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Abstract: There is evidence to suggest that Cu(II) is involved in the natural spinning process of a silkworm helping to convert the concentrated silk fibroin (SF) solution (or dope) into tough insoluble threads. To investigate the interaction between SF and Cu(II), a series of regenerated SF (RSF) films with different mass ratios of Cu(II) to SF were prepared. X-ray photoelectron spectroscopy (XPS) was employed to study the chemical interaction between Cu(II) and SF in these Cu(II)–RSF films. A significant change in the binding energy of Cu 2p3/2 demonstrated that the chemical state of Cu(II) in the Cu(II)–RSF films was affected by the interaction between Cu(II) and SF. Moreover, chemical shifts of N 1s and O 1s of SF were also detected, implying that Cu(II) may coordinate with both N and O atoms in the SF. In addition, Raman spectra of the same series of Cu(II)–RSF films recorded the conformation transition of SF that may occur by the coordination of Cu(II) and SF macromolecular chains. © 2006 Wiley Periodicals, Inc. Biopolymers 82: 144–151, 2006

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INTRODUCTION

People used to think that the extraordinary mechanical properties of natural fibers, for example, spider dragline silk and silkworm silk fibers, which are comparable or even superior to those of high performance synthetic polymers, mainly depend on their unique amino acid sequence.¹–⁴ However, in the past few
years, it has been possible to produce recombinant 
silk proteins with precisely specified amino acid 
sequences that match those of natural silk proteins, 
yet spinning these materials through various process-
ing routes yields silk fibers with mechanical prop-
erties much worse than those of natural silks.7,8 
Hence it can be reasonably supposed that the actual 
mechanism by which a natural silk fiber is formed is 
equally important in the production of high perform-
ance fibers. This is further supported by the recent 
finding of Shao and Vollrath that the mechanical 
properties of silkworm silk can approach those of 
Nephila edulis spider dragline silk merely by control-
ing the movement of the silkworm’s head and keep-
ing a constant reeling velocity for drawing the fiber. 
Therefore the associated weakness and lower extensi-
bility of silkworm silk are a result of the method of 
spinning, highlighting the need to understand and thus 
control the relationship between the spinning mecha-

nism and the mechanical properties of natural fibers.

Many studies have revealed that silkworms and 
spiders spin their fibers from a liquid crystalline aque-
ous protein solution, with the proteins undergoing a 
conformational change from random coil and/or heli-
cal conformation into a β-sheet crystalline structure 
upon application of a shear or extensional flow.4,9,10 
Other factors, such as pH and the presence of various 
metallic ions, were found to play a part in the spinning 
process.11–16 It is known that metallic ions, in 
particular Cu(II) ions, cause a conformation change 
from helical conformation to β-sheet in several im-
portant and well-studied proteins, such as Prion pro-
teins (PrP) and amyloid β-peptides (Aβ).17–23 For this 
reason we have followed the changes in the local elec-
tronic structure of Cu(II) ions in SF films using a new 
X-ray photoelectron spectroscopy (XPS) technique.

XPS has been widely used for the structural char-
acterization of metal–polymer films involving both 
synthetic and natural polymers.24–27 In addition, it 
permits the sensitive detection of the ability to adsorb 
proteins onto polymer surfaces,28–32 as well as the 
metal sorption by polysaccharides (chitin or chitosan) 
or proteins (collagen or globular proteins).33–38 XPS 
enables the observation of the oxidation state and 
chemical environment of metallic ion by detection of 
the binding energy (BE) of the inner electrons of the 
metallic ion.39 This additional information will com-
plement the structural determination already gained of 
the interaction of SF with metallic ions by Raman, 
NMR, FTIR, and CD.40–44 By examining regenerated 
SF (RSF) films with the different ratios of Cu(II) ions, 
we hope to provide an insight of how Cu(II) may 
change the conformation of SF. Conventional Raman 
spectroscopy provides supporting evidence of the 
conformational change of the SF in the cast Cu/SF 
films. This information will help us to understand the 
spinning mechanism of the Bombyx mori silkworm.

