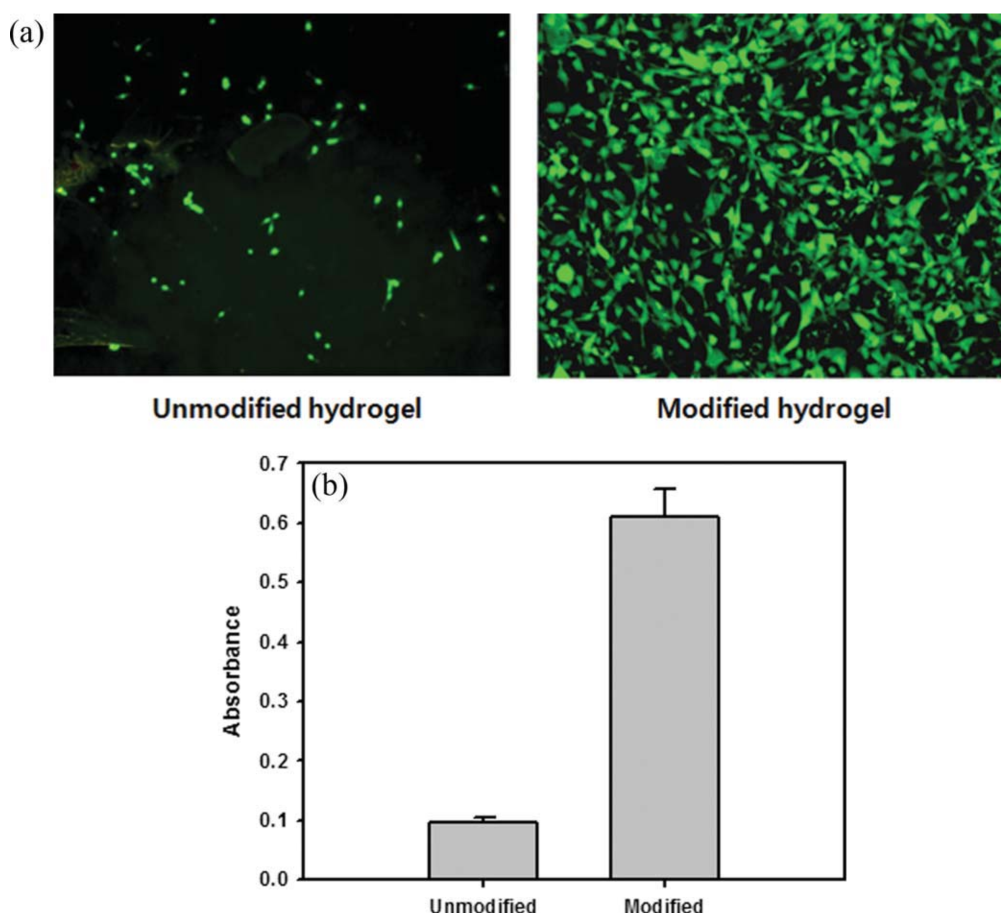

Figure 6 Adhesion of corneal epithelial cells on the modified and unmodified IPN hydrogels. (a) Fluorescence images of adhered cells, (b) Result of MTT assays on the adhered cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

many more green spots (living cells) than red spots (dead cells), indicating that most of the cells remained viable in the presence of IPN hydrogels. The viability of cells was further quantified using a MTT assay that measures mitochondrial dehydrogenase activity (MTS) within cells. Here, colorimetric measurement of formazan dye was performed on a spectrophotometer with an optical density reading at 490 nm, where the absorbance value is proportional to the number of cells that remain viable. Results were expressed as the relative absorbance value compared with the control condition (cells cultured in the absence of a hydrogel). As shown in Figure 5(b), MTS activity was not significantly different between HCECs cultured in the presence or absence of hydrogels, indicating that IPN hydrogels do not affect cell viability. These results demonstrate that the surface-modified IPN hydrogels were nontoxic to cells and that no toxic molecules leached from the hydrogel after the synthesis and surface modification processes.

After the cytocompatibilty of the IPN hydrogels was verified, cell adhesion and growth were evaluated. Figure 6a shows the adhesion of HCECs to collagen-modified and unmodified IPN hydrogels after 24-h incubation. Adherent cells were stained with calcein-AM for visualization. As expected, few cells adhered and cell spreading was not observed on the surfaces of unmodified hydrogels due to the exclusionary effect of the PEG and PHEMA against proteins and cells. In contrast, many cells adhered and spread on the hydrogel surfaces with covalently immobilized collagen, indicating that our surface modification strategy was effective at promoting robust bioactivity on otherwise inert hydrogel surfaces. Calcein-AM diffuses through the membrane of living cells and reacts with intracellular esterase to produce a green fluorescence. Therefore, green fluorescence not only allows the morphology of cells to be visualized but also indicates that the cells are viable and have enzymatic activity. The MTT assay further confirmed that more viable cells adhered to the surface-modified IPN hydrogels than unmodified IPN hydrogels [Fig. 6(b)]. Although more thorough investigations of the optimal cell growth conditions on hydrogel surfaces are needed, our preliminary results are very encouraging. Future work will be focused on further investigation of the long-term cell

viability, migration, and proliferation of cells on hydrogel surfaces modified using various cell adhesion molecules such as fibronectin, vitronectin, or their peptide analogues, as well as collagen.

CONCLUSIONS

We prepared IPN hydrogels by crosslinking PHEMA inside PEG hydrogels to improve the mechanical strength and allow surface modification of the resulting hydrogels. IPN hydrogels were synthesized by a two-step sequential UV polymerization process, which resulted in the formation of hydrogels consisting of two different networks physically entangling one other. The incorporation of PHEMA produced mechanically stronger hydrogels than single PEG hydrogels with a water content greater than 50%. The optical transparency of the resultant IPN hydrogels was strongly dependent on the amount of PHEMA incorporated; the more PHEMA incorporated, the higher the transparency of the hydrogel. The surfaces of IPN hydrogels were successfully modified by covalent immobilization of collagen via covalent links to the hydroxyl groups of PHEMA, which greatly improved cell adhesion and spreading on the hydrogel surfaces. These *in vitro* results provide the foundation for future optimization of this prototype for subsequent implantation *in vivo*, and indicate that PEG/PHEMA IPN hydrogels are promising materials for artificial corneas.

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