Characterization and Enzymatic Degradation of PEG-Cross-Linked Chitosan Hydrogel Films

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ABSTRACT: In our previous study (Kiuch et al., J Appl Polym Sci 2008, 107, 3823), poly(ethylene glycol) (PEG) cross-linked chitosan hydrogel films with various PEG to chitosan ratio and PEG molecular weight have been successfully prepared and their thermal, mechanical and swelling properties at acidic pH were studied. These films are potential biodegradable polymeric materials, because their constituents, that is, chitosan and PEG, are well known as biodegradable polymers. In this study, glass transition temperature (T_g), contact angle, swelling behavior at physiological conditions and *in vitro* enzymatic degradation behavior were investigated for the PEG-cross-linked chitosan hydrogel films. These properties of chitosan hydrogel were found to change significantly upon introducing the PEG-cross-links. According to differential scanning calorimeter analysis, the T_g value increased with the PEG content in the hydrogel films, indicating the densification of the film by PEG-crosslinking. The contact angle was decreased with increasing the number of cross-links, reflecting that introduction of the cross-linked structure induced the reduction of chitosan ordered structure. The swelling behavior depended on the PEG to chitosan ratio and the cross-linked structure. The water content was changed with the number of PEG-crosslinks. The rate of lysozyme-catalyzed degradation was also influenced by the introduction of PEG-cross-linked structure. The surface morphology of the film was observed by the scanning electron microscopy. It was confirmed that the PEG-cross-linked chitosan hydrogel film was degraded by lysozyme from its surface area. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 1902–1907, 2009

Key words: biodegradable; crosslinking; degradation; enzymes; chitosan

INTRODUCTION

Chitosan is composed of β(1-4)-linked *N*-acetyl-D-glucosamine(GlcNAc) and D-glucosamine subunits and is obtained via deacetylation procedure from chitin. Chitin is the second abundant natural polysaccharide next to cellulose and it is contained in the exoskeletons of arthropods such as shrimp and crab. Chitosan is not soluble in aqueous solution at neutral or alkaline pH but is soluble in slight acidic aqueous solution. This solution is viscous and becomes gel form when its longer-chain has entanglement. Recently, chitosan has attracted much attention in many biomedical applications because of its biocompatibility, biodegradability, non-toxicity, and antimicrobial activities.[1] Chitosan hydrogels are usable as the matrix material of biodegradable drug delivery systems under physiological conditions.[2,3]

Hydrogels of biocompatible and biodegradable polymers are of great interest in many biomedical fields such as tissue engineering, wound healing, and drug delivery.[4] So a variety of hydrogels made of natural polymers have been investigated.[5–10] These investigation involved several kinds of materials including grafted polymers and cross-linked polymers. In these investigations, the crosslinks, which are expected to result in better mechanical properties, are mainly classified into two types, that is, chemical and physical crosslinks. The former one is covalently bridged and the latter is physical interactions such as hydrogen bonding, ionic bonding, and hydrophobic interaction. From the viewpoint of sustainable drug release, covalently cross-linked hydrogel is favorable because of expectation of its long-term drug release.[11,12] One of the disadvantages of some hydrogels is caused by toxicity of crosslinking agent (cross-linkers), such as glutaraldehyde. Thus, using non-toxic cross-linker is desirable for preparing hydrogels for biomedical uses.

Poly(ethylene glycol) (PEG) shows hydrophilicity, less toxicity, biocompatibility, and biodegradability, and it has been used in many kinds of biomedical applications.[13] PEG with molecular weight lower than 20 kDa but higher than 400Da is cleared immediately without structural change in the urea. Whereas low molecular-weight oligomers of PEG of about 400Da or less is degraded *in vivo* by alcohol dehydrogenase to toxic metabolites, but PEG with molecular weight above 1000Da is safety, and so, non-toxic.[13,14]

