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メタデータ	言語: English 出版者: 公開日: 2009-08-28 キーワード (Ja): キーワード (En): 作成者: TANIGAWA, Junichi, MIYOSHI, Norio, SAKURAI, Kensuke メールアドレス: 所属:
URL	http://hdl.handle.net/10098/2123

Characterization of chitosan/citrate and chitosan/acetate films and applications for wound healing

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Abstract

In this work, we aimed to develop a scaffold of chitosan (CS) with a porous sponge structure for an artificial skin. The scaffolds were prepared from both CS/citric and CS/acetic solutions. In addition, the cast films were also prepared from the same solutions to compare some properties of them. They were characterized using WAXD, FT-IR, DSC, tensile measurements and SEM observation. It was found that CS/acetate had low crystallinity but CS/citrate was in an amorphous state, resulting in a large ductility with rubbery softness. Despite the different morphologies of CS/citrate and CS/acetate scaffolds, both scaffolds exhibited the wound healing effect available for tissue engineering.

Keyword: scaffold; chitosan; wound healing; microstructure;

INTRODUCTION

Chitin is a basic polysaccharide existing in nature, having a chemical structure similar to that of cellulose. It is contained mainly in crustaceans such as crabs, shrimps and krills and insects, as complexes with proteins and calcium carbonate, and plays the roles of frame formation in living bodies. [1,2] Chitosan, which is deacetylated chitin, dissolves in some common organic and inorganic acid solutions, and can be readily processed into films, fibers, beads, gels, and sponges for various applications. [3] Chitosan is biodegradable, antibacterial, and non-toxic and thus it has been considered to be a candidate material for medicinal application, e.g., for tissue engineering. [4] The living body affinity, antibacterial properties, and wound recovering effect that chitosan possesses have drawn a lot of interest in recent years. [5-8] The scaffold can be developed using either natural or synthetic polymers but as compared with synthetic polymer, natural macromolecules facilitate cell affinity. Therefore, the material of the natural polymer is often used for organization regeneration. Hence, this kind of scaffolds must have a good biocompatibility, high porosity, and appropriate mechanical properties. [9-11] Many researchers have reported regarding the preparations of chitosan scaffolds from acetic acid and the relationship between a pore size within the scaffold and the effect in tissue engineering. [12] In addition, it has been found to be possible to control the pore size by changing freeze-drying temperature. [13] In this work, we prepared cast films as well as scaffolds with a porous sponge structure from the chitosan dissolved in aqueous citric or acetic acid solution. The cast films and scaffolds were characterized and then the latter ones were tried to apply to an artificial skin. The effect of such scaffolds on the wound healing was estimated and discussed by comparing the scaffolds of CS/citrate and CS/acetate.

EXPERIMENTAL

Materials

Chitosan (Mw 1,000,000 and degree of deacetylation of 96%) was kindly supplied by Katakura Chikkarin Co. Ltd. Citric acid and acetic acid (reagent grade) were used as purchased.

Preparation of cast films

Chitosan was dissolved in a 2% aqueous citric acid solution and/or a 0.5% acetic acid solution at 60 °C to prepare 1%(w/v) chitosan solution. The solution was poured into a petri dish and dried in an oven at 70 °C for ca. 12 h to obtain a cast film.

Preparation of scaffolds

Chitosan scaffolds were prepared by a freeze-drying technique. Here, the two kinds of chitosan solutions were used; 1wt% chitosan in 2% citric acid solution and 1wt% chitosan in 0.5% or 2.0% acetic acid solution. They were frozen at -27 °C for about one day and lyophilized for three days to obtain the microporous films of the scaffolds. (this type of microporous film denoted as a scaffold, here)

X-ray measurements

Wide-angle X-ray diffractions (WAXD) intensity curves were obtained with a Rigaku Denki model Rint 2100 X-ray diffractometer equipped with a scintillation counter. The X-ray source

was nickel-filtered CuK α radiation (40kV,20mA). The scan region was $2\theta=2\sim40^\circ$. The scan speed was 1°/min. WAXD photographs were recorded by TOSHIBA model XC-40H using nickel-filtered CuK α radiation (40kV,20mA). The sample films were piled up to the thickness of about 0.5mm. The exposure time was six hours.

FT-IR Measurements

FT-IR spectra were recorded with a Magna 560 FT-IR spectrometer (Nicore USA).

Citric acid, chitosan powder and two films were measured by a KBr method.

DSC measurements

Differential scanning calorimetry (DSC) experiments were conducted on a RIGAKU DSC 8230L in nitrogen atmosphere at a heating and cooling rate of 10 °C/min in the temperature region of -100 °C to 50 °C.

Tensile measurements

Tensile experiments were performed by Shimazu Autograph AGS-J. The sample was 40mm long, 5mm wide, and the thickness was ca.1.5mm and it was stretched at 10cm/min under the relative humidity of 65% at 25 °C.

Scanning electron microscope (SEM) observation

The porous structures of scaffolds prepared by freeze-drying were observed with scanning electron microscope (SEM, HITACHI S-2600HS). The surface and the cross-section fractured in liquid nitrogen of scaffolds were observed.

Water adsorption of scaffolds

Prior to a measurement, the scaffold was neutralized with a 5% NaOH solution for 1 hour, rinsed completely with water and dried under reduced pressure. A given amount of the scaffold was immersed in the distilled water for 3 h. The scaffold absorbing water was weighed after carefully removing water on the surface of the scaffold. The degree of water adsorption was calculated from the following equation:

$$DW = \frac{W_1 - W_0}{W_0} \times 100 \quad (\%) \quad (1)$$

where W_1 represents the weight of the scaffold absorbing water and W_0 is the weight of the dry scaffold before absorbing.

