Preparation of Metal-Containing Protein Fibers and Their Antimicrobial Properties

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ABSTRACT: *Bombyx mori* silk, *Antheraea pernyi* silk, and wool fibers were chemically modified by treatment with tannic acid (TA) or by acylation with ethylenediaminetetraacetic (EDTA) dianhydride. Kinetics of TA loading or acylation with EDTA-dianhydride varied from fiber to fiber. *B. mori* silk and wool displayed the highest weight gains with TA and EDTA-dianhydride, respectively. The uptake of different metal ions (Ag^{+}, Cu^{2+}, Co^{2+}) by protein fibers, either untreated or chemically modified, was studied as a function of weight gain and pH of the aqueous metal solution. Below pH 7, absorption of metal ions by untreated and TA-treated fibers was negligible. Acylation with EDTA-dianhydride enabled protein fibers to absorb and bind significant amounts of metal ions in the acidic and neutral pH range. The levels of metal desorption at acidic pH depended on the fiber-metal combination. Untreated protein fibers usually displayed the lowest stability of the metal complex. Metal complexes with protein fibers exhibited prominent antimicrobial activity against the plant pathogen *Cornebacterium*. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 89: 638–644, 2003

Key words: fibers; proteins; metal-polymer complexes

INTRODUCTION

Protein fibers treated with metal ion solutions form protein-metal complexes, whose properties depend on many different variables, such as type of cation and counter-ion, concentration, temperature, and pH. Several studies have dealt with the kinetics and reaction mechanisms of metals and protein fibers.[1a,2] Depending on the treatment conditions, various kinds of metal complexes are formed. Free carboxyl groups of aspartyl and glutamyl residues are considered the most likely binding sites over a wide pH range, from acidic to alkaline conditions. Deprotonated amino and amide nitrogen atoms are thought to play a role in coordinating metal ions at more alkaline pH values. Under these conditions, the sulphur amino acid cystine present in keratin fibers is also active in binding metal ions, leading to the formation of stable mercaptides, especially at high temperature.

Studies on the treatment of protein fiber with metal ions have provided insight into various chemical, physical and structural features of the fibers.[3] In addition, the possibility of inducing useful changes in fiber properties and developing functional protein-metal complexes has attracted the interest of scientists. In their review, McLaren and Milligan[1a] showed that several reactions of metal ions with keratin fibers effectively improved dyeability, wrinkle recovery, shrinkage, abrasion, and flame resistance of wool. However, they pointed out that the application of these treatments on an industrial level has alaways been seriously limited due to technical and environmental problems. Fukatsu[4–6] studied the reaction of wool with Cu^{2+} ions and investigated the catalytic activity of the wool-Cu complexes as a model of metalloenzymatic reactions. More recently, Goto et al.[7] proposed the exploitation of the metal absorption capacity of protein fibers for the development of new types of polymer-based sorbents, applicable in the removal of heavy metal pollutants from industrial effluents and in the purification of contaminated water supplies. The treatment of silk with various metal ions has proven to be effective in improving fiber strength, resistance to photodegradation,[8] and dyeability.[9] Chen et al.[10] reported the optimum conditions for the preparation of metal-containing silk fibers with enhanced antimicrobial activity. Fibers were pretreated with tannic acid, which acted as the preferential ligand for the metal ion.

In response to the growing consumer awareness of safe and healthy lifestyles, the textile industry has recently developed and marketed a new range of man-made fibers with odor-removing and antimicrobial activity, which are used for manufacturing underwear

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and sport articles. The new functionality is imparted on fibers by incorporating antimicrobial agents (metal particles or chemicals) during the spinning process or by applying suitable post-treatments. We have been using the latter approach with the aim of developing textiles with functional performances based on protein fibers with an enhanced barrier effect against microorganisms.[11,12] To achieve this goal, silk and wool fibers were preliminarily modified by reaction with an acylating agent, ethylenediaminetetraacetic (EDTA) diahydride, which exhibits chelating activity toward metal ions. This treatment enabled the fibers to absorb and coordinate Ag^+ and Cu^{2+} ions over a wide range of pH conditions, because the coordination sites organized within the protein fibers became the combining sites for the metals. All the metal-containing fibers exhibited enhanced antimicrobial activity against plant and animal pathogens.

