Absorption of Metal Cations by Modified *B. mori* Silk and Preparation of Fabrics with Antimicrobial Activity

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ABSTRACT: Silk fabrics were modified by treatment with tannic acid (TA) solution or by acylation with ethylenediaminetetraacetic (EDTA) dianhydride. Kinetics of modification with TA and acylation with EDTA–dianhydride was investigated. The physico-mechanical properties of silk fabrics acylated with EDTA–dianhydride remained unchanged regardless of chemical modification. The absorption of metal cations (Ag$^+$, Cu^{2+}) by untreated and modified silk fabrics was studied as a function of the kind of modifying agent, weight gain, and pH of the metal solution. The absorption of Cu^{2+} at alkaline pH was not significantly influenced by chemical modification of the silk substrate. The absorption of Ag$^+$ by acylated silk remained at a level as low as untreated silk, while was enhanced by TA. The higher the content of TA, the higher the absorption of Ag$^+$. With respect to the pH of the metal solution, the acylation with EDTA–dianhydride enabled silk to absorb and bind metal cations even in the acidic and neutral pH range, where tannic acid had no effect. Medium to high levels of metal desorption were exhibited by untreated and modified silk fabrics towards the metal cations, with the only exception of the silk–tannic acid–Ag complex, which displayed an extraordinary stability. All metal-containing silks exhibited significant antibacterial activity. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 80: 297–303, 2001

Key words: silk fabrics; tannic acid; ethylenediaminetetraacetic dianhydride; acylation; metal absorption; antimicrobial activity

INTRODUCTION

As a protein fiber, *Bombyx mori* silk is amphoteric in nature, because it possesses ionizable groups on the side chain of various amino acid residues whose dissociation state depends on the surrounding pH conditions. This characteristic makes silk able to absorb and bind other charged molecules, such as metal ions, as it has been well documented in the literature.[1–7] When immersed in aqueous solution of metal salts, silk exhibits the tendency to absorb metal cations, the rate and extent of uptake depending on various factors, such as the kind of metal and its valence state, the solution pH, time, temperature, etc. The most likely binding sites on the silk fibroin backbone able to form complexes with metal cations are the free carboxyl groups of aspartic and glutamic acid occurring in the amorphous polypeptide sequences. These acidic groups have a pK_a of about 4–4.8, and are almost completely dissociated at pH 7, therefore providing negative charged groups available for binding metal cations over a

wide pH range. The protein–metal interaction is, in principle, reversible, because in suitable conditions (usually at acidic pH) the metal cation can be desorbed due to the strong competition of hydrogen ions for the same binding sites.

The interaction between metals and protein fibers, such as silk and wool, can be exploited for various applications. It has been reported that many metal salts produce useful changes in wool properties, such as flame resistance, wrinkle recovery, improved shrinkage and abrasion resistance.[8] Some years ago there has been a great interest in using wool as sorbent to remove heavy metal pollutants from industrial effluents and to purify contaminated water supplies.[9] The great capacity of silk of absorbing mineral matters has been industrially exploited for a process known as mineral weighting, consisting in loading degummed silk fibers with variable amounts of tin–phosphate–silicate salts in order to compensate for the loss of weight during degumming and for improving fiber volume, handle, and luster.[10] Moreover, the treatment with various metal cations have been found to improve strength, resistance to photodegradation,[1] and dyeability.[7] Recently, Chen et al.[6] reported the optimum conditions for the preparation of metal containing silk fibers with good antimicrobial activity. The metal treatment was carried out in alkaline ammonia solution. The metal was absorbed and bound by silk either untreated or pretreated with tannic acid, which acted as the preferential ligand for the metal cation. The potential application of metal-containing silk as functional biomaterial was also discussed.[11]

In this study the absorption and binding of metal cations (Ag^+, Cu^{2+}) onto silk fabrics, either untreated or modified by treatment with tannic acid (TA) or by acylation with ethylenediaminetetraacetic (EDTA) dianhydride, was investigated as a function of fiber weight gain and pH of the metal solution. The antimicrobial activity of metal-treated silk fabrics was evaluated as a function of fiber modification and amount of metal absorbed, the aim being to explore the possible application of silk–metal complexes for the preparation of textile-based devices with enhanced barrier properties against micro-organisms.

