Preparation and Properties of Polyelectrolyte Complex Sponges Composed of Hyaluronic Acid and Chitosan and Their Biological Behaviors

Sang Bong Lee,[1] Young Moo Lee,[1] Kang Won Song,[2] Moon Hyang Park[2]

[1]*School of Chemical Engineering, College of Engineering, Hanyang University, Seoul 133-791, Korea*
[2]*Department of Pathology, College of Medicine, Hanyang University, Seoul 133-791, Korea*

Received 9 September 2002; accepted 9 December 2002

ABSTRACT: Polyelectrolyte complexes (PECs) composed of chitosan and hyaluronic acid (HA) were prepared in various pH regions and at different weight ratios. At low pHs, there was a strong ionic interaction between NH_3^+ groups in chitosan and both COO^- and COOH groups in HA due to the deprotonation of HA, whereas weak linkages were formed at high pHs because only the carboxyl groups of HA could interact with NH_3^+ groups in chitosan. The formation of PECs resulted in a decrease in the crystallinity and thermal stability caused by the interactions between polyions. With variations in the degree of ionization of polyions at various pH conditions, novel PEC sponges were prepared by the freeze drying of PEC solutions. Furthermore, for the evaluation of the wound-healing effect of PEC sponges with or without an antimicrobial agent (silver sulfadiazine), they were applied to a full-skin defect of a Wistar rat *in vivo*. The histology and computerized morphometric analysis of the epidermal healing confirmed the proliferation of fibroblasts in the wound bed and a distinct reduction in infectious agents. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 90: 925–932, 2003

Key words: polyelectrolytes; polysaccharides; macroporous polymers

INTRODUCTION

Hyaluronic acid (HA) consists of 2-acetamide-2-deoxy-β-D-glucose and β-D-glucuronic acid residues linked by alternate (1–3) and (1–4) glycoside bonds [see Fig. 1(a)]. HA, one of the components of the extracellular matrix, has a high capacity for lubrication, water adsorption, and water retention and is also known to influence several cellular functions, such as migration, adhesion, and proliferation.[1] Recent biomedical applications of HA have included ophthalmic surgery, arthritis treatment, wound healing, coatings, and components of implant materials.[2–4] Also, chitosan, a deacetylated form of chitin, has a subunit of (1,4)-linked 2-amino-2-deoxy-β-D-glucan [see Fig. 1(b)]. Because of its viable bioactivity, its applications are increasing in areas such as hematology, immunology, wound healing, drug delivery, and cosmetics.[5–8]

On the basis of the types of interactions, polymer complexes can be classified as hydrogen-bonding complexes, polyelectrolyte complexes (PECs), stereocomplexes, and charge-transfer complexes.[9] PECs are formed by the reaction of oppositely charged polymers and constitute an important class of polymer materials used in many applications, such as membranes, medical prosthetics, antistatic coatings, environmental sensors, artificial tissue encapsulators, and chemical detectors.[10–12]

However, once formed, PECs cannot easily be fabricated into desired forms such as films, fibers, and sponges because precipitated PECs are not easily dissolved in organic solvents or in aqueous media on account of their strong interactions.[13] In a previous study, the biological properties of PECs between HA and chitosan were investigated with films because of the difficulty in fabricating sponges, although sponges are more favorable than films for wound-dressing materials because of their flexibility, durability, adherence, capacity to absorb wound debris, and protection of the lesion from dehydration.[14]

Therefore, PECs formed between HA and chitosan, both of which are bioactive materials, could be used in a greater number of applications if a sponge form of PECs could easily be prepared. Because chitosan is an abundant natural polymer and is relatively cheap, the cost of the final product containing expensive HA would be reduced and more economically feasible.

The purpose of this work was to investigate the properties of PECs composed of HA and chitosan at various pH conditions. With variations in the degree of interaction of PECs at each pH, PEC sponges composed of HA and chitosan were prepared by the mod-

Correspondence to: Y. M. Lee (ymlee@hanyang.ac.kr).
Contract grant sponsor: Korea Food and Drug Administration.
Contract grant sponsor: BK21 Program (to S.B.L.).

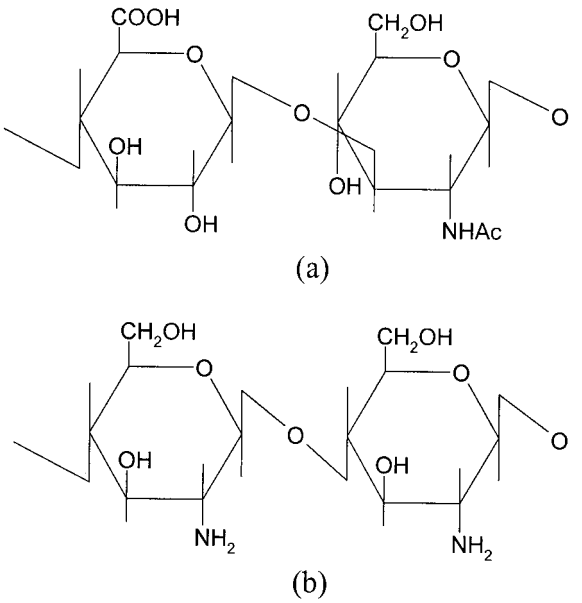

Figure 1 Molecular structures of (a) HA and (b) chitosan.

ulation of the pH of the solution. Furthermore, an *in vivo* animal test was conducted to evaluate the PEC sponge as a wound-dressing material.

