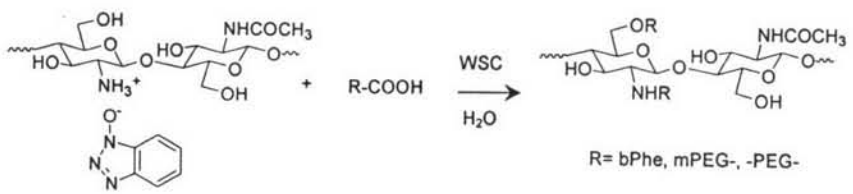
# CHAPTER V

# CHITOSAN-HYDROXYBENZOTRIAZOLE AQUEOUS SOLUTION: A NOVEL WATER-BASED SYSTEM FOR CHITOSAN FUNCTIONALIZATION


## Abstract

Chitosan-hydroxybenzotriazole (HOBt) aqueous solution prepared by simply mixing chitosan and HOBt in water provides an effective system to functionalize chitosan in aqueous. This aqueous solution in combination with water-soluble carbodiimide (WSC) allows the conjugation of functional groups onto chitosan in mild conditions without requiring any organic solvents or acids and heat. In this contribution, we demonstrate a series of model reactions via a novel water based system of chitosan to functionalize chitosan with boc-L-phenylalanine, poly(ethylene glycol) methyl ether, and dicarboxylated poly(ethylene glycol).


## Graphical Abstract




Conjugation of functional group onto chitosan via chitosan-HOBt aqueous solution.


Keywords: Chitosan gel; Hydroxybenzotriazole; Phenylalanine; Poly(ethylene glycol); Water-soluble chitosan

## 1. Introduction

Chitin-chitosan is the second most abundant naturally-occurring polysaccharide consisting of β-(1-4)-2-acetamido-2-deoxy-β-D-glucose and β-(1-4)-2-amino-deoxy-β-D-glucose units. In nature, chitin-chitosan exists in the exoskeleton of crustaceans, insects, and fungi. Chitin-chitosan is known for the antimicrobial activity,[1] biodegradability,[2-3] biocompatibility,[4-5] and non-toxicity.[6] Chitosan is a derivative of chitin obtained from chitin deacetylation and the amino group content obtained is more than 70%. Comparing to chitin, chitosan has received much more attention as it has the reactive amino group together with hydroxyl group for functionalization and it is soluble in acid aqueous solutions. In recent years with a rapid progress in instrumentation, the studies on chitosan at molecular level and the consequence nano-structure have received much attention. The advanced researches on the scaffolds for tissue engineering,[7-8] nanoparticles for drug carrier,[9-11] drug targeting,[12] vaccines[13-14] and genes[15-16] delivery system, are actively carrying out.

It is important to note that in modifying chitosan for those advanced applications, effective chemical reactions have to be considered. For chitosan, the chemical modification is difficult due to the lack of solubility in water and most of organic solvents as a consequence form its strong inter and intramolecular hydrogen bonds network. Most reactions of chitosan have to be done in heterogeneous system or in some specific acid solutions, and this limits the satisfactory of the substitution degree and/or the variety of the reactions. In addition, there might be the cases where using chitosan in acid aqueous solutions involves the side reactions between the acids and the reactants, especially the reactions using the coupling or conjugating agent such as carbodiimide derivatives. Various derivatives to improve chitosan solubility either in water or organic solvents, e.g. *O*-carboxymethylchitosan[17] or *N*-carboxymethylchitosan,[18] *N*-phthaloylchitosan,[19] chitosan-g-PEG,[20] etc., have been developed and the potential applications including the further functionalization for the uses in biomedical and pharmaceutical fields were variously reported.[21,25-27]

As we found a unique but simple way to dissolve chitosan in water, i.e. the mixture of chitosan and HOBt, we then aim to show how the chitosan-HOBt aqueous

system is useful for the functionalization of chitosan. It is known that HOBt is a conjugating additive, here, we expect that the system is practical for conjugation of chitosan with the functional molecules, e.g. drugs, protein, genes, etc., via the hydroxyl, amine, and carboxyl groups, by using the water-soluble conjugating agent. The advantages of chitosan-HOBt are also related to the most problems related to the functionalization of chitosan such as the uses of organic solvents, the heterogeneous conditions, and the harsh conditions. In addition, as the system is carrying out in water, it provides us the chitosan derivatives without the trace amount of solvents.

Based on this viewpoint, herein, we demonstrate the conjugation of chitosan using WSC in water at room temperature via the carboxyl groups of a series of model compounds, i.e. boc-L-phenylalanine, mPEG, and dicarboxylated PEG.

