

CHAPTER III

CHITOSAN GEL VIA CHITOSAN-EPOXY AND ITS HYDROXYAPATITE MINERALIZATION

Abstract

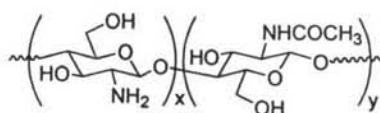
A novel hybrid material of chitosan-hydroxyapatite (HAp) composite gel is proposed. The introduction of epoxy ring onto chitosan via phthaloylchitosan to obtain a precursor from homogeneous DMF solution is demonstrated. The reaction of the precursor with hydrazine in water gives the recovery of amino groups as well as the ring opening reaction of oxirane for gelation. An alternate soaking with calcium and phosphate solution develops HAp mineralization and initiates the gel strength. Cell culture studies reveal 50% cell viability, whereas the mineralization with HAp reduces the cell adhesion.

Keywords: Chitosan; Epoxy; Hydroxyapatite

1. Introduction

Chitin-chitosan is the second-most abundant naturally-occurring polysaccharide. Chitin-chitosan consists of β (1-4)-2-acetamido-2-deoxy- β -D-glucose and β (1-4)-2-amino-2-deoxy- β -D-glucose units and exists in crustacean shells (Scheme 1). For more than three decades, fundamental studies on chitin-chitosan copolymer led to an understanding about its unique amino polysaccharide properties, such as complexation with metal ions [1], antibacterial and antimicrobial activities [2], tissue and cell compatibilities [3], and biodegradability by the enzymes chitinase [4], chitosanase [5], and lysozyme [6], etc. In order to apply chitin-chitosan, with those specific properties, to practical products, various materializations have been developed for decades. Many fabrications, such as fibers [7], films [8], and beads [9], are recognized as successful examples. Chemical modifications with some functional groups to make it water soluble [10], organic solvent soluble [11], and/or colloidal nanosphere derivatives [12], are known as alternative materializations. In recent years, advanced applications in the biomedical field (such as gels for wound dressing [13], prodrugs for drug delivery system [14], fibers for antimicrobial textiles [15], etc.) have received much attention.

Scheme 1



Chitosan is a derivative of chitin obtained from chitin deacetylation. It is important to note that chitosan is known for stimulation of bone formation (osteoconductivity) [16], moreover, it is reported for hybridization with inorganic compounds [17]. Since one of the inorganic components in bone is hydroxyapatite (HAp), the formation of chitosan-HAp is considered to be a way to use chitosan as composite material for bone tissue engineering including bone paste and glue. In addition, it was found that lysozyme degradation in the human body changes chitosan to *N*-acetylglucosamine and glucosamine oligomers [18]. As the structures of these oligomers are resemble to extra cellular matrix of bone and cartilage, it was

found that these oligomers provide the major components for connective bone tissues and mucous membranes [16]. In the past, several mineralizations of chitin-chitosan were reported [19-25]. For example, Tokura *et al.* reported composites of *O*-carboxymethyl-chitin with HAp accelerate the bone tissue formation [24], whereas Tachaboonyakiat *et al.* demonstrated that non-crystalline HAp is formed in chitosan gel by soaking gel alternatively in calcium and phosphate solutions [25]. Based on these viewpoints, it is a challenging theme to focus on chitosan gel with HAp for bone tissue engineering.

In order to obtain chitosan gel, one has to admit that the traditional gel formation by dissolving chitosan in acetic acid and adding glutaraldehyde [26] consume amino groups of chitosan to result in the loss of aminosaccharide properties. The present article, therefore, focuses on a unique chitosan gel where the amino groups remain in the structure. The introduction of oxirane ring by conjugating with epichlorohydrin is designed as a precursor to crosslink with amide derivatives. Herein, we report (i) a chitosan-epoxy crosslink network with amino group remaining in the chitosan unit, (ii) the homogeneous reaction of chitosan in water to form gel, (iii) the mineralization of HAp in chitosan-epoxy gel, and (iv) the cell affinity of chitosan-epoxy gel and chitosan-epoxy gel/HAp hybrid composite.

2. Experimental

2.1. Materials

Chitosan (%DD = 89, $M_v = 10^6$ g/mol) was locally supplied from Seafresh Chitosan (Lab) Company Limited, Thailand. Calcium chloride, disodium hydrogen phosphate, methanol and tris(hydroxymethyl)aminomethane (Tris) were purchased from Carlo Erba Regenti, Italy. Sodium acetate, *N,N*-dimethylformamide (DMF), isopropanol and potassium hydroxide were obtained from Univar, Australia. Phthalic anhydride was obtained from Fluka Chemika, Switzerland. Epichlorohydrin was purchased from Acros Organics, Belgium. Hydrazine monohydrate was purchased from Nacalai Tesque., Inc., Japan. All chemicals were used without further purification.

