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Preparation and spectroscopic characterization of methoxy poly(ethylene glycol)-grafted water-soluble chitosan

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Abstract—The object of this study was to test the solubility of a methoxy poly(ethylene glycol) (MPEG)-grafted chitosan copolymer in organic solvents and aqueous solution. Water-soluble chitosan with low molecular weight (LMWSC) was used in a PEG-graft copolymerization. The MPEG was conjugated to chitosan using 4-dicyclohexylcarbodimide (DCC), and *N*-hydroxysuccimide (NHS). Introduction of PEG was confirmed by ^{1}H and ^{13}C NMR spectroscopy and FT-IR spectroscopy. The degree of substitution (DS) of MPEG into chitosan was calculated from ^{1}H NMR data and also by estimating the molecular weight (MW) using gel permeation chromatography (GPC). The DS values obtained from ^{1}H NMR spectroscopy and GPC were similar, indicating that MPEG-grafted LMWSC was synthesized and properly characterized. Furthermore, the introduction of PEG into chitosan increases the solubility in aqueous solutions over a range of pH values (4.0–11.0) and organic solvents such as DMF, DMSO, ethanol, and acetone.

Keywords: Water-soluble chitosan; Poly(ethylene glycol); Aqueous solubility; Organic solvent solubility; Graft copolymer

1. Introduction

Chitosan is a natural polysaccharide derived from chitin by deacetylation. Because chitosan is regarded as a biocompatible, biodegradable, non-toxic, and cationic polymer, it has been extensively investigated in biotechnological, biomedical, and environmental fields.[1–4] In particular, chitosan has been reported to enhance drug delivery across the nasal or mucosal layer without damage.[5] The cationic properties of chitosan offer valuable properties for drug delivery systems, gene delivery systems, and tissue engineering, that is, the formation of ion complexes between chitosan and anionic drugs or DNA can be used as a delivery vehicle.[6–8] However, acidic solutions are required to improve the aqueous solubility of chitosan. Because many biomolecules, for example, DNA, protein or peptide drugs, and anticancer drugs are unstable in acidic solutions, the poor aqueous solubility of chitosan under physiological conditions and in organic solvents reduces its potential in the biomedical field.

To overcome these drawbacks, various derivatives of chitosan have been developed.[9–15] Among them, PEGylation of chitosan was reported by several authors to increase its aqueous solubility.[8,9,16–18] Ouchi et al. reported that PEGylation of chitosan resulted in increased solubility in aqueous solutions or organic solvents, and led to self-aggregation properties in aqueous solution. The PEGylation of chitosan derivatives is also favorable for improving biocompatibility and increasing cell viability compared to trimethyl chitosan.[17] Other candidates for the modification of chitosan is to graft poly(L-lactide) or polycaprolactone to give an amphiphilic molecule, that is, a hydrophilic chitosan domain and a hydrophobic polyester domain.[19,20] Recently, nanoparticles of chitosan-g-polyester with

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particle sizes below 100 nm have been prepared and these materials showed the self-assembling properties of copolymers.[19,20]

In the previous report, we demonstrated that water-soluble chitosan with a low molecular weight (abbreviated as LMWSC) has promise as a drug or DNA carrier due to its good aqueous solubility and increased numbers of free amino groups.[21,22] Because LMWSC has a high content of free amino groups in the chitosan chain, it is distinguished from other kinds of chitosan by increased affinity to DNA or anionic drugs.[21] Furthermore, PEG-graft LMWSC can be used as an excellent drug or gene carrier.[8,23] Hu et al.[24] reported that PEG-grafted chitosan has increased solubility in aqueous solution and organic solvents such as dimethylformamide (DMF) and dimethylsulfoxide (DMSO). However, they did not show aqueous solubility at various pH values or in other organic solvents. Increased solubility in aqueous solution at a range of pH values, and in organic solvents, will increase the application of chitosan derivatives in biomedical field.

In this study, we synthesized methoxy PEG-grafted LMWSC (abbreviated as ChitoPEG) and characterized the material using FT-IR spectroscopy, ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, and GPC. Furthermore, the solubility of ChitoPEG was investigated in aqueous solution at various pH ranges and in organic solvents such as DMF, DMSO, acetone, acetonitrile, and ethanol. The attachment of PEG to chitosan will increase its solubility and in turn its ability to be processed and applied. The introduction of PEG into chitosan chain has been previously studied,[9,17,18,24] but its full characterization has not yet been achieved.

