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Preparation and Characterization of Silk Fibroin Nanoparticles

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Silk fibroin was conjugated with methoxypoly(ethylene glycol) derivatives to prepare silk nanoparicles. Conjugation of SF with PEG was examined with various instrumental analyses. Nuclear magnetic resonance spectrometry and amino acid analysis showed that serine and tyrosine residues in SF were reacted with PEG and resulted in increasing molecular weight. The sizes and shapes of SF nanoparticles observed by transmission electronmicroscope were ranged about 150-400 nm in diameter and spherical morphology. UV/VIS spectrometry showed SF nanoparticles might be outer PEG and inner SF structure.

Key words: Silk fibroin, nanoparticles, poly(ethylene glycol)

Introduction

Silk fibroin (SF) is a typical natural polymer produced by silkworm *Bombyx mori*. Traditionally SF has been used as textile fiber and surgical suture with human beings. SF has good mechanical and biological properties including low inflammatory reaction, good water vapor and oxygen permeability (Kweon *et al.*, 2001; Minoura *et al.*, 1990), blood compatibility (Sakabe *et al.*, 1989), acceleration of collagen formation, and proliferation of cultured human skin fibroblasts (Yeo *et al.*, 2000; Yamada *et al.*, 2004). Therefore, SF has been attempted in wide varieties of biomedical applications such as matrix for mammalian cell culture and enzyme immobilization (Minoura *et al.*, 1995), scaffold for bone substitution (Sofia *et al.*, 2001), and drug delivery carrier (Hanawa *et al.*, 1995).

In polymer science, polymeric nanoparicles have been investigated as vehicle for drug, gene and so on (Tan *et al.*, 2010; Khvedelidze *et al.*, 2010). Generally polymeric nanoparticles manufactured from block or graft copolymer with balanced hydrophilic and hydrophobic ratio. SF is amphiphilic because it is characterized as hydrophobic crystalline region and hydrophilic amorphous region. However, amphiphilic balance of SF is not sufficient to form self-assembled nanoparticle. Therefore, hydrophilic modification of SF is needed to prepare SF nanoparticles. Poly(ethylene glycol) (PEG) is used as biomedical and pharmaceutical materials due to its good biological properties including minimal toxicity, antigenecity, immunological properties, and good solubility in water and common solvents (Harris 1992, Harris and Zalipsky 1997, Nucci *et al.*, 1991).

We would like to prepare PEG-modified SF with methoxy PEG derivatives to form SF nanoparticles. Various instrumental analysis including nuclear magnetic resonance spectrometry, amino acid analysis, molecular weight analysis, transmission electromicropy, and ultraviolet spectrometry were performed.

Materials and Methods

Materials

Raw silk was degummed twice with 0.5% on the weight of fiber (o.w.f.) marseilles soap and 0.3% o.w.f. sodium carbonate solution at 100°C for 1 h and then washed with distilled water. Degummed silk was hydrolyzed with 0.5 N of hydrochloric acid, neutralized, and then desalted. Lyophilized SF stored in refrigerator prior to use.

Methoxy poly(ethylene glycol) activated with cyanuric chloride, p-nitrophenyl carbonate, and tresylate were purchased from Sigma-Adrich, Korea. And other chemicals used in this study were purchased from Sigma without

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further purification.

Preparation of SF nanoparticles

Silk fibroin-PEG conjugate was prepared according to our previous method (Cho *et al.*, 2003; Gotoh *et al.*, 1993) described as follows; actPEG was added to 0.1% SF aqueous solution containing 0.1 M sodium borate and then stirred smoothly at 4°C overnight. Subsequently the solution was dialyzed against distilled water using dialysis membrane (MWCO 12,000~14,000) for 2 days. A lyophilized SF-PEG conjugate was dissolved in ethanol and then dialyzed against distilled water using a dialysis membrane (MWCO 12,000~14,000) for 2 days.

Characterization

^{1}H NMR spectra were obtained at 25°C using AVANCE 600 spectrometer.

Amino acid composition analysis was carried out using Biochrom 20 Amino Acid Analyser (Amersham Pharmacia Biotech. Co., Sweden). The 10 mg of samples were hydrolyzed in 6N HCl at 110°C for 18hrs. The filtrate was loaded on the analyzer after 0.2 um PVDF Acrodisc LC 13 syringe filter.

The molecular weight of SF conjugated with PEG was measured by gel permeable chromatography (GPC) with a TSK-gel G2000 SWXL column (300× 7.8 mm). The mobile phase was distilled water (pH 7.0). The operation condition is followed; flow rate 0.5 ml/min, column temperature 37°C.

