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Preparation and characterization of PEG-cross-linked chitosan hydrogel films with controllable swelling and enzymatic degradation behavior

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ABSTRACT

The PEG-cross-linked chitosan hydrogel films with different content and molecular weight of PEG were prepared. The formation of the PEG-cross-linked structure was confirmed by FT-IR measurement. The swelling ratio increases with the decrease of pH value of the surrounding buffer solution and molecular weight of PEG with the same content sample. The reversible temperature dependence was observed for swelling behavior of the PEG-cross-linked chitosan hydrogel films. The degradation rate of chitosan component by lysozyme was found to be influenced by the content and molecular weight of PEG. An increase in total PEG content resulted in a considerable increase of the degradation rate, that is, the weight loss of the sample including 45% PEG was amount to more than 15% after 24 days degradation, and lowering the molecular weight of PEG led to the fast degradation. From the SEM observation of the film surface, it was confirmed that the PEG-cross-linked chitosan hydrogel film was degraded by lysozyme from its surface to internal area.

1. Introduction

Chitin and chitosan have attracted much attention over many years. Chitin is the second abundant natural polysaccharide next to cellulose and it is contained in the exoskeletons of arthropods such as lobster, shrimp and crab (Rinaudo, 2006). Chitin is not soluble in various general solvents because of strong inter- and intramolecular hydrogen bonds. Chitosan is obtained via partial deacetylation procedure from chitin and is not soluble in aqueous solution at neutral or alkaline pH, but is soluble in dilute acidic aqueous solution (Rinaudo, 2006). Chitosan has many advantageous properties such as biocompatibility, biodegradability, nontoxicity, accelerated wound healing properties and antimicrobial activities (Kurita, 1998; Rinaudo, 2006). Chitosan also has film forming properties and the mechanical properties change depending on several parameters such as solvent pH, the type of solvent, chemical crosslink, annealing treatment and the presence of plasticizer like water and polyol. (Alexeev, Kelberg, & Evmenenko, 2000; Arvanitoyannis, Kolokuris, Nakayama, Yamamoto, & Aiba, 1997; Arvanitoyannis, Nakayama, & Aiba, 1998; Zivanovic, Li, Davidson, & Kit, 2007). Chitosan can bind negatively charged compound such as DNA, glycosaminoglycans and protein without harsh and denaturing organic solvent because of the protonated amino groups. Thus, chitosan has been widely investigated in the pharmaceutical industry for its potential use as the controlled release implant systems (Itoh, Matsusaki, Kida, & Akashi, 2006; Oh, Siegwart, & Matyjaszewski, 2007; Wang, Dong, Du, & Kennedy, 2007; Zhang, Mardyani, Chen, & Kumacheva, 2006).

Poly(ethyleneglycol) (PEG) is fascinating synthetic polymer. It shows biodegradability, biocompatibility, less toxicity and hydrophilicity and has been used in many kinds of applications (Roberts, Bentley, & Harris, 2002). The mechanical properties of chitosan film were improved by blending with PEG oligomer as plasticizer (Suyatma, Tighzert, & Copinet, 2005). When incorporated into human body, PEG with molecular weight lower than 20 kDa but higher than 400 Da is cleared immediately without structural change in the urea. Whereas low molecular-weight oligomers of PEG of about 400 Da or less is degraded *in vivo* by alcohol dehydrogenase to toxic metabolites, but PEG with molecular weight above 1000 Da is safety, and so, non-toxic (Clark et al., 1996; Roberts et al., 2002).

In our previous paper (Kiuchi, Kai, & Inoue, 2008), PEG-cross-linked chitosan hydrogel films have been prepared and their thermal and mechanical properties and swelling behavior have been studied. It was found that an introduction of the PEG-cross-linked structure in chitosan network improves mechanical properties of chitosan hydrogel films. We have also reported glass transition temperature, contact angle, swelling behavior at physiological conditions and *in vitro* enzymatic degradation behavior of PEG-cross-linked chitosan hydrogel films (Tanuma, Kiuchi, Kai, Yazawa, & Inoue, 2009).

