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# A strategy for improving the mechanical properties of silk fiber by directly injection of ferric ions into silkworm

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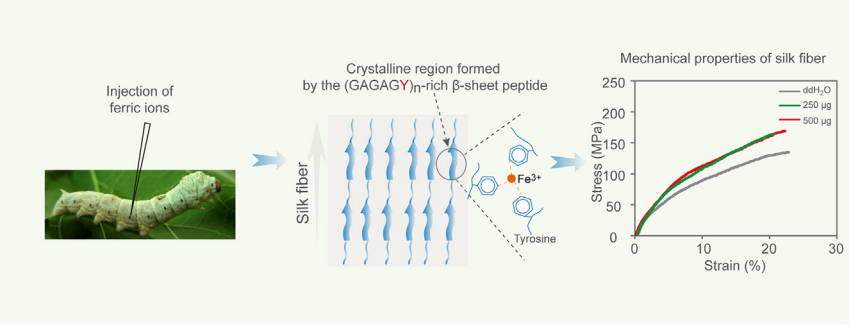
## HIGHLIGHTS

- We reported a new strategy for modifying the mechanical properties of silk fibers.
- Injection of $Fe^{3+}$ improved the mechanical properties of silk fibers.
- $Fe^{3+}$ induces the conformational transition of silk via interacting with tyrosine and tryptophan.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Silk fibers produced by the silkworm, *Bombyx mori*, are widely used bio-materials due to their superior toughness and elongation. However, the stress and stiffness of silkworm silk are not comparable to the synthetic fiber or other natural silk such as the spider silk. How to improve the mechanical properties of silk fibers has been of great interest. In this paper, we developed a new strategy for improving the mechanical properties of silk fibers by directly injecting ferric ions ($Fe^{3+}$) into silkworm. The $Fe^{3+}$-injected silkworms could produce robust silk fibers with improved stress, stiffness and toughness. Secondary structural analysis indicated that $Fe^{3+}$ promoted the conformation transition of silk from random coil or helical structure to β-sheet structure. These evidences might explain why these fibers became stronger, stiffer and tougher. Trying to unravel the molecular foundation behind this interesting phenomenon, turbidity assays and fluorescence spectroscopy were introduced. The results indicated that $Fe^{3+}$ was able to interact with tyrosine and tryptophan residues within the silk. The crosslinking might act as the "bridge" to form the β-sheet structure, thus increasing the mechanical properties of silk fiber. These findings suggested that $Fe^{3+}$ could be a promising target to modify the mechanical properties of silk fibers.



## 1. Introduction

Silk fiber, spun by silkworm, is a widely used material for textile, medical, military and other applications because its abundance and good mechanical properties such as toughness and elongation. However, the stress and stiffness of silkworm silk are not comparable to the synthetic fiber or other natural silk such as the spider silk [1].

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The defects in mechanical performance of silk thus restrain the use of silk in high performance areas. The studies for improving silk mechanical properties are very active and various methods have been developed during the past decades. Forced reeling is a good way to produce a wide range of silk fibers with improved mechanical properties [2–4]. In addition, silk fibers with special features can be directly obtained by feeding silkworm with inorganic nanoparticles such as single-walled carbon nanotubes, nanocarbon, $TiO_2$ nanoparticles, and graphene [5,6]. On the other hand, the mechanical properties of silk fibers can be significantly improved by genetic modification of the silk by overexpression of spider silk gene [5–8]. Recently, emerging evidences have revealed that the metal ions are able to induce the conformational transition of silk proteins [9]. $Ca^{2+}$can induce a stable protein network in silk fibroin, while $Na^+$ and $K^+$ have an opposite effect in silk protein [10]. $Mg^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ can induce the conformation transition of silk fibroin from random coils to β-sheet [9,11–13]. Inspired by these findings, our group previously discovered the positive effects of calcium ions, potassium ions and copper ions on silk conformational transition and mechanical properties in vivo [10,14]. Furthermore, over-expression of a calcium-transporting protein (SERCA) in the spinning duct of silkworm to disrupt the $Ca^{2+}$ content in silk significantly increases the strength, toughness and stiffness of silk fibers [15].

Ferric ion ($Fe^{3+}$) is trivalent ion with a strong electronegativity. It is suggested that the positive charged ions (such as $K^+$ and $Ca^{2+}$) may have a strong electrostatic interaction with carboxylic acids side chain of silk [14,16]. Thus, $Fe^{3+}$ might play a special role in the conformational transition and mechanical properties of silk. However, there are few reports focused on the relationship between $Fe^{3+}$ and silk. Most of these studies are carried on in vitro [9,16]. Although evidences have shown that $Fe^{3+}$ can induce a conformational change from helix to β-sheet in silk fibroin [17], the effects of $Fe^{3+}$ on silk fibers formation and mechanical properties in vivo have not yet been reported.

