CHAPTER IV

Nanostructured Chitosan for Drug Targeting System

Abstract

The conjugation of two types of amino acids, i.e. tryptophan and asparagine onto chitosan whisker is proposed. The reactions are successful by carrying out in water at room temperature using chitosan whisker, water soluble conjugating agent (WSC) and conjugating additive. Although the amino acids need amino group protection, the quali and quantitative analyses by FTIR and NMR indicate that no polyamino acids were formed. The morphological study by transmission electron microscope (TEM) shows that the chitosan conjugated with tryptophan is in spherical shape with 160-280 nm in size.

Keywords: Drug targeting; Chitin-chitosan; Chitosan whisker; Amino acids

1. Introduction

Drug delivery system is a system that the drugs are controlled and released by phagocytosis and endocytosis mechanism into the cell or organ all of the body. The main problems currently associated with systemic drug administration are the distribution of pharmaceuticals in the body, the drug specific affinity toward the pathological site, the dose amount of drug to achieve high local concentration, the non-specific toxicity and the other adverse side-effects due to high drug doses. Drug targeting is, thus, proposed as a solution for specific drug administration route.[1]

Drug targeting shows the advantages on the ability of the drug to accumulate in the target organ or tissue selectively and quantitatively. The molecules for drug targeting should be biocompatible, non-toxic, inert (to the immune response), and capable for drug loading. Polymer-based systems are designed under considerations of the type of disease, the drug properties, the therapy approaches, the administration route, including the characteristic of the polymeric materials used, and their mechanisms during the targeting. Polylactides (PLA), polyglycolides (PGA), poly(lactide-co-glycolides) (PLGA), polyanhydrides, polyorthoesters, poly(ethylene glycol) (PEG) and polysaccharides, including chitin-chitosan[2] have been actively investigated for the past decades.

It is known that chitosan is a biodegradable and bioadhesive polysaccharide. It is non-toxic and soft tissue compatible in a range of toxicity tests.[3] It has been widely used in pharmaceutical researches and in industry as a carrier for drug delivery[4] and as biomedical materials.[5] Chitosan is considered to be a good candidate for the gene delivery system since it is already known as a biocompatible, biodegradable, and non-toxic material with high cationic charge potential.[6] Illum *et al.* (1998) proposed chitosan nanoparticles and showed its recognized mucoadhesivity and mucosal surface penetrating ability.[7]

Kimura *et al.* (2001) showed the conjugation of mitomycin C (MMC) with CD 105 monoclonal antibody as the targeting molecule using dextran as the carrier. The increase of MMC activity and the reduction of drug loss and side effects imply the successful drug targeting for choroidal neovascularization.[8]

It is important to note that chitosan has the reactive groups especially hydroxyl and amino groups to conjugate with specific molecules and obtain targeting

system. Amino acid and sugar are recognized as the targeting molecules to interact with specific organ such as heart, lung, liver including tumor cells. For example, asparagine and tryptophan are the non-essential amino acids existed only in tumor cells. It can be expected that by introducing amino acids on chitosan, we may achieve the drug targeting system.

The use of chitosan is also the point to be considered. The present work focuses on using the low molecular weight chitosan in the form of chitosan whisker (or nanoscaffold) to increase the water solubility and enhance the reactivity in conjugating reaction.

2. Experimental

2.1 Materials

Chitosan (%DD = 95) was supplied from Seafresh Chitosan (Lab) Company Limited, Thailand. 1-Hydroxybenzotriazole monohydrate (HOBt·H_2O) and water-soluble carbodiimide hydrochloride (WSC·HCl) were purchased from Wako Pure Chemical Industries Co. Ltd., Japan. L-asparagine monohydrate and L(-)-tryptophan were obtained from Fluka Chemika, Switzerland. Sodium hydroxide and hydrochloric acid were purchased from Lab-Scan, Ireland. All chemicals were used without further purification.

2.2 Instruments

Qualitative and quantitative Fourier transform infrared spectra were obtained from a Thermo Nicolet Nexus 670 with 32 scans at a resolution of 2 cm^{-1} in a frequency range of 4000-400 cm^{-1} using a deuterated triglycinesulfate detector (DTGS). Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained from a 400 MHz JEOL JNM-GSX spectrometer. The morphology was investigated by using a JEOL JSM-5200 scanning electron microscopy (SEM) at 15 kV.

2.3 Preparation of chitin whisker, **2** (Scheme 1)

Chitin flakes, **1** (1.00 g), were treated in 3 *N* hydrochloric acid (HCl) (100 mL) and stirred at reflux for 3 h before centrifugation. The treatment with 3 *N* HCl

was repeated three times. Finally, the residues were collected and dialyzed in distilled water until neutral to obtain chitin whiskers, **2**.

