An Investigation Into Transition Metal Ion Binding Properties of Silk Fibers and Particles Using Radioisotopes

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ABSTRACT: Silk is a structural protein fiber that is stable over a wide pH range making it attractive for use in medical and environmental applications. Variation in amino acid composition has the potential for selective binding for ions under varying conditions. Here we report on the metal ion separation potential of Mulberry and Eri silk fibers and powders over a range of pH. Highly sensitive radiotracer probes, $^{64}Cu^{2+}$, $^{109}Cd^{2+}$, and $^{57}Co^{2+}$ were used to study the absorption of their respective stable metal ions Cu^{2+}, Cd^{2+}, and Co^{2+} into and from the silk sorbents. The total amount of each metal ion absorbed and time taken to reach equilibrium occurred in the following order: $Cu^{2+} > Cd^{2+} > Co^{2+}$. In all cases the silk powders absorbed metal ions faster than their respective silk fibers. Intensive degumming of the fibers and powders significantly reduced the time to absorb respective metal ions and the time to reach equilibrium was reduced from hours to 5–15 min at pH 8. Once bound, 45–100% of the metal ions were released from the sorbents after exposure to pH 3 buffer for 30 min. The transition metal ion loading capacity for the silk sorbents was considerably higher than that found for commercial ion exchange resins (AG MP-50 and AG 50W-X2) under similar conditions. Interestingly, total Cu^{2+} bound was found to be higher than theoretically predicted values based on known specific Cu^{2+} binding sites (AHGGYSGY), suggesting that additional (new) sites for transition metal ion binding sites are present in silk fibers. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 3630–3639, 2011

Key words: absorption; silk; fibers; powder; separation of metal ions

INTRODUCTION

Silk is a structural protein composed of highly repetitive sequences of amino acids spun by silk producing arthropods, primarily silkworms, and spiders.[1] The diversity of amino acid side chains in silk protein (known as fibroin) provides options to bind a range of ions for various applications. For instance, to improve weight, handling, and luster in silk fabric, it was a common practice in the textile industry to load the silk with tin-phosphate-silicate salts.[2,3] In recent years, silk fabrics have been treated with Cu^{2+}, Co^{2+}, and Ag^+ to induce antibacterial properties.[4,5] The binding of sulfate and sulfonate groups has been found to render anticoagulant and antithrombogenic properties to the silk material,[6,7] while binding of doxycycline and ciprofloxacin allows it to be used as an antibiotic delivery vehicle.[8]

Silk has also been used as a sorbent to effectively separate transition metal and actinide ions.[5,9,10] It is expected that novel silk materials in the form of particles would increase their potential as a sorbent due to their high surface area and versatility in product design. For instance, a column filled with chopped silk fiber was investigated for separation of Cu^{2+}.[11] Likewise, polyphenol (an antioxidant from olive leaf extracts) was separated and delivered using silk powder.[12,13]

There are numerous commercially available silk varieties; however, the absorption of silk has been mainly investigated using the well known Mulberry silk fibers produced by the domestic silkworm *Bombyx mori* (*B.mori*). Other commercially available silk fibers such as Eri (produced by *Philosamia cynthia ricini* silkworms) are different from Mulberry silk in terms of their amino acid composition, sequence, and fine structure. Table I summarizes the composition of key amino acids (aa) in Mulberry and Eri silks that have been previously identified to be involved in binding transition metal ions.[4,14–20] There is a distinct difference in aa composition between the two silks and it is of interest to see if these differences might show variation in their respective metal ion binding properties. Furthermore,

TABLE I
Composition of Selected Amino Acids in Silk (% Moles)

Amino acid	Mulberry (B.mori)				Eri (P.c ricini)			
Reference	Ref: 41	Ref: 42	Ref: 43	Ref: 44	Ref: 41	Ref: 42	Ref: 43	Ref: 44
Tyrosine	4.80	5.07	5.42	5.58	4.50	5.56	5.37	5.84
Aspartic acid	1.90	1.28	1.51	1.49	2.70	3.53	3.52	3.89
Arginine	0.50	1.83	1.90	1.13	1.70	1.87	6.95	4.12
Hystidine	0.20	0.53	0.36	0.13	1.0	1.4	1.53	0.75
Glutamic acid	1.40	1.0	1.07	1.77	0.70	0.79	0.66	1.31
Lysine	0.40	0.63	0.60	0.23	0.20	0.34	0.3	0.23
Phenylanine	0.70	0.62	0.50	0.14	0.20	0.11	0.64	0.23
Tryptophan	–	0.33	0.39	0.73	0.30	–	0.52	1.68

[a] Protein collected from silkworm's gland.

different silk varieties are also known to have quite different proportions of micro- and macro-voids in their fibers,[21–26] which may also influence ion diffusion properties.

Many different techniques have been used to measure metal ion binding. These include proton induced X-ray emission (PIXE),[27,28] neutral red fading method (NR),[28] inductively coupled plasma mass spectroscopy (ICP-MS),[9,27] inductively coupled plasma-atomic emission spectroscopy (ICP-AES),[4,5] and atomic absorption spectroscopy (AAS).[10,27] For this study, we have used radioisotopes to track the metal ion movement as they offer a more sensitive (up to 10^{-14} M) signal to noise ratio and faster processing. Moreover, smaller quantities of materials can be used for these types of experiments. Because the gamma signal (detection limit of 50 Bq) from the radioisotopes is largely insensitive to media (i.e., solid or liquid), negligible processing of solution is required and therefore more accurate assessment of metal binding may be achieved.

In this study, transition metal ion binding behavior of silk sorbents is conducted in buffered solutions to prevent the formation of metal hydroxide species. By understanding the effect of pH on the metal ion binding of the silk materials, we hope to identify how these materials can be applied as sorbents in industrial and environmental applications. A deeper understanding of the metal-silk fibroin interactions and the influence of pH is also useful for gaining an insight into the mechanism of silk spinning and structure development.[27,29,30] Therefore it may shed light on how metal ions can potentially be used to engineer ultra high strength green fibers from recombinant proteins.

