J Mater Sci: Mater Med (2010) 21:2425–2433
DOI 10.1007/s10856-010-4094-5

Immobilization of type-I collagen and basic fibroblast growth factor (bFGF) onto poly (HEMA-co-MMA) hydrogel surface and its cytotoxicity study

Tuo Yan · Rong Sun · Chun Li · Baihua Tan · Xuan Mao · Ningjian Ao

Received: 25 January 2010 / Accepted: 5 May 2010 / Published online: 26 May 2010
© Springer Science+Business Media, LLC 2010

Abstract Type-I collagen and bFGF were immobilized onto the surface of poly (HEMA-co-MMA) hydrogel by grafting and coating methods to improve its cytotoxicity. The multi-layered structure of the biocompatible layer was confirmed by FTIR, AFM and static water contact angles. The layers were stable in body-like environment (pH 7.4). Human skin fibroblast cells (HSFC) were seeded onto Col/bFGF-poly (HEMA-co-MMA), Col-poly (HEMA-co-MMA) and poly (HEMA-co-MMA) films for 1, 3 and 5 day. MTT assay was performed to evaluate the extraction toxicity of the materials. Results showed that the cell attachment, proliferation and differentiation on Col/bFGF-poly (HEMA-co-MMA) film were higher than those of the control group, which indicated the improvement of cell-material interaction. The extraction toxicity of the modified materials was also lower than that of the unmodified group. The protein and bFGF immobilized poly (HEMA-co-MMA) hydrogel might hold great promise to be a biocompatible material.

1 Introduction

Synthetic hydrogels such as poly (vinyl alcohol) (PVA), poly (2-hydroxyethyl methacrylate) (PHEMA), and poly (acrylamide) (PA) are attractive biomaterials due to their tissue-like elasticity, high diffusion capability, and high water content[1–4]. However, they also demonstrate relatively low protein adsorption and cell adhesion. The biological performance of biomaterials depends largely on protein adsorption, which subsequently induces responses such as cell adhesion and proliferation [5, 6]. Hydrogels are usually difficult to induce cellular responses due to their weak driving force associated with their hydrophilic surface [7]. Therefore, adequate surface modification approaches are needed to improve the biocompatibility of hydrogels.

The surface of hydrogels must be modified to support protein immobilization before it can be used to promote cell adhesion [8–10]. Here, two kinds of bimolecules, type-I collagen and basic fibroblast growth factor (bFGF), were chosen to modify the hydrogel. Type-I collagen is a robust fiber protein and the main component of the extracellular matrix of most tissues. It is commonly immobilized on different substrates for surface modification of biomaterials to improve their biocompatibility [11–14]. Cell growth factors are also necessary to be grafted onto the materials to enhance the cell growth rate and to adjust the cell functions. bFGF is a potent angiogenic factor in vivo and in vitro, playing an important role in regulating the proliferation, differentiation and migration for both fibroblast and endothelial cell [15].

PHEMA is widely used in many fields, such as: artificial cornea and cardiac tissue engineering, but it shows poor mechanical property and its protein adsorption and cell adhesion are low as well. The mechanical properties of hydrogels can be modified by co-polymerization. Co-polymerization with methyl methacrylate (MMA) is a possible way to improve the strength of PHEMA. In our previous work, we copolymerized HEMA and MMA with a

T. Yan · B. Tan · X. Mao · N. Ao (✉)
Department of Biomedical Engineering, Jinan University, No. 601, HuangPu Road, Guangzhou 510632, China
e-mail: taonj@jnu.edu.cn

T. Yan
e-mail: hgyant@163.com

R. Sun · C. Li
Department of Chemistry, Jinan University, No. 601, HuangPu Road, Guangzhou 510632, China

series of contents, the tear resistance increased from 262 to 568 J/cm^2 when the MMA content rose from 0 to 10% (v%). However the copolymer still remained a relatively high hydrophilic [16, 17].

In this study, we co-polymerized HEMA and MMA with a MMA content of 10 (v%), and type-I collagen was covalently immobilized onto the copolymer surface by carbonyldiimidazole (CDI) treatment, which is an easy, effective, and rapid way for surface modification, with little effect on the bulk of the materials [18–22]. The grafting and coating method was adopted to coat the collagen and bFGF onto the surface of poly (HEMA-co-MMA). The cytotoxicity was investigated by cell adhesion test and MTT assay.

2 Materials and method

2.1 Materials

Type-I collagen, HEMA, methyl methacrylate (MMA), CDI, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and bFGF were purchased from Sigma (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Beijing Newsprobe Biotechnology Co. Ltd. (China). Fetal bovine serum (FBS), penicillin and streptomycin were supplied by Hangzhou Sijiqing Bioengineering Material Company (China). All reagents used in the experiments were analytical grade.

2.2 Preparation of collagen/bFGF-coated poly (HEMA-co-MMA) film

2.2.1 Preparation of poly (HEMA-co-MMA)

MMA was mixed with HEMA with a content of 10 v%, and then the photo-initiator benzoin (1.0 g) and the crosslinking agent divinyl glycol (DVG) (0.2 ml) were added. The mixture was copolymerized in N_2 atmosphere and irradiated with UV light for 90 min. Then the film was put in an oven at 70°C for 4 h to accomplish the reaction. Finally, the film was rinsed for 3 times with deionized water to remove the unreacted monomer.

2.2.2 Poly (HEMA-co-MMA) surface activated by CDI

CDI was dissolved in 1,4-dioxane (20 mM). The poly (HEMA-co-MMA) sample was rinsed for 3 times with 1,4-dioxane to remove the remained water, and dried under vacuum before incubated in CDI solution at 37°C for 2.5 h. The sample was subsequently rinsed with 1,4-dioxane for 3 times to remove the unreacted CDI. The hydroxyl groups on the surface of the sample were activated by CDI, and an intermediate compound was formed (see Fig. 1a).