2.4 Preparation of chitosan whisker, **3** (Scheme 1)

Compound **2** (20 mL) was stirred in NaOH aq. (40% w/v 100 mL) at reflux for 7 h before leaving at room temperature overnight. The treatment with 40% NaOH aq. was repeated three times. The crude product was dialyzed until neutral in distilled water to obtain **3**.

2.5 Synthesis of chitosan-L-asparagine, **5** (Scheme 1)

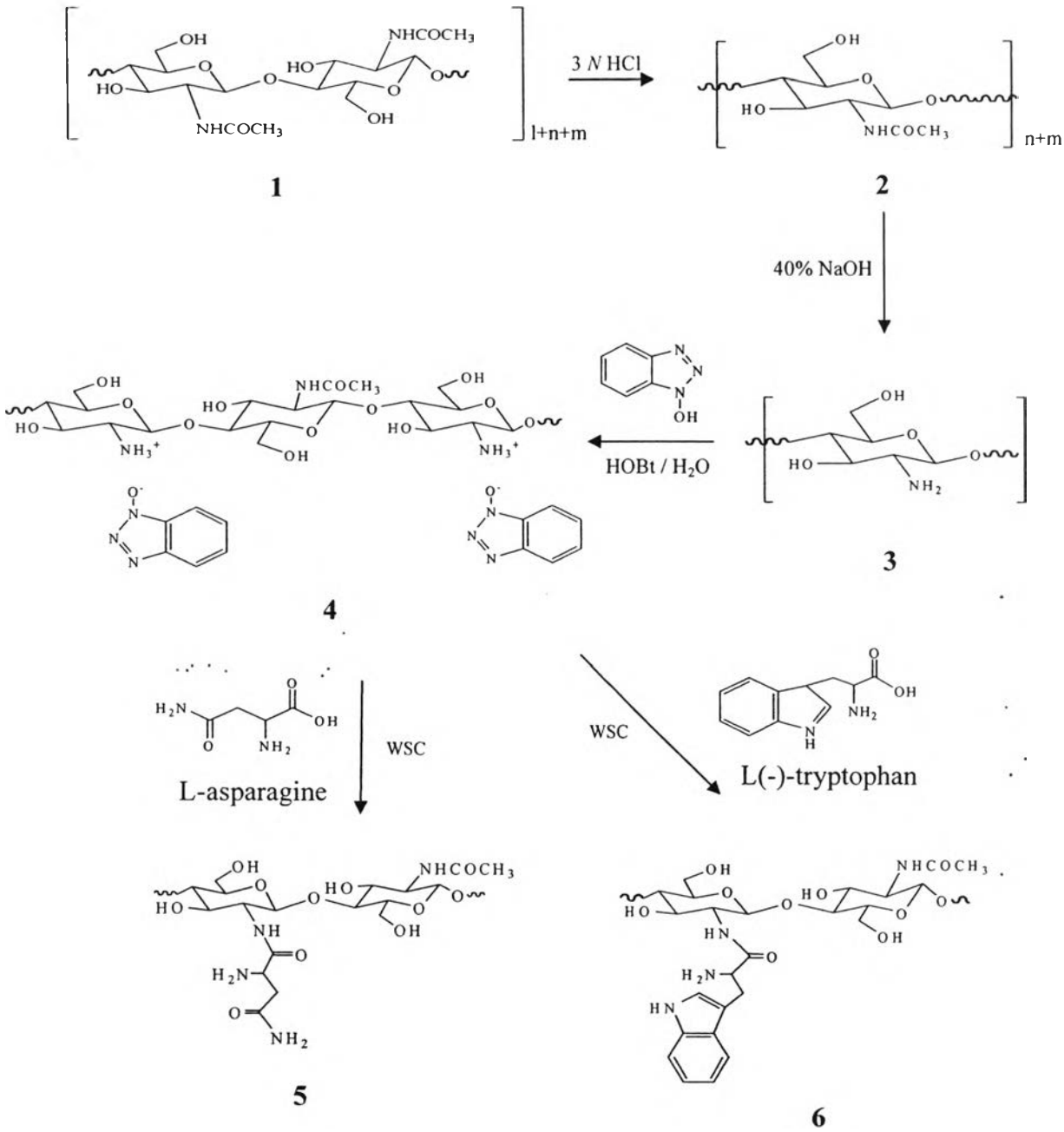
Chitosan whisker (0.1 g, 0.61 mmol) was vigorously stirred with HOBt·H_2O (0.09 g, 0.61 mmol) to that of chitosan in deionized water 8 mL at ambient temperature until the clear solution, **4**, was obtained. The solution **4** (40 mL) was mixed with L-asparagine (0.29 g, 0.61 mmol) followed by adding WSC·HCl (0.37 g, 0.61 mmol). The reaction was carried out at room temperature for overnight. The crude product was dialyzed and lyophilized to obtain **5**.