## 2. Experimental

### 2.1. Materials

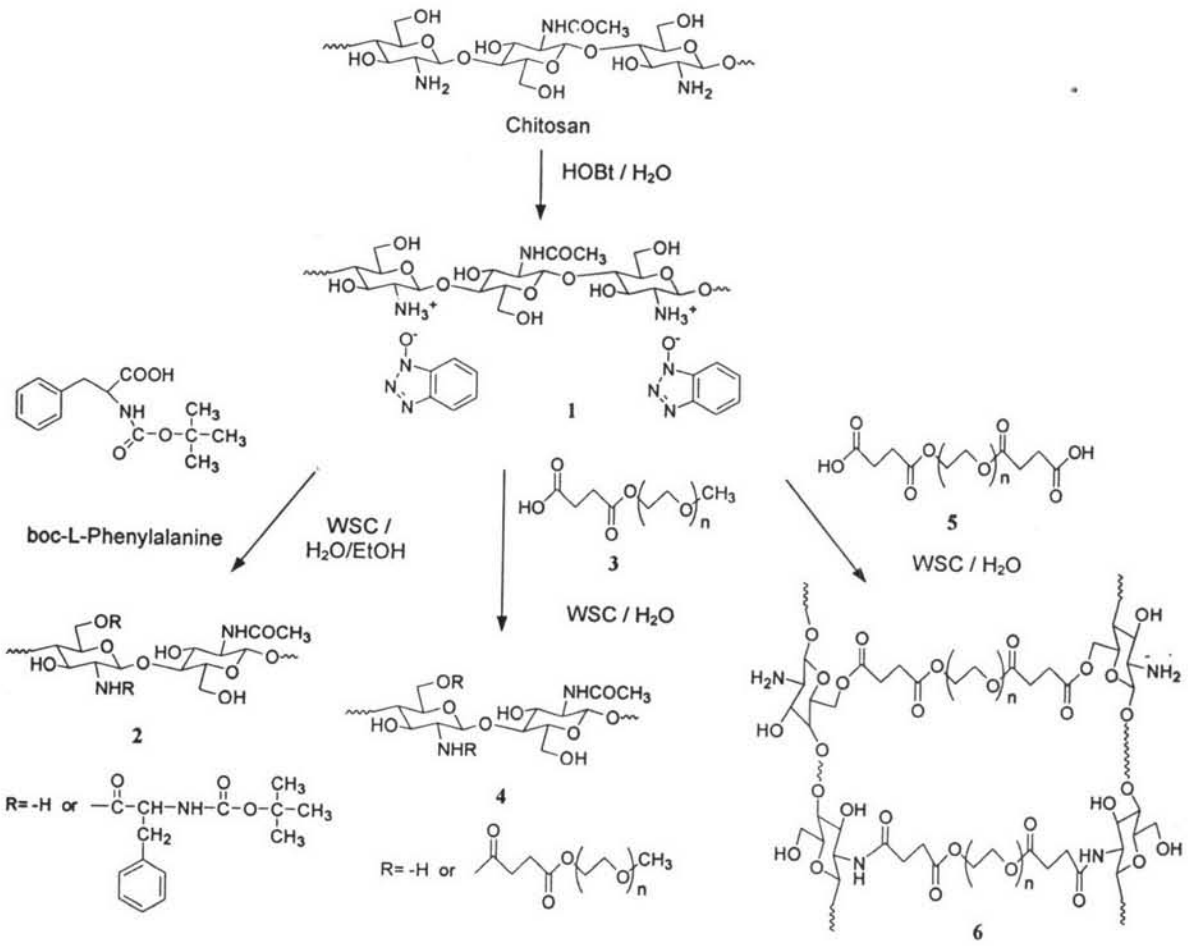
Chitosan (95%DD, $M_v$ of $5.6\times10^5$) was supplied from Seafresh Chitosan (Lab) company limited, Thailand. 1-Hydroxybenzotriazole monohydrate (HOBt·$H_2O$) and 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide) hydrochloride (EDC·HCl) or water-soluble carbodiimide hydrochloride (WSC·HCl) were purchased from Wako Pure Chemical Industries Co. Ltd., Japan. *N*-(tert-butoxycarbonyl)-L-Phenylalanine (bPhe) was obtained from Tokyo Kasei Kogyo Co., Ltd., Japan. Succinic anhydride was provided from Fluka Chemika, Switzerland. Poly(ethylene glycol) methyl ether (mPEG, $M_n$ 2000 Da) and poly(ethylene glycol) (PEG, $M_n$ 1450 Da) were purchased from Sigma-Aldrich, Inc., USA. All chemicals were used without further purification.

