CHAPTER IV
CHITINEOUS GEL VIA EPOXY GROUP INCORPORATION AND ITS HYDROXYAPATITE FORMATION

Abstract

A derivative of epoxy-chitosan gel for composite with hydroxyapatite is developed. The reaction of epoxy group with chitosan is focused at the hydroxyl group (C-3 and/or C-6) while the amino group is retained. Alternate soaking in calcium chloride and sodium hydrogen phosphate is applied to form hydroxyapatite in epoxy-chitosan matrix. The chemical functionalization is clarified by FTIR, TGA, and XRD.

Keywords: Epoxy-chitosan, Hydroxyapatite, Chitineous gel, Alternate soaking

Introduction

At present, artificial bone joint materials play an important role in bone surgery. In most cases, polymeric materials with biocompatibility are suitable due to the requirement about less tissue irritation. Considering some specific cases of bone therapy, the first aid is to fix up the fracture bone followed by the generation of the bone tissue. In this case, the first aid needs material with biodegradability, biocompatibility, and osteoconductivity including adhesiveness. Thus, the artificial glue compound for bone therapy is one of the effective approaches.

Chitin-chitosan is an attractive material to apply for bone therapy, with the properties of biocompatibility[1], biodegradability[2], non-toxicity[3] and osteoconductivity[4]. Chitin-chitosan also provides crosslinkable sites, i.e., hydroxyl and amino groups that are useful for producing a gel. In addition, the amino group in chitosan tends to form cationic species, which provides stable ionic interaction. Since hydroxyapatite (HA) is the main inorganic component in bone, the formation of chitosan-HA via ionic interaction is considered to be a practical pathway to obtain composite material. Up to now, various methods of chitosan/HA have been reported, for examples, coprecipitation of chitosan with H_3PO_4 and $Ca(OH)_2$ by heat treatment in saturated steam[5], and alternate soaking of chitosan gel with $CaCl_2$ and Na_2HPO_4 to allow hydroxyapatite formed in the matrix[6].

It should be noted that chitosan/HA composite proposed in the past is in the forms of gel[6] or powder[7], which the applications are based on the treatment rather than the bone fixation. On this viewpoint, the development of chitineous glue is a potential way to obtain a new compound for bone therapy in the step of bone fixation. The strategy of glue type of chitosan/HA by functionalizing chitosan with epoxy group. The ring opening reaction of epoxy as well as the crosslinking with amino groups can be expected for the adhesion properties. The alternate soaking process[6] is considered for the formation of hydroxyapatite in the chitosan-epoxy matrix.

Experimental Section

Materials. Chitosan with a degree of deacetylation (%DD) of 70 was locally supplied from the SEAFRESH (Lab) Company Limited, Bangkok, Thailand. Acetic acid, hydrochloric acid, calcium chloride, disodium hydrogenphosphate, methanol and tris(hydroxymethyl)aminomethane (Tris) were purchased from Carlo Erba Regenti, Italy. Sodium acetate, *N,N*-dimethyformamide (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.

Instruments and Equipment. Qualitative and quantitative Fourier transform infrared spectra were obtained from a Bruker Equinox 55/S with 32 scans at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed using a deuterated triglycinesulfate detector (DTGS) with a specific detectivity, D^*, of 1×10^9 $cm.Hz^{1/2}$ w^{-1}. A Dupont thermal gravimetric analyzer was applied using a N_2 flowing rate of 20 mL/min with a heating rate of 20°C/min from 30°C to 600°C. X-ray diffraction patterns were obtained from a RIGAKU RINT 2000, using CuK_α (λ=0.154 nm) as an X-ray source with 2θ of 5-50° operating at 40 kV, 30 mA with Ni filter. Differential scanning calorimetry was performed on a Perkin-Elmer DSC7. The sample was sealed in a closed aluminum sample liquid pan and heated from 25°C to 120°C at a heating rate of 20°C/min. Intrinsic viscosity [η] was measured with a calibrated viscometer Cannon-Ubbelohde (No.2, A149) in 0.1 M sodium acetate/ 0.2 M acetic acid aqueous solution at 30±0.05°C. Molecular weight was calculated using the Mark-Houwink equation with $K=1.64\times10^{-30}\times DD^{14}$ and $a=(-1.02\times10^{-2}\times DD)+1.82$.[8]

***N*-Phthaloyl-Chitosan.** The amino group protection of chitosan was carried out according to Kurita *et al.*[9]

Chitosan (1.00 g) was reacted with phthalic anhydride (4.27 g, 5 mol equivalent to pyranose rings) in DMF (20 mL) at 100°C under nitrogen for 6 h. The temperature was reduced to 60°C, and the mixture was left overnight. The solution was concentrated to obtain yellowish viscous product. The crude

product was reprecipitated in ice water. The precipitate was collected, washed with methanol several times, and dried *in vacuo* to give *N*-Phathaloyl-Chitosan, **2** (Scheme 1).