2.2. Instruments and equipment

Qualitative and quantitative Fourier transform infrared spectra (FTIR) analyses were obtained from a Bruker Equinox 55/S with 32 scans at a resolution of 4 cm^{-1} . A frequency range of $4000\text{--}400\text{ cm}^{-1}$ was observed by using a deuterated triglycinesulfate detector (DTGS) with a specific detectivity, D^* , of $1 \times 10^9\text{ cm}\cdot\text{Hz}^{1/2}\text{ w}^{-1}$. An Opus/IR (version 3.0) was used for the FTIR curve fitting. Proton nuclear magnetic resonance ($^1\text{H NMR}$) spectra were recorded using a Varian Mercury-400BB spectrometer. Thermal analysis was carried out by using a TGA 2950 Dupont using N_2 with a flow rate of 20 mL/min and a heating rate of $20\text{ }^\circ\text{C/min}$ from $30\text{ }^\circ\text{C}$ to $600\text{ }^\circ\text{C}$. A JSM-35CF scanning electron microscope was applied at an accelerate voltage of 25 kV . Fluid rheology studies were performed by using a Rheometric ARES with parallel plate geometry 25 mm in diameter equipped with a water bath temperature controller at $30.0 \pm 1.0\text{ }^\circ\text{C}$. Cell adhesion was observed by a Nikon electron microscope, at $20\times$ by phase contrast. Intrinsic viscosity $[\eta]$ was measured with a Cannon-Ubbelohde (No.2, A149) in 0.1 M sodium acetate/ 0.2 M acetic acid aqueous solution at $30 \pm 0.05\text{ }^\circ\text{C}$. Molecular weight was calculated by using the Mark-Houwink equation with $K = 1.64 \times 10^{-30} \times \text{DD}^{14}$ and $a = (-1.02 \times 10^{-2} \times \text{DD}) + 1.82$ [27].

2.3. Synthesis of *N*-phthaloylchitosan, 2

Compound **2** was prepared from chitosan by previously reported method [11,28].

Chitosan (1.00 g) was reacted with phthalic anhydride (4.47 g , 3 moles equivalent to pyranose ring) in DMF (20 mL) at $100\text{ }^\circ\text{C}$ under nitrogen for 6 h. The temperature was reduced to $60\text{ }^\circ\text{C}$, and the mixture was left overnight. The solution was concentrated to obtain a yellowish viscous product. The crude product was reprecipitated in ice water. The precipitate was collected, washed with methanol several times, and dried *in vacuo*.

Anal. Calcd. for **2**, $(\text{C}_{14}\text{H}_{13}\text{O}_6\text{N})_{0.69} (\text{C}_{22}\text{H}_{17}\text{O}_9\text{N})_{0.20} (\text{C}_8\text{H}_{13}\text{O}_5\text{N})_{0.11}$ (%): C, 56.90; H, 4.62; and N, 4.98. Found (%): C, 55.74; H, 4.58; and N, 3.99. FTIR (KBr, cm^{-1}): 3472 (OH), 1776 and 1714 (C=O anhydride), and 721 (aromatic ring). ^{13}C

CP/MAS NMR (δ , ppm): 23.3 (CH₃), 57.0 (C-2), 64.7 (C-6), 73.2 (C-3, C-5), 80.5 (C-4), 100.4 (C-1), 131.1 (aromatic ring), and 169.1 (C=O). ¹H NMR (DMSO-d₆, ppm): 1.7 (CH₃ in acetamide), 3.4-5.0 (pyranose ring), and 7.6-7.7 (aromatic ring).

2.4. Epoxy-*N*-phthaloylchitosan, **3**

Compound **2** (1.00 g) was dissolved in DMF (20 mL) and heated to 60 °C. After 30 min, a catalytic amount of potassium hydroxide-isopropanol solution and epichlorohydrin (2.78 mL, 10 moles equivalent to pyranose ring) were added. The reaction was carried out *in vacuo* at 60 °C for 12 h. The solution was concentrated and reprecipitated in cold water. The precipitate was collected and washed with methanol several times, followed by drying *in vacuo*.

Anal. Calcd. for **3**, (C₁₇H₁₇O₇N)_{0.64} (C₂₂H₁₇O₉N)_{0.2} (C₁₁H₁₇O₆N)_{0.11} (C₁₄H₁₃O₆N)_{0.05} (%): C, 58.49; H, 4.78; and N, 3.94. Found (%): C, 56.58; H, 4.66; and N, 4.04. FTIR (KBr, cm⁻¹): 3472 (OH), 1766 and 1714 (C=O anhydride), 907 (oxirane ring), 721 and (aromatic ring). ¹H NMR (DMSO-d₆, ppm): 1.7 (CH₃ in acetamide), 2.5-2.9 (oxirane ring), 3.4-5.7 (pyranose ring), and 7.6-7.7 (aromatic ring).

