Preparation and characterization of silk fibroin as a biomaterial with potential for drug delivery

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Abstract

Background: Degummed silk fibroin from Bombyx mori (silkworm) has potential carrier capabilities for drug delivery in humans; however, the processing methods have yet to be comparatively analyzed to determine the differential effects on the silk protein properties, including crystalline structure and activity.

Methods: In this study, we treated degummed silk with four kinds of calcium-alcohol solutions, and performed secondary structure measurements and enzyme activity test to distinguish the differences between the regenerated fibroins and degummed silk fibroin.

Results: Gel electrophoresis analysis revealed that Ca(NO3)2-methanol, Ca(NO3)2-ethanol, or CaCl2-methanol treatments produced more lower molecular weights of silk fibroin than CaCl2-ethanol. X-ray diffraction and Fourier-transform infrared spectroscopy showed that CaCl2-ethanol produced a crystalline structure with more silk I (α-form, type II β-turn), while the other treatments produced more silk II (β-form, anti-parallel β-pleated sheet). Solid-State 13C cross polarization and magic angle spinning-nuclear magnetic resonance measurements suggested that regenerated fibroins from CaCl2-ethanol were nearly identical to degummed silk fibroin, while the other treatments produced fibroins with significantly different chemical shifts. Finally, enzyme activity test indicated that silk fibroins from CaCl2-ethanol had higher activity when linked to a known chemotherapeutic drug, L-asparaginase, than the fibroins from other treatments.

Conclusions: Collectively, these results suggest that the CaCl2-ethanol processing method produces silk fibroin with biomaterial properties that are appropriate for drug delivery.

Keywords: Silk fibroin, Calcium-alcohol solutions, Crystalline structure, Drug delivery, Biomaterial

Background

Silk fibers produced by silkworms are widely used in our daily life. While they have occupied an important niche in the textile industry for thousands of years, their potential as biomaterials has been recognized and developed only over the past decade [1]. Being non-toxic, non-immunogenic, and biocompatible with a broad range of animal species has allowed for the adherent properties of silk fibroin and silk-like proteins to be exploited for biomedical purposes. To date, silk fibroins have mainly been applied to wound healing, successfully performing as man-made blood-vessels [2], surgical sutures [3], and repair materials [4]. New processing strategies for silk fibers and proteins have expanded the biomedical utility of these molecules. For example, the gel spun silk-based matrix derived from silk fibroin was successfully applied for bladder augmentation in a murine model [5]. More recently, scientists determined that the cocoons from Bombyx mori harbor antioxidant and hypolipidemic properties and that the crude silk extracts have bioactivity against hypercholesterolemia and atherosclerosis [6].

Recently, the regenerated silk fibroin has been proved as an attractive candidate of a carrier for drug or therapeutic proteins delivery and is the focus of much ongoing research. Attachment of bioactive molecules or therapeutic proteins to silk fibroin has many benefits to enhance the properties of bioactive molecules in solution for delivery both in vitro and in vivo, including the therapeutic efficacy in the body, thermal stability, storage stability, and lengthens the circulatory half-life and...
decreases immunogenicity and antigenicity [3]. For instance, bioconjugations of insulin, glucose oxidase, L-asparaginase (L-ASNase), lipase and phenylalanine ammonia-lyase with the regenerated silk fibroin greatly improved their biological stability, reduced the immunogenicity and toxicity of the drug [7-11]. Moreover, The SELP (silk-elastinlike protein polymer)-controlled gene delivery approach could potentially improve activity of adenoviral-mediated gene therapy of head and neck cancer and limit viral spread to normal organs at the same time [12].

It has been known that the properties of silk-matrix are controlled by a combination of the chemistry and the spinning process, which directly affect the activity and stability of the enzymes attached. Spinning conditions, such as temperature, drawing rate, time, and specific type of silkworm, can modulate biomaterial features. In addition, chemistry, such as ion concentration, type of ion, and solution pH, can also affect the mechanical properties of silk fibroins [1]. In previous studies, degummed fibroin has generally been treated with aqueous solutions of hexafluoro-isopropanol (HFIP) [13], methanol [8], CaCl2-ethanol [7,9], or Ca(NO3)2-methanol [14]. Lu et al. has reported glucose oxidase attached to the regenerated silk fibroin film without treated with methanol remain more activity but lower stability than that treated with methanol [8]. After cross-linking L-ASNase with regenerated silk fibroin prepared with concentrated CaCl2 mixture solution with ethanol and water (1:2:8 mol), the immunogenicity and toxicity of the drug significantly reduced, and its circulatory half-life lengthened in vitro [9].