Wound healing test

A nude mouse that has no hair on the skin was used. The epidermis of the skin was scraped off by rubbing a part of the back, which was applied with depilatory, using a cotton swab. As a result, a wound with the size of ca. 1cm^2 was made. Further, three line cuts a little less than 1mm deep were made by a razor blade in the wounded part, which reached a dermis layer. This procedure was carefully carried out so that the subcutaneous tissue under the dermis should not be damaged. The scaffold prepared here was stuck on the wound part, in which the healing condition was observed for 10 days. After 10 days, the wounded part of the skin was cut off with scissors and dyed with Hematoxylin-Eosin (HE), and the recovery condition of the cell was estimated from an optical microscopic observation.

RESULTS AND DISCUSSION

WAXD profiles

Figure 1 shows WAXD profiles of a chitosan (CS) powder, and the cast films from the CS/citric acid and the CS/acetic acid solutions (denoted as CS/citrate and CS/acetate films, respectively). Once the CS powder is dissolved in the acetic acid solution and then cast, the crystal form of CS/acetic salt [14] different from that of CS itself appears and besides, a large reduction occurs in the crystallinity as seen in Figure 1.

The original sample of CS powder clearly shows crystalline diffractions due to a high crystallinity and the CS/acetate film also gives crystalline diffractions though they are not so strong. On the other hand, no crystalline diffraction peak can be seen in the profile of the CS/citrate film. Only the broad scattering peak is observed at 2θ of 19° , which corresponds to a so-called amorphous halo due to the molecules in the amorphous state. These facts imply that the molecules in the CS/citrate film should be in the amorphous state.

FT-IR spectra

It is well known that when chitosan is dissolved in an aqueous carboxylic acid such as formic, acetic, propionic, etc., chitosan is associated to acid molecules due to the electrostatic interaction, resulting in a salt formation. [15] Such a chitosan salt remains in the solid state after chitosan/acid solution is cast. These facts have been confirmed by FT-IR measurements. [15, 16] In addition, several kinds of chitosan salts have been analyzed by x-ray crystallography [17-19], when the chitosan salt possesses crystallinity. In this work, FT-IR

measurements were carried out to confirm that the interactions between chitosan and citric acid as well as acetic acid really occur through the $-\text{NH}_3^+$ and $-\text{COO}^-$ functions.

Figure 2 shows FT-IR spectra of citric acid, CS/citrate and CS/acetate cast films. In Fig. 2a citric acid gives a strong absorption peak at about 1740 cm^{-1} due to carboxyl groups because citric acid has three carboxyl groups within a molecule. Fig. 2b shows FT-IR spectra of CS/citrate film, in which the peaks appear at 1630 cm^{-1} and 1400 cm^{-1} assigned to $-\text{NH}_3^+$ and $-\text{COO}^-$, respectively. [16] In addition, a strong peak at 1730 cm^{-1} also appears, suggesting the existence of a lot of free $-\text{COOH}$ groups. These results reveal that an ionic complex or the CS/citrate salt is formed between chitosan and citric acid molecules, and many carboxyl groups of citric acid, which cannot contribute to the complex formation, are present. In Fig. 2c the CS/acetate film shows the absorption peaks at 1560 cm^{-1} and 1400 cm^{-1} and the shoulder at 1630 cm^{-1} , which are assigned to amide II band, $-\text{COO}^-$ and $-\text{NH}_3^+$, respectively. This indicates that the salt of chitosan and acetic acid or the CS/acetate salt is formed, as well known, mentioned above.

Stress-strain curves

Usually, chitosan films indicate a characteristic behavior of small draw-ability against stretching, when they are prepared from the common acidic solution of chitosan and allowed to stand in an ambience at room temperature. Namely, it is not easy to stretch the chitosan films. [20] Both films of CS/acetate and CS/citrate prepared here were hard just after casting and the former was somewhat more flexible than the latter. However, when they were kept in the controlled atmosphere at the relative humidity of 65% at $25\text{ }^\circ\text{C}$ for three days, the latter rather became much softer than the former. This change should be attributed to high moisture

adsorption by CS/citrate film, since this film contains lots of free carboxyl groups. Stress-strain curves are shown in Figure 3. The elongation of CS/acetate film was about 49%, but, in contrast, CS/citrate film could be extended more than 240%. In addition, judging from the shape of the stress-strain (S-S) curves and the absolute values of stress and strain, the CS/citrate film shows the rubbery behavior while the CS/acetate provides the typical S-S behavior of a plastic film with a clear yield point. It is, thus, recognized that the moisture controlled CS/citrate film should be in a rubbery state. This unique feature led us to try to apply the porous sponge of CS/citrate, i.e., the scaffold of CS/citrate to an artificial skin, which will be discussed below.

DSC measurements

DSC measurements were carried out on the samples of CS/citrate and CS/acetate films to determine the glass transition temperature (T_g). It was reported that chitosan had T_g of 203 °C in a dry state [21]. However, it is well known that the T_g is much influenced by moisture sorption, if the polymer is hydrophilic. In fact, Ogura et al. [22] reported that the moisture-controlled chitosan possessed the T_g of about 120 °C from the DMA measurement.

The two chitosan salt films were kept under the relative humidity of 65% at 25 °C for three days to control the moisture sorption of the samples prior to DSC measurements. The T_g of CS/citrate film was determined to be -22.5 °C based on the midpoint method for the inclination of baseline caused by the glass transition, as shown in Figure 4. But the slope of baseline for CS/acetate film did not change in the temperature region of -100 °C to 50 °C (Fig. 4a). This suggests that the T_g of CS/acetate should be higher than 50 °C, even if CS/acetate film suffered the moisture control. These facts reveal that the CS/citrate is much more

hygroscopic than the CS/acetate and the former absorbs much moisture to lead a drastic reduction in the Tg compared with that of pure chitosan in the dry state. As a result, the moisture-controlled CS/citrate film is in a rubbery state at room temperature of ca.25 °C. In addition, as is seen in WAXD measurements, CS/citrate is almost amorphous, though CS/acetate shows the low crystallinity. Thus, it is not surprising that the CS/citrate film possesses quite large draw-ability after moisture controlled.