EXPERIMENTAL

Material preparation

Raw cocoons of indoor reared Eri and Mulberry silk were purchased from cocoon production centers in Northeast India. The cocoons were processed to obtain different silk materials in the following manner.

Normally degummed Eri fiber

Eri silk cocoons (1 kg per batch) were degummed for 120 min in a laboratory dyeing machine (from Thies) using laboratory grade 2 g/L Na_2CO_3 and 0.6 g/L sodium dodecyl sulfate (from Sigma Aldrich) at 100°C with a material (kg) to liquor (L) ratio 1 : 25. After degumming, the cocoons were thoroughly washed with warm distilled water followed by copious amounts of cold distilled water. Degummed silk fibers were dried at 60°C overnight.

Normally degummed Mulberry fiber

Mulberry silk cocoons were degummed as for normally degummed mulberry fiber (ND-E-F), except that the time of degumming was reduced to 20 min, as silk gum from Mulberry silk could be removed faster compared to Eri silk under similar conditions.

Intensively degummed Eri fiber and Mulberry fiber

ID-E-F and ID-M-F were prepared from respective cocoons and processed in an identical manner. A harsher degumming treatment was used by increasing the concentration of Na_2CO_3 to 10 g/L and temperature to 120°C. Material to liquor ratio was changed to 1 : 10. Other conditions were the same as used for ND-E-F.

Normally degummed Eri powder and mulberry powder

The details of powder production were reported in an earlier publication.[31] Briefly, normally degummed Eri fibers (ND-E-F) and Mulberry fibers (ND-M-F) were chopped into snippets using a cutter mill (Pulverisette 19 from Fritsch). A stirred ball mill (1S Attritor from Union Process) was used for wet

grinding of the silk snippets using 20 kg yttrium treated zirconium oxide grinding media (5 mm) in a 9.5-L tank. The stirrer speed was 280 rpm. Time of milling was 12 h. Distilled water was used in wet grinding operation. Dry powders from wet milled silk slurry were recovered by a laboratory spray dryer (B-290 from Buchi Labortechnik AG). A laboratory air jet mill (Sturtevant) was then used (grinding air pressure of 110 kg/cm^2) to further reduce the size of attritor milled silk particles.

Material characterization

Particle size measurement

A Malvern Instruments Mastersizer 2000 (a laser diffraction-based particle size analyser) with a Hydro 2000S side feeder was used. Dispersion medium was 2- Propanol (Sigma-Aldrich). A refractive index of 1.542 and imaginary refractive index of 0.01 were used for necessary calculations by the software, Mastersizer 2000 (ver 5.21).

BET (Brunauer, Emmett, Teller) surface area analysis

Micromeritics ASAP 2400 was used to measure surface area of silk materials by BET technique using nitrogen gas adsorption. Approximately 0.5 g of specimen was placed in a glass analysis tube and conditioned under vacuum at 50°C for 16 h. The specimens were checked on the conditioning rig to confirm that the surface was cleaned and sorbents were ready for analysis. The analysis tubes were then weighed and vacuum tested prior to analysis by the BET nitrogen adsorption technique.

Scanning electron micrographs

Cross sections of silk fibers and morphology of silk particles were studied after gold sputter coating with a scanning electron microscope (LEO 1530 FEG-SEM) at 2 kV accelerated voltage, and 2–4 mm working distance. To prepare the fiber cross sections, slightly tensioned fibers were embedded in resin (Technovit kit) and sliced perpendicular to the fiber axis by a microtome (Cut 5062, SLEE).

Absorption studies

Reagents

All reagents and solvents used (without further purification) were of analytical grade and obtained from commercial sources. All water used was Milli-Q grade. High specific activity (SA) radioisotopes $^{57}Co^{2+}$ ($t_{1/2}$ = 271.8 days; SA = 185 GBq/mg), $^{64}Cu^{2+}$ ($t_{1/2}$ = 12.7 h; SA = 500 GBq/mg) and $^{109}Cd^{2+}$ ($t_{1/2}$ = 461 days; SA = 0.12 GBq/mg) were purchased from MDS Nordion, ANSTO Radiopharmaceuticals and Industrials and Perkin–Elmer, respectively. The gamma emissions used to monitor each radioisotope were 122.1 and 136.5 keV for ^{57}Co, 511 keV for ^{64}Cu, and 88.0 keV for ^{109}Cd. Analytical standard solutions of $CuCl_2$ (0.01M) and $Cd(NO_3)_2.4H_2O$ (1M) were supplied by Riedel de Haen and $CoCl_2$ (0.01M) by Sigma-Aldrich.

Binding study: Effect of pH

The binding affinity and rate of uptake of metal ions by silk materials were monitored over the pH range 3–9 using various buffer solutions; 0.1M glycine/0.1M NaCl adjusted with HCl for pH 3; 0.1M sodium succinate for pH 4; 0.1M 4-morpholinoethanesulfonic acid (MES) for pH 5 adjusted with NaOH; 0.1M sodium phosphate dibasic for pH 6, 7, and 8, adjusted with NaOH; 0.1M glycine/0.1M NaCl for pH 9, adjusted with NaOH. A typical procedure involved incubating approximately 10 mg (accurately weighed in quadruplicate) silk materials at 21°C in the appropriate buffer (about 900 μL). The relevant metal ion (Co^{2+}, Cu^{2+}, or Cd^{2+}) doped with radioisotope ($^{57}Co^{2+}$, $^{64}Cu^{2+}$, or $^{109}Cd^{2+}$, respectively) was added to this suspension. The final volume of the reaction mixture was adjusted to 1 mL using buffer and the final concentration of metal ions was $10^{-4}M$. The mixture was then vortexed (SLRM-2M Intelli Mixer rotomix) and left to rotate at 60 rpm. At set intervals (t = 5, 15, 45, and 120 min and 24 and 72 h) the mixture was centrifuged at 5000 rpm for 15 min (Eppendorf 5804R) and the supernatant sampled (triplicate of 20 μL). The radioactivity count in each aliquot was measured in a gamma counter (Wallac Wizard 1480) with a detection limit of 50 Bq. Percentage of radiation count absorbed was then calculated using the following equation at each time interval:

$$\% \text{ Absorption at time } (t) = \left(C_{ab,t}/C_0\right) \times 100 \qquad (1)$$

Where: C_{srt} is Count in the aliquot at time (t) treated with sorbent
C_0 is Count in the standard aliquot at time = 0
$C_{ab,t}$ is Count absorbed at time t
$C_{ab,t} = C_0 - C_{srt}$.