2.5. Chitosan-epoxy gel, **4**

Compound **3** (1.00 g) was dissolved in hydrazine aqueous solution (20 mL) under the variables of reactant molar ratio, reaction time, and temperature. After the reaction, the crude gel was washed thoroughly with water and methanol several times to obtain **4**.

FTIR (KBr, cm⁻¹): 3412 (OH), 1654 (amide I), 1549 (amide II), and 895 (pyranose ring).

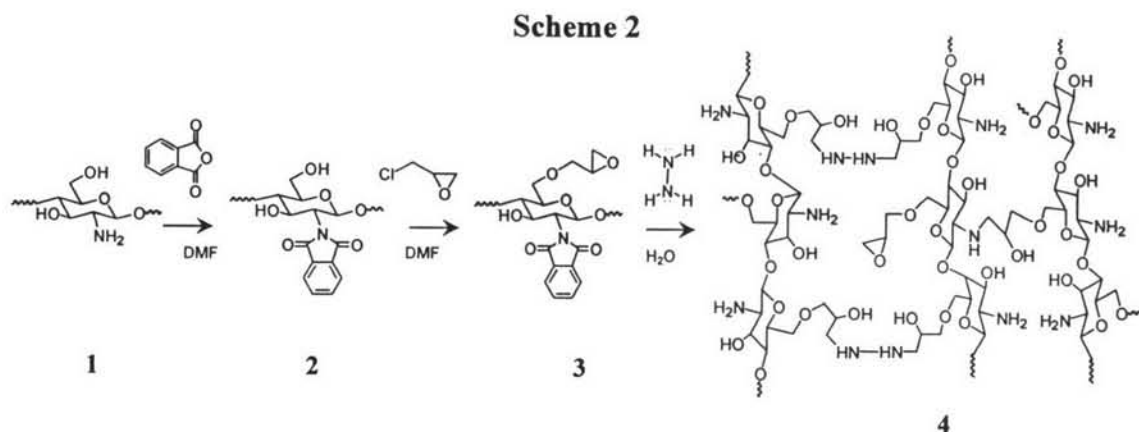
2.6. Chitosan-epoxy gel/HAp hybrid composite [25]

Gel **4** (1.00 g) was soaked in CaCl₂ (200mM)/ Tris-HCl (pH 7.4) aqueous solution (20 mL) at 37 °C for 2 hours, followed by rinsing thoroughly several times with deionized water. The product was soaked in Na₂HPO₄ (120 mM) aqueous solution (20 mL) at 37 °C for 2 hours and washed thoroughly with water. An alternate soaking was carried out for several times.

FTIR (KBr, cm^{-1}): 3412 (OH), 1654 (amide I), 1549 (amide II), 895 (pyranose ring) and 561 (PO_4^{3-}).

2.7. Cell culture

Mouse fibroblast cells (L929) were cultured on a tissue culture dish in an Eagle minimum essential medium and incubated at 37 °C to produce a reference standard of viable cells. L929 cells were seeded on **4** and chitosan-epoxy gel/HAp hybrid composite at a density of 1×10^5 cells/well. The samples were incubated at 37 °C in 5% CO_2 for 24 hours. After washing with phosphate buffered saline solution (PBS), the number of cells on the hydrogels was counted by using tetrazolium compound, WST-1, a sodium salt of 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate [29]. Adhered cells were observed by using an electron microscope.



3. Results and Discussion

As chitosan is hardly dissolved in common organic solvents, the crosslink by simply mixing chitosan with di-epoxide or epichlorohydrin is not practical. Although chitosan shows good solubility in aqueous acetic acid, the introduction of oxirane ring by reacting with epichlorohydrin in acid solution is not effective. Here, the synthetic route via phthaloylation [11] was applied as the starting compound since the derivative is an amino-protected one and gives the homogeneous reaction in DMF and/or DMSO (Scheme 2).

