

Biosynthesis of insulin-silk fibroin nanoparticles conjugates and in vitro evaluation of a drug delivery system

Hai-Bo Yan · Yu-Qing Zhang · Yong-Lei Ma ·
Li-Xia Zhou

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Abstract Silk fibroin derived from *Bombyx mori* is a biomacromolecular protein with outstanding biocompatibility. When it was dissolved in highly concentrated CaCl_2 solution and then the mixture of the protein and salt was subjected to desalting treatments for long time in flowing water, the resulting liquid silk was water-soluble polypeptides with different molecular masses, ranging from 8 to 70 kDa. When the liquid silk was introduced rapidly into acetone, silk protein nanoparticles with a range of 40–120 nm in diameter could be obtained. The crystalline silk nanoparticles could be conjugated covalently with insulin alone with cross-linking reagent glutaraldehyde. In vitro properties of the insulin-silk fibroin nanoparticles (Ins-SFN) bioconjugates were determined by Enzyme-Linked Immunosorbent Assay (ELISA). The optimal conditions for the biosynthesis of Ins-SFN bioconjugates were investigated. The Ins-SFN constructs obtained by 8 h of covalent cross-linking with 0.7% cross-linking reagent and the proportion of insulin and SFN being 30 IU: 15 mg

showed much higher recoveries (90–115%). When insulin was coupled covalently with silk nanoparticles, the resistance of the modified insulin to trypsin digestion and in vitro stability in human serum were greatly enhanced as compared with insulin alone. The results in human serum indicated that the half-life in vitro of the biosynthesized Ins-SFN derivatives was about 2.5 times more than that of native insulin. Therefore, the silk protein nanoparticles have the potential values for being studied and developed as a new bioconjugate for enzyme/polypeptide drug delivery system.

Keywords Silk fibroin nanoparticles · Insulin · Biosynthesis · Conjugation · ELISA · Cross-linking · Nanobiotechnology · Biocompatibility

Introduction

Insulin was first found to be an important polypeptide drug to lower the levels of blood glucose in diabetes. Since 1922, insulin crude derived from mammal pancreas has been used to treat diabetes (Yalow and Berson 1959). Over the following eight decades, the purity of medical insulin has been enhanced continuously with the development of biotechnology. There were also a lot of studies on the delivery methods of insulin for diabetic treatment. Almost all delivery ways from the earliest injection to oral administration, skin penetrating, lung adsorption, plant in skin,

H.-B. Yan · Y.-Q. Zhang (✉) · Y.-L. Ma · L.-X. Zhou
The State Engineering Laboratory of Modern Silk,
Soochow University, Suzhou 215123,
People's Republic of China
e-mail: yqzhang@public1.sz.js.cn; sericult@suda.edu.cn

H.-B. Yan · Y.-Q. Zhang · Y.-L. Ma · L.-X. Zhou
Silk Biotechnology Key Laboratory of Suzhou City,
Medical College of Soochow University, Suzhou 215123,
People's Republic of China

and so on have been tried. No matter how great the progress in diabetic treatment and insulin preparation is, hypodermic injection of insulin is still an effective drug delivery method as far as bioavailability is concerned. But the drug faces the serious hydrolyses of protease and the loss of its activity in blood within a very short period of time (2–3 h generally). Thus, diabetes patients need to accept frequent injections for a long time. To reduce patient's agony and the inconvenience of frequent injections, long effect drugs are developed mostly through the chemical modifications of insulin molecules (Asada et al. 1994; Asada et al. 1995; Baudys et al. 1995), for example, nanoparticles shelled with Chitosan (Lin et al. 2007), alginate microparticles as novel carrier (Reis et al. 2007), polysaccharide nanoparticles for oral insulin delivery (Sarmiento et al. 2006), thiolated chitosan microparticles for nasal peptide drug delivery (Krauland et al. 2006), chitosan/cyclodextrin nanoparticles as macromolecular drug delivery system (Krauland and Alonso 2007), and complexation hydrogels as delivery vehicles for the insulin-transferrin conjugates. Almost all these studies are still in the stages of research or development (Kavimandan et al. 2006). Few drugs are in clinical use. The injection is still the first-selected delivery way for the treatment of type I diabetes. The middle and long effect drugs such as neutral milt protein Zn insulin and milt protein Zn insulin could be maintained for about 24 h and 24–36 h, respectively. Diabetes patients suffer from frequent injections. Therefore, the synthesized nanoparticles or nano-vesicle from biomacromolecular materials was still a focus of studies of long effect injection. For instance, the synthesized nanoparticles (100 nm in size) of poly-cyano acrylic acid monomer by emulsion polymerization method were used as an injection of insulin nano-vesicle (Yin and Lu 2000). There were a few reports on protein-based nanoparticles as vehicle for drug delivery system owing to its biocompatibility. These studies focus mainly on the albumin originated mostly from human or bovine serum. Basically three different methods for particle preparation have been described, i.e., emulsion formation, coacervation, and desolvation (Lin et al. 1993; Müller et al. 1996; Weber et al. 2000). A disadvantage of these methods is the need to apply surfactants required for emulsion stabilization or glutaraldehyde for particles cross-linking. In fact, the protein-based nanoparticles were colloidal and

cross-linked albumin nanoparticles. Up to now, there have been few reports on the protein nanoparticles as a bioconjugate or vehicle for insulin delivery system.