FT-IR (KBr, cm^{-1}): 1641 cm^{-1} (amide I). ^1H NMR (δ, ppm): 2.74 (-CH_2-) , 2.03 (NHAc), 3.01 (H2 of GluN unit in chitosan), and 3.16-3.75 (H3-H6 of pyranose ring).

2.6 Synthesis of chitosan-L(-)-tryptophan, **6** (Scheme 1)

Compound **6** was prepared similar to **5** but using L(-)-tryptophan.

FT-IR (KBr, cm^{-1}): 1640 cm^{-1} (amide I), 749 cm^{-1} (benzene ring). ^1H NMR (δ, ppm): 1.90 (NHAc), 3.00 (H2 of GluN unit in chitosan), 3.18-3.29 (-CH_2-), 2.90-3.74 (-NH-CH_2- and H3-H6 of pyranose ring), and 7.43-7.72 (-CH- of benzene ring in tryptophan).



Scheme 1. Synthesis of chitosan-L-asparagine and L-(-)-tryptophan.

3. Results and Discussion

3.1 Chitosan-L-asparagine, **5**, and L(-)-tryptophan, **6**

Up to now, syntheses of polymers with amino acid pendants have been reported with various purposes, for example, poly(D,L-lactic-co-glycolic acid) conjugated with N-(9-fluorenylmethoxycarbonyl-N-tert-butoxycarbonyl-L-tryptophan) for drug controlled release model, chitosan with different amino acids

(glycine, L-lysine, L-isoleucine, and L-glutamic acid) for enhancing the heavy metal ions adsorption, and chitosan having L- and D-phynylalanine spacer arms for polymer-suppoted asymmetric reducing agents.[9]

It is important to note that asparagine and tryptophan are the non-essential amino acids involving with the targeting molecules existed only in tumor cells. The conjugation of asparagine and tryptophan can be expected for the targeting molecules in the drug targeting system. Here, L-asparagine, and L(-)-tryptophan were selected as model compounds for conjugation via chitosan whisker-HOBt system (Scheme 1). The reactions were carried out in homogeneous system at room temperature for overnight with WSC and the reactions were followed by FTIR. As shown in Figure 1 (c), chitosan-asparagine, **5**, after the reaction proceeded for overnight, the characteristic peak C=O belonging to amide I (1641 cm^{-1}) was significant. Figure 2 (e) shows the important peaks of chitosan-tryptophan, **6**, at 1640 and 749 cm^{-1} corresponding to amide I, and C-H (symmetric out of plane) bending of benzene ring respectively.

The ^1H NMR spectrum of chitosan whisker gives the 1.90 ppm (NHAc), 3.00 ppm (H2 of GluN unit in chitosan), and 3.54-3.73 ppm (H3-H6 of pyranose ring) (Figure 2 (a)). The integral of NHAc peak indicates the acetyl group for 4%, or in other words the degree of deactylation is 96%. Figure 2 (b) shows a typical ^1H NMR pattern of HOBt in D_2O with the triplet peaks at 7.41, and 7.51 ppm for H-b and two doublet peaks at 7.60, and 7.66 ppm for H-a.

The ^1H NMR operating at room temperature was further analyzed to confirm the structure of **5** and **6**. Figure 3 (a) shows the chemical shift of **5** with 2.03 ppm (NHAc), 2.74 ppm ($-CH_2-$), 3.01 (H2 of GluN unit in chitosan), and 3.16-3.75 (H3-H6 of pyranose ring). The degree of substitution (DS) was calculated by using the integration of H2 of GluN unit in chitosan as an internal standard peak. The DS was found to be 25% based on the $-CH_2-$ integration. The ^1H NMR carried out at room temperature confirms the structure of **6** (Figure 3 (b)) from the chemical shifts at 1.90 ppm (NHAc), 3.00 ppm (H2 of GluN unit in chitosan), 3.18-3.29 ppm ($-CH_2-$), 2.90-3.74 ppm ($-NH-CH_2-$ and H3-H6 of pyranose ring), and 7.43-7.72 ppm (-CH- of benzene ring in tryptophan and HOBt). The quantitative analysis for the degree of substitution of L(-)-trytophan onto chitosan chain was determined by using

H2 of GluN unit in chitosan as an internal standard peak. The DS was found to be 4% based on the -CH_2- integration.