Morphology of scaffolds

SEM photographs of CS/acetate and CS/citrate scaffolds are shown in Figures 5, 6. These scaffolds were prepared by the freeze dried method that gave a sponge consisting of microporous structures [23]. In this work, we prepared the two kinds of CS/acetate and one kind of CS/citrate scaffolds from the 1 wt% chitosan solutions, in which 0.5% and 2.0% aqueous acetic acid solutions were used (denoted as CS/acetate(0.5%) and CS/acetate(2.0%)) and 2.0% aqueous citric acid solution was employed. The concentration of 0.5% acetic and 2.0% citric acids were the lowest concentration required to dissolve chitosan in each system, respectively. The concentrations of 0.5% and 2.0% acetic acids correspond to the molar ratios of 1.34 and 5.36 to the repeating unit of chitosan in those solutions, while 2.0% citric acid gives that of 1.68, assuming that the acid molecule is not evaporated in the freeze dried process. The pore structure may be affected by this molar ratio as well as the acid nature [23], e.g., acidity, stereo-structure, etc., derived from the molecular structure of acid.

The surfaces of the scaffolds are shown in Figure 5. The CS/acetate(0.5%) had elliptic pore structures from 100 to 200 μm long shown in Fig. 5a, when it was prepared from 0.5% acid solution, corresponding to the molar ratio of 1.34. Prepared from the usage of 2.0%

acetic acid solution (or the molar ratio of 5.36), the pore size became a little smaller to be around 50 to 150 μm long (Fig.5b). It was noticed that the pore structure formed in the CS/acetate scaffold did not largely change even if the molar ratio changed four times, though the pore size, indeed, became smaller a little. While the CS/citrate possessed nearly circular pores with a still smaller diameter of about 50 μm than the CS/acetate(2.0%) scaffold (Fig.5c). In this case the molar ratio of citric acid to chitosan was 1.68, which is nearly close to 1.34 rather than 5.36 in the case of the CS/acetate scaffolds, and therefore this pore structure may depend on not only the molar ratio but also the acid nature, as expected.

Definite difference can be seen in the cross-sections of the scaffolds. The fractured cross-sections of the scaffolds are shown in Figure 6. A cell wall surrounding each pore with an average pore size of 60 to 90 μm is formed in the CS/acetate(0.5%) scaffold (Fig.6a). The CS/acetate(2.0%) had also the similar pore structure, though the pore size became smaller to be about half of that in the CS/acetate(0.5%). The formation of this type of pore structure has been reported. [12, 24] However, the CS/citrate provided the structure much different from the pore structure of the CS/acetate. The pore structure having a cell wall is no longer observed but a porous structure consisting of fibrous networks like a nest is formed, which has never been reported and may be caused mainly by the nature of citric acid.

Water adsorption of scaffolds

The ability to preserve water in a scaffold is one of the most important aspects for skin tissue engineering. [25-27] The water adsorption of the scaffold which consists of a microporous structure was measured. Before the discussion, it is necessary to see the behavior of water adsorption of the chitosan/acid salt film (or the cast film), which is prepared by casting the

chitosan solution to form the dense film without micropores. When the cast film is neutralized, this chitosan film would present almost the same water adsorption due to the swelling even if any acid was used as a solvent. However, the scaffold with microporous structures seems to show the difference in the water adsorption depending on the porous structure, which is attributed to the water retained within pores by capillarity accompanied by the swelling of the material of chitosan itself. Among them, the water adsorption must be governed by the microporous structure of the scaffold.

Before the measurement, the scaffold of chitosan acid salt was neutralized in a 5% NaOH solution and washed with distilled water, since it became dissolved in water without neutralization. At this moment, the shape of CS/acetate scaffold hardly changed, but the large contraction occurred in the CS/citrate scaffold. It may be due to a removal of bulky citric acid from the CS/citrate scaffold, in which a porous structure is consisted of fibrous networks, in the neutralization process, since the molecular weight of citric acid is much larger than that of acetic acid. From the SEM observation, it was found that the original pore size formed in the CS/citrate scaffold changed to become very small after neutralization. The measured water adsorption of these scaffolds neutralized is shown in Table 1. The CS/acetate scaffold showed the very large value more than 1000%. It might be readily supposed from the result mentioned above. On the other hand, CS/citrate scaffold gave a comparatively small value of about 280%. These results should depend on the pore structure. The CS/acetate scaffold can hold water in each pore (Fig. 6c) surrounded by cell walls and besides it has quite a similar pore structure to that of the hyaluronic acid scaffold, which possesses a similar water adsorption. [28] However, it may be difficult to keep much water in pores of CS/citrate scaffold, in which the pores are formed by the fibrous networks like a nest instead of cell walls in the case of

CS/acetate scaffold. (Fig. 6c) It was found that the degree of water adsorption (DW) was closely related to such pore structures in the chitosan scaffold.

Wound healing test using scaffolds

In order to estimate the effect as an artificial skin of scaffolds prepared here, the animal experiment was conducted using nude mice. The healing state was observed at an interval of every three days, and finally the cross-section of skin was observed ten days after the wound was made and immediately covered tightly with the scaffold. A photograph of each stage was taken to see the recovery condition.

CS/acetic scaffold

In the passage of three days after the wound skin was tightly covered with the scaffold, the new skin did not regenerate yet but bleeding was still seen. The scab which was a first stage of recovery was not formed yet.(Fig. 7a) The scab was found to be formed in the passage of six days and bleeding was no longer seen around there. It was found that the new and thin skin had been regenerated and the scar disappeared on the tenth day. These results showed that CS/acetate scaffold was effective in a wound healing and regeneration of the outer skin.

