CHAPTER IV

ONE-POT SYNTHESIS IN AQUEOUS SYSTEM FOR WATER-SOLUBLE CHITOSAN-GRAFT-POLY(ETHYLENE GLYCOL) METHYL ETHER

Abstract

Chitosan is functionalized with poly(ethylene glycol) methyl ether (mPEG) at the amino and hydroxyl groups via a single step reaction in a homogeneous aqueous system. A chitosan aqueous solution obtained from the mixture of chitosan and hydroxybenzotriazole (HOBt) in water is a key factor in providing mild conditions to conjugate mPEG by using a carbodiimide conjugating agent. The reaction at ambient temperature for 24 hours gives chitosan-g-mPEG with water solubility with mPEG content as high as 42%. This work demonstrates that a water-soluble chitosan-HOBt complex is an effective system for the preparation of chitosan derivatives via the aqueous system without the uses of acids or organic solvents.

Keyword: Chitosan; One-pot synthesis; Poly(ethylene glycol); Aqueous system; Carbodiimide

1. Introduction

Chitin-chitosan (Scheme 1) 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 with specific properties on biodegradability,[1-2] biocompatibility,[3-4] and non-toxicity.[5] From the structural viewpoint, it has reactive hydroxyl and amino groups for further derivatization. As chitin-chitosan is not soluble either in water or in organic solvents except aqueous acids, the applications, especially, on biomedical fields are very limited. Up to now, modifying chitosan to water soluble derivatives,[6-8] lowering the molecular weight to oligomers,[9-11] and conjugating chitosan with drugs,[12] vaccines,[13] and genes[14] have been challenging.

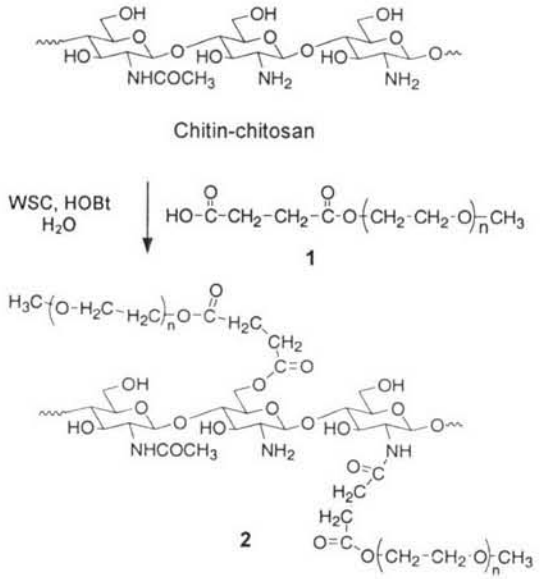
It should be noted that although poly(ethylene glycol) (PEG) is a synthetic polymer, it is known for its uses in pharmaceutical and biomedical fields.[15] In recent years, the grafting of PEG onto chitosan has received much attention as an alternative approach to achieve water soluble derivatives with the requirements satisfied for drug carriers,[16] thermoreversible hydrogel,[17] etc. Previously, Harris *et al.* reported the reaction of chitosan with PEG-aldehyde via the reduction by sodium cyanoborohydride ($NaCNBH_3$) in a mixture of aqueous acetic acid and methanol. Although the reaction was successful in acetic acid, the products showed low water solubility with the degree of substitution (%DS) approximately 5-6%.[18] Sugimoto *et al.* modified the procedures by gradually adding $NaCNBH_3$ to avoid precipitation. The DS obtained was improved to 37% and the product showed an increase in water-solubility.[19] Ouchi *et al.* reported the use of 6-*O*-triphenylmethyl-chitosan in mPEG conjugation to achieve chitosan-g-PEG with DS from 10 to 55% and solubility in organic solvents and water.[20] Mao *et al.* synthesized trimethyl chitosan (TMC)-g-PEG in DMSO to obtain the substitution of PEG for as high as 89%. The TMC-g-PEG copolymers were completely water-soluble over the entire pH range even though the graft density was as low as 10%.[16]

Although there are many pathways to obtain chitosan-g-PEG, the points on the multiple steps reaction,[20] the needs to priorly change chitosan to other derivatives beforehand,[16,20] the use of organic or acid solvents with harsh conditions,[16-21]

products with poor water solubility,[18] including the difficulty in controlling the substitution degree,[18] etc., are still in further study.