However, these studies have used only one treatment per experiment and, up to now, the systematic comparative analysis to distinguish the difference of those treatments has not yet been reported, thus we do not know which one is the best choice for future potential application. Here, we describe our systematic comparative analysis of silk fibroins prepared with four of the commonly used preparative solutions, Ca(NO3)2-methanol, Ca(NO3)2-ethanol, CaCl2-methanol, and CaCl2-ethanol. The results could help to reveal the mechanisms of properties of silk-derived matrix under different treating conditions and provide evidence to choose right solution to prepare silk fibroins for potential drug delivery applications.

**Materials and methods**

**Materials**

L-asparaginase (L-ASNase) from *E. coli* (10,000 IU) was purchased from Changzhou Qianhong Bio-Pharma Co., Ltd. (Jiangsu Province, China). L-asparagines (anhydrous) was purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Trichloroactic acid (TCA) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Methanol, ethanol, calcium nitrate tetrahydrate (Ca(NO3)2·4H2O), calcium chloride (CaCl2), and HgI2, all analytical reagent grade, were purchased from Chengdu Kelong Chemical Reagent Factory (Sichuan Province, China).

**Preparation of degummed silk fibroin**

Cocoons from *B. mori* were degummed by incubating in a mixture of sodium dodecyl sulfate (SDS; 0.25%,w/v) and sodium carbonate (0.25%,w/v) at 98°C for 30 min. The samples were then cooled to room temperature, rinsed three times with deionized water, and dried at 65°C overnight. The ratio of cocoons and solution was 1:100 (w/v). The degummed silk fibroins were isolated, along with another silk protein, sericin.

**Calcium-alcohol solvents treatment of silk fibers**

The isolated fibroin fibers were separately dissolved in concentrated CaCl2 solution mixed with ethanol or methanol and water (1:2:8 mol), and separately dissolved in concentrated Ca(NO3)2·4H2O solution mixed with ethanol or methanol (1:2 mol) at 65°C in a water bath for 1 h. The ratio of the silk fibers and solution was 1:20 (m/v). The aqueous solution of silk fibroin was obtained by dialyzing against flowing water. After that, the resulting dialyzed solutions were lyophilized. The dry silk powder (fibroins treated with CaCl2-ethanol solution) or pieces (from the other three solutions) were stored at 4°C until use.

**SEM**

The silk fibroins were vacuum-coated with a 20 nm layer of gold. The surface morphology of each silk fibroin was observed with a scanning electron microscope (S-3400N SEM; Hitachi, Japan) and photographed at a voltage of 15 kV and room temperature.

**SDS-polyacrylamide gel electrophoresis (PAGE)**

The silk fibroins separately treated with Ca(NO3)2-methanol, Ca(NO3)2-ethanol, CaCl2-methanol, and CaCl2-ethanol solution were analyzed by SDS-PAGE to determine the corresponding molecular weights of the protein. Samples were resolved on 12% acrylamide gel and 4% condensing gel, and protein bands were visualized by staining with 0.25% Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO, USA).

**FTIR spectroscopy**

The infrared spectra of each fibroin produced with Ca(NO3)2-methanol, Ca(NO3)2-ethanol, CaCl2-methanol, and CaCl2-ethanol solution, and degummed fibroins (as control), were measured on a FTIR spectrometer using KBr pellets (Tensor 27 FTIR; Bruker, Ettlingen, Germany). Spectra, with a resolution of 4 cm⁻¹, were
recorded and subtracted from the sample readings. All samples were measured in reflection mode; for this, the silk fibroin powder treated with CaCl₂-ethanol solution had been transformed into tablet form. The results are presented as the average of 64 repeated 4000 ~ 400 cm⁻¹ scans.

**WAXD**
The crystalline structure of the silk fibroins produced with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, CaCl₂-methanol, and CaCl₂-ethanol solution, and of degummed fibroins, were determined by WAXD using a Siemens type F X-ray diffractometer (Siemens, Munich, Germany) with Ni-filtered Cu Kα radiation. The voltage and current of the X-ray source were 30KV and 20 mA, respectively. The wavelength, λ, was 0.15406 nm. The samples were mounted on aluminum frames and scanned from 5° to 40° (2θ) at a speed of 2°/min. The D-spacing was calculated by the following equation: D = λ/ (2 × sin(θ)), i.e. D = 0.0752/(sin θ) nm. For example, if the scanning angle was 28° = 20°, then D = 0.0752/(sin 10°) nm, and the D-space was 0.43 nm.