**EXPERIMENTAL**

**Sample Preparation**

Raw B. mori silk fibers were treated with boiling aqueous 
Na2CO3 (0.5 wt %) solution for 30 min to remove sericin. 
The RSF solution was prepared by dissolving 10 g deg-
gummed silk in 100 mL 9.3 mol/L LiBr solution at room 
temperature and dialyzed against deionized water for 3 
days. After dialysis, the RSF solution (4.0 wt %) was mixed 
in the ratio 3:1 (v/v) with CuCl2 aqueous solution (with dif-
derent CuCl2 content) to ensure complete mixing of the SF 
with the Cu(II) ions. The mixtures were cast onto poly sty-
renes disks and allowed to dry at room temperature for 
3 days, thereby obtaining a series RSF–Cu(II) films with 
different mass ratios of Cu(II) to SF. All films show good 
homogeneity by optical and SEM observation.

**XPS Analysis**

XPS experiments were performed on a PHI 5000C ESCA 
system (Perkin Elmer, USA) equipped with an X-ray source of Mg/Al dual anodes. In general, the X-ray anode was run 
at 250 W and the high voltage was kept at 14.0 kV with a 
detection angle of 54°. Complete XP energy spectra in 
the range 1200 to 0 eV were acquired with Al Kα radiation 
(hν = 1486.6 eV) and 93.90 eV pass energy, while spectra 
of a selected narrow energy range for each of the elements 
studied were recorded at a pass energy of 23.50 eV to 
ensure both sufficient sensitivity and higher resolution. 
The base pressure of the analyzer chamber was about 5 × 10⁻⁷ 
Pa. The position of the carbon peak (284.6 eV) for C 1s in 
—CH₂ was used to calibrate the XPS BE scale for all sub-
strates.33,38 XPS data fitting was performed using commer-
cially available software with 100% Gaussian curve fitting. 
A linear background subtraction was used for C 1s, N 1s, 
and O 1s curves, and a Shirley background subtraction was 
used for Cu 2p3/2. It was preferred to perform the fitting 
while allowing the full-width half maximum (FWHM) to 
var.

**Raman Spectroscopy**

Raman spectra were recorded using a Dilor LabRam-1B 
Raman microscope (Dilor, France), operating at a resolu-
tion of 1 cm⁻¹. A 3 mW He–Ne laser (of wavelength 
632.81 nm) was focused with a 100× objective (0.8 NA-
Olympus, Olympus, Japan) to a diameter of approximately 
0.7 µm. The spectra were collected 200 s for each exposure 
using a 1-inch open electrode Peltier-cooled CCD detector 
(1024 × 256 pixels). The region of 1600–1720 cm⁻¹ for 
amide I (stretching vibration of C=O) was investigated 
because of its high intensity and lack of overlap with other 
molecular vibrations. In addition, this band is widely used
RESULTS AND DISCUSSION

XPS Analysis of Neat SF Film

SF consists of heavy (391 kDa) and light chain (28 kDa) polypeptides joined by a disulfide link. In addition, another polypeptide called P25 (25 kDa) associates with the heavy–light chain complex primarily by hydrophobic interactions. The heavy chain has 5263 amino acid residues composed of 45.9% glycine (Gly), 30.3% alanine (Ala), 12.1% serine (Ser), 5.3% tyrosine (Tyr), 1.8% valine (Val), and 0.25% tryptophan (Trp) repeats with alternating hydrophobic and hydrophilic side groups along the chain.52,53 Chemical analysis using XPS gives the atomic fraction of C, N, and O in SF as 0.52, 0.31 and 0.17, respectively (Table I), which coincided well with the data calculated from the amino acid composition of SF.