In this article, the PEG-cross-linked chitosan hydrogel film samples with several molecular weight and content of PEG are prepared. For these samples, more direct method than previous

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thermal analysis, that is, FT-IR will be applied to investigate the formation of the PEG-cross-linked structure. The swelling property sensitive to pH and temperature is considerably important capacity for the biomedical application such as artificial muscles or switches and drug delivery systems. So, the swelling properties of the PEG-cross-linked chitosan hydrogel films at various pH conditions near neutral, temperature-dependent reversible swelling behavior will be studied. Then, the enzymatic degradation behavior at physiological conditions using hen egg white (HEW) lysozyme will be also investigated for these films. Enzymatic degradability should be one of indispensable properties of these films to be applied as biomaterials (Etienne et al., 2005; Freier, Koh, Kazazian, & Shoichet, 2005; Neamnark et al., 2007).

2. Materials and methods

2.1. Materials

Chitosan powder was kindly supplied by Yaidzu Suisan Ltd., Shizuoka, Japan and was used as received. The degree of deacetylation was determined to be 58% by FT-IR spectroscopy. PEG2000 (M_w = 2000), PEG20,000 (M_w = 20,000), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Nacalai Tesque Inc., 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., Ltd, Tokyo Japan and used as received. Lysozyme from hen egg white (50,000 U/mg) was purchased from Merck Co., Ltd., Tokyo, Japan and was used without further purification.

2.2. Preparation of PEG-cross-linked chitosan hydrogel films

The PEG-cross-linked chitosan was prepared by cross-linking chitosan with diepoxyPEG, which has the epoxy rings at both the ends of the PEG chain. The diepoxyPEG was synthesized according to the method reported by Laine et al. (2004). The PEG-cross-linked chitosan hydrogel films were synthesized according to the method reported by Kiuchi et al. (2008). The sample codes, molecular weight of PEG and reactant compositions are shown in Table 1. The chitosan/diepoxyPEG blend film for FT-IR measurement was also prepared by casting method from 0.4% acetic acid solution, and its sample codes and PEG content are also shown in Table 1. The samples for all analysis were repeatedly washed in methanol for 24 h to remove the acetic acid and unreacted diepoxyPEG. Subsequently, they were freeze-dried for 48 h to remove water and weighed.

2.3. FT-IR analysis

The Fourier transformed infrared (FT-IR) spectra were recorded on the AIM-8800FTIR spectrometer (Shimazu Co., Kyoto, Japan) to analyze the chemical structure of the PEG-cross-linked chitosan films. The spectra were recorded with the sum of 16 scans at a resolution of 4 cm^{-1}. The sample for FT-IR measurement was prepared by slicing the film along its thickness direction using microtome.

Table 1
Sample code, the molecular weight and content of diepoxyPEGs and the peak intensity ratio of the IR bands at 1410 versus 1380 cm^{-1} of hydrogel film samples.

Sample code	PEG molecular weight of diepoxyPEG (g/mol)	PEG content (wt.%)	A_{1410}/A_{1380}
Chitosan		0	1.289
L15	2000	15	0.820
L30	2000	30	0.798
L45	2000	44	0.677
LH30	20,000	30	0.751
Blend	2000	30	1.250

2.4. Swelling test

The swelling behavior of the dried sample films (10 × 10 mm^2) with the thickness of about 0.15 mm was observed in phosphate buffer saline solution at pH 7.4, 6.8 and 6.2 at 25 °C. These buffer solutions with different pH values were prepared using sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4). After the dried films were weighed, they were conditioned at 25 °C in the buffer solution at each pH condition. The samples were taken from the medium when they were reached to the equilibrium swelling, wiped with filter paper and weighed. The water content of the film sample 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 temperature dependence and reversibility of swelling behavior were observed in phosphate buffer saline solution at pH 7.4. At first, the film samples were immersed in phosphate buffer saline solution at 2 °C. After they reached the equilibrium swelling ratio, they were taken from the medium, wiped and weighed. Then, they were immersed in the buffer solution at 37 °C. When they reached the equilibrium swelling ratio at 37 °C, they were taken from the medium, wiped and weighed. Further, they were immersed again in the buffer solution at 2 °C. These processes repeated two times.

