Carbohydrate Polymers 76 (2009) 410–416

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Preparation and characteristics of novel porous hydrogel films based on chitosan and glycerophosphate

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ARTICLE INFO

Article history:
Received 9 September 2008
Received in revised form 31 October 2008
Accepted 3 November 2008
Available online 21 November 2008

Keywords:
Chitosan
Glycerophosphate
Hydrogel films
Protein adsorption
Cell culture

ABSTRACT

In this study, we prepared thermosensitive hydrogels by adding α-β-glycerophosphate (α-β-GP) to chitosan (CS) solutions. Then the hydrogels were dried to form films at room temperature. Scanning electron microscope (SEM) revealed that the hydrogel films had rough surfaces and porous cross-sections. Compared with pure chitosan films, the CS/GP hydrogel films showed better elasticity and lower tensile strength. Contact angle studies indicated that all these materials have good hydrophilicity. The CS/GP hydrogel films exhibited higher protein adsorption against both negatively charged protein (bovine serum albumin) and positively charged protein (lysozyme) than pure chitosan films. The results of MTT assay performed with the extracts of the CS/GP hydrogel films revealed the films had nontoxicity. The mouse embryonic fibroblast cells cultured on the CS/GP hydrogel films had good spreading and no apparent impairment of cell morphology. The results indicated that the CS/GP hydrogel film could be a promising candidate biomaterial for biomedicine applications.

1. Introduction

Porous biomaterials often attract scientist's particular concern in biomedicine applications. In the filed of tissue engineering, porous polymer scaffolds can provide a frame work for the seeded cells until they are well organized into a functioning tissue, (Kaufmann, Heimrath, Kim, & Mooney, 1997; Madihally & Matthew, 1999), especially in bone regenerative therapy (Park et al., 2000; Yaylaoğlu et al., 1999). In the filed of surgical, the porous biomaterial is usually used as wound dressing to absorb wound fluid and promote healing (Deng et al., 2007).

In recent years, considerable attention has been given to porous membrane materials based on chitosan. Chitosan, derived from chitin found in crustacean shell, is the second most abundant polysaccharide in nature after cellulose. It has both reactive amino and hydroxyl groups, which can be used to chemically alter its properties under mild reaction conditions. With good biocompatibility, biodegradability, antibacterial and wound-healing property, and the ability to be formulated in a variety of forms including powders, films, beads (Chen et al., 2006; Guo et al., 2005) hydrogels (Molinaro, Leroux, Damas, & Adam, 2002; Obara et al., 2003), tubular (Mironov et al., 2005), conduits (Wang et al., 2006), and porous scaffolds (Madihally & Matthew, 1999). Chitosan is widely used as a supporting material for tissue engineering applications (Kim et al., 2008), cell culture, nerve regeneration (Li et al., 1999; Wang et al., 2006) and so on.

Chitosan/glycerophosphate (CS/GP) hydrogel, a novel thermosensitive hydrogel, first reported by Chenite et al. (2000) is an important system due to its sol–gel transition at body temperature. The system remained to be solution at physiological pH (7.0) and room temperature, but changed into a gel upon heating at physiological temperature (37 °C). Many works had reported this thermosensitive hydrogel can be used in pharmaceutical areas (Gariépy & Leroux, 2004; Sharma et al., 2007), as scaffolds for cell culture (Crompton et al., 2007; Roughley et al., 2006). Recently, Human mesenchymal stem cells was seeded in CS/GP hydrogels, and found the stem cells can be differentiated to NP-like cells (Richardson et al., 2008). α-β-Glycerophosphate (α-β-GP) is a mixture of α-glycerophosphate (α-GP) and β-glycerophosphate (β-GP), and α-GP has linear chain structure and shows less steric hindrance than β-GP. Wu, Su, and Ma (2006) had reported a transparent thermo- and pH-sensitivity hydrogel based on quaternized chitosan (HTCC)/α-β-GP and concluded that α-β-GP had better gelation capacity compared with β-GP. When the HTCC/GP hydrogel cross-linked by PEG (Wu, Wei, Wang, Su, & Ma, 2007), it can be dropped or sprayed easily at room temperature, but transformed into viscous hydrogel at body temperature. Zhou et al. (2008) had prepared a thermosensitive hydrogel with chitosan and α-β-glycerophosphate (α-β-GP) which could be transited from solution into gel at 37 °C. In conclusion, chitosan (or chitosan derivatives) solution was neutralized by glycerophosphate can be transformed

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0144-8617/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2008.11.020

into macroporous thermosensitive hydrogel. The former methods to engineer porous scaffold include foaming, freeze drying (Arpornmaeklong, Suwatwirote, Pripatnanont, & Oungbho, 2007), salt leaching (Kim, Park, Kim, Wada, & Kaplan, 2005), 3D printing (Park et al., 1997; Wan, Creber, Peppley, & Bui, 2003) and spinning (Liu & Bai, 2005) techniques. Till now, there was no report on the porous films based on chitosan/glycerophosphate hydrogels.