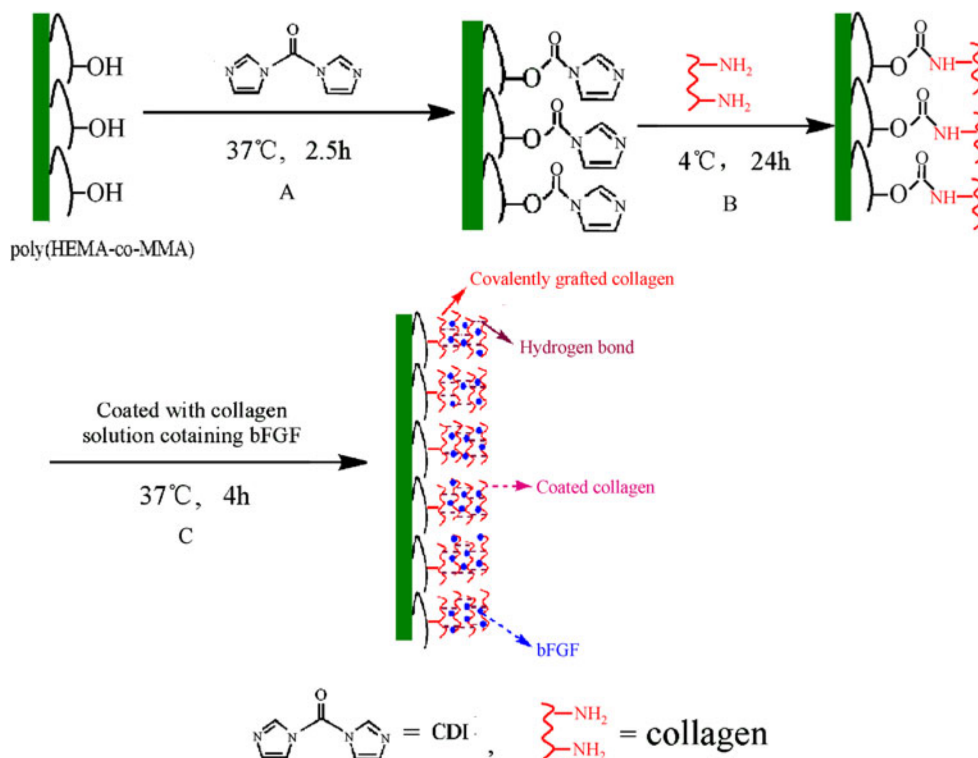

Fig. 1 Reaction scheme for immobilization collagen and bFGF onto the poly (HEMA-co-MMA) surface

2.2.3 Preparation of collagen-grafted poly (HEMA-co-MMA)

The activated film was washed three times with deionized water to remove the remaining CDI molecule. The collagen-grafted poly (HEMA-co-MMA) film was achieved by immersing the CDI-activated film in collagen solution (2.5 mg/ml, in 0.3% acetic acid) for 24 h at 4°C. The sample was then rinsed with phosphate buffer solution (PBS) for 3 times to remove the none-grafted (physically adsorbed) collagen and dried under vacuum. CDI plays a role as a condensing agent to promote the condensation between –OH and $-NH_2$ to immobilize protein molecules onto the polymer surface via covalent bonds (see Fig. 1b). The collagen was chemically grafted onto the surface of poly (HEMA-co-MMA).

2.2.4 Preparation of collagen/bFGF-poly (HEMA-co-MMA)

The collagen-grafted poly (HEMA-co-MMA) film were immersed at 37°C for 4 h in 0.3% acetic acid solution containing both collagen (2.5 mg/ml) and bFGF (1000 U/ml), and then dried under vacuum. The collagen and bFGF were physically coated onto the grafted collagen layer due to the strong hydrogen bonds between each other (see Fig. 1c). The only collagen-coated film was also prepared as a contrast by immersing the collagen-grafted poly (HEMA-co-MMA) film in collagen solution (2.5 mg/ml, in 0.3% acetic acid) at the same conditions as the bFGF-containing one.

2.3 Surface characterization

FTIR spectra of the collagen, poly (HEMA-co-MMA), Col/bFGF-poly (HEMA-co-MMA) were obtained with an FTIR spectrophotometer (EQUINOX55, Germany). Surfaces morphology was investigated using an atomic force microscope (AFM, CP-Research) in contact mode in air. The scanning range of AFM scanner was 4 μm × 4 μm, and the images were flattened with the software (Proscan Image Processing Software Version2.1) to eliminate low-frequency background noise in scanning direction. The material of tip is UL20B doped Si and the resonance frequency is 255–315 kHz. The wettability of the films was characterized by static water contact angles (Cam-plus LR9224). The values of contact angle were obtained by averaging the results of 10–12 measurements on each sample. At least five samples were tested in each group.

2.4 Stability of grafted and coated collagen layers

The word ‘stable' was used to express that the immobilized biocompatible layers adhered rather tightly on the films and were not easy to elute from the film surface. A good stability of the layers was crucial to perform its biological functions.