2.5. Enzymatic degradation

The *in vitro* degradation of the 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, 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 for 48 h and weighed. To distinguish enzymatic degradation from the dissolution, the control samples were tested under the same condition as described above, but without adding lysozyme. Each of weight loss data reported was actually the average of at least three measurements using these films.

2.6. SEM morphology

Scanning electron microscope (SEM) observation of the morphology for the film samples was carried out on a SEM 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.

3. Results and discussion

3.1. Characterization of PEG-cross-linked chitosan hydrogel films

The formation of the PEG-cross-linked structure was investigated by the FT-IR analysis. Fig. 1 shows the FT-IR spectra of diepoxyPEG, chitosan film, chitosan/PEG blend film and PEG-cross-linked chitosan hydrogel film. The IR spectrum of chitosan film (Fig. 1(b)) shows characteristic bands at 1650 cm^{-1} (amide I) and 1580 cm^{-1} (amide II), which can be assigned as the C=O stretching

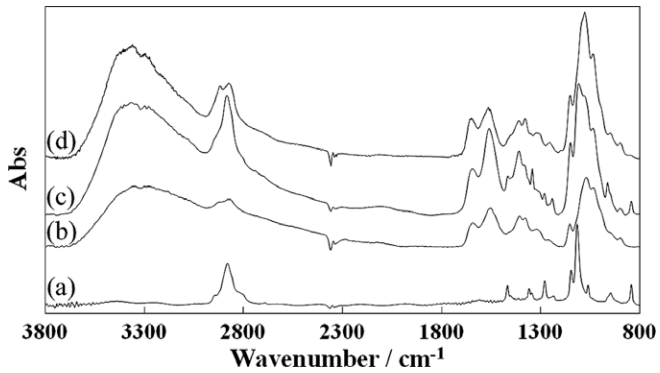

Fig. 1. FT-IR spectra of the films of (a) diepoxyPEG, (b) chitosan, (c) chitosan/PEG blend and (d) PEG-cross-linked chitosan hydrogel.

vibration and the N–H deformation vibration, respectively (Cao et al., 2005). The IR spectrum of diepoxyPEG (Fig. 1(c)) shows characteristic bands at 1280, 947 and 843 cm^{-1} (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005). In the spectrum of the blend film (Fig. 1(c)), the relative intensity of the absorption peak at 1580 cm^{-1} was increased compared to that at 1650 cm^{-1}, though there are no absorption peaks in the range from 1700 to 1500 cm^{-1} in the pure diepoxyPEG spectrum (Fig. 1(a)). In this blend system, PEG and chitosan may form inter-molecular hydrogen bonds and this inter-polymer interaction influence the amine deformation vibration band at 1580 cm^{-1}. On the other hand, this absorption peak was not so strong in the spectrum of the PEG-cross-linked chitosan hydrogel film (L30) compared to that in the spectrum of the blend film. In other words, compared to the amide I peak at 1650 cm^{-1}, the peak intensity of the amide II once increased by the inter-polymer interaction and then largely decreased, indicating that the chemical structure of chitosan amino groups was changed by the ring-opening reaction of diepoxyPEG. Thus, the absorption peak of the N–H deformation vibration of L30 was decreased. From this IR result, the formation of the cross-linked structure was confirmed.

In addition, the formation of the cross-linked structure was also confirmed by the absorption peak at 1380 cm^{-1} (Fig. 2). The intensity at 1380 cm^{-1} increased with the content of diepoxyPEG of the PEG-cross-linked chitosan hydrogel films while those of the chitosan and the chitosan/PEG blend film did not increase. This 1380 cm^{-1} absorption is caused by the PEG CH_2 deformation vibration, because the number of the CH_2 groups was increased with proceeding the cross-linked reaction. The relative peak intensity ratio of the 1380 cm^{-1} band to the 1410 cm^{-1} band was also shown in Table 1. The ratio decreased with increasing the diepoxyPEG content, indicating that the number of the PEG-cross-links increased with the diepoxyPEG content. Thus, the formation of the PEG-cross-linked structure was also confirmed.