2. Experimental

2.1. Materials

Water-soluble chitosan with a low molecular weight (MW = 10,000 Da, deacetylation degree = 97.0%, abbreviated as LMWSC) was a gift from Chittolife Co. Ltd, Seoul, Korea. Water-insoluble chitosan was purchased from Wako Pure Chemical, Co. Ltd, Japan (chitosan 10, abbreviated as IS-chitosan). Methoxy poly(ethylene glycol) (MPEG) (MW = 2000 g/mol) was purchased from SunBio Co. Korea, *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), succinic anhydride, dimethylformamide (DMF), dimethylsulfoxide (DMSO), diethylether, methylene chloride (CH_2Cl_2), ethanol, acetonitrile (ACN), and acetone as HPLC grade were purchased from Sigma Co. Ltd, USA. DMSO, deuteriochloroform ($CDCl_3$), deuterium oxide (D_2O), DCl, and NaOD were purchased from Aldrich Co., USA. Dialysis tubing (Molecular weight cut-off (MWCO) = 12,000 g/mol) was purchased from SpectraPor Co. Ltd, USA. Before use, the dialysis tubing was treated with hot water (100 °C) for 30 min and then washed with tap water for 3 h.

2.2. Synthesis of MPEG–NHS

To synthesize the copolymer, the hydroxyl group of MPEG was modified to a carboxylic acid and converted to the NHS-activated form as shown in Figure 1. MPEG was dissolved in dry CH_2Cl_2 and an excess of succinic anhydride was added to this solution. This solution was heated at reflux for 3 h at 60 °C. The resulting

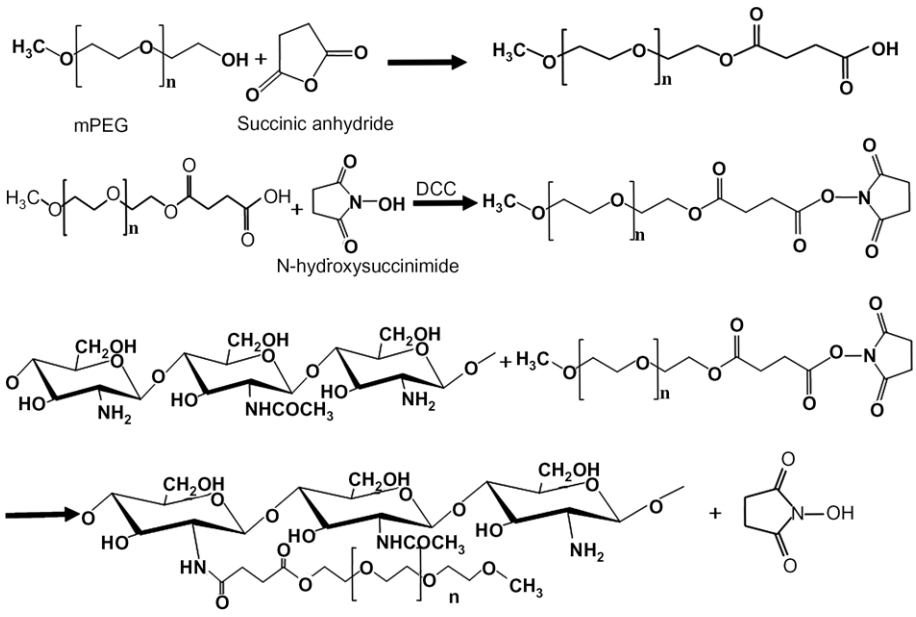

Figure 1. Synthesis of the ChitoPEG copolymer.

solution was precipitated by diethyl ether and filtered. The white powder thus obtained was dried in vacuum oven for 3 days. The dried material was dissolved in deionized water and dialyzed against deionized water for 2 days to remove unreacted succinic anhydride and the solution was lyophilized for 3 days. The yield of MPEG–COOH was greater than 98% as confirmed by ^{1}H NMR spectroscopy. MPEG–COOH was activated to the NHS form as follows: MPEG–COOH was dissolved in dry CH_2Cl_2 and 1.2 equiv of DCC–NHS was added. This reaction was carried out for 6 h and the solution was then filtered. The product was precipitated with diethyl ether, harvested by filtration, and then vacuum dried for 3 days. The resulting product, the NHS-activated form of MPEG (MPEG–NHS), was used in the graft copolymerization with chitosan. The yield of NHS activation was higher than 97% as determined by ^{1}H NMR spectroscopy.