### 2.2. Instruments

Fourier transform infrared (FTIR) spectra were performed using the attenuated total reflection (ATR) accessory equipped on a Perkin Elmer Spectrum 1. The analysis was carried out with 32 scans at a resolution of 4 $cm^{-1}$ over the frequency range of 4000-400 $cm^{-1}$. Proton nuclear magnetic resonance ($^1H$ NMR) spectra were obtained from a 400 MHz JEOL JNM-GSX spectrometer. Viscometry

measurements were recorded on a Brookfield Model DV-III Programmable Rheometer using a spindle head number 27 at 100 rpm.

**Scheme 1**




2.3. Chitosan-HOBt aqueous solution, **1** (Scheme 1)

Chitosan (0.1 g, 0.61 mmol) was vigorously stirred with twice moles of HOBt·$H_2O$ (0.188 g, 1.22 mmol) to that of chitosan in deionized water 10 mL at ambient temperature until the clear solution, **1**, was obtained.

2.4. Synthesis of chitosan-boc-L-phenylalanine, **2** (Scheme 1)

Solution **1** was mixed with boc-L-phenylalanine (0.156 g, 0.61 mmol) in ethanol (20 mL) followed by adding the solution of WSC·HCl in ethanol (0.235 g,

1.22 mmol, 10 mL) dropwise. The reaction was carried out at ambient temperature for overnight. The crude product was dialyzed and lyophilized to obtain **2** for 98% yield.

ATR-FTIR ($cm^{-1}$): 1657 (amide I), 699 (benzene ring).

$^1H$ NMR ($CD_3COOD/D_2O$, ppm): 1.15 (H-a), 2.70 (H-b), 4.21 (H-c), 7.00-7.40 (H-d), 1.89 (H-Ac), 2.99 (H-2 of pyranose ring), 3.20-4.00 (H-3 to H-6 of pyranose ring).

2.5. Synthesis of chitosan-*graft*-mPEG, **4** (Scheme 1)

Carboxyl terminated poly(ethylene glycol) methyl ether (mPEG-COOH), **3**, was prepared as reported previously.[21] In brief, mPEG ($M_n$ 2000, 20.00 g, 10 mmol) was reacted with succinic anhydride (1.001 g, 10 mmol) in the presence of a catalytic amount of pyridine at 60°C for 24 hours. The mixture was reprecipitated in diethyl ether, washed several times with diethyl ether and dried *in vacuo* to obtain **3**, for 61% yield.

To the solution of **1** (10 mL), **3** (1.287 g, 0.61 mmol, 10 mL) and WSC·HCl (0.235 g, 1.22 mmol, 10 mL) were added with distilled water. The reaction was allowed at ambient temperature for overnight. The solution obtained was concentrated and poured into acetone to obtain the crude product. The product was thoroughly washed by acetone and ethanol for several times, followed by drying *in vacuo* to obtain **4** for 60% yield.

ATR-FTIR ($cm^{-1}$): 2870 (CH stretching), 1732 (C=O ester), 1650 (amide I), 1559 (amide II).

$^1H$ NMR ($D_2O$, ppm): 3.02 (H-a), 3.78-4.35 (H-b and H-3 to H-6 of pyranose ring), 3.73 (H-c), 2.40 (H-Ac), 4.98 (H-1 of pyranose ring), 3.25 (H-2 of pyranose ring).

2.6. Synthesis of PEG-crosslinked chitosan, **6** (Scheme 1)

PEG ($M_n$ 1450, 20.00 g, 13.8 mmol) was reacted with succinic anhydride (2.7622 g, 27.6 mmol) in the presence of a catalytic amount of pyridine at 60°C for 24 hours. The crude product was purified by reprecipitating in diethyl ether, washing

for several times and drying *in vacuo* to obtain carboxyl terminated poly(ethylene glycol) (COOH-PEG-COOH), **5,** for 55% yield.

To the solution of **1** (10 mL), **5** (0.479 g, 0.305 mmol, 10 mL) and WSC·HCl (0.235 g, 1.22 mmol, 10 mL) were added with distilled water. The reaction was carried out at ambient temperature to obtain gel. The crude gel was dialyzed and lyophilized to obtain **6** for 40% yield.

ATR-FTIR ($cm^{-1}$): 2870 (CH stretching), 1732 (C=O ester), 1650 (amide I), 1560 (amide II).

$^1H$ NMR ($CD_3COOD/D_2O$, ppm): 2.95 (H-a), 3.78-4.35 (H-b and H-3 to H-6 of pyranose ring), 2.33 (H-Ac), 5.18 (H-1 of pyranose ring), 3.48 (H-2 of pyranose ring).