EXPERIMENTAL

Materials

Degummed habutae silk fabric (plain weave, ca. 60 g/m^2) was used as the substrate for the tests carried out in this study. Tannic acid (TA) and ethylenediaminetetraacetic (EDTA) dianhydride were purchased from Wako Pure Chemical Industries, Ltd., and used without further purification.

Loading with tannic acid was performed by immersing the silk fabric into a 4.7% (w/v) aqueous solution of the reagent (material-to-liquor ratio: 1 : 100), at 70°C, for different times (15–90 min). Silk was then washed with running water, rinsed with distilled water, and dried at room temperature before metal absorption experiments.

Acylation of silk was performed with 10% (w/v) EDTA-dianhydride in *N,N*-dimethylformamide (material-to-liquor ratio: 1 : 40), at 75°C, for different times (from 1 to 8 h), to obtain samples with increasing weight gains.[12]

For the preparation of the silk–metal complexes, either untreated or modified silk fabrics were immersed in aqueous solutions of 0.5 mM metal salts [$AgNO_3$, $Cu(NO_3)_2$], containing KNO_3, at 25°C, for 30 h. The solution pH was adjusted with acetic acid or ammonia.

Metal desorption experiments were performed by immersing the silk–metal complexes in an aqueous solution at pH 3.8 by acetic acid, at room temperature for 120 h.

The various silk–metal complexes prepared in this study are identified by the following symbols: Silk–Ag (or: —Cu): untreated silk fabrics containing the metal cation; Silk–EDTA–Ag (or: —Cu): acylated silk fabrics containing the metal cation; Silk–TA–Ag (or: —Cu): silk fabrics pretreated with tannic acid containing the metal cation.

Measurements

Moisture regain was determined on dried samples kept at 20°C and 65% R.H. for 7 days and expressed as grams of moisture/100g silk fiber.

The tensile properties were measured with a Tensilon UTM-II (Toyo Boldwin Co.), using the standard technique at 20°C and 65% R.H. at a gauge length of 100 mm and strain rate of 40 mm/min.

The amount of metal cations absorbed by silk was quantitatively determined by using an Inductive Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) Mod. Plasma 400, Perkin-Elmer. Samples of 5–20 mg were completely digested with HNO_3 65% (2 mL), by using a Microwave Digestion System Mod. MDS-81D (CEM), and then diluted to 10 mL with distilled water before ICP-AES analysis. The results are the average of duplicate tests.
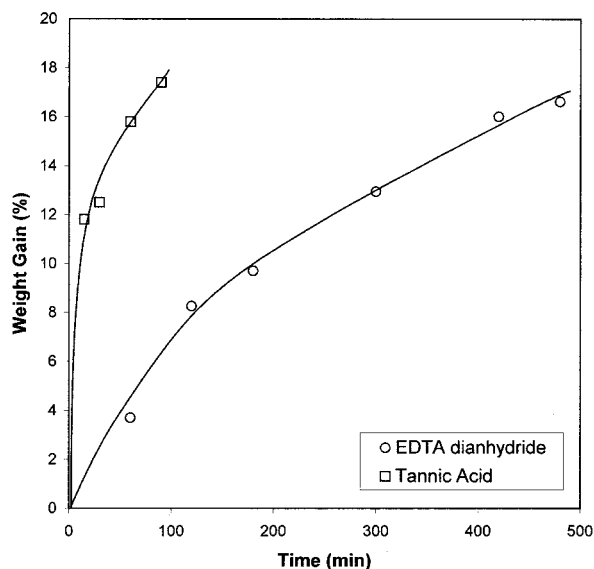
Figure 1 Weight gain of silk modified with tannic acid and acylated with ethylenediaminetetraacetic dianhydride as a function of reaction time.