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In our previous article,[15] poly (ethyleneglycol) (PEG)-cross-linked chitosan hydrogel films have been prepared and their thermal and mechanical properties and swelling behavior at acidic pH were studied. It was found that an introduction of the cross-linked structure in chitosan network improves mechanical properties of chitosan hydrogel films.[15] In this article, the cross-linked structure and the behavior of enzymatic degradation at physiological conditions using hen egg white (HEW) lysozyme will be investigated for the PEG-cross-linked chitosan hydrogel films. As the β-(1-4)-linked GlcNAc sequence of chitosan is degraded by HEW lysozyme as well as human lysozyme,[16,17] the PEG-cross-linked chitosan hydrogel is expected to be degraded by lysozyme. Enzymatic degradability should be one of indispensable properties of the hydrogel films to be applied as biomedical materials such as drug delivery systems.[18–20]

TABLE I
Sample Code, the Molecular Weight of DiepoxyPEGs, the Amount of diepoxyPEGs and Chitosan, Peg Ratio of PEG-cross-linked Chitosan Hydrogels and PEG/Chitosan Blends

Sample code	Molecular weight of diepoxyPEG (g/mol)	Amount of diepoxyPEG (g)	Amount of chitosan (g)	PEG ratio (wt %)
Cross-linked films				
f2000-0.2	2000	0.2	1.0	17
f2000-0.4	2000	0.4	1.0	29
f2000-0.6	2000	0.6	1.0	38
f2000-0.8	2000	0.8	1.0	44
f4000-0.4	4000	0.4	1.0	29
f4000-0.8	4000	0.8	1.0	44
Blend films				
blend04	2000	0.4	1.0	29
blend06	2000	0.6	1.0	38
blend08	2000	0.8	1.0	44

EXPERIMENTAL SECTION

Materials

Chitosan powder was kindly supplied by Yaidzu Suisan, Shizuoka, Japan and was used as received. The degree of deacetylation was determined to be 58% by FT-IR spectroscopy. PEG2000 ($M_w = 2000$) and PEG4000 ($M_w = 4000$) were purchased from Nacalai Tesque, Kyoto, Japan, and used without further purification. Phosphate buffer saline (PBS, pH 7.4), sodium hydride (NaH), and epichlorohydrin were purchased from Kanto Kagaku Co., Japan and used as received. Lysozyme from hen egg white (50,000 U/mg) was purchased from Merck Co., Japan and was used without further purification. The diepoxyPEGs were synthesized according to the method reported by Laine et al.[21] The PEG-cross-linked chitosan hydrogel films were synthesized according to the method reported by Kiuchi et al.[15] The sample codes, molecular weight of PEG and reactant compositions are shown in Table I. The chitosan/diepoxyPEG blend films for DSC measurement were prepared by casting method from 0.4% acetic acid solution, and their sample codes and blend ratio are also shown in Table I.

Characterization

Differential scanning calorimeter (DSC) measurements were performed on a Seiko DSC-220U equipped with a SSC-5300 control system (Seiko Instruments Co, Tokyo, Japan). The sample (3 ~ 5 mg) sealed in an aluminum sample pan was heated from –10 °C to 160°C at a heating rate 10°C/min. The melting enthalpy (ΔH) of diepoxyPEG was determined from the first heating scan. The value of glass transition temperature (T_g) was determined from the second heating scan (from −50°C to −10°C at a heating rate 2 °C/min) after the first heating scan and quenching. The rate of second heating scan enables to detect the small change. The water contact angle was determined for the chitosan hydrogel films using the sessile drop method with a contact angle meter CA-X (Kyowa Interface Science Co., Tokyo, Japan) at 25°C in the atmosphere. The water droplet was limited to about 0.5 mL to prevent gravitational distortion of its spherical profile. Each reported contact angle was actually the average of at least five measurements.

The swelling behavior of the hydrogel films were observed in phosphate buffer saline solution at pH 7.4 and 25°C. The dried disk-like sample films with the radius of about 3 mm and the thickness of about 0.15 mm were conditioned in a vacuum oven for 24 h. After the dried films were weighed, they were conditioned at 20–25 °C in the buffer solution of pH 7.4 and the time dependence of their weights was analyzed. The water content of the samples was determined according to the following equation:

$$\text{Water content } (\%) = (W_t - W_0)/W_0 \times 100(\%),$$

where W_t and W_0 represent the weights of swollen and dried state samples, respectively.