As the radioactive isotopes have the same chemistry as the respective nonradioactive metal ions, the percentage absorption of activity (count) calculated by the above formula was equal to the percentage absorption of metal ions. From the initial concentration of metal ions (1 × $10^{-4}M$), actual sorbent weight, total volume of solution (1 mL) and the molar mass of respective metal ions, the metal ions

absorbed per unit weight of sorbent were calculated. The volume lost due to measurements was ignored as it was less than 6%.

Loading capacity and desorption study

The loading capacity of silk sorbents was performed by measuring binding over a range of metal ion concentrations, $10^{-5}M$ to $10^{-3}M$. Total volume of buffer was at least 900 μL in the 1 mL total solution used for each silk sorbent ($\sim$ 10 mg). Procedure of solution preparation, total volume, doping with radioisotopes, sampling (triplicate of 20 μL from each concentration) and measurement in gamma counter and calculations of ions absorbed were the same as for the binding study. pH 8 was maintained in all loading experiments. Loading capacity was measured at $t = 5, 15, 120$ min and 24 h. After 24 h of loading, samples were centrifuged (5000 rpm for 15 min) and the supernatant removed. Totally, 1 mL of pH 3 buffer was then added to each sample. The mixture was vortexed and then centrifuged and supernatant removed (20 μL $\times$ 3) at the set time intervals (30 and 120 min and 24 h). The radioactivity count in each aliquot was measured using gamma counter. The percentage desorption of metal ions was calculated as follows:

$$\% \text{ Desorption at time } (dt)$$
$$= (C_{r,dt}/C_{ab,t=24} \times CF_{dt}) \times 100 \quad (2)$$

Where: $C_{ab,t=24}$ is Count absorbed (at $t = 24$ h) prior to desorption

CF_{dt} is Count correction factor due to decay at desorption time (dt)

$C_{r,dt}$ is Count released at desorption time (dt).

RESULTS AND DISCUSSION

Preparation of silk sorbents

Silk filament consists of a stable core formed by fibroin protein covered by a structurally less stable gummy protein known as sericin. Sericin is soluble at alkaline pH at high temperature. The process of sericin removal from silk is known as degumming. Our previous work showed that fibroin structure was substantially changed during degumming depending on the intensity of alkali treatment.[32] Degumming intensity also influences milling efficiency of silk powder.[31] Thus both Eri (E) and Mulberry (M) silk fibers were degummed using normal degumming (ND) and intensive degumming (ID) protocols and their effect on metal ion binding was investigated. To understand the influence of surface area on metal ion binding, fine milled silk particles (P) were prepared through wet attritor milling followed by air jet milling and their binding efficiency was compared with their parent fibers (F).

pH dependent binding by silk sorbents

Prior to conducting metal binding studies all solutions were tested for the formation of colloids by thin layer chromatography (TLC). Typically each buffered solution containing metal ions was spotted on to a TLC paper and exposed to a mobile phase that separated free metal ion species from metal hydroxide species. These TLC were monitored by using a gamma counter for all concentrations of metal ions. In all cases there was no evidence of colloid after 1 h over the entire pH range studied.

Figure 1 shows the amount of metal ions absorbed at 24 h (in equilibrium) by selected Mulberry and Eri silk fibers. The data show that for all silk sample types the total amount of Cu^{2+} bound is similar at optimum pH (5–8), but varied at pH 4 and 9. For Co^{2+} binding, the behavior is similar in trend, however there is a significant enhancement ($2\times$) of the amount of Co^{2+} bound for ND-E-F and ID-E-F samples compared with ID-M-F at pH 8. A similar story is apparent for Cd^{2+} binding however only a slight increase in total amount bound is present for ND-E-F at pH 7 compared with the ID-M-F and ID-E-F samples.

Interestingly, Figure 1 illustrates how the metal binding behavior for each silk type is similar in trend with respect to change in pH, but quite different for each metal ion. This tends to indicate that the interactions between the metal ions and the amino acid of the silk materials are different at each pH. Further that preferred coordination chemistry for each metal ion is dictating much of the interaction. For example, the binding of Cu^{2+} has a broad absorption maxima at pH 5–8, and Cd^{2+} was in the range 7–9. The absorption peak for Co^{2+} was comparatively sharp at pH 8. At pH 3 there is negligible absorption of any of the metal ions to the silk materials. As the pH of the solution is increased to pH 4 and further there is substantial increase in Cu^{2+} binding. The binding is quite different for Cd^{2+} remaining weak at pH 4–6 with a dramatic increase at pH 7. The isoelectric point of fibroin is reported to be between pH 3.6–4.2[33,34] and the apparent increase in binding of the metal ions at pH > 4 correlates well. The variation in the total amount of each metal ion absorbed supports the hypothesis that the binding is reflective of the difference in the specific coordination donor preferences and kinetics of metal ion complexation.