The stability of collagen layer in PBS (pH 7.4) and acetic acid (4%, w/v) solutions was studied respectively. Five samples were adopted in each group. The samples were cut into 1 cm × 1 cm, and immersed in the two solutions above at 4°C for different periods, and the amount of the collagen remained on the sample was quantified by coomassie brilliant blue method [23, 24]. To 0.1 ml mixture, 5 ml Coomassie brilliant blue solution was added and stood for 10 min. The absorbance of the solution was measured at 595 nm (A_{595}) with a UV–vis spectrophotometer. The standard curve was obtained using bovine serum albumin (BSA) as the control protein. A series of BSA solutions with the final concentrations of 0, 2, 4, 6 and 8 μg/ml, respectively, were mixed with 5 ml Coomassie brilliant blue solution. Relational equation (Eq. 1) was obtained by linear regression calculation, and the regression coefficient (R^2) was 99.8% (Fig. 2). According to Eq. 1, the amount of collagen immobilized on the surface could be calculated.

$$A_{595} = 0.00441\ C \tag{1}$$

2.5 Cell culture

Human skin fibroblast cells (HSFC) were supplied by the Medical department of Jinan University (Guangzhou, 510632). The cells were cultured in DMEM medium supplemented with 10% FBS and 100 U/cm^3 penicillin and streptomycin. The cultured medium was changed each second day. When cell confluence was achieved, the cells were detached by incubation with 0.25% trypsin/EDTA.

2.6 Cell adhesion assay

The Col/bFGF-poly (HEMA-co-MMA), Col-poly (HEMA-co-MMA) and poly (HEMA-co-MMA) films were placed in the bottom of tissue culture plate (24-well). The cell suspension with a density of 6×10^4 cells/well was immediately seeded onto each sample, and incubated at 37°C and 5% CO_2. The cell attachment and morphology were characterized at 1, 3 and 5 days respectively by a phase contrast light microscope. The numbers of the cells attached on each samples were counted under a haemacytometer after harvesting the adhered cell by trypsinization. Three samples were counted in each group.

2.7 MTT assay

MTT assay was carried out to evaluate the extraction toxicity of modified and unmodified materials. The films with a surface area/extracting vehicle ratio of 6 cm^2/ml, were extracted according to the ISO 10993 guideline. The extraction was performed in DMEM culture medium at

37 ± 2°C for 24 ± 2 h. The extract solutions, after filtration to eliminate any contamination, were stored at −20°C less than a week. The cells were adjusted to a density of 3×10^4 cells/ml. 100 µl cell suspensions were added into each well (96-well plates). After cells adhered to the plates, the culture medium was replaced with the extract solutions of modified and unmodified groups, and the TCPS group replaced with fresh DMEM culture medium as control. After incubated for 3, 6 and 9 days, 20 µl MTT was added at a concentration of 0.5 mg/ml, and incubated at 37°C for 4 h. Then 100 µl 10% SDS was added into each well. Optical densities (OD) were measured at 492 nm using a plate reader (Multiskan Mk3). Mean values were obtained from five wells per group.

To further evaluate the cytotoxicity, cell proliferation inhibition index (CPII) was determined using the following equation:

$$\mathrm{CPII} = 100\% - \frac{\text{Tested culture OD}_{492\mathrm{nm}}}{\text{TCPS culture OD}_{492\mathrm{nm}}} \times 100\% \qquad (2)$$

2.8 Statistical analysis

Data from all studies were analyzed by SPSS software. Statistical comparisons were made by one-way analysis of variance (ANOVA) (*T*-test) to determine whether there were any statistically significant differences. *P*-values < 0.05 were considered statistically significant.

3 Results and discussion

3.1 Characterization of modified films

FTIR spectra of poly (HEMA-co-MMA), Col/bFGF-poly (HEMA-co-MMA), and collagen were shown in Fig. 3.

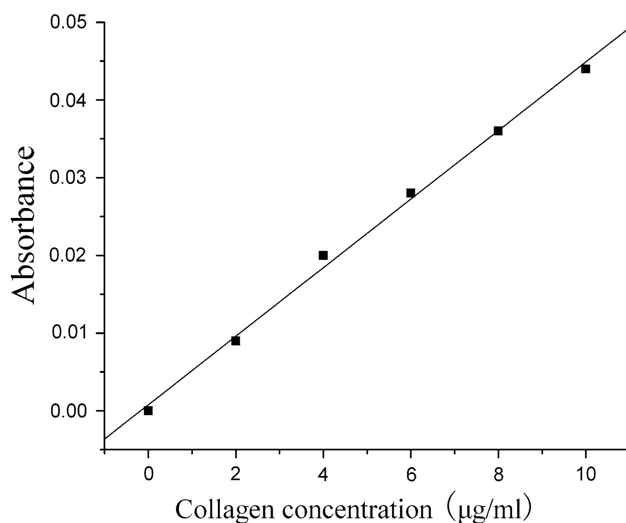

Fig. 2 Standard curve of coomassie brilliant blue method

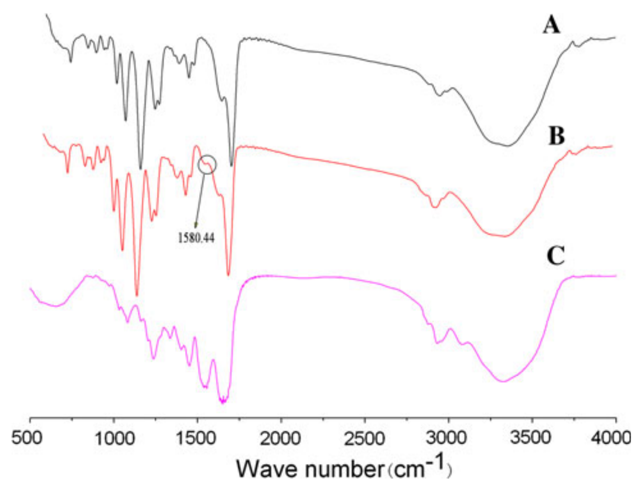

Fig. 3 FTIR spectra of (*a*) poly (HEMA-co-MMA), (*b*) Col/bFGF-poly (HEMA-co-MMA), and (*c*) collagen

The broad adsorption in the range of 3250–3500 cm^{-1} was assigned to the stretching vibration of O–H or N–H from –NH_2, –$CONH_2$, –OH or –COOH group in poly (HEMA-co-MMA) or collagen. The adsorption at 1580.44 cm^{-1}, assigned to the amide groups in collagen, can be observed in the spectra of Col/bFGF-poly (HEMA-co-MMA), but not in the poly (HEMA-co-MMA), which indicated that the collagen was successfully immobilized onto the surface of the poly (HEMA-co-MMA) film.