This study aims to improve the mechanical properties of silk fibers by $Fe^{3+}$ and investigate the effect of $Fe^{3+}$ on conformation transition in silk in vivo. To address this goal, $Fe^{3+}$ was injected into the hemolymph of silkworm. Then, we measured the mechanical properties and the secondary structure of silk fibers spun by injected silkworm by tensile testing and synchrotron Fourier-transform infrared spectroscopy (S-FTIR), respectively. Furthermore, we found that $Fe^{3+}$ was able to interact with tyrosine residues and induced the conformation transition of silk fibroin.

## 2. Materials and methods

### 2.1. Insect rearing and sample preparation

Silkworms (strain *21–872*) were kindly provided by the State Key Laboratory of Silkworm Genome Biology, Southwest University. The larvae were reared in lab conditions (27 ± 1 °C, 60% RH) and fed with mulberry leaves until spinning. The spinning larvae were used for dissection and injection. The others were allowed to spun cocoons naturally for subsequent analysis.

### 2.2. Injection of $FeCl_3$

Silkworms weighing 3.0 ± 0.1 g were divided into three groups (each group had 60 larvae) randomly for the experiments. We chose the spiracles in postmedian of the silkworm for injection. Silkworms were injected with 10 μL different doses (500 μg/larva and 250 μg/larva) of $FeCl_3$ solution into the hemolymph by spiracle. The larvae from control group were injected with 10 μL $ddH_2O$·The injected silkworms were allowed to make cocoons naturally in the same conditions (27 ± 1 °C, 60% RH). After the cocoons were constructed successfully, they were randomly chosen for S-FTIR analysis and mechanical testing as described below.

### 2.3. Mechanical testing

After removed the cocoon floss, the single fibers with two brins were reeled from the cocoons in hot $NaHCO_3$ solution (80–100 °C) and dried at room temperature. Mechanical tests were performed according to the method previously reported [10]. The first 50 m of the single fibers were collected and cut into 2 cm fragments for diameter measurement and mechanical testing. The samples were sputter coated in MP-19020NCTR Neocoater (JEOL, Japan) and then transferred to the JCM-5000 scanning electron microscope (JEOL, Japan) for diameter measurement. The diameters were used to calculate the cross-sectional areas of the single fibers. Mechanical tests were carried out using an AG-X plus instrument (Shimadzu, Japan) with a stretch speed of 2 mm/min. The results were recorded in TRAPEZIUM 1.26 software (Shimadzu).

### 2.4. S-FTIR analysis

Five cocoons were randomly chosen for S-FTIR analysis. The cocoons were firstly put into 0.5% (*w*/*v*) $NaHCO_3$ solution for degumming, then dried completely at room temperature. Experiments were performed at BL01B in the Shanghai Synchrotron Radiation Facility (SSRF) with the Nicolet 6700 Fourier transform infrared spectrometer, infrared microscopy, and imaging systems. Infrared spectra were collected in the mid-infrared (MIR) range of 800–3800 $cm^{-1}$ at a resolution of 4 $cm^{-1}$ with 256 coadded scans. The spectra data collection and processing were using OMINC 9 software (Thermoscientific). The curve fitting was carried out using the PEAKfit4.0 software. Secondary structure analysis was performed afterwards using the previous reported protocol [18]. It should be noted that each spectrum reported in this article was from a single experiment, which just represents the results of second derivative of one spectrum. But the data obtained from the spectra (e.g., second structure content, etc.) reported in this article were the average results taken from at least nine separate samples.

### 2.5. Observation of the spinning behavior of silkworms after injection

We chose the spinning speed as an index to investigate the spinning behavior of silkworms after injection. Five wandering larvae from each injected group ($ddH_2O$, 250 μg, and 500 μg $FeCl_3$) and untreated group were chosen to perform this experiment. The silkworms were placed on a clean desktop to spin. The spinning length of the silkworms in one minute were measured at the time point of 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 8 h, 12 h after injection to calculate the spinning speed of silkworm.

### 2.6. Preparation of regenerated silk fibroin (RSF) solution

Clean cocoons were chosen and cut into small pieces. Then the cocoon pieces were boiled twice for 30-min in 0.5% (*w*/*v*) $NaHCO_3$ solution until degumming thoroughly. The degummed fibers were allowed to dry in a fume hood overnight. The dry degummed fibers were dissolved in 9.3 mol/L LiBr solution, then dialyzed in deionized water for 2 days [19].

### 2.7. Turbidity measurement

Turbidity measurement was carried out by adding different concentrations of $FeCl_3$ into the RSF. Measurements were performed by using a UV visible spectrophotometer (DU800, Beckman Coulter) with the absorbance at 600 nm.

### 2.8. Circular dichroism (CD) analysis

The circular dichroism spectra of the RSF were recorded by a MOS-500 CD spectrophotometer (Biologic, France) with a scanning range from 190 nm to 250 nm. The experiments were repeated three times