In this study, we used the reported method to prepare CS/GP hydrogel. The hydrogel films were obtained through evaporation. The properties of resultant hydrogel films, such as morphology, hydrophilicity, mechanical properties, protein absorption, and biocompatibility, were carefully tested to determine whether it was an ideal material for biomedicine application.

2. Materials and methods

2.1. Materials

Chitosan, derived from crab shell, molecular weight 1360 kDa; deacetylation degree 75.6%, prepared as previously by the method of degradation with acetic acid according toChen et al. (2002). α-β-Glycerophosphate (α-β-GP), lactic acid (LA), acetic acid (HAC), hydrochloric (HCL) were all analytical grade (Sigma Co., St. Louis, USA). Dulbecco's modification of Eagle's medium (DMEM), Bovine serum albumin (BSA), lysozyme, Bicinchoninic acid assay kit and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Sigma Co., St. Louis, MO, USA).

2.2. Preparation of hydrogel films

About 1.8 g of chitosan was dissolved in 90 ml, 0.1 M acetic acid solution (acetic acid, hydrochloric, and lactic acid) with stirring until complete dissolution. Then the chitosan solution was chilled to 4 °C for 20 min. Fifty percent w/v aqueous α-β-GP (10 ml) was prepared in distilled water and chilled along with the chitosan solution to 4 °C. Then the α-β-GP solution was added dropwise to the chitosan solution under stirring and the final chitosan-α-β-GP solution was mixed for another 20 min. Then poured the chitosan-α-β-GP solution on a stainless steel plate and incubated at 37 °C. The formed hydrogel was maintained at room temperature to evaporate as much water as possible till the hydrogel film formed. The as-prepared films was further neutralized with 1% NaOH solution, excessively washed with distilled water, and dried in vacuo at room temperature for 24 h.

The hydrogel films prepared with different solvent named CS_{LA}/GP, CS_{HAC}/GP, CS_{HCL}/GP, respectively. The pure chitosan films were prepared by dissolve chitosan in different solvent, and casting the chitosan solution on a stainless steel plate let films formed, identified as CS_{LA}, CS_{HAC}, CS_{HCL}, respectively.

2.3. Scanning electron microscope (SEM) observation

The surface and cross-section morphologies of films were observed by scanning electron microscopy. The films were gently rinsed with distilled water to reach an equilibrium hydration. After lyophilize, the film samples were then coated with gold under vacuum using SCD 004 Balzers sputter coater (Balzers, Liechtenstein) and the surfaces were investigated scanning electron microscopy (KYKY2800B, KYKY Technology Development Ltd., Beijing, China).

2.4. Swelling index

Films were shaken in PBS (pH 7.4) solution at 37 °C in a horizontal shaker until maximum weight was reached. The swelling index, Sw, of the prepared films was determined according to the following equation:

$$Sw = \frac{W_s - W_d}{W_d}$$

where W_s and W_d represent the weights of the fully hydrated and the dry film, respectively.