It was shown by AFM images that the coated collagen fibrils were attached in a flattened state to the surface of Col/bFGF-poly (HEMA-co-MMA). A mass of fibrils could be seen, and many fibrils were also self-assembled to form massive fibrils (Fig. 4)

Figure 5 showed the water contact angle of unmodified and modified poly (HEMA-co-MMA) film. The modified poly (HEMA-co-MMA) film exhibits a higher water contact angle (about 84°) than the unmodified one (about 70°) does, which indicated a slight decrease in hydrophilicity of Col/bFGF-poly (HEMA-co-MMA) film [25]. It is probably because PHEMA hydrogels are more hydrophilic than collagen.

3.2 Stability of the collagen layers

Figure 6 showed the significant difference of collagen amount after being immersed in two different solutions (*$P < 0.05$), which had a close relationship with the special biocompatible two-layered structure.

After being immersed in PBS, the outmost layer of collagen was washed into PBS solution, so a slightly weight loss was detected at initial hours, but no significant weight loss was observed in the following periods. The results suggested that the physically coated collagen was rather stable in body-like environment, and not apt to

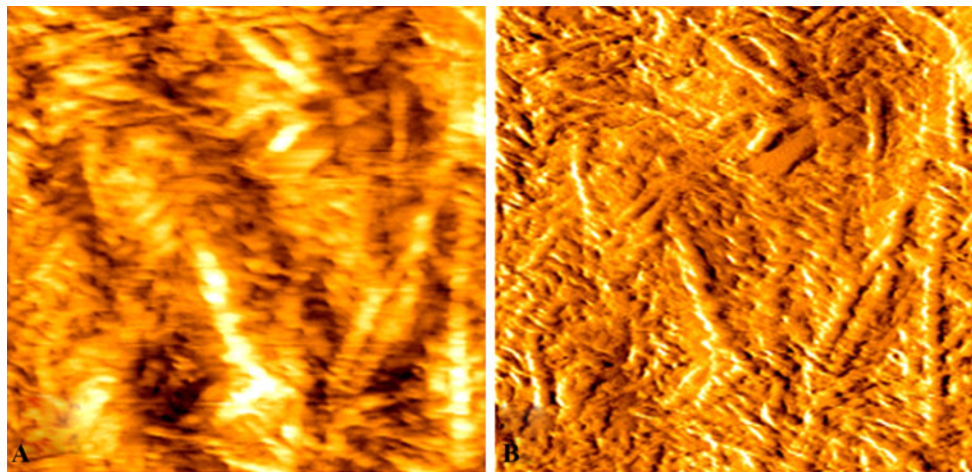

Fig. 4 AFM images of Col/bFGF-poly (HEMA-co-MMA) surface: (**a**) Topography and (**b**) Error signal morphology

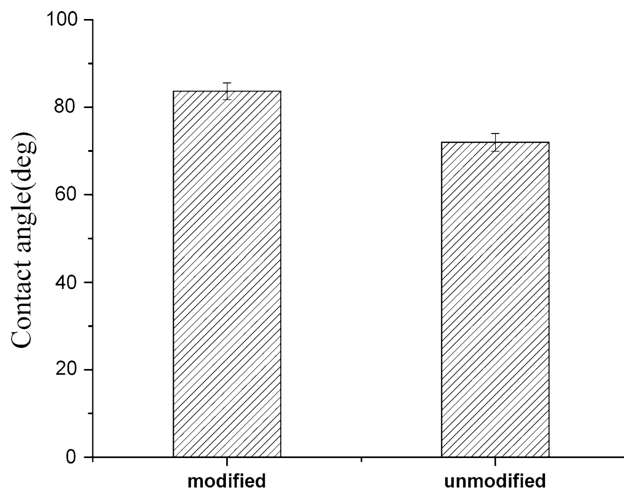

Fig. 5 Water contact angle of unmodified and modified films

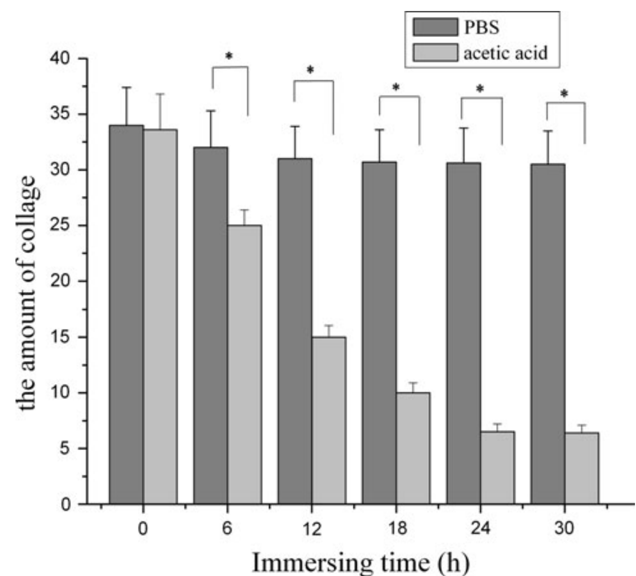

Fig. 6 The amount of collagen after the materials being immersed in PBS and acetic acid in different time periods

detach from the surface of the polymer, which was significant for implantation materials.