EXPERIMENTAL

Materials

Chitosan (viscosity average molecular weight [M_v] = 500,000, degree of deacetylation = 76%) was purchased from Tokyo Kasei Co. (Tokyo, Japan). HA, in a sodium form (M_v = 1,700,000), was obtained from Pacific Chemical Co., Ltd. (Ansan, Korea). Silver sulfadiazine (AgSD) was kindly supplied by Dong Wha Pharmaceutical Co., Ltd. (Seoul, Korea). Water was distilled and deionized with a Milli-Q System (Millipore, Billerica, MA). Other chemicals were reagent-grade and were used without any further purification.

Formation of PECs within various pH regions

HA and chitosan were dried in a vacuum oven for 1 day. Chitosan was dissolved in a 0.05*N* HCl aqueous solution to a 1 wt % concentration. The dissolved chitosan solution was filtered with a glass filter. HA was dissolved in double-distilled water to a 0.5 wt % concentration. The two solutions were mixed at various weight ratios for 30 min after the pH of each solution was adjusted with diluted NaOH or HCl. Then, the newly formed precipitates were centrifuged for 30 min at 12,000 rpm and were washed with water for the removal of any residual chitosan and HA that had not participated in the PEC formation. The treated precipitate was kept at −76°C for 1 day and then freeze-dried.

Preparation of porous PEC sponges

HA and chitosan with various weight ratios were dissolved in a 15% aqueous formic acid solution. The concentration of chitosan and HA in the solution was 1 wt %. Distilled water was added to the solution containing HA and chitosan until precipitates were formed. In this case, the pH of the solution was increased to form precipitates at pH 2–3. The precipitates were centrifuged for 30 min at 12,000 rpm. The water added to form the precipitates was then removed as a supernatant to adjust the solution concentration to be 1 wt %, and the solution was homogeneously mixed with a homogenizer (Diax 900, Heidolph, Cinnaminson, NJ) at 10,000 rpm for 10 min. The resulting product was poured into a petri dish and frozen in a −76°C refrigerator for 6 h. The frozen petri dish was then dipped into a 0.01*N* NaOH solution for the removal of any remaining formic acid. After being washed with water, the precipitates were kept at −76°C for 1 day and then freeze-dried.

Measurements

The pH was measured with a pH meter (SP-701, Suntex Co., Taipei, Taiwan). A Fourier transform infrared (FTIR) spectrometer (Magna IR 550, Nicolet, Madison, WI) was used to confirm the ionic state in chitosan, HA, and PECs. Wide-angle X-ray diffraction (WAXD) patterns were recorded by the reflection method with nickel-filtered Cu Kα radiation with a Rigaku Denki X-ray diffractometer (Tokyo, Japan) operated at 50 kV and 180 mA in the 2θ scanning mode between 5 and 40°. The thermal stability was investigated with a DuPont Instruments (New Castle, DE) 951 thermogravimetric analyzer from 50 to 700°C at a heating rate of 10°C/min. The morphology of the sponges was investigated with scanning electron microscopy (6340F, JEOL, Tokyo, Japan). Specimens were placed on a copper mount and were coated with a gold-coating apparatus. The porosity and the average diameter of the pores were investigated with geometrical measurements of scanning electron micrographs with an image analyzer (Bum Mi Universe Co., Ltd., Seoul, Korea). The mechanical properties of the PEC sponges were measured with an automated materials tester (Instron 4465, Canton, MA) after the PEC sponges (10 cm × 1 cm) were pressed at 210 kgf/cm^2 for 1 min to make films. The constant deformation rate was 0.5 mm/min.

In vivo study

Under intraperitoneal anesthesia with Pentobal, full-thickness excisions 10 mm in diameter were prepared on the dorsa of 5-week-old Wistar rats. After the wounds were disinfected with Potadine, the excised

wounds were covered with various wound-dressing materials with or without AgSD. As a control, Vaseline gauze was applied on the skin wounds. Then, sterilized elastic bands were used to fix the covered sponges. At 5 and 12 days after the operation, the Wistar rats were sacrificed by anesthetic overdose and were preserved in 10% formaldehyde. The skin wound tissue was cut from the central regions of the wound or margins, embedded in paraffin wax, sectioned (4 μm), and stained with hematoxylin and eosin staining. The wound-healing effects were histologically investigated.