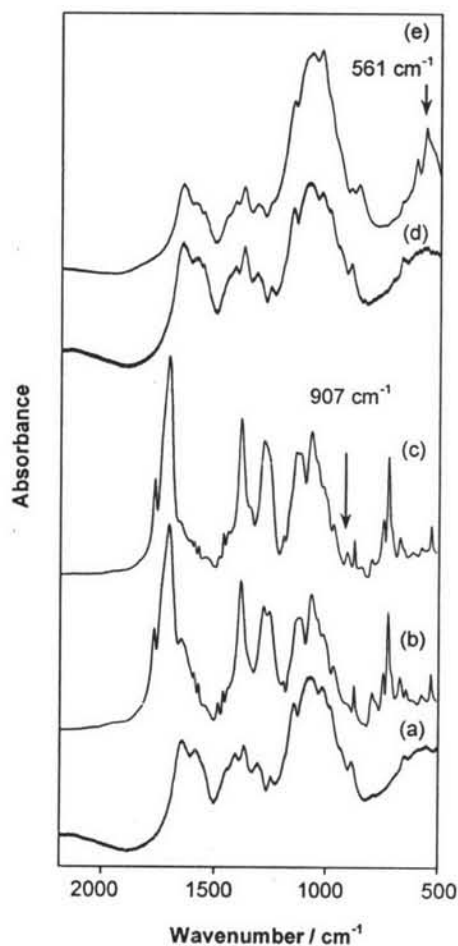


Figure 1. FTIR spectra of (a) **1**, (b) **2**, (c) **3**, (d) **4**, and (e) **4** after alternate soaking in $\text{CaCl}_2/\text{Na}_2\text{HPO}_4$ for 5 times.

For **2**, the structure was confirmed by the characteristic peaks of phthalimido group ($1776, 1714 \text{ cm}^{-1}$ ($\text{C}=\text{O}$ anhydride)), and aromatic ring (721 cm^{-1}) (Figure 1 (b)). In order to evaluate the phthalimido substitution, elemental analysis was applied. Kurita *et al.* demonstrated that C/N ratio is good for evaluation the substitution on chitosan [30]. As there might be incomplete chitosan combustion due to the possible contamination of inorganic salt, here we applied the C/N ratio to estimate the phthalimido substitution. In the case of **2**, the substitution is possible at both amino and hydroxyl groups [30]. Based on the %DD, the content of amino group is equal to 0.89. If all the amino groups are substituted by phthalimido groups, the calculated C/N ratio should be 11.42. However, the found C/N ratio was 13.97 to

imply that not only the amino group but also some of hydroxyl group at C-6 and/or C-3 are substituted. The result is relevant to that of Kurita *et al* [30].

3.1. Synthesis of epoxy-*N*-phthaloylchitosan, 3

Although sodium hydroxide is a general base used in the preparation of epoxy resins, in this work, potassium hydroxide-isopropanol solution is applied as a catalyst in the reaction of epichlorohydrin with *N*-phthaloylchitosan in DMF. The product obtained was confirmed by FTIR from the oxirane ring peak (907 cm^{-1}) (Figure 1 (c)). It is important to note that the reaction mechanism with epichlorohydrin in the existence of KOH results in the chloride salt [31]. Here, the crude product was washed several times in water and methanol to confirm that there was no trace amount of unreacted epichlorohydrin remaining in the final product. The FTIR was carefully analyzed to confirm that the final product did not give the C-Cl peak ($700\text{-}800\text{ cm}^{-1}$) or any other peaks referred to epichlorohydrin (Figure 1 (c)). The ^1H NMR shows the methylene proton of oxirane ring at 2.5-2.9 ppm. This implies the oxirane ring introduction. The characterization by ^1H NMR was also used to determine the oxirane ring substitution. However, this peak was overlapped with the broad peak of pyranose ring. Considering the structure of chitosan, the reaction might mainly occur at the primary alcohol (C-6) followed by the secondary hydroxyl group at C-3. For simple evaluation of oxirane ring substitution, the substitution of epoxy group was assumed to occur at only C-6 to give the chemical formula $(\text{C}_{17}\text{H}_{17}\text{O}_7\text{N})_{0.69}(\text{C}_{22}\text{H}_{17}\text{O}_9\text{N})_{0.2}(\text{C}_{11}\text{H}_{17}\text{O}_6\text{N})_{0.11}$. Based on the phthalimido substitution degree of compound 2, the content of hydroxyl groups remain for 0.8. If all the hydroxyl groups are substituted by oxirane rings, the calculated C/N ratio should be 14.85. Here, the C/N ratio was found to be 14.00 or in other words the substitution of oxirane rings was approximately $14/14.85 \times 0.8$ or 0.75.

3.2. Ring opening reaction of epoxy-*N*-phthaloylchitosan, 4

Nishimura *et al.* [11] reported the deprotection of the amino group by using an excess amount of hydrazine (100 moles equivalent to pyranose ring) at a reaction temperature $100\text{ }^\circ\text{C}$ for 15 hours. In our case, the reaction with hydrazine was expected to be effective due to the high amorphous derivative resulting from the