2.3. Synthesis of ChitoPEG copolymer

The synthesis of the ChitoPEG graft copolymer (Fig. 1) was carried out as follows: 100 mg of LMWSC was dissolved in 0.2 mL of deionized water and diluted with 9.8 mL of DMSO. A solution of MPEG–NHS dissolved in 2 mL of DMSO was added and the mixture was kept overnight under a nitrogen atmosphere. The resulting solution was dialyzed for 2 days against an excess of deionized water followed by lyophilization. The lyophilized solid was resuspended three times in excess CH_2Cl_2 to remove unreacted MPEG–NHS and then fractionated into deionized water followed by lyophilization.

2.4. Measurement using gel permeation chromatography (GPC) for determination molecular weight of ChitoPEG graft copolymer

The absolute molecular weight and MW distribution, represented as the polydispersity index (PD), of the MPEG-g-chitosan copolymers were measured using a gel permeation chromatograph equipped with a multi-angle laser light scattering detector (GPC-MALLS, 18 angle detector, Wyatt, USA), as previously reported by Son et al.[12] The samples were dissolved in 0.5 M ammonium acetate buffer (pH 5.5, at more than 5 different concentrations ranging from 0 to 1.0 mg/mL) and the change in the reflective index (dn/dc) was measured by means of a Pot-LAB reflectometer (Wyatt, USA). Then, the absolute MW and MW distribution of the ChitoPEG copolymer were obtained from the GPC chromatogram with light scattering data (Debye plot regressions). The mobile phase was 0.5 M ammonium acetate buffer (pH 5.5) and the flow rate was 0.5 mL/min. The injection volume was 0.2 mL (10 mg/mL). The standard for determination of copolymer MW was poly(ethylene glycol) (PEG).

2.5. ^{1}H NMR, ^{13}C NMR, and FT-IR spectroscopy

The ^{1}H and ^{13}C NMR spectra of the copolymer and polymeric micelle were measured in D_2O or DMSO (*d*-form) using a 400 MHz NMR spectrometer (AVANCE 400FT-NMR 400 MHz, Bruker). The chemical structure of WSC and chitoPEG copolymer was analyzed by FT-IR spectroscopy (Shimadzu, FT-IR 8700, Osaka, Japan).

2.6. Solubility test

The solubility of the ChitoPEG copolymer, was carried out by dissolving 5 mg of LMWSC or ChitoPEG copolymer in 0.1 mL of deionized water followed by the addition of 4.9 mL of an aqueous solution or organic solvent (1.0 mg/mL). The aqueous solutions employed were phosphate-buffered saline (PBS), pH 7.0 and 7.4; acetate buffer, pH 4.0; HCl solution, pH 2.0; carbonate buffer, pH 9.0. The organic solvents used were DMF, DMSO, ethanol, acetonitrile, and acetone. To determine the solubility of the ChitoPEG graft copolymer, the turbidity was measured using a UV-spectrophotometer at 600 nm (UV-1601, Shimadzu Co. Ltd, Osaka, Japan).[25]

2.7. Zeta potential measurement

For zeta potential measurements, LMESC and ChitoPEG copolymers were dissolved in deionized water (1.0 mg/mL) and measured at a wavelength of 632.8 nm at 25 °C with an ELS-8000 electrophoretic LS spectrophotometer (NICOMP 380 ZLS zeta potential/particle sizer, Otsuka electronics Inc., Japan) equipped with a He–Ne laser beam (scattering angle of 90°).

3. Results and discussion

3.1. Characterization of ChitoPEG graft copolymer

The introduction of MPEG to chitosan was performed using the DCC–NHS system. Because one end of MPEG contains a methoxy group and the other end a hydroxyl group, activation is required before the starting of copolymerization. First, the hydroxyl end of MPEG was converted to a carboxylic acid (yield >98%) and this group was then activated using DCC–NHS system (yield of NHS-ester: >97%) as shown in Figure 1. The yield of the conversion of the hydroxyl group to a carboxylic group was calculated by the integration of the peak ratio between methoxy group (Fig. 2c, 3.31 ppm—peak 1) and the peak specific to succinic acid (Fig. 2c, 2.95 ppm—peak 9). The conversion yield of the carboxylic acid to NHS-activated form