The antimicrobial activity of metal-treated silk fabrics against *Cornebacterium* was evaluated according to the following procedure. *Cornebacterium* was chosen due to its association with plant pathogenicity, its relative sensitivity to antibiotics and nonpathogenicity to humans. From a *Cornebacterium* cell culture with a cell density of 100 cells/mL, 2 mL were taken and mixed with 25 mL agarose containing King B growth medium (Wako Pure Chemical Industries, Ltd.) at 55°C, poured into a glass Petri dish and allowed to solidify at 25°C. The metal-treated silk fabrics (5 $\times$ 5 mm) were placed onto the surface of the solid gel and incubated at 25°C for 2 days. The antimicrobial activity was evaluated by measuring the size of the zone of growth inhibition.

RESULTS AND DISCUSSION

Chemical Modification of Silk

The absorption of tannic acid (TA) and the reaction with ethylenediaminetetraacetic (EDTA) dianhydride was studied as a function of time (Fig. 1).

As it is well known, silk exhibits a noticeable affinity for vegetable matters such as tannic acid. In fact, the treatment with tannic acid has long been used as a method for vegetable loading of silk.[13] Tannic acid is physically absorbed and held

within the silk matrix through chemical interactions other than covalent bonds. In our experimental conditions, impregnation with tannic acid proceeded quite rapidly. An immersion time of 90 min was sufficient to attain a weight gain of about 18%. Comparable weight gains were obtained by reaction with EDTA–dianhydride, but it was necessary to extend the reaction time until 8 h. The reactivity of various acid anhydrides towards silk depends on both steric and chemical factors related to the characteristics of the anhydride substituent.[12] The bulkier the side chain, the lower the reactivity, although the presence of electronegative groups usually enhances the rate of reaction. Amine groups of basic amino acid residues (lysine, histidine, and arginine), as well as hydroxy and phenol groups of serine, threonine, and tyrosine are the most important reactive sites for anhydrides. While basic amino acids are mostly present in the accessible amorphous regions, a significant part of the hydroxy amino acids are blocked in the ordered crystalline regions.[14] Assuming that the accessible hydroxy groups account for about 40% of the total, the reactive sites potentially available for reaction with anhydrides can be estimated to be about 100–110 mol/10^5 g of silk. Because the maximum weight gain reached with EDTA–dianhydride was 16.6%, corresponding to an acyl content of 64.8 mol/10^5 g, we can estimate that about 60–64% of the potentially reactive sites available on the silk fibroin backbone reacted with the modifying agent. Higher extents of acylation were attained by using other aliphatic acid anhydrides, such as succinic and glutaric anhydrides.[15]

On the basis of the kinetic data shown in Figure 1, samples of silk fabrics modified with TA or acylated with EDTA–dianhydride with similar weight gains (5, 12, and 17%) were prepared for the metal uptake tests.

Physical Properties of Acylated Silk

The treatment of silk with tannic acid is known to leave the intrinsic physico-mechanical properties of silk almost unchanged,[13,16] especially at the low weight gain values reached in this study. The same behavior was reported for silk modified with different anhydrides.[12] However, it has been thought of interest to evaluate the physico-mechanical properties of silk acylated with EDTA–dianhydride, in consideration of the quite long reaction times needed to reach the weight gain values required.

Table I Moisture Regain and Tensile Properties of Silk Fabrics Acylated with Ethylenediaminetetraacetic Dianhydride

Weight Gain (%)	Moisture Regain (%)	Breaking Load (gf)	Strength (gf/den)	Elongation at Break (%)	Energy (gfxmm)
Control	6.6	439 ± 21	3.95 ± 0.2	18.7 ± 1.2	3302 ± 576
4.5	7.7	473 ± 9	3.70 ± 0.1	17.3 ± 0.5	3136 ± 215
8.3	7.3	464 ± 44	3.68 ± 0.4	16.8 ± 1.4	3042 ± 682
11.1	7.3	471 ± 39	3.62 ± 0.3	15.9 ± 1.2	2945 ± 550

Table I lists the moisture regain and the tensile properties of silk acylated with EDTA-dianhydride with increasing weight gain.