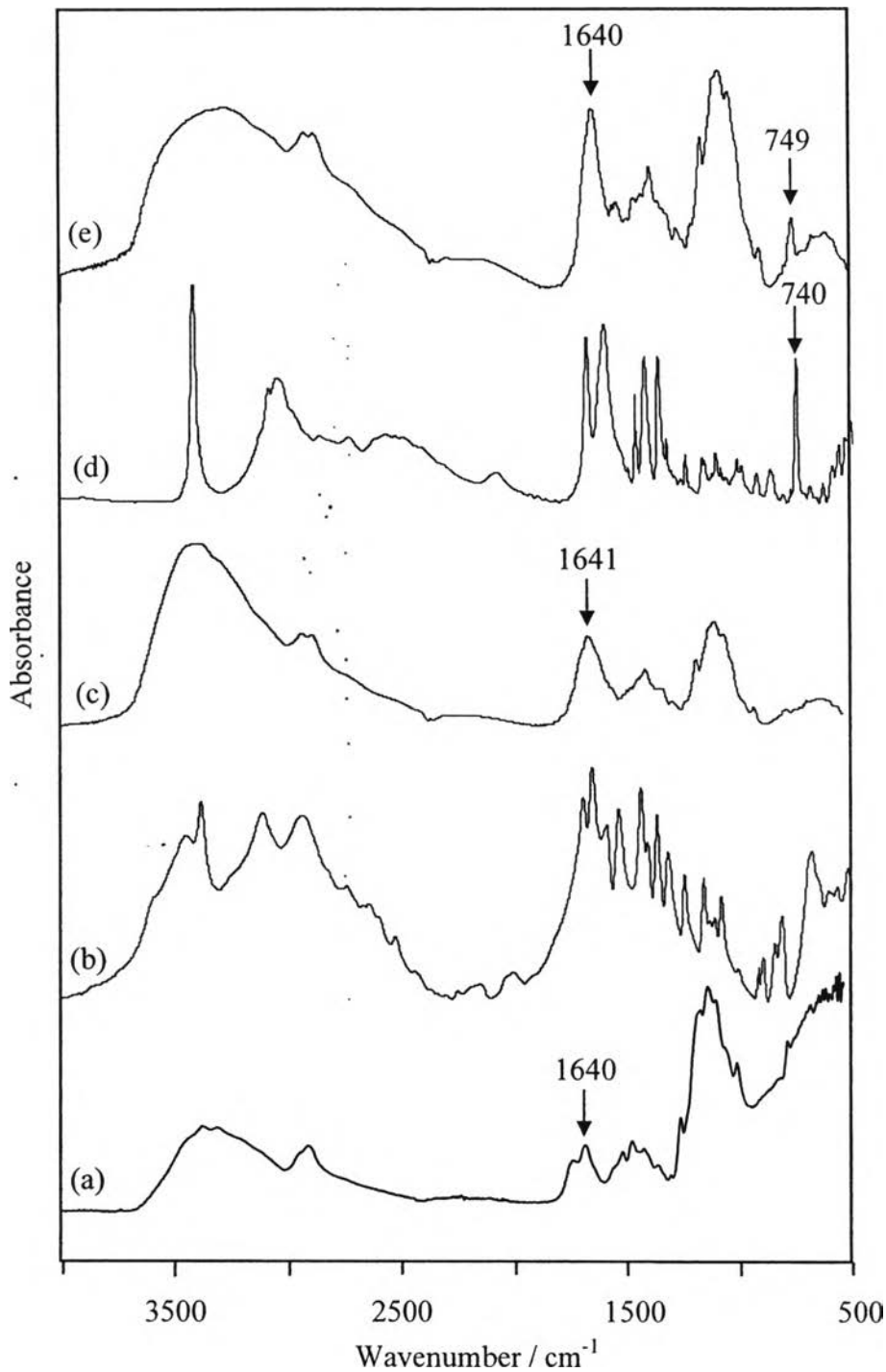


Figure 1. FTIR spectra of; (a) chitosan whisker, (b) asparagines, (c) **5**, (d) tryptophan, and (e) **6**.