Neutralized CS/acetate scaffold (CS scaffold)

A pure CS scaffold was obtained by neutralizing CS/acetate scaffold. In the same way as the case of CS/acetate scaffold, bleeding was still seen on the third day and the scab was formed in six days.(Fig. 7b) In addition, pus mixed with blood was observed in the passage of three

days. The dressing by this scaffold could not regenerate the new skin completely but provided the formation of scab on the tenth day. This may be related to the lack of acetic acid caused by the procedure of neutralization. From these results, it was found that the pure CS scaffold was not necessarily adequate for the healing of wound.

CS/citrate scaffold

CS/citrate scaffold provided much different results from others mentioned above. Bleeding is no longer seen and the scab is already formed in the passage of three days, and the new skin was made at the six days. (Fig. 7c) These facts remind us that citric acid is much hydroscopic and once the CS/citrate film absorbs moisture, it becomes softened to be drawn easily, as mentioned in the above sections. Consequently, CS/citrate scaffold could be stuck close to the wounded part and absorb blood immediately, leading to the stop of bleeding and the rapid regeneration of the skin. In ten days, the new skin was regenerated and the wound healed completely. A remarkable facilitation of wound healing occurred compared with other two scaffolds. As is discussed above, the film of CS/citrate is amorphous but those of CS/acetate and the pure chitosan are crystalline, which might also influence on it. As a result, CS/citrate scaffold was found to possess a good performance in the wound healing.

Skin cross-section observations

The state of the skin cross-sections on the tenth day after the formation of wound is shown in Figure 8. The epidermis layer and the dermis layer of the skin are found to be dyed violet, though the former is thicker than the latter, which enables us to distinguish them. In addition, both layers have the enough thickness similar to the original ones before making the wound,

whether CS/citrate scaffold or CS/acetate one is used. Needless to say, just after the skin was scraped off by rubbing, the epidermis layer could not be seen in the skin section. These facts show that regeneration of the skin occurs in ten days in both cases using CS/citrate or CS/acetate scaffolds, though there is a difference in a recovery rate of the skin between them, mentioned above.

Finally, it is suggested that an infectious disease has not occurred around the wounded part of the skin for ten days when these scaffolds are used like a bandage, judging from the observations of the surface and the section of the skin. These results lead us to conclude that CS/citrate and CS/acetate scaffolds are suitable for an artificial skin quite effective to regeneration of the outer skin.

CONCLUSION

CS/citrate provided an amorphous state, while CS/acetate showed a low crystalline state. When these films were controlled under the moisture condition, CS/citrate indicated a low glass transition temperature of -22.5 °C, resulting in rubbery and large draw-ability at room temperature, but CS/acetate did not show such properties. The scaffold, prepared by a lyophilization method, gave quite a distinct pore structure especially inside it, depending on the kind of the acid used for the preparation of chitosan solution.; the pore consisted of fibrous networks appeared in CS/citrate, while the pore surrounded by cell walls occurred in CS/acetate. Despite the large difference in the pore structure, both scaffolds were effective in regeneration of the outer skin. However, it is noticed that the CS/citrate scaffold provides better facilitation in wound healing than the CS/acetate one.

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Table 1 Degree of water adsorption, DW

Sample Scaffold	dry weight W_0 (g)	Absorbed weight W_1 (g)	DW (%)
CS/acetate*	0.058	0.859	1,381
CS/citrate*	0.034	0.128	276

*) after neutralization

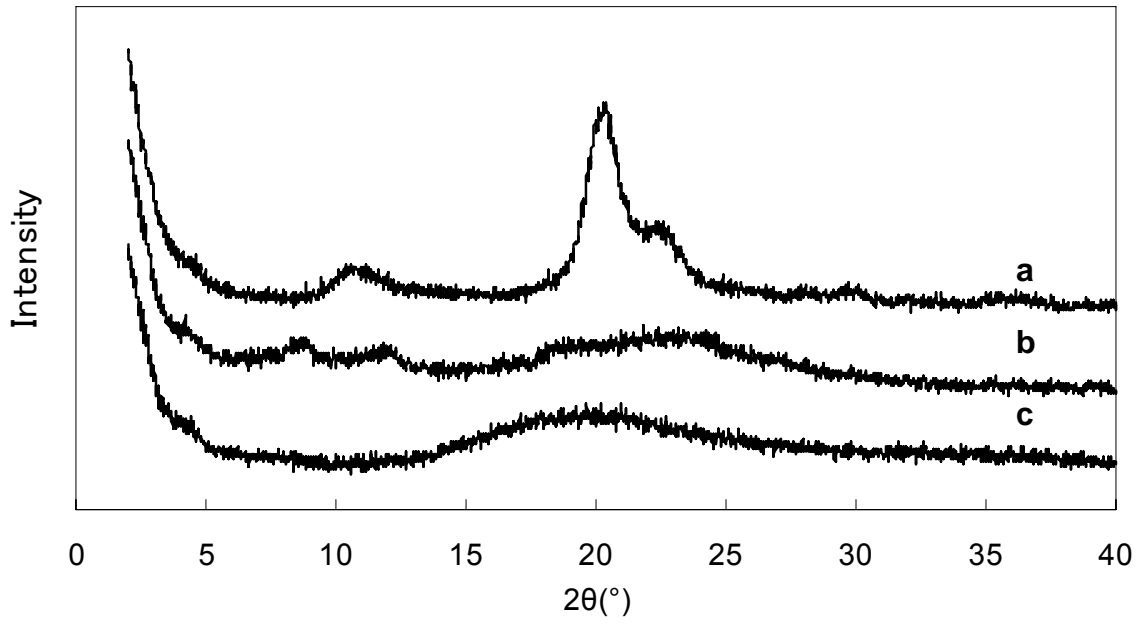


Figure 1 WAXD profiles of (a) CS powder, (b) CS/acetate film and (c) CS/citrate film.