Following acylation moisture regain increased slightly, probably due to the presence of free carboxyl groups introduced into the fiber matrix, which may act as water-binding sites. The tensile behavior of acylated silk did not differ significantly from that of the control sample. The values of breaking load remained unchanged while the tensile strength decreased slightly due to the increase in fiber size induced by loading silk with the modifying agent. Judging from the values of elongation at break, silk became slightly stiffer, especially at above 10% weight gain. However, the changes observed can be considered quite small, suggesting that the reaction did not induce adverse effects towards the intrinsic physical properties of silk.

Absorption of Metal Cations: Effect of Chemical Modification of Silk

The absorption of Ag^+ and Cu^{2+} by modified silk fabrics with increasing weight gains was preliminarily investigated at alkaline pH, in the presence of ammonia. In these conditions, it has been reported that metal cations form stable metal–amine complexes within silk.[6] The results obtained are shown in Figure 2.

The absorption of Cu^{2+} by untreated silk was 8–10 times as big as that of Ag^+. This may depend on the properties of the metal cation itself, as well as on kinetic parameters of the metal–amine complex (equilibrium constant). Modification with TA or acylation with EDTA–dianhydride induced only slight changes in the absorption of Cu^{2+} as a function of weight gain.

The absorption of Ag^+ by silk acylated with EDTA–dianhydride remained somewhat lower than that of the divalent cation over the entire weight gain range explored. On the other hand,

loading with tannic acid resulted in a sharp increase in the absorption of Ag^+, whose amount rose steadily and almost linearly with the weight gain.

These results indicate that the absorption of Ag^+ at alkaline pH was significantly enhanced by modification of silk with tannic acid, in agreement with literature data.[6] The effect of acylation with EDTA–dianhydride was much lower, although the amount of Ag^+ absorbed at 17% weight gain was four times as big as that of the untreated sample. As previously observed, the absorption of Cu^{2+} at alkaline pH was not significantly influenced by the modification of the silk substrate.

Absorption of Metal Cations: Effect of Solution pH

In an attempt to go deeper into the behavior of modified silk fibers towards the absorption of
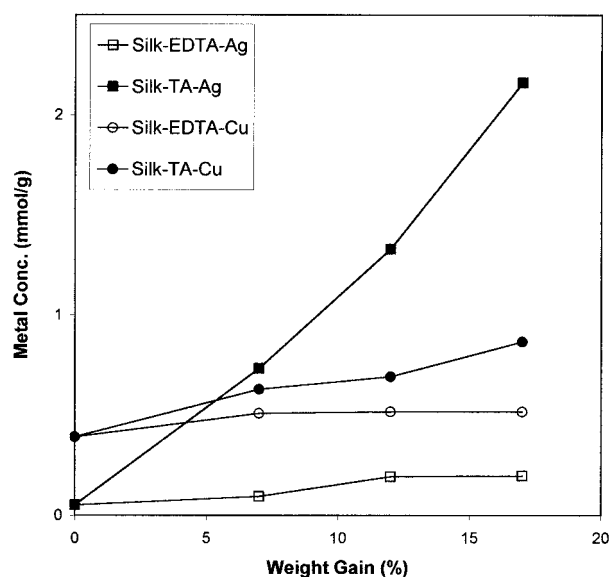