**Solid-state ¹³C CP/MAS-NMR spectra measurement**
Solid state ¹³C CP/MAS-NMR has been successfully used to analyze the secondary structure of proteins [15], and was similarly applied in our study. The ¹³C CP/MAS-NMR spectra were recorded on a Bruker AVANCE III 400 WB spectrometer equipped with a 4 mm standard bore CP/MAS probe head, whose X channel was tuned to 100.62 MHz for ¹³C and the other channel was tuned to 400.18 MHz for broad band ¹H decoupling. A magnetic field of 9.39T at 297 K was used. The dried and finely powdered samples were packed in a ZrO₂ rotor that was sealed with an Kel-F cap and spun at 12 kHz rate. The experiments were conducted at a contact time of 2 ms. A total of 3000 scans were recorded with 6 s recycle delay for each sample. All ¹³C CP/MAS chemical shifts were referenced to the resonances of the adamantane (C10H16) standard (δCH₂ = 38.5).

**Enzyme cross-linking and activity test**
L-ASNase immobilization was performed according to the method previously described by Zhang et al. [16] with minor modifications. An aliquot (50 mg) of each fibroin produced with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, CaCl₂-methanol, and CaCl₂-ethanol solution, and degummed fibroins (as control), were placed into plastic centrifuge tubes and mixed with 2mL L-ASNase (Paradigm; Beckman-Coulter). Data are presented as mean ± SD and evaluated using the Student's t-test (SPSS 13.0, SPSS Inc.). P < 0.05 was considered to be statistically significant. The mobilization and activity detection of glucose oxidase were performed by referring to the published literature [8].

**Results and discussion**

**Morphology of silk fibroins**
The silk fibroins treated with Ca(NO₃)₂·4H₂O-methanol, Ca(NO₃)₂·4H₂O-ethanol, CaCl₂-methanol-H₂O, and CaCl₂-ethanol solution were separately dissolved. After lyophilized, the surface morphology of degummed silk fibroins and regenerated silk fibroins was observed with SEM (Figure 1). The size and shape of the degummed silk fibroins were normal, with diameters of 6–8 μm (Figure 1A). In contrast, the regenerated silk fibroins were spherical or irregular shapes. This shape may have resulted from the merger of smaller micelles that occurred in the aqueous solutions of Ca(NO₃)₂·4H₂O-methanol (Figure 1B), Ca(NO₃)₂·4H₂O-ethanol (Figure 1C), and CaCl₂-methanol-H₂O (Figure 1D), and CaCl₂-ethanol (Figure 1E).

**Molecular weight ranges of silk fibroins**
The silkworm’s cocoon is composed of two kinds of silk protein, the silk sericin, which makes up the membrane, and the silk fibroin, which makes up the inner portion. The silk sericin is a glue-like mixture of glycoproteins.
with varying molecular mass, and is removed by the degumming and rinsing steps. The silk fibroin protein of *B. mori* is rich in alanine, glycine and serine residues [17], and is ~400 kDa, with 300 kDa making up a heavy chain (H-chain), 26 kDa making up a light chain (L-chain), L-chain and H-chain linked by disulfide bond(s) and about 30 kDa making up a P25 glycoprotein that associates with the H-L complex primarily by hydrophobic interactions [18].

The silk fibroins produced with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, CaCl₂-methanol, and CaCl₂-ethanol solutions were dissolved, and the molecular weights were measured by SDS-PAGE. As shown in Figure 2, the regenerated silk fibroins treated with Ca(NO₃)₂- methanol had a molecular weight from about 95 kDa to over 170 kDa, but Ca(NO₃)₂-ethanol from about 100 kDa to over 170 kDa. The CaCl₂-methanol solution fibroins ranged from about 140 to over 170 kDa, while the CaCl₂-ethanol fibroins ranged from about 100 to nearly 300 kDa. Two low molecular weight bands, ~17 and ~26 kDa, were obviously present in these regenerated silk fibroins, but the silk fibroins produced with CaCl₂-ethanol showed relatively faint low molecular weight bands at these positions. In addition, the degummed silk fibroins are poorly soluble, except in the chemistry solution and organic solvents, we could not observe obvious bands in the gel.