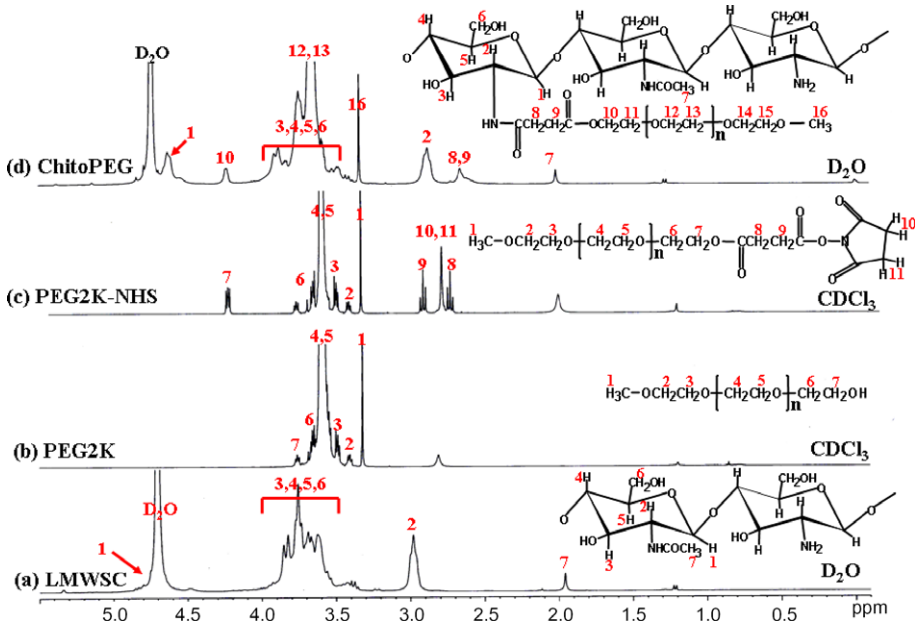

Figure 2. ^{1}H spectra of MPEG, MPEG–NHS, LMWSC 10 K, and ChitoPEG copolymer in D_2O.

was estimated from the integration ratio between methoxy group peak (Fig. 2c, 3.31 ppm—peak 1) and the specific peaks of NHS (Fig. 2c, 2.80 ppm—peaks 10 and 11).

The activated form of MPEG was used in a graft copolymerization with chitosan. The chitoPEG copolymer comprises a non-ionic hydrophilic MPEG side chain and a cationic chitosan main chain as shown in Figure 1. To characterize the ChitoPEG copolymers, MPEG, MPEG–NHS, LMWSC 10 K, and ChitoPEG each was dissolved in D_2O and subjected to analysis by NMR spectroscopy. As shown in Figure 2, specific peaks of MPEG are present between 3.5 and 3.7 ppm. In the activated form, MPEG–NHS, the peaks of carboxylic group and NHS group are present between 2.5 and 3.0 ppm (peak numbers 8–11). The peaks specific to chitosan (LMWSC 10 K) appeared between 1.8 and 5.0 ppm. In the ChitoPEG copolymer, peak assignments were as follows: H1, 4.6 ppm; H2, 2.9–3.0; H3–H6, 3.5–4.0 ppm; the methyl group of MPEG appeared about ~3.7 ppm. Figure 3 shows the ^{13}C NMR spectrum of the ChitoPEG copolymer. The MPEG peaks appeared between 55 and 80 ppm and those for the carboxylic group and NHS groups were present at 25–30 ppm and 168–172 ppm, respectively. The peaks for chitosan (LMWSC 10 K) appeared at 100 ppm (C1), 57 ppm (C2), 72–78 ppm (C3), and 61–62 ppm (C6). In the ChitoPEG copolymer, peaks associated with LMWSC 10 K and MPEG both appeared. The ^{1}H–^{13}C HSQC map of LMWSC 10 K and the ChitoPEG copolymer is shown in Figure 4. The peak assignments and shifts of LMWSC 10 K (Fig. 4a) were as follows: C1, 100 ppm; C2, 57 ppm; C3, 72 ppm; C4, 78 ppm; C5, 76 ppm; C6, 61–62 ppm and acetyl group, 23–24 ppm. In the ChitoPEG copolymer (Fig. 4b), C1 (100 ppm) and C2 (57 ppm) appeared clearly, but C3–C6 of LMWSC 10 K overlapped with the methyl group of MPEG. The acetyl group appeared at ~23 ppm.

Four different kinds of ChitoPEG copolymer were synthesized, each having a different content of MPEG side chains, as summarized in Table 1. The higher MW ChitoPEG copolymer was synthesized by increasing the amount of activated MPEG. To remove unreacted MPEG, the resulting copolymer was purified by precipitation with DCM, as MPEG is freely soluble in DCM, while chitosan is insoluble in it. The substitution degree (DS) of MPEG to the chitosan was estimated from the integration ratio between H1 (4.6 ppm) and acetyl group (1.9–2.0 ppm) of chitosan and the methyl group of MPEG (3.6–3.7 ppm). The DS of MPEG was calculated using the integration ratio between H1 position/acetyl group of chitosan and the methyl group of MPEG. This approach allowed us to calculate the DS of MPEG and the molecular weight of chitoPEG copolymer. We aimed to produce material with a MPEG substitution of 5, 10, 15, and 20 mol %, and the resulting DS calculated from ^{1}H NMR spectroscopy was 4.6, 9.4, 14.7, and 19.1 mol %, respectively. As shown in the GPC results in Figure 5, the retention time of MPEG and LMWSC 10 K appeared at ~34 min and 30 min, respectively. As the substitution degree of MPEG increased, the retention time of the ChitoPEG copolymer gradually decreased. The estimated MW of the ChitoPEG copolymer from GPC is also summarized in Table 1. These results show that the DS of MPEG from GPC is similar to the value obtained from ^{1}H