The aim of this study is to widen the range of protein substrates suitable for metal absorption, focusing mainly on tussah silk from the wild silkworm species *Antheraea pernyi*, and to exploit new metal ions, such as cobalt, as antimicrobial agents in addition to conventional Ag^+ and Cu^{2+}, which were described in our previous papers.[11,12] Like the other protein fibers (*Bombyx mori* silk and wool), tussah silk was modified by treatment with tannic acid or by acylation with EDTA-dianhydride, and the metal absorption capacity was studied as a function of solution pH and extent of chemical modification. Besides the traditional antimicrobial metals, Ag^+ and Cu^{2+}, Co^{2+} was used in this study because its complexes are known to exhibit durable antimicrobial activity, which is enhanced by light irradiation.[13] The challange of preparing durable and environmentally safe metal-containing fibers is the need to use minimal amounts of metal and to preserve the stability of the metal complexes formed within the fibers. In this study, metal desorption tests were performed in order to address the application of the new functional materials. Finally, the antimicrobial activity of the different protein-metal complexes was evaluated against plant and animal pathogens.

EXPERIMENTAL

Materials

White wool, preliminarily degreased by solvent extraction, and degummed *B. mori* and tussah silk fibers were used as the substrates for chemical modification and metal uptake tests. Tannic acid (TA), ethylenediaminetetraacetic (EDTA) diahydride, and $Co^{2\pm}$-metal complexes with organic ligands were purchased from Wako Pure Chemicals (Tokyo, Japan) and used without further purification.

Loading with TA was performed by immersing the protein fibers into a 4.7% (weight / volume) aqueous solution of the reagent, with a material-to-liquor ratio of 1 : 100, at 70°C for various times. The fibers were then washed with running water, rinsed with distilled water, and dried at room temperature.

Acylation of *B. mori* silk and wool with EDTA-dianhydride was performed in *N,N*-dimethylformamide (DMF), with a material-to-liquor ratio of 1 : 40, at 75°C, for different times, to obtain samples with increasing weight gains. Tussah silk was pretreatred with LiSCN and then acylated in a dimethyl sulfoxide (DMSO) solvent, as described elsewhere.[14]

To prepare metal complexes, protein fibers, either untreated or modified with TA or EDTA-dianhydride, were immersed in an aqueous solutions of 33.3 *mM* $AgNO_3$, or $Cu(NO_3)_2$, or $Co(NO_3)_2$, containing 46.7 *mM* KNO_3, at 25°C for 30 h. The solution pH was adjusted with acetic acid or ammonia. Precipitates formed at alkaline pH were removed by filtration before the metal uptake experiments.

Metal desorption experiments were performed by immersing the metal-containing protein fibers in an aqueous solution at pH 3.8 by acetic acid, at room temperature for 120 h.

Measurements

The increase in weight (weight gain) of fibers acylated with EDTA-dianhydride or modified with tannic acid was calculated from the difference in the weight of fibers before and after the reaction.

The amount of metal ions absorbed was quantitatively determined by using an Inductive Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) mod Plasma 400 (Perkin Elmer Cetus Instruments, Norwalk, CP). Samples of 5–20 mg were completely digested with a 65% HNO_3 solution (2 mL), by using a microwave digestion system model MDS-81D (CEM Corporation, Matthews, NC, USA), and then diluted to 10 mL with distilled water before ICP-AES analysis. The results listed represent the averages of duplicate tests.

The antimicrobial activity of metal-containing protein fibers was determined by means of the agar-based test and expressed in terms of size of the zone of growth inhibition (mm). Experimental conditions were similar to those described elsewhere.[11,12] Light exposed samples were placed 30 cm below a fluorescent lamp (National GL type, 10W). Non-exposed samples were shielded with a black cloth throughout the test.