3.2. Swelling behavior

Fig. 3 shows the result of the swelling ratio at equilibrium under different pH conditions. The equilibrium swelling ratio at pH 7.4 was small. Under middle pH condition (pH 6.8), it becomes a little larger than that at pH 7.4. The largest equilibrium swelling ratio was observed when the pH value was the lowest among pH conditions investigated here. Thus, the equilibrium swelling ratio was observed for all samples to increase with the decrease of pH value of the surrounding buffer solution. This result suggests that the swelling mechanisms of these samples are almost the same, i.e., the amino groups of chitosan molecules were protonated in acidic pH and the matrix was expanded by the electrostatic repulsion among these positively charged groups.

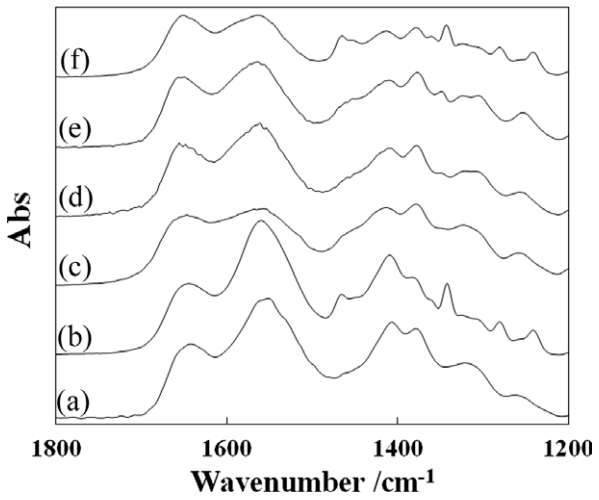

Fig. 2. FT-IR spectra of the films of (a) chitosan, (b) chitosan/PEG blend, and PEG-cross-linked chitosan hydrogel of (c) L15, (d) L30, (e) L45 and (f) LH30.

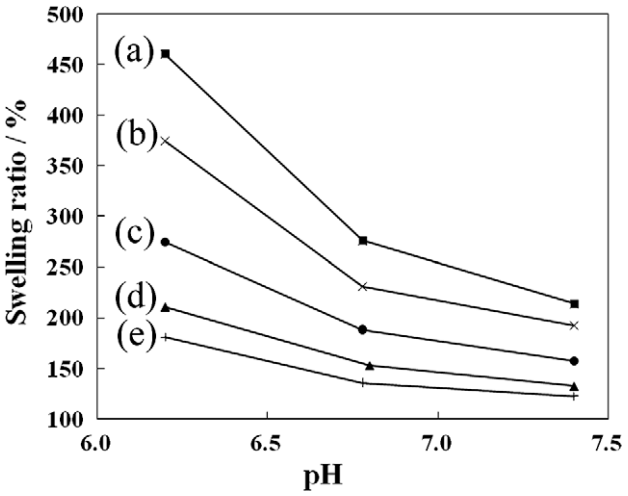

Fig. 3. Swelling behavior of the hydrogel films in the various phosphate buffer solutions (pH 6.2, 6.8, 7.4) at 25 °C. (a) L45, (b) LH30, (c) L30, (d) L15, (e) chitosan film.

The swelling ratio of L30 was higher and lower than those of L15 and L45, respectively, indicating that the swelling ratio of the sample with the same PEG molecular weight (L15, L30, L45) increased with the PEG content. If the swelling ratio was only influenced by the amino groups of chitosan molecules, L15 should be the most swelled because it has more amino groups not participated in the cross-linking reaction. But, L15 shows less water content, indicating that the swelling of the hydrogel film was largely governed by the presence and the content of the PEG chains. This is at least partly due to the fact that the incorporation of the PEG cross-links reduces the number of the free and intramolecular hydrogen bonded amino groups in chitosan molecules.