Deprotonated nitrogens in the side and main chains of the protein fiber are known to be the
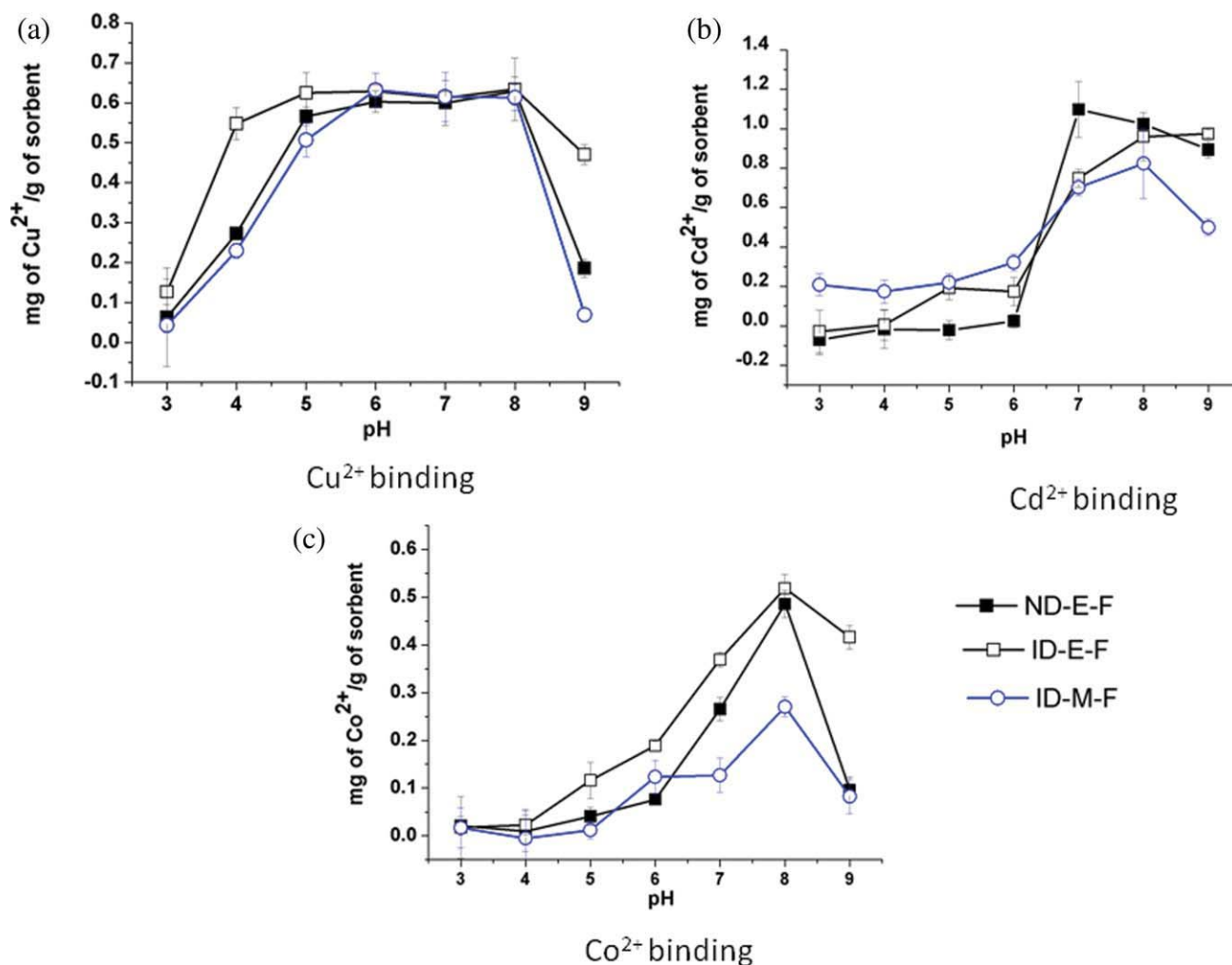
Figure 1 Binding by silk sorbents (24 h) at pH 3–9; Concentration of metal ions in solution: $10^{-4}M$; $N = 12$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

preferred donor atoms for binding of transition metal ions such as Cu^{2+} and Zn^{2+}.[35] Free carboxylic acids of C-terminal end and amino acid side chains are considered favorable sites to bind metal ions.[4,17,18] Silk has two large hydrophilic blocks with irregular amino acid sequences at each chain end. The terminal end of these sequences are carboxylic acid groups (pKa = 4) and are quite likely to be the primary binding sites for metal ions at pH $\geq$ 4. Within the protein structure, the carboxylate groups of aspartic and glutamic acid are also potential sites for weak metal ion binding.[36,37]

As the pH is increased the histidine group will start to play a more significant role in metal ion binding to the silk powders. Researchers have demonstrated that the N atom of the histidine imidazole and the N, O atoms of glycine are the preferred sites for Cu^{2+} binding.[28] The mode in which the metal ion binds to these specific sites depends on the pH. For example, Mulberry silk has only one nitrogen reported to be bound to the Cu^{2+} ion, (i.e., as Cu-1N3O) at pH 4. As the pH is increased more nitro-

gen atoms are engaged in the coordination sphere of the Cu^{2+} ion, such as Cu-2N2O at pH 5.2, Cu-3N1O at 7.5 and Cu-4N, at 8.[38]

At the pH decreases from neutral/alkaline to weakly acidic, the ratio of Cu^{2+}-histidine is reduced from 1 : 1 to 1 : 2. Therefore only half the amount of Cu^{2+} can be bound at low pH. Such changes appear to be linked to the changes in the quaternary structure of the protein, from α to β conformation.[39,38] These conformational changes for prior protein, PrP, have been linked to neurodegenerative diseases,[40] and for silk there is notable increase in mechanical properties associated with conformation changes to β-sheets.[27,29,30]

Overall the metal binding properties of the silk correlate well with protein sequencing information and pKa of dominant amino acids groups present. The decrease in binding at pH 8 and 9 indicates that under conditions studied the buffer solution has a stronger binding affinity for the metal compared to the silk. The enhanced binding of Cu^{2+} by ID-E-F at pH 4 indicates that the degumming process increases accessibility of donor groups probably because of increased porosity.