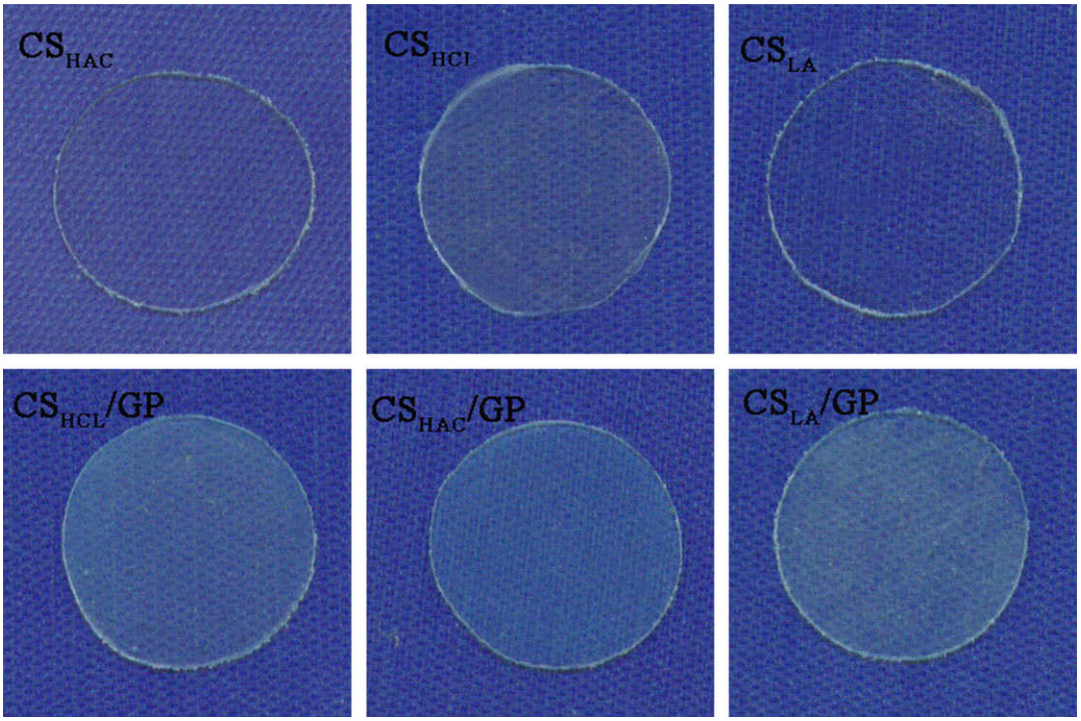

Fig. 1. The appearance of different films.

