Polyelectrolyte Complex Hydrogel Composed of Chitosan and Poly(γ-Glutamic Acid) for Biological Application: Preparation, Physical Properties, and Cytocompatibility

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ABSTRACT: Polyelectrolyte complex (PEC) hydrogels composed of chitosan as a cationic polyelectrolyte and poly (γ-glutamic acid) (γ-PGA) as an anionic polyelectrolyte were prepared from PEC dispersions based on a chitosan solution to which different amounts of γ-PGA solutions were added to charge equivalency. The chemical structures of the PEC hydrogels were investigated by Fourier transform infrared spectroscopy. The physical properties, fixed charge concentration, crystallinity, mechanical properties, micromorphology, and swelling properties of the PEC hydrogels were also investigated. The total fixed charge concentration of the PEC hydrogels varied as a function of pH on the pK intervals between chitosan (pK = 6.5) and γ-PGA (pK = 2.27). The isoelectric points (IEP) were shifted to a lower pH with a higher weight ratio of γ-PGA to chitosan. The elastic modulus was decreased with the weight ratio increasing from 0 : 1 to 1 : 1 (γ-PGA/chitosan) by ionic crosslinking between the amino groups of chitosan and the carboxyl groups of γ-PGA. The results of the swelling study showed that the swelling properties of PEC hydrogels were more affected by the change in the elastic restoring force than by the change in the fixed charge concentration depending on the pH. Also, the cytotoxicity of the PEC hydrogels was investigated using normal human dermal fibroblast (NHDF) cell lines, and the results showed the PEC hydrogels were not toxic. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 386–394, 2007

Key words: hydrogels; polyelectrolytes; polysaccharides; polyamides

INTRODUCTION

Polyelectrolyte complex (PEC) hydrogels are the networked structure of polymer chains crosslinked to each other and surrounded by an aqueous solution. The polymer chains contain acidic and basic groups bound to them. The acidic groups on the chains deprotonate at a high pH, whereas the basic groups protonate at a low pH. In the presence of a salt solution, the polymer chains absorb water, and the association and dissociation of various ions to and from polymer chains determines the degree of hydrogel swelling. The structure and properties of PEC hydrogels are similar to those of many biological tissues, such as cartilage and the corneal stroma in the eye.[1,2] PEC hydrogels are capable of undergoing large, reversible deformations in response to changes in several environmental factors.[3] For example, hydrogel volume is sensitive to solution pH, salt concentration, temperature, and electric fields. The sensitivity of hydrogels to a large number of physical factors makes them candidates for a broad range of biological applications including control of microfluidic flow,[4] musclelike actuators,[5,6] and drug delivery.[7,8]

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Chitosan [β-(1-4)-2-amino-2-deoxy-D-glucose] is a partially deacetylated derivative of chitin [β-1-4-2-actamido-2-deoxy-D-glucose] (Fig. 1),[9,10] the primary structural polymer in arthropod exoskeletons. Chitosan is a crystalline polysaccharide and normally insoluble in aqueous solutions above pH 7. However, the free amino groups of chitosan are protonated in dilute acids, and the molecule becomes soluble.[11] The high positive charge density of chitosan in a dilute acid solution allows the formation of insoluble PEC with a wide variety of water-soluble polyanionic species.[11] Complex formation has been documented with anionic polysaccharides such as GAGs and alginates, as well as synthetic polyanions such as poly(acrylic acid).[12–16] Because the charge density of chitosan is pH dependent, the ionic complexes at physiological pH can result in the dissociation of a portion of the polyanions. This property can be used for a drug delivery system.[17]

Poly(γ-glutamic acid) (γ-PGA) is an unusual anionic, naturally occurring homopolyamide that is made of D- and L-glutamic acid units connected by amide linkages between the α-amino and γ-carboxylic acid groups (Fig. 1).[18] Its anionic nature and high charge density allows γ-PGA to form PECs with chitosan at an appropriate pH. Attractive properties of γ-PGA are that it is water soluble, anionic, biodegradable, and edible. These and other features

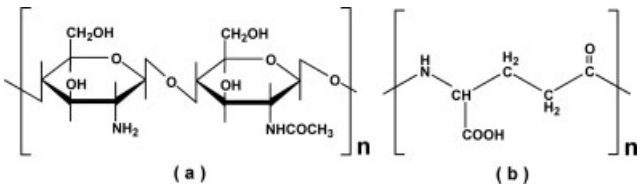

Figure 1 Molecular structures of (a) chitosan and (b) γ-PGA.

make it of interest for biological applications. The application of γ-PGA is versatile, safe, and environmentally friendly. Furthermore, the production of γ-PGA has already been established on an industrial scale because it can be produced easily and extracellularly in high yield by the culturing of bacteria in a fermenter. Therefore, the development of this material is both economically and environmentally valuable.[18]

The aim of this study was to control the degree of hydrogel swelling by using a PEC system. For this purpose, we prepared PEC hydrogels with chitosan as the polycation and γ-PGA as the polyanion and tried to control the degree of hydrogel swelling by changing two factors, pH and the mixing weight ratio of two polymers. Also, to explain the mechanism of swelling control, we characterized their chemical and physical properties and evaluated their cytocompatibility for biological applications.

EXPERIMENTAL

Materials

Chitosan powder, which has a degree of deacetylation of 85% and a weight-average molecular weight of 400,000, was provided by Jakwang (Korea). γ-PGA solution (Phyto collage; the main active component of Phyto collage is γ-PGA obtained from fermented soybeans) was provided by Ichimaru Pharcos Co. Ltd. (Shinsei, Japan). NHDF cell lines were provided by the Korea Cancer Center Hospital. Dulbecco's modified Eagle's medium–F12 (DMEM-F12) growth media, fetal bovine serum (FBS), and penicillin/streptomycin and trypsin-EDTA (2.5 mg/mL trypsin, 200 μg/mL EDTA, in PBS) were purchased from GIBCO® BRL Co. (USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Thiazoyl blue] was purchased from Sigma Chemical Co. All other chemicals were purchased from Sigma Chemical Co.