The *in vitro* degradation of chitosan hydrogel films (10×10 mm^2) was followed in 2 mL phosphate buffered solution (PBS, pH = 7.4) at 37°C containing 1 mg/mL lysozyme (hen egg white). The samples were preliminarily washed in methanol for 24 h so that the remains of unreacted diepoxyPEG were disappeared from the samples. Subsequently, they were freeze-dried for 48 h to remove water and weighed. The samples, after some minutes of degradation,

were removed from the medium, rinsed with soaking in methanol for 3 h, dried overnight under atmosphere, then freeze-dried and weighed. To distinguish enzymatic degradation from the dissolution, the control samples were tested under the same condition as described above, but without the addition of lysozyme. Each of weight-loss data reported was actually the average of at least three measurements.

Scanning electron microscope (SEM) observation of the morphology for the hydrogel samples was carried out on a scanning electron microscope JSM-5200 (JEOL. Co, Tokyo, Japan). The SEM sample was prepared by freeze-drying method. The sample was frozen at –70°C in refrigerator and was dried under vacuum for 24 h. After that, the sample was coated with gold.

RESULTS AND DISCUSSION

DSC analysis can provide useful information on the thermal properties of the hydrogel films, from which the glass transition temperature and the endothermic enthalpy were calculated. For DSC analysis, several cross-linked chitosan hydrogels films were prepared by reacting the chitosan (1 g) with increasing amounts of diepoxyPEG from 0.2 g to 0.8 g. The sample of PEG-cross-linked chitosan and PEG/chitosan blend used in this study are listed in Table I.

The DSC thermograms of diepoxyPEG, chitosan and PEG-cross-linked chitosan hydrogel film (Table I, sample f2000–0.6) are shown in Figure 1. The hydrogel film and chitosan samples are registered wide endothermic peak centered between 40 and 130°C. This wide peak was caused by the overall endothermic process connected with the evaporation of bound water. In general, polysaccharides have a strong affinity for water and can be easily hydrated in the solid state.[1] For this reason, the broad peak was also observed for the hydrogel film. Both the hydrogel film and diepoxyPEG samples show a sharp endothermic peak centered between 40 and 50°C. This is connected with the melting of the PEG moiety. The intensity of this endothermic peak of the hydrogel film was largely decreased compared with that of diepoxyPEG. DiepoxyPEG which was involved as cross-linker in the hydrogel film could not crystallize, because both the terminals of diepoxyPEG were used in crosslinking reaction and it was immobilized with stiff chitosan structure, suppressing the mobility, and the crystallization of the PEG chain. So the formation of crystalline structure was difficult. As a result, diepoxyPEG used in crosslinking reaction remained in the amorphous state. The intensity decrease and lower shift of the peak top of this endothermic peak indicates indirectly that the hydrogel films have crosslinked structure.

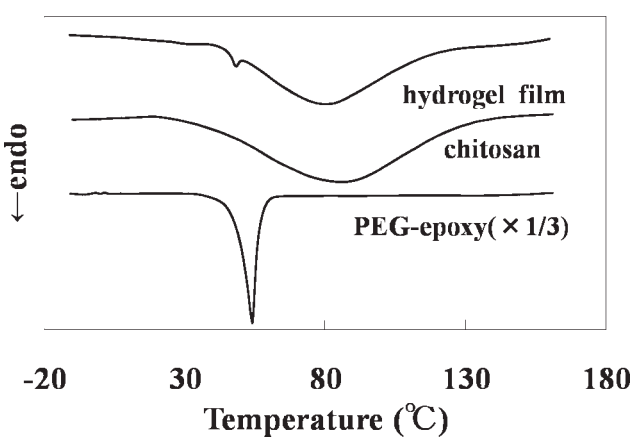

Figure 1 DSC first heating curves of diepoxyPEG (×1/3), chitosan and hydrogel film (f2000–0.6).