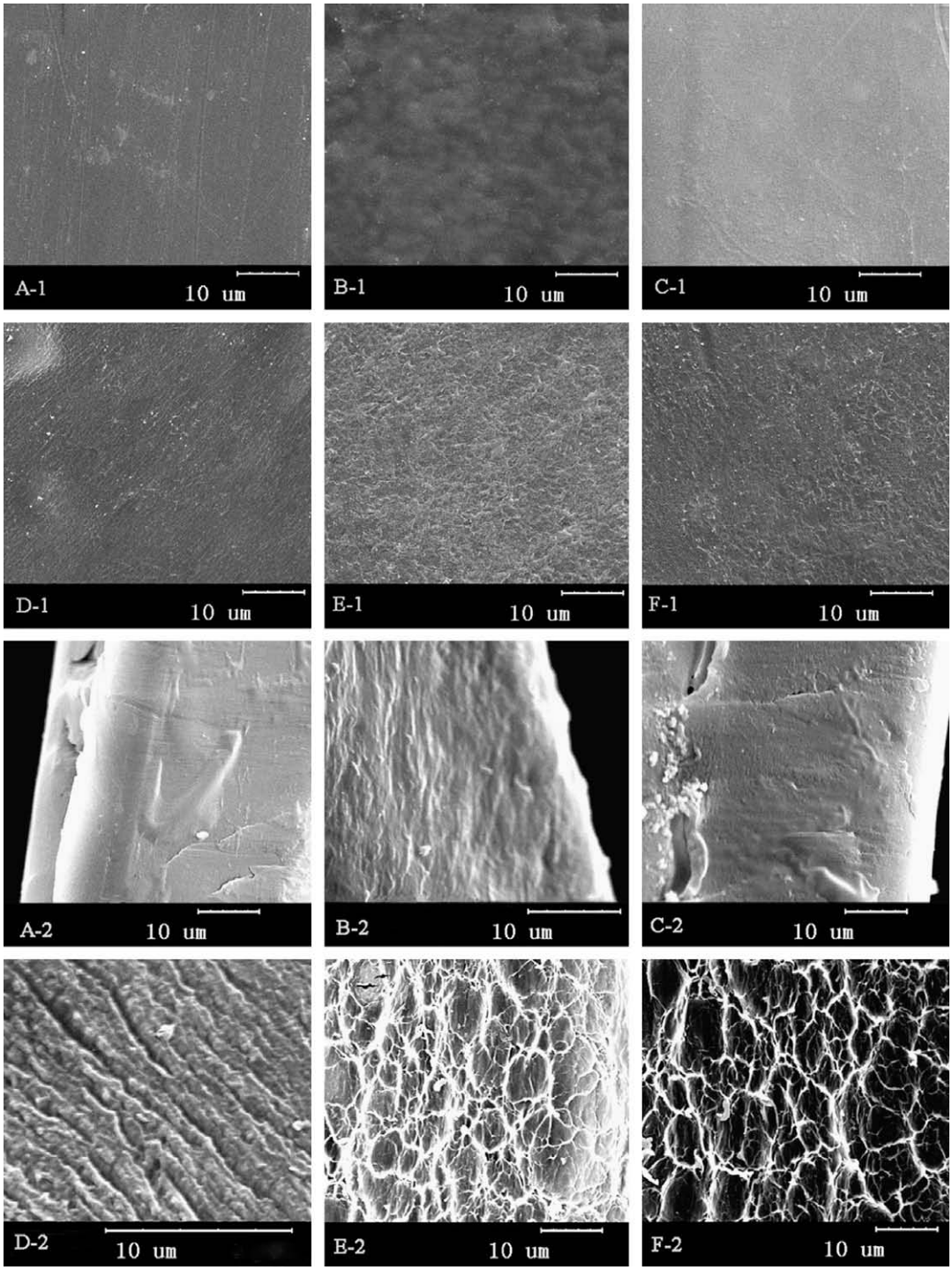

Fig. 2. SEM observations of the surface and cross-section of the membranes: (A-1) CS_{HAC} (surface); (A-2) CS_{HAC} (cross-section); (B-1) CS_{HCL} (surface); (B-2) CS_{HCL} (cross-section); (C-1) CS_{LA} (surface); (C-2) CS_{LA} (cross-section); (D-1) CS_{HAC}/GP (surface); (D-2) CS_{HAC}/GP (cross-section); (E-1) CS_{HCL}/GP (surface); (E-2) CS_{HCL}/GP (cross-section); (F-1) CS_{LA}/GP (surface); (F-2) CS_{LA}/GP (cross-section).

Table 1
Static contact angle (°) with water and swelling index at the equilibrium (%) in PBS (pH 7.4) at 37 °C of different films.

	Films					
	CS_{HAC}	CS_{HCL}	CS_{LA}	CS_{HAC}/GP	CS_{HCL}/GP	CS_{LA}/GP
Contact angle (°)	76 (1.0)	81 (2.5)	70 (2.5)	66 (1.6)	69 (2.1)	65 (1.2)
Swelling index (%)	192	159	203	242	214	256

Standard deviation in parentheses.

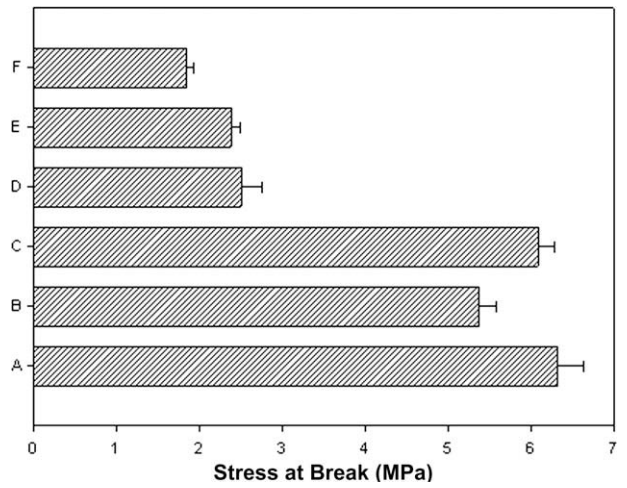

Fig. 3. Strain–stress of different films. (A) CS_{HAC}; (B) CS_{HCL}; (C) CS_{LA}; (D) CS_{HAC}/GP; (E) CS_{HCL}/GP; (F) CS_{LA}/GP.

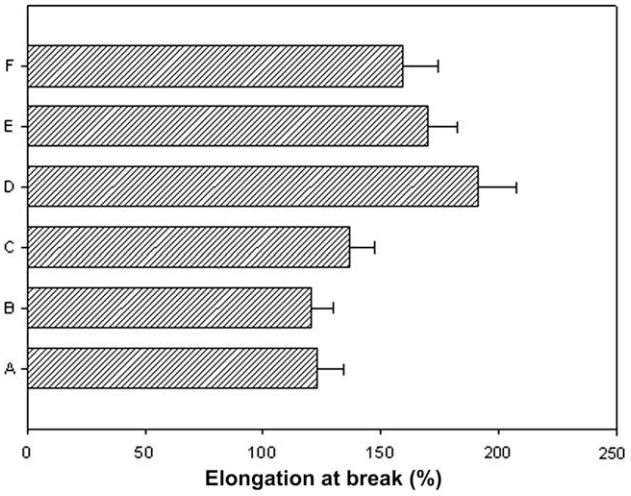

Fig. 4. Elongation at break of different films. (A) CS_{HAC}; (B) CS_{HCL}; (C) CS_{LA}; (D) CS_{HAC}/GP; (E) CS_{HCL}/GP; (F) CS_{LA}/GP.