## 3. Results and Discussion

### 3.1. Chitosan-HOBt aqueous solution, 1

It is important to note that by simply mixing chitosan with HOBt in water, chitosan is completely dissolved even at room temperature. This indicates the organic complexation between two. Here, $^1H$ NMR and ATR-FTIR were applied to confirm the complex. Figure 1A 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. Figure 1B demonstrates the chitosan and HOBt for the mole ratio of 1:2 (equimolar to pyranose ring). The peaks of chitosan appear at 1.87 ppm (H-Ac), 2.83 ppm (H-2), and 3.2-4.0 ppm (H-3 to H-6). The integral of H-Ac peak indicates the acetyl group for 5%, or in other words the degree of deactylation is 95%. The peaks at 7.3 and 7.6 ppm refer to the benzene protons of HOBt at H-b and H-a, respectively. Comparing Figure 1A to Figure 1B, the shifts of the benzene protons in HOBt indicate the complex formation with chitosan as organic salt, **1**, demonstrated in Scheme 1.

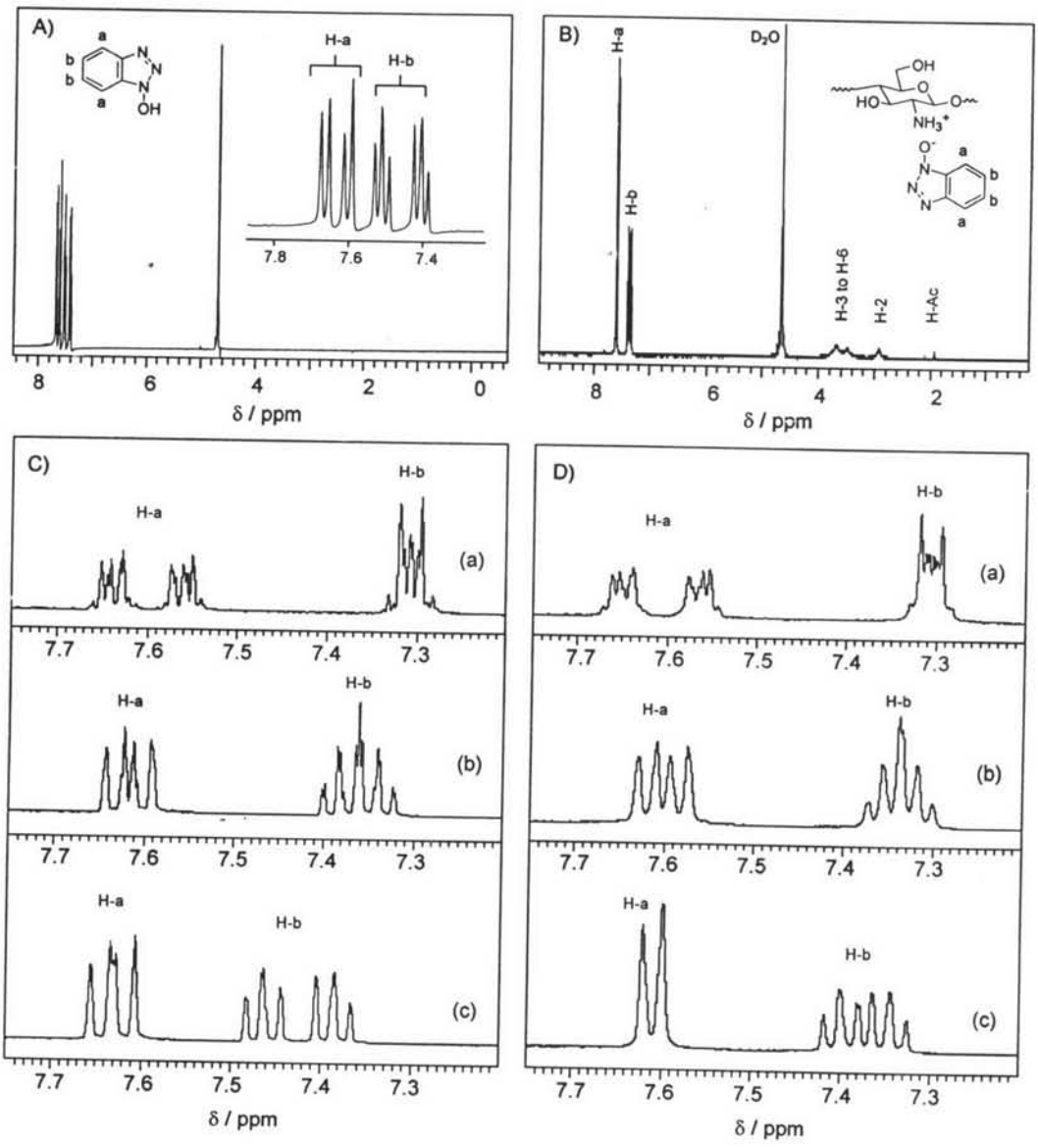
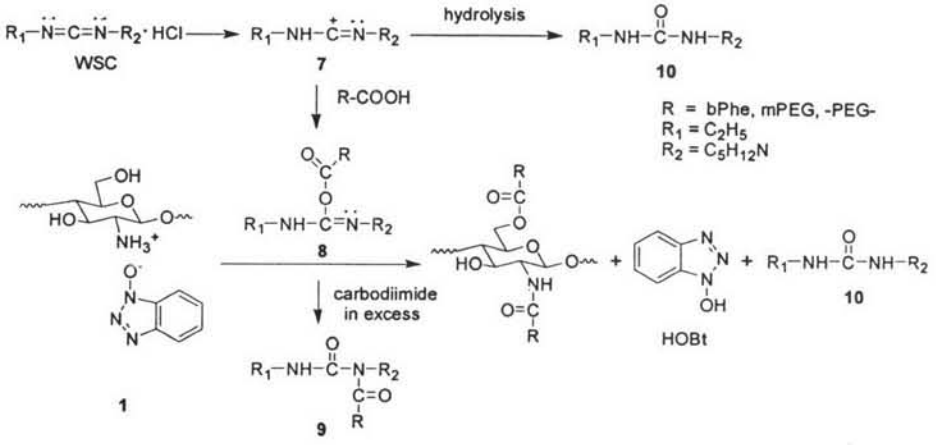