RESULTS AND DISCUSSION

Chemical modification of protein fibers

The chemical and kinetic aspects of the reaction between acylating agents and protein fibers have been

TABLE I
Kinetics of Chemical Modification of Protein Fibers with Tannic Acid and Ethylenediaminetetraacetic Dianhydride

	EDTA-dianhydride		Tannic acid	
Sample	Acyl content[a] (μmol/g)	Rate of acylation (μmol/g × min)	Weight gain[b] (%)	Rate of loading (g/g × min)
B. mori silk	505	2.63	17.4	0.79
Tussah silk	213	0.80	10.3	0.14
Wool	790	4.84	4.3	0.05

[a] Reaction time: 5 hr.
[b] Reaction time: 1.5 hr.

extensively investigated by Tsukada and coworkers.[15] Table I lists the results obtained in this study by using EDTA-dianhydride for the acylation of silk and wool fibers. The rate and extent of acylation varied from fiber to fiber. Under the reaction conditions adopted, wool attained the highest values of weight gain and acyl content, followed by *B. mori* and tussah silk fibers. The rate of acylation, calculated from the initial slope of the acyl content *vs.* reaction time plots, fell in the following order: wool > *B. mori* silk > tussah silk, in agreement with the equilibrium results. Although the number of basic and hydroxyl groups present in the proteins are similar (2.8, and 2.5 mmol/g for wool, and the two silk fibers, respectively), modification has occurred to different extents among these fibers. This behavior can be explained by taking into account the fact that the reaction yield in these heterogeneous systems is dependent not only on the number of potentially reactive sites, but also on their location, distribution, and accessibility within the fiber matrix. Fiber fine structure and morphology may exert a great influence on the rate and extent of diffusion of chemicals toward the reactive sites. It is well known, for example, that most serine hydroxyl groups of *B. mori* silk fibroin are not available for the reaction because they are buried within the more ordered fiber domains.[11] Unlike silk, wool has a high degree of histological heterogeneity, and it has been shown that different keratin fractions (high-sulfur and high-glycine-tyrosine proteins) that form the most accessible fiber domains (interfibrillar matrix and intercellular cement) are particularly rich in reactive amino acid residues, such as tyrosine, serine, threonine, and arginine.[1b] These features may be responsible for the higher reactivity of wool fibers observed in this study.

It is worth noting the significantly lower degree of acylation attained by tussah silk compared to *B. mori* silk, despite the similar amount of potentially reactive amino acid residues. It has also been reported that tussah silk possesses a degree of internal porosity higher than *B. mori* silk,[16,17] a feature predicted to favor internal diffusion of modifying reagents toward potentially reactive amino acid residues. One would therefore expect acylation to proceed with at least the same, if not a higher, rate. However, the necessity of a swelling pretreatment with a salt solution seems to indicate the presence of a surface barrier that prevents penetration and diffusion of the reagents into tussah fibers, as was recently elucidated by Tsukada et al.[14]

Loading with TA proceeded rapidly for domestic and wild silk fibers, owing to their well known affinity for vegetable matters.[11] In particular, *B. mori* silk attained about a 17% weight gain in 90 min, displaying the highest rate of loading (Table I). On the other hand, the uptake of TA onto wool proceeded at a markedly lower rate, probably due to its complex cellular structure, with different structural and morphological internal compartments, as well as to the presence of a highly hydrophobic external layer (epicuticle). It is noteworthy that the amount of TA absorbed by wool did not increase significantly even when the soaking time was extended by several hours.[12]

Uptake of Ag and Cu ions by tussah silk

The uptake of Ag^+ and Cu^{2+} by untreated and modified tussah silk fibers was studied at an alkaline pH, where the binding groups of the protein substrate and those of the chelating agents are almost completely deprotonated and may function as active ligands for the cations. As listed in Table II, untreated tussah silk absorbed 0.485 and 0.115 mmol/g of Cu^{2+} and Ag^+, respectively. The pattern of metal absorption closely resembled that of *B. mori* silk, while wool behaved differently, showing a much stronger binding capacity for Ag^+.

TABLE II
Amount of Metal Ions Absorbed by Untreated Protein Fibers at Alkaline pH (11.4)

	Ion content (mmol/g)		
Sample	Ag^+	Cu^{2+}	Co^{2+}
B. mori silk	0.052	0.393	0.483
Tussah silk	0.115	0.485	0.620
Wool	0.750	0.546	0.275