Rate of binding and equilibrium binding

Only selected data for metal ion bound to silk sorbents at pH 8 is presented in Figure 2 Incubation for more that 24 h did not yield significant changes in total amount of metal ion bound. This result is consistent with that of Goto and Suyama, who reported equilibrium for Cu^{2+}, Cd^{2+}, and Co^{2+} absorbed on different animal fiber proteins was also reached within 24 h.[10]

Influence of metal ion

In the metal binding studies, almost all (1×10^{-7} moles) the metal ions were bound to the sorbent (10 mg) present. Data in Figure 2(a) show that the moles absorbed/mg was > 97% for Cu^{2+} ions at equilibrium. Greater than 80% of the Cu^{2+} was absorbed within 5 min for all samples except ND-M-F. For the latter, it took a further 85 min to reach 80% absorption and equilibrium at 24 h. A comparison of Cu^{2+} binding with Co^{2+} and Cd^{2+} (in moles of metal ion bound per mg of sorbent) show the absorption rate was greatest for Cu^{2+} and slowest for Co^{2+} as following the trend $Cu^{2+} > Cd^{2+} > Co^{2+}$. A competitive binding study by Ishikawa et al., comparing the binding of the same metal ions reported similar behavior for Mulberry silk.[9]

Mulberry silk versus Eri silk

Figure 2 illustrated the effect of silk species on the rate of uptake. Significant enhancement in the rate and amount of metal ion bound can be seen for some samples. For example, mulberry silk fibers (ND-M-F) absorbed 40% less Cd^{2+} and 58% less Co^{2+} ions at 24 h (equilibrium) compared with normally degummed Eri silk fibers (ND-E-F). Furthermore, absorption by Eri silk was significantly faster than Mulberry silk; attaining higher moles of metal ion (all three) bound at 5 and 15 min compared with Mulberry silk. The amount of Co^{2+} and Cd^{2+} bound to the mulberry silks (ID-M-F) was far less than that for all Eri silk samples. In contrast, there was no difference in the rate of Cu^{2+} ions taken up by ID-E-F compared with ID-M-F.

According to the amino acid composition chart compiled from past studies (Table I), it is evident that the most important metal binding ligands, Histidine and Tryptophan are at higher ratios in Eri silk than found in Mulberry silk.[41–44] Other amino acids in these proteins that are reported to play a role in metal binding include phenylalanine, tyrosine,[19] arginine, and lysine[18,19] and aspartic and glutamic acids.[4,17,18] It is clear from Table I that there are no significant differences in phenylalanine and tyrosine contents between the two silk varieties, but arginine content is much higher in Eri silk. The lysine content
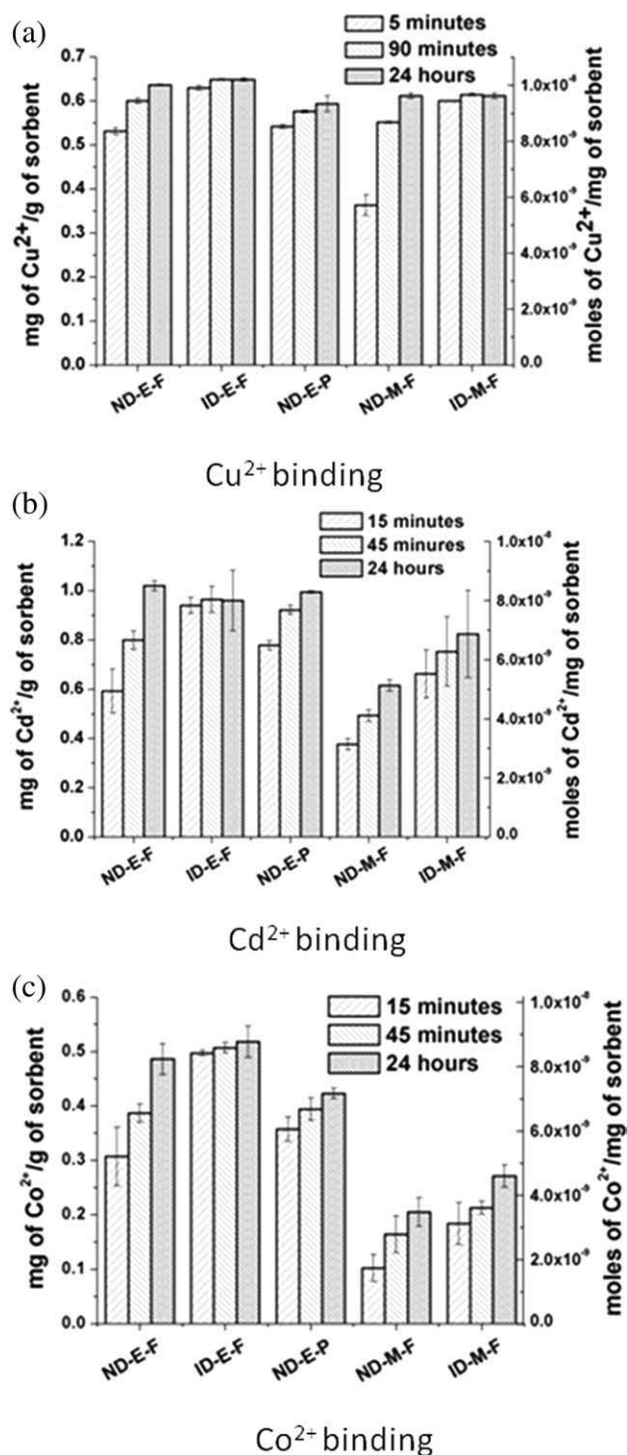

Figure 2 Binding by silk sorbents at different times; Concentration of metal ions in Solution: $10^{-4}M$, pH 8; $N = 12$.