Figure 1 (curve a) shows the whole XP spectra of a pure SF film. The C 1s peak can actually be separated into three peaks, by careful curve fitting; the triplet of peaks implies the existence of multiple bonding states for carbon. According to assignments in the literature,28–31,33,35,38 the C 1s peak consists of a peak at 284.6 eV being attributed to C – C and C – H, one at 285.7 eV related to C – N, and one at 287.6 eV related to O = C – N, in the ratios 0.55, 0.23, and 0.22, respectively (Table I). Thus the BE assignments are concluded to be reliable because it is easy to deduce from the chemical structure of protein backbone that the C – N and O = C – N groups are equal. In addition, the BE of O 1s is 531.0 eV33,38 and of N 1s is 399.0 eV.31,54 All the above results indicate that XPS is a practical technique not just for chemical analysis but also to provide structural information about SF. Therefore the technique was used to determine the interaction between Cu(II) and SF in RSF–Cu(II) films.

XPS Analysis of RSF–Cu(II) Films

The complete energy ranges XP spectra of RSF–Cu(II) films are shown in Figure 1. Besides the BE peaks of C 1s, N 1s, and O 1s, two other BE peaks corresponding to Cu 2p1/2 and Cu 2p3/2 occur. It is found that, with an increase of the Cu(II)/SF mass ratio (hereafter simply termed Cu(II)/SF ratio), the BE peaks intensities of N 1s and O 1s decrease. This is shown more clearly in Table II: the N/C and O/C ratios decrease with an increase of the Cu(II)/SF ratio. For a ratio of Cu(II)/SF = 0.01, the N/C ratio (that is the fraction of N) decreases while the O/C ratio remains the same as that of pure SF (Cu(II)/SF = 0). A further increase of the Cu(II)/SF ratio causes both N/C and O/C to decrease. Since the quantity of N and O relative to C remains constant as there is no change in the chemical composition of the SF, the decrease of N/C and O/C ratios reflects a ‘‘shielding’’ of N and O by other atoms (i.e., a concealment of the N and/or O from exposure to the X-rays). This can only be caused by the Cu present in the films interacting with the N and/or O. Moreover, Cu seems to interact preferentially (or at least first) with the N atoms in SF because only the N/C ratio decreases when the Cu(II) content in the Cu(II)–RSF films is low (for Cu(II)/SF = 0.01).

Other evidence of the interaction between N, O, and Cu is the chemical shifts of N 1s, O 1s, and Cu 2p. The BE of N 1s and O 1s shifted significantly to a higher position (0.6 eV for N 1s and 0.5 eV for O 1s) when the ratio of Cu(II)/SF was 1 (Figure 2). Similar shifts for N 1s and O 1s have been reported for chitosan in the presence of Cu(II); the BE of both N 1s and O 1s were shifted to a higher energy (0.8 ~ 0.9 eV).

Table I  Binding Energy and Atomic Fraction of C, N, and O in Pure RSF Film

<table>
<thead>
<tr>
<th>Element</th>
<th>Binding Energy (fraction)</th>
<th>Assignment</th>
<th>Atomic Fraction by XPS</th>
<th>Atomic Fraction by Theoretical Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1s</td>
<td>284.6 eV (0.55)</td>
<td>C – C, C – H</td>
<td>0.52</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>285.7 eV (0.23)</td>
<td>C – N</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>287.6 eV (0.22)</td>
<td>O = C – N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O 1s</td>
<td>531.0 eV (1.00)</td>
<td>O in amide</td>
<td>0.31</td>
<td>0.24</td>
</tr>
<tr>
<td>N 1s</td>
<td>399.0 eV (1.00)</td>
<td>N in amide</td>
<td>0.17</td>
<td>0.21</td>
</tr>
</tbody>
</table>