Epoxy-*N*-Phathaloyl-Chitosan. Compound **2** (1.00 g) was dissolved in DMF (20 mL) and heated to 60°C in vacuo. After 30 min, a catalytic amount of potassium hydroxide-isopropanol solution and epichlorohydrin (3.50 g, 10 mol equivalent to pyranose rings) were added. The reaction was carried out at 60°C for 5 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*.

Epoxy-Chitosan. The deprotection of amino group was carried out as reported by Kurita *et al.*[9]

Compound **3** (1.00 g) and hydrazine monohydrate (3.12 g, 20 mol equivalent to pyranose rings) were mixed in water (20 mL) and heated to 60°C under atmosphere. After 15 hours, the product was concentrated, washed thoroughly with methanol several times, and dried *in vacuo*.

Epoxy-Chitosan/Hydroxyapatite (HA) Composite. The chitosan/HA composite was obtained by an alternate soaking process as reported by Tachaboonyakiat *et al.*[6]

Compound **4** (1.00 g) was immersed in $CaCl_2$ (200mM)/ Tris-HCl (pH 7.4) aqueous solution (20 mL) at 37°C for 2 hours, followed by rinsing with distilled water. The product was immersed in Na_2HPO_4 (120 mM) aqueous solution (20 mL) at 37°C for 2 hours and washed thoroughly with water. The soaking was repeated alternatively several times, followed by drying *in vacuo*.

Results and Disscussion

The molecular weight (M_v) of chitosan starting material was found to be 1.18×10^6 as characterized by Ubbelohde viscosity measurement. In order to obtain a chitineous gel based epoxy, the reaction was focused on reacting epichlorohydrin with hydroxyl groups of chitosan.

***N*-Phthaloylation of Chitosan.** Kurita *et al.*[9] reported that the phthaloylation is a reaction to improve the solubility of chitosan in most organic solvents, such as dimethylformamide (DMF), dimethylacetamide (DMAc), dimethyl sulfoxide (DMSO), and pyridine. The phthaloylation is also effective since there is no side reaction occurred at hydroxyl groups of the C-3 and C-6 positions. The present work, phthalimido protection was initially carried out to improve its solubility.

The compound obtained was confirmed by FTIR. Figure 1 (b) shows the characteristic peaks due to phthalimido groups at 1776 and 1714 cm^{-1} and aromatic ring at 721 cm^{-1}. The packing structure confirmed by XRD shows a broad peak at 20° 2θ (Figure 2 (b)) that might be related to the reduction of inter- and intramolecular hydrogen distances of the chitosan chain as a result from the bulky phthalimido groups. Figure 3 ((a) and (b)) show the thermal stability of **2** as compared to chitosan. In either case, the weight loss starts from 60°C to 100°C referred to the moisture on surface and water content. Compound 2 shows the broad degradation starting from 200°C to 400°C. Considering the degradation peak of chitosan in the range from 300-350°C, it can be speculated that the introduction of *N*-phthaloyl group effects to the chain packing and crystallinity, as a result, the degradation starts at only 200°C. However, the result that the weight loss of **2** proceeded continuously to as high as 400°C should be related to the stability of phthaloyl group. Comparing the ash content of chitosan with *N*-phthaloyl-chitosan, it should be noted that chitosan has high ash content above 40%, while *N*-phthaloyl-chitosan has low ash content below 20%. This might be related to the strong inter- and intra-molecular hydrogen bonding and some inorganic content remaining in chitosan starting material. Therefore, the TGA result shows the incomplete degradation of chitosan. For *N*-phthaloyl-chitosan, the reason that TGA result shows the nearly complete degradation might be related to the changing of its packing

structure from high crystallinity to more amorphous structure. This speculation is relevant to the XRD result.

Synthesis of Epoxy-*N*-Phthaloyl Chitosan. Although sodium hydroxide is generally used as a base catalyst in the preparation of an epoxy resin, it cannot be dissolved in organic solvent. In this work, the reaction of epichlorohydrin with *N*-phthaloyl chitosan was done in DMF with a catalytic amount of potassium hydroxide in isopropanol. The reaction was done successfully at 60°C for 5 hours *in vacuo*. The product obtained shows a characteristic peak at 907 cm^{-1} for an oxirane ring (Figure 1 (c)). The XRD pattern shows the broader peak at 20° 2θ implying the decrease in crystallinity. The broader peak reflected the loose packing structure via the side chain of epoxy groups (Figure 2 (c)). The thermal stability by TGA showed a single step of continuous weight loss starting from 300°C to 400°C (Figure 3 (c)). This implies the unity of its structure. The XRD showed the amorphous structure which supports the speculation. The weight loss ended at 400°C might be related to the degradation of phthaloyl group and epoxy group. The ash content is below 10% implying amorphous structure as also confirmed by XRD.