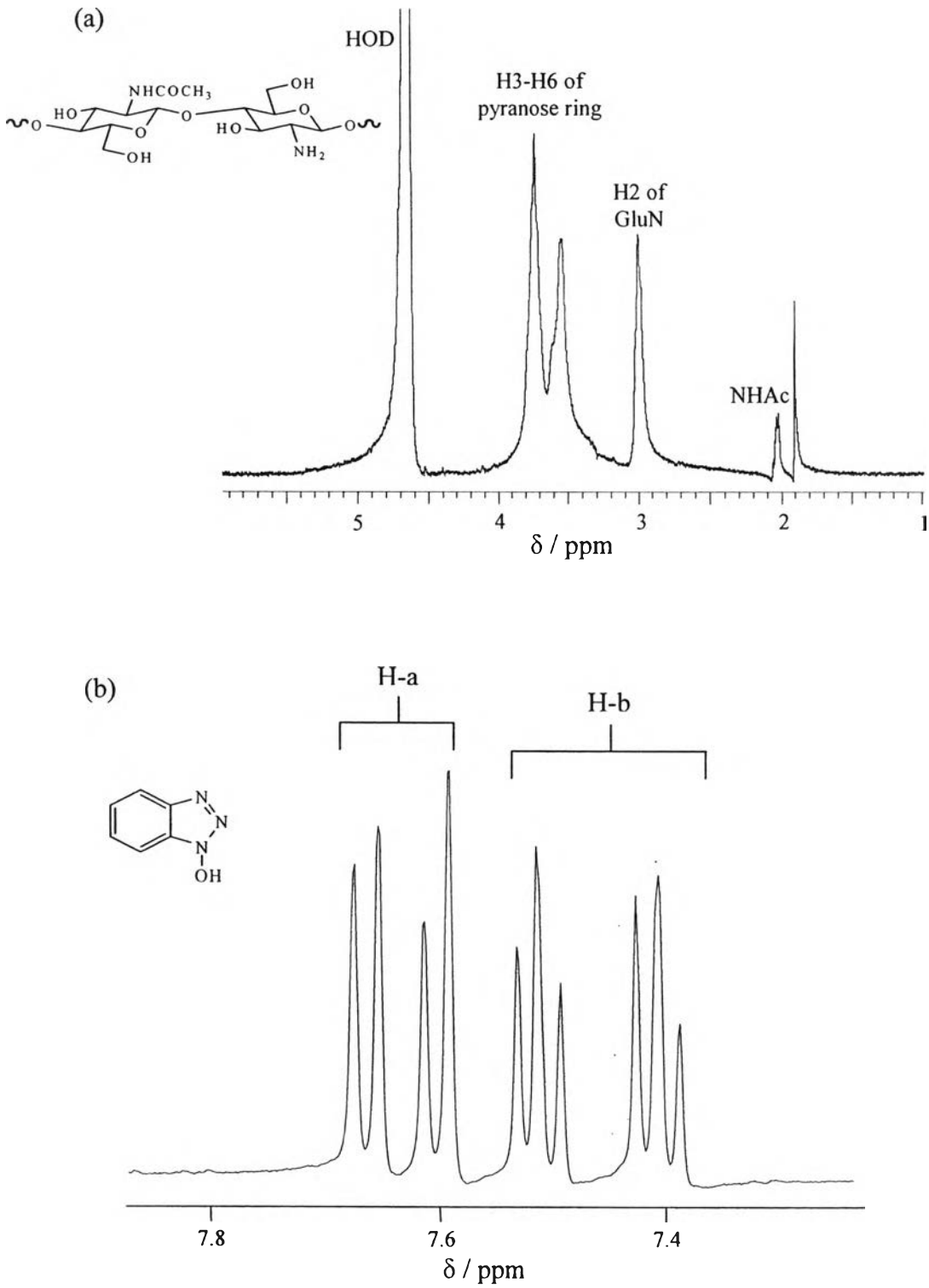


Figure 2. ^{1}H NMR spectra of; (a) chitosan whisker, and (b) HOBt in CD_3COOD/D_2O at room temperature.