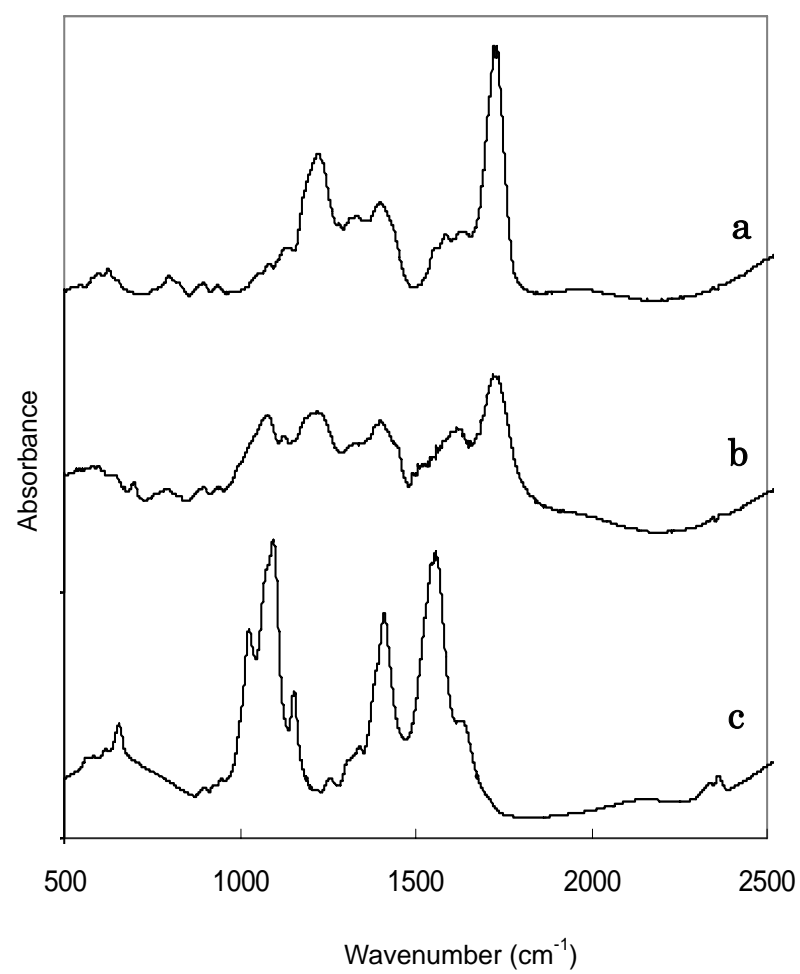
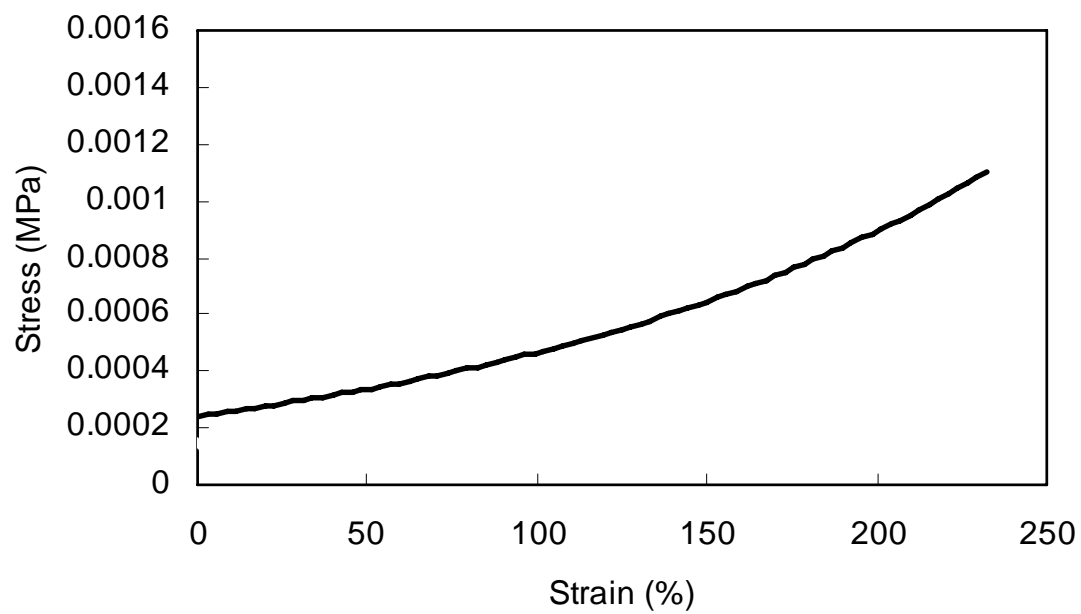
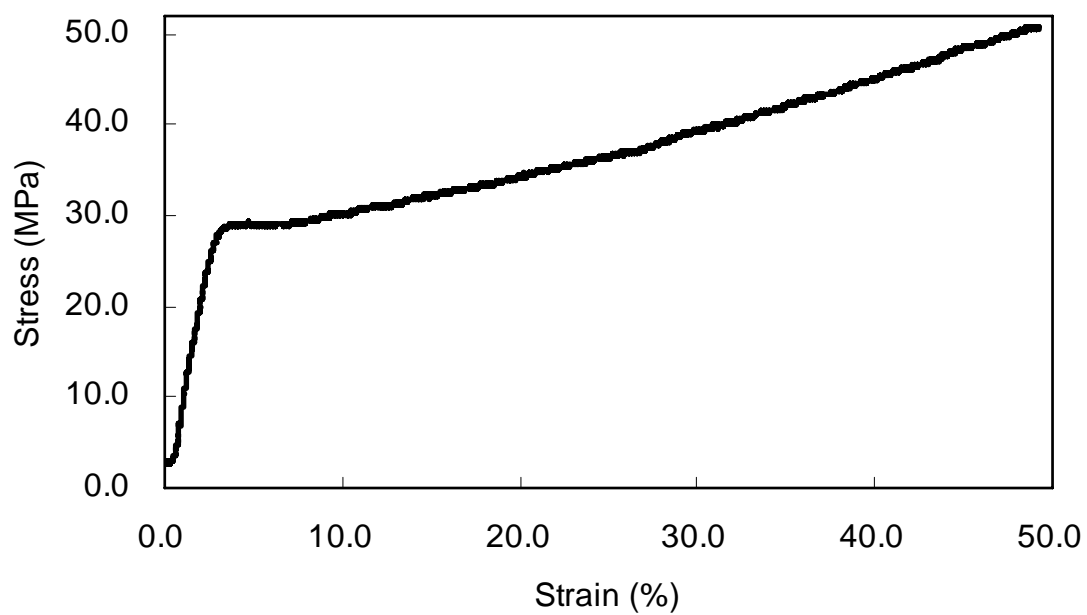