Dephthaloylation of Chitosan Derivatives. The removal of phthaloyl group was efficiently carried out by treatment with hydrazine monohydrate at 60°C in water. The FTIR spectrum shows the peak at 1595 cm^{-1} due to primary amino groups. The peaks at 1776 and 1714 cm^{-1} for phthalimido groups disappeared completely suggesting that the deprotection was successful (Figure 1 (d)). After the deprotection of phthalimido group, compound **4** shows an XRD pattern with a little sharper peak at 20° 2θ (Figure 2 (d)). This implies that the elimination of bulky phthalimido groups induces the increasing of its packing structure. Theoretically, hydrazine will not only function for deprotection of amino group but also initiation the oxirane ring opening reaction to crosslink with other epoxy-chitosan chains. Here, the reaction was confirmed by FTIR curve fitting. Figure 4 show the FTIR spectra and FTIR curve fitting of Epoxy-*N*-Phthaloyl-Chitosan reacting with hydrazine monohydrate with various times. Figure 5 shows the plot of oxirane peak at 907 cm^{-1} as a function of reaction time. The result implies that the crosslinking occurred quantitatively. Considering the molar ratio of hydrazine to deprotect the

amino group, it can be well explained that the excess amount of hydrazine initiates the crosslinking. Figure 3 shows the thermal stability of **4** that has the same chain degradation as chitosan. This might be due to the performing of epoxy group as a crosslink chain. The ash content up to 45% also reflects the stabilization of the structure via crosslink network. Figure 6 shows the exothermic peak implying the heat used to generate the network. After crosslinking was completed, the exothermic peak disappeared completely.

Hydroxyapatite Formation in the Swollen Chitosan Hydrogels. Tachaboonyakiat *et al.*[6] demonstrated hydroxyapatite formation by alternate soaking process. The process can be done by immersing in calcium chloride and sodium hydrogen phosphate alternatively. In this way, production of small crystalline hydroxyapatite similar to bone hydroxyapatite can be achieved. The stoichiometric reaction is shown below.

$$10\ CaCl_2 + 6\ Na_2HPO_4 + 2\ H_2O \rightarrow Ca_2(PO_4)_6(OH)_2 + 12\ NaCl + 8\ HCl$$

Since chitosan is a cationic polymer, calcium ion is known to interact among the chains at amino groups. In order to make the alternate soaking provide hydroxyapatite formation, the immersing in calcium chloride solution has to be done in advance. The calcium ions interaction with amino group in chitosan chain will repulse each chitosan chain and provide the space for hydroxyapatite formation.

After repeating alternate soakings for four times, the compound obtained was observed by FTIR and XRD. The FTIR result shows the phosphate group in hydroxyapatite at 561 cm^{-1} (Figure 1 (e)). Figure 2 (e) shows the XRD pattern of the compound obtained with the small peaks at 26° and 32° 2θ which can be assigned to the hydroxyapatite peaks. The peak at 20° 2θ confirms the epoxy-chitosan chain. In order to evaluate the amount of hydroxyapatite formed in the chitosan network, TGA was applied and the ash content was observed. As shown in Figure 7, the amount of hydroxyapatite was found to increase with an increasing in the number of alternate soaking cycles. After the four times of alternate soaking, the ash content was found to increase for 10%. This reflected the amount and the stability of hydroxyapatite formed in the epoxy-chitosan network.