Preparation of PEC hydrogels

PEC dispersions containing varied weight ratios of chitosan and γ-PGA (γ-PGA/chitosan = 0.2, 0.4, 0.6, 0.8, and 1.0) were prepared by mixing chitosan and γ-PGA in a 1% (v/v) acetic acid solution (pH 4.3); the final concentration of the PEC dispersions was 2% (w/v). These solutions were agitated for 24 h. Viscous PEC dispersions were frozen at −20°C and then lyophilized.[19] The dried PEC hydrogels were immersed in absolute ethanol for 1 h and in 70% and 50% (v/v) ethanol for 2 h in order to stabilize them. Then the PEC hydrogels were equilibrated with deionized water, and finally they were relyophilized. These prepared PEC hydrogel samples are listed in Table I.

Characterization

FTIR

Fourier transform-infrared (FTIR) spectra were recorded on an FTIR instrument (Model 8400S, Shimazu Corp., Japan) in order to observe the chemical structure of the PEC hydrogels. The powdered samples were mixed with exhaustively dried KBr, and transparent discs were prepared by compression.

Wide-angle X-ray diffraction

Wide-angle X-ray diffraction (WAXD) patterns were recorded by the reflection method with nickel-filtered Cu Kα radiation with an X-ray diffractometer (Model XDS2000, Scintag, USA) operated at 40 kV and 35 mA in the 2θ scanning mode between 5° and 40°.

Total fixed charge quantity

The total fixed charge quantity of the PEC hydrogels was calculated by the following equation[20] induced from the dissociation constant of chitosan (pK = 6.5) and γ-PGA (pK = 2.27):

$$N_f = N_{BT}\left(\frac{C_H^g}{K_B + C_H^g}\right) - N_{AT}\left(\frac{K_A}{K_A + C_H^g}\right) \qquad (1)$$

where N_f is the total fixed charge quantity of the PEC hydrogel; N_{BT} is the charge quantity of the cationic polymer, chitosan; N_{AT} is the charge quantity of the anionic polymer, γ-PGA; C_H^g is the concentration

TABLE I
Sample Preparation and Designation of PEC Hydrogels

Designation	Weight ratio [γ-PGA]/[chitosan]	Molar ratio [COOH]/[NH_2]
PEC 0.2	2 : 10	11 : 45
PEC 0.4	4 : 10	22 : 45
PEC 0.6	6 : 10	33 : 45
PEC 0.8	8 : 10	43 : 45
PEC 1.0	1 : 1	54 : 45

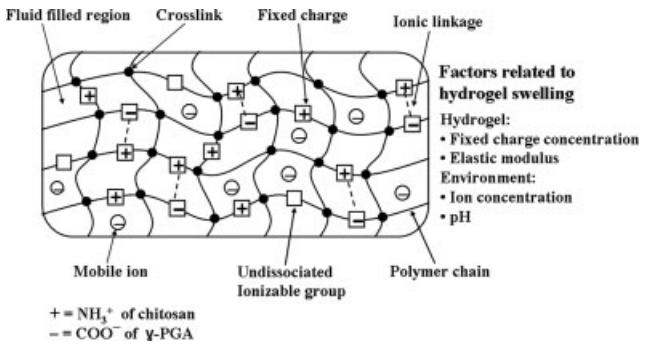

Figure 2 Structure of the PEC hydrogels and factors related to PEC hydrogel swelling.

of hydrogen ions in the gel; K_B is the dissociation constant of the positively charged group, the amino group, in the chitosan ($—NH_2 + H^+ \leftrightarrow —NH_3^+$); and K_A is the dissociation constant of the negatively charged group, the carboxyl group, in the γ-PGA ($—COO^- + H^+ \leftrightarrow —COOH$).

Mechanical properties

The mechanical properties were characterized by a texture recorder (Model TAHDi, TAHD, USA). The specimens were rectangular disks (4 cm × 1 cm × T, where T is the thickness of the PEC hydrogel). The crosshead speed was 5 mm/min. The ultimate tensile strength and fraction stress elongation at break were determined. The elastic modulus (E) was calculated by the following equation:

$$\sigma = E\varepsilon \quad (2)$$

where σ is the ultimate tensile strength, E is the Young's modulus or the elastic modulus, and ε is the elongation of the PEC hydrogel.

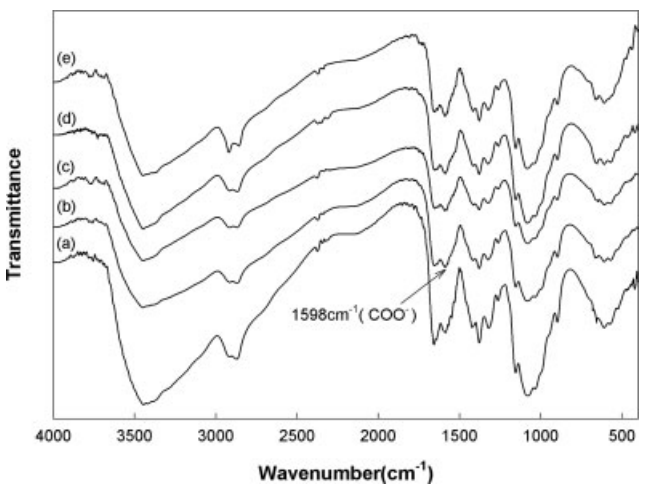

Figure 3 FTIR spectra of the PECs: (a) chitosan, (b) PEC0.2 (c) PEC0.4, (d) PEC0.6, and (e) PEC0.8.