Silk fibroin is a native biopolymer with a molecular mass of 370 kDa. The protein fiber has been used as a surgical suture for a long time due to its biodegradable and non-antigenic protein like albumin (Sakabe et al. 1989; Sadayuk et al. 1999; Santin et al. 1999). When the silk protein is dissolved in highly concentrated neutral salts, the resulting liquid silk fibroin can be made into various forms of fibroin including film, gel, powder, and fiber. Biotechnological and biomedical applications of the silk protein, which mainly attribute to its excellent properties, have been widely reported. Recently, the authors have prepared both water-soluble silk sericin and fibroin as the modifiers or bioconjugates for insulin modification (Zhang et al. 2006a, b). The in vivo experiments also showed that the bioconjugates of the two silk proteins with insulin had no antigenicity in rats and rabbits. When native insulin was bonded covalently with both water-soluble silk sericin and fibroin, the bioconjugation improved evidently insulin's biological stability and activity in vitro and in vivo. When bioconjugated with insulin, the insulin-silk protein conjugates could be used as an injection for diabetes, but it also suffers from the protease digestion in vivo because both silk proteins are water soluble. More recently, the authors have again developed a novel method for processing silk nanoparticles (Zhang et al. 2007). These silk fibroin nanoparticles are the globules with a fine crystallinity that may offer various possibilities for surface modification and covalent drug attachment. The purpose of the present study is to prepare the crystalline silk protein nanoparticles as vehicles for insulin bioconjugation and investigate and evaluate in vitro the physicochemical properties and stability of the synthesized insulin in detail by means of ELISA.

Materials and methods

Materials and compounds

The cocoons of silkworm *Bombyx mori* were provided by Department of Agriculture Science and Technology, Medical College of Soochow University, Suzhou, P. R. China. Pupa and its covering in the cocoon are

removed and the cocoon shells cut into small pieces of about 1.0 cm² for the following experiments.

Insulin injection (40 IU/mL) is made in Biochemical Pharmaceutical Factory of Shanghai First Pharmacy Co. (Shanghai, P. R. China). Glutaraldehyde is purchased from Sigma Co. (St. Louis, MO, USA). Human serum is obtained from the No. 1 People's Hospital of Soochow University. Alloxan monohydrate is supplied from Fluka Chemie GmbH CH-9471 Buchs, Switzerland. Other reagents are of analytical grade and used without further purification. The Insulin ELISA kit (INS-ELISA, KAP1215) for the quantity measurement of insulin is purchased from BioSource Europe S. A (8, Rue de l'Industrie, B-1400 Nivelles, Belgium). Na₂CO₃, CaCl₂, and acetone are all analytical grade of reagents, purchased from Shanghai Chemicals Factory (P. R. China). Sodium dodecyl sulfate (SDS), TEMED, acrylamide, and Coomassie Brilliant Blue R-250 are purchased from Sigma, USA. Protein ladder is purchased from Fermentas Co., Lithuania.

Regenerated liquid silk fibroin

The cocoon shell of silkworm *Bombyx mori* was degummed twice in boiling solution of 0.5% Na₂CO₃ for 0.5 h, and the resulting degummed fiber was subsequently dissolved in a ternary system, i.e., a mixed solution of calcium chloride, ethanol, and water (CaCl₂ · C₂H₅OH · H₂O: 1:2:8 mole ratio), at 70 °C for 2 h, respectively. After the silk fibroin-salts solution was centrifuged at 8,000 rpm for 10 min, the supernatant was dialyzed continuously for 48 h against running pure water to remove CaCl₂, smaller molecules, and some impurities using a cellulose semi-permeable membrane whose molecular weight cutoff is 10 kDa. The resulting liquid silk fibroin was stored at 4 °C and used in the following experiments for the preparation of silk fibroin nanoparticles.