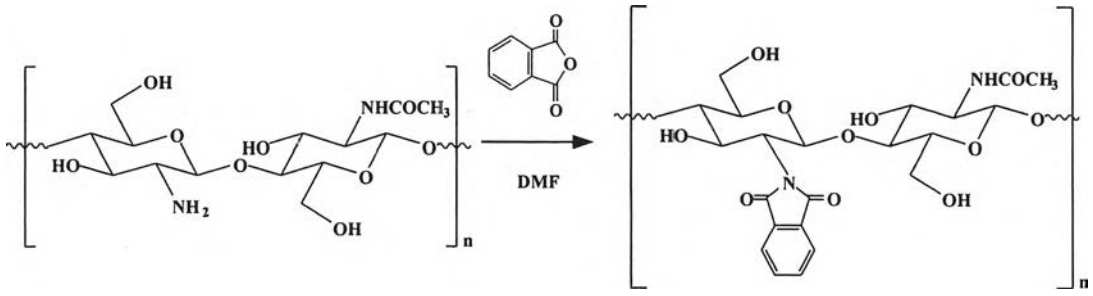
Conclusions

The epoxy-chitosan gel can be prepared by conjugating chitosan with epichlorohydrin. To improve its solubility, the amino group was protected initially. The introduction of epichlorohydrin was acheived mainly at hydroxyl group of C-6. The quantitative FTIR confirmed that the deprotection and the crosslinking were successful by the reaction with hydrazine. The alternate soaking in calcium chloride with sodium hydrogen phosphate helps to form hydroxyapatite in epoxy-chitosan matrix as confirmed by FTIR and XRD. The amount of hydroxyapatite was formed for 10% as confirmed by TGA.

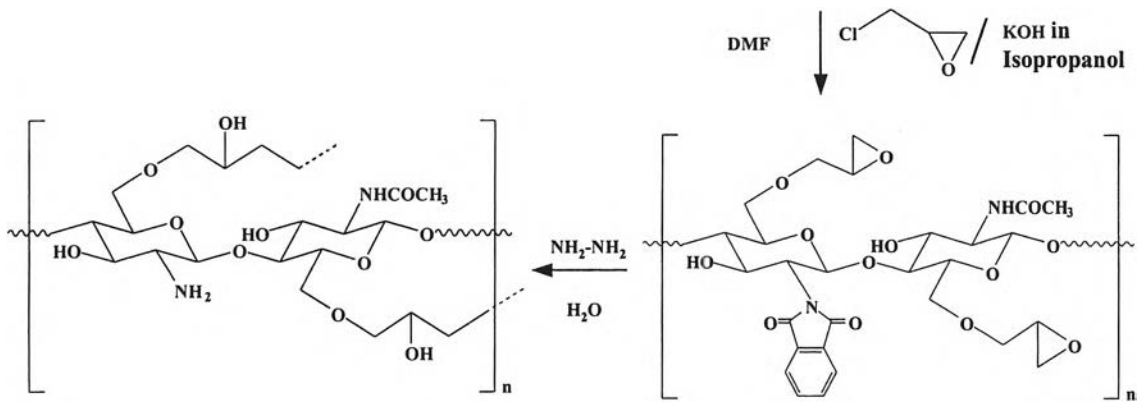
Acknowledgements. The authors thank Seafreash Chitosan (LAB) Company Limited, Thailand for chitosan material.

References

1. Singh, D.K. and Ray, A.R. *Journal of Applied Polymer Science*. **1994**, 53, 1115-1121.
2. Mark, H.F., Bikales, N.M., Overberger, C. G., and Menges, G. *Encyclopedia of Polymer Science and Engineering*; John Wiley&Sons: New York, **1985**; 430-440.
3. Chandy, T. and Sharma, C.P. *Biomaterials*. **1992**, 13(13), 949-952.
4. Lahiji, A., Sohrabi, A., Hungerford, D.S., and Frondoza, C.G. *Journal of Biomedical Materials Research*. **2000**, 51, 586-595.
5. Yamaguchi, I., Tokuchi, K., Fukuzaki, H., Koyama, Y., Takakuda, K., Monma, H., and Tanaka, J. *Journal of Biomedical Materials Research*. **2001**, 55, 20-27.
6. Tachaboonyakiat, W., Serizawa, T., and Akashi, M. *Polymer Journal*. **2001**, 33:(2), 177-181.
7. Tokura, S. and Tamura, H. *Biomacromolecules*. **2001**, 2, 417-421.
8. Wang, W., Bo, S., Li, S., and Qin, W. *International Journal of Biological Macromolecule*. **1991**, 13, 281-285.
9. Kurita, K., Nishimura, S.I., and Kohgo, O. *Macromolecules*. **1991**, 24, 4745-4748.



Chitosan, 1 **N-Phthaloyl-Chitosan, 2**



Epoxy-Chitosan, 4 **Epoxy-*N*-Phthaloyl-Chitosan, 3**

Scheme I. (Jutatip et al.)

Figure Captions

Figure 1. FTIR spectra of: (a) **1**, (b) **2**, (c) **3**, (d) **4**, and (e) epoxy-chitosan/HA composite.

Figure 2. X-ray diffractograms of: (a) **1**, (b) **2**, (c) **3**, (d) **4**, and (e) epoxy-chitosan/HA composite.

Figure 3. TGA diagrams of: (a) **1**, (b) **2**, (c) **3**, and (d) **4**.

Figure 4. FTIR spectra and curve fitting of **3** with hydrazine monohydrate under the curing at room temperature: (a) 4 minutes, (b) 12 minutes, (c) 20 minutes, and (d) 28 minutes.