Swelling behavior

Known-weight PEC hydrogels were placed in PBS for 12 h for the measurement of the equilibrium swelling ratio (ESR). The wet weight of the PEC hydrogels was determined by first blotting the hydrogel surface with filter paper to remove adsorbed water and then weighing them immediately. The ESR of the PEC hydrogels was calculated by the following equation:

$$\text{Equilibrium swelling ratio } (\%) = (W_s - W_d)/W_d \quad (3)$$

where W_d is the weight of the dry hydrogel and W_s is the weight of the swollen hydrogel.

Morphologies of PEC hydrogels

The morphologies of the PEC hydrogels surface and cross section were observed with a scanning electron microscope (SEM; Model S-3500N, Hitachi, Japan) operated at an accelerating voltage of 20 kV. The hydrogel samples were coated with an ultrathin layer of Au/Pt in an ion sputter (Model E-1010, Hitachi, Japan).

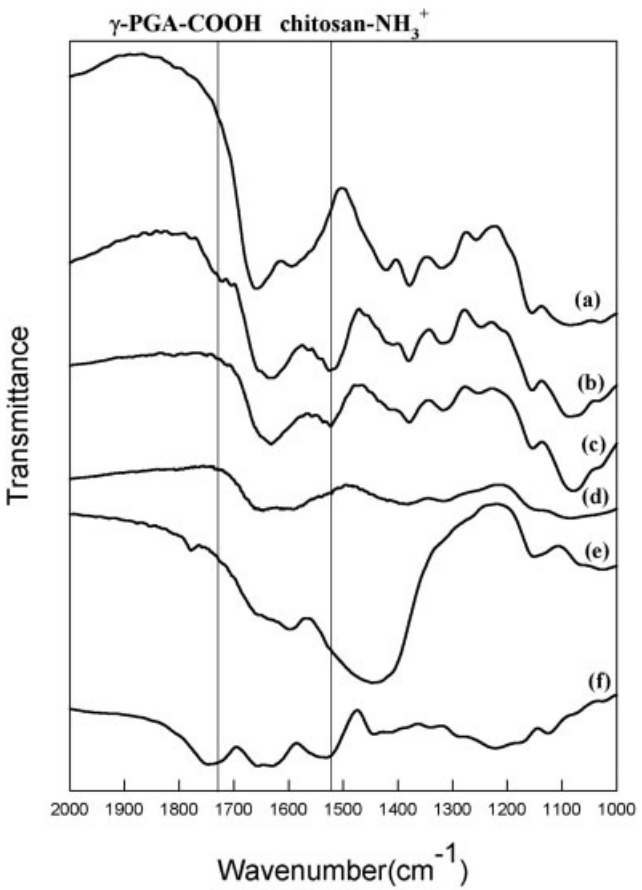

Figure 4 FTIR spectra of PEC hydrogels: (a) chitosan, (b) PEC1.0 at equilibrium at pH 2.0, (c) PEC1.0 at equilibrium at pH 4.5, (d) PEC1.0 at equilibrium at pH 7.4, (e) PEC1.0 at equilibrium at pH 9.0, and (f) γ-PGA.

Cytocompatibility

The cell proliferation test was carried out by direct contact with the NHDF cell lines. The PEC hydrogel samples were immersed in 70% (v/v) ethanol for 24 h for sterilization and washed with PBS. Then 1×10^6 NHDF cell lines were seeded into the PEC hydrogels and were cultivated for 14 days at 37°C in air with 5% CO_2 and 100% humidity in DMEM F-12 media containing 10% (v/v) FBS and 1% (v/v) penicillin and streptomycin. Cell viability was evaluated by the MTT assay.

RESULTS AND DISCUSSION

Figure 2 shows the structure of the PEC hydrogel and several factors related to hydrogel swelling. A PEC hydrogel is composed of a solid and a liquid phase. The solid portion of the gel consists of a crosslinked polymer network with acidic and basic groups bound to the polymer chains. When immersed in an aqueous solvent, the chains in the network become solvated. Crosslinks prevent complete mixing of the polymer chains and the solvent by providing an elastic restoring force that counters the expansion of the network. A PEC hydrogel is a combination of the polymer network and the internal solution that surrounds the chains. In the pH-sensitive hydrogels investigated in this study, basic amino and acidic carboxyl groups were bound to the chains. The mixing ratio of these two groups and the pH determined the total fixed charge concentration, a positive factor, and elastic modulus, a negative factor, on the pH-sensitive swelling of the PEC hydrogels.

In this study, we first prepared stable PEC dispersions for hydrogel formation by mixing two polymers in appropriate proportions, weight ratios of γ-