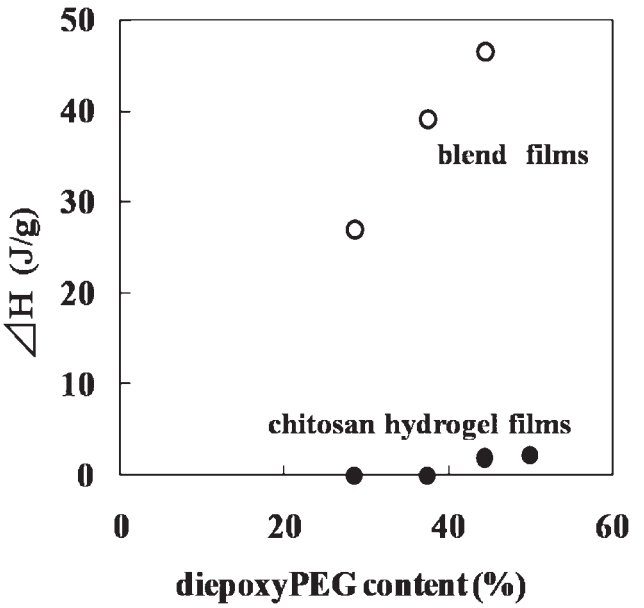

Figure 2 Exothermic enthalpy ΔH (J/g) of diepoxyPEG melting vs. diepoxyPEG content of the hydrogel films (●) and the chitosan/diepoxyPEG blend films (○).

The conversion of diepoxy-end of diepoxyPEG as the cross-linker was qualitatively evaluated by the DSC measurement. The endothermic enthalpy (ΔH) calculated from the area of endothermic peak caused by the melting of the PEG chain crystal was plotted against the diepoxyPEG content as shown in Figure 2. The endothermic enthalpy of the blend sample was proportional to the amount of diepoxyPEG. In contrast, the values of endothermic enthalpy of the hydrogel film samples were very small compared with those of the blend film with the same diepoxyPEG content. If the hydrogel films do not have any difference in their structures, the endothermic enthalpy must be the same value as those of the blend films. So, these differences in ΔH value indicated that the crosslinking reaction proceeded. Thus, it

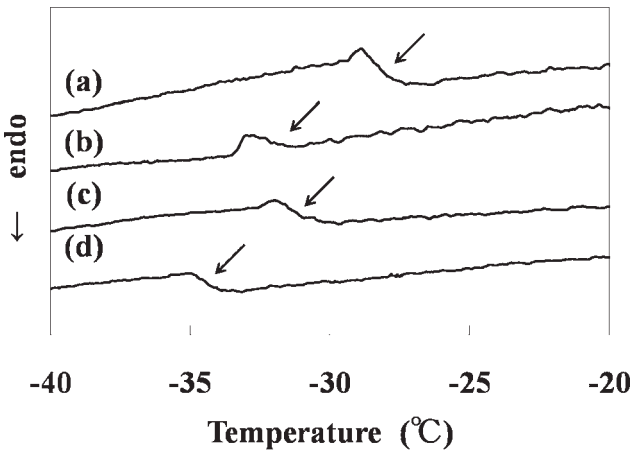

Figure 3 DSC second heating curves of the cross-linked hydrogel films with various fraction of diepoxyPEG; (a) f2000–0.8, (b) f2000-0.6, (c) f2000–0.4, (d) f2000–0.2.

was found that the reactivity of the diepoxy-end was very high.

Figure 3 shows the second run DSC curves of the hydrogel films cross-linked with various amounts of diepoxyPEG 2000, indicating the glass transition. If respective hydrogel films have no difference in the chain flexibility of PEG component, the value of glass transition temperature of the hydrogel films was expected to decrease with increasing the fraction of diepoxyPEG, approaching to that of pure diepoxyPEG (about −50 °C) with increasing the content of diepoxyPEG, as predicted by the Flory-Fox equation.[22–24] In this study, however, the T_g values increased with the content of diepoxyPEG. This may be related to the density of the internal matrix which was formed by cross-linking reaction. With increasing the diepoxyPEG content, the number of crosslinks increases, and the increase of the number of crosslinks would make the matrix more rigid and dense. When the density becomes high, the mobility of PEG polymer chain was constrained by the stiff chitosan chain, thus the T_g would increase.