Figure 2 FT-IR spectra of (a) citric powder, (b) CS/ citrate film and (c) CS/acetate film.



(a) CS/citrate film



(b) CS/acetate film

Figure 3 Stress-strain curves of (a) CS/citrate film and (b) CS/acetate film.

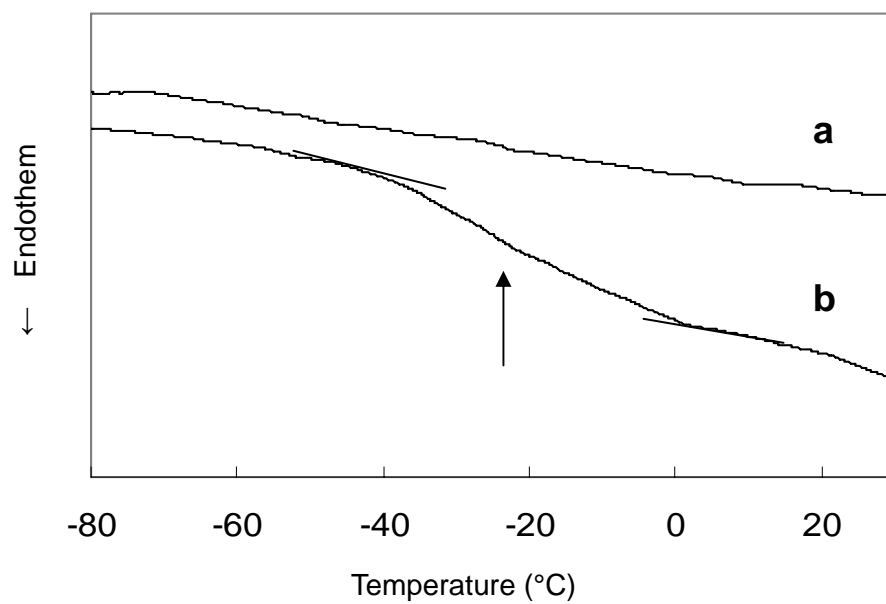


Figure 4 DSC thermograms of films in the second heating run:
(a) CS/acetate, (b) CS/citrate.

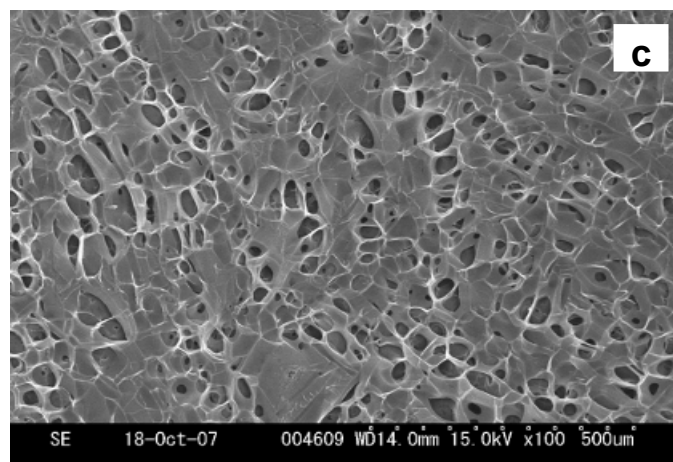
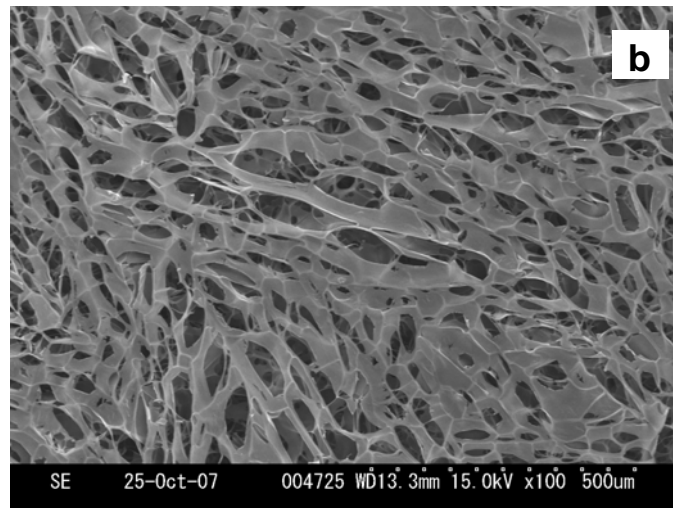
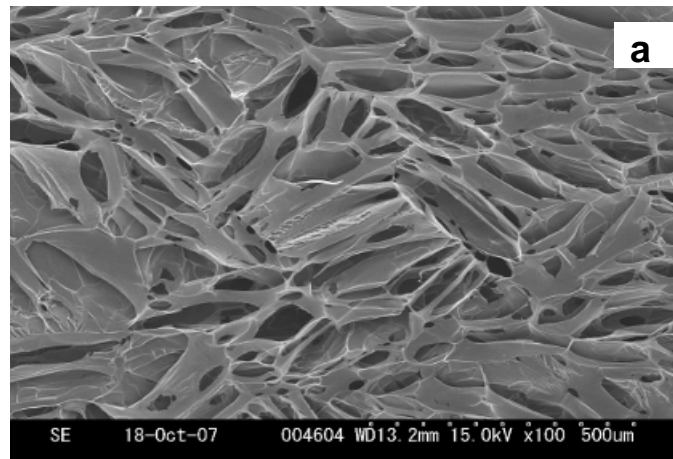