The swelling ratio of the film sample with high molecular-weight PEG (LH30) was higher than that with low molecular-weight PEG (L30). Compared to the lower molecular-weight PEG chain, the higher one may exclude ordered chitosan structure maintained by the intramolecular hydrogen bonds and expands internal three dimensional structures to hold more water molecules inside the space. The swelling ratio of LH30 was lower than that of L45. Thus, there are influences of molecular weight and content of PEG and these two factors show opposite effects on the swelling behavior.

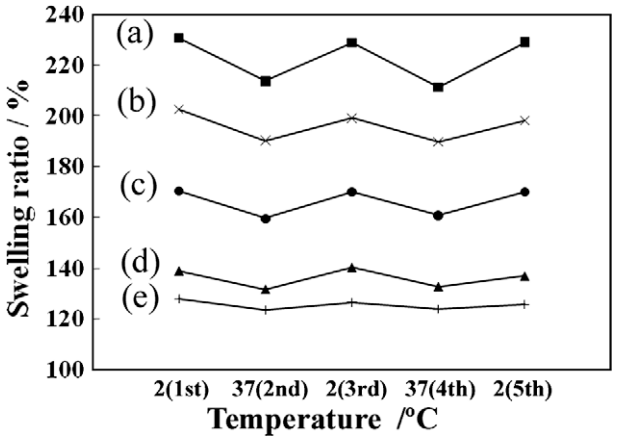

Fig. 4. Swelling ratio of (a) L45, (b) LH30, (c) L30, (d) L15, (e) chitosan film at a equilibrium swelling states under repeated temperature change (2 and 37 °C).

Temperature-dependent reversible swelling behavior was found, as shown in Fig. 4. At first run, the equilibrium swelling ratio was measured at 2 °C. Then, the equilibrium swelling experiment was done at 37 °C for the same film sample. The equilibrium swelling ratio decreased with increasing the temperature to 37 °C. When the temperature was lowered to 2 °C again (3rd run), the equilibrium swelling ratio increased to the level of the first run at 2 °C. These heating/cooling runs were repeated two times. The similar reversible and reproducible temperature dependence of swelling–deswelling behavior was observed for all samples. Thus, all samples have almost the same temperature dependency, higher swelling ratio at lower temperature and lower at higher temperature. This swelling–deswelling behavior is mainly due to the interaction between polymer and water molecules. In general, the strength and extent of this interaction decrease with increasing the temperature. The sample with higher PEG content is more sensitive to the temperature change, showing more distinctive temperature-dependent swelling–deswelling response. It would be a desirable character for controlled-drug release system with swelling property controllable by pH and temperature. So, it can be expected that these hydrogel films are of great interest for biomedical application such as artificial muscles or switches and drug delivery systems.

3.3. Enzymatic degradation

Chitosan is mainly degraded by lysozyme in human body. The *in vitro* degradation behavior of chitosan has been usually investigated by using hen egg white (HEW) lysozyme (Etienne et al., 2005; Freier et al., 2005; Neamnark et al., 2007), because HEW lysozyme as well as human lysozyme (Nordtveit, Varum, & Smidsrod, 1996) cleavages the β(1-4)-linked GlcNAc and GlcN subunits of chitosan.

In this study, the degradation behavior of the hydrogel film in the presence and in the absence of lysozyme was investigated in PBS at 37 °C (Fig. 5). Fig. 5(a) shows the degradation behavior of the L15 film sample. The difference between the weight loss of the sample degraded in the PBS medium with and without lysozyme was not so clear. On the other hand, the weight loss of L30 remains at most 9% even after 32 days in PBS without lysozyme, while in the presence of 1 mg/ml lysozyme, the weight loss reached to more than 21% after 32 days. Besides the degradation rate of the hydrogel films is proportional to the degradation time. Similarly, the weight loss of L45 remains at most 5% even after 24 days in PBS without lysozyme, while in the presence of 1 mg/ml lysozyme, the weight loss was more than 15% after 24 days and it is proportional to the degradation time. The weight loss of