**Figure 1.** $^{1}H$ NMR spectra of A) HOBt in $D_2O$ (pH=2.8), B) chitosan-HOBt with the mole ratios of chitosan:HOBt for 1:2, C) HOBt with NaOD at pH of: (a) 4.9, (b) 4.4, (c) 3.8; and D) chitosan-HOBt with the mole ratios of chitosan:HOBt for: (a) 1:1 at pH 4.9, (b) 1:1.5 at pH 4.3, (c) 1:2 at pH 4.0; in $D_2O$ at room temperature.

It is important to note that the pH for chitosan-HOBt in Figure 1B was 4.0. In order to confirm the complexation and observe its stability, the $^1$H NMR of HOBt in various pHs and chitosan-HOBt with various mole ratios were comparatively studied. A series of $^1$H NMR spectra belonging to the HOBt solution with various pHs by adding NaOD in $D_2O$ are shown in Figure 1C. The solution of HOBt in $D_2O$ originally gives the pH for 2.8 (Figure 1A). At that time the peaks of the aromatic protons appear with the triplet peaks at 7.41 and 7.51 ppm, and doublet peaks at 7.60 and 7.66 ppm. After adding an amount of NaOD to adjust the pH to be 4.9 (a), 4.4 (b), and 3.8 (c), the peaks of the aromatic protons are significantly shifted and split in different patterns as shown in Figure 1C. This implied that the HOBt and NaOD form acid-base interaction and each condition initiates the change in chemical shifts of H-a, and H-b. Figure 1D shows $^1$H NMR spectra of the mixture of chitosan and HOBt in $D_2O$ with the mole ratio of chitosan:HOBt for (a) 1:1, (b) 1:1.5, and (c) 1:2. At these points, the pH of each was (a) 4.9, (b) 4.3, and (c) 4.0, respectively. It should be noted that the benzene protons (H-a and H-b) of chitosan-HOBt aqueous solution (Figure 1D (a) to (c)) shows the similar chemical shift to HOBt (Figure 1C (a) to (c)) at the same pH range. This implied that HOBt and chitosan form a type of salt, and the acid-base interaction is dependent on the molar ratio.

**Scheme 2**




Here, the amino group of chitosan might be protonated by HOBt to form **1** in aqueous solution (Scheme 1). The mixture of chitosan and HOBt at 1:2 mole ratio