*Data calculated from the 10 main amino acid residues (Gly, Ala, Ser, Tyr, Val, Asp/Asn, Thr, Glu/Gln, Phe, and Ile) that comprise 98.1% of the total composition of the heavy chain.
shift), indicating the formation of a complex by the coordination of Cu(II) to –NH2 and –OH groups in chitosan.34 In addition to the chemical shifts of N 1s and O 1s, a more significant chemical shift of Cu 2p further demonstrates the coordination of Cu(II) to the SF macromolecular chains. Figure 3 displays the variation of Cu 2p XP spectra of RSF–Cu(II) films for the same Cu(II)/SF ratios. The BE of Cu 2p3/2 appeared at 932.0 ~ 932.5 eV and Cu 2p1/2 appeared at 951.6 ~ 952.0 eV, for which both are shifted greatly compared with CuCl2·2H2O (934.2 and 954.2 eV, respectively34,36). This gives the strongest indication of the coordination of Cu(II) and SF in RSF–Cu(II) films.

With increasing Cu(II)/SF ratio, the XP spectra of both Cu 2p3/2 and Cu 2p1/2 become asymmetric. A shoulder at 934.4 eV similar to Cu 2p3/2 in CuCl2·2H2O appears (the same is found in Cu 2p1/2), which may be due to the excess Cu(II) not coordinated to N and/or O at high Cu(II)/SF ratios. Thus, it is possible to deconvolute the Cu 2p3/2 curves into two components, one at 932.0 eV and the other at 934.2 eV, that correspond to the coordinated and excess “free” Cu(II), respectively. In addition, a further peak appears at 942.2 eV with the increase of Cu(II)/SF ratio, also indicating an increase of free Cu(II). The ratio of the intensity at 932.0 eV to the total intensities at 932.0, 934.2, and 942.2 eV [i.e., I932.0/(I932.0 + I934.2 + I942.2)] may indicate the fraction of the coordinated Cu(II) in Cu(II)–RSF films. Figure 4 shows that such a fraction decreases with the increase of Cu(II)/SF ratio, indicating that, for a lower concentration of Cu(II) in the Cu(II)–RSF films, the more Cu(II) is coordinated with SF. The intensity of Cu 2p is too weak to be detected when Cu(II)/SF < 0.0025, so the fraction of coordinated Cu(II) could not be determined. Nevertheless, by extrapolation of the experimental data, we may conclude that, for a Cu(II)/SF ratio of about 0.001, all the Cu(II) is coordinated to SF, which is in good agreement with the value obtained by spectrophotometry in our previous work.41

**Raman Spectra of RSF–Cu(II) Films**

The results of the XPS analysis shown above indicate that Cu(II) coordinates to N and O atoms in SF macromolecular chains, indicating the formation of a complex by the coordination of Cu(II) to –NH2 and –OH groups in chitosan.34 In addition to the chemical shifts of N 1s and O 1s, a more significant chemical shift of Cu 2p further demonstrates the coordination of Cu(II) to the SF macromolecular chains. Figure 3 displays the variation of Cu 2p XP spectra of RSF–Cu(II) films for the same Cu(II)/SF ratios. The BE of Cu 2p3/2 appeared at 932.0 ~ 932.5 eV and Cu 2p1/2 appeared at 951.6 ~ 952.0 eV, for which both are shifted greatly compared with CuCl2·2H2O (934.2 and 954.2 eV, respectively34,36). This gives the strongest indication of the coordination of Cu(II) and SF in RSF–Cu(II) films.

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### Table II  Atomic Fractions and N/C, O/C Ratios in Different Cu(II)–SF Films

<table>
<thead>
<tr>
<th>Cu(II)/SF</th>
<th>C</th>
<th>N</th>
<th>O</th>
<th>N/C</th>
<th>O/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.52</td>
<td>0.17</td>
<td>0.31</td>
<td>0.32</td>
<td>0.59</td>
</tr>
<tr>
<td>0.01</td>
<td>0.53</td>
<td>0.15</td>
<td>0.32</td>
<td>0.27</td>
<td>0.60</td>
</tr>
<tr>
<td>0.1</td>
<td>0.56</td>
<td>0.14</td>
<td>0.30</td>
<td>0.25</td>
<td>0.55</td>
</tr>
<tr>
<td>1</td>
<td>0.60</td>
<td>0.13</td>
<td>0.27</td>
<td>0.22</td>
<td>0.46</td>
</tr>
</tbody>
</table>