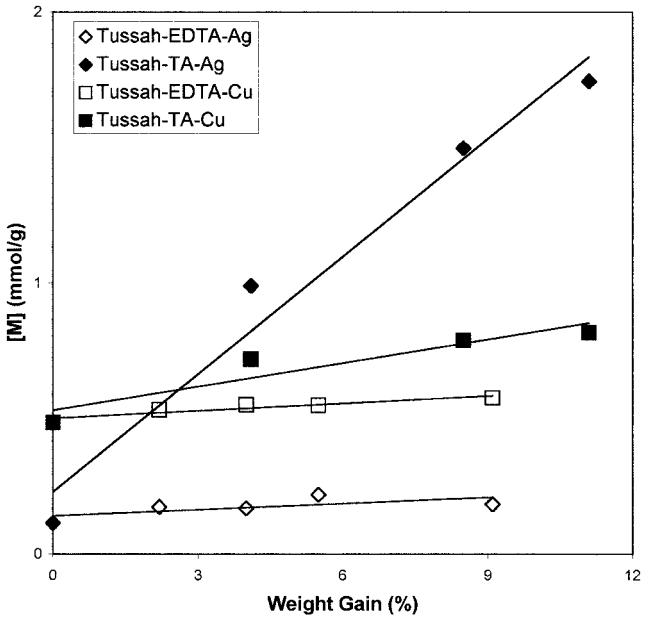

Figure 1 Absorption of Ag^+ and Cu^{2+} at alkaline pH (11.4) by tussah silk fibers chemically modified with TA or EDTA-dianhydride as a function of weight gain (w.g).

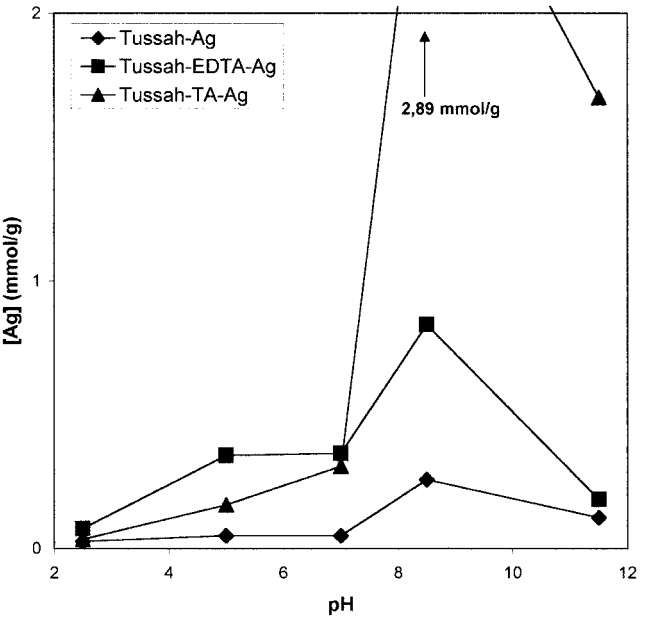

Figure 2 Absorption of Ag^+ by tussah silk fibers chemically modified with TA (11.1% w.g.) or EDTA-dianhydride (9.1% w.g.) as a function of solution pH.

The presence of increasing amounts of the two chelating agents within tussah silk fibers had different effects on metal uptake (Fig. 1). EDTA-dianhydride did not seem to have any appreciable effect on the uptake of Ag^+ and Cu^{2+} at an alkaline pH. On the other hand, the higher the amount of TA, the higher the uptake of Ag^+ and Cu^{2+}, although the latter increased to a lower extent.

To elucidate the effect of pH on the absorption of metal ions, uptake experiments were performed at different pH values. The pH dependence of Ag^+ uptake showed a turning point at pH 7 (Fig. 2). EDTA-dianhydride enhanced the absorption of the metal ion in the neutral-acidic range, while TA was much more effective in the alkaline range. Untreated tussah silk displayed low levels of Ag^+ uptake over the entire pH range. It is noteworthy that the two chelating agents reached a maximum uptake at a pH of 8.5 (2.89 and 0.84 mmol/g for tussah-TA and tussah-EDTA, respectively). The absorption of Cu^{2+} as a function of pH (Fig. 3) denotes constantly higher levels of uptake for tussah-EDTA, compared to both tussah-TA and untreated tussah silk fibers. These results are consistent with those previously reported for both *B. mori* silk and wool.[11,12] The trends of Ag^+ and Cu^{2+} uptake are basically confirmed, indicating that the effectiveness of the chelating agent depends on both the pH of the metal solution and the kind of metal ion used.