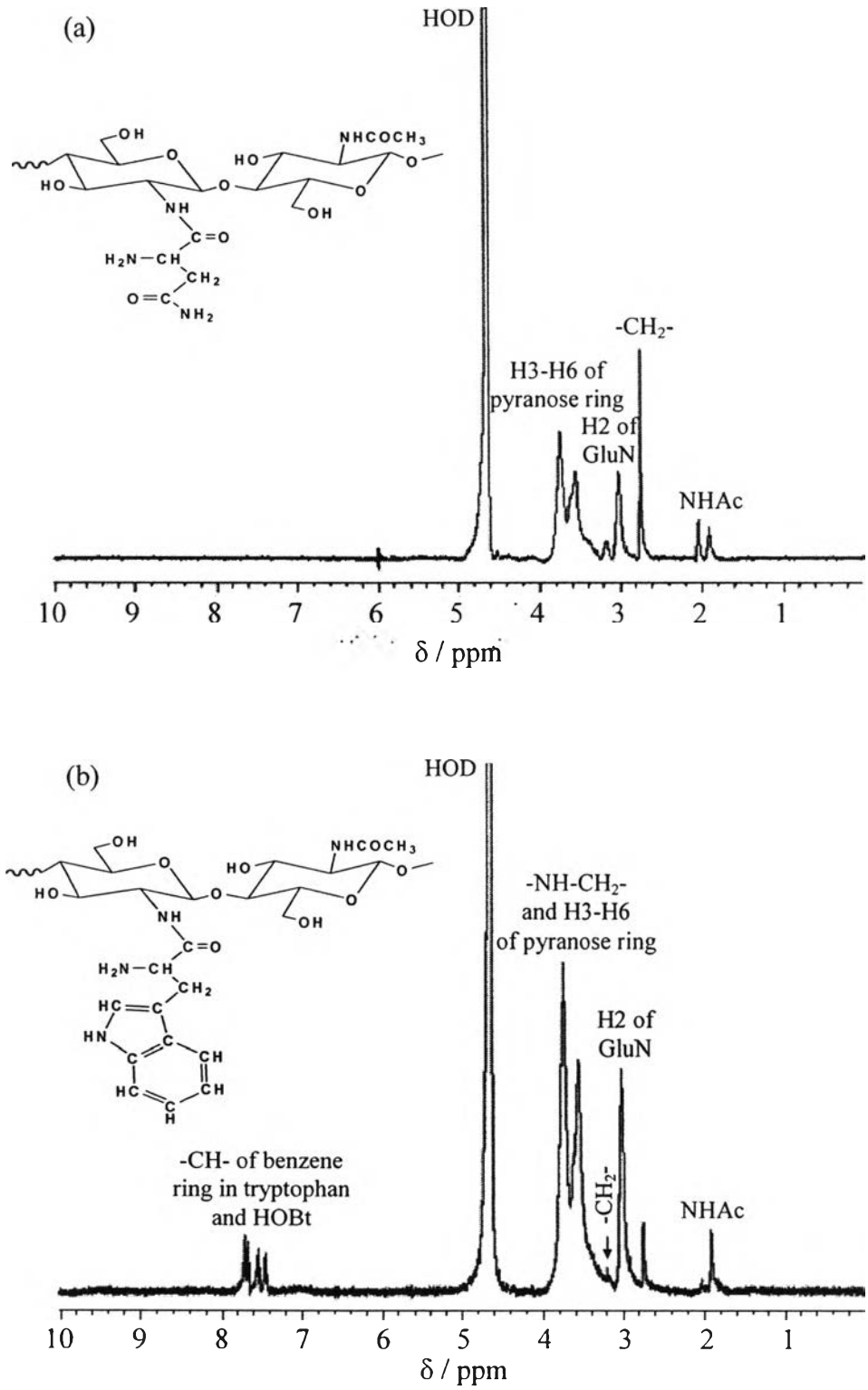


Figure 3. ^{1}H NMR spectra of; (a) **5**, and (b) **6** in CD_3COOD/D_2O at room temperature.

3.2 Thermal Stability

Asparagine (Figure 4 (a)) gives the degradation at 79-340°C whereas tryptophan (Figure 4 (b)) gives it at 290-400 °C. The degradation temperatures of chitosan whisker, **5**, and **6** were identified. Chitosan whisker (Figure 4 (c)) shows the degradation temperature between 280°C to 320°C under nitrogen. The stability of chitosan whisker changes after functionalization with asparagine and tryptophan as seen from the shift of the degradation temperature. The degradation temperatures of 5 and 6 (Figure 4 (d) and (e)) appear at 190-250°C. This implies that the amino acids obstruct the packing structure of chitosan whisker.

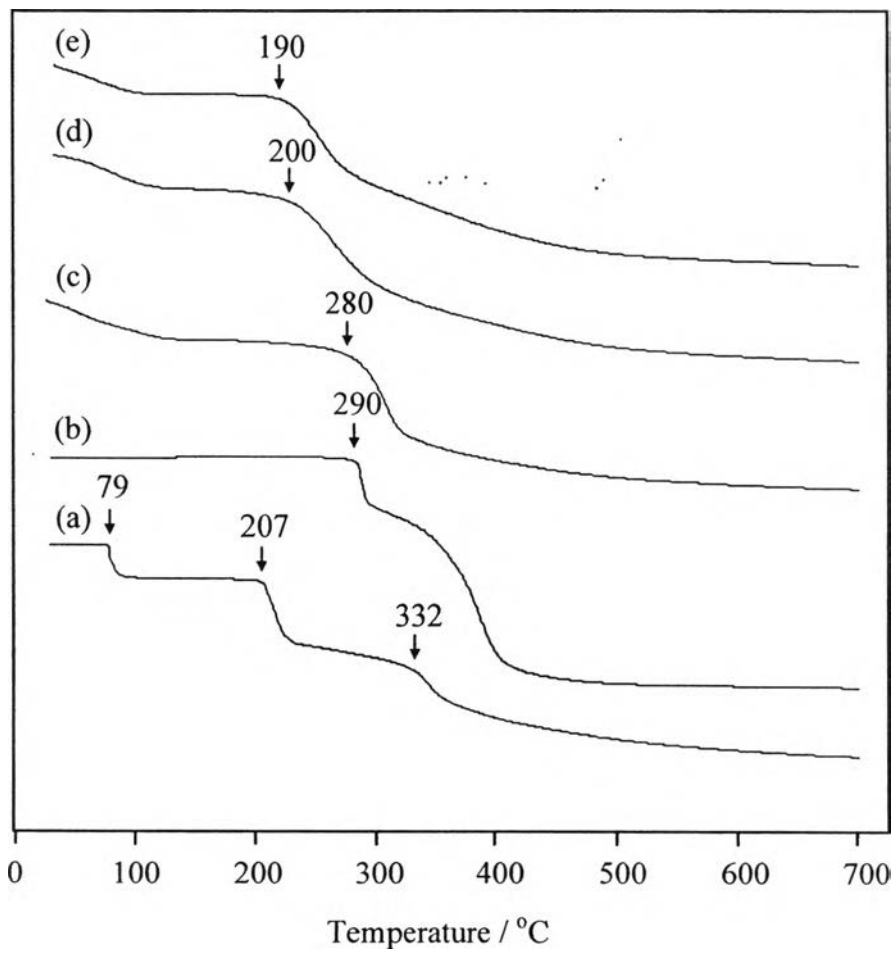
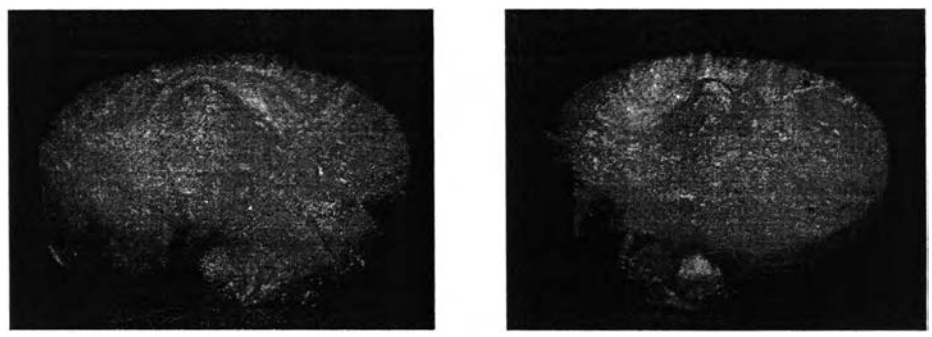