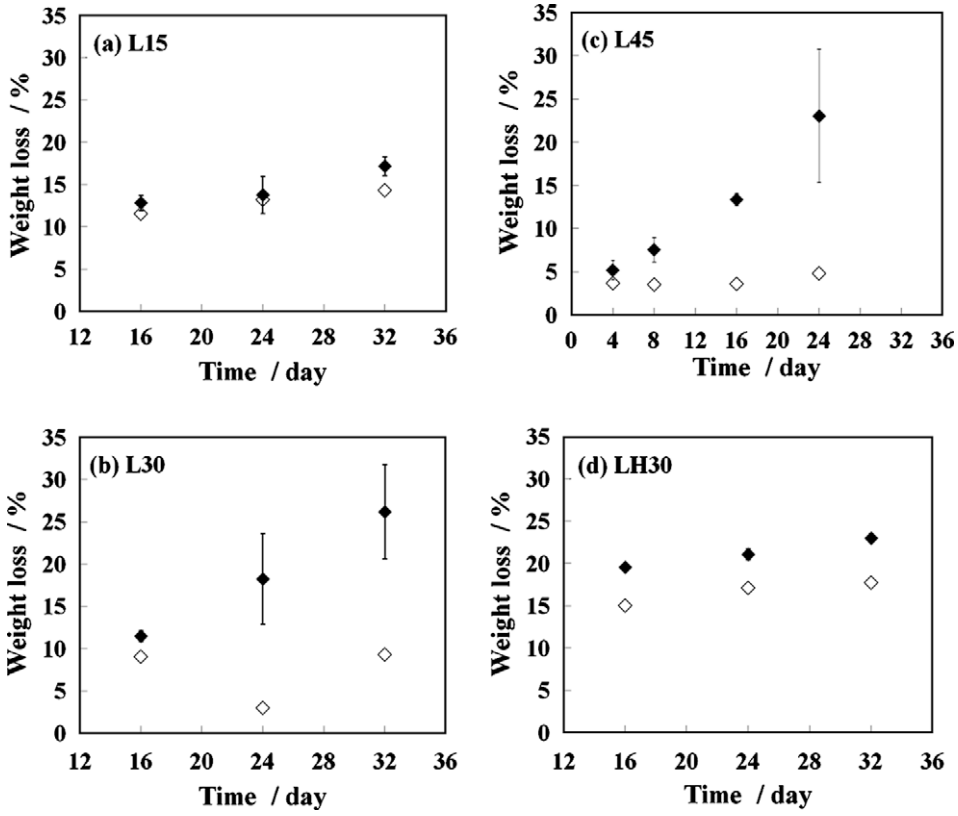

Fig. 5. Weight loss of hydrogel films in 1 mg/ml lysozyme/PBS and in PBS without lysozyme at 37 °C as a function of time. Filled and empty mark shows the sample of with and without lysozyme, respectively. (a) L15 (b) L30 (c) L45 (d) LH30.