It should be noted that as we found a unique but simple way to dissolve chitosan in water, i.e. the mixture of chitosan and HOBt, we then challenged the reactions of chitosan in an aqueous system. Herein we demonstrate an effective pathway to achieve water-soluble chitosan-*graft*-poly(ethylene glycol) methyl ether (chitosan-g-mPEG) by using a single step reaction at room temperature in an aqueous system. The one-pot synthesis is under mild conditions to be carried out in water with no organic solvents required. This assures us that the chitosan derivative obtained performs its safety for the potential biomedical applications.

Scheme 1



2. Materials and Methods

2.1. Chitosan-*graft*-poly(ethylene glycol) methyl ether

Chitosan (95%DD, M_v of 5.6×10^5) was supplied from Seafresh Chitosan (Lab) Company Limited, Bangkok, Thailand. Carboxyl terminated poly(ethylene glycol) methyl ether (mPEG-COOH), **1**, was prepared as reported previously.[22] In brief, poly(ethylene glycol) methyl ether (mPEG, M_n 2000, 20.00 g, 10 mmol) was reacted with succinic anhydride (1.0008 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 for two times with diethyl ether, and dried in vacuo to obtain **1**.

Chitosan (0.25 g, 1.53 mmol) was stirred with 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) (0.4693 g, 3.06 mmol) in deionized water 20 mL until a clear solution was obtained. Compound **1** (3.2183 g, 1.53 mmol) and deionized water (15 mL) was added to the chitosan solution followed by the addition of an aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide) hydrochloride (EDC·HCl) (0.5875 g, 3.06 mmol, 15 mL) (Scheme 1). The reaction was carried out in ambient temperature for 24 hours. The water was removed and the solution obtained was poured into acetone to obtain the crude gel. The gel was thoroughly washed by acetone and ethanol for several times, followed by drying *in vacuo* to obtain **2d** for 60% yield. Compounds **2a-2f** were prepared by varying the ratios of chitosan : mPEG-COOH : HOBt : EDC as detailed in Table 1.

2.2. Instruments

Qualitative and quantitative Fourier transform infrared spectra (FTIR) analyses were obtained from a Bruker Equinox 55/S (Ettlingen, Germany) with 32 scans at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed by using a deuterated triglycinesulfate detector (DTGS) with a specific detectivity, D*, of 1×10^9 $cm.Hz^{1/2}$ w^{-1}. An Opus/IR spectroscopic software (version 3.0) was used for the FTIR curve fitting. Proton neuclear magnetic resonance (^1H NMR) spectra were obtained from a Varian Mercury-400BB spectrometer (Palo Alto, CA, USA) using D_2O at room temperature and 70°C for **3** and **2**, respectively. The transmittance was measured by a Perkin Elmer UV-VIS spectrometer Lambda 16 (Norwalk, CT, USA) in the range of visible light (500-600 nm) to evaluate the solubility in water of the derivatives.

3. Results and Discussion

The reactivity of mPEG was enhanced by activating the chain end with a carboxyl group via the esterification on the hydroxyl group of mPEG with succinic anhydride. As methyl-terminated PEG has only single side for esterification, the

crosslink network with chitosan formed via esterification with diacid groups can be avoided. In order to graft mPEG-COOH onto the chitosan chain in an aqueous system, EDC, a water-soluble carbodiimide (WSC), and HOBt were used.

3.1. Chitosan-HOBt in aqueous system

It is known that chitosan barely dissolves in organic solvents but in acid aqueous solutions. Acetic acid is recognized as a good solvent for chitosan and gives the successful reactions such as the reaction of chitosan with aldehyde to produce Schiff-base or with anhydride in acetylation, etc. However, there might be cases where using chitosan in acetic acid solution involves the side reactions between the reactants and acetic acid, especially the reactions using coupling or conjugating reagents, e.g. 1,3-dicyclohexylcarbodiimide (DCC), carbonyldiimidazole (CDI), etc. Nishimura *et al.*[23] developed organo-soluble chitosan, such as *N*-phthaloyl-chitosan and 6-*O*-triphenylmethyl-chitosan to provide homogeneous reactions which satisfy the coupling and conjugating reactions.[20,22]