Figure 2 Absorption of metal cations at alkaline pH (11.4) by silk modified with tannic acid and acylated with ethylenediaminetetraacetic dianhydride as a function of weight gain.
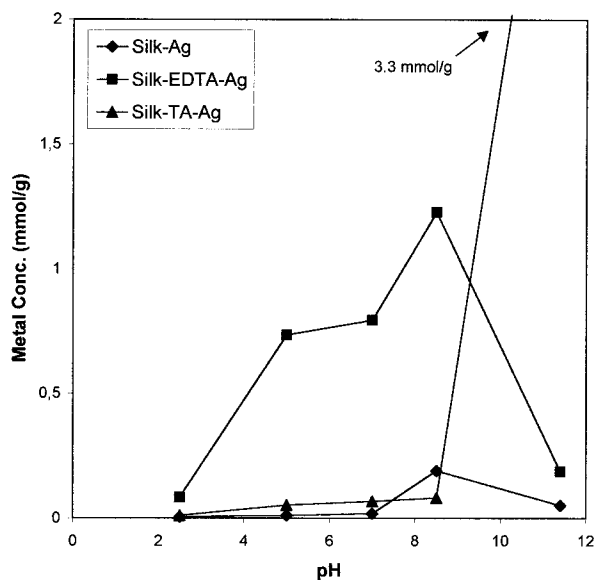
Figure 3 Absorption of Ag$^+$ by silk untreated, modified with tannic acid (20% weight gain), and acylated with ethylenediaminetetraacetic dianhydride (17% weight gain) as a function of solution pH.

metal cations, the effect of pH on metal absorption was investigated. Tests were performed by immersing untreated, acylated, and TA-modified silk fabrics in metal solutions at pH values of 2.5, 5, 7, 8.5, and 11.4.

Figure 3 shows that the absorption of Ag$^+$ by untreated silk remained at a rather low level over the entire pH range examined. Silk modified with tannic acid showed the same trend until pH 8.5, then the absorption of the metal cation rose abruptly at pH 11.4 attaining 3.3 mmol/g of silk, one of the highest levels registered in this study. Silk acylated with EDTA–dianhydride showed a different behavior. Ag$^+$ was effectively absorbed at acidic, neutral and slightly alkaline pH, where untreated and TA-modified silks absorbed only negligible amounts of metal. The maximum was reached at pH 8.5. A further increase of pH induced a sharp drop in the absorption of Ag$^+$, suggesting that EDTA–dianhydride was not as effective as TA in promoting the formation of the Ag$^+$–amine complex.

Cu^{2+} behaved almost similarly, as demonstrated by the trend of the corresponding curves shown in Figure 4. Modification with tannic acid enabled silk to absorb the highest amounts of metal in the alkaline pH range. However, acylation with EDTA–dianhydride resulted in the absorption of metal cations even at acidic an neutral pH.

The most attractive aspect of these results is that modification of silk with EDTA–dianhydride was effective in inducing the absorption and binding of metal cations even in the acidic and neutral pH range, where tannic acid had no effect. This property must be primarily related to the chemical characteristics of the acylating agent. Following opening of the anhydride rings at least three free carboxyl groups are formed (one is supposed to bind to a reactive site located onto the silk fibroin chains). The pK$_a$ values of the four carboxyl groups of ethylendiaminetetraacetic acid are 1.99, 2.67, 6.16, and 10.36. Therefore, strong negatively charged carboxyl groups are available for binding metal cations even in a pH range where aspartic and glutamic acid side chains are only partly dissociated, and the hydroxy groups of tannic acid are still undissociated.