This phenomenon suggested that some of the disulfide linkages and hydrophobic bonds, between silk fibroin...
molecules may have been destroyed by Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, or CaCl₂-methanol treatments. The solvent of CaCl₂-ethanol appeared to be sufficiently gentle to produce silk fibroins with less obvious damage to the secondary bonds. Thus, the CaCl₂-ethanol solution may be superior to the other solutions in its ability to protect the integrity of the fibroin secondary structure. The regenerated silk protein treated with these calcium-alcohol solvents were water-soluble, this results consistent with published reports that the silk proteins prepared from Ca(NO₃)₂-methanol were water-soluble, indicating that the regenerating coagulants affected the crystallinity and conformation of the fibroin [14].

Fourier-transform infrared spectroscopic analysis of the silk fibroins’ crystalline structure

Due to the presence of amide groups in silk protein, the characteristic vibration bands around 1620 cm⁻¹ were assigned to the absorption peak of the peptide backbone of amide I (C = O stretching), bands around 1513 cm⁻¹ to amide II (N-H bending), the bands around 1230 and 1444 cm⁻¹ to amide III (C-N stretching) [15], and 694 cm⁻¹ to amide IV [19,20]. All these characteristic absorbance peaks indicate the existence of a hydrogen-bonded NH group [21]. The molecular conformation of B. mori silk fibroin is characterized by β-sheet absorption peaks around 1630, 1530 and 1240 cm⁻¹, random coil conformation absorption peaks at 1650 or 1645, 1550 and 1230 cm⁻¹, and an α-helix absorption peak around 1655 cm⁻¹ [15,22]. Tang and colleagues had previously reported that the intensity of peaks around 3300 cm⁻¹ (data not shown here) fluctuate in response to hydrogen bonds [23].

In Figure 3, the β-sheet conformation was indicated by shifts of absorption peaks as follows: 1625-1630 cm⁻¹ (amide I), 1520-1530 cm⁻¹ (amide II), and 1265-1270 cm⁻¹ (amide III). FTIR spectra of the regenerated silk fibroins showed intense absorption peaks around 1620 cm⁻¹, 1514 cm⁻¹, and 1230 cm⁻¹, which are the characteristic absorption peaks of β-sheet. The detected crystalline structure of CaCl₂-ethanol silk fibroin showed more silk I (α-form, type II β-turn), while that of the other three fibroins showed more silk II (β-form, anti-parallel β-pleated sheet).

Wide-angle X-ray diffraction analysis of the silk fibroins’ crystalline structure

The toughness of silk fibers is dependent on their β-sheet composition. In spider and cocoon silk, the β-sheet consists of a poly-alanine or a GAGAGAGAAS sequence, arranged in an anti-parallel or parallel conformation. A previous study by Lu et al. demonstrated that the corresponding D-spacings of silk I (α-form, type II β-turn) were 0.74 nm, 0.56 nm, 0.44 nm, 0.41 nm, 0.36 nm, 0.32 nm, and 0.28 nm, and of silk II (β-form, anti parallel β-pleated sheet) were 0.98 nm, 0.48 nm, and 0.43 nm [24].

Figure 4 shows the WAXD data of the regenerated silk fibroins produced in our study with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, CaCl₂-methanol, and CaCl₂-ethanol solutions. The degummed fibroin and fibroins produced with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, and CaCl₂-ethanol showed similar 2θ diffraction peaks, which corresponded to silk II crystalline spacing of 0.47 nm (2θ = 18.4°), and silk I crystalline spacing of 0.39 nm (2θ = 22.4°). In contrast, fibroin treated with CaCl₂-
ethanol showed four obvious diffraction peaks at 2θ, namely 19.4°, 20.3°, 24.6°, and 29.3°, which corresponded to silk I crystalline spacing of 0.44 nm, 0.41 nm, 0.35 nm, and 0.30 nm, respectively. No typical diffraction peaks of silk II were found for this regenerated silk fibroin. The mean peak at 2θ = 18.4° for fibroins treated with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, or CaCl₂-methanol was not as sharp as that for degummed fibroin. This finding indicated that these solutions decreased the crystallization ability of fibroin, and the sharp peak observed at 2θ = 20.3° of CaCl₂-ethanol fibroins indicated an increased crystallization ability of fibroin.