Figure 5 SEM observation of the surfaces of scaffolds:

(a) CS/acetate (0.5%), (b) CS/acetate (2.0%), (c) CS/citrate (2.0%).

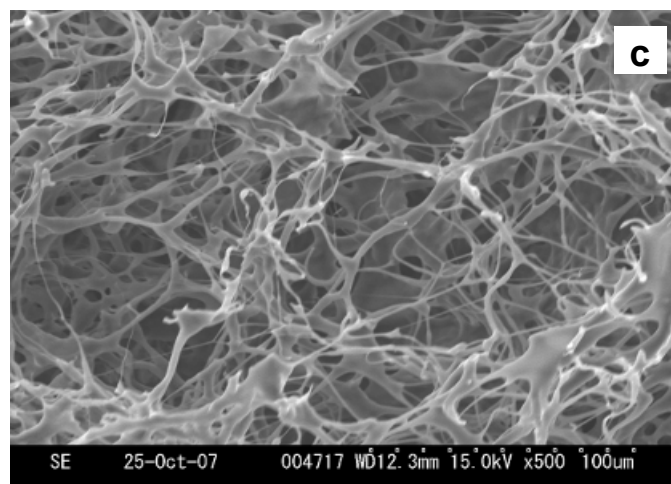
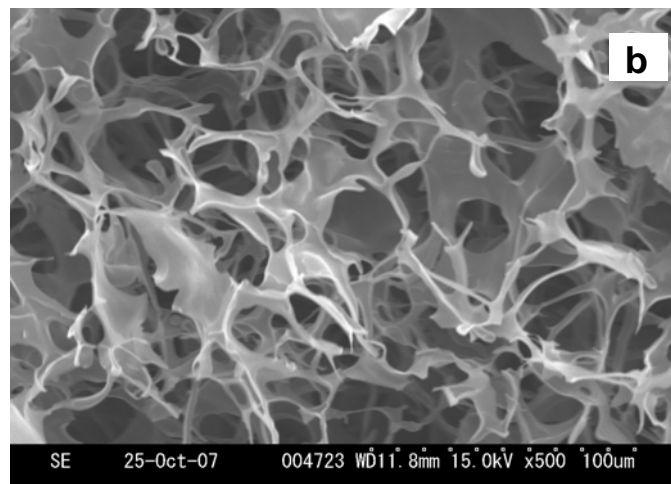
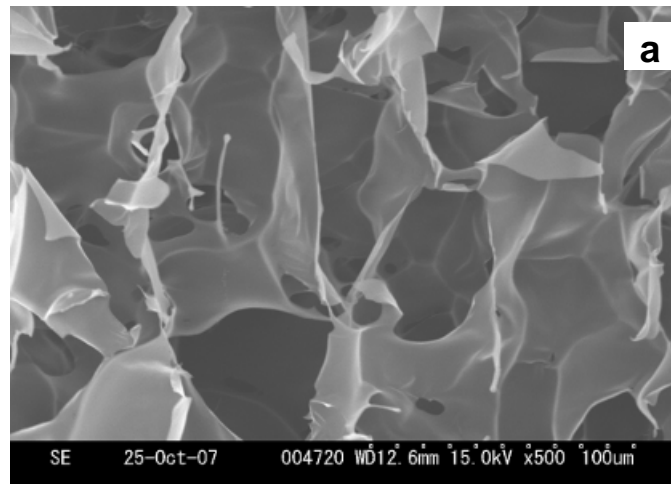


Figure 6 SEM observation of the cross-sections of scaffolds:
 (a) CS/acetate (0.5%), (b) CS/acetate (2.0%), (c) CS/citrate (2.0%).