Figure 4. TGA thermograms of; (a) asparagine, (b) tryptophan, (c) chitosan whisker (d) **5**, and (e) **6**.

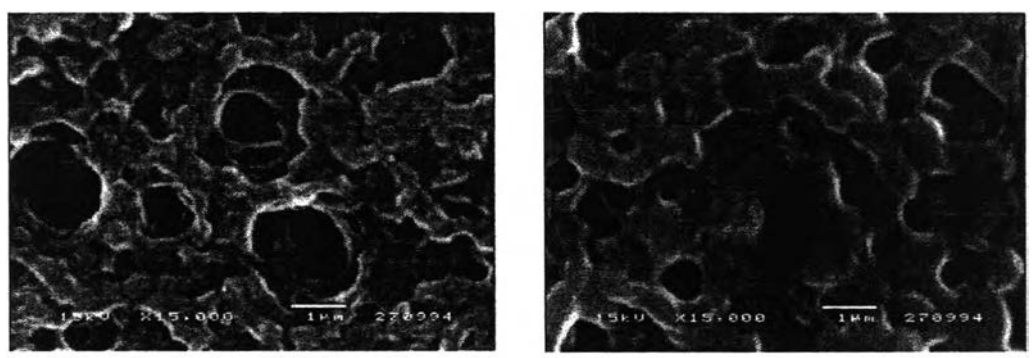
3.3 Morphological Studies

Compounds **5** and **6** show the soft cotton like appearance after lyophilized (Figure 5). The appearance is similar to chitosan whisker functionalized with lactose and maltose as reported by Phongying *et al.* (2006). Figure 6 shows the morphology of **5** and **6** as observed by SEM. It is important to note that by functionalizing either with asparagine or tryptophan, chitosan changes the morphology to the porous network. Here, futher investingation whether the porous network was the result from the chitosan whisker from the functionalization with amino acid needs to be done. For example, the comparative studies between chitosan and chitosan whisker in functionalizing with asparagine and tryptophan.



(a) (b)

Figure 5. Appearances of; (a) **5**, and (b) **6** after lyophilization.



(b)

Figure 6. SEM micrographs of; (a) **5** (× 15,000), and (b) **6** (× 15,000).

3.4 Solubility

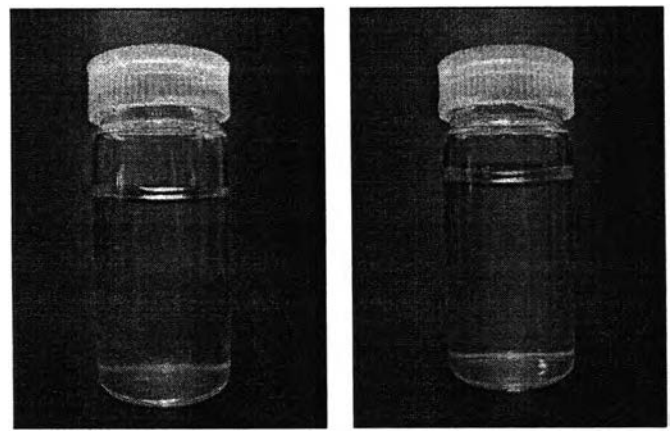
As chitosan-amino acids are the potential materials for drug targeting applications, their solubility in various solvents, especially aqueous-based, are important. Here, compounds **5** and **6** were dissolved in the range of polarity index of various solvents (Table 1). Compound **5** also shows good swelling in ethanol, chloroform, toluene, and n-hexane. Compounds **5** and **6** show good solubility in high polar solvents i.e. water as shown in Figure 8. It is also important to note that the solution of **5** and **6** in water is not completely transparent implying the possibility of the nanoparticles existence. Figure 8 (a) shows the fibril of **5** whereas Figure 8 (b) shows the droplets of **6** which confirms the 160-280 nm nano-scaled spheres.