Morphometric analysis of the epidermal wound healing

Each wound section was placed on a microscope stage, and the image was displayed on a video screen via a closed circuit digital (CCD) camera (Mitsubishi, Tokyo, Japan) interfaced to a computer. The percentage of wound resurfacing was determined by the on-screen measurement of the distance from the right wound margin to the left wound margin with an image analyzer program (Bmi Plus 1.14, Bum Mi Universe). Then, the length of newly regenerated epithelium across the surface of the wound was determined. This length was defined as the sum of the new epidermis growing out from the left and right margins of the wound.

RESULTS AND DISCUSSION

pH regions for the formation of PECs

PECs are formed between pK_a of the polyanion and pK_b of the polycation. pK_a of HA is 2.9 $\pm$ 0.1, and pK_b of chitosan is 6.5.[13,15] Therefore, if a PEC is formed between pK_a of HA and pK_b of chitosan, the following equation is established:

$$[\text{HA–COO}^-] + [\text{Chitosan–NH}_3^+] \rightleftarrows [\text{HA–COO}^-][\text{NH}_3^+\text{–Chitosan}] \quad (1)$$

However, Denuziere et al.[13] reported that a PEC composed of HA and chitosan could be formed below pK_a of HA because the ionic interaction between the polyions competes with the protonation of HA. In this case, the PEC is formed passing through two steps. The first step is the deprotonation of the carboxyl groups in HA, and the second step is the reaction between the groups of ammonium salt in chitosan and carboxyl groups in HA:

$$[\text{HA–COOH}] \rightleftarrows [\text{HA–COO}^-] + [\text{H}^+] \quad (2a)$$

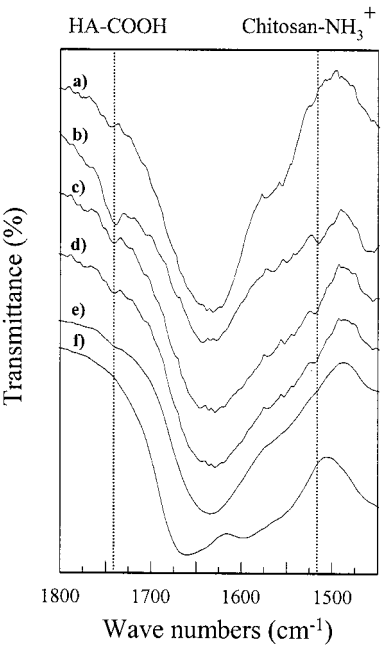

Figure 2 FTIR spectra of (a) HA, (b) PEC mixed at pH 3.2, (c) PEC mixed at pH 4.1, (d) PEC mixed at pH 5.1, (e) PEC mixed at pH 5.9, and (f) chitosan.

$$[\text{HA–COO}^-] + [\text{Chitosan–NH}_3^+] \rightleftarrows [\text{HA–COO}^-][\text{Chitosan–NH}_3^+] \quad (2b)$$

In this study, the pH for PEC formation was only limited to be below pK_b of chitosan (6.5) because of the precipitation of chitosan.

Degree of the interactive strength between polyions in various pH regions

FTIR spectra

Figure 2 shows the FTIR spectra of HA, chitosan, and PECs formed with a 1:1 weight ratio under various pH conditions. The characteristic peaks of chitosan appear at 1650 and 1550 cm^{-1} due to amide I and II bands, respectively.[16] The characteristic peaks of HA (sodium salt form) can be seen at 1630 and 1570 cm^{-1}. The PECs show absorption bands at 1515 and 1735 cm^{-1} due to —NH_3^+ of chitosan and —COOH of HA, respectively.[17] The peaks do not appear in either chitosan or HA. In the absorption bands of PECs, the peak intensities of the —COOH group in HA and the NH_3^+ group in chitosan increase with decreasing pH.

From the peak intensity of PEC, the degree of ionization was investigated at each pH because the degree of ionic interaction between the polycations and polyanions depended on the degree of ionization and the ionic strength of the alkaline or acidic sites attached to the polymer main chain. Therefore, PECs with strong interactions were expected if the anions and cations contained strong acids and bases or if

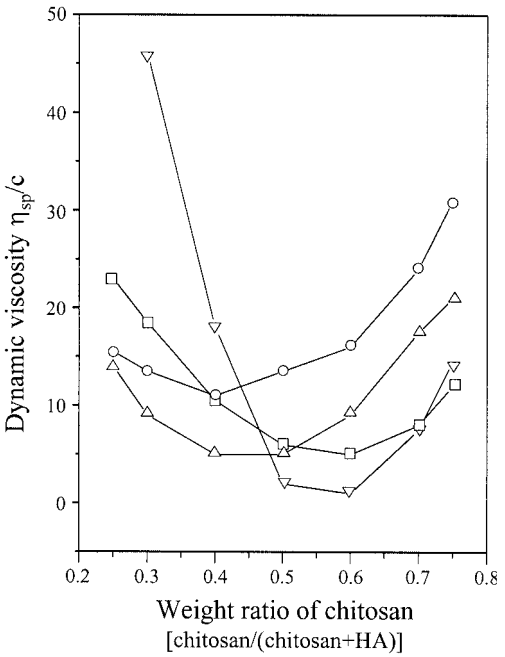

Figure 3 Viscosity of the supernatant mixed at (○) pH 3.2, (△) pH 4.1, (□) pH 5.1, and (▽) pH 5.9.

