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PEGylation of Silk Fibroin Model Peptide

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Silk fibroin model peptide, alanine pentamer was synthesized through solid-phase method and modified with poly(ethylene glycol). Nuclear magnetic resonance spectrometry and Fourier-transform infrared spectroscopy showed the conformation of alanine pentamer, β-sheet structure and random coil conformation were not changed with PEGylation. Differential scanning calorimetry showed that relatively strong exothermic peak around 180ºC by PEGylation. No cytotoxicity of PEGylated pentamer was observed by L929 cell proliferation test.

Key Words: Silk fibroin, Alanine pentamer, Solid-phase peptide synthesis, Poly(ethylene glycol), Cytotoxicity

Introduction

Silk fibroin (SF) is a typical natural polymer spun by silkworm *Bombyx mori* L. Traditionally SF has been used as a textile fiber and a 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).

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In general, hydrophobic polymer has been modified with hydrophilic moieties throughout block or graft copolymerization for pharmaceutical applications such as drug or gene vehicles (Tan *et al.,* 2010; Khvedelidze *et al.,* 2010). In polymer science, SF has been classified with hydrophobic polymer due to its major hydrophobic amino acid composition; alanine and glycine. Therefore, the crystalline hydrophobic region of SF has been modified with hydrophilic biomaterial, poly(ethylene glycol), to widen the application fields. Poly(ethylene glycol) (PEG) is a well known biomedical and pharmaceutical material 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 SF model peptide, alanine pentamer, through solid-phase synthesis and modified with PEG. Various instrumental analysis including nuclear magnetic resonance spectrometry, Fourier-transform infrared spectroscopy, and differential scanning calorimetry were performed. Biocompatibility of PEGylated alanine pentamer was examined with L929 cell culture.

Materials and Methods

Materials

Alanine-preloaded 2-chlorotrityl chloride resin and Fmoc alanine were purchased from BeadTech (Seoul, Korea). Piperidine, diisopropylethylamine (DIPEA), dicyclohexylcarbodiimide (DCC), trifluoroethanol, 1-hydroxybenzotriazole monohydrate (HOBt) were obtained from Sigma-Aldrich Korea.

Methoxy poly(ethylene glycol) activated with cyanuric chloride was purchased from Sigma-Adrich, Korea. And other chemicals used in this study were purchased from Sigma without further purification.

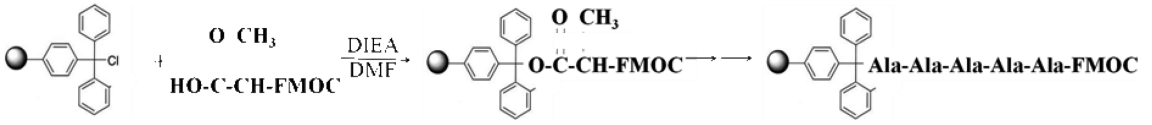

Fig. 1. Synthetic scheme of alanine pentamer.

Synthetic procedure of pentapeptide

Pentapeptide synthesis of alanine was performed manually on a Libra tube. 2-chlorotrityl chloride resin preloaded alanine was used as a solid support, and Fmoc-protected alanine was coupled in the presence of DCC according to the standard Fmoc-solid phase peptide synthesis (Carpino and Han 1972, Sheppard 1986, Miranda and Alewood 1999). Detailed synthesis procedures are illustrated in Fig. 1.

Complete reaction was assumed on the basis of a negative Kaiser assay as follows; Solution A: KCN 0.2 g was dissolved in distilled water 200 ml and then the aqueous KCN solution 0.2 ml was diluted to a volume of 10 ml with pyridine. B: ninhydrin 0.89 g was dissolved in ethanol 10 ml. C: 3.76 g phenol was dissolved in 10 ml EtOH. Then each solution was added to a test tube containing the sample and boiled for 5 min in a waterbath.

Alanine pentamer was obtained by cleavage reaction with mixture reagents, i.e., trifluoroethanol (TFE), acetic acid, and dichloromethane (DCM) in a volumetric ratio of 1:1:8. The mixture of the reagents was added to the Libra tube, stirred for 30 min, and subsequently filtered off via suction twice. Remaining acetic acid was then driven off under high vacuum. The pentapeptide was dissolved in MeOH followed by slow addition of diethyl ether to induce precipitation of the product.