**Biopolymers DOI 10.1002/bip**
romolecular chains. To investigate the influence of such coordination on the conformation changes of SF, Raman spectroscopy was applied in this study. Figure 5 shows the amide I region of Raman spectra with the same Cu(II)/SF ratios as used in the films for the XPS analysis. Pure RSF films (curve a) show a maximum wavenumber of amide I band at 1659 cm\(^{-1}\) corresponding to the random coil and/or helical conformation (hereafter shortened to “random coil”).\(^{45,46,50}\) After the addition of small amount of Cu(II) [Cu(II)/SF = 0.001, curve b], the maximum of amide I band shifts away from 1659 cm\(^{-1}\). With continuing increase of the Cu(II)/SF ratio to 0.1 (curve c and d), the maximum of the amide I band shifts to 1667 cm\(^{-1}\), which is usually characteristic of \(\beta\)-sheet.\(^{45,50,51}\) This may indicate that the conformation of SF in the films were changed and part of them probably converted from “random coil” to \(\beta\)-sheet. We assume such a transition because \(B.\), mori SF has a primary structure that is largely a regular alternation of glycine with alanine or serine, which has the intrinsic propensity to form a \(\beta\)-sheet structure under favorable conditions.\(^{55}\) Our previous study of RSF–Cu(II) films with \(^{13}\)C CP/MAS NMR spectra also showed an increase of \(\beta\)-sheet content when Cu(II)/SF ratio was about 0.001.\(^{20}\) Moreover, a similar conformation transition was observed as well in our studies on the effects of Cu(II) on natural SF dope and concentrated RSF aqueous solutions (with the same protein concentration as the natural spinning dope).\(^{56}\) The critical Cu(II)/SF ratio for the onset of the conformation transition in concentrated RSF solution is also around 0.001, which is consistent with the results shown above. However, because of the broadness of the amide I band in the Raman spectra, other conformations and conformation changes may also be present. For instance, a component near 1685 cm\(^{-1}\) that was shown in the concentrated RSF solution\(^{56}\) can be also seen in the Raman spectra presented here. Such a component

\[\text{FIGURE 3} \quad \text{Cu2p XP spectra of RSF–Cu(II) films and the related deconvolution curves (a–d) as well as CuCl}_2 \cdot 2\text{H}_2\text{O (e). Cu(II)/SF ratio (w/w): (a) 1/400; (b) 1/100; (c) 1/10; (d) 1/1.}\]

\[\text{FIGURE 4} \quad \text{Effect of Cu(II)/SF ratio on the fraction of coordinated Cu(II) in RSF–Cu(II) films, calculated from the ratio of the intensity of the peak at 932.0 eV to the summed intensity of the peaks at 932.0, 934.2, and 942.2 eV, respectively, related to the total Cu(II) in the Cu(II)–RSF films.}\]

\[\text{FIGURE 5} \quad \text{Amide I region of Raman spectra of RSF–Cu(II) films. Cu(II)/SF ratio (w/w): (a) 0; (b) 1/1,000; (c) 1/100; (d) 1/10.}\]
may due to turns and was regarded as an “intermediate conformation” between “random coil” and β-sheet in our previous paper. It is possible that the shift of the amide I band to 1667 cm⁻¹ was a consequence of an increase intensity of this “intermediate conformation” mainly composed of turns. In addition, the shift of the peak maximum with the increase of Cu(II)/SF ratio may only be due to the global disordering or the loosening of the overall protein secondary structure. Further work and more evidence are needed to clarify this point.