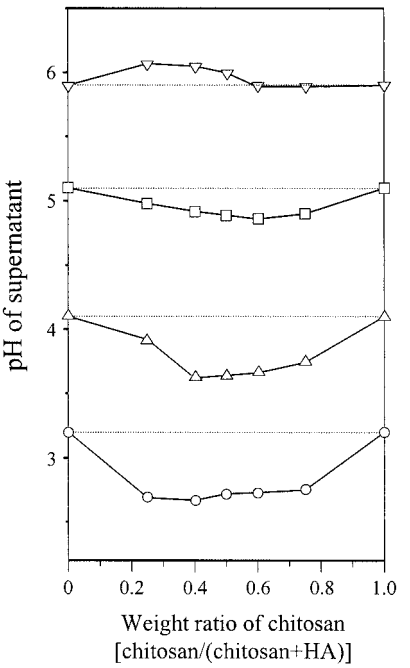

Figure 5 pH changes in the supernatant mixed at (○) pH 3.2, (△) pH 4.1, (□) pH 5.1, and (▽) pH 5.9.

polyions existed in their fully ionized forms. However, according to eq. (1), a strong interaction would not be obtained at any pH because the relative numbers of $—NH_3^+$ groups in chitosan and —COOH groups in HA were high at low pHs, and the opposite trend appeared at high pHs (see Fig. 2). That is, as the number of one ionizing group increased, the number of the other group decreased. However, a solubility test showed that the ionic interaction was stronger at a low pH. The PEC formed at a low pH could only be dissolved in relatively strong acid solution such as formic acid, whereas the precipitate produced at a high pH was easily dissolved in a weak acid such as acetic acid.

Viscosity behavior

The viscosity was used to investigate the compatibility of the polymer mixture and the association between polymers containing the interpolymer complexes in solution. If the viscosity, concentration, and weight

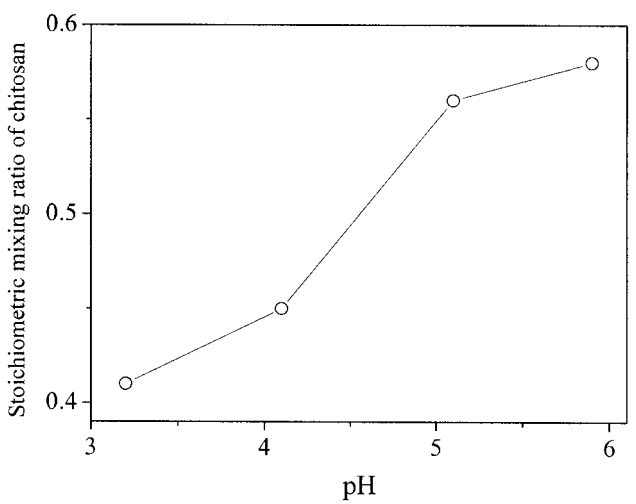

Figure 4 Effect of pH on the stoichiometric mixing ratio of chitosan.

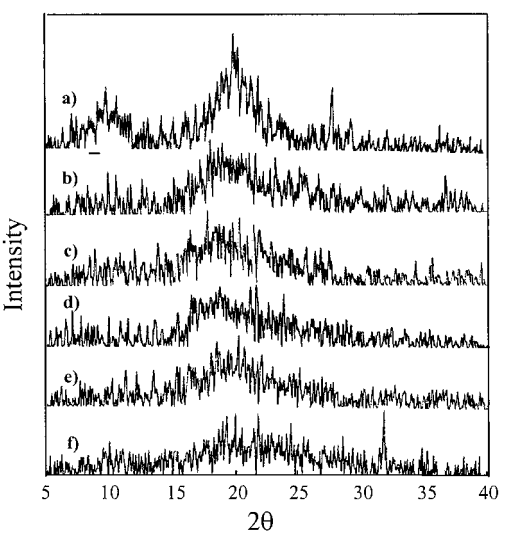

Figure 6 X-ray diffraction patterns of (a) chitosan, (b) PEC formed at pH 3.2, (c) PEC formed at pH 4.1, (d) PEC formed at pH 5.1, (e) PEC formed at pH 5.9, and (f) HA.