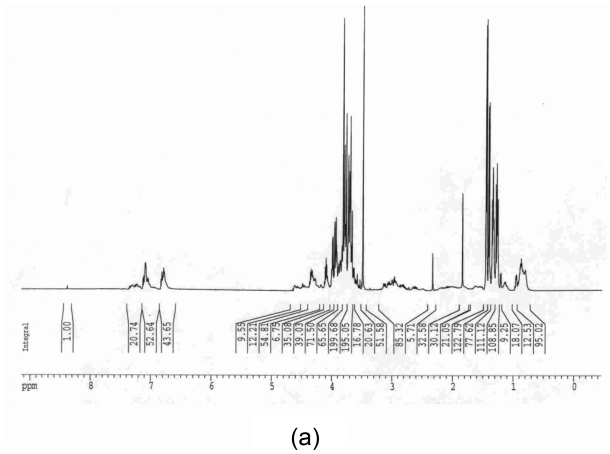
Morphology of SF nanoparticles was observed through a transmission electron microscope (JEM 1010, JEOL, Japan). One drop of the nanoparticles was placed on a copper grid and stained with phosphotungstic acid for 30 s. The grid was allowed to dry further for 10 min and examined with TEM.

UV/VIS spectra were obtained by UV/VIS spectrometer (Lambda 10, Perkin Elmer, USA). Turbidity was measured as the absorbance of 280, 400, and 600 nm according to the pH using a cell with a path length of 1 cm.

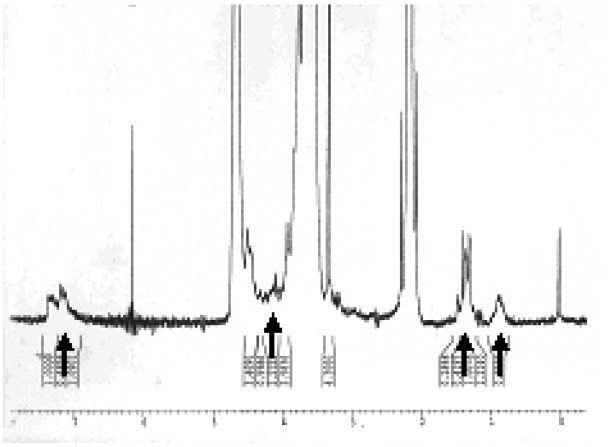
Results and Discussion

Preparation of SF-PEG conjugate

Fig. 1 shows ^{1}H NMR spectra of SF and SF-PEG conjugate. The results indicate that the proton peaks at 6.76 and 7.05 ppm of the tyrosine residue in SF shifted downfield to 7.18 and 7.32 ppm, respectively, which suggested the change in the molecular environment of the tyrosine residue caused by the modification; that is, the shift is the result of the shielding effect of the triazine ring on the



(a)



(b)

Fig. 1. NMR spectra of SF(a) and SF/PEG conjugate(b).

tyrosine residue (Jackman and Sternhell, 1969). Due to the electron-withdrawing effects of the PEG-triazine ring, the valence electron density around the protons attached to the carbon decreased. From the result of NMR, it can be said that the tyrosine residues of the SF reacted with PEG. Cyanuric chloride reacted with nucleophilic groups such as amino, imino, and hydroxyl groups (Vallee and Riordan, 1969; Tandford and Hauenstein, 1956). Therefore, the amino group of the lysine residue and the imidazole group of the histidine residue in SF could react with PEG (Vallee and Riordan, 1969; Tandford and Hauenstein, 1956). However, the peaks of these residues could not be detected by NMR measurement due to their very low contents in SF.

Amino acid composition of SF nanoparticle

To elucidate which amino acids were reacted with PEG, amino acid analysis were performed and represented in Table 1. SF is known a protein composed of 18 amino acids, but its composition is unique. The major amino

Table 1. Amino acid analysis of SF/PEG conjugate

Sample	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
SF(%)	3.10	1.85	12.72	2.55	33.50	22.81	3.26	-	1.25	0.90	6.56	1.50	0.62	1.05	0.06
treSF(%)	1.00	0.73	2.67	0.50	14.32	7.14	2.30	0.55	0.67	0.56	1.40	0.41	2.53	2.77	1.20
cyaSF(%)	1.44	-	5.09	0.90	26.37	11.92	2.64	0.42	0.71	0.54	4.06	0.66	1.07	2.20	0.60
nitroSF(%)	1.36	0.75	3.31	0.75	29.62	11.93	3.23	0.77	0.77	0.64	2.88	1.59	5.08	4.34	1.28