was thus freeze-dried for characterization by ATR-FTIR. As shown in Figure 2, chitosan (curve (a)) shows a peaks at 1646 $cm^{-1}$ (amide I), 1589 $cm^{-1}$ (amide II), whereas **1** (curve (b)) gives a peak at 1534 $cm^{-1}$ implying the protonated form of $NH_3^+$. In similar, chitosan with 1-hydroxy-7-azabenzotriazole (HOAt) or *N*-hydroxysuccinimide (HOSu) also dissolves well in water. Although carboxylic acids such as formic and acetic acids are the good solvents for chitosan, the unpleasant odor and the side reactions of reactants with solvent molecules are the points to be aware of. As HOBt, HOAt, and HOSu are the conjugating additives, they initiate the conjugation and allow the reaction of chitosan in water when they combine with water soluble conjugating agents. The mechanism showing how chitosan-HOBt effectively conjugated with R-COOH by using water-soluble carbodiimide (WSC) is summarized in Scheme 2. A carbodiimide molecule is protonated by HCl co-existing in the reagent to form carbocation, **7**, then, reacted with R-COOH to form *O*-acylisourea, **8**. In the case of unless carboxyl group is present, **7** will be hydrolyzed by water into the urea derivative, **10**. If carbodiimide is in excess, the react ion with **8** gives *N*-acylurea, **9**. Here, the use of HOBt in combination with WSC is to reduce **9** and improve the reaction rate. The conjugation is possibly at either C-2 or C-6 to obtain amide or ester linkages. At the end of the reaction, the urea by-product and HOBt were regenerated and they were removed by washing thoroughly with good solvent, i.e. ethanol and acetone.

### 3.2. Synthesis of chitosan-boc-L-phenylalanine, **2**

Up to now, the synthesis of polymers with amino acid pendants has been reported with various purposes, for example, poly(D,L-lactic-co-glycolic acid) conjugated with *N*-(9-fluorenylmethoxycarbonyl-*N*-*tert*-butoxycarbonyl-L-tryptophan (Fmoc-Trp(Boc)) for drug controlled release model,[22] chitosan with different amino acids (glycine, L-lysine, L-isoleucine, and L-glutamic acid) for enhancing the heavy metal ions adsorption,[23] and chitosan having L- and D-phenylalanine spacer arms for polymer-supported aymmetric reducing agents.[24] As phenylalanine is known as an essential amino acid involving with the nervous system, the conjugation of phenylalanine can be expected for the controlled release

with the specific targeting. Here, boc-L-phenylalanine was selected as a model compound for conjugation via chitosan-HOBt system (Scheme 1). The reaction was carried out in homogeneous system at room temperature for overnight with WSC and the reaction was followed by ATR-FTIR. As shown in Figure 2 (c), after the reaction proceeded for overnight, the characteristic peak C=O belonging to amide I (1657 $cm^{-1}$) was significant and C-H (symmetric out of plane) bending of benzene ring (699 $cm^{-1}$) was identified. The $^{1}H$ NMR operating at room temperature was further analyzed to confirm the structure of **2** and evaluate the boc-L-phenylalanine substitution. Figure 3 (a) shows the chemical shift corresponding to H-a at 1.15 ppm, H-b at 2.70 ppm, H-c at 4.21 ppm, H-d at 7.00-7.40 ppm, H-3 to H-6 belonging to pyranose ring at 3.20-4.00 ppm, and H-2 at 2.99 ppm. The substitution degree was calculated by using the integration of H-2 as an internal standard peak. Two calculations based on H-a and H-d protons were done to find the same substitution degree at for 28%.