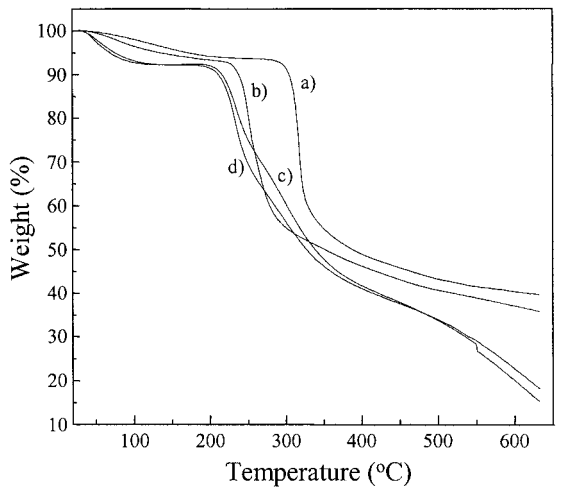

Figure 7 Thermogram analysis of (a) chitosan, (b) HA, (c) PEC formed at pH 5.1, and (d) PEC formed at pH 3.2.

ratios of the two polymers are known, the following equation can be established:

$$(\eta/c)_m = w_1(\eta_1/c_1) + w_2(\eta_2/c_2) \quad (3)$$

where subscripts 1 and 2 refer to HA and chitosan, respectively; c is the total polymer concentration; w_1 and w_2 are the weight ratios of the two polymers in the mixture; η_1/c_1 is the dynamic viscosity of HA at concentration c_1; and η_2/c_2 is the dynamic viscosity of chitosan at concentration c_2. A positive deviation in the solutions, which shows that the viscosity is higher than the calculated viscosity from eq. (3) at a particular weight ratio, imparts good compatibility or gel-like association between two polymers. However, a negative deviation shows poor compatibility or the formation of a compact interpolymer complex.[18,19]

The effect of pH on the viscosity of PECs is shown in Figure 3. The viscosity of the supernatant decreased because the precipitates were formed as chitosan and HA interacted between the oppositely charged polyions, and this resulted in the shrinkage of the PEC chains and the prevailing of hydrophobic forces that favored the precipitation of a compact complex. Therefore, the viscosity decreased with more compact complex formation, and the strongest complex was stoichiometrically formed at the minimum viscosity at each pH. For the formation of PECs in low-pH regions, a low weight ratio of chitosan was necessary in a stoichiometric mixing ratio with the polyions of HA because of the large number of protonated amine groups of chitosan, whereas a high weight ratio of chitosan was added in high-pH regions.

Figure 4 shows at each pH the stoichiometric mixing ratio obtained when the dynamic viscosity was the lowest at each pH and formed the strongest interaction between polyions at each pH. The stoichiometric mixing ratio of chitosan increased with pH, and this indicated that more chitosan was necessary to form the more compact PECs because of a decrease in the number of protonated amino groups at high pHs.

pH changes of the supernatant

Figure 5 shows the pH changes of the supernatant after mixing at each pH with various weight ratios of chitosan and HA. The pH of the supernatant after the mixing of two solutions was lower than the initial pH of the solution, except when the mixing was performed at pH 5.9. The decrease in pH in the supernatant can be explained by eq. (4), if we assume that the pH of the supernatant is only influenced by the presence of an excess of acid extracted during the formation of the PEC, without the addition of acid:

$$[\text{HA–COOH}] + [\text{Chitosan–NH}_3^+] \rightleftarrows [\text{HA–COO}^-][\text{Chitosan–NH}_3^+] + [\text{H}^+] \quad (4)$$

According to eq. (4), the protons separated from carboxyl groups move to the supernatant during the formation of the complex by the deprotonation of HA.

However, at pH 5.9, most amino groups and carboxyl groups in chitosan and HA are in the —NH_2 and —COO^- forms, respectively:

$$[\text{HA–COO}^-] + [\text{Chitosan–NH}_2] + [\text{H}^+] \rightleftarrows [\text{HA–COO}^-][\text{Chitosan–NH}_3^+] \quad (5)$$

TABLE I
Physical and Morphological Characteristics of the Tested Sponges

Sample[a]	Porosity (%)[b]	Average pore size (μm)[c]	Water uptake (g of H_2O/g of sponge)[d]
CH-46	42.5 ± 0.2	25 ± 3.4	10 ± 4.4
CH-37	41.2 ± 0.5	24 ± 1.2	12 ± 5.3
CH-28	40.3 ± 0.3	21 ± 5.4	15 ± 3.4

[a] CH-*AB:* weight ratio of chitosan = $A \times 10\%$; weight ratio of HA = $B \times 10\%$.
[b] The porosity was obtained from area analysis between the pore zone and the matrix zone by the image analyzer program.
[c] The average pore size was calculated by measurement of the size of 30 pores with the image analyzer tools.
[d] Water uptake ability was calculated from the weight difference between the wet and dry sponges.