is slightly less compared with Mulberry.[41–44] While the glutamic acid content is low in Eri silk, the high values for aspartic acid may explain the enhance binding.[41–44] The difference in the ratio and type of amino acids content in the silks may explain the difference in the metal binding preference for Eri silk compared to Mulberry silk as seen in this study.
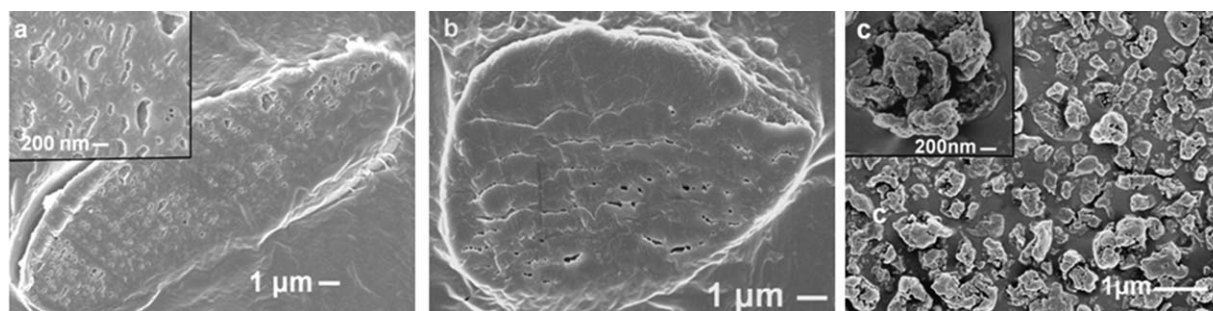
Figure 3 SEM micrographs of silk sorbents: (a) Cross section of Eri silk fiber; (b) Cross section of Mulberry silk fiber; (c) Eri silk particle.

The porous structure of sorbents is likely to influence metal ion diffusion and determine the available surface area for binding. The cross-sectional images of silk fibers in Figure 3 show that a large number of macro-voids are present in Eri, but not so much in Mulberry. A few holes visible in Mulberry silk are probably artifacts of material preparation. Past studies also suggested a lack of macro-voids in Mulberry silk.[24,44] Non-Mulberry silk fibers, Tasar and Muga, from the same family as Eri (*Saturdiine*) are also known to have elongated cavities (average 200 nm diameter in cross section),[24,25,45] the size of which matches with those seen in Eri silk (Fig. 3). Silk fibers are also known to have micro-voids ($\sim$ 10 nm or less) and their number and size distribution vary between Mulberry and Non-Mulberry (*Saturdiine*) silk fibers.[2] Many fine structural features of Eri silk match with other Non-Mulberry (*Saturdiine*) silks but differ significantly from Mulberry silk.[14,43,44,46,47] As voids provide large internal surface area, their content and size distribution could have influenced diffusion rate and volume of ion absorption.[21] It is also worth noting that Eri silk has a higher amorphous fraction compared to Mulberry silk.[46,47] The amorphous domains in polymers are considered the most efficient binding sites, therefore it is reasonable to assume similar behavior could explain the result obtained for Eri silks. Further analysis will be required to shed more light on the influence of microstructure and porous structure on absorption behavior.

Effect of degumming treatment

The normal industrial degumming conditions were sufficient to remove all gums present in silk fibers. However to induce severe alkali hydrolysis during degumming, time of Na_2CO_3 treatment, temperature, and Na_2CO_3 concentration were increased during intensive degumming. Although the total amount of metal ion absorbed at equilibrium is the same for all samples, there is a significant difference in the kinetics of metal binding between processed samples. For example, there is an increase in the rate of binding of metal ion by the silk powder after intensive degumming as illustrated in Figure 2. At 15 min the total amount of Co^{2+} and Cd^{2+} bound to ND-E-F was 51.4% and 55.2% respectively. After processing (i.e., ID-E-F) the amount of metal ion bound over the 15 min was increased to 86.5% and 84.3%, respectively, for the same metal ions. Similarly, ID-E-F showed a rapid binding rate for Cu^{2+} and absorbed nearly all available ions in 5 min. (Equilibrium binding data in Figure 2 represent absorption of more than 90% available ions). Similarly, both uptake rate and equilibrium ion bound increased for Mulberry silk after intensive degumming. However for Cu^{2+}, which showed the fastest binding kinetics, the effect of binding was not significantly enhanced on degumming.

Degumming process is known to reduce the molecular weight of silk fibroin.[48–50] Intensive degumming (ID) is therefore expected to reduce the molecular weight substantially. Decrease in molecular weight could mean a rise in the number of free carboxylic acids of C-terminal ends which would increase ion binding. This could be the reason for higher binding in intensively degummed Eri (ID-E-F) and intensively degummed Mulberry (ID-M-F) over normally degummed Eri (ND-E-F) and normally degummed Mulberry (ND-M-F) fibers respectively. In that sense, the influence of longer degumming time in Eri silk (ND-E-F) compared with Mulberry (ND-M-F) on higher ion absorption efficiency of the former as discussed earlier can not be ruled out. In contrast, degumming conditions were identical for ID-E-F and ID-M-F, and there was still a distinct difference between metal binding properties suggesting amino acid and porosity play a significant role for the differences in binding efficiency between the two silk species.

Effect of milling

To study the effects of surface area, degummed fibers (ND-E-F) were milled into fine particles. The volume median particle size of milled Eri silk powder (ND-E-P) is 0.9 μm and their BET surface area is 18.4 $\pm$ 0.3 m^2/g.

Figure 3(c) shows the SEM image of particles, which have large cracks and pores and hence a large surface area. On the other hand, the equivalent diameter of the parent fibers (ND-E-F) is about 15 μm (according to calculations from its cross-sectional area and known density[46]) and they have a BET surface area of 1.29 ± 0.17 m^2/g. The marked difference in surface area between fibers and particles is obvious. The change in surface area could accelerate ion absorption rate in particles compared to parent fibers. However, the increase was less dramatic than the change induced by intensity of degumming for Eri silk.