Furthermore, the fibroins produced with CaCl₂-ethanol were composed of more silk I (α-form, type II β-turn) than the other three regenerated fibroins and the degummed fibroin, which had more silk II (β-form, anti-parallel β-pleated sheet). As reported previously, the standard orientation methods, such as rolling and drawing, are able to transform the metastable silk I into silk II [25]. Therefore, it is possible that the CaCl₂-ethanol solution is superior to the other solutions in its ability to protect the integrity of the fibroin, maintaining more silk I.

**Solid-state ¹³C CP/MAS-NMR analysis of the conformational and inter-molecular arrangement of silk fibroins**

As shown in Figure 5, the peaks on the dotted line marked Ala C₆ (16.8 ppm) correspond to the representative peaks of random coil or distorted β-turn, where the torsion angles of a backbone chain are distributed largely around the averaged angles of silk II structure [26]. The other two peaks that were observed, at 20.2 and 22.6 ppm, correspond to anti-parallel β-sheets [27]. However, the presence of peaks of Ala C₈ at 15.2 ppm and Ala Cα at 52.4 ppm suggested that the residues strongly favor an ordered structure, most likely a helical structure [28]. According to previous studies, the silk protein consists of many repeated motif sequences, such as AGSGAG [29], AGYGAG, AGVGYGAG and GAAS [30]; since glycine and alanine can readily form peptide bonds, this is a likely event in the regenerated silk fibroins.

The peaks of alanine and serine carbons that were observed in our study samples suggest that all of them contain random coils or distorted β-turns (16.8 ppm peak). An increased amount of these peaks was found in the fibroins produced with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, and CaCl₂-methanol, as compared with degummed silk fibroins and silk fibroins treated with CaCl₂-ethanol (Figure 5).

The peak position of the Cα and Cβ carbons from alanine and serine residues indicate clearly that these samples had a β-sheet structure. The silk fibroins produced with Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, and CaCl₂-methanol solutions showed increased peaks for alanine Cα and decreased peaks for glycine with C = O (169.1 ppm peak) interactions between them. Furthermore, the peaks of silk fibroin produced with CaCl₂-ethanol were nearly identical to those for the degummed silk fibroin sample. This finding may be related to the different levels of chemical shift that were produced by the Ca(NO₃)₂-methanol, Ca(NO₃)₂-ethanol, and CaCl₂-methanol solutions. Regardless, the regenerated fibroin produced with CaCl₂-ethanol solution appeared to be the best method to protect the conformational and intermolecular arrangement of the silk fibroin chains of degummed fibroin.

**Enzymatic activity of L-ASNase when conjugated with silk fibroins**

L-asparaginase is a well-established chemotherapeutic agent in routine use to treat acute lymphoblastic leukemia. However, treatment withdrawal due to side effects, some life-threatening and immunological reactions is not uncommon [31]. In addition, circulation half-life is short, necessitating longer and larger doses of the drug.

In this study, we tested whether any of the four silk fibroins produced by the different solutions had more beneficial effects on a bioconjugated enzyme that is relevant for human therapy. The L-ASNase enzyme was chosen for these in vitro experiments, along with its substrate L-asparagine. Since L-ASNase hydrolysis of L-
asparagine produces NH$_3$. Nessler’s reagent, which turns yellow in the presence of NH$_3$ was chosen to measure L-ASNase activity. According to the results, the activity of L-ASNase attached to the regenerated fibroins produced with CaCl$_2$-ethanol solution was higher than the other fibroins (Figure 6A). The highest activity of glucose oxidase-linked to the four silk fibroins was observed in CaCl$_2$-ethanol group too, very similar to that observed for L-ASNase (Figure 6B). Therefore, the CaCl$_2$-ethanol solution appear to be the most appropriate methods by which to prepare regenerated silk fibroins for use as drug delivery carriers, at least for these two particular enzymes. However, the immunogenicity and biocompatibility properties of these regenerated silk fibroins produced with CaCl$_2$-ethanol have yet to be determined and require further investigation in an animal model before clinical application.