Reversibility of Metal Absorption

The absorption of metal cations by protein fibers is known to be a reversible process.[17] This is mainly due to the competition between metal cations and hydrogen ions for the same binding sites. On this basis it is reasonable to suppose that if the silk–metal complex is immersed in acidic solution, variable amounts of metal cations are released, the extent depending on solution pH and strength of the silk–metal bond. For example, in
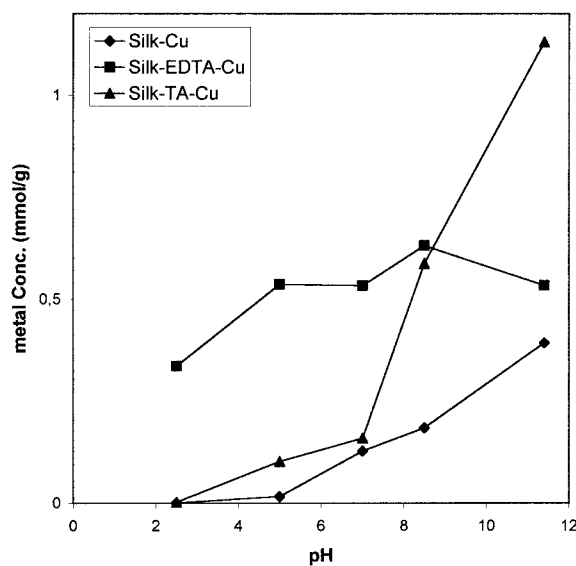

Figure 4 Absorption of Cu^{2+} by silk untreated, modified with tannic acid (20% weight gain), and acylated with ethylenediaminetetraacetic dianhydride (17% weight gain) as a function of solution pH.

the case of the Ag$^+$–wool system, Ag$^+$ was quantitatively desorbed in 0.1 N sulfuric acid.[17] The small amount of Ag$^+$ retained was that bound to cystine residues, which formed unsoluble silver mercaptides.

The results of desorption tests conducted at pH 3.8 on various silk–metal complexes are listed in Table II. The extent of release varied among the samples as a function of the kind of metal and of the properties of the fibrous substrate (untreated, modified with TA, acylated with EDTA–dianhydride). Untreated silk showed the lowest binding strength for Cu^{2+}, which was almost completely released, while the loss of Ag$^+$ was significantly lower. The two metal cations were released at almost the same extent by silk acylated with EDTA–dianhydride. TA-modified silk showed an extraordinarily high stability of the Ag$^+$ complex, whose concentration within the fiber remained almost unchanged, while desorption of Cu^{2+} occurred at significantly higher extent.

The observed desorption behavior accounts for the difference in strength of the silk–metal bonds, which is influenced by both cation properties and kind of chemical modification performed onto the silk substrate. The more or less pronounced capacity of metal-treated silk to release the metal must be regarded as an important functional parameter that may address its application as antimicrobial material.

Antimicrobial Activity

The antimicrobial activity of metal-treated silk fabrics was preliminarily evaluated by using the agar-based zone of inhibition test. In this test, an effective antimicrobial treatment will prevent growth not only on the textile surface, but also on

Table II   Release of Metal Cations by Immersion of Silk–Metal Complexes in Aqueous Solution at pH 3.8 for 120 h

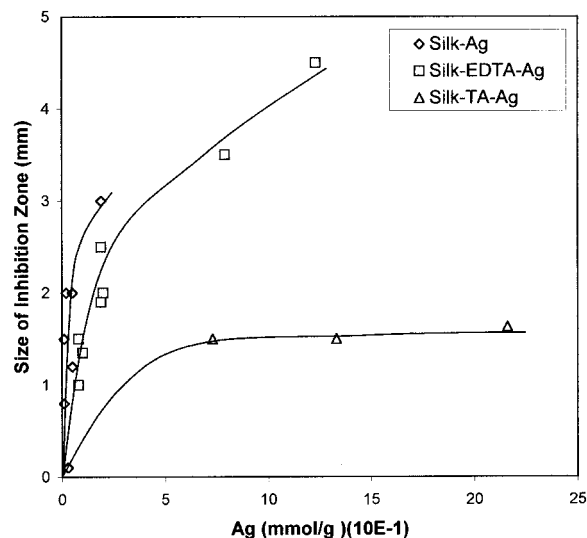| Sample	Metal Content (mmol/g)		
	Before	After	$\Delta(\%)$
Silk-Ag	0.052	0.031	−40.2
Silk-EDTA-Ag	0.198	0.075	−61.8
Silk-TA-Ag	2.161	2.143	−0.8
Silk-Cu	0.393	0.046	−88.2
Silk-EDTA-Cu	0.518	0.157	−69.7
Silk-TA-Cu	0.867	0.535	−38.3