Preparation of silk fibroin nanoparticles

The preparation of silk fibroin nanoparticles was carried out according to the method described previously by the authors with slight modification (Zhang et al. 2007). The regenerated silk fibroin solution containing 5.0% by weight was rapidly introduced into at least 70% (v/v) of the final mixture

volume of water-miscible acetone by using a sample pipette at room temperature. Milk-like silk protein particles were formed at once and suspended in the mixture comprising water and organic solvent. These protein particles were water insoluble and went down slowly due to the gathering of microparticles. The precipitates of silk protein nanoparticles were collected and purified from the mixture by repeated centrifugation at 30,000 rpm (Beckman Avanti J30I) to separate these particles from the solvent. When the precipitate in water was subjected to a supersonic treatment (Output Watts: 16) for 2 min × 5 (Ultrasonic Processor, SONICS & MATERIALS INC, 53 Church Hill RD, Newtown, CT. USA), the nanoparticles dispersed evenly in 50 mmol/L phosphate buffer or water and stored at 4 °C in refrigerator for the following experiments.

SEM observation and size distribution

The SFN solution mentioned above was subjected to ultrasonic treatment for 2 min × 2 and then diluted prior to SEM observation and determination of size distribution. SEM images (×10 K) of silk fibroin nanoparticles were obtained using a Hitachi S-570 Scanning Electron Microscope. Size distribution of silk fibroin nanoparticles was measured using a Zetasizer 3000HSa (Malvern Co. Ltd; UK).

Preparation of Ins-SFN bioconjugates

A given volume of silk nanoparticles (20 mg/mL) was added into a plastic flask, and mixed with insulin solution at 4 °C. Over mild homogenization, a given volume of 25% glutaraldehyde solution was gently added into the mixture. The flask was stoppered tightly and placed in an orbital shaker at 4 °C for cross-linking reaction. At a certain period of time after the reaction, glycine solution (about 100 mg) was added to end the reaction. The reaction mixture was then centrifuged repeatedly at 30,000 rpm (Beckman Avanti J30I) to remove impurities, unreacted reagents, and even uncross-linked insulin. After that, the Ins-SFN bioconjugates were taken out and volumed to 1,000 mL with distilled water. Finally, the purified insulin bioconjugates were maintained at 4 °C and used for the following experiments of insulin measurement.

SDS-PAGE

The molecular mass range of regenerated silk fibroin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described previously by Laemmli with 12% acrylamide gel and 5% condensing gel (Laemmli 1970), which was stained with 0.25% Coomassie Brilliant Blue R-250 (Aldrich, USA). The samples were calibrated against a wide range of molecular weight markers.

Determination of insulin content

The insulin contents in bioconjugates and native insulin as control were measured according to the operation guide of insulin ELISA test kit [instruction of the INS-ELISA, BioSource Europe S. A]. The reagent kit provides materials for the quantitative measurement of insulin in serum or plasma. This assay is intended for *in vitro* diagnostic use. All specimens and reagents are allowed to reach room temperature ($\sim 25\text{ }^{\circ}\text{C}$) and mixed roughly by gentle inversion before use. Standards, controls, and unknowns should be assayed in duplicate.

In vitro experiments

About 100 μL of the insulin alone or Ins-SFN bioconjugates was added to 900 μL normal human serum. After mild homogenization, the serum mixture

was incubated at $37 \pm 1\text{ }^{\circ}\text{C}$ incubator. A little serum mixture taken by a given time interval was measured repeatedly three times by ELISA and the survival level of native insulin and its bioconjugates in human serum was calculated.

Results and discussion

Characterization of silk protein nanoparticles

When the degummed fiber of silk fibroin was solubilized in the ternary system of CaCl_2 -ethanol- H_2O at $70\text{ }^{\circ}\text{C}$ for 2 h, lots of amide bond in the molecular chains of silk fibroin were broken down at different sites. SDS-PAGE showed that the molecular masses and distributions of the regenerated silks suffered from the solubilization conditions of silk fiber in CaCl_2 ternary solvent system (Fig. 1a). In fact, the regenerated liquid silk was a mixture of gradually degraded product of fibroin polypeptides. The distribution ranges of the liquid silk are about 8–70 kDa, and the average value was about 25 kDa. When a little liquid silk solution was rapidly introduced into excessive acetone, the milk-white suspension of silk protein formed at once. The observation of scanning electronic microscopy (SEM) showed that the silk particles were globular granules (Fig. 1b). The maximal and minimal granules were about 120 and 40 nm in diameter. Because of the easy aggregation of nanoparticles, especially protein granules, the size distribution of silk