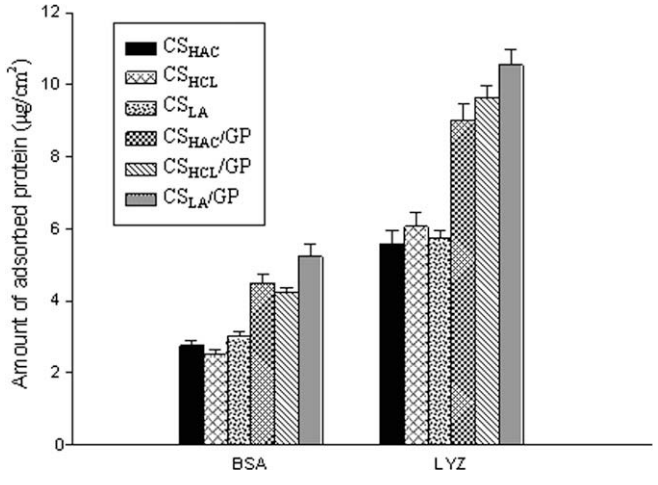

Fig. 5. Amount of adsorbed BSA and LYZ on different films.

2.5. Contact angle measurement

Contact angle measurements were performed at room temperature with a contact angle measuring device (model JC2000A, Power each Digital Equipment, Shanghai, China). A drop of double distilled water was dropped onto the surface of the dry film. Images of the water droplet were recorded within 5 s. Digital pictures were analyzed by computer for angle determination. The contact angles were measured on both sides of the films and averaged. The results presented are the mean values of six independent measurements.

2.6. Mechanical properties (tensile strength and elongation at break)

The tensile strength and elongation at break of the films were measured with a tensile tester (AI-7000M, Taiwan Gao Tie Technology, China) at room temperature. Films in the fully hydrated state were tested at constant rate (100 mm/min) until break. Test samples were cut into dumbbell model with length 75 mm, width 4 mm. The results presented were the mean values of six independent measurements.

2.7. Protein adsorption study

Bovine serum albumin (BSA) and lysozyme (LYZ) were selected for the protein adsorption study. Bicinchoninic acid (BCA) assay was used to determine the amount of adsorbed protein on the films.

Protein solutions were freshly prepared by dissolving BSA (LYZ) in PBS at pH 7.4 to give a final concentration of 0.1 mg/ml. All the films were cut into circular disc specimens, of which diameter was 20 mm.

To reach an equilibrium hydration, the film substrate was immersed in the PBS solution overnight prior to adsorption. Each sample was removed from PBS solution, then dipped in 3 ml protein solution and incubated at 37 °C for 24 h. Then the films were removed and rinsed with PBS solution, followed by sonication in 2 ml of 1 wt% sodium dodecylsulfate (SDS) to remove reversibly adsorbed protein. The amount of protein adsorbed on the substrates was determined by the micro-BCA protein assay. The absorbance of the solution was measured at 562 nm by UV–VIS spectroscopy. The amount of adsorbed protein was determined by comparison the absorbance of the samples with a calibration curve. Three repetitions were performed for all samples.

2.8. Cell culture

Before cell culture, the circular films were sterilized with 70% ethanol for 30 min, then washed three times with fresh culture medium and incubated at 37 °C in fresh culture medium for 24 h. Mouse embryonic fibroblasts (MEF) suspensions were diluted to a concentration of 1×10^4 cells/ml, as counted with a hemacytometer. Then the diluted cell suspension was added to 12-well tissue culture clusters (Costar) coated with different membranes. The clusters were incubated in DMEM containing 100 mg/ml penicillin and streptomycin, 10% fetal bovine serum at 37 °C with 5% CO_2 for 48 h before observation.

2.9. Extraction method

The sterilized hydrogel films (6 cm^2/ml of culture medium) were soaked in serum-supplemented culture medium (DMED) for 72 h at 37 °C according to a previously reported procedure (Ye, Kennedy, Li, & Xie, 2006). Then the extracts were diluted with culture medium, a series of the extract dilutions (50%, 25%, 12.5%,