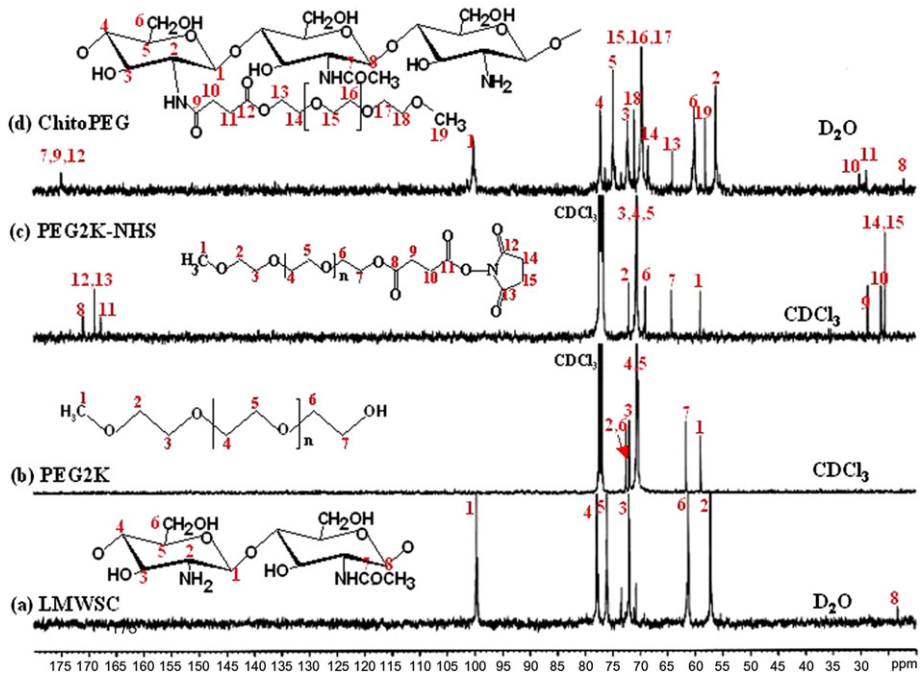

Figure 3. ^{13}C NMR spectra of MPEG, MPEG–NHS, LMWSC 10 K, and ChitoPEG copolymer in D_2O.

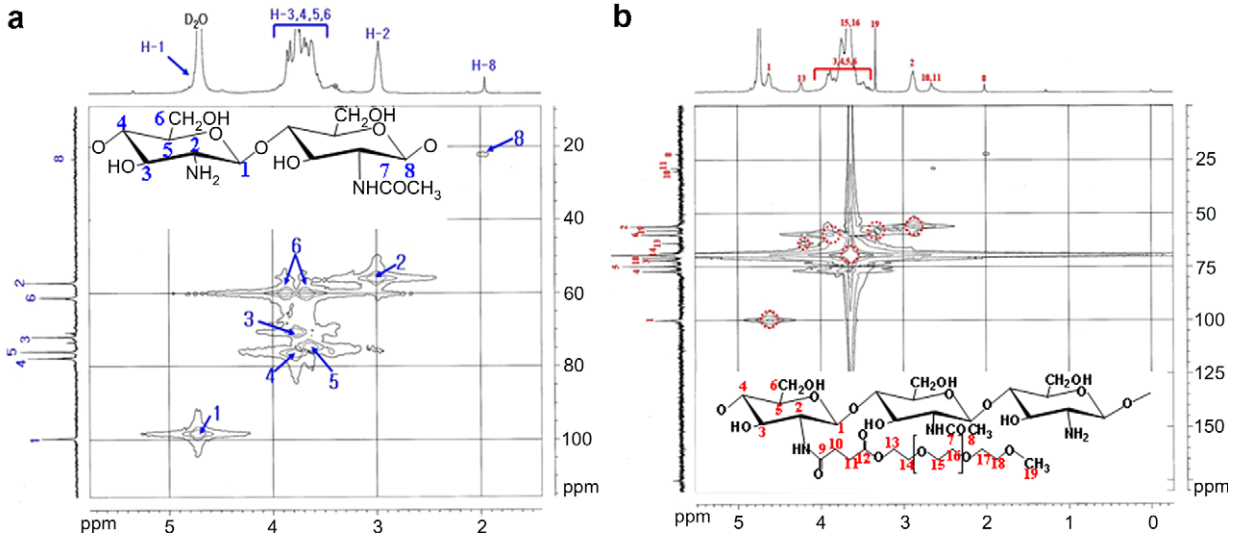

Figure 4. C–H single bond correlation (HMQC) of (a) LMWSC 10 K and (b) ChitoPEG copolymer.