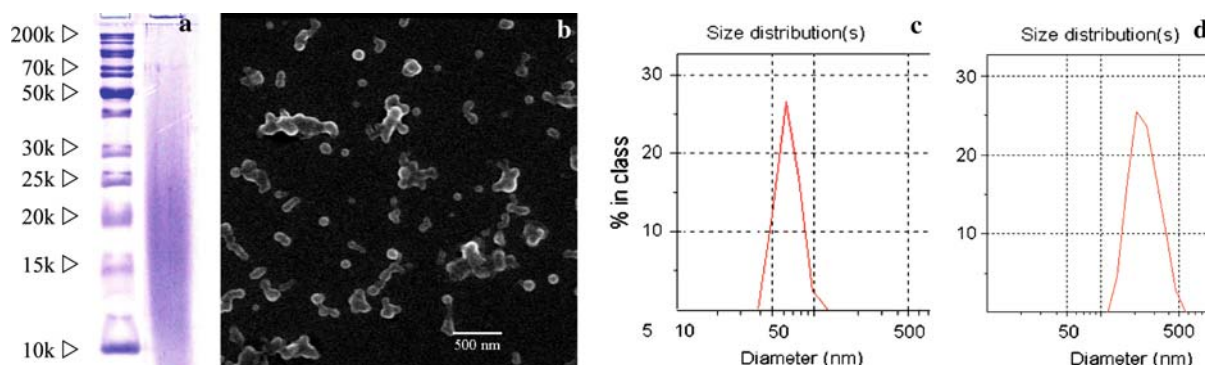


Fig. 1 SDS-PAGE of aqueous silk fibroin and SEM images and size of silk fibroin nanoparticles. **a** SDS-PAGE (12% gel) of aqueous silk fibroin samples dissolved in CaCl_2 ternary solvent at $70\text{ }^{\circ}\text{C}$ for 2 h. Protein Ladder 10–200 kDa was purchased from Fermentas Co. Ltd. **b** SEM images ($\times 10\text{ K}$) of

silk fibroin nanoparticles obtained by Hitachi S-570 Scanning Electronic Microscope. **(c and d)** are size distributions of silk fibroin nanoparticles before and after ultrasonic treatment obtained by Zetasizer 3000HSa (Malvern), respectively

protein nanoparticles in water ranged from 115 to 570.7 nm before ultrasonic treatment (Fig. 1d). When the SFN was subjected to ultrasonic treatment for 2 min \times 5, the size distribution in water ranged between 40 and 120 nm, as observed using Laser Sizer (Fig. 1c). Their values of polydispersity are 38.1 nm (2.98%), 49.4 nm (20.21%), 62.1 nm (44.42%), 78.2 nm (27.97%), 98.5 nm (3.88%), and 124.0 nm (0.47%), respectively. The average size of the silk nanoparticle was about 70 nm. Over one-half of the ϵ -amino groups exist around the protein nanoparticles as observed using a trinitrobenzenesulfonic acid (TNBS) method. Raman spectra shows the tyrosine residues on the surface of the globules are more exposed than those on native silk fibers (Zhang et al. 2007). The crystalline polymorph and conformation transition of the silk nanoparticles from random-coil and α -helix form (Silk I) into anti-parallel β -sheet form (Silk II) are investigated in detail by using infrared, fluorescence, and Raman spectroscopy, DSC, ^{13}C CP-MAS NMR, and electron diffraction. X-ray diffraction of the silk nanoparticles shows that the nanoparticles crystallinity is about four-fifths of the native fiber. Therefore, these characteristics of silk nanoparticles are in favor of the bioconjugation of enzyme/polypeptide drug as a novel vector.

Effects of cross-linker concentration and reaction time on insulin bioconjugation

According to processing procedure described earlier, insulin bioconjugates with SFN were prepared. The effects of glutaraldehyde concentration in cross-linking reaction on the conjugation of insulin with SFN were first investigated. As shown in Fig. 2, the effect of glutaraldehyde on insulin bioconjugation was very evident. When its concentration rose from 0.1 to 0.6%, the absorbance value of the bioconjugates enhanced along with it and reached a peak value at 0.7%. Then, it decreased gradually with increasing concentration of glutaraldehyde. It is well known that glutaraldehyde is not only a cross-linker but also a denaturant. An excess of cross-linker in the cross-linking reaction system results in insulin denaturation and a deficiency of it leads to insufficient cross-linking of insulin with silk fibroin nanoparticles. Therefore, in the following experiments of cross-linking time, all cross-linking reactions of insulin with SFN were carried out in 0.7% glutaraldehyde concentration (Fig. 3). The

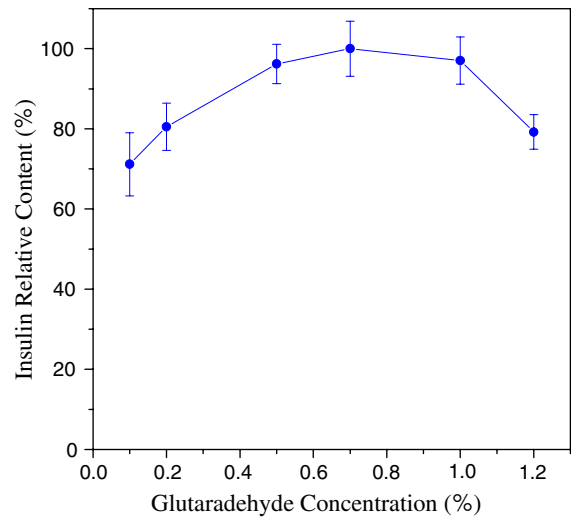


Fig. 2 Effects of glutaraldehyde concentration on the bioconjugation of insulin with SFN