Collagen is insoluble in PBS (pH 7.4), but soluble in acetic acid solution. Since the physically coated collagen could be dissolved by acetic acid solution, the amount of collagen continuously declined while being immersed in acetic acid solution. Until the coated collagen was completely dissolved, only the chemical grafted collagen was left, the amount reached a very low constant amount after 24 h. Therefore, the amount of the coated collagen could be obtained from Eq. 3:

$$M_{\text{physical coated collagen}} = M_{\text{initial amount collagen}} - M_{\text{chemical grafted collagen}}. \quad (3)$$

$M_{\text{initial amount collagen}}$ and $M_{\text{chemical grafted collagen}}$ could be obtained from the vaules of the samples immersed in acetic acid solution for 0 and 30 h respectively. From the datas of Fig. 6, we could easily found that the $M_{\text{initial amount collagen}}$ was about 34 μg/cm^2, and $M_{\text{chemical grafted collagen}}$ was about 6.5 μg/cm^2, so $M_{\text{physical coated collagen}}$ was about 27.5 μg/cm^2 (Fig. 6)

3.3 Adhesion and proliferation of cells on materials

The response of cells to different surfaces was obviously different (Fig. 7). The uncoated poly (HEMA-co-MMA) could not promote the cell attachment and proliferation during the culturing period. The cell attachment of collagen coated surface (Col-poly (HEMA-co-MMA) and Col/bFGF-poly (HEMA-co-MMA)) was relatively higher than that of the uncoated surface after 1 day, which indicated that the coated collagen functionalized to improve cell attachment of polymer surface. After 3 days, a large number of cells began

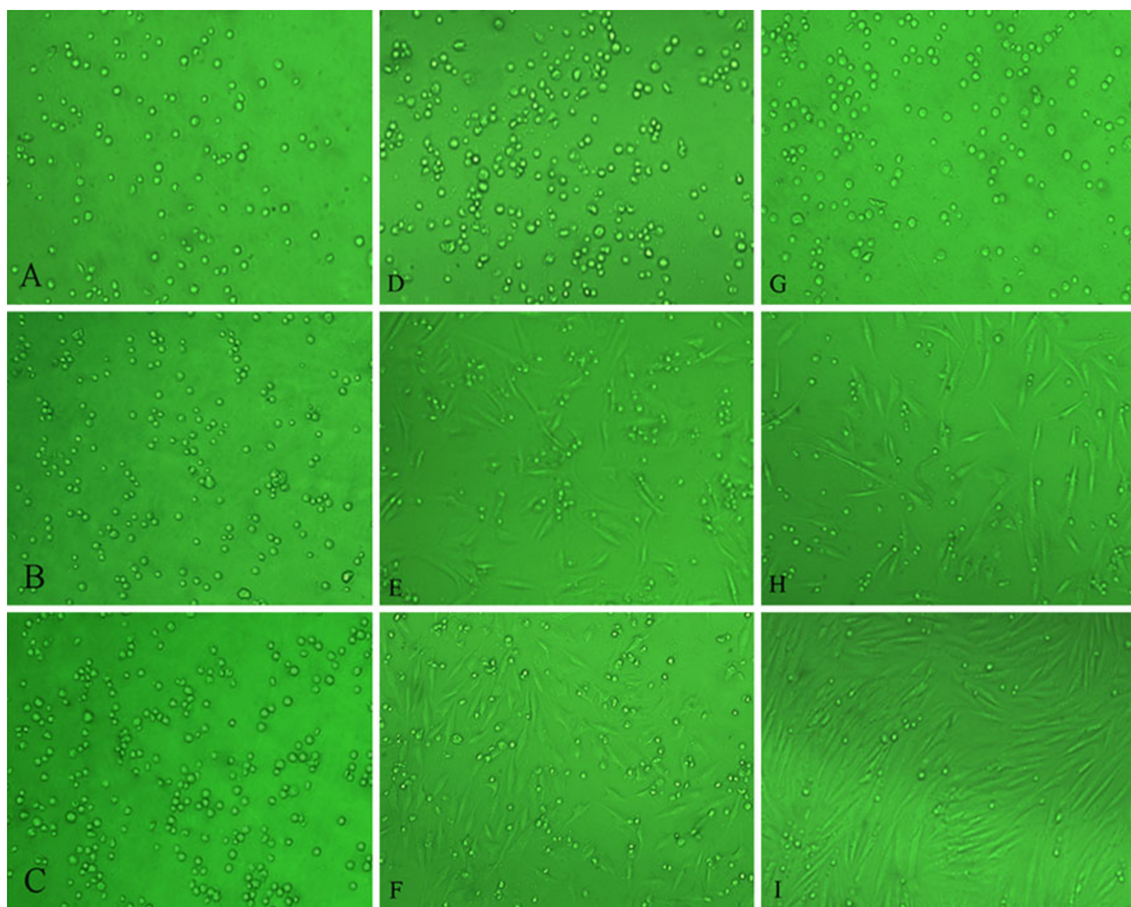

Fig. 7 Representative micrographs of HSFC cultured on poly (HEMA-co-MMA) for (**a**) 1 day; (**b**) 3 day, (**c**) 5 day; Col-poly (HEMA-co-MMA) for (**d**) 1 day, (**e**) 3 day, (**f**) 5 day; and Col/bFGF-poly (HEMA-co-MMA) for (**g**) 1 day, (**h**) 3 day, (**i**) 5 day