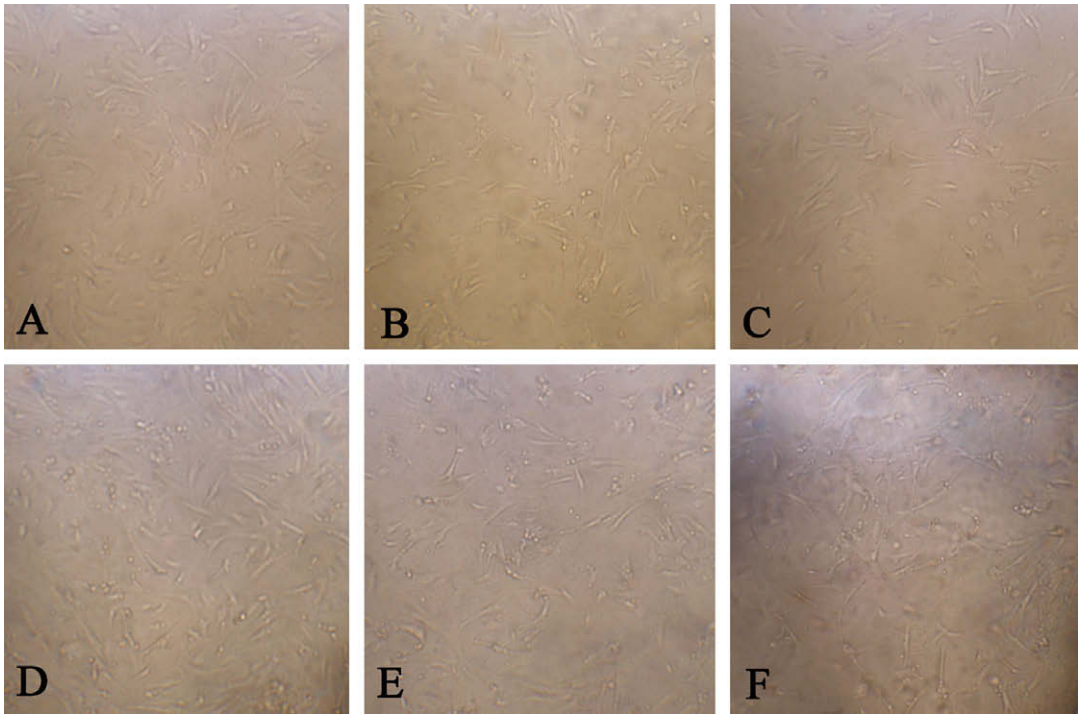

Fig. 6. Photographs of MEF cells cultured for 48 h on different films (light micrograph, 100×). (A) CS_{HAC}; (B) CS_{HCL}; (C) CS_{LA}; (D) CS_{HAC}/GP; (E) CS_{HCL}/GP; (F) CS_{LA}/GP.

and 6.25% (v/v)) was collected. The cells were seeded into 96-well plates at a density of 1×10^5 cells/ml. The culture medium (100 μl) was replaced with each concentration extract dilutions after 24 h. After 24, 48, and 72 h incubation, the viability of every group was assessed by the MTT assay. Serum-supplemented culture medium (DMED) was used as control groups.

2.10. MTT assay

MTT (25 μl) was added to each well and the cultures were incubated at 37 °C for additional 4 h. The cells were then washed gently with PBS of pH 7.5 to remove untransformed MTT and sample residues. DMSO (150 μl) was subsequently added to each well to dissolve the MTT formazan purple crystals. Absorbency of the solution was measured at 490 nm using an enzyme linked immunosorbent assay (ELISA) Reader (MODEL550, Bio-Rad, USA).

The relative cell growth (%) related to control cells containing cell culture medium without extracts was calculated from the following equation:

$$\text{Relative cell growth}(\%) = \frac{[\text{OD}]_{\text{test}}}{[\text{OD}]_{\text{control}}} 100\%$$

3. Result and discussion

3.1. Morphology studies

The chitosan/glycerophosphate hydrogels were prepared with different solvent, acetic acid, hydrochloric, and lactic acid. All the hydrogels were injectable viscous liquids below 37 °C, while they would transform into non-flowing gel at 37 °C. Both the CS_{HAC}/GP hydrogel and CS_{HCL}/GP hydrogel were soft and transparent. As the two hydrogels were heated from 20 to 80 °C, their transparence remained the same. But the CS_{LA}/GP hydrogel was ivory-white and relatively hard when the temperature increased from 20 to 80 °C.

The appearance of different hydrogel films are showed in Fig. 1. The pure chiosan films were transparent. Compared with pure chitosan films, the CS/GP hydrogel films were ivory-white in appearance.

Scanning electron microscope (SEM) microphotographs of the surfaces and cross-section of different films are showed in Fig. 2. The surfaces of pure chitosan films were relatively flat, homogeneous, and smooth. The cross-section of the samples was dense, non-porous. However, the surface of chitosan/GP hydrogel films were roughness, and the cross-section showed porous network microstructure. This is attributed to the formation of 3D macroporous network structure, which also exists in resultant films, in the gelation process of the chitosan/GP solutions.