Figure 5 Size of the inhibition zone as a function of Ag$^+$ concentration of silk untreated, modified with tannic acid, and acylated with ethylenediaminetetraacetic dianhydride.

the surrounding agar matrix, the size of the zone of inhibition depending on the extent of diffusion of the antimicrobial agent from the fabric into the agar. The size of the zone of inhibition displayed by untreated, TA-modified and acylated silk fabrics containing Ag$^+$ was measured and plotted as a function of Ag$^+$ concentration (Fig. 5).

Untreated and acylated silks were highly effective in killing micro-organisms even at low metal content, in agreement with the data of metal desorption listed in Table II. In fact, the higher the level of metal desorption the larger the size of the inhibition zone. Accordingly, silk fabrics treated with tannic acid gave small-size inhibition zones even at very high concentration of Ag$^+$ due to the high stability of the Ag$^+$–silk complex towards desorption. The relation between metal content, metal release, and size of the inhibition zone was also confirmed by the silk samples containing Cu^{2+}.

Figure 6 shows that TA-modified silk fabrics exhibited the lowest average size of inhibition zone, even though the content of metal was higher than untreated and acylated silks. The latter displayed the highest effectiveness in killing micro-organisms.

CONCLUSIONS

Both the modification of silk with tannic acid (performed according to the technique proposed
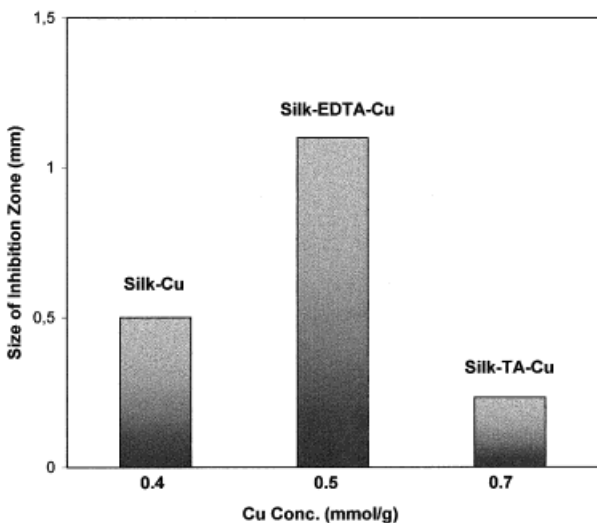
Figure 6 Size of the inhibition zone as a function of Cu^{2+} concentration of silk untreated, modified with tannic acid, and acylated with ethylenediaminetetraacetic dianhydride.

by Chen at al.[6]) and the reaction of silk with EDTA–dianhydride enhanced the capacity of the fiber to absorb and bind metal cations. In particular, the newly proposed acylation technique displayed interesting features, because the metal uptake became effective even in the acidic and neutral pH range, where untreated and TA-modified silks absorbed only negligible amounts of metal.

These results are of chief importance in view of developing a safe and effective processing technique for producing metal-containing silk fibers. In fact, acylated silk can be safely treated with the metal solution at pH values close to neutrality, where damages of the fiber properties are unlikely to occur. Moreover, the amount of metal absorbed can be modulated over a wide range of concentration according to end-use requirements, especially in the case of Ag^+, by simply changing the solution pH.

Finally, the results of desorption tests and measurement of antimicrobial activity must be regarded as a useful starting point for developing textile materials with variable durability of the metal treatment for meeting various application requirements. For example, in some cases immobilization of the antimicrobial agent is needed to avoid toxic effects towards users, while in other cases migration of the agent from the textile to the environment can be exploited as a key factor to enhance the effectiveness of the material against micro-organisms.[18]

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