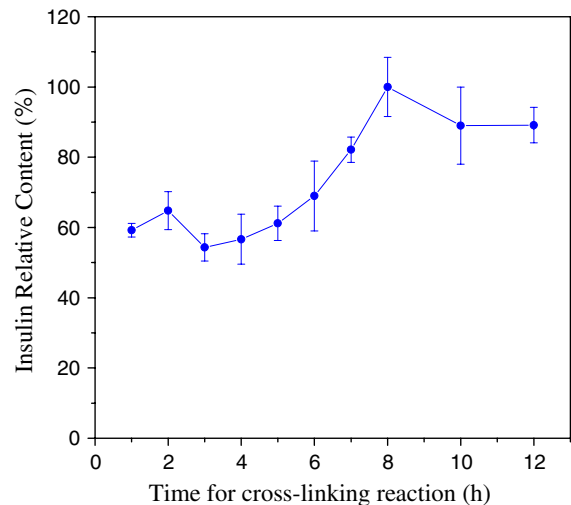


Fig. 3 Effect of reaction time on the insulin bioconjugation with SFN. The highest value in the figure was set as 100%

experimental result showed that the absorbance of insulin bioconjugates with SFN appeared at a lower level of period from the cross-linking time of 2–5 h, and subsequently increased rapidly with the delay of cross-linking time. Finally, it reached a peak value (ABS 0.8) at 8 h of cross-linking time and went down after 8 h. This indicates that excessively long cross-linking time also brought about insulin denaturation as excessive cross-linker in the reaction system. Therefore, all samples of insulin bioconjugates with SFN were prepared at 0.7% glutaraldehyde

concentration for 8 h of cross-linking time in the following experiments.

The optimizing experiment for the cross-linking proportion of insulin and SFN

Silk protein is not toxic to human body. Considering the cost of silk, we should choose the least amount of silk protein to cross-link with the maximum amount of insulin. 10 IU of insulin was attached covalently with a series of different amounts of SFN (1, 5, 10, 50, and 100 mg) at the same conditions of 0.7% glutaraldehyde concentration and 8 h of reaction time. As shown in Fig. 4, when 10 IU of insulin was cross-linked with 5 mg of SFN, the highest insulin recovery (ABS 0.6) can be obtained. If the reaction system contained too much or too little silk fibroin nanoparticles, the synthesized insulin will significantly reduce its activity. When 10 IU of insulin was attached covalently with 5 mg of SFN for 8 h in 5 mL reaction system and 0.7% glutaraldehyde concentration, the insulin construct had the highest recovery (120%). There were two possible reasons for insulin recoveries more than 100%. Firstly, it is well known that the molecules of the native insulin as a standard sample in solution easily aggregate by themselves. The self-aggregation of insulin molecules resulted in the slightly lower content of the native insulin by ELISA. Secondly, in the continuously stirred reaction system including glutaraldehyde as cross-linking reagent the

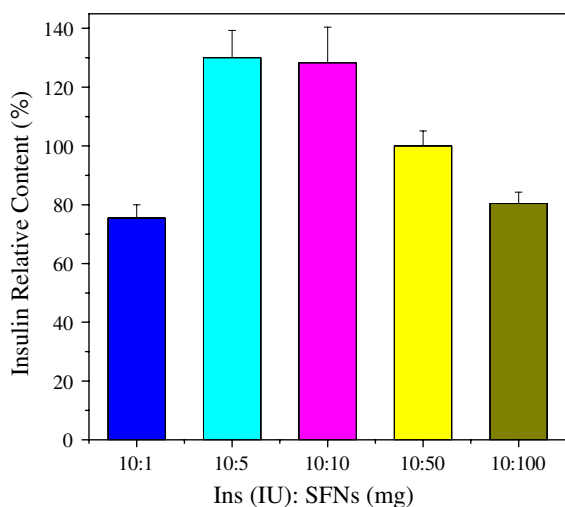


Fig. 4 Optimizing experiment for the cross-linking proportion of insulin and SFN. The highest value in the figure was set as 100%

self-aggregated phenomenon of native insulin molecules is not likely to occur and these molecules should be separately bonded with those ϵ -amino group of the lysine on the surface of silk nanoparticles. However, the recovery of L-asparaginase occurred only at about 44% when the native enzyme was conjugated covalently with the same silk nanoparticles under the same reaction conditions (Zhang et al. 2008a). In general, the silk protein nanoparticle is preferred to the bioconjugation of insulin for delaying its action time *in vivo*.