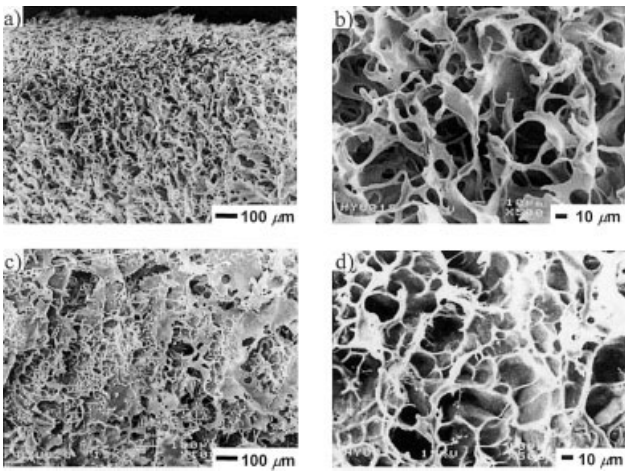

Figure 8 Cross-sectional morphologies of (a,b) CH-46 and (c,d) CH-28.

For PECs to be formed as in eq. (5), protons are essential. Protons should be incorporated from the supernatant, and this results in an increase in the supernatant pH.

In addition, compared with the measurement of the viscosity in Figure 3, the pH of supernatant was lowest at the stoichiometric mixing ratio (see Fig. 5). This means that the strongest interaction between polyions was formed for PECs with the stoichiometric mixing ratio at each pH. At the stoichiometric mixing ratio, most protonated carboxyl groups reacted with ionizing amino groups in chitosan according to eq. (4). Therefore, the pH of the supernatant was lowest at the stoichiometric mixing ratio.

Structural deformation of PEC

Figure 6 shows WAXD patterns of chitosan, HA, and PECs. Chitosan exhibited crystalline peaks around $2\theta = 9.8$ and $2\theta = 19.3°$ due to the presence of (020) and a mixture of (110) and (040), respectively.[20] However, HA showed a weak peak intensity around $2\theta = 20°$ on account of its low crystallinity. In PECs, the peak at $2\theta = 9.8°$ disappeared, and the peak at $2\theta = 19.3°$ decreased under all pH conditions. This means that the carboxylic groups of HA were expected to participate in the formation of ionic linkages with the amino groups of chitosan and that the PECs showed a relatively low-crystalline or loosely ordered structure.

Figure 7 shows thermograms of the PECs, chitosan, and the sodium form of HA. The thermal degradation of PECs composed of HA and chitosan started at a lower temperature than that of HA and chitosan alone. The weight losses of these PECs started around 200°C, whereas chitosan and HA degraded at 290 and 220°C, respectively. The shift to a lower temperature in the thermal degradation of the PECs indicated that there existed an absence of organization of the PECs probably due to the crystalline structure being broken as ionic bonds between polyions were formed.

PECs exhibited a decreased intensity in WAXD patterns at $2\theta = 19.3°$ and a drop in thermal stability, as elucidated in thermogravimetric analysis thermograms; this means that the crystalline structure of chitosan was interrupted as it was ionically complexed.

Preparation and properties of the PEC sponge

In this study, a novel PEC sponge composed of HA and chitosan was prepared by the modulation of the degree of interaction between polyions. At the initial ionic state of polyions dissolved in formic acid, the amino groups of chitosan interacted with formic acid, and the carboxyl groups of HA existed in a protonated form. As the pH of the solution increased, protonated carboxyl groups competed with the precomplexed amine groups of chitosan with formic acid to form an ionic linkage between HA and chitosan. The precipitates formed in this procedure had a weak interaction between HA and chitosan because formic acid was still attached in many parts to amine groups in chitosan. After the desired shape was molded, formic acid interacting with chitosan was removed by NaOH. At this time, the dimensions of the sponge were reduced as more ionic linkages formed between polyions.

Table I and Figure 8 show physical and morphological characteristics of the sponges. The porosity of the sponges ranged from 40.3 to 42.5 μm, with an average pore size of 21–25 μm. The morphology of these sponges seemed to be dependent on the mixing ratios. The capacity for water uptake increased with the amount of HA, whereas the porosities and average pore sizes decreased because excess HA that had not participated in the formation of PEC was confined to large pores of PEC on account of the high viscosity of HA. The residual HA was attached between large pores of PEC after freeze drying.

In addition, Figure 9 shows the mechanical properties of the sponges after pressing. The stiffness and

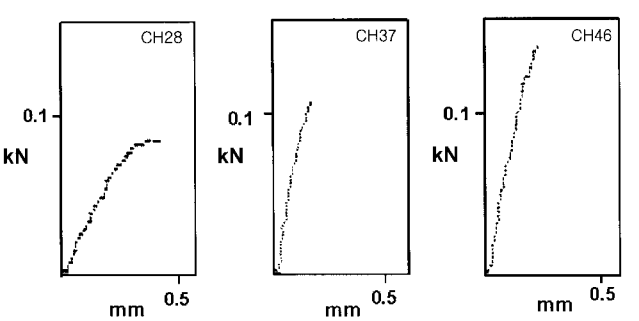

Figure 9 Mechanical strength of PEC sponges after being pressed at 210 kgf/cm^2 for 1 min.