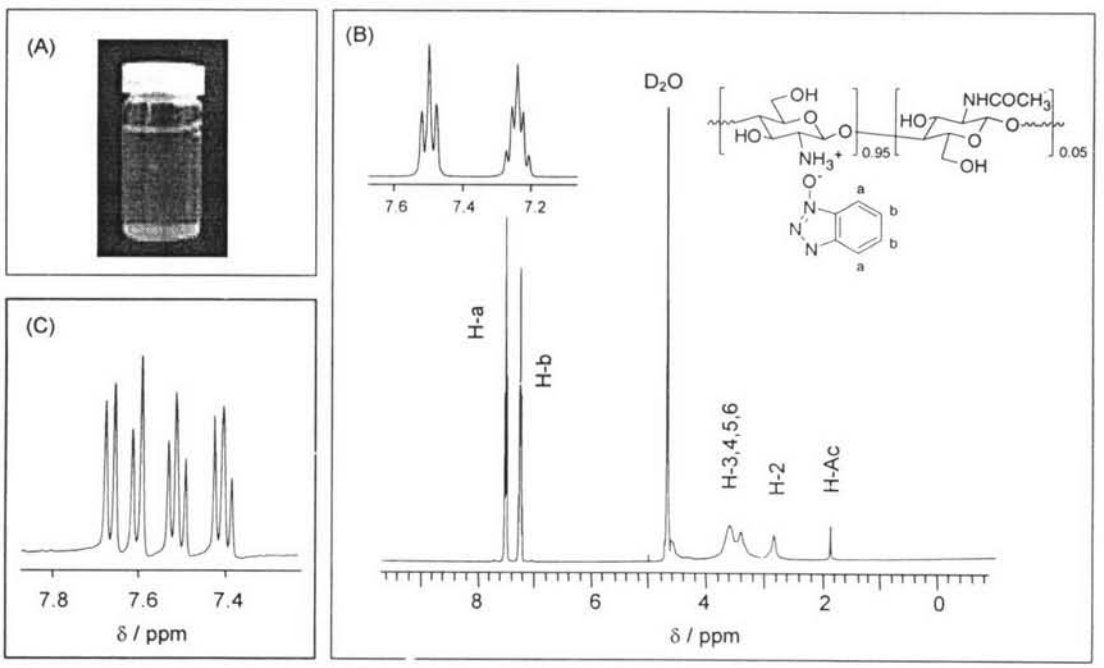


Figure 1. (A) Appearance of HOBt and chitosan (2:1 molar ratio) in water, (B) ^{1}H NMR spectrum of HOBt and chitosan (2:1 molar ratio), and (C) ^{1}H NMR spectrum of HOBt in D_2O at room temperature.

It should be noted that HOBt is difficult to dissolve in water similar to chitosan, but, to our surprise, when HOBt and chitosan were mixed, especially at 2:1 molar ratio, the mixture became transparent (Figure 1 (A)). At that time, the pH of the solution was found to be ~ 4.4. This indicates organic complexation between the two. Here, ^{1}H NMR was applied at room temperature to investigate the complex. Figure 1 (B) shows the peak of chitosan at 1.87, 2.83, 3.2-4.0 ppm for the protons of acetamide group (H-Ac), proton of pyranose ring at H-2, and H-3 to H-6, respectively. The CH_3 of the acetamide group indicates the acetyl group is 5%, or in other words the degree of deacetylation is 95%. The pentet peaks at 7.24 ppm and triplet peak at 7.50 ppm refer to the benzene protons of HOBt at H-b and H-a, respectively. It should be noted that when the ^{1}H NMR was carried out at room temperature, the peak at δ=4.98 ppm (H-1 in pyranose ring) is hard to observe which might be due to the high viscosity of the solution. Figure 1 (C) shows the typical ^{1}H 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. Comparing Figure 1 (B) to 1 (C), the shifts of the benzene protons indicate the complexation of chitosan-HOBt. The proposed structure may be ascribable to the salt formation between the basic chitosan and the acidic HOBt as **3** in Scheme 2.

As chitosan is dissolved well in water with HOBt to form **3**, this homogeneous solution provides the conjugation with mPEG-COOH on the amino and hydroxyl groups of the chitosan in water. The success of the reaction will offer the advantage for the preparation of chitosan derivatives via an aqueous system without the use of organic solvents when we focus on the chitosan derivative for bio-system purposes.