The hydrophilicity is one of the most basic properties for biomedical application of biodegradable hydrogel films. The value of water contact angle was measured to evaluate the hydrophilicity of the film surfaces, as listed in Table II. The hydrophilicity of the hydrogel film surface was enhanced with addition of the diepoxyPEG. However, excess of PEG, that is the sample f2000–06 and f2000–08, does not increase much the hydrophilicity. In general, the values of water contact angle of chitosan film are high.[25] Furthermore, it is known that an introduction of cross-linked or grafted structure into chitosan molecules increases hydrophilicity,[25] because, in the chitosan film there are strong intermolecular and intramolecular hydrogen bonds and the incorporation of chemical modifications like above leads to break out these interactions.[25-27] For these reasons, the hydrophilicity of the hydrogel films increased. Thus, the contact angle of cross-linked films was small compared to the blend films.

TABLE II
Contact Angle of Chitosan Film and Chitosan Hydrogel Films

Sample	Contact angle (°)
Chitosan	80 ± 4.6
f2000–0.2	75 ± 3.0
f2000–0.4	22 ± 4.9
f2000–0.6	21 ± 0.5
f2000–0.8	20 ± 2.6
f4000–0.4	28 ± 1.4
f4000–0.8	26 ± 1.3
Blend04	19 ± 3.3
Blend06	76 ± 2.9
Blend08	80 ± 3.7

The dependence of contact angle on molecular weight and diepoxyPEG content appeared. With respect to molecular weight, the contact angles of the hydrogel film prepared by using diepoxyPEG of molecular weight 2000 are slightly smaller than that of molecular weight 4000. There are two reasons. One is that the more the number of cross-linked point increase, the more the intermolecular and intramolecular hydrogen bond of chitosan break, so the sample of molecular weight 2000 becomes hydrophilic. The other is that the hydrophilicity of diepoxyPEG itself has much effect on the hydrophilicity of chitosan hydrogel film.

Figure 4 shows the result of the swelling tests for the hydrogel films with different diepoxyPEG content. At first, each sample swelled rapidly in PBS solution, then, the swelling rate was slowing down gradually. At last, the water content reached

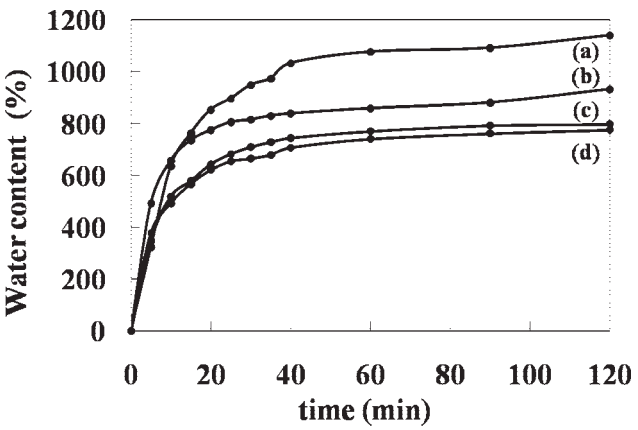

Figure 4 Swelling behavior of the hydrogel films in the phosphate buffer solution (pH 7.4) at 25°C. (a) f4000–0.4, (b) f2000–0.4, (c) f4000–0.8, (d) f2000–0.8.

equilibrium value. This result indicates that the swelling mechanisms of these samples are almost the same. But, it was found some variations in the values of equilibrium water content. For example, the samples with less content of diepoxyPEG and with high molecular-weight PEG chain took in much water. This means that the water content of these hydrogel films mainly depends on the ionic bond between the water molecules and the amino groups of chitosan (positive charge)[7], because the sample of less content and high molecular weight of diepoxy-PEG have many amino groups that was not used in the cross-linked reaction. The number of cross-linked points has also effect on the swelling property, because it decreased with proceeding the cross-linked reaction; the cross-linked points of f2000–0.4 are twice as many as that of f4000–0.4, so the number of the amino groups of chitosan component of f2000–0.4 is smaller.