Figure 5. FTIR curve fitting of **3** with hydrazine monohydrate using intensity of the oxirane peak at 907 cm^{-1} and pyranose ring peak at 1026 cm^{-1} with various times.

Figure 6. DSC diagrams of: (a) curing of **3**, and (b) after curing of **3.**

Figure 7. TGA diagrams of epoxy-chitosan/HA composite after (a) 1 cycle, (b) 2 cycles, (c) 3 cycles, and (d) 4 cycles of alternate soaking of calcium and phosphate solution.

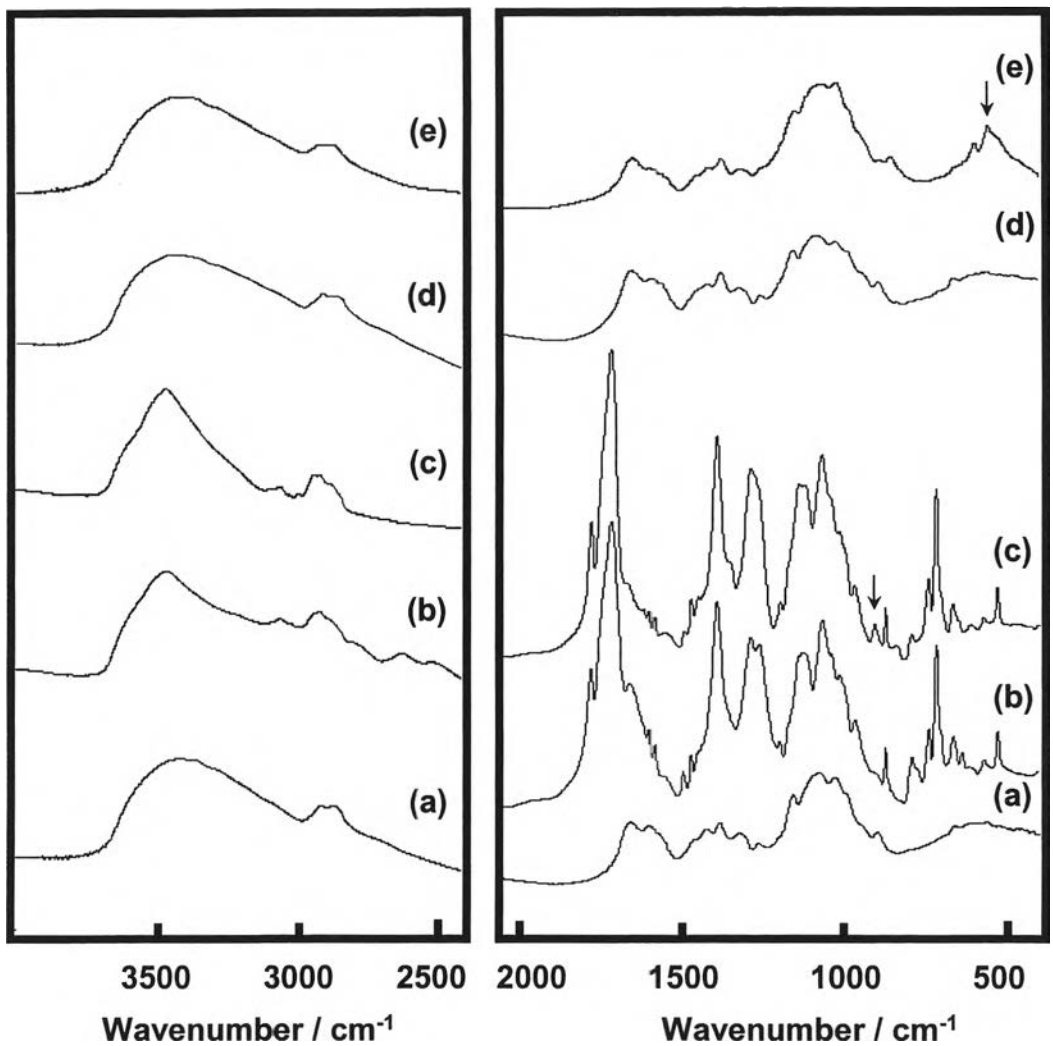


Figure 1. (Jutatip et al.)