Table 1. Characterization of ChitoPEG copolymers

	MPEG feeding ratio (mol %)	MW by GPC			DS of MPEG* (mol %)	
		M_n	M_w	Polydispersity	NMR[a]	GPC[b]
Chitosan	—	9435	11,830	1.253 ± 0.615		
ChitoPEG-1	5	14,530	17,920	1.233 ± 0.383	4.6	4.3
ChitoPEG-2	10	19,910	25,640	1.287 ± 0.597	9.4	8.9
ChitoPEG-3	15	24,680	31,360	1.271 ± 0.492	14.7	13.0
ChitoPEG-4	20	31,140	39,280	1.261 ± 0.372	19.1	18.5

[a] Degree of substitution (DS) of MPEG was evaluated as follows: $DS = \frac{\text{Proton integration ratio of methyl group of MPEG}/3}{\text{Proton integration ratio of H1 of chitosan}+(\text{Proton integration ratio of acetyl group of chitosan}/3)} * 100$.

[b] DS of MPEG evaluated by GPC was as follows: $DS = \frac{(M_n\text{of ChitoPEG copolymer by GPC} - M_n\text{of chitosan})/2000}{\text{Degree of polymerization(DP)of Chitosan}} * 100$.

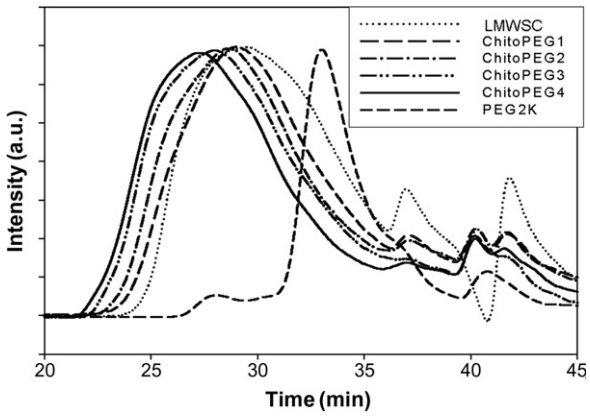

Figure 5. GPC chromatogram of ChitoPEG copolymer.

NMR spectroscopy. Figure 6 shows the FT-IR spectra of the ChitoPEG copolymers, which also confirmed the introduction of the PEG. In the ChitoPEG copolymer, absorption peaks associated with MPEG appeared at ~840, 950, 2850 cm^{-1}. From these combined results, it is clear that MPEG introduction was successfully accomplished and that the DS of MPEG was similar to the theoretical value.

3.2. Solubility of ChitoPEG copolymer into water and organic solvent

Commercial water-insoluble chitosan was used to compare the water and the organic solubility of LMWSC 10 K and the ChitoPEG copolymers. The solubility of chitosan was measured by the transparency of a solution using a UV spectrophotometer. A higher solution transparency indicates the higher solubility of chitosan and its derivatives while a low transparency reflects aggregation or precipitation. As shown in Figure 7, the transparency of IS-chitosan solutions gradually decreased from high at pH 6.0 to practically insoluble at pH higher than 7.4. On the other hand, solutions of LMWSC 10 K maintained good transparency at all pH ranges. Furthermore, solutions of all ChitoPEG copolymers showed good transparency at all pH ranges even if the transparency of ChitoPEG 10 was slightly decreased. Figure 8 shows the solubility of chitosan and its deriva-

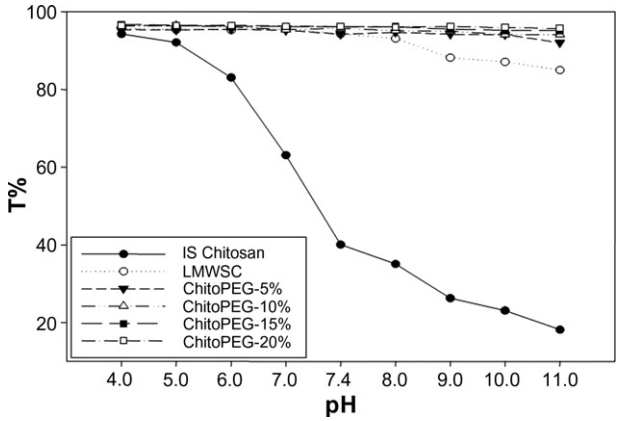

Figure 7. Solubility of ChitoPEG copolymer in the aqueous solution as a function of pH changes in the buffer solution.