Silk is a unique protein biopolymer, with a block co-polymer structure dominated by large hydrophobic domains and small hydrophilic spacers. This primary structure, upon folding into assembled silk structures, leads to organized crystalline domains (β-sheets) and less organized more flexible domains (more hydrated). This assembly leads to localized nanoscale pockets where other proteins may be entrapped with limited but sufficient hydration [8]. The silk biomaterial offers some important features that suggest utility as a stabilization matrix. In addition, methanol and ethanol treatment of silk fibroin resulted in a gradual transition from silk I to silk II [32,33]. The analyses also indicated that formation of aggregated strands among extended sericin chains induced by ethanol treatment is the key to generating molecular orientation [34]. Other research showed that the chimeric protein which formed by a clone encoding consensus repeats from the major protein in the spider dragline silk of Nephila clavipes fused to the carboxyl terminal domain of dentin matrix protein 1 (CDMP1) was incubated with CaCl$_2$, the secondary structure shifted from random coil to α-helix and β-sheet, due to the interactions between the CDMP1 domain and Ca$^{2+}$ [35]. The results confirmed that concentrated neutral salts such as Ca$^{2+}$, or organic solvents including methanol and ethanol can affect the crystallinity and conformation of the fibroin. However, the molecular mechanisms for these effects have to be clarified in the future.

![Figure 6](http://www.translational-medicine.com/content/10/1/117)

Figure 6 Enzyme activity tests for silk fibroins prepared with various solutions. L-ASNase (A) and glucose oxidase (B) were separately immobilized to degummed silk fibroin or regenerated fibroins prepared from four different calcium-alcohol solutions. The activities of these enzymes attached to the fibroins were calculated as a change in optical density at 450 or 460 nm measured on a microplate reader, accordingly. Results are presented as mean ± SD (n = 3 assays of triplicate samples). P < 0.05 was considered to be statistically significant.

The degradation rate of a matrix is an important parameter for a biomaterial designed to be used for tissue engineering applications. The properties of silk-matrix also directly affect the enzymatic degradation of the enzymes attached. Random coil and α-helical structures formed of the biospun fibroin accelerate the process of degradation in both PBS and enzyme solutions in comparison with β-sheets [36]. Zhang et al. reported that cross-linking L-ASNase with regenerated silk fibroin treated with CaCl$_2$-ethanol solution significantly increased heat and storage stability and resistance to trypsin digestion, and its longer half-life (63 h) than that of control L-ASNase (33 h) [9]. These observations also suggested that the silk-based matrix prepared with CaCl$_2$-ethanol solution formed more crystalline domains (β-sheets) potentially help to decrease the degradation rate.

It’s concluded that regenerated silk fibroin can be used as an immobilization matrix for enzymes or therapeutic proteins. The properties of the regenerated silk-matrix directly effect the activity and stability of the enzymes attached. In the present research, Fourier-transform infrared spectroscopy and X-ray diffraction showed that the regenerated silk-matrix treated with CaCl$_2$-ethanol has a crystalline structure with more silk I (α-form, type II β-turn), while the silk-matrice treated with other solutions have more silk II (β-form, anti-parallel β-pleated sheet). Solid-State $^{13}$C CP/MAS-NMR analysis also suggested that the silk-matrix regenerated from CaCl$_2$-ethanol were nearly identical to degummed silk fibroin, while the others show significantly different
chemical shifts. These results suggested that the silk-based matrix prepared with CaCl2-ethanol solution formed more crystalline domains (β-sheets) than others, which potentially helps to enhance the stability and improve activity of drug or therapeutic proteins. Furthermore, the properties of the regenerated silk-matrix can satisfy the needs of modern carrier materials, ruling out the use of most synthetic polymer materials, thus the carrier materials of silk fibroin treated with CaCl2-ethanol could be widely applicable.

In addition, a range of medical needs such as silk sutures, drug delivery systems, and fiber-based tissue products that exploit the mechanical properties of silks can be envisioned for ligament, bone, and other tissue repairs may become more and more popular in the next few years [1]. These materials based on silk fiber can lead to multifunctional material platforms that integrate with living systems for medical materials, industrial material and a host of other applications.

Conclusions
Preparation of B. mori degummed silk fibroin by CaCl2-ethanol preserved the best original protein structure and produced a better affinity to the enzyme drug L-ASNase than the Ca(NO3)2-methanol, Ca(NO3)2-ethanol and CaCl2-methanol treatments. The CaCl2-ethanol solution may represent the most appropriate method by which to prepare silk fibroins for use as biomaterials, especially as carriers for drug delivery.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Hao Zhang and Ling-ling Li mainly performed research; in addition, Hao Zhang analyzed data and wrote the paper. Xia Yang designed research, analyzed data and wrote the paper. Fang-yin Dai, Hao-hao Zhang and Wei Zhou helped to prepare the materials and performed research. Bing Ni revised the manuscript. Yu-zhang Wu participated in the design of the study and provided administrative support. All authors have contributed and approved the final manuscript.

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