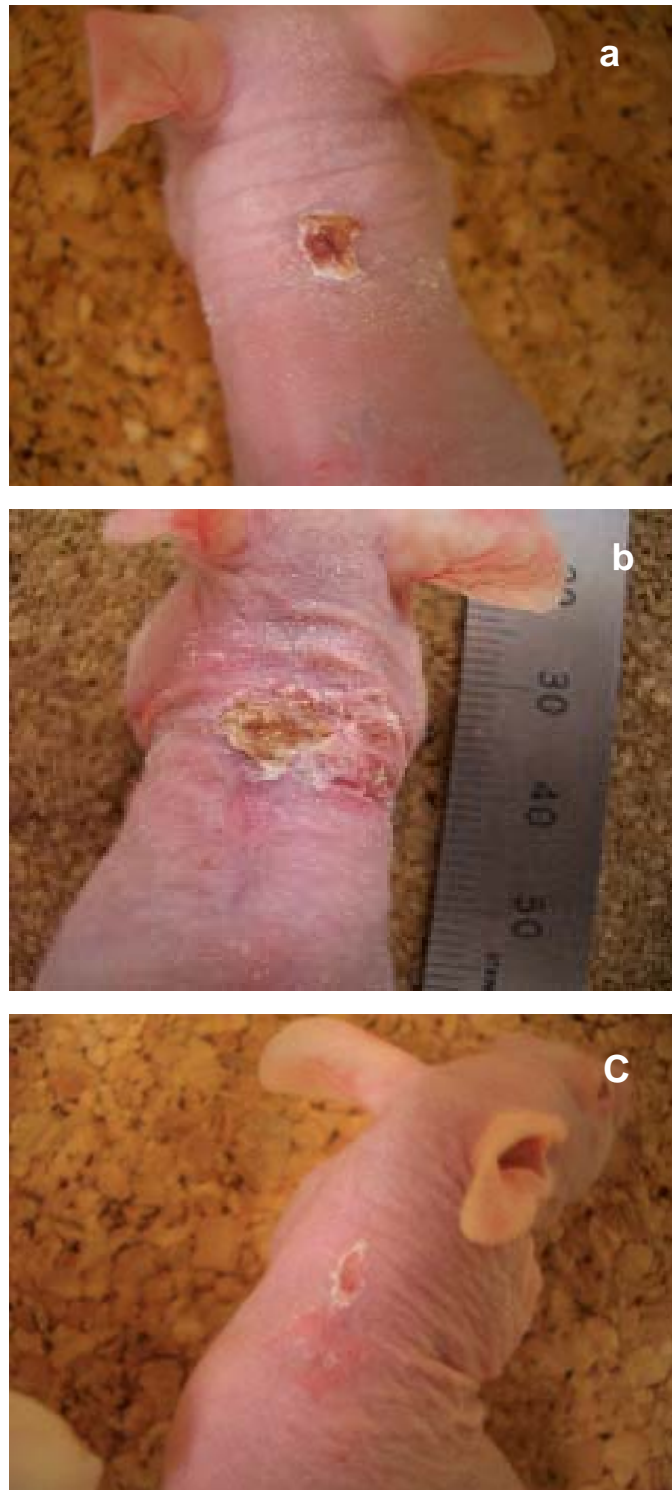


Figure 7 wound healing conditions after 6 days:
(a) CS/acetate scaffold, (b) CS scaffold (neutralized),
(c) CS/citrate scaffold.

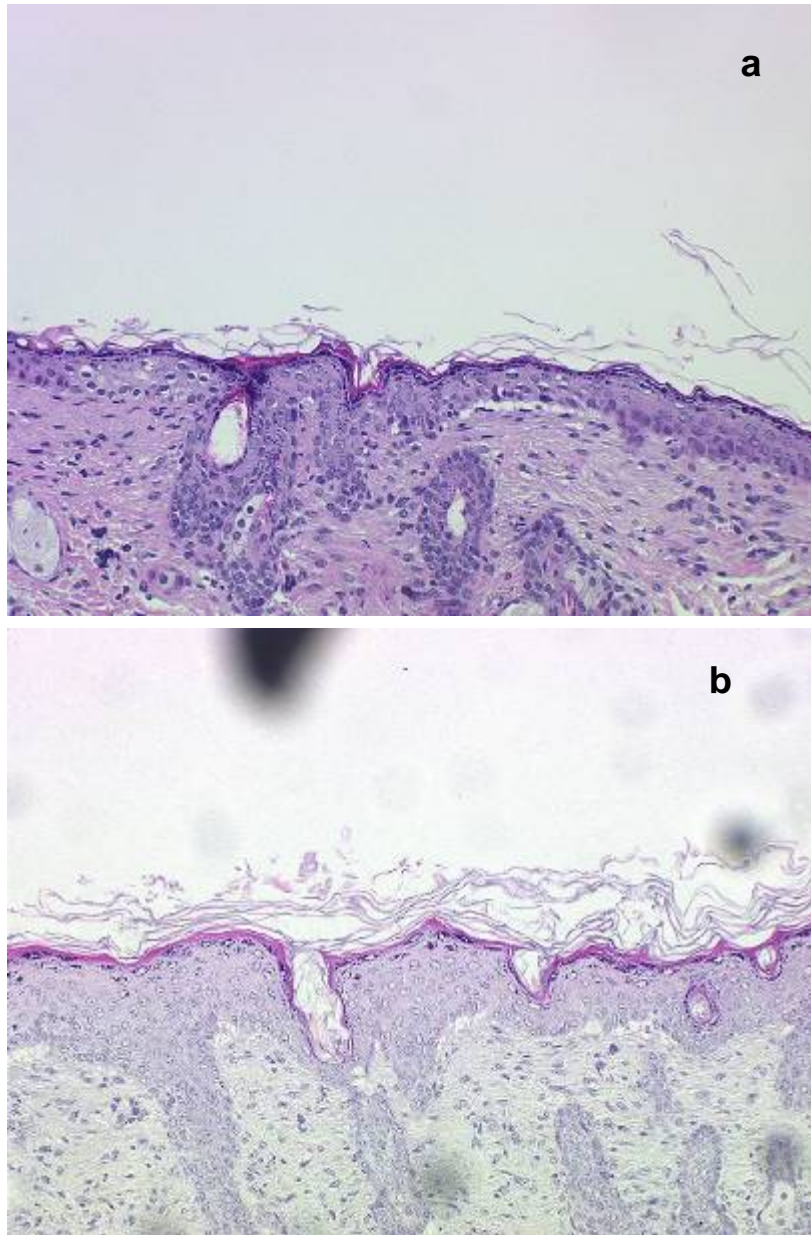


Figure 8 Micrographs of skin sections sample after 10 days ($\times 100$):
(a) CS/citrate scaffold, (b) CS/acetate scaffold