PEGylation

PEGylation of alanine pentamer was performed according to modified previous method (Kweon *et al.,* 2010; Cho *et al.,* 2003; Gotoh *et al.,* 1993) described as follows; 1 g of Methoxy poly(ethylene glycol) activated with cyanuric chloride was added to 100 mg of pentapeptide suspension in 0.1 M sodium tetraborate and then stirred smoothly at 4°C overnight. Subsequently the solution was dialyzed against distilled water using dialysis membrane (MWCO 3,500) for 2 days. A lyophilized PEGylated peptide was dissolved in ethanol and then dialyzed against distilled water using a dialysis membrane (MWCO 3,500) 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 18 hrs. The filtrate was loaded on the analyzer after 0.2 um PVDF Acrodisc LC 13 syringe filter.

Differential calorimetric properties were measured with a DSC 2910 differential scanning calorimeter (TA instruments Co., USA). The measurements were carried out in the range from 50 to 450°C with a scanning rate of 10°C/min.

Cytotoxicity of PEGylated pentamer was examined using L929 mouse murine fibroblast. Briefly, L929 murine fibroblasts (ATCC, Rockville, MD) were maintained in 75 cm^2 flasks in a RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 50 Uml^{-1} penicillin and 50 $ugml^{-1}$ streptomycin. The proliferation was measured by MTT method in triplicate.

Results and Discussion

Synthesis of pentapeptide

In general, peptide can be synthesized using solid phase peptide synthesis and liquid phase peptide synthesis. The highly acid-labile 2-chlorotrityl chloride resin (Barlos *et al.,* 1988) was chosen as solid support. The typical features of this resin in comparison to alcohol-based resin are free of raceminzation, highly compatible with the base-labile protecting groups (Fmoc and nosyl), and mild cleavage condition (Barlos *et al.,* 1991).

The reaction was monitored by using Kaiser reagents (Fig. 2). The Kaiser test was efficient for detection of free amino groups on solid phase resin. The ninhydrin reaction was developed by Moore and Stein (1948) and adapted for detection of amino groups on solid phases by Kaiser *et al.* (1970) to detect amino groups on solid phases. A negative Kaiser test confirmed the coupling reaction completed (Hollink *et al.,* 2005). The representative color of the resins was changed from blue, incompletely coupled reaction, to yellow, complete coupling reaction.

Structural analysis: NMR, FT-IR

The ^{1}H NMR spectrum of PEGylated alanine pentamer is shown in Fig. 3. The characteristic peaks of poly(ethylene glycol) can be seen clearly at ~3.38 and ~3.6 ppm due to protons of methoxy (-OCH_3) and methylene (-OCH_2-CH_2-), respectively (Tan *et al.,* 2010). The characteristic

Fig. 2. Representative characterizations of the resins using the Kaiser test: characterization indicative of a complete coupling reaction (left) and an incompletely coupled resin (right).

peaks of alanine pentamer were shown at 1.2 and 4.0 ppm. Kimura *et al* (2000) reported that ^{1}H NMR spectrum of Tussah silkworm, *A. pernyi* fibroin is roughly similar to that of a-helix PLA (Shoji *et al.,* 1996). The peak at 4.0 ppm assigned as of a-helix conformation of alanine pentamer, due to alanine is a main component of *A. pernyi* fibroin. However, the peak at 1.2 ppm, assigned as b-sheet conformation (Suzuki *et al.,* 2009; Zainuddin *et al* 2008), due to the PEGylation.

Amino acid composition of alanine pentamer and PEGylated pentamer were performed and confirmed the presence of alanine regardless of PEGylation as expected (data not shown).