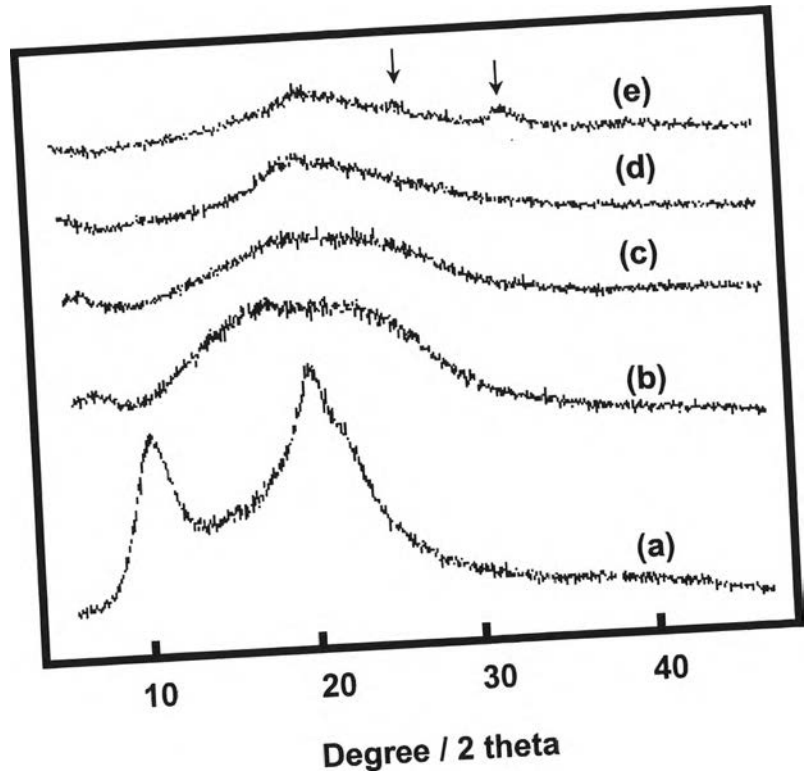


Figure 2. (Jutatip et al.)

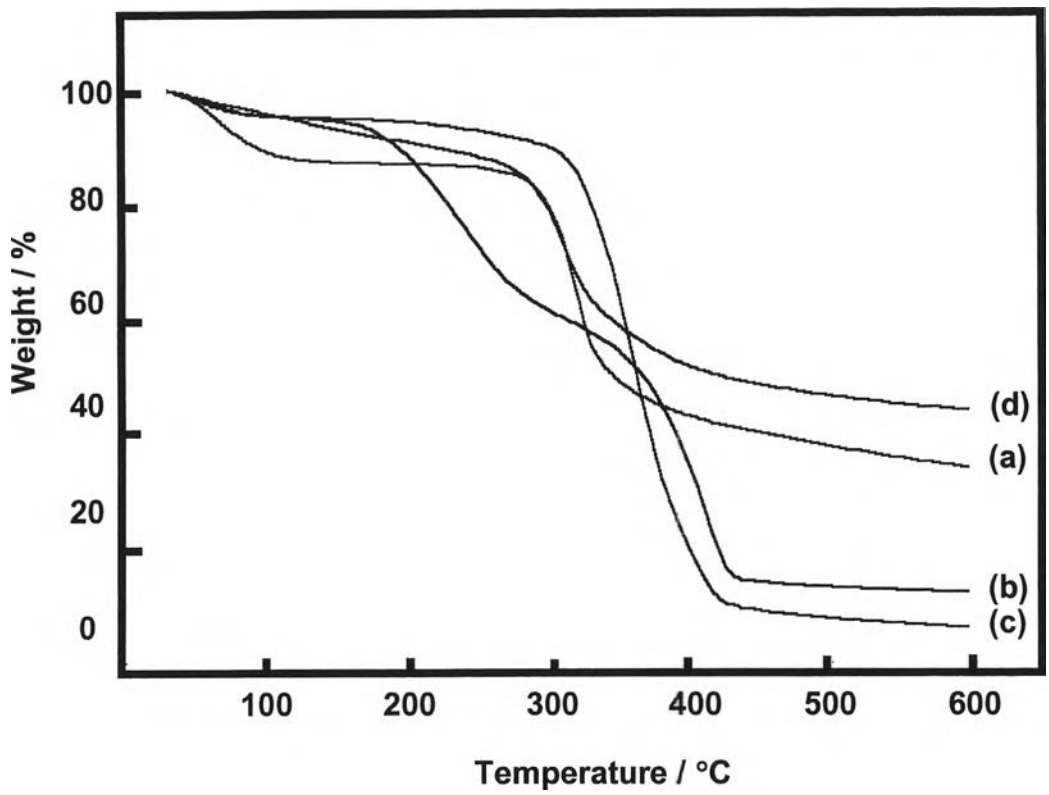


Figure 3. (Jutatip et al.)