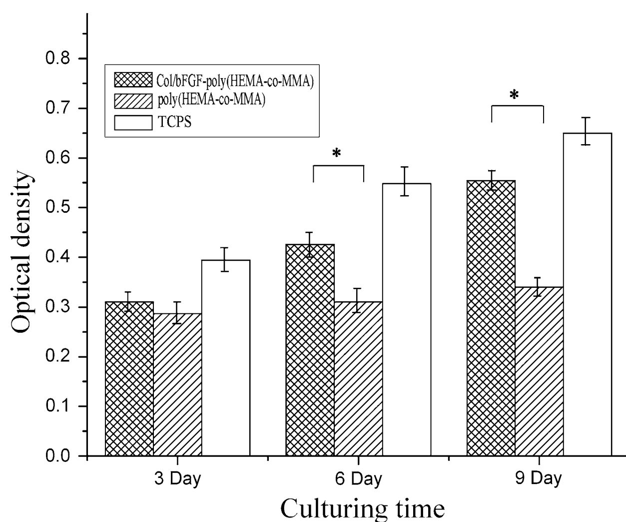

Fig. 8 MTT viability of cells cultured on control and modified films

to spread and proliferate on the surface of Col-poly (HEMA-co-MMA) and Col/bFGF-poly (HEMA-co-MMA). The proliferation rate of cells cultured on Col/bFGF-poly (HEMA-co-MMA) was higher compared to the other groups. After 5 days, more cells were found on the surface of Col/bFGF-poly (HEMA-co-MMA) and the cells exhibited a more spindle-like morphology, compared to those on the Col-poly (HEMA-co-MMA) films, which demonstrated that the coated bFGF, introduced by grafting and coating methods, maintained a high bioactivity, and further promoted the proliferation and differentiation of cell cultured on Col/bFGF-poly (HEMA-co-MMA) films.

3.4 Extraction toxicity of materials

Figure 8 showed the extraction toxicity of poly (HEMA-co-MMA) and Col/bFGF-poly (HEMA-co-MMA) films. After 3 days' culture, a higher value of optical density was observed for Col/bFGF-poly (HEMA-co-MMA) in comparison with poly (HEMA-co-MMA). After 6 and 9 days'culture, the difference in optical density was significant ($*P < 0.05$), which indicated that the extraction toxicity of Col/bFGF-poly (HEMA-co-MMA) was obviously lower compared to that of the unmodified group.

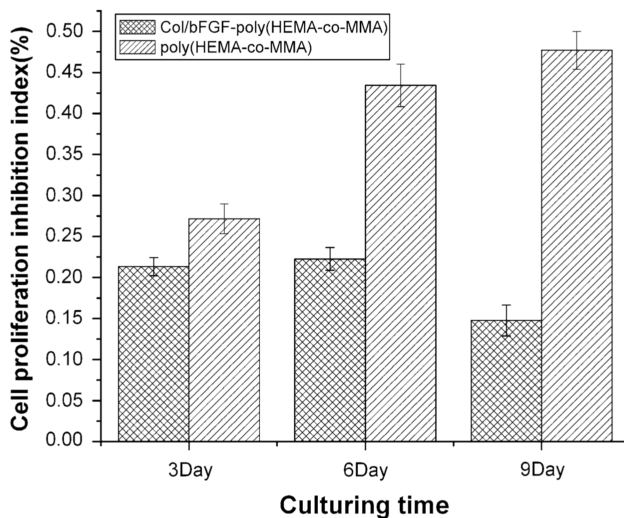

Fig. 9 Cell proliferation inhibition index (CPII) after 3, 6 and 9 days

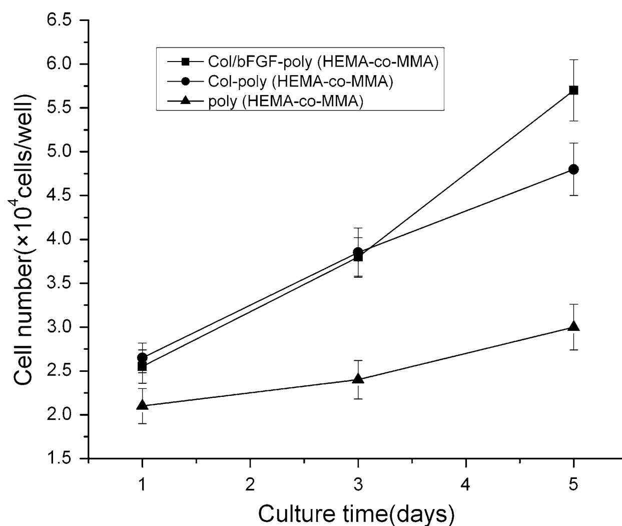

Fig. 10 Numbers of HSFC cultured on differently coated poly (HEMA-co-MMA)

Commonly, the high value of CPII indicated the extraction cytotoxicity of the materials. Figure 9 showed the CPII values of Col/bFGF-poly (HEMA-co-MMA) and poly (HEMA-co-MMA) after culturing for 3, 6 and 9 days. It was clearly that the CPII values of poly (HEMA-co-MMA) hydrogel were constantly higher than those of Col/bFGF-poly (HEMA-co-MMA) hydrogels ($P < 0.05$), i.e., the Col/bFGF-poly (HEMA-co-MMA) films had lower extraction cytotoxicity than the unmodified ones.