The swelling behavior of two samples with the 0.8 g diepoxyPEG content (f4000–0.8 and f2000–0.8) shows a little difference. These two samples have almost the same water content after equilibrium. This indicated the presence of a maximum number of cross-linked points, and the thickness of the internal structure was saturated. Thus the value of equilibrium water content could be almost the same. From Table II, it was found that the hydrophilicity of the sample f2000–0.4 was higher than that of sample f4000–0.4, but water content of the sample f4000–0.4 was much higher than that of the sample f2000–0.4. It is known that the presence of PEG provides porous structure because of exclusion volume of polymer (PEG) and phase separation.[28] So, these results indicated that influences of porous structure and hydrophilicity compete with each other. If the structure of hydrogel's internal network became thick, the structure can not hold enough water. And the increase of hydrophilicity of hydrogel films surface was connected with decrease of the number of cross-linked points.

To mimic the *in vivo* biodegradation performance, degradation behaviors of the hydrogel film in the presence and in the absence of lysozyme were investigated in PBS (Fig. 5). The degradation of the chitosan films was not so clear and its degradation rate was very slow. This is due to the formation of intra and intermolecular-hydrogen bonds which make the thick structure and may prevent lysozyme from accessing to the binding site. Thus, there was a little difference in degradation behavior between in the presence and in the absence of lysozyme. In contrast, the degradation of the hydrogel films shows clear result; the weight loss of the hydrogel film remains at most 4% even after 16 days in PBS without lysozyme, whereas in the presence of 1 mg/mL lysozyme, the weight loss was at least 12% after 16 days.

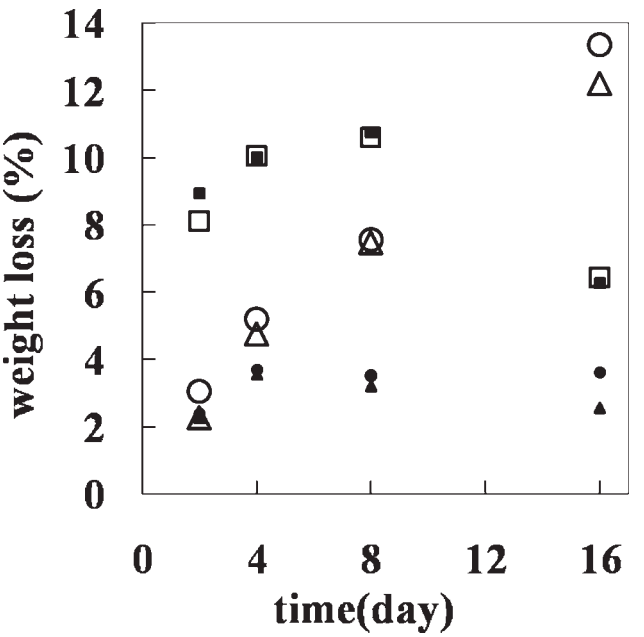

Figure 5 Weight loss of hydrogel films in 1 mg/mL lysozyme/PBS and in PBS without lysozyme at 37°C as a function of time. (□: chitosan/lysozyme, ○: f2000–0.8/lysozyme, Δ: f4000–0.8/lysozyme, ■: chitosan, ●: f2000-0.8, ▲: f4000–0.8).