Recoveries of insulin in Ins-SFN bioconjugates

From the results of the optimizing experiment described above, we knew that the optimal bioconjugation proportion of insulin and SFN was 10:5, i.e., there were 10 IU of insulin and 5 mg of SFN in the cross-linking reaction system (0.7% glutaraldehyde conc.) for 8 h of reaction time. But we didn't know how many amounts of both insulin and SFN in a given volume of cross-linking reaction system was best. Considering lower amount of SFN as a medical injection of insulin bioconjugates, we tried the following experiment on the recoveries of the modified insulin in Ins-SFN bioconjugates. The recovery of the covalently bound insulin in the bioconjugate was estimated based on the amount of native insulin.

Insulin was conjugated covalently with SFN in cross-linking proportion of 2:1 (IU/mg) in 5 mL of reaction system. The quantitatively cross-linking reaction of insulin with SFN was carried out in the presence of 0.7% glutaraldehyde for 8 h in a series of different concentrations of 2:1, 6:3, 10:5, 14:7, 18:9, 22:11, 26:13, 30:15, and 34:17 (IU/mg) insulin and SFN (Fig. 5). The results showed that the recoveries of Ins-SFN bioconjugates ranged from 80 to 120% in a series of different concentrations of insulin and SFN. These recoveries could be fitted into a good linear equation ($y = 112.08 - 2.69119x$) by using a software of Origin 6.0. Their recoveries ranged from 90 to 115%. As shown in Fig. 5, Ins-SFN bioconjugates derived from the cross-linking proportion (30:15 in the figure) of 30 IU insulin and 15 mg SFN contained in the reaction system had a recovery of about 90%. In other words, a bottle (10 mL, 40 IU/mL) of insulin injection containing 400 IU of total insulin content could be cross-linked covalently with only 200 mg silk fibroin nanoparticles.

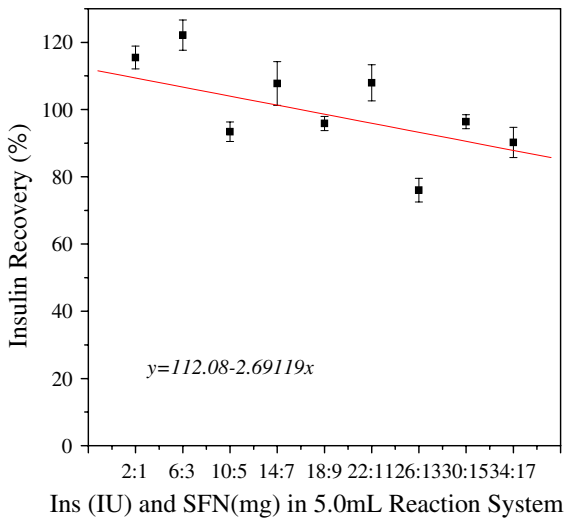


Fig. 5 Insulin recoveries in Ins-SFN bioconjugates

The activity comparison of three kinds of Ins-SFN bioconjugates

More recently, the authors have described that silk fibroin nanoparticles could be used for L-asparaginase immobilization as a support (Zhang et al. 2008a). We have also found the activity differences during the synthesized L-asparaginase bioconjugates with three kinds of SFN derived from the liquid silk fibroin with a different molecular mass. The activity of the modified L-asparaginase bioconjugated with SFN derived from the liquid silk with a range of molecular mass of 40 to >200 kDa was 4 times higher than that of the modified L-asparaginase bioconjugated with SFN derived from the liquid silk with a range of 15–50 kDa. Therefore, we did the same experiment as described above. Three kinds of SFN were prepared with three kinds of liquid silk by dissolving in CaCl₂ ternary solvent at 90 °C for 6 h (Sample 1) and at 70 °C for 2 h (Sample 2) and in 9.3 mol/L LiBr solution at 50 °C for 2 h (Sample 3). The molecular ranges of the three liquid silk are 15–50 kDa, 10–200 kDa and 40 to >200 kDa, respectively. The resulting silk nanoparticles (Samples 1, 2, and 3) are very similar in size. However, their surface characteristics such as the number of ε-amino group are different from one another and result in the difference in the number of L-asparaginase covalently bonded on silk particles (Zhang et al. 2008a). Fortunately, three kinds of insulin constructs synthesized by using above

Table 1 Activity comparison of three kinds of Ins-SFN bioconjugates

Samples	ABS Values/n = 3			Means	±SD
1	0.224	0.205	0.215	0.215	0.010
2	0.198	0.191	0.208	0.199	0.009
3	0.199	0.216	0.248	0.221	0.025

Samples 1, 2, and 3 were prepared with three kinds of the liquid silks from dissolving in CaCl₂ ternary solvent at 90 °C for 6 h and at 70 °C for 2 h and in 9.3 mol/L LiBr solution at 50 °C for 2 h, respectively

three kinds of liquid silk under the same conditions have no evident differences in the amount of insulin covalently coupled on silk nanoparticles by ELISA analysis (Table 1). This indicated that the bioconjugation of insulin with the silk nanoparticles was preferable to that of L-asparaginase.