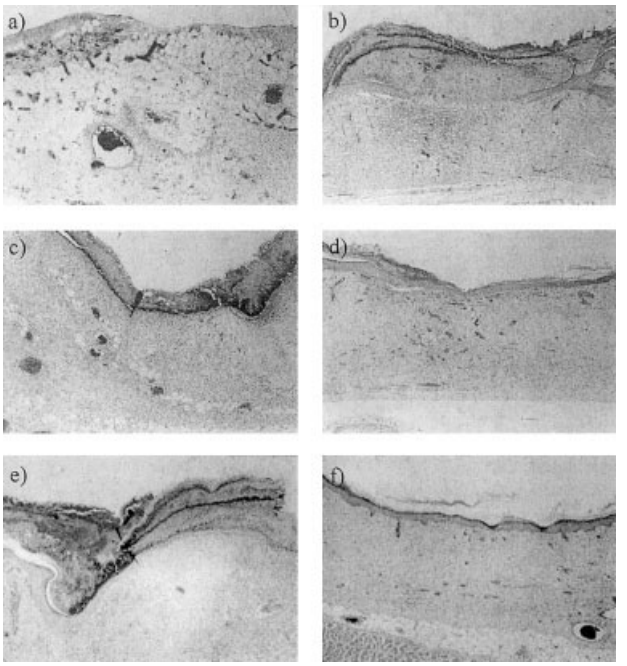

Figure 10 Histological cross sections of a rat skin wound 5 and 12 days after the operation (hematoxylin and eosin staining; 40×): (a,b) CH-37 without AgSD, (c,d) CH-37 with AgSD, and (e,f) Vaseline gauze.

hardness decreased with an increase in the weight ratio of HA because of the soft mechanical properties of HA.

Histological studies

Figure 10 exhibits the histological results of HA–chitosan PEC sponges applied to the dorsal skin wounds of Wistar rats. Histological cross sections of PEC sponges, with or without AgSD and with Vaseline gauze after 5 and 12 days of covering Wistar rat wounds are shown.

In Figure 10(a), sections of the PEC sponge containing 70 wt % HA (CH-37) without AgSD after 5 days show that the surface was covered with thick, neutrophilic exudates and fibrinous fluid containing blood. A thick granulation tissue was present in the dermis. Twelve days after the operation [Fig. 10(b)], the wound was completely reepithelialized, and the skin appendages were destroyed. The underlying granulation tissue revealed minimal leukocytic infiltration edema and increased vascularity. In the case of CH-37 with AgSD [Fig. 10(c)] after 5 days, the wound was covered with a fibrinous acute inflammatory exudate. The dermis showed early granulation tissue, numerous blood vessels, and fibroblastic cells. After 12 days, the dermis was covered with a newly formed thin epidermis. The skin appendages were lacking. The dermis showed a healing fibrous scar with mononuclear cells.

As a control, conventional Vaseline gauze was employed for wound dressing. Figure 10(e,f) shows histological cross sections of the wound covered with

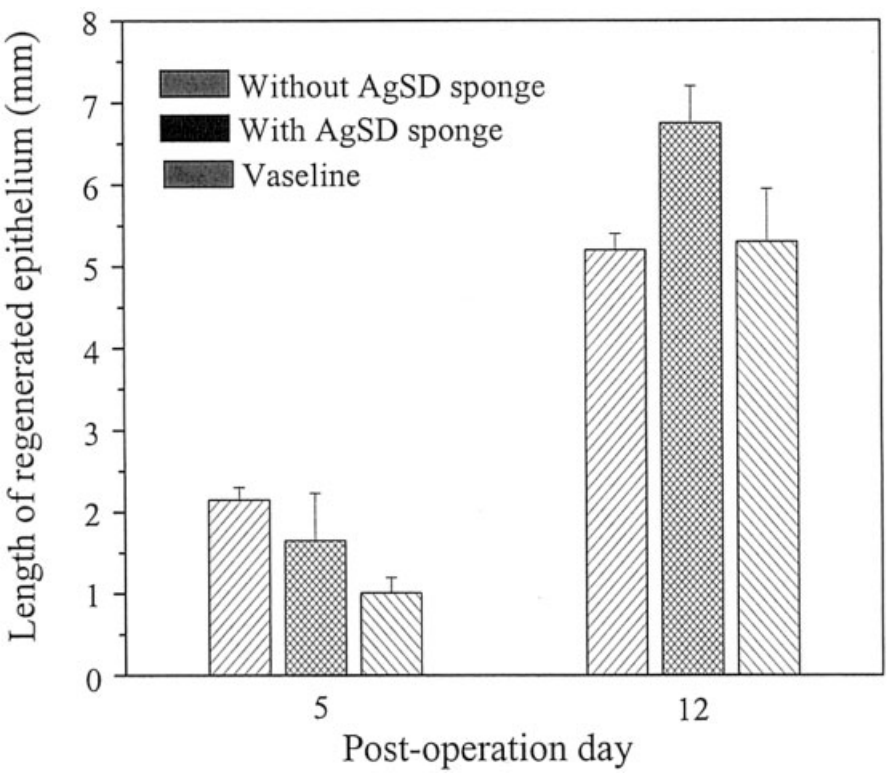

Figure 11 Morphometric analysis of epidermal wound healing by CH-37 with or without AgSD and Vaseline gauze in full-skin thickness wounds with a diameter of 1 cm 5 and 12 days after the operation ($n = 3$).

conventional Vaseline gauze after 5 and 12 days, respectively. As shown in Figure 10(e), the skin defect was covered with a blood fibrinous exudate rich in neutrophils and macrophages. The granulation tissue progressively invaded the skin defect. After 12 days, the skin defect was covered with an intact epidermis. Abundant granulation tissue grew from the margin.