To further verify the effect of milling on improved binding, we studied Mulberry silk particles (ND-M-P). The study was restricted to Co^{2+} binding only as its migration into Mulberry fibers (ND-M-F) was very slow and poor. Results showed that absorption of Co^{2+} at pH 8 increased from 0.10 ± 0.02 mg/g to 0.21 ± 0.01 mg/g at 15 min and from 0.16 ± 0.03 mg to 0.22 ± 0.01 mg/g at 45 min after milling into particles with a volume $d(0.5)$ of 700 nm. Comparing this result with data presented in Figure 2(c), it is obvious that the Mulberry silk particles could match the improvement in binding kinetics obtained through intensive degumming, but they have a considerably lower binding capacity for Co^{2+} compared with Eri silk powder and fibers.

Loading of metal ions

Figure 4 shows the proportional increase in equilibrium metal ions uptake at 24 h because of an increase in concentration of solutions from $1 \times 10^{-5}M$ to $1 \times 10^{-3}M$ at pH 8. Concentration was restricted to $1 \times 10^{-3}M$ as at higher ion concentration at that pH, we noted colloid formation by TLC. The loading results are summarized as follows.

Cu^{2+} loading

Figure 4(a) demonstrates that for all sorbents, percentage Cu^{2+} ions absorbed remained above 90% while the concentration was raised. There was a linear (log-log) relationship between ions absorbed and amount available for binding. The maximum binding by Mulberry silk fibers (ND-M-F) within the experimental concentration range was 6.2 mg Cu^{2+}/g. There is scope to further increase the loading as silk materials were not saturated according to Figure 4(a). It is worth noting that Cu^{2+} loading is much higher than suggested by other researchers. For instance, Zhou et al. reported that a maximum of 4 mg Cu^{2+} was absorbed by 1 g regenerated silk fibroin solution.[27] Similarly, Ki et al. showed that 1 g electrospun silk fibroin mat could bind up to 1.65 mg Cu^{2+} at pH 7, while Chen et al., using cut degummed fibers achieved 3.66 mg loading/g of
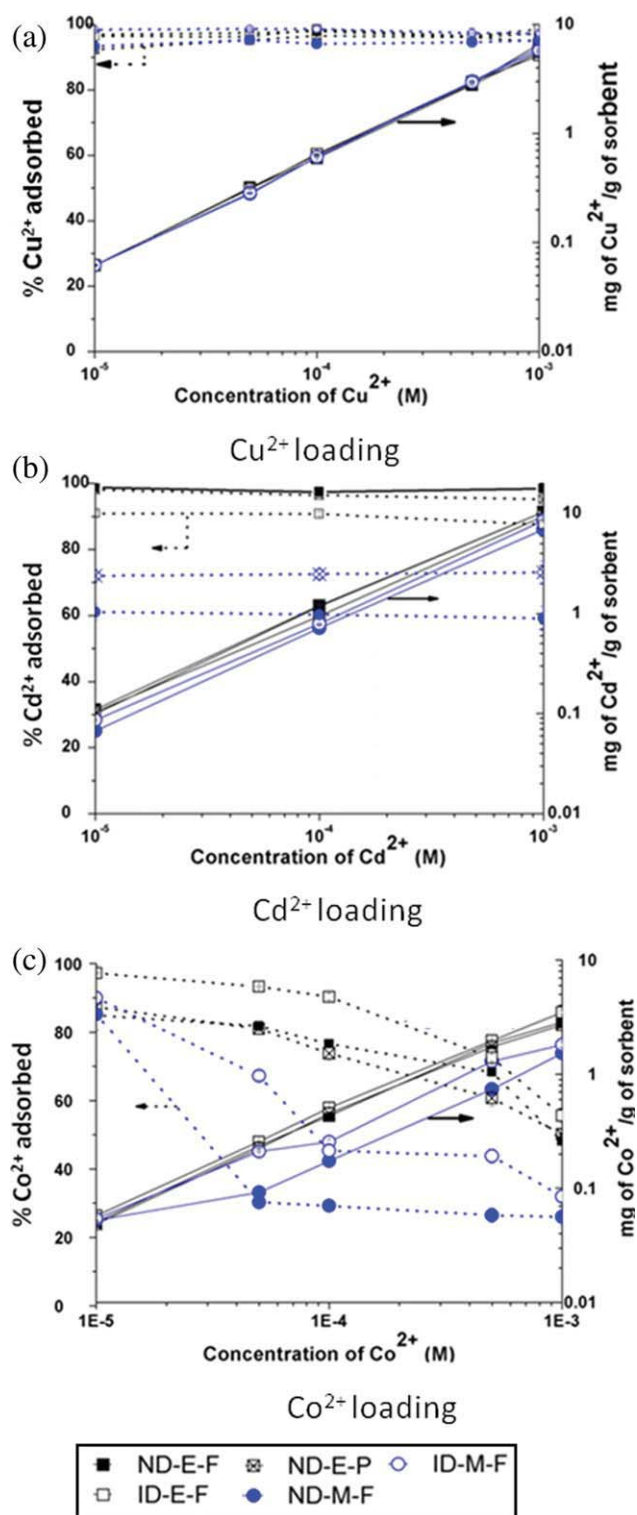

Figure 4 Loading capacity (at equilibrium binding) of silk sorbents at pH 8; $N = 4$. Dotted line: % ion absorbed; Solid line: mg of metal ion/g of sorbent. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

silk at pH 6.[17,11] The higher binding in this study could be because of the form of silk (fiber versus regenerated or chopped silk) or variation in the degumming process. The difference in pH used for