Resistance to trypsin digestion

A given amount of native or modified insulin in 0.2 mL 0.05 mol/L Tris–HCl buffer (pH 8.6) was mixed with 30 μL trypsin solution (1.0 mg/mL) for proteolysis. After the sample solution had been incubated at given intervals, the activities were determined by ELISA. Figure 6 showed the resistances of free insulin and Ins-SFN bioconjugates to trypsin digestion. It is obvious that the resistance of the modified insulin to trypsin digestion was greatly

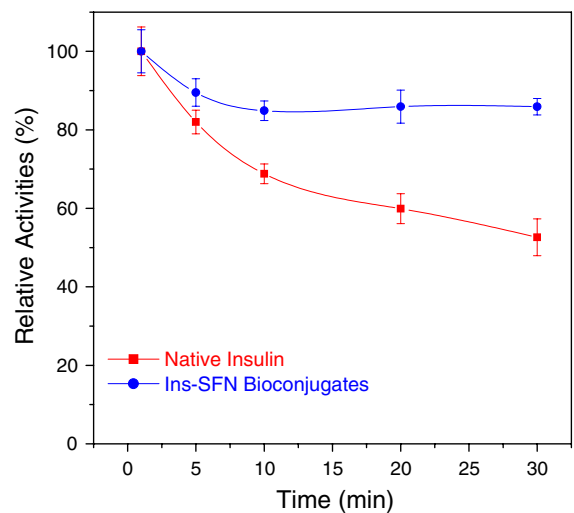


Fig. 6 Resistance of native insulin and Ins-SFN bioconjugates against trypsin digestion

improved in comparison with that of the native form. After being hydrolyzed for 10 min by trypsin, the activity of the modified insulin retained about 75% of the original activity while the unmodified drug retained about 65% of the original activity. Even though the incubation time in trypsin solution was lengthened to 30 min, native insulin had only retained about one-half of their original activity and Ins-SFN bioconjugates could maintain about 85% of the original activity.

In vitro stabilities in human serum

The stabilities and in vitro half-lives of both native insulin and insulin bioconjugates with SFN in human serum were determined by ELISA. The stabilities of insulin alone or bioconjugates in human serum are summarized in Fig. 7. The native insulin in human serum without diabetes exhibited a reduction of more than 70% of original level after 24 h due to the presence of proteases capable of making native insulin inactive in human serum. When incubated in the same manner as the physiological solution at 37 °C for 24 h, Ins-SFN bioconjugates could retain more than 60% of their respective initial levels. When incubated respectively, in human serum for 60 h, native insulin almost lost all its activity and the modified insulin with SFN could maintain about 40% of its initial levels. Plasmatic half-lives in vitro of insulin alone and Ins-SFN constructs in human serum

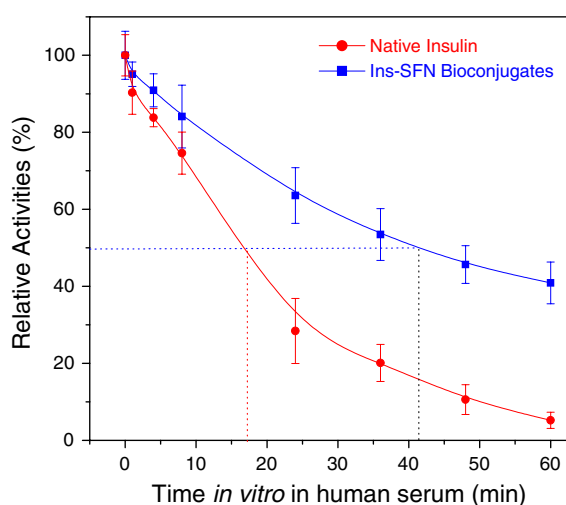


Fig. 7 In vitro stabilities of Ins-SFN bioconjugates in human serum

are 17 and 42 h, respectively. The result suggests that the modification of insulin with the globular silk fibroin nanoparticles protects insulin with steric hindrance to the enzymatic hydrolysis caused by proteases in serum.