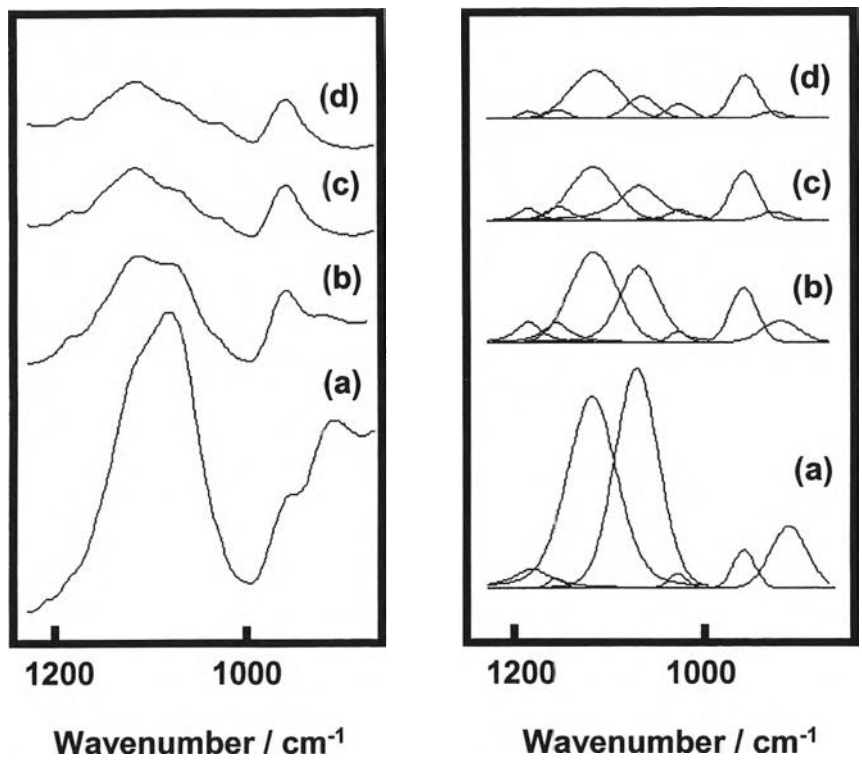


Figure 4. (Jutatip et al.)

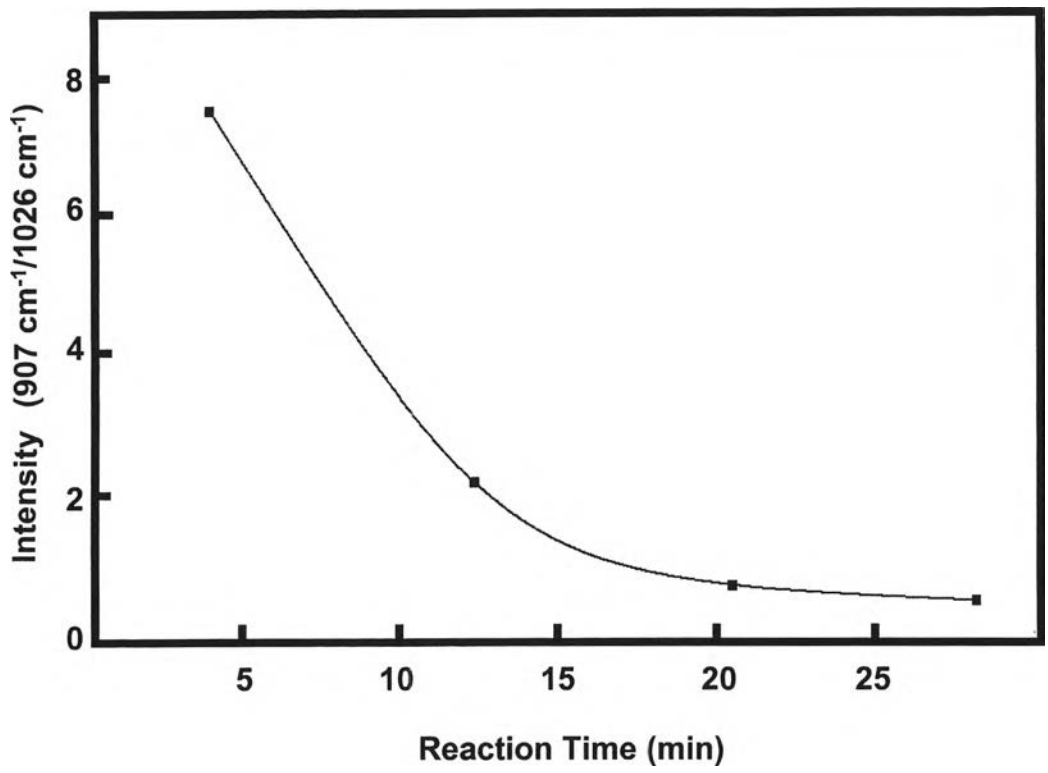


Figure 5. (Jutatip et al.)