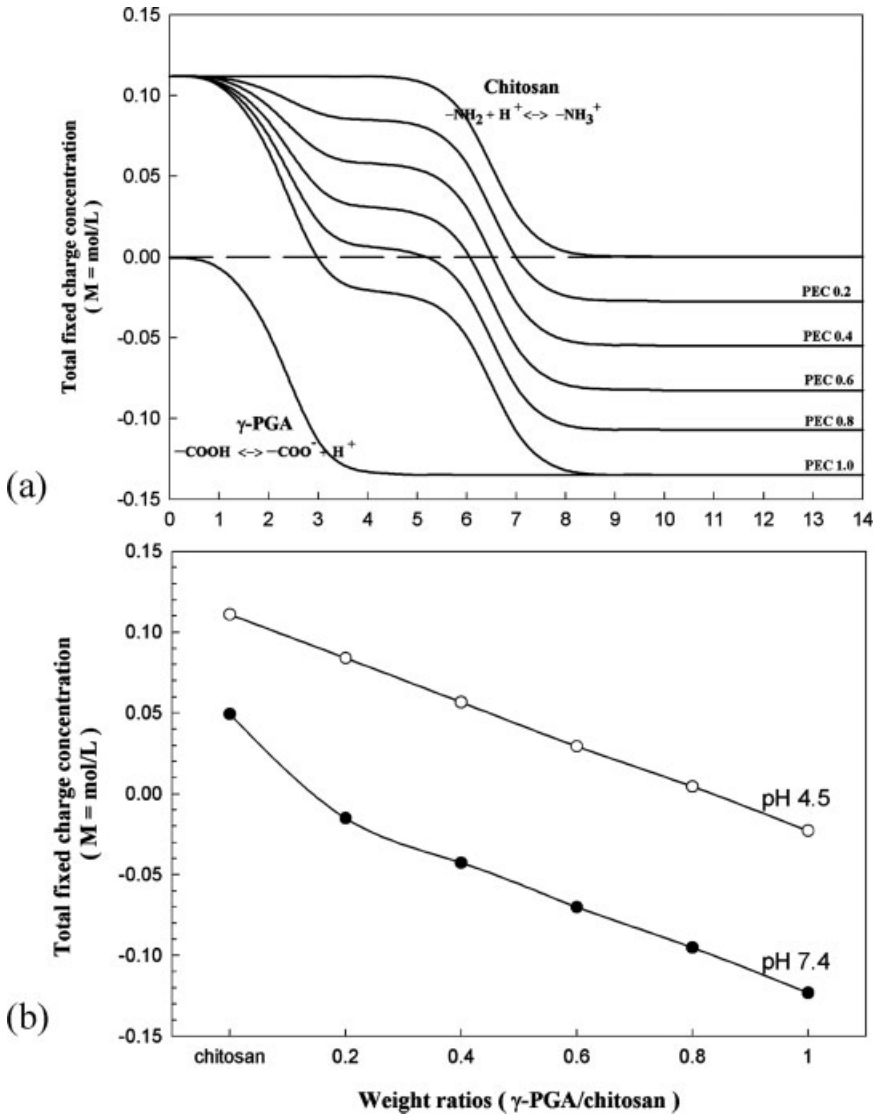

Figure 5 Total fixed charge concentration of PEC hydrogels: (a) relation between total fixed charge concentration and pH and (b) relation between total fixed charge concentration and mixing weight ratio.

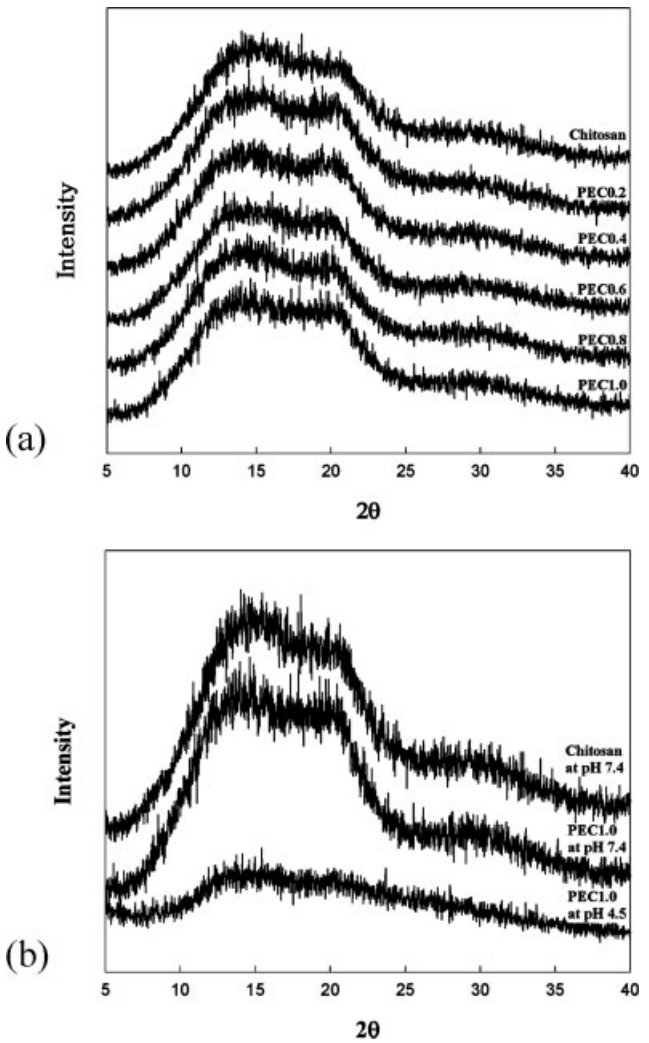

Figure 6 WAXD patterns of PEC hydrogels composed of chitosan and γ-PGA.

PGA/chitosan from 1 : 0 to 1 : 1. In the PEC formation process, we fixed chitosan polymer concentration and increased γ-PGA concentration because the change in chitosan concentration could have a bigger influence on the physical properties of the hydrogels than could PEC. Also, the range of mixing weight ratios was determined by the results of the turbidity test (data not shown), which showed that above a 1 : 1 proportion, unstable PEC dispersions were formed. With these stable PEC dispersions, we then prepared the PEC hydrogels using the freeze-drying technique.

FTIR spectra

FTIR spectra of the PEC hydrogels prepared at different weight ratios of chitosan and γ-PGA are shown in Figure 3. Characteristic peaks of chitosan appeared at 3500–3450 cm^{-1}, stretching peaks of the hydroxyl groups, and at 1640 and 1560 cm^{-1}, because of amides I and II, respectively.[21] Also, the peak at 1525 cm^{-1} in the spectra of the PEC hydrogels was assigned as a symmetric $—NH_3^+$ deformation, whereas the peak at 1591 cm^{-1} was attributed to a carboxylate salt.[22] FTIR spectra (1000–2000 cm^{-1} range) of dried PEC1.0 hydrogel after swelling in different pH buffer solutions for 24 h are shown in Figure 4. The —COOH and the $—NH_3^+$ peaks at pH 2.0 appeared at 1720 and 1525 cm^{-1}, respectively.