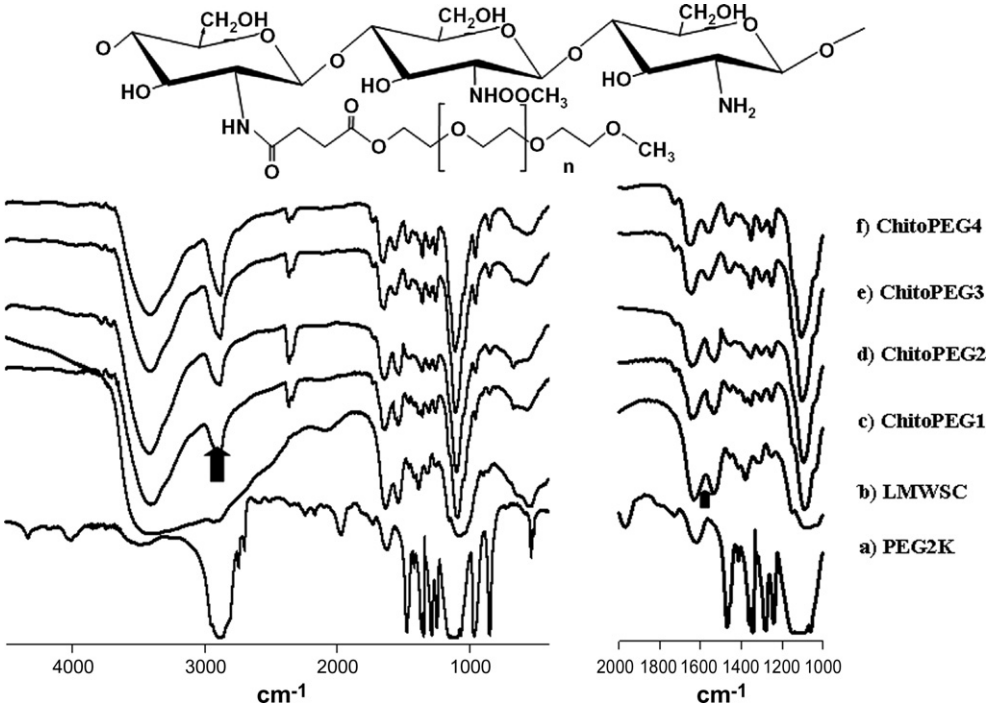

Figure 6. FT-IR measurements of MPEG, MPEG–NHS, LMWSC 10 K, and ChitoPEG copolymer.

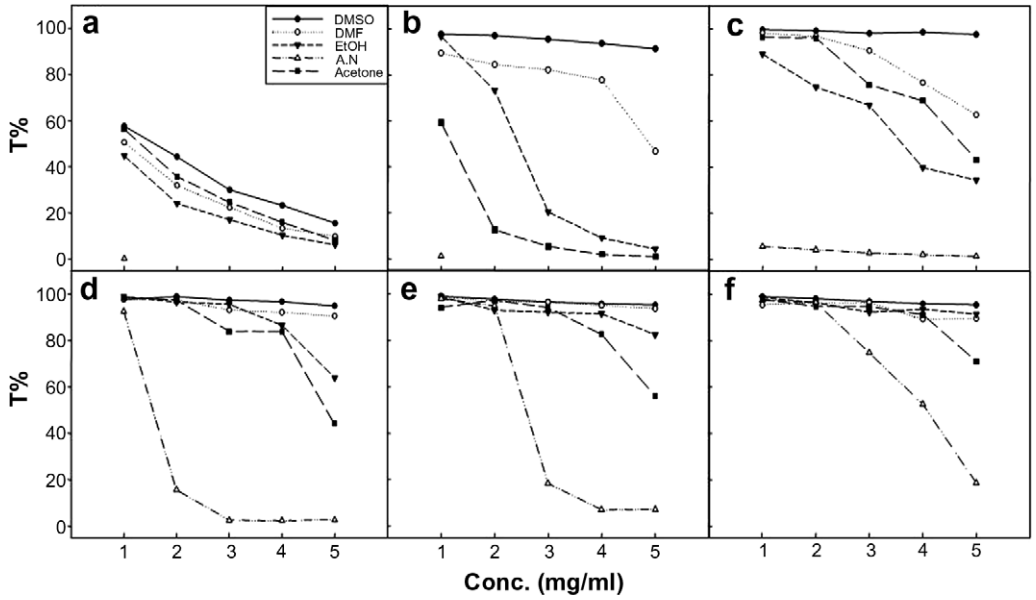

Figure 8. Solubility of ChitoPEG copolymer in organic solvents. (a) IS-chitosan, (b) LMWSC 10 K, (c) ChitoPEG-5, (d) ChitoPEG-10, (e) ChitoPEG-15, and (f) ChitoPEG-20.