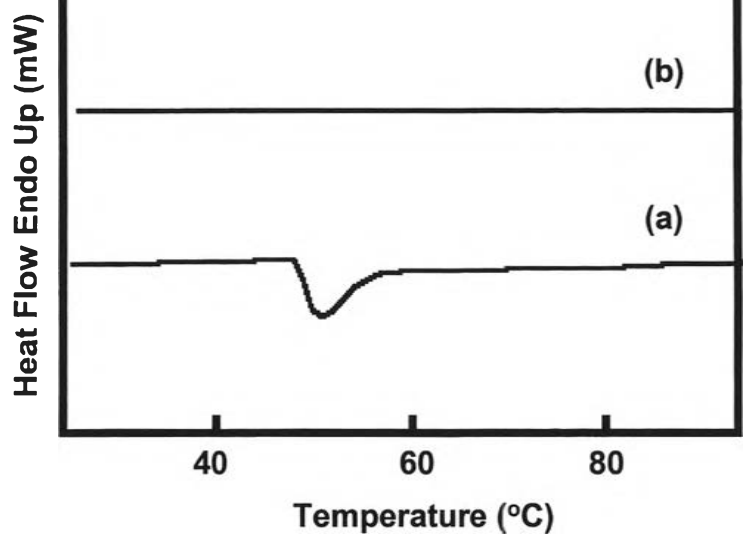


Figure 6. (Jutatip et al.)

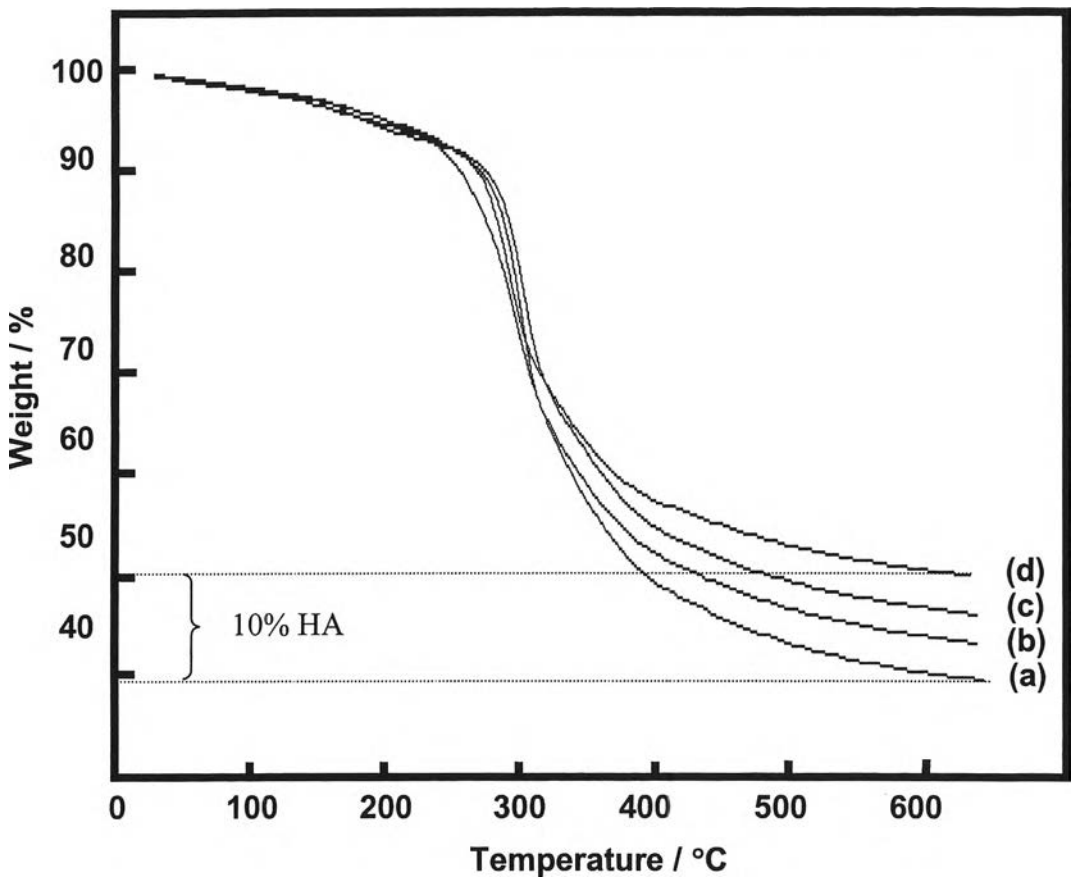


Figure 7. (Jutatip et al.)