3.2. Conjugation of chitosan with mPEG in aqueous system

From the reaction mechanism (Scheme 2), EDC is protonated by HCl co-existing in the reagent to form carbocation, **4**. Compound **4** is further reacted with mPEG-COOH to form *O*-acylisourea, **6**. In our reaction, EDC was added dropwise into the solution of chitosan-HOBt and mPEG-COOH so as to avoid the instability of EDC which can be hydrolyzed to form the corresponding urea, **5**. If EDC is in

excess, the react ion with **6** gives *N*-acylurea, **7**. Here, the use of HOBt in combination with EDC is to reduce **7** and improve the reaction rate.[24] The conjugation is possibly at either C-2 to obtain amide or C-3 and C-6 to obtain ester linkages.[24] For C-3, although the hydroxyl group is reactive because of the fact that it has steric hindrance with its secondary hydroxyl species, we omit the possibility of the reaction at C-3 for ease of discussion. In the next step, the urea by-product **5** and HOBt were regenerated. In our reaction, they were removed by washing thoroughly with good solvents, i.e. ethanol and acetone.

Scheme 2

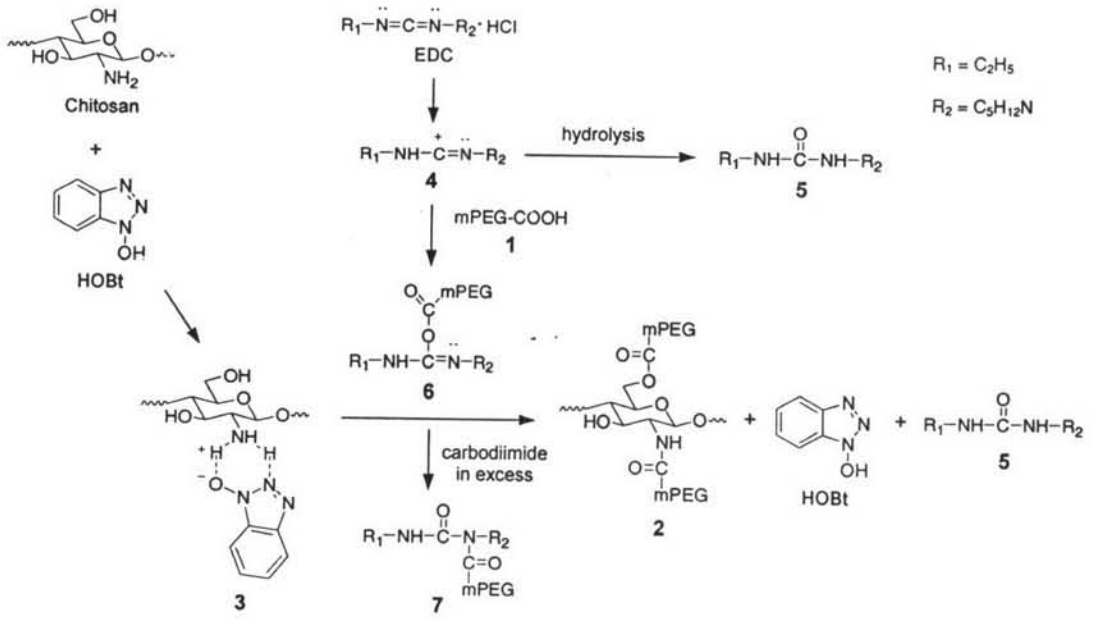


Figure 2 represents the results of **2** in the case of the condition **2d**. As shown in Figure 2 (A), chitosan (curve (a)) shows the peaks at 1650 cm^{-1} (amide I), 1594 cm^{-1} (amide II), and 899 cm^{-1} (pyranose ring). For **2d** (curve (b)), the new peak at 1735 cm^{-1} for the ester group and the increase of 1650 cm^{-1} for the amide bond and the significant 2871 cm^{-1} for CH stretching indicate that the substitution of mPEG at C-2 or C-6 of chitosan to obtain **2** was successful. The ^1H NMR (Figure 2 (B)) at 70 °C confirms the chemical shifts corresponding to H-c at δ=4.05 ppm, H-d at δ=3.73

ppm, H-b at δ=3.02 ppm, H-2 of pyranose ring at δ=3.25 ppm, H-3 to H-6 of pyranose ring at δ=3.78-4.35 ppm, and H-1 of pyranose ring at δ=4.98 ppm.