Uptake of Co ions by protein fibers

The same experimental scheme applied to Ag^+ and Cu^{2+} was used to study the absorption and binding of Co^{2+} onto protein fibers. The uptake of Co^{2+} by untreated fibers was determined to be in the following order: tussah silk > *B. mori* silk > wool (Table II). Wool displayed the lowest level of binding capacity for Co^{2+}, in striking contrast to the results of Ag^+ and Cu^{2+}. Similar results were reported by Masri et al.[18]

The effect of using increasing amounts of the chelating agent (EDTA-dianhydride or TA) on the uptake of Co^{2+} at alkaline pH is shown in the graph of Figure

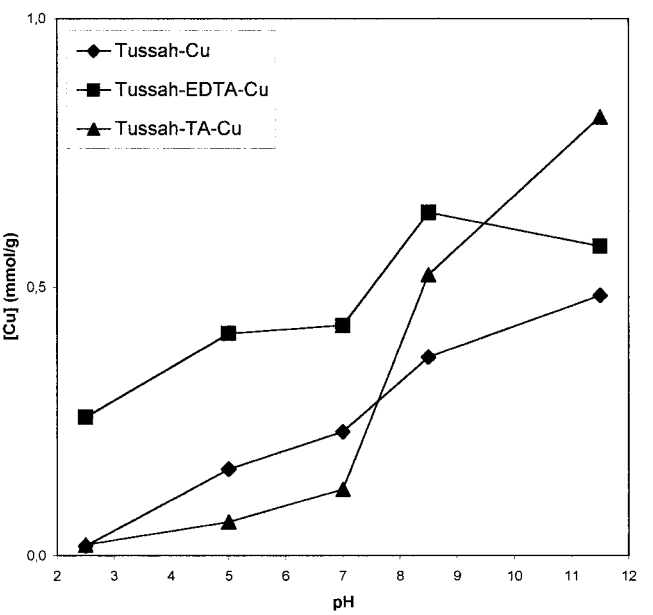

Figure 3 Absorption of Cu^{2+} by tussah silk fibers chemically modified with TA (11.1% w.g.) or EDTA-dianhydride (9.1% w.g.) as a function of solution pH.

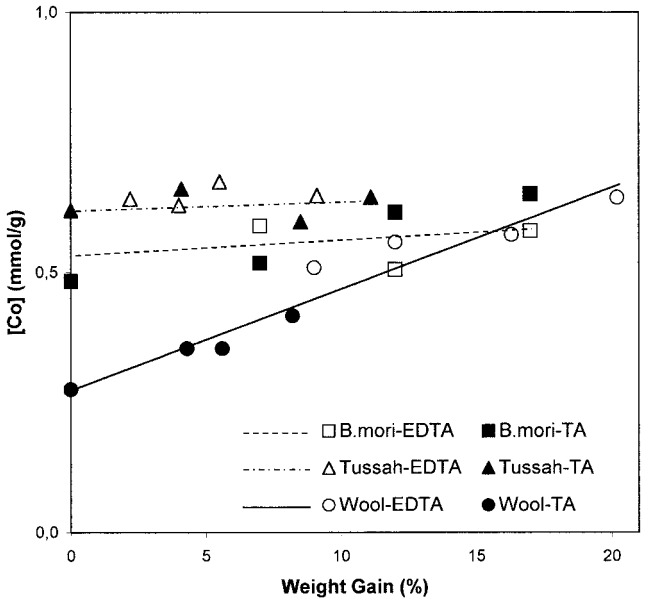

Figure 4 Absorption of Co^{2+} at alkaline pH (11.4) by *B. mori* silk, tussah silk, and wool fibers chemically modified with TA or EDTA-dianhydride as a function of weight gain.