The length of the regenerated epidermis at 5 and 12 days is shown in Figure 11. At day 5, the length of regenerated epithelium in the PEC sponges was longer than that under a Vaseline gauze. A PEC sponge containing AgSD was superior to Vaseline gauze and to a sponge without AgSD. Moreover, a PEC sponge containing AgSD showed the best proliferation of fibroblasts in the wound bed of a Wistar rat after 12 days.

CONCLUSIONS

The generation of PEC sponges composed of HA and chitosan for wound healing is a difficult process. In this study, sponges were prepared after the properties of the ionic linkages between the polyions under various pH conditions were investigated. In low-pH regions, a large number of ionic linkages were formed between the high number of NH_3^+ groups in chitosan and both protonated and deprotonated carboxyl groups in HA, whereas a relatively small number of ionic linkages were formed at a high pH because the carboxyl groups of HA interacted with a low number of NH_3^+ groups of chitosan. In addition, PECs exhibited a decrease in the crystallinity of chitosan and a drop in thermal stability due to the ionic interactions between the main chains of polyions. On the basis of the degree of interaction between the polyions at each pH, PEC sponges were prepared. The porosity of the sponges ranged between 40 and 43 μm, with an average pore size of 21–25 μm. The stiffness and hardness of the sponges decreased with an increasing weight ratio of HA to chitosan because of the softness of HA. Histological studies of the novel sponge materials containing AgSD confirmed the greater proliferation of fibroblasts in the wound bed of the Wistar rats after 12 days and a reduction in the number of infectious cells in comparison with the Vaseline gauze. This study reveals that novel PEC sponges composed of HA and chitosan may be good candidates for wound-dressing materials.

References

1. Sbarbati, R.; Guerra, D.; Cascone, M. G.; Barbani, N.; Lazzeri, L. J Mater Sci: Mater Med 1994, 5, 613.
2. Seggiani, M.; Lazzeri, L.; Giusti, P. J Mater Sci: Mater Med 1994, 5, 877.
3. Choi, Y. S.; Hong, S. R.; Lee, Y. M.; Song, K. W.; Park, M. H.; Nam, Y. S. J Biomed Mater Res 1999, 48, 631.
4. Hong, S. R.; Lee, S. J.; Shim, J. W.; Choi, Y. S.; Lee, Y. M.; Song, K. W.; Park, M. H.; Nam, Y. S.; Lee, S. I. Biomaterials 2001, 22, 2777.
5. Muzzarelli, R. A. A. Carbohydr Polym 1993, 20, 7.
6. Brine, C. J.; Sanford, D. A.; Zikakis, J. P. Advanced Chitin and Chitosan; Elsevier Science: Amsterdam, 1992; p 9.
7. Mi, F. L.; Shyu, S. S.; Wu, Y. B.; Lee, S. T.; Shyong, J. Y.; Huang, R. N. Biomaterials 2001, 22, 165.
8. Kumar, M. N. V. R. React Funct Polym 2000, 46, 1.
9. Qiu, X.; Jiang, M. Polymer 1995, 36, 5084.
10. Michaels, A. S. Ind Eng Chem 1965, 57, 32.
11. Blumstein, A.; Kakivaya, S. R.; Salamone, J. C. J Polym Sci Polym Lett Ed 1974, 12, 651.
12. Sittinger, M.; Lukanoff, B.; Burmester, G. R.; Dautzenberg, H. Biomaterials 1996, 17, 1049.
13. Denuziere, A.; Ferrier, D.; Domard, A. Carbohydr Polym 1996, 29, 317.
14. Denuziere, A.; Ferrier, D.; Damour, O.; Domard, A. Biomaterials 1998, 19, 1275.
15. Cleland, R. L.; Wang, J. L.; Detweiler, D. M. Macromolecules 1993, 15, 386.
16. Taravel, M. N.; Dormard, A. Biomaterials 1995, 16, 865.
17. Kikuchi, Y. J Appl Polym Sci 1976, 20, 2561.
18. Staikos, G.; Tsitsilianis, C. J Appl Polym Sci 1991, 42, 867.
19. Staikos, G.; Bokias, G.; Tsitsilianis, C. J Appl Polym Sci 1993, 48, 215.
20. NMR and X-ray studies of chitin and chitosan in solid state; Skjak-brak, G., Anthonsen, T., Sandford, P. A., Eds.; In Chitin and Chitosan; Elsevier Applied Science: London.