phthalimido groups and oxirane rings. Here, an attempt to clarify the minimal hydrazine amount required for our system was done by varying the hydrazine from 10 to 50 moles equivalent to pyranose ring. It is important to note that the addition of hydrazine brings **3** to dissolve in water and after a few hours it ends up as a gel. It was found that the soft gel was obtained when the hydrazine was about 10 moles equivalent to pyranose ring of **3** at the reaction temperature of 60 °C. With the similar condition, but using 20 moles of hydrazine, the gel became harder as compared to that of 10 moles hydrazine. The gel was washed several times with water and methanol to remove unreacted hydrazine and the deprotected phthalimido group to obtain **4**. Figure 1 (d) shows the disappearance of 1776 and 1714 cm^{-1} , implying successful deprotection. It should be noted that the oxirane ring peak at 907 cm^{-1} also disappeared. This confirms that (i) hydrazine functions not only for the deprotection of amino group for chitosan, but also for crosslinking after the ring opening reaction of epoxy group, and (ii) the hydrazine amount of 20 moles equivalent to pyranose ring of **3** is an optimal amount for gelation. In order to ensure the removal of hydrazine, the product obtained was carefully analyzed from the disappearance of the characteristic peaks of hydrazine, i.e. the strong peaks at 3350 and 1620 cm^{-1} . Based on the degree of oxirane ring substitution for 0.75 (see Synthesis of epoxy-*N*-phthaloylchitosan, **3**), the high amount of hydrazine (20 moles equivalent) required for gelation might come from the fact that there is a large amount of oxirane rings present in the chitosan unit. When the amount of hydrazine was as high as 50 moles (equivalent to pyranose ring) a hard and brittle gel was obtained.

Gel formation at room temperature was also studied. It should be noted that the crude product of **3** (before drying *in vacuo*) can be dissolved easily in hydrazine, and after that the gel will be formed. Here, the gelation and the change in chemical structure were traced by the FTIR at periodic intervals using the curve fitting technique [32]. The ring opening reaction was quantitatively analyzed from the decrease of the oxirane ring peak (907 cm^{-1}) during the reaction times of 4, 12, 20, and 28 minutes (Figure 2 (A) and (B)). By using the C-O-C peak (1026 cm^{-1}) in the pyranose ring as an internal standard, a kinetic scheme of the reaction as a function of time could be determined (Figure 2 (C)). It was found that the oxirane ring almost

disappeared after 20 minutes. This implied that the crosslink at room temperature was accomplished at around this time. The successful crosslink at room temperature suggests that the chitosan derivative might be a candidate for paste or glue used in biosystem.

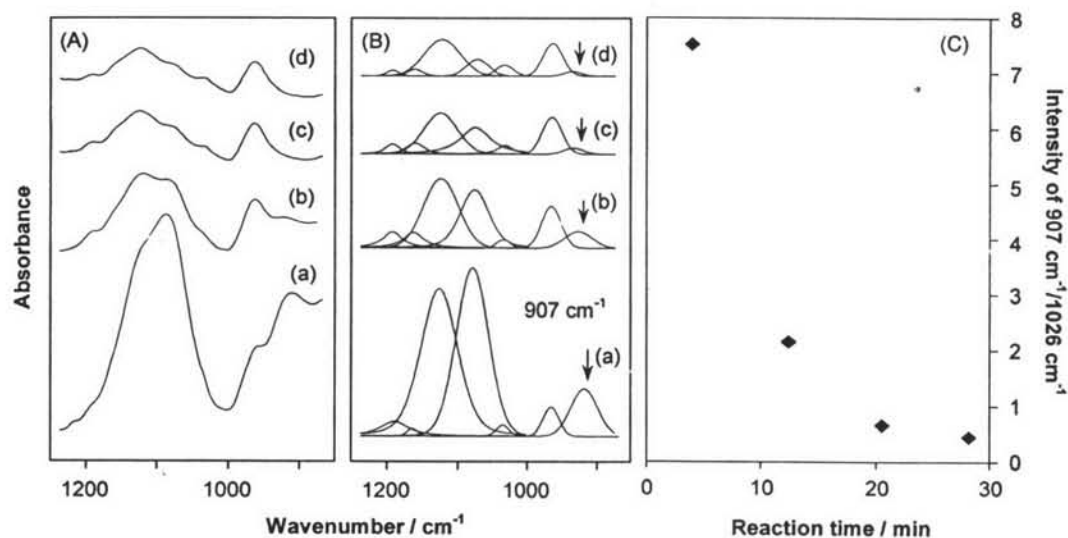


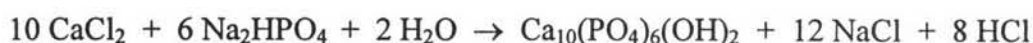
Figure 2. FTIR spectra of (A) before and (B) after curve fitting of **3** reacted with hydrazine 20 moles equivalent to pyranose ring at room temperature: for (a) 4 minutes, (b) 12 minutes, (c) 20 minutes, (d) 28 minutes; and (C) Gelation time of **4** as quantified by the oxirane peak at 907 cm⁻¹ and pyranose ring peak at 1026 cm⁻¹.