tives in various organic solvents. The transparency of IS-chitosan was lower than 60% in all organic solvents. The transparency of IS-chitosan solutions gradually decreased with an increase in chitosan. In practice, IS-chitosan is insoluble in any solvent other than aqueous acid. On the other hand, solutions of LMWSC 10 K at all concentrations had good transparency in DMSO; practically no insoluble fragments were observed. Furthermore, DMF was a good solvent at lower than 4 mg/mL concentration. However, the transparency of the solutions of LMWSC 10 K was significantly decreased at increased concentrations of ethanol and acetone. LMWSC 10 K was practically insoluble in ethanol and acetone and was insoluble in acetonitrile at any concentration.

As expected, the introduction of MPEG increased the solubility of LMWSC 10 K as shown in Figure 8c–f. All of the ChitoPEG copolymers were easily dissolved in DMSO and their solutions showed good transparency, that is, higher than 90%. Solutions of the ChitoPEG copolymer had good transparency in DMF with the exception of ChitoPEG 5, which gradually decreased with increased concentration of organic solvent. Furthermore, ChitoPEG-5 showed low solubility in acetone and ethanol, that is, solution transparency decreased according to an increase in the concentration of ChitoPEG copolymer, and this material was practically insoluble in acetonitrile. Solutions of ChitoPEG-10, -15, -20 showed good transparency in ethanol and acetone at concentrations lower than 4 mg/mL. The ChitoPEG copolymers are difficult to solubilize in acetonitrile. Solutions of the ChitoPEG-10, -15, -20 copolymers had low transparency, although ChitoPEG-15, -20 copolymer was soluble below 2 mg/mL. These results indicate that acetonitrile is not suitable for dissolving chitosan and its derivatives.

The introduction of PEG into LMWSC 10 K does increase aqueous solubility at any pH. Furthermore, LMWSC 10 K itself also showed good aqueous solubility at various pH values while IS-chitosan was insoluble. The organic solubility of LMWSC 10 K was better than IS-chitosan although DMSO was a good solvent at any concentration. The introduction of MPEG into chitosan significantly increased the solubility of LMWSC 10 K to all organic solvents except acetonitrile.

The increased organic solubility of chitosan by the introduction of PEG has also been reported by other researchers.[9,24] Ouch et al.[9] reported that PEG-grafted chitosan was soluble in DMF and DMSO. The effect of PEG introduction to chitosan to increase water- and organic-solubility was also investigated by Hu et al.[24] They showed that PEG-grafted chitosan can dissolve in phosphate-buffered saline (0.1 M, pH 7.4) and organic solvent such as DMF or DMSO. However, their PEG-grafted chitosan had a greater than 24% degree of PEG substitution before it dissolved in DMF and DMSO. Introduction of PEG has double-faced properties, that is, a higher content of PEG values is beneficial to solubility but it can decrease the cationic properties of chitosan. Otherwise, LMWSC itself showed superior aqueous solubility when compared to IS-chitosan. Furthermore, MPEG introduction into LMWSC induces a significant increase in solubility in DMF, DMSO, acetone, and EtOH. Furthermore, an increase in PEG content in ChitoPEG copolymer also increased solubility in acetonitrile.

4. Conclusions

ChitoPEG copolymers were successfully synthesized and characterized. MPEG was conjugated to LMWSC using DCC–NHS system. The formation of the NHS-activated MPEG and ChitoPEG copolymer was confirmed by ^{1}H and ^{13}C NMR spectroscopy and FT-IR. Four different kinds of ChitoPEG copolymers were synthesized with different amounts of MPEG–NHS. The degree of substitution of MPEG calculated from ^{1}H NMR spectroscopy was similar to the values obtained from GPC. LMWSC has good solubility in aqueous solution at any pH. Furthermore, the introduction of PEG into LMWSC resulted in increased solubility in aqueous solution at various pH values (4.0–11.0) and organic solvents such as DMF, DMSO, ethanol, and acetone.

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