**Interactions between Cu(II) and SF Macromolecular Chains**

Much effort has been made previously by us to understand the possible role of Cu(II) ions in the spinning process of silkworm in vivo. We first demonstrated that the Cu(II) content increased from the posterior part of the silk gland to the anterior part and further increased in the silk fiber. The coordination between Cu(II) and SF has been shown to occur in dilute RSF solution by means of spectrophotometry and electronic paramagnetic resonance. The conformation transition of SF from “random coil” to β-sheet induced by the addition of Cu(II) ions in natural SF dope, concentrated RSF solution, and RSF membrane was detected with ¹³C CP/MAS NMR (for membranes) and Raman spectroscopy (for solutions). All of these results may indicate that Cu(II) ions aid the in vivo formation of the β-sheet structure, and hence why the Cu(II) content is higher in the silk fiber than in the gland and duct. However, the effect of Cu(II) ions may be only one of the factors in the complicated process of spinning biopolymers. In our previous paper, we attributed such a Cu(II) effect to the stable coordination of Cu(II) with SF heavy chain via the AHGGYSGY motif, but here we wish to modify this assumption according to the semiquantitative analysis of XPS data. In general, the most probable amino acid residues that could coordinate with Cu(II) are histidine (H), tryptophan (W), tyrosine (Y), and serine (S). As shown above, from the Cu(II)/SF ratio for the onset of the conformation transition of about 0.001, we can deduce that one SF heavy chain may be coordinated by 6 ~ 7 Cu(II) ions at such a Cu(II)/SF ratio. Also there are 3 repeats of the AHGGYSGY motif and 11 repeats of the GYEYAWSS motif in SF heavy chain that have the potential to coordinate Cu(II). Previously, we suggested that the histidine-containing motif AHGGYSGY may be the coordination site of Cu(II) since it is somewhat similar to the highly conserved PHGGGWGQ in PrP thought to be particularly important for binding Cu(II) ions. However, only 3 binding sites among 5263 amino acid residues might be too little to induce a conformation transition of SF. If we consider tryptophan in the GYEYAWSS motif as another potential binding site of Cu(II) as in PrP, the total binding sites in SF heavy chain increase to 14. Furthermore, it would be highly possible that one Cu(II) binds two amino acid residues from either different SF chains or different parts of the same SF chain. Therefore, the number of amino acid residues coordinated by Cu(II) when Cu(II)/SF = 0.001 may be between 12 and 14. From the discussion above, we may suggest that the conformation transition of SF induced by Cu(II) in vitro is aided by the coordination of Cu(II) to SF macromolecular chain, in which one Cu(II) binds two amino acid residues of histidine and/or tryptophan in the AHGGYSGY or the GYEYAWSS motifs. The His(Typ)–Cu(II)–His(Trp) bridges formed between different SF chains or different parts of the same SF chain may make them parallel to each other to enable the formation of intermolecular hydrogen bonds and hence induce the formation of β-sheet. Whether this may be dramatically different from the natural spinning process in vivo, in which the Cu(II)/SF ratio is much less (~ 2 × 10⁻⁶) and the main driving force for conformation transition is shear and/or extensional flow is a cause of speculation.

**CONCLUSION**

The interaction between Cu(II) and SF in the RSF–Cu(II) films with different Cu(II)/SF ratio were investigated by means of XPS. The BE of O 1s, N 1s, and Cu 2p changed with increasing Cu(II) content, indicating a coordination between the Cu(II) ions and SF macromolecular chains; at lower Cu(II)/SF ratios in the film, a greater proportion of the Cu(II) was coordinated as observed from the Cu 2p peaks in the XP spectra. The coordination of Cu(II) and SF result in the conformation transition of SF, which was monitored by Raman spectroscopy. From both XPS and Raman analysis, we may suggest that the Cu(II)/SF ratio for the initialization of the conformation transition was about 0.001, where all of Cu(II) may coordinate to SF macromolecular chains. This value accorded well with that of our previous work using different methods and different samples.

These findings may have important implications for our understanding of Cu(II) effect on the natural spinning process of silkworm and may assist in the ultimate development of artificial methods for spinning strong fibers from silk-like proteins.
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