TABLE II
Percentage Release of Metal Ions by Silk Sorbents

Silk Sorbent	Co^{2+}	Cu^{2+}	Cd^{2+}	pH	Time (h)	Ref.
ND-E-F	86.9 ± 5.1	93.2 ± 7.2	91.5 ± 1	3	0.5	Present work
ID-E-F	51.2 ± 7.6	44.7 ± 4.6	80.4 ± 6.5			
ND-E-P	78.3 ± 8.3	88.5 ± 5.5	90.8 ± 6.6			
ND-M-F	99.3 ± 11.6	75.3 ± 2.6	99.1 ± 8.5			
ID-M-F	82.9 ± 5.9	61.6 ± 3.6	80.5 ± 7.6			
Mulberry	44.2	88.2	–	3.8	120	5,4
Regenerated Mulberry	–	102		–	0.5	17
Tasar	46.4	47.6	–	3.8	120	4

measurement could also play a role. However for Cu^{2+}, the differences in equilibrium binding between pH 5, 6, 7, and 8 were found to be insignificant [Fig. 1(a)]. Previous studies were conducted using techniques such as ICPMS, AAS, and ICP-AES. The effect of sample preparation and low sensitivity may also explain the difference found in this study.

We have calculated theoretically that 1 g silk can bind only 0.47 mg of Cu^{2+}. The calculation is based on average molecular weight of amino acids in Mulberry silk as 78[38] and molar percentage of histidine present in specific Cu^{2+} binding octapeptide sequence (AHGGYSGY) as 5.7×10^{-4}.[51] The calculation also assumes that Cu^{2+} binds to this sequence in ratio 1 : 1 at pH 8.[39,38] On the other hand, if the assumption includes all histidine residues in silk and percentage mole of histidine is taken as 0.2% for Mulberry silk (from Table I), then the theoretical Cu^{2+} binding is increased to 1.6 mg/g. The values are significantly less than achieved compared to studies reported here, suggesting that many other amino acids described earlier are involved in metal binding. Studies using other proteins also revealed unidentified high-affinity Cu^{2+} binding sites outside the octapeptide repeat region.[52] Further studies are required to understand the loading potential of each ion based on specific binding sites.

Cd^{2+} and Co^{2+} loading

It is seen in Figure 4(b) that, like Cu^{2+}, there was no change in percentage absorption of Cd^{2+} as the concentration of ion increased. However unlike Cu^{2+}, clear difference in loading between silk sorbents in the entire concentration range is evident. All Eri silk sorbents absorbed more than 90% available Cd^{2+} at equilibrium, while Mulberry sorbents picked up 60–70%. Clear differences between sorbents are also reflected in Co^{2+} loading profiles [Fig. 4(c)]. In addition, unlike the other two ions, the percentage of Co^{2+} absorbed declined with increasing ion concentration. Saturation could not be reached for Co^{2+} under the condition studied due to precipitation of insoluble salts. The loading study confirm studies reported

above show the amount of metal ion bound followed the following trend, $Cu^{2+} > Cd^{2+} > Co^{2+}$.

The Co^{2+} loading study was performed on strong cation exchange commercial resins AG MP-50 and AG 50W-X2 with concentration of metal ions up to $10^{-4}M$ under identical conditions. The maximum binding in mg/g of sorbents was ~ 0.176. These resins are Polystyrene-divinylbenzene sulfonic acid and used for water purification, solute concentration and analytical determination of mixed cationic solutions. In this study, (0.170–0.504) mg and (1.52–3.46) mg Co^{2+} was loaded per g of sorbent in solution of $10^{-4}M$ and $10^{-3}M$ concentration, respectively. Under the same solution conditions, the silk materials have a significantly higher loading capacity than the commercial resin further supporting the extraordinary qualities of this natural resource.

Desorption of metal ions

In this study, metal ions absorbed by all sorbents at pH 8 were re-exposed to pH 3 for 30 min to examine their desorption behavior. Desorption pH was selected according to Figure 1. Table II compares the percentage release of metal ions obtained from this study with those reported in the literature. Normally degummed fibers of both silk species released more than 75% of metal ions within 30 min. However, desorption was low in intensively degummed silk sorbents particularly in ID-E-F. The results suggest that the high amount of Cu^{2+} absorbed in ID-E-F at pH ~ 4 (near the isoelectric point) was the result of a change in microstructure due to the degumming process; suggesting enhanced porosity and therefore increased diffusion of solutes.

The high loading capacity and fast kinetics of metal ion absorption to the silk sorbents described in this study support the application of these materials into nontraditional areas, such as remediation of metal contaminated sites and or separation and recycle of metal ion waste in industry. For example, the ability to form ultrafine particles from the silk protein fibers makes them attractive for use in ion-exchange applications. Similarly, their biocompatibility and biodegradability support their use in biological systems and the environment,.[53,54]

CONCLUSIONS

The metal ion binding profiles of Cu^{2+}, Cd^{2+}, and Co^{2+} by Eri and Mulberry silk varieties were found to be different over the pH range 3–9. Maximum binding for Cu^{2+} was over the pH range 6–8, whereas for Cd^{2+} it was at pH 7–8 and Co^{2+} at pH 8. Absorption remained low at pH 3–4 for the metal ions and silk types investigated. Overall, the equilibrium binding as well as rate of binding followed the trend $Cu^{2+} > Cd^{2+} > Co^{2+}$. The rate of binding for all metal ions was substantially higher for Eri compared with Mulberry silk. Such differences appear to be governed by the presence of more metal binding sites or metal binding amino acids such as histidine, tryptophan, and aspartic acid, as well as optimum porosity and microstructure in Eri silk. The intensity of silk fiber degumming has a significant effect on the binding and release of ions. High surface area of ultrafine silk particles also increased the uptake rate. Comparative loading capacity of the silk sorbents with commercial resins under similar conditions showed that the former absorbed metal ions more efficiently. In addition, release of up to 100% of the absorbed metal ions within 30 min using mild conditions indicates that silk sorbents have prospects in metal ion recovery and recycling applications.

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