Although the network of chitosan gel is difficult to illustrate correctly, we suspected that the reaction of hydrazine might result in the recovery of amino group and the generation of crosslinks, i.e. between (i) the two oxirane rings, and (ii) oxirane ring and hydroxyl group or amino group of chitosan, as shown in Scheme 2. Here, the C-N band at 1075-1110 cm⁻¹ is useful information to confirm the crosslink network; however, the pyranose ring (C-O-C at 1040-1110 cm⁻¹) obtained overlaps this peak and obstructs our structural analysis (Figure 1 (d)).

3.3. Mineralization with hydroxyapatite in **4**

It is important to note that the attractive points to modify chitosan with epoxy group are not only for gel formation but also for amino group protection to

effectively mineralize with an inorganic compound. The amino group is expected for the interaction with inorganic compound in mineralization and cell adhesion ability. Previously, Tachaboonyakiat *et al.* [25] reported that hydroxyapatite (HAp) was formed through the ionic charge of the amino group on chitosan by alternate soaking with calcium chloride and sodium hydrogen phosphate. The HAp formation was expected as the following stoichiometric relationship [25]:



It was found that the first soaking of **4** in phosphate solution leads to significant gel shrinkage. This might be due to the adsorption of phosphate ions with the amino groups on chitosan. Here, **4** was soaked in a calcium chloride solution in the first step to replace water molecules adsorbed in the gel, followed by soaking in Na_2HPO_4 solution. FTIR and WAXD were used to trace the HAp formation. It was found that after soaking for five cycles, the phosphate group (561 cm^{-1}) was identified, implying the existence of HAp (Fig. 1e). Figure 3 (c) shows the peaks at $26^\circ 2\theta$ and $32^\circ 2\theta$, similar to that reported by Tachaboonyakiat *et al.* [25]. This confirms crystallization of HAp in the gel.

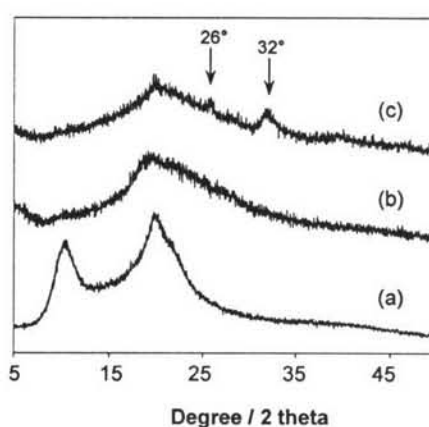


Figure 3. X-ray diffractograms of (a) **1**, (b) **4**, and (c) **4** after alternate soaking in $\text{CaCl}_2/\text{Na}_2\text{HPO}_4$ for 5 times.

TGA was applied to evaluate the amount of HAp in **4**. Here, it was assumed that the increase in ash content of the gel with HAp (Figure 4 (b)-(f)) as compared to the chitosan gel without HAp (Figure 4 (a)) reflected the HAp amount formed in the gel. Figure 4 shows that the ash content of **4** increases as the number of soaking cycles increased. The HAp amounts of the first and second cycle (Figure 4 (b) and (c)) are 9% and 17%, respectively. The HAp content is saturated at 20% after soaking four to five times (Figure 4 (e) and (f)). It is important to note that HAp mineralization initiated the gel strength significantly (see Gel strength of chitosan-epoxy gel).

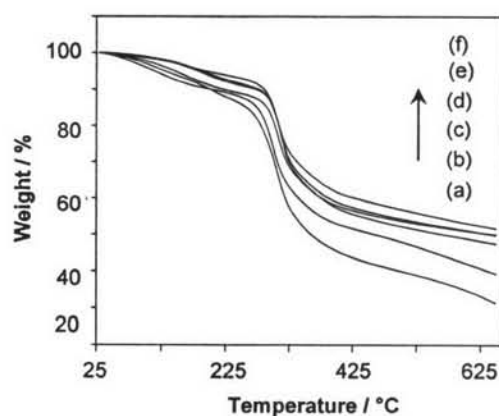


Figure 4. TGA thermograms of (a) **4**, and **4** after alternate soaking in $\text{CaCl}_2/\text{Na}_2\text{HPO}_4$ for the (b) 1st cycle, (c) 2nd cycle, (d) 3rd cycle, (e) 4th cycle, and (f) 5th cycle.

SEM was applied to observe the growth and dispersion of HAp in the gel. Fig. 5a represents the gel obtained from the reaction of **3** with 20 moles of hydrazine equivalent to pyranose ring of **3**. After a cycle of alternate soaking (Figure 5 (b)), some uniform particles are observed all through the gel. The uniform growth of HAp not only means that there is an effective mineralization, but it also implies a micro-porous structure to allow the hybridization of inorganic compound. The particle size of HAp was found to be more significant after the soaking for 5 times (Figure 5 (c)), at that time, the average size was 4 μm .