4 Discussion

Once cells contact with the materials, the initial and primary step was the adhesion of the cells onto the surface. To improve the biocompatibility of biomaterials, the improvement of their cell adhesion rate is the first issue to be considered. The adhesion process is highly complicated, which includes two steps [26, 27]: (i) cells attach to the surface via pseudopod; (ii) cells adhere to the surface via focal contact. The two steps both depend on the interaction between protein ligands from the surface and the membrane receptors from the cells. Therefore, the adsorption of specific proteins onto the material is the precondition of the cell adhesion. As for poly (HEMA-co-MMA) hydrogels, their low protein adsorption often leads to poor cell adhesion. So it is necessary to engineer specific protein to the surface of the material, to control the interactions between the ligands and the receptors, and to provide good cell adhesion for longer tissue integration.

Type-I collagen is one of the component proteins of human extracellular matrix (ECM). The peptide chain of type-I collagen comprised some short functional peptide sequence, such as RGD and DGEA peptides [28], which act as ligands in the cell adhesion process. Thus, the immobilization of type-I collagen may be able to control the ligands and receptors interactions when the cells attach to the material.

The following behaviors of cell-material interactions are cell proliferation, migration, and differentiation. FGF, one of the components of ECM, which plays an important role in controlling biological functions of cells, is also required to provide more bioactive substrate.

Until now, various technologies have been explored to immobilize collagen and bFGF onto the polymeric surface. For example, Li et al.[29] successfully attched collagen onto PU surface via oxygen plasma treatment; Hong et al.[30] immobilized bFGF onto PLGA surface by CO_2 plasma treatment; Ma et al. [31] grafed collagen on the PLLA surface by photo-oxidization method; Solitaire et al. [32] combined bFGF with PEG hydrogel by chemical grafting method. The results in these articles demonstrated that the immobilized collagen and bFGF could improve the adhesion, growth and migration of the cells on the surface. The materials modified by either collagen or bFGF showed better cytocompatibility than the unmodified groups.

However, the methods reported always resulted in very few amounts (less than 10 μg/cm^2) of successfully-grafted collagen [33, 34]. The efficiency of the immobilization was very low. On the other hand, few methods could immobilize collagen and bFGF on the polymer surface simultaneously were reported. In order to solve those problems, the grafting and coating methods were both adopted in our work. By the two-stepped grafting and coating methods, the collagen-grafted layer was formed firstly on the surface of the polymer, and then the collagen and bFGF mixed-layer was subsequently coated onto the grafted layer via strong hydrogen bonds between the grafted and the coated

collagen molecules. bFGF were coated on the material surface along with the immobilization of type-I collagen. The results of the stability test demonstrated that the efficiency of the immobilization and the stability of the physically-coated collagen layer were improved simultaneously by grafting and coating method, compared to those only through either chemical grafting method or directly coating method.

After the fibroblasts were seeded on the materials for 1 day, the numbers of cells adhered on collagen modified groups became much higher than those on the unmodified group (see Fig. 10). After 5 days, the proliferation rate of the cells cultured on collagen/bFGF modified material was higher than those of the groups only modified by collagen and the unmodified ones (see Fig. 10). The results proved that the immobilized collagen, which provided more ligands, could attract more cells to adhere onto the materials, and successfully improved the adhesion rate of the cells; bFGF, as a signal molecule, further promoted the adhered cells to grow, differentiate, and migrate on the polymer. Both of the proteins coated on the material functioned as signal transduction intermediate at the interface of the material and the biological system. The signals from outside of the cell film transmitted into the cytoplasmic components or DNA to induce cell response or gene expression.

The novel hydrogel is promising for artificial cornea due to its good cytocompatibility. The ‘‘core-and-skirt structure with a porous skirt was the most commonly and comparatively successful style of artificial cornea. Porous skirt could allow host tissue growing into the skirt and thus provide long-term implantation. A good biocompatibility of skirt material for the host keratocytes is required. The novel Col/bFGF-p (HEMA-co-MMA) hydrogel has shown much better cytocompatibility than unmodified one and may hold a great promise in this field.

5 Conclusions

In this study, collagen and bFGF were successfully immobilized onto the surface of poly (HEMA-co-MMA) hydrogel. With specific grafting and coating methods, a collagen-coated layer containing bFGF was obtained. The existence of the biocompatible layer was confirmed by FTIR and AFM. The amount of collagen attached only decreased slightly in PBS (pH 7.4), while an obvious decrease occurred in acetic acid. It indicated that the layer was rather stable in weak alkaline, similar to human body environment. Cell assay also demonstrated that HSFC exhibited better attachment, proliferation and differentiation on the surface of Col/bFGF-poly (HEMA-co-MMA) than that of Col-poly (HEMA-co-MMA) and poly (HEMA-co-MMA), which suggested the cooperative improvement of both collagen and bFGF on the cytotoxicity of materials. In a word, Col/bFGF-poly (HEMA-co-MMA) is an ideal and potential material for artificial organ.

Acknowledgment The study was supported by the National Natural Science Foundation of China (NO. 209760868/B060805).

Conflict of interest The authors claim no conflicts of interest.