Table 1 Evaluation of solubility in various solvents related to polarity index

Solvent	Polarity Index[a]	Appearance[b]	
		5	**6**
Water	9.0	+	+
Methanol	6.6	-	-
N,N-Dimethylformamide	6.4	-	-
Acetone	5.4	-	-
Ethanol	5.2	±	-
Chloroform	4.4	±	-
Toluene	2.3	±	-
n-Hexane	0	±	-

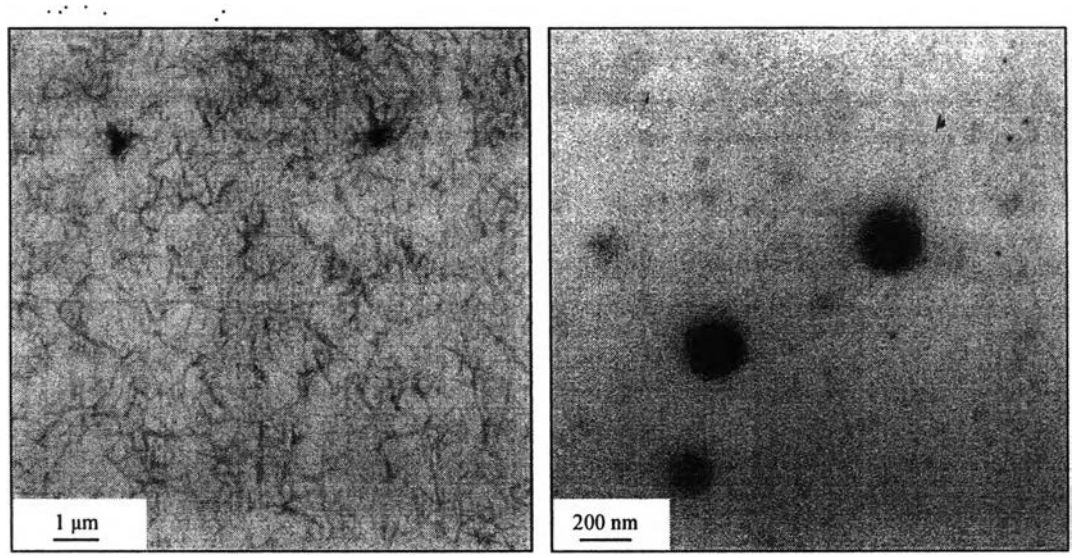
[a] from Synder (1974).

[b] + soluble; ± swelled; - partial swelled.



(a) (b)

Figure 7. Appearances of; (a) **5**, and (b) **6** in water.



(a) (b)

Figure 8. TEM micrographs of; (a) **5**, and (b) **6**.

4. Conclusions

The present work demonstrated an effective conjugation of chitosan whisker in simple conditions, i.e. in water at room temperature, using the water soluble chitosan whisker-HOBt aqueous solution, and WSC conjugating agent. The conjugation of chitosan whisker with amino acids of L-asparagine, and L(-)-

tryptophan was successful as confirmed by FTIR and ^{1}H NMR. The compounds obtained showed the unique soft and cotton-like appearance. The morphology of the compounds was in porous network. For **6**, the water dispersity was so excellent. It was also found that **6** formed nanospheres with the size of 160-280 nm.

References

1. P. Torchilin, *European Journal of Pharmaceutical Science* **2000**, 11, 81.
2. M. Ottenbrite, *Polymeric Drugs and Drug Administration* **1994**, 5, 436.
3. K. Aspden, E. Lim, *Biomaterials* **2003**, 24, 2339.
4. A. Sunil, N. Nadagouda, M. Tejraj, *J. Controlled Release* **2004**, 100, 5.
5. J. Mao, H. Liu, Y. Yin, K. Yao, *Biomaterials* **2003**, 24, 1621.
6. X. Kim, D. Yumin, J. *Pharmaceutics* **2003**, 250, 215.
7. M. Illum, Z. Xia, A. Serreqi, M. Berrada, A. Rodrigues, D. Wang, D. Buschmann, A.Gupta, *J. Pharmaceut Biomed* **1998**, 32, 1149.
8. N. Kimura, D. Edmonson, O. Veiseh, F. Matsen, M. Zhang, *Biomaterials* **2001**, 26, 6176.
9. K. Nair, A. Dufresne, *Biomacromolecules* **2003**, 4, 666.