3.2. Hydrophilicity and swelling properties

As far as the surface characteristics are concerned, the hydrophilicity of materials has been shown to be an important factor for cell attachment (Bumgardner et al., 2003), and protein adsorption (Burns, Holmberg, & Brink, 1996).

The result of contact angles and the swelling index were listed in Table 1. The contact angles of pure chitosan films prepared with HAC, HCL, and LA were 76, 81, and 70, while the relevant hydrogel films were 66, 69, and 65. The chitosan/GP hydrogel films showed higher hydrophilicity than the films prepared with pure chitosan. But the contact angles for all the materials were less than 90, indicating that the surface of all these materials had good hydrophilicity.

The chitosan/GP hydrogel films showed higher swelling index than the membranes of pure chitosan. Because almost only electrostatic interaction between the amino groups and hydroxyl groups contributed to the swelling property in CS films, while in CS/GP hydrogel the contributive interactions included electrostatic attraction, hydrogen bonding, and hydrophobic interactions (Garié-py & Leroux, 2004).

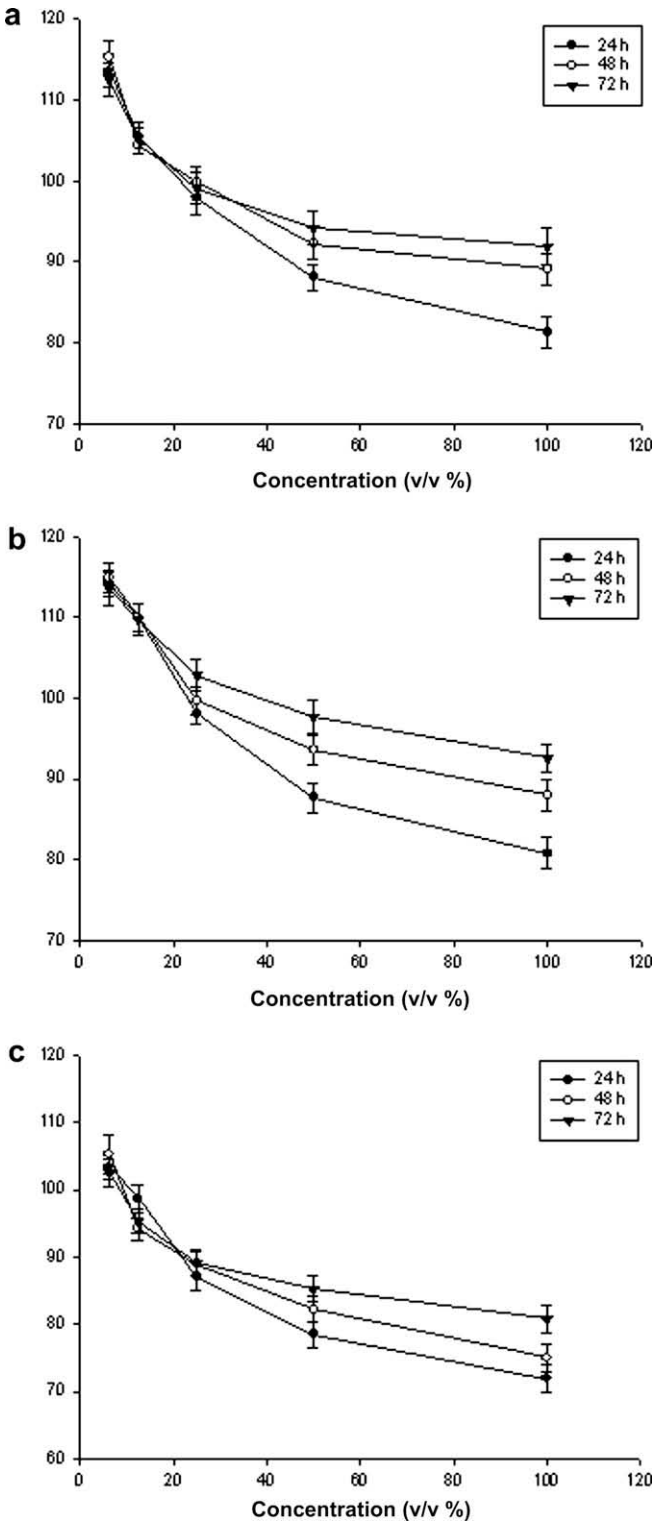

Fig. 7. Effects of the concentration of the extracts of CS/GP hydrogel films on the relative cell growth of MEF cells after 24, 48, and 72 h. (a) CS_{HAC}/GP; (b) CS_{HCL}/GP; (c) CS_{LA}/GP.