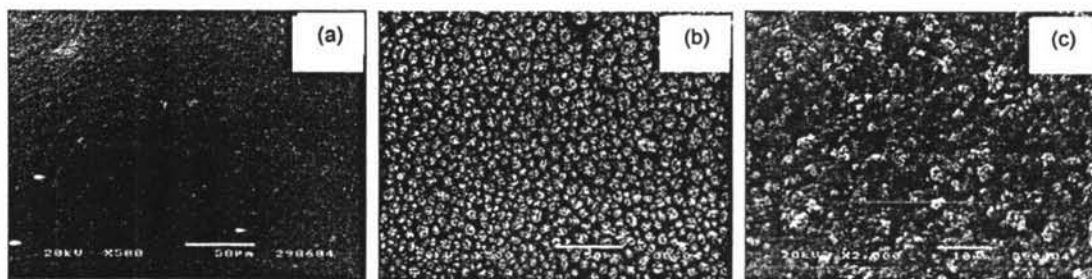


Figure 5. Scanning electron microscopy photographs at 25 kV of (a) **4** with 20 moles of hydrazine ($\times 500$), and **4** after alternate soaking in $\text{CaCl}_2/\text{Na}_2\text{HPO}_4$ at 37°C for (b) the 1st cycle ($\times 500$), and (c) the 5th cycle ($\times 2000$).

3.4. Gel strength

Gel strength characterized by rheological measurement was observed at the maximum shear stress in the plot between shear stress versus time [33] to reveal the deformation behaviors of gels before and after mineralization. Fig. 6A shows the gel strength increases with an increase of hydrazine amount to achieve a maximum shear stress of 261 Pa when hydrazine was 50 moles equivalent to pyranose ring. This result reflects that the reaction of hydrazine induces the gelation via the ring opening reaction of the oxirane ring in **3**. Water content is another factor in the evaluation of hydrophilicity related to gel strength. TGA results reveal that the gel obtained gives water content above 85% for all samples (Figure 6).

The mineralization with hydroxyapatite was also evaluated. Here, the gel obtained from 20 mole equivalent to pyranose ring was focused on. In this case, the change of gel strength from the effect of mineralization can be easily observed. Fig. 6B shows an increase of gel strength as the soaking cycles increase. The soaking at the 5th cycle gives the gel strength of the maximum value of 1346 Pa. It should be noted that when the soaking was more than 5 times, HAp crystallized on the gel surface.

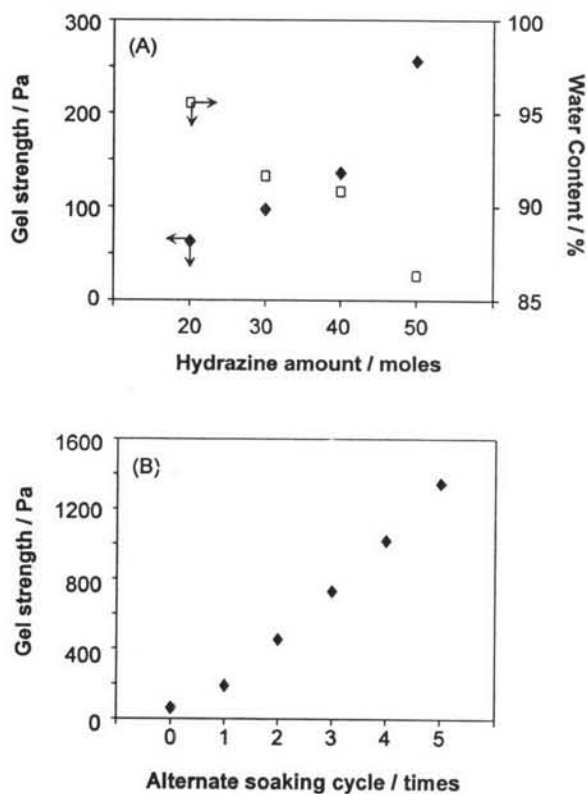


Figure 6. (A) Gel strength and water content of **4** under various hydrazine molar ratios, (B) Gel strength of (a) **4** with 20 times of hydrazine and **4** after alternate soaking in $\text{CaCl}_2/\text{Na}_2\text{HPO}_4$ for the (b) 1st cycle, (c) 2nd cycle, (d) 3rd cycle, (e) 4th cycle, and (f) 5th cycle.