References

1. Anna CJ, Conley Wake M, Mikos AG. Review: hydrogels for cell immobilization. Biotechnol Bioeng. 1996;50:357–64.
2. Hisatoshi K, Masabumi K, Tetsushi T, et al. Collagen immobilized PVA hydrogel-hydroxyapatite composites prepared by kneading methods as a material for peripheral cuff of artificial cornea. Mater Sci Eng C. 2004;24:729–35.
3. Haydar A, Serpil A, Hayrettin T. Covalent immobilization of amylase on chemically activated poly (styrene-2- hydroxyethyl methacrylate) microbeads. J Food Biochem. 2008;32:299–315.
4. Lai JY, Hsiue GH. Functional biomedical polymers for corneal regenerative medicine. React Funct Polym. 2007;67:1284–91.
5. Jin HL, Ju YM, Kim DM. Platelet adhesion onto segmented polyurethane film surfaces modified by addition and crosslinking of PEO-containing block copolymers. Biomaterials. 2000;21:683–91.
6. Mehrdad R, Takeshi M, Feng FL. Surface modification of collagen-based artificial cornea for reduced endothelialization. J Biomed Mater Res A. 2009;88:755–68.
7. Rocha Aracely, Hahn Mariah, Liang Hong. Critical fluid shear stress analysis for cell–polymer adhesion. J Mater Sci. 2010;45:811–7.
8. Zhong KW, Li B, Hong C, Lin Y, et al. Immobilization of proteins on metal ion chelated polymer surfaces. Colloid Surf B. 2009;69:71–6.
9. Skopinska WJ, Sionkowska A, Kaminska. Surface characterization of collagen/elastin based biomaterials for tissue regeneration. Appl Surf Sci. 2009;255:8286–92.
10. Chen H, Lin Y, Wei S. Biocompatible polymer materials: Role of protein–surface interactions. Prog Polym Sci. 2008;33:1059–87.
11. Lidia AS, Mirta M, Caraballo, Alexandre MR. Synthesis and characterization of biocomposites with different hydroxyapatite–collagen ratios. J Mater Sci Mater Med. 2009;20:2395–400.
12. Suh H, Hwang Y, Lee JE, Han CD, Park JC. Behavior of osteoblasts on a type I atelocollagen grafted ozone oxidized poly-L-lactic acid membrane. Biomaterials. 2001;22:219–30.
13. Lee DA, Assoke E, Doyle V. A specific quantitative assay for collagen synthesis by cells seeded in collagen-based biomaterials using sirius red F3B precipitation. J Mater Sci Mater Med. 1998;9:47–51.
14. Bhuvanesh G, Hilborn J, Christopher P. Thermal crosslinking of collagen immobilized on poly(acrylic acid) grafted poly(ethylene terephthalate) films. J Appl Polym Sci. 2002;85:1874–80.
15. Yao CC, Ping Y, Wu H, Zha ZG. Absorbable collagen sponge combined with recombinant human basic fibroblast growth factor promotes nerve regeneration in rat sciatic nerve. J Mater Sci: Mater Med. 2007;18:1969–72.
16. Qin BH, Huang H, Di JF. The cytotoxic measurement of copolymer PHEMA-MMA. Chin J Med Phys. 2003;20:186–8.
17. Qin BH, Huang H, Zhou H. Study on the PHEMA-MMA copolymer. J Biomed Eng. 1996;16:145–6.

18. Shi Q, Chen XS, Lu TC. The immobilization of proteins on biodegradable polymer fibers via click chemistry. Biomaterials. 2008;29:1118–26.
19. Russell HT, Sylvie MF, Brigitte LS. Covalent protein immobilization on glass surfaces: application to alkaline phosphatase. J Biotechnol. 2005;118:265–9.
20. Green D, Walsh SM. The potential of biomimesis in bone tissue engineering: lessons from the design and synthesis of invertebrate skeletons. Bone. 2002;30:810–5.
21. Stephanie MM, Ramesh G, Tae KK, Deborah L, Cecilia MG, Buddy DR. Characterization and analysis of osteopontin-immobilized poly(2-hydroxyethyl methacrylate) surfaces. J Biomed Mater Res A. 2003;67:334–43.
22. Sally LM, Michael WH, Viola Vogel, David GC. Covalent coupling and characterization of supported lipid layers. Langmuir. 2003;19:8316–24.
23. Li YH, Huang YD. Preparation of collagen–polyurethane composite film and its subcutaneous implantation in rats: the improvement of tissue compatibility. J Appl Polym Sci. 2006; 99:1832–41.
24. Lu XY, Li DJ, Yan H. Application of a modified Coomassie brilliant blue protein assay in the study of protein adsorption on carbon thin films. Surface Coat Tech. 2007;201:6843–6.
25. Ma ZW, Gao CY, Gong YH, Jian J. Immobilization of natural macromolecules on poly-L-lactic acid membrane surface in order to improve its cytocompatibility. J Biomed Mater Res. 2002;63: 838–47.
26. Elbert DL, Hubbell JA. Surface treatment of polymers for biocompatibility. Annu Rev Mater Sci. 1996;26:365–94.
27. Mosher DF. Adhesive proteins and their cellular receptors. Cardiovasc Pathol. 1993;23:149–55.
28. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature. 1984;309:30–3.
29. Li YH, Huang YD. The study of collagen immobilization on polyurethane by oxygen plasma treatment to enhance cell adhesion and growth. Surf Coat Tech. 2007;201:5124–7.
30. Hong S, Hu XX, Bei JZ, Wang SG. The immobilization of basic fibroblast growth factor on plasma-treated poly (lactide-co-glycolide). Biomaterials. 2008;29:2388–99.
31. Ma ZW, Gao CY, Jian Ji, Shen JC. Protein immobilization on the surface of poly-L-lactic acid films for improvement of cellular interactions. J Eur Polym J. 2002;38:2279–84.
32. Solitaire AD, James JM, Jennifer LW. Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. Biomaterials. 2005;26:3227–34.
33. Stephanie MM, Jeffrey LS, Cecilia MG, Buddy DR. Enhancing the biological activity of immobilized osteopontin using a type-1 collagen affinity coating. J Biomed Mater Res A. 2004;70:10–9.
34. Yokoyama Y, Kobayashi, Iwaki M. Evaluation of collagen immobilized to silicon plates by ion beam. Nucl Instrum Methods Phys Res Sect B. 2006;242:37–40.