3.3. Mechanical properties

The mechanical properties of hydrated films are showed in Figs. 3 and 4. Compared with the pure chitosan films, CS/GP hydrogel films exhibited lower tensile strength, which was attributed to the three-dimensional porous structure of the hydrogel films. And with a denser cross-section, the elongation property of CS films is lower than that of CS/GP hydrogel films. Interestingly, the CS_{HAC}/GP hydrogel films showed higher tensile strength and elongation property than CS_{LA}/GP and CS_{HCL}/GP hydrogel films.

3.4. Protein adsorption study

Bovine serum albumin (BSA) and lysozyme (LYZ) were selected as models of negatively charged proteins and positively charged proteins. BSA (pI = 4.7, M_w = 68 kDa) is an amphiphilic protein due to the presence of a NH_2 and a COOH group in its molecular structure. The pH of the environment has a great effect on BSA adsorption (Peng, Hidajat, & Uddin, 2004), and under the experimental condition (pH 7.4 buffer) in this study it carries a negative charge. While lysozyme (pI = 11, M_w = 14.6 kDa), a globular protein, contains a large number of OH and NH_2 groups showed positive charge.

Fig. 5 shows the amount of protein adsorbed on the membrane surface. The CS/GP hydrogel films adsorbed more proteins than the chitosan films both for BSA and LYZ. Among the three kinds of hydrogel films, the CS_{LA}/GP hydrogel film showed the highest adsorptive behavior. Several interactions of chitosan/GP hydrogel films may be involved in controlling this behavior. The high protein absorption ability of CS/GP hydrogel films may be attributed to the rough and high hydrophilic surface, the porous network of cross-section, the less positive charge and so on.

The ability of surface protein adsorption is considered as an important factor contributing to the biocompatibility of materials, as it could influence cell adhesion, proliferation, differentiation (Wilson, Clegg, Leavesley, & Pearcy, 2005), and the conformation variation of the adsorbed protein (Zhang et al., 2002). Protein adsorption is often controlled by hydrophobic interaction, hydrogen bonding, and electrostatic interaction, simultaneously, especially by the hydrophilicity and surface charge of materials (Burns et al., 1996).

3.5. Biocompatibility of the hydrogel films

Photographs of the MEF cells cultured for 48 h on the various materials are shown in Fig. 6. The cells remained relatively flat and well spread in the light microscope. The cells attached and spread on the hydrogel film surfaces without apparent impairment of cell morphology.

Fig. 7a–c shows effects of the concentration of the extracts of CS/GP hydrogel films on the relative cell growth after 24, 48, and 72 h. The relative cell growth for CS_{HAC}/GP and CS_{HCL}/GP extracts and dilutions were more than 80% after 24, 48, and 72 h, while the CS_{LA}/GP extract only 70%. The reason may be that lactic acid is a metabolite in cell growth, which can do harm to cells. On the whole, we can find the relative cell growth increased with the decrease of extracts concentration. In addition, the relative cell growth increased with time.

From the results of MTT assay and cell morphology evaluation, we could see that the CS/GP hydrogel films had good biocompatibility. Cell cultured on the surface of films, must require the films have low interfacial tension that allow cells to move across the material boundary. With the formation of 3D macroporous network scaffold at the first step, the hydrogel films prepared in this paper have similar mechanical properties to soft tissue. As the water evaporation, the network shrank, but the macroporous structure remains in the system. And the network of the dried hydrogel films could partly absorb free water in culture medium, thus allowing diffusion of oxygen, nutrients, and waste throughout the scaffold. In this way, the GP/CS hydrogel films are ideal for cell culture.

4. Conclusion

The present processing method was demonstrated to be successful for preparing porous and soft CS/GP hydrogel films. The photos of SEM showed the resultant hydrogel films possess of rough surface and porous cross-section. Compared with pure chitosan films, the hydrogel films exhibit better elasticity, lower tensile strength, and higher hydrophilicity. Cell culture and protein absorption experiments showed the CS/GP hydrogel films had non-toxicity and high protein adsorption. The EMF cells can be attached and spread on the surface of the hydrogel films without impairment. In addition, with their high porosity, high capacity of protein adsorption and good biocompatibility, they are more adapted to the delivery of drugs, proteins, and enzymes, the hydrogel films are a promising candidate biomaterial for pharmaceutical and tissue-engineering applications.

Acknowledgements

We thank the financial support of Ministry of Education of the People's Republic of China (20070423013) and the Natural Science Foundation of Shandong Province (No. Y2006C110).

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