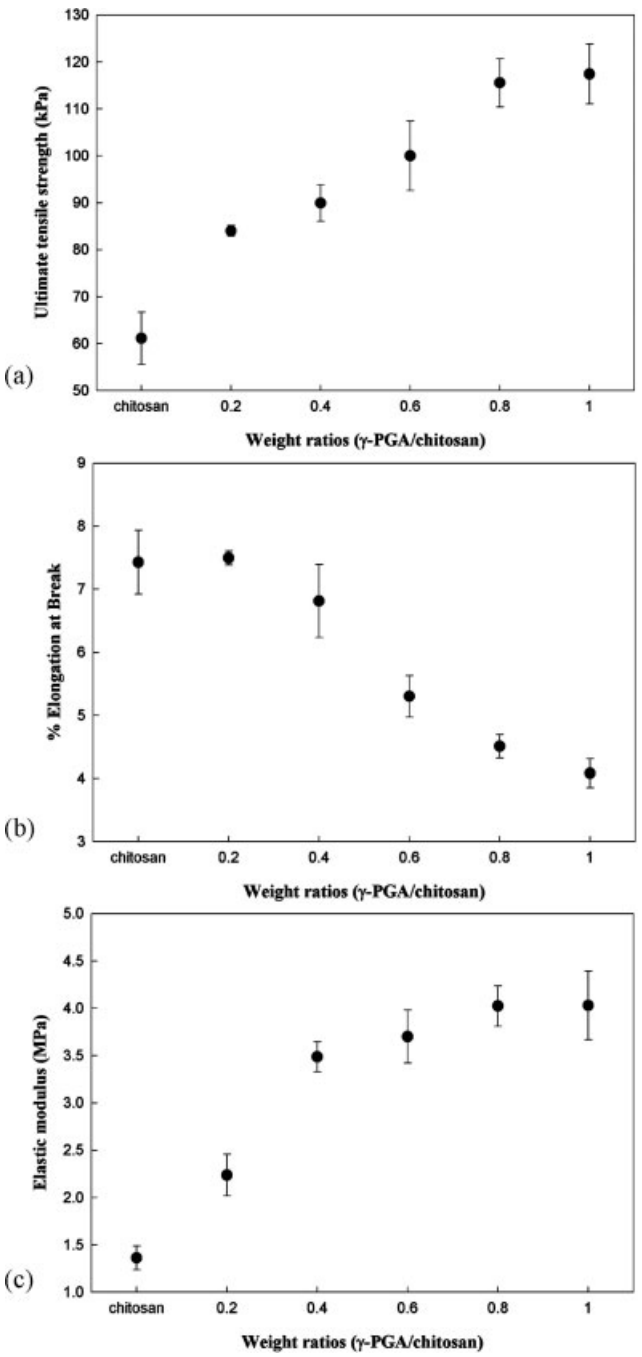

Figure 7 Mechanical properties of the PEC hydrogels at pH 4.5: (a) ultimate tensile strength, (b) elongation, and (c) elastic modulus.

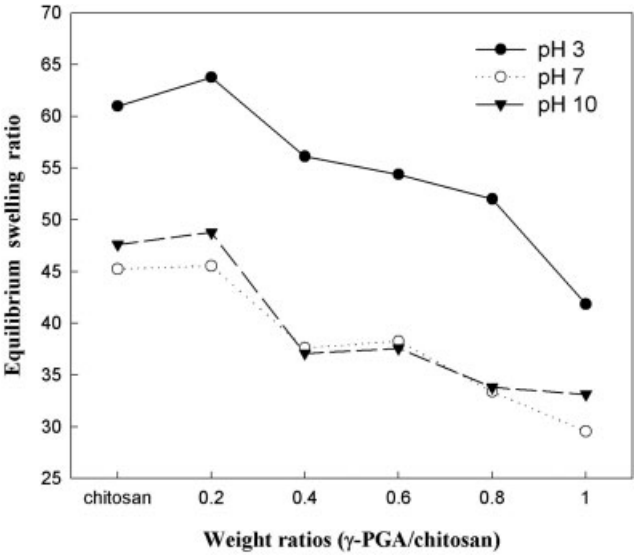

Figure 8 Equilibrium swelling ratios of the PEC hydrogel as a function of weight ratio.

However, the —COOH peak of γ-PGA and the $—NH_3^+$ peak of the chitosan disappeared at pH 4.5 and pH 7.4, respectively, because the carboxyl groups and the ammonium ion groups were deprotonated above the pKs of γ-PGA (2.27) and chitosan (6.5). These results suggest that PEC formation between chitosan and γ-PGA is possible in weak acidic condition (pH 2.27–4.5, pK intervals of chitosan and γ-PGA).

Total fixed charge quantity

A PEC hydrogel composed of chitosan and γ-PGA contains just two types of ionizable groups: chitosan, which has a weak base group (amino) with pK $= -\log K_B = 6.5$, and γ-PGA, which has a weak acid group (carboxyl) with pK $= -\log K_A = 2.27$. The total quantity of weak acid and weak base groups in the PEC hydrogel is determined by changing the weight ratio of chitosan to γ-PGA, whereas the concentration of fixed charge in the hydrogel is dependent on the pH (Fig. 5). Figure 5(a) shows the isoelectric points (IEPs) of PEC hydrogels in which the net charge of the hydrogel is zero because equal numbers of ionized amino and carboxyl groups are dependent on the weight ratio of chitosan to γ-PGA. The IEP of PEC0.2 was close to the pK point of chitosan. As the content of γ-PGA increased, the IEP of the PEC hydrogels shifted to a lower pH range. However, at a low pH, the binding of hydrogen ions neutralized the charge of the carboxyl groups and favored the ionization of the amino groups, thus conveying a net positive charge to the hydrogel. At a high pH, the ionization of amino groups was suppressed and the ionization of carboxyl groups enhanced, thus conveying a net negative fixed charge to the hydrogel. Figure 5(b) demonstrates the relation between the fixed charge concentration and the weight ratio (γ-PGA/chitosan) of the PEC hydrogel at pHs of 4.5 and 7.4. As shown in Figure 5(b), the fixed charge concentration of the PEC hydrogel was zero at pH 7.4 when the weight ratio was about 0.1 (γ-PGA/chitosan), and the fixed charge concentration of the PEC hydrogel at pH 4.5 decreased with higher weight ratios.