Fig. 4 shows FTIR spectra of PEG (a), PEGylated alanine pentamer (b), and alanine pentamer (c). As shown in Fig. 3(a), PEG exhibited strong absorption band at 1468, 1360, 1342, 1280, 1242, 1150, 1114, 1060, 964, and 842 cm^{-1}. The strong band at 2886 (data not shown) and 1105–~1060 cm^{-1} were assigned to –CH_2- stretching and –C-O-C- stretching, respectively, attributed to the PEG chain (Kweon *et al.,* 2003). Alanine pentamer, shown in Fig. 3(c), showed an absorption band at 1629 (amide I), 1541 (amide II), and 690 cm^{-1} (amide V). In general, silk fibroin shows typical absorption bands sensitive to the molecular conformation. The absorption bands at 1660 (amide I), 1540 (amide II), and 1235 cm^{-1} (amide III) are assigned to the random coil conformation of silk fibroin and the bands at 1630, 1530, and 1265 cm^{-1} are assigned to b-sheet conformation (Kweon *et al.,* 2001; Tsukada *et al.,* 1994; Canetti *et al.,* 1989; Iizuka and Yang, 1968). Therefore, FT-IR showed that alanine pentamer showed random and b-sheet conformation. PEGylated pentamer exhibited complicated amide I and II bands and 1413 cm^{-1} absorption due to the environmental change by PEGylation. The conformation of alanine pentamer and other

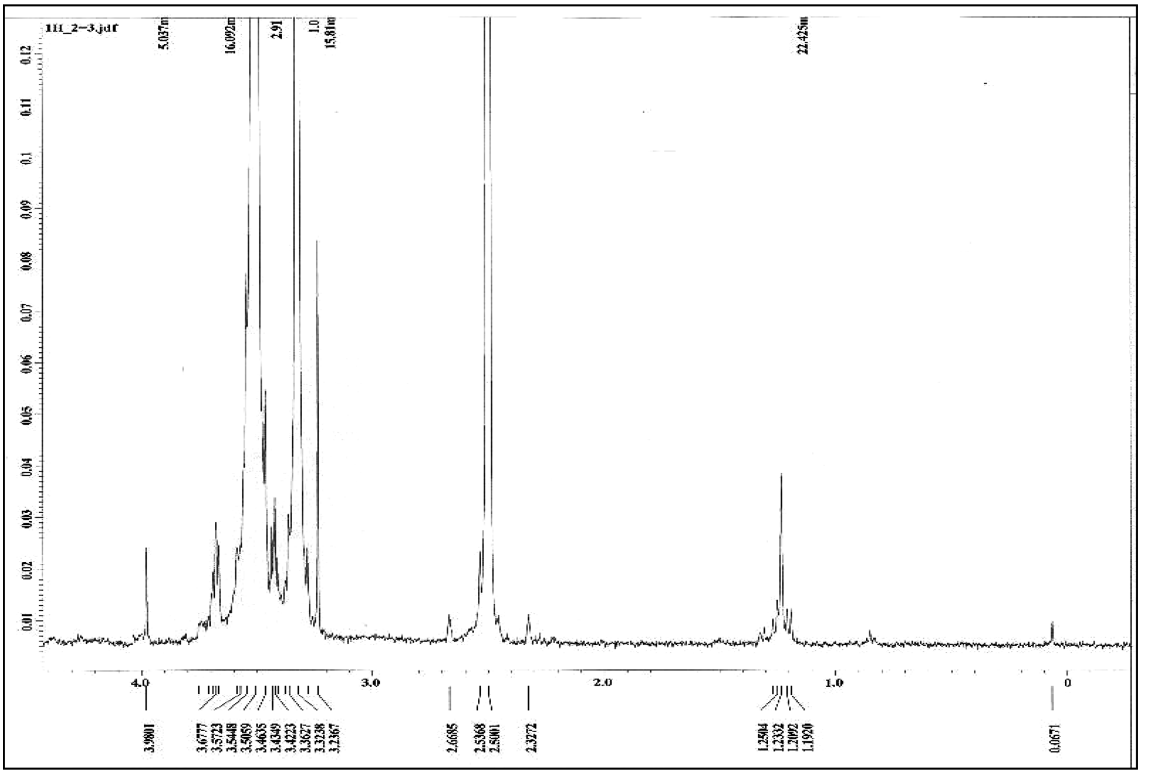

Fig. 3. NMR spectrum of PEGylated alanine pentamer.