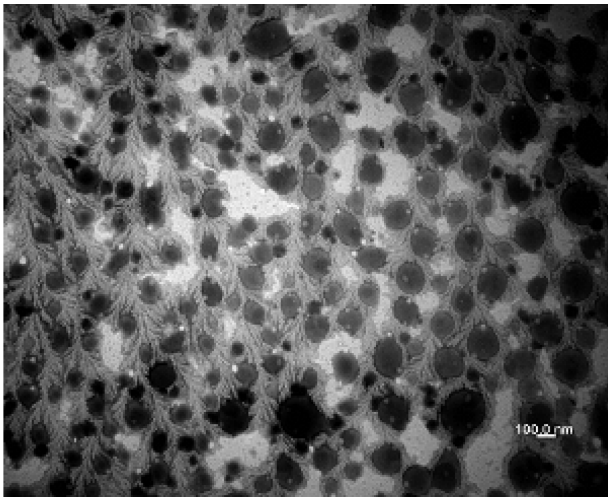


Fig. 2. Typical transmission microscopic photographs of SF nanoparticles.

acids of SF are glycine(33.5%), alanine(22.8%), and serine(12.7%). Conjugated SF showed significant decrease in serine contents with PEGylation; tresylated SF 2.67%, cyanulated SF 5.09%, and nitrophenylated SF 3.31%. The results indicate that serine residues in SF reacted with PEG. Cho et al (2003) reported similar results that serine residues in silk sericin were reacted cyanuric chloride activated PEG.

Molecular weight of SF and SF-PEG conjugate

To confirm the reaction with SF and PEG, molecular weight of SF itself and its PEG conjugates measured by GPC are shown in Table 2. The molecular weight of SF, 1,210, was changed to over 5,000 with the modification method; SF-conjugates with nitrophenyl PEG 6640, tresyl PEG 8620, and cyanuric chloride activated PEG 12500. It indicates that SF was conjugated with activated PEG.

Transmission microscopic observation of SF nanoparticles

SF nanoparticles of various sizes from 150 to 400 nm prepared by diafilteration method were shown in Fig. 2. The self-assembled polymeric nanoparticles were prepared from SF-PEG conjugate consisting of SF and PEG as the hydrophobic and hydrophilic parts, respectively. The shapes of almost SF nanoparticles were observed as spherical.

Table 2. Molecular weight of SF conjugate

Sample	SF	nitroSF	treSF	cyaSF
Molecular Weight (Da)	1,210	6,640	8,620	12,500

*nitroSF : SF conjugated with nitrophenyl poly(ethylene glycol)
treSF : SF conjugated with tresylated poly(ethylene glycol)
cyaSF: SF conjugated with cyanuric chloride activated poly (ethylene glycol)

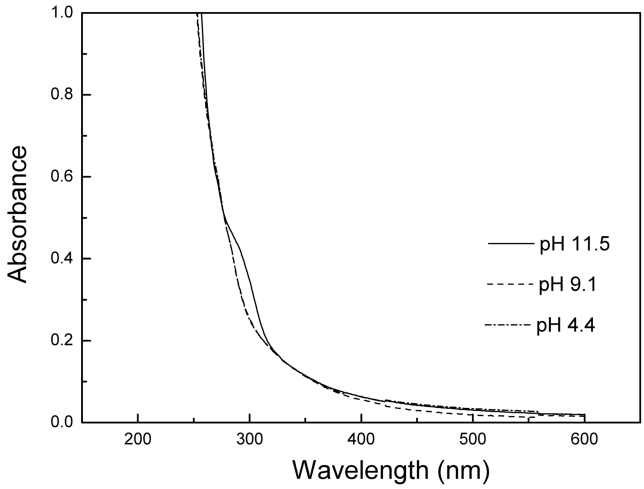


Fig. 3. The effect of pH on the UV/VIS spectra of SF nanoparticles.

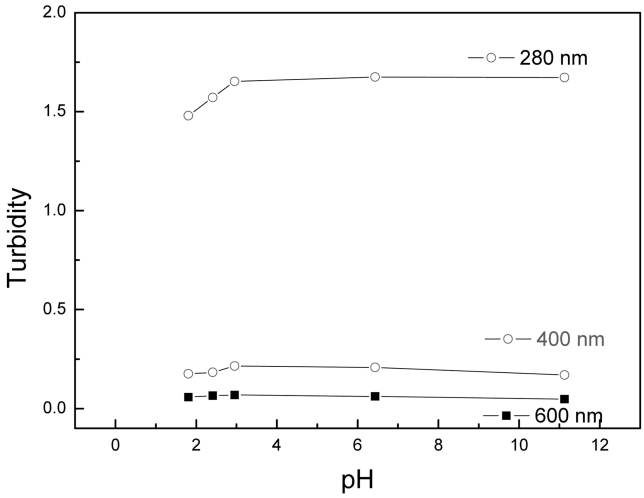


Fig. 4. Turbidity of SF nanoparticles at different wavelengths.

Turbidity of SF nanoparticles

UV/VIS spectra were measured and shown in Fig. 3. Turbidity of SF nanoparticles was observed according to the pH (Fig. 4). The optical density of SF nanoparticles at 280 nm was increased with an increase of pH upto 3 and then did not significantly change with pH. This is explained that the surface of SF nanoparticles is composed of PEG and the inner part of SF nanoparticles are composed of SF.

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