X-ray diffraction patterns

The WAXD patterns of the PEC hydrogels are shown in Figure 6. Chitosan exhibited typical crystalline peaks, which appeared around $2\theta = 12^\circ$ and 20° because of the presence of (020) and a mixture of (110) and (040), respectively.[23] At pH 7.4, all PEC hydrogel samples exhibited the same crystalline peak as that of a chitosan hydrogel. The peaks of $2\theta = 20^\circ$ and 12° at pH 4.5 disappeared and decreased, respectively, suggesting that the carboxyl groups of γ-PGA participated in the formation of ionic linkages with the amino groups of chitosan and that the PECs had a relatively low-crystalline or loosely ordered structure in a weak acidic condition (pK intervals of chitosan and γ-PGA). However, these results could be explained by the crystal forming within the chitosan itself, without any evidence of an interaction with the γ-PGA.

Mechanical properties

Ultimate tensile strength, elongation, and elastic modulus at break for the PEC hydrogels are shown

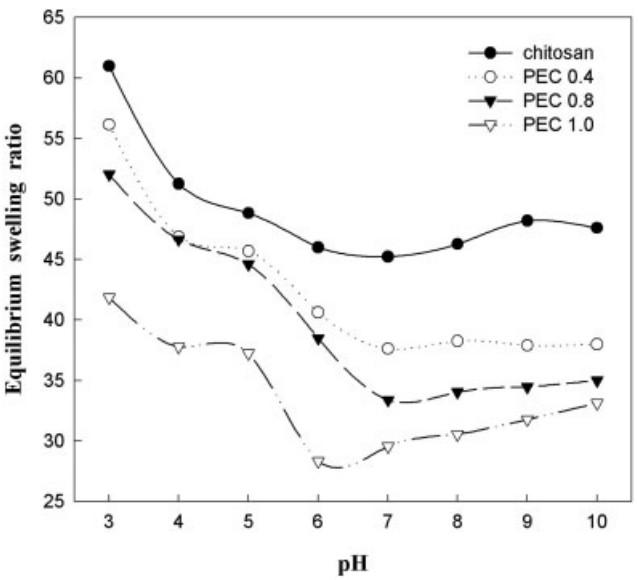

Figure 9 Equilibrium swelling ratios of the PEC hydrogels as a function of buffer pH.

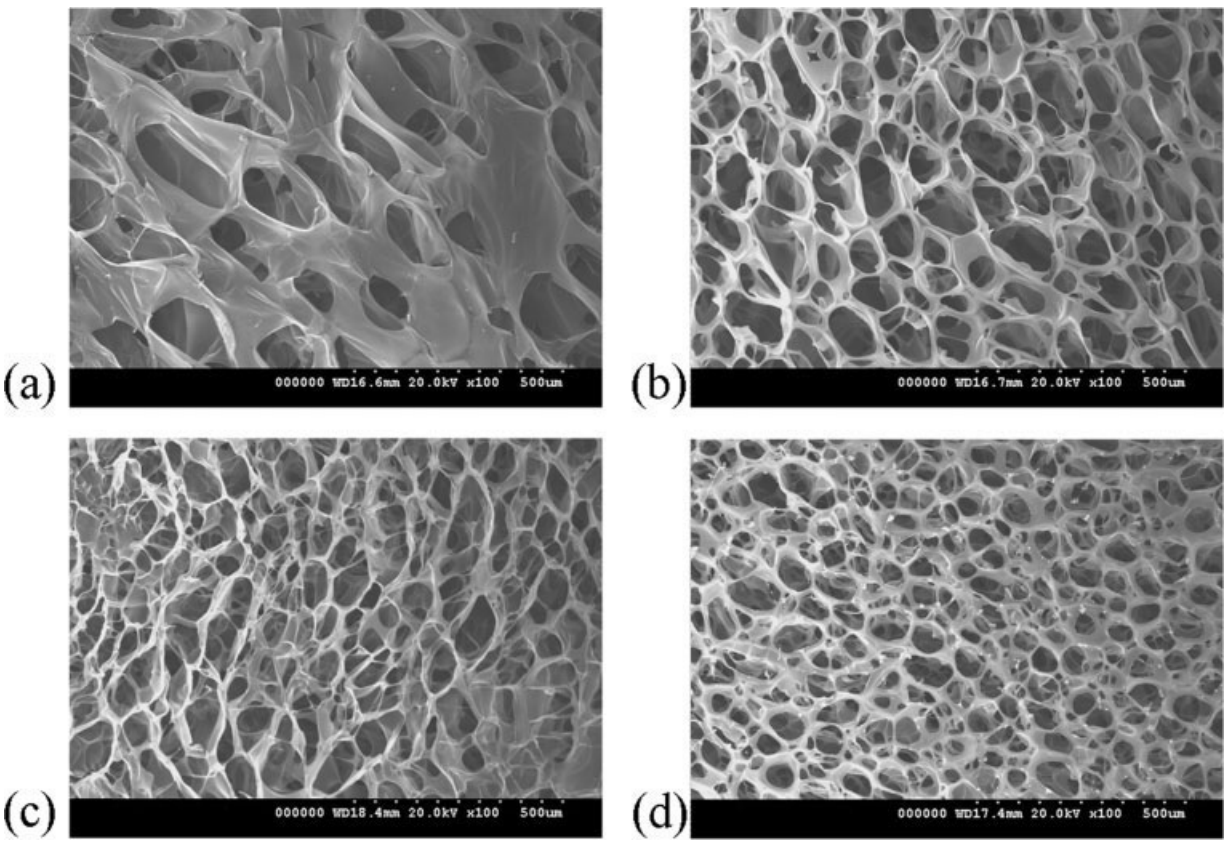

Figure 10 SEM micrographs of the surface of PEC hydrogel composed of chitosan and γ-PGA: (a) chitosan hydrogel, (b) PEC hydrogels with a 0.2 weight ratio (γ-PGA/chitosan), (c) PEC hydrogel with a 0.6 weight ratio, and (d) PEC hydrogel with a 1.0 weight ratio.