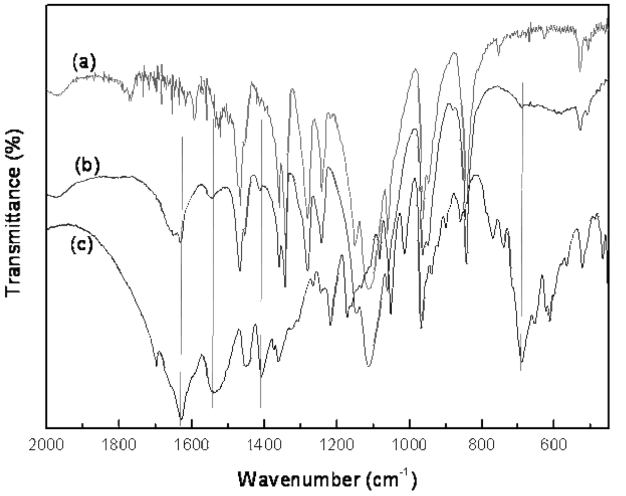

Fig. 4. FT-IR spectra of PEG (a), PEGylated pentamer (b), and alanine pentamer (c).

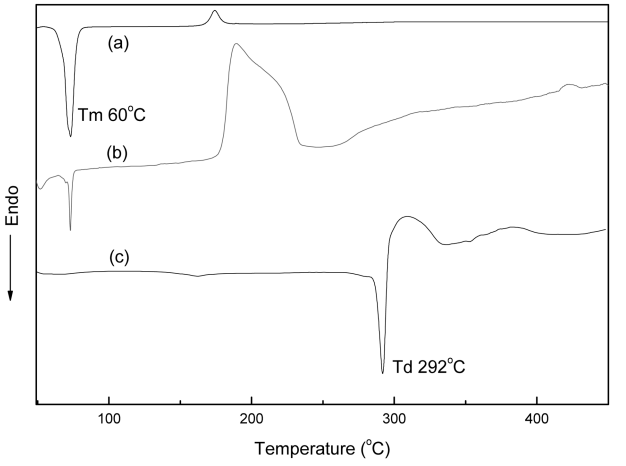

Fig. 5. DSC thermograms of PEG (a), PEGylated pentamer (b), and alanine pentamer (c).

absorption band originated from PEG did not affect by PEGylation.

Thermal properties: DSC

Fig. 5 shows the thermograms of PEG (a), PEGylated pentamer (b), and pentamer itself (c). PEG showed melting peaks at 60°C and an exothermic peak at 165°C. Alanine pentamer showed thermal decomposition peak at 290°C. PEGylated pentamer showed strong exotherm at 188°C and relatively weak melting peak at 60°C due to the PEGylation.

Cytotoxicity of alanine pentamer PEGylated was estimated by a cell proliferation assay using L929 mouse murine fibroblast cell line. Fig. 6 showed cell proliferation of L929 checked by MTT assay at 24 and 48 hrs. Compared with the plane medium, PEGylated pentamer showed available biocompatibility above 80%. Recently Kweon *et al.* (2008) reported that Semi-interpenetrating polymer networks composed of SF and PEG has no cytotoxicity by flow cytometic analysis compared with the plane medium. Gotoh *et al.* (1997) reported the surface interaction between L929 cells and silk fibroin conjugated with poly(ethylene glycol). According to the Gotoh *et al.* (1997) cell attachment and growth ratio of the conjugate film were lower than those of SF itself due to the hydrophilicity of PEG.

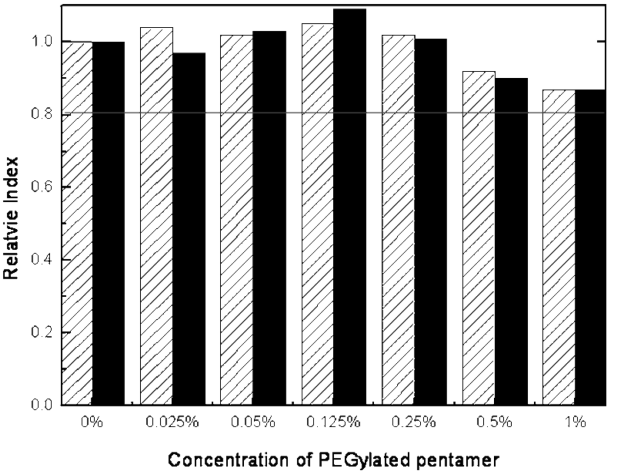

Fig. 6. Cytotoxicity analysis of PEGylated pentamer with L929 cells after 24 (sparse) and 48 hrs (solid).

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