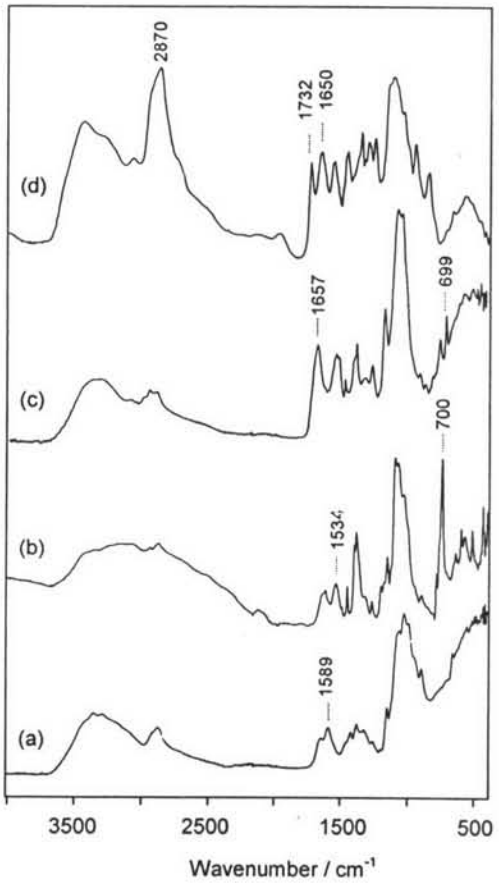



**Figure 2.** ATR-FTIR spectra of (a) chitosan, (b) **1**, (c) **2**, and (d) **4**.

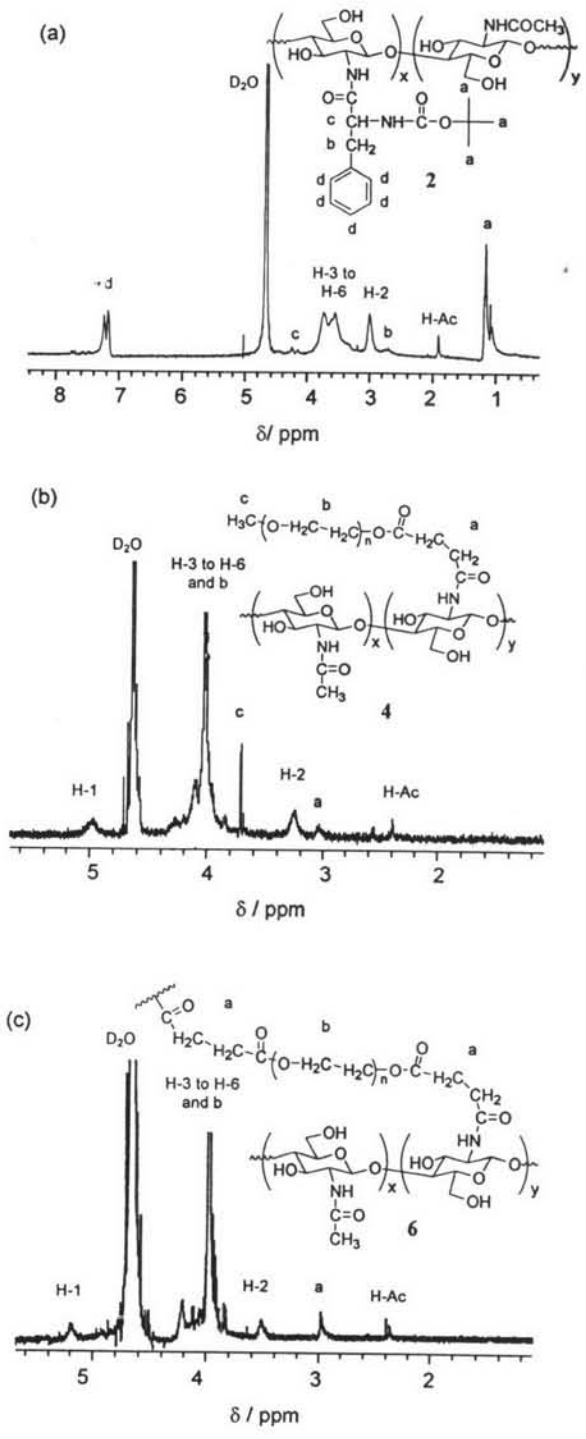



**Figure 3.** $^1$H NMR spectra of (a) **2** in $CD_3COOD/D_2O$ at room temperature, (b) **4** in $D_2O$ at 70 °C, and (c) **6** in $CD_3COOD/D_2O$ at 70 °C.

3.3. Synthesis of chitosan-*graft*-mPEG, **4**

As chitosan grafted PEG is one of the important derivatives satisfying the requirements for drug carriers,[25-27] thermoreversible hydrogel,[28] etc., various preparations of chitosan-*graft*-PEG have been reported. However, the reactions are related to the multiple steps reaction, the needs to priory change of chitosan to an intermediate, the use of organic or acid solvents, the harsh conditions, etc., and as a result, more studies are in progress. Here, the use of chitosan-HOBt aqueous solution to conjugate the PEG chain by using WSC might be one of the possibilities. The one-pot reaction in aqueous at ambient temperature by adding mPEG-COOH in an equimolar to pyranose ring with WSC is simple and practical. Here, after the reaction of **1** and **3** (Scheme 1) was carried out for overnight, the product was purified by washing thoroughly with ethanol and acetone to remove unreacted mPEG-COOH, urea by-products and HOBt. As shown in Figure 2 (d), the ATR-FTIR spectrum of **4** with the new peak at 1732 $cm^{-1}$ for ester group, the increase of the peak at 1650 $cm^{-1}$ for amide I, and the significant increase of the peak at 2870 $cm^{-1}$ for CH stretching implies the success of the reaction. The $^1H$ NMR carried out at 70°C confirms the structure of **4** (Figure 3 (b)) from the chemical shifts corresponding to H-a at 3.02 ppm, H-c at 3.73 ppm, H-Ac at 2.40, H-1 of pyranose ring at 4.98 ppm, H-2 of pyranose ring at 3.25 ppm. The H-b is overlapped with those of H-3 to H-6 of pyranose ring at 3.78-4.35 ppm. The quantitative analysis for the degree of substitution (DS) of mPEG onto the chitosan chain was determined by using H-1 as an internal standard peak. The DS was found to be 36% based on the H-c integration. In addition, when the amount of mPEG-COOH was as high as 2 moles of chitosan, the substitution was found to reach 42%. Comparing **4** to chitosan grafting with PEG-aldehyde reported by Sugimoto *et al.* (1998),[20] our system gives a comparable degree of substitution together with the water-solubility.