in Figure 7. It is known that polymer chains that have electrostatic interactions with another polymer chain experience a restriction in mobility because of ionic linkage, resulting in a shift in the glass-transition temperature (T_g) and rigidity. The PEC hydrogel composed of chitosan and γ-PGA exhibited increased tensile strength, whereas elongation at break and elastic modulus decreased compared with chitosan hydrogel. These results suggest that the ionic linkage between polyelectrolyte polymers influences the change in the mechanical properties by a cross-linking effect.

Swelling properties

Figures 8 and 9 show the results of ESR at 37°C for the PEC hydrogels in a PBS solution. The swelling equilibrium of the PEC hydrogels was determined by a balance of two primary forces: (1) the elastic retractile responses of the networks and (2) the net osmotic pressure with the networks resulting from the mobile counterions surrounding the fixed charge groups.

As shown in Figure 9, the equilibrium swelling ratios of four hydrogels depended on the pH. Although the hydrogels consisted of various proportions of chitosan and γ-PGA, they showed the same trend, regardless of weight ratio. As the pH increased from 3, the ESR decreased steadily, reaching a minimal value at a specific pH, and then rising again with further increases in pH. The results shown in Figure 8 indicate that the ESR decreased as the weight ratio of the PEC hydrogel increased because of the increased ionic interaction, the cross-linking effect, between chitosan and γ-PGA.

The coulombic interactions between opposite charges on the networks of hydrogels caused them to collapse. However, the hydrogels could maintain electrical neutrality with fewer electrolytes present. Osmotic pressure with the hydrogel networks was smallest at the IEP. These two factors led to minimal ESR values at the IEP. In our results, pH values at which the ESR of the hydrogel was at the minimum was not the same with the IEP. This indicates that in this experiment, the ideal swelling pattern of the PEC hydrogel was not shown because the change in the elastic restoring force that was dominated by ionic interactions between chitosan and γ-PGA and hydrogen bonds between chitosan polymer chains was more affected by the hydrogel swelling than by the change in the total fixed charge concentration. Accordingly, at both in neutral and acidic pHs, the

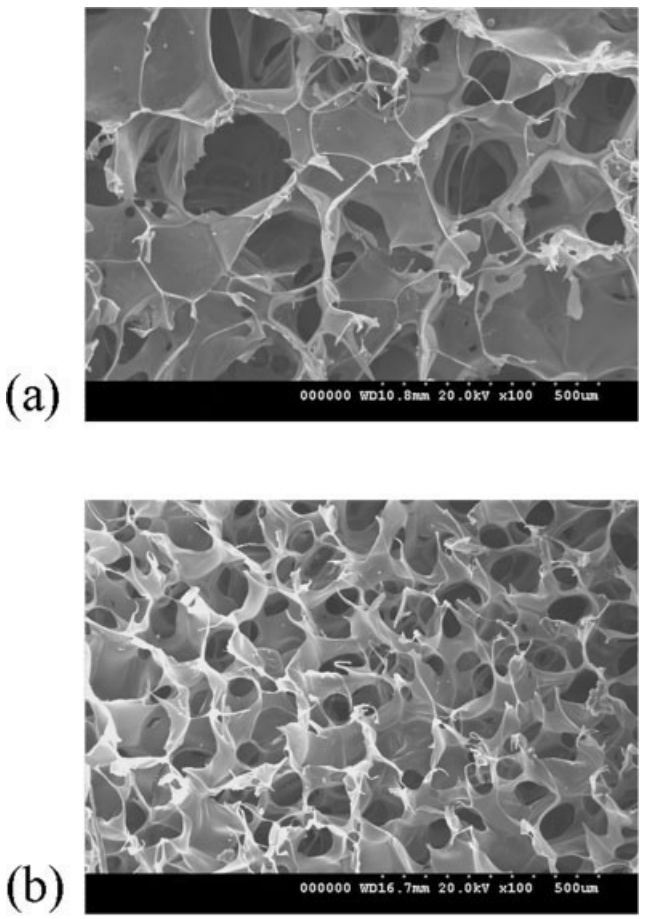

Figure 11 SEM micrographs of the PEC hydrogel composed of chitosan and γ-PGA: (a) cross section of chitosan hydrogel and (b) cross section of PEC hydrogels with a 0.6 weight ratio (γ-PGA/chitosan).

PEC hydrogels showed a smaller degree of swelling degree than the chitosan hydrogels because of the crosslinking effect of the polyelectrolyte complex after PEC formation. Therefore, the ESR studies suggested that to obtain an ideal swelling pattern of PEC hydrogels in response to pH, change in the elastic restoring force has to be minimized for the successful pH sensitivity of the PEC hydrogel. These results mean that chitosan is a good biomaterial because it has good biocompatibility and pH-dependent swelling properties, but its high swelling properties are a limitation in biological applications of this hydrogel. Actually, in an acidic pH the swelling properties of the chitosan hydrogel were too high and its mechanical strength too low. To resolve this problem, chemical crosslinking methods have been introduced such as adding glutaraldehyde, and by changing the concentration of the crosslinking agent, the swelling of chitosan hydrogels can be controlled, but this crosslinking agent is very toxic for biological applications. However, no crosslinking agents were needed in the PEC hydrogels, and the reaction was generally performed in an aqueous solution, which represents the main advantage over covalently crosslinked networks and thus favoring biocompatibility and avoiding purification before administration. So we were able to control the hydrogel swelling ratios by changing the mixing weight ratios without chemical crosslinking.