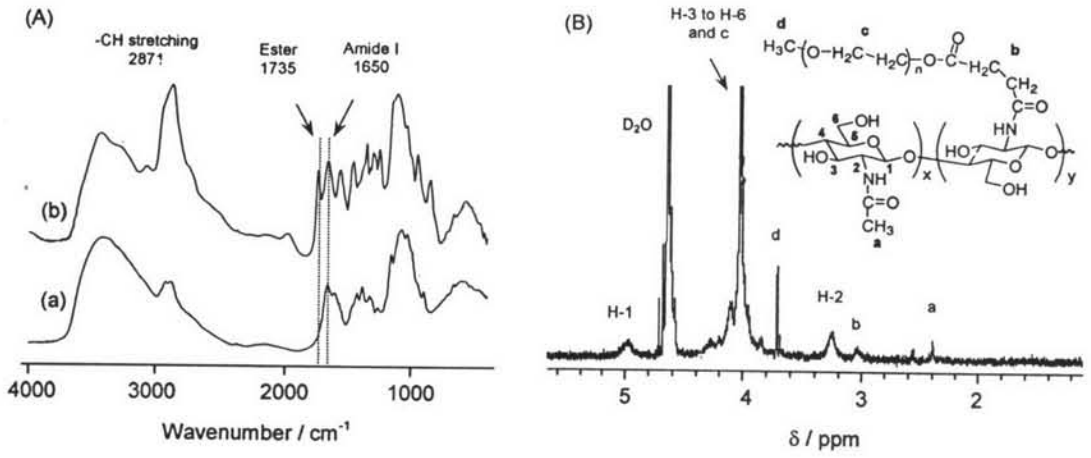


Figure 2. (A) FTIR spectra of (a) chitosan and (b) **2d**, (B) ^{1}H NMR spectrum of **2d** in D_2O at 70°C.

Here, the quantitative analysis by ^{1}H NMR to determine the degree of substitution (DS) of mPEG onto the chitosan chain was also carried out. The integral peaks for H-d and H-1 of the pyranose ring were the peaks of interest. In the analysis step, the integration of H-1 (pyranose ring) was set to 1 and assumed that the conjugation of mPEG occurred at C-2. In the case of Figure 2 (B), the integral ratio of H-d to H-1 was 1.084/1 and to determine the degree of substitution it is necessary to divide again by 3 due to the fact there are three H atoms in the methyl group. This confirms that **2d** obtained shows the mPEG substitution for 36%. The mole ratio of EDC, HOBt, mPEG-COOH to chitosan were varied to clarify the optimal conditions. Table 1 summarizes the degree of substitution of mPEG for **2a-2f**. When only the mole ratio of mPEG-COOH increased (**2a-2c**), the %DS was found to increase. However, the substitution was saturated at 19% (**2c**) even the mole ratio of mPEG-COOH was increased to 1 mole equivalent to pyranose ring. The substitution was increased significantly (from 19% to 36%) in the case of adding HOBt and EDC for twofold chitosan and mPEG-COOH. When the reaction was reduced from 24 hours (**2d**) to 5 hours (**2e**), the substitution was found to decrease slightly. This condition

(**2e**) might be considered as an effective condition in conjugating mPEG by this one-pot synthesis method. In the case of **2f**, when the amount of mPEG-COOH was increased for 2 times of chitosan, the substitution was further increased to 42%.

Table 1 Degree of substitution and yield of **2a-2f**

Sample code	Mole ratio of Chitosan:mPEG-COOH:HOBt:EDC	DS [a, b] (%)	Yield (%)
2a	1: 0.1 : 1 : 1	10	80
2b	1: 0.5 : 1 : 1	19	73
2c	1: 1 : 1 : 1	19	47
2d	1: 1 : 2 : 2	36	60
2e	1: 1 : 2 : 2 [c]	30	44
2f	1 : 2 : 2 : 2	42	43

[a] all substitutions are assumed at only C-2
[b] as determined from ^{1}H NMR
[c] the reaction time is 5 hours.

From the structural viewpoint, mPEG-COOH has the possibility to react with either C-2 or C-6 to result in the amide and ester linkages as indicated in Scheme 1. It is important to note that the structure of mPEG-COOH also consists of an ester group which brings the ester peak in FTIR, and, as a result, simple observation from FTIR spectra is difficult to determine whether the ester peak is from mPEG-COOH or from the esterification at C-6 position. Here, in order to investigate how significant the amidization or esterification was, FTIR quantitative analysis by using the integral peaks of C=O ester (1735 cm^{-1}) and amide I (1650 cm^{-1}) was carried out. If the conjugation was satisfied only at C-2, the peak intensity ratio of ester/amide might be constant for all %DS. Figure 3 (B) identifies the curve fitting of each spectrum from Figure 3 (A) using OPUS software. Figure 3 (C) shows the peak ratio of ester (1735 cm^{-1})/amide (1650 cm^{-1}) related to the %DS obtained from the ^{1}H NMR. It is clear that the peak ratio is not constant, but increases with an increase of %DS. The slope of the curves can be divided into two levels. At initial (for DS 10%-19% or **2a-2b**), the increase in the peak ratio to %DS is not as significant as the ones above 30% DS (**2d-2f**). This implies that although the substitution might occur at both positions, the substitution at C-6 drastically

increased as the feed amount of mPEG increased. At present, ^{13}C NMR studies to follow the reaction on C-2 and C-6 are in progress.