3.4. Synthesis of PEG-crosslinked chitosan, **6**

Chitosan hydrogels have received much attention for a variety of biomedical applications, i.e. drug delivery,[29] and tissue engineering.[30] However, we have to aware of the contamination of the solvents or dialdehyde crosslinker including its

toxicity to the bio-system. The crosslink in water using chitosan-HOBt might be a good way to produce gel without the problems about removing organic solvents. Here, dicarboxylated poly(ethyleneglycol) was applied as it provides two functional groups for the crosslinking. The reaction was carried out in water at ambient temperature to give **6** (Figure 4 (a)). Gel **6** was dialyzed and freeze-dried before analyzing by ATR-FTIR. The product obtained showed the peaks similar to that of **2**. The important peaks were at 2870, 1732, and 1650 $cm^{-1}$ corresponding to CH stretching, C=O ester, and amide I, respectively. Although it is difficult to confirm the structure of the gel material by NMR, an attempt to characterize the structure of **6** by swelling it in $CH_3COOD/D_2O$ was done as it shown higher swelling property in this solvent than in pure water. This might be due to the free amino groups of chitosan is protonated by acetic acid and repulse each other to obtain higher swelling property. The $^1H$ NMR spectrum of **6** at 70°C (Figure 3 (c)) shows H-a at 2.95 ppm, and H-b overlapped with H-3 to H-6 of pyranose ring at 3.78-4.35 ppm.

In order to investigate the gelation time, the viscosity was traced by Brookfield viscometer. A constant low-viscosity for the first 5-7 minutes was observed (Figure 4 (b)). After that, a rapid increase in viscosity was seen to give a gel onset at 10 minutes. It should be noted that **6** shows a reversible gelation since it doesn't show any cracking after removing water, and the re-swelling was occurred homogeneously. This information is useful when we consider the gel for reversible purpose.

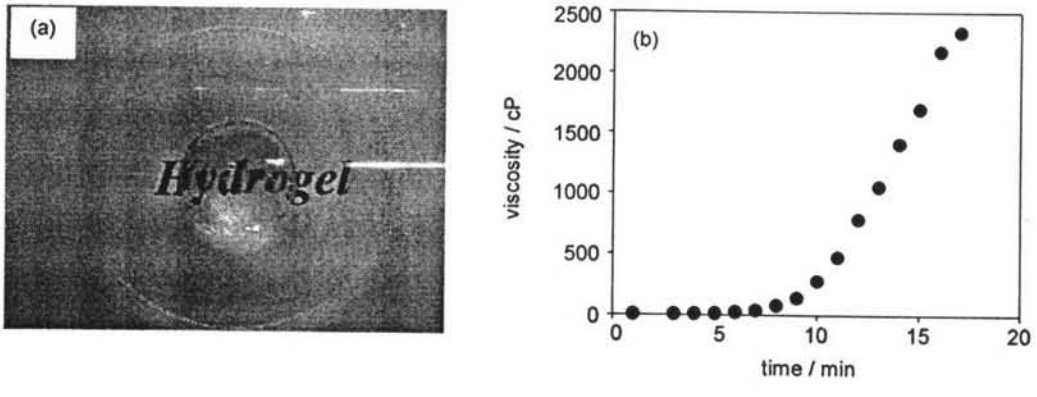



**Figure 4.** (a) Gel **6** and (b) viscosity and gelation time during the gelation of **6**.

## 4. Conclusion

Chitosan forms an organic salt with HOBt in water as identified by $^1$H NMR. The aqueous systems of chitosan-HOBt gave a successful conjugation at room temperature by using WSC conjugating agent. The conjugation of chitosan with the carboxyl group was demonstrated by using the model compounds of boc-L-phenylalanine, mPEG, and dicarboxylated PEG. The reactions were successful based on the qualitative and quantitative analyses by ATR-FTIR and $^1$H NMR. The degree of substitution of boc-L-phenylalanine was as high as 28% whereas that of mPEG was up to 42%. In the case of dicarboxylated PEG, the chitosan performed the gelation in 10 minutes. The present work demonstrated an effective conjugation of chitosan in simple conditions, i.e. in water at room temperature, using the water soluble chitosan-HOBt aqueous solution, and WSC conjugating agent.

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