3.5. Cell adhesion on chitosan-epoxy gel

Fischer *et al.* reported that cell adhesion was dependent on the hydrophobic/hydrophilic property, electric charge, surface morphology, and the surface functional groups of a biomaterial [34]. In our case, cell adhesion might be affected by the presence of amino group and surface morphology. Here, mouse fibroblasts (L929) were cultured on the various gel types to evaluate their cell adhesion. Considering the micrographs of gel A ~ C, it can be concluded that the number of cells decreases after HAP mineralization (Figure 7). Figure 8 shows the quantitative results of cell adhesion onto various gels. Comparing gel A to chitosan-glutaraldehyde gel (gel D) [35], it was found that gel A showed a higher percentage (~10%) of the adhered cell. This supported our speculation that gel A has more

amino groups than gel D to allow the effective electrostatic interaction with the negatively charged surface of the cell membrane [34].

Unexpectedly, after the gel was mineralized with HAp, a significant decrease in cell adhesion was observed. Our speculation is that HAp initiated the rough granular surface to the gel, as observed by SEM (Figure 5), and this probably obstructed cell adhesion. As a result, when HAp was saturated (in this case, after soaking for 5 times), the cell adhesion was as low as 8%. Comparing to that of chitosan-glutaraldehyde gel with HAp (gel E), the loss in cell adhesion as found in gel C might be the point to improve in the next step.

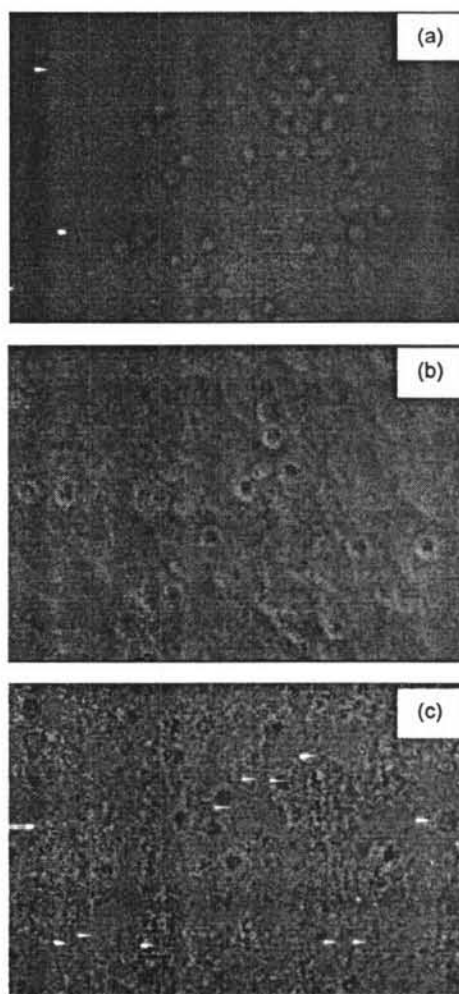


Figure 7. Micrographs of fibroblast adhesion cultured for 1 day on (a) 4, and 4 after alternate soaking in $\text{CaCl}_2/\text{Na}_2\text{HPO}_4$ for the (b) 1st cycle, and (c) 5th cycle.

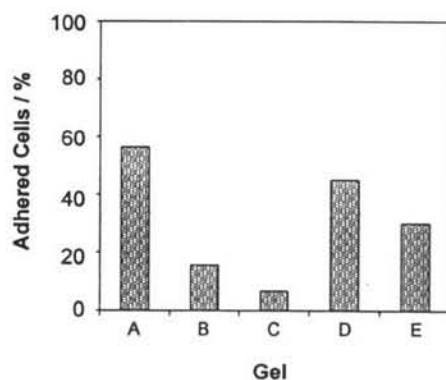


Figure 8. Fibroblast adhesion cultured for 1 day on 4 (Gel A), 4 after alternate soaking in $\text{CaCl}_2/\text{Na}_2\text{HPO}_4$ for the 1st cycle (Gel B), the 5th cycle (Gel C), chitosan-glutaraldehyde gel (Gel D), and chitosan-glutaraldehyde gel/HAp hybrid composite (Gel E).

4. Conclusion

The present work demonstrated a novel hybrid chitosan-HAp composite gel in water. The molecular design was based on introducing oxirane rings onto the chitosan chain and forming a crosslinked network via subsequent reaction between the oxirane rings and hydrazine. The epoxy group was successfully introduced onto the hydroxyl group by reacting epichlorohydrin with *N*-phthaloylchitosan. Here, *N*-phthaloylchitosan was applied not only for the homogeneous reaction but also for the amino group protection. The amino group deprotection and the crosslinking to obtain gel were accomplished by a single step reaction with hydrazine in water at room temperature. Cell adhesion studies showed significant cell viability on chitosan gel. The alternate soaking process with calcium and phosphate salts brought about the crystallization of HAp in gel. The gel with HAp showed a lower cell adhesion property than that of the gel without HAp.

Acknowledgments

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