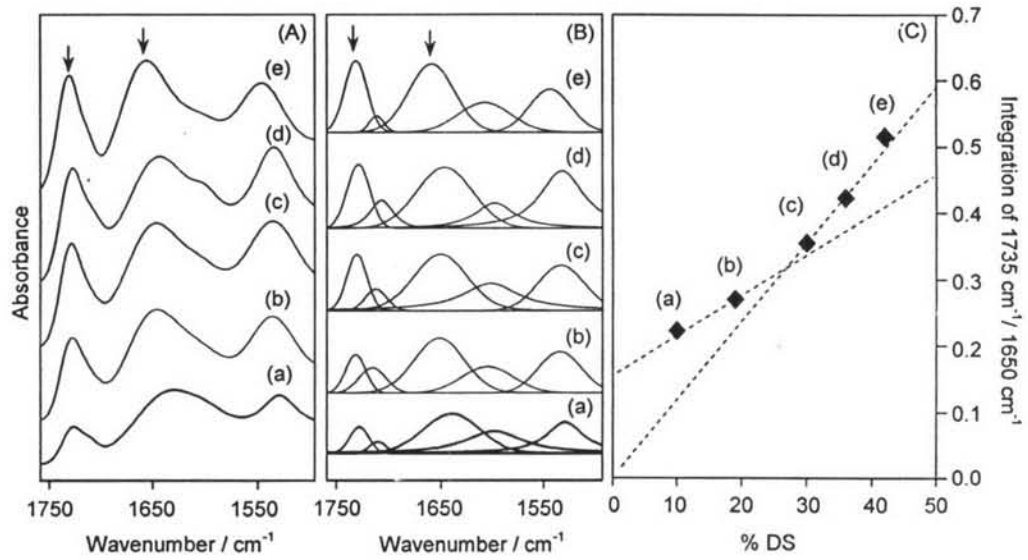


Figure 3. (A) FTIR spectra of (a) **2a**, (b) **2b**, (c) **2d**, (d) **2e**, (e) **2f**, (B) curve fitting of the spectra in (A), and (C) plots of the integral ratio of ester peak at1735 cm^{-1} to amide peak at 1650 cm^{-1} relating to %DS.

3.3. Solubility

As chitosan-g-mPEG is a potential material for biomedical applications, its solubility in various solvents, especially aqueous-based ones, is important. Here, compounds **2a-2f** were dissolved in water (4 mg/mL) to observe the transmittance. The results show that the transmittance was over 90% for all of the samples implying the dissolution of **2a-2f**. However, when mPEG substitution is higher (as **2d-2f**), the compounds show the swelling before dissolving. The pH was also varied to evaluate the solubility of **2a-2f** in water. It was found that all derivatives were soluble in the range of pH 2~10. Table 2 summarizes the average solubility of **2a-2f** in a series of solvents differing in their polarity indices. Compounds **2a-2f** show good solubility in high polar solvents, i.e. water; good swelling in methanol and chloroform, and partial swelling in ethanol and acetone.

Table 2 Evaluation of solubility in various solvents relating to polarity index

Solvents	Polarity index[a]	Appearance[b]
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 Snyder (1974)[25]
[b] + soluble; ± swelled; - partial swelled.

4. Conclusion

Chitosan-PEG is a potential material for biomedical and pharmaceutical purposes. The present work reported an effective pathway to synthesize the water-soluble chitosan-g-mPEG in an aqueous system via a one-pot homogeneous reaction at room temperature. The aqueous reaction condition is achieved from the unique chitosan-HOBt complex in water. The chitosan-g-mPEG showed the %DS as high as 42%. The compounds show good solubility in water for pH 2~10, swelling in methanol and chloroform; which is a useful guideline for further studies in applying chitosan-g-mPEG for drug carriers, etc.

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