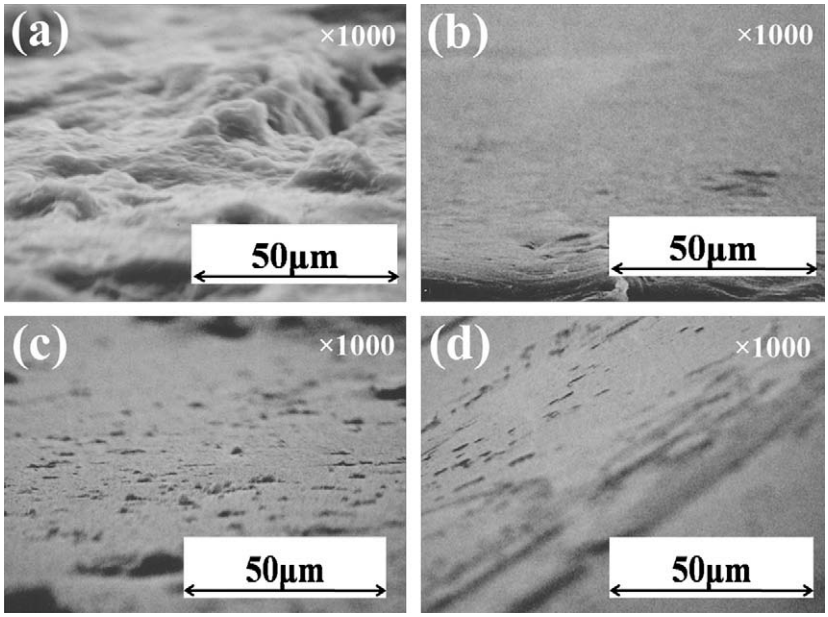

Fig. 6. SEM photographs of (a) L30 after degradation with lysozyme in PBS, (b) L30 after degradation without lysozyme in PBS, (c) LH30 after degradation with lysozyme in PBS and (d) LH30 after degradation with lysozyme in PBS for 24 day at 37 °C. Scale bar is 50 μm.

the sample L45 after 32 days degradation was not shown in Fig. 5(c), because the degradation largely proceeded and the film was broken down. What it comes down to is that the degradation rate was increased with the PEG content, indicating that the introduction of the PEG-cross-linked structure severed the formation of the intra and inter-molecular hydrogen bonds and may facilitate the access of lysozyme to the binding site. In other words, the intra and inter-molecular hydrogen bonds of the L15 sample is stronger than the L30 and L45 ones.

The degradation behavior of LH30 was shown in Fig. 5(d). The weight loss of LH30 degraded with 1 mg/ml lysozyme was more than 23% after 32 days, that is, the differences between the degradations with and without lysozyme is not so large. This result indicated that the degradation of LH30 sample was slow, as compared to L30 sample. From these results, the tendency of the enzymatic degradation rate is found to be paralleled with that of the swelling ratio in view of the samples with the same molecular weight; the higher the value of equilibrium swelling ratio at pH 7.4 was, the faster the enzymatic degradation rate was. On the other hand, this tendency is unequal in view of the samples with different molecular weight. This is because the L30 has more dispersed cross-linked point and lysozyme may easily access the binding site of chitosan molecular chain. Thus, the swelling ratio and the internal structure influenced the enzymatic degradation.

The surface morphologies of the film of PEG-cross-linked chitosan hydrogel L30 and LH30 after enzymatic degradation and their control samples were observed by SEM, as shown in Fig. 6. The surface of the sample L30 after 24 days degradation in 1 mg/ml lysozyme/PBS medium was very rough, while that after degradation without lysozyme was smooth. The surfaces of the L45 films degraded under the same condition shows similar tendency to that of the sample L30. On the other hand, Fig. 6(c and d) show the picture of the surface of the LH30 film after 24 days degradation in 1 mg/ml lysozyme/PBS and without lysozyme. These two pictures show little difference. The sample degraded in PBS including lysozyme shows slightly rough surface. These results indicate that the enzymatic degradation of these films occurred not only on its surface but also in the internal surface caused by swelling.

4. Conclusions

The PEG-cross-linked chitosan hydrogel films with different contents and molecular weight of PEG were prepared successfully. The formation of the PEG-cross-linked structure was confirmed by comparing absorption peaks of amide I (1650 cm^{-1}) and amide II (1580 cm^{-1}). The swelling ratio increased with the decrease of pH value of the surrounding buffer solution and decreased with the increase of molecular weight of PEG. All films also showed similar reversible temperature-dependent swelling behavior; the high swelling ratio under low temperature. The weight loss of the sample including 45% PEG was more than 15% after 24 days degradation, and PEG with low molecular weight led the faster degradation than that with high molecular weight. We can conclude that the hydrogel sample with lower molecular weight and higher content of PEG is more sensitive to the enzymatic degradation. The degradation rate would be controllable by changing the content and molecular weight of PEG, so this PEG-cross-linked chitosan hydrogel film will be useful in the field of controlled-drug release device which enable sustainable drug release with proceeding in degradation of film.

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