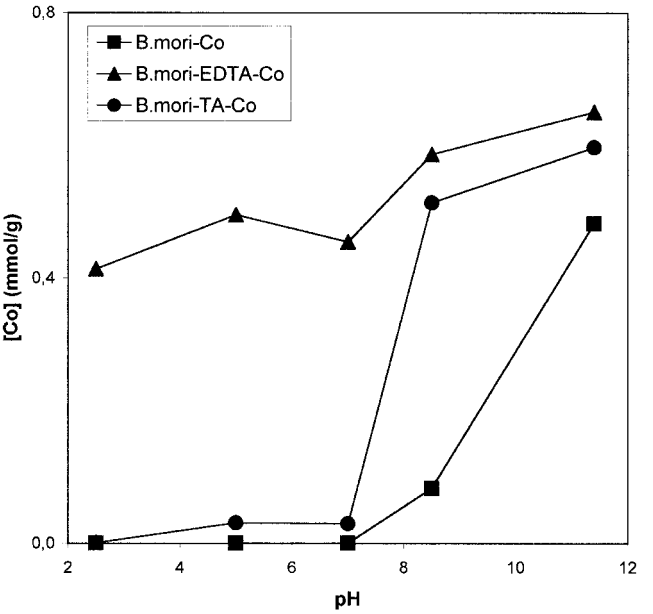

Figure 5 Absorption of Co^{2+} by *B. mori* silk fibers chemically modified with TA (17.4% w.g.) or EDTA-dianhydride (20% w.g.) as a function of solution pH.

4. Data points for wool, *B. mori*, and tussah silk fibers fell within a narrow range of metal concentration, irrespective of the kind of modifying agent used and the weight gain attained, showing a more or less pronounced tendency for increasing values of metal uptake as a function of weight gain. The observed trend is essentially similar to that reported for the uptake of Cu^{2+} onto wool,[12] *B. mori* silk,[11] and tussah silk (this study), under the same treatment conditions.

The plots of Co^{2+} uptake *vs.* pH of the metal solution are shown in Figures 5, 6, and 7 for untreated and modified *B. mori*, tussah, and wool fibers, respectively. Compared to untreated and TA-treated fibers, whose binding capacity for Co^{2+} became detectable only at pH $>$ 7, those acylated with EDTA-dianhydride displayed constantly higher levels of metal uptake over the entire pH range. It is well known that changes in the pH of the metal solution may have a strong effect on the rate and extent of metal absorption, owing to modification of the pattern of charged groups within the fiber substrate. In addition to this, the presence of a chelating agent is expected to exert a strong influence as well, depending on the pK_a values of the dissociating groups. Following acylation with EDTA-dianhydride, the internal balance of electrostatic charges and their density and distribution were drastically changed. Amine functions of basic amino acids were blocked, while new acidic groups, with pK_a values ranging from 2 to 10, were introduced into the fibers. The availability of negatively charged groups over a wide pH range allowed fibers to absorb and bind metal cations even at acidic pH, where the binding capacity of untreated fibers was almost null. These results are in agreement with those previously reported for the absorption of Ag^{+} and Cu^{2+},[11,12] confirming the advantage offered by EDTA-dianhydride over TA as the chelating agent for the preparation of metal complexes based on protein fibers.

In reference to the uptake of Co ions at pH $>$ 7, it is worth noting that their oxidation state may change from Co^{2+} to Co^{3+} due to the tendency displayed by

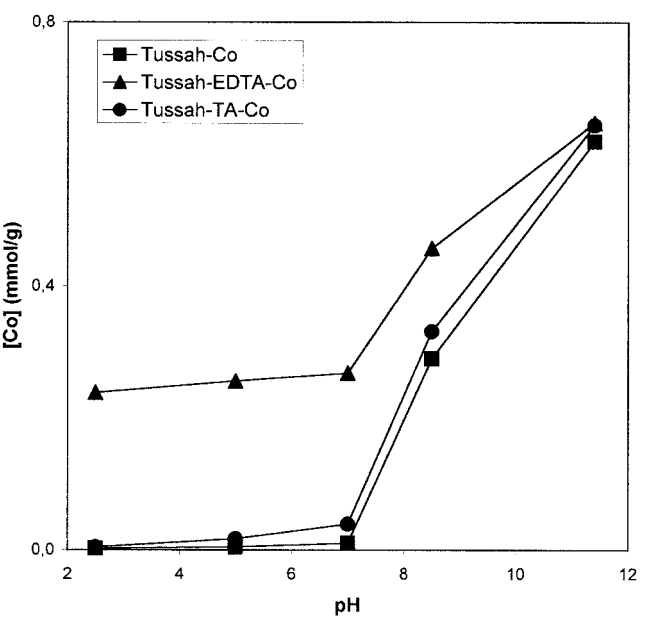

Figure 6 Absorption of Co^{2+} by tussah silk fibers chemically modified with TA (11.1% w.g.) or EDTA-dianhydride (9.1% w.g.) as a function of solution pH.