Discussion

As is well known, PEG is a water-soluble synthesized polymer with no immunogenicity in human body and is expelled from in vivo human via kidney. When bioconjugated with this polymer, enzymes or polypeptide drugs could delay their half-lives in vitro or in vivo, decrease or eliminate their immunogenicity, and greatly reduce side effect. PEG-modified L-asparaginase (Pegaspargase) made in Enzon Inc (Piscataway, NJ, USA) is in commercial use for clinical anti-leukemia therapy (Holle 1997). A blemish in an otherwise perfect thing is that the linear molecule of PEG was conjugated with only one molecule of enzyme or polypeptide drug in one end. The branched PEG could be joined with two or three molecules. The umbrella-like structure of branched PEG gives better protection than linear PEG toward approaching proteolytic enzymes, antibodies, phagocytic cells, etc. It has been shown recently that of spherical PEG molecules of nanosize, which is composed of a spherical core of hydrophobic polymer and the hydrophilic PEG chains on the surface, could conjugate with many active biomolecules. In fact, the silk fibroin nanoparticles used in the present experiment are very similar to the spherical PEG molecules mentioned above.

Recently, lots of studies focus mostly on the lipid/chitosan nanoparticles and polysaccharide nanoparticles or PEG-grafted chitosan nanoparticles as pulmonary, peroral, and nasal absorption delivery systems of insulin (Grenha et al. 2008; Sarmiento et al. 2007a, b; Zhang et al. 2008b; Jintapattanakit et al. 2007). These delivery systems of nanoparticles almost wrapped insulin molecules. On the other hand, the studies on protein-based nanoparticles as drug delivery system focus mainly on the albumin originated mostly from human or bovine serum owing to its biocompatibility. Basically three different methods for particle preparation have been described, i.e., emulsion formation, coacervation, and desolvation (Müller et al. 1996; Lin et al. 1993; Weber et al.

2000). A disadvantage of these methods is the need to apply surfactants to emulsion stabilization or glutaraldehyde to the cross-linking of particles. In fact, the protein-based nanoparticles were cross-linked albumin nanoparticles. But there was little investigation on the global protein as a drug delivery system of insulin injection in clinical use.

Silk fibroin is a biodegradable and non-antigenic protein like albumin. When the regenerated liquid silk fibroin was bioconjugated with insulin molecules by glutaraldehyde, the insulin bioconjugates also had no immunogenicity in rabbits in our previous experiments (Zhang et al. 2006b). Here, we prepared globular silk fibroin nanoparticles (SFN) with a fine crystallinity from regenerated liquid silk by means of water-miscible protic and polar aprotic organic solvents. Then, these silk nanoparticles with an average size of about 70 nm in diameter coupled covalently with insulin molecules acted as a steric barrier to retard the interaction of the insulin with proteases as well as protease hydrolysis. Moreover, these silk nanoparticles themselves were crystalline globular nanoparticles difficult to be hydrolyzed by proteases. In the experiment of the resistance of Ins-SFN bioconjugates or SFN alone to trypsin digestion (Sect. 3.6), we have not found any silk peptide or amino acid presence in the protease digestion system (data not shown). Thus, prolongation of insulin action *in vitro* was realized by silk nanoparticles bioconjugation. So, the primary test in the present paper indicated that silk nanoparticles were safe and could be used for the bioconjugation of active polypeptides such as insulin. Further studies will focus on the optimization of the synthetic procedure to prepare Ins-SFN conjugates, and finally, pharmacodynamic properties of the optimized bioconjugates will be evaluated *in vivo* by using plenty of diabetic model animals. The bioconjugation technology of silk nanoparticles offers a promising strategy for the enhancement of the therapeutic value of insulin and other pharmacologically active peptides.

Conclusions

1. Silk fibroin is a biocompatible protein-based polymer. When dissolved in highly concentrated salts and dialyzed for desalination, the resulting

regenerated liquid silk can be made into crystalline silk nanoparticles by means of the water-soluble organic solvents. The crystalline spherical nanoparticles, an average size of about 70 nm, were not soluble in water but could disperse in water or buffer. The ε -NH₃ present on the surface of spherical nanoparticles was very suitable for the immobilization or bioconjugation of enzymes or polypeptides drugs.

2. Insulin was coupled covalently with the crystalline silk spherical nanoparticles by cross-linking reagent glutaraldehyde. ELISA analysis showed that the recovery of Ins-SFN bioconjugates ranged from 90 to 115%.
3. The optimal preparation conditions of insulin bioconjugation with silk fibroin nanoparticles are 0.7% of glutaraldehyde concentration, a ratio of 30:15 of insulin (IU) and silk nanoparticles (mg), and 8 h of reaction time in 4 °C.
4. The bioconjugation of insulin with silk fibroin nanoparticles strikingly improved the *in vitro* stabilities of the polypeptide in both human serum and trypsin solution.
5. Silk fibroin nanoparticles as a vector or conjugate for the modification or conjugation of enzyme or polypeptide drug had a prominent merit that the purification of the reaction product Ins-SFN bioconjugates is very easy and repeated centrifugation at 30,000 rpm makes it possible to obtain high-purity modified insulin sample.

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