Besides the degradation rate of the hydrogel films is proportional to degradation time. So, the hydrogel film was found to be degraded easily by lysozyme. The weight loss of sample f2000–0.8 was slightly larger than the sample f4000–0.8 in each day. Given the condition that the number of cross-linked points of the sample f2000–0.8 is larger than that of the sample f4000-0.8, the sample f4000–0.8 is likely to degrade faster than the sample f2000–0.8, but the result is not so obvious. From the viewpoint of hydrophilicity, the sample f2000–0.8 is more hydrophilic, as indicated by the contact angle. So, these results indicated that enzymatic degradation by lysozyme is mainly influenced by the hydrophilicity of film's surface. The degradation mechanism of chitosan film and the hydrogel film could be different. Lysozyme cleavages the β(1-4)-linked GlcNAc and Glc subunits. The chitosan film has no PEG-cross-linkage but entanglement of chitosan molecular chains. However, the hydrogel film has both PEG-cross-linkages and entanglement so that the weight loss of the hydrogel film in the initial state is small.

The surface morphologies of the chitosan hydrogel films after enzymatic degradation and its control samples were observed by SEM, as shown in Figure 6(a,b). The surface of the hydrogel film after 1 day degradation in 1 mg/mL lysozyme/PBS was rough, while that without lysozyme was smooth. So, we can say the hydrogel film was degraded by lysozyme and its degradation occurred from its surface.

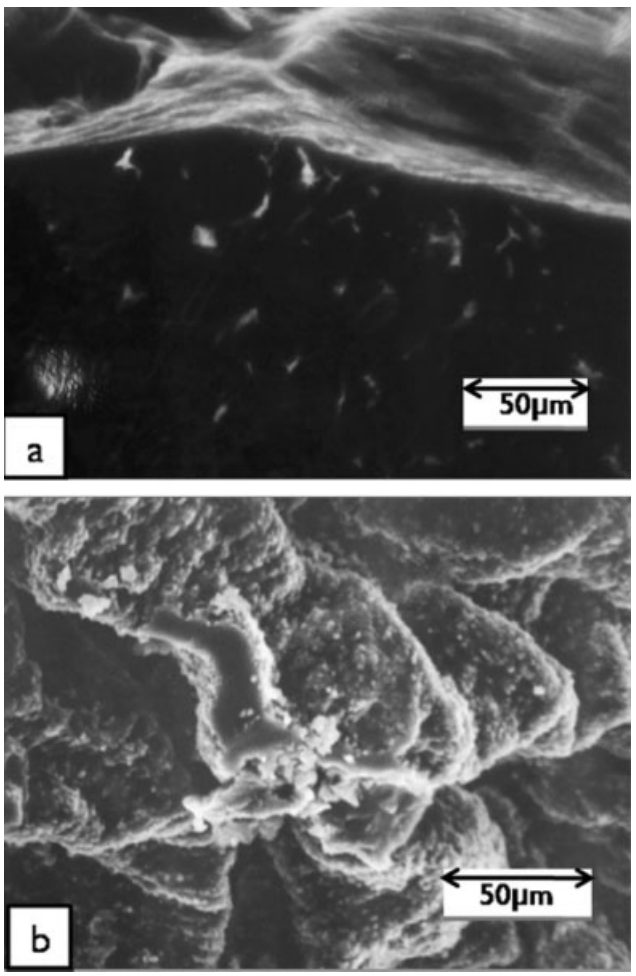

Figure 6 SEM image of the hydrogel film degraded in (a) PBS without lysozyme and (b) in 1 mg/mL lysozyme/PBS for 1 day at 37°C. Scale bar is 50 μm.

CONCLUSION

The PEG-cross-linked chitosan hydrogel films were prepared successfully. By measuring the glass transition temperature by DSC, the internal three dimensional structure of cross-linked chitosan hydrogel film was turn out to become dense as the increase of diepoxyPEG content. The three dimensional structure of PEG-cross-linked chitosan hydrogel film has much effect on its swelling behavior; the thick structure prevented from retaining much water in its structure. The hydrophilicity of hydrogel film depends on the molecular weight and the content of diepoxyPEG. It was confirmed that the cross-linked chitosan hydrogel films was degraded by lysozyme under physiological condition. In addition, the degradation rate depends on the hydrophilicity. It might be possible that adjustment of several condition, such as the content and the molecular weight of diepoxyPEG, lead this cross-linked chitosan hydrogel film to use the drug control release, for example, as the drug loading hydrogel films which gradually releases drug with its degradation.

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