Morphology

The porous structure of the material will be beneficial to diverting the fluid when applied to biomaterials. The surface and cross-sectional morphologies of the hydrogel before and after PEC formation are shown in Figures 10 and 11. The interconnected 3-D porous structure of the hydrogels was retained after PEC formation. However, some other significant changes occurred in pore size and the morphology.

Mean pore size of the PEC hydrogel (Fig. 12) was smaller than that of the chitosan hydrogel. Accompanying the reduction in the fibers between the pores was the appearance of a more sheetlike structure together with the condensed walls. These results indicate the difference in the morphology was mainly caused by PEC formation and the topological change tjat occurred. Because chitosan has strong intermolecular hydrogen bonds, it is aggregated in the process of hydrogel preparation, and a bulky surface forms between each pore in the hydrogel. But PEC hydrogels have no bulky surfaces because of the strong intermolecular hydrogen bonds disturbed by the ionic interaction with γ-PGA.

Cytocompatibility

To investigate the cytocompatibility of PEC hydrogels, cell proliferation was evaluated with NHDF cell lines as shown in Figure 13. PEC hydrogels with

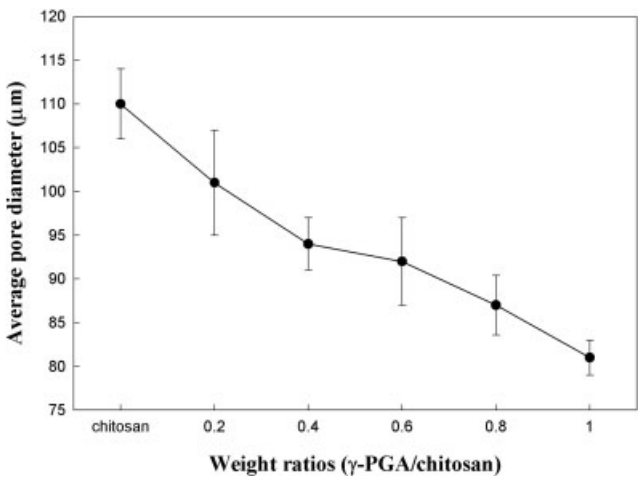

Figure 12 Mean pore size distribution of the PEC hydrogels as a function of weight ratio.

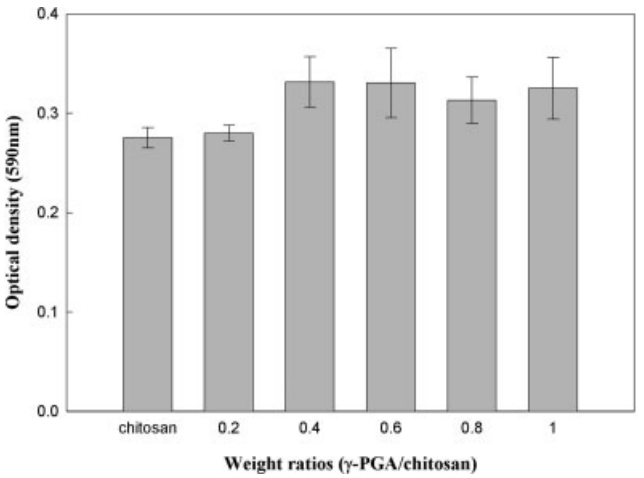

Figure 13 Cytocompatibility of the PEC hydrogels as a function of weight ratio.

a weight ratio above 0.4 exhibited a slightly increased affinity for NHDF cell lines compared with chitosan hydrogels. Both chitosan and γ-PGA previously have been demonstrated as biocompatible substrates for various types of cells, but the ability to promote cell proliferation has been not demonstrated. Therefore, it was considered that the difference in cell proliferation could be affected by PEC formation. The ability of PEC hydrogels to support cell proliferation could be attributed to its microstructure. Because chitosan has a homogeneous structure from PEC formation with γ-PGA and cells are spread homogeneously on PEC hydrogels, the cell density that is crucial for cell proliferation has uniformity and cell proliferation can be promoted. However, the bulky structure of chitosan hydrogels does not have such a high uniformity that the cell density may be formed locally, and so some contact inhibition occurred.

CONCLUSIONS

In this study, we prepared PEC hydrogels by the direct mixing of chitosan as a cationic polyelectrolyte and γ-PGA as an anionic polyelectrolyte. Because we used PEC hydrogels, we expected that the PECs would be formed in pK intervals of the chitosan, pK = 6.5 and γ-PGA, pK = 2.27, and that the total fixed charge concentration in this region would be changed as a function of pH, so swelling ratios could be sensitively controlled by changing the pH. However, the experimental results showed that the swelling ratios of the PEC hydrogels were more dependent on elasticity than on the total fixed charge concentration. Accordingly, the swelling ratios of the PEC hydrogel showed the minimum values at close to a neutral pH. In acidic conditions, the swelling ratios increased rapidly by the breaking of hydrogen bonds of chitosan and elasticity also increased. But with a basic pH, the swelling ratios increased slightly. As the weight ratios increased, the swelling ratios of the PEC hydrogels decreased over the entire pH range studied because of the crosslinking effects induced by ionic linkage between chitosan and γ-PGA. From the results, it was considered that to acquire an ideal swelling pattern of PEC hydrogels composed of chitosan and γ-PGA, the effect of the change in the elastic restoring force in response to a change in pH would have to be minimized in all pH regions. Now, we are trying to develop the method to minimize the effect of the elastic restoring force and to maximize the effect of the fixed charge concentration to have successful pH-sensitive PEC hydrogels. However, the meaning of this study is that swelling ratios of the hydrogels can be controlled by controlling the weight ratios using the PEC system, which is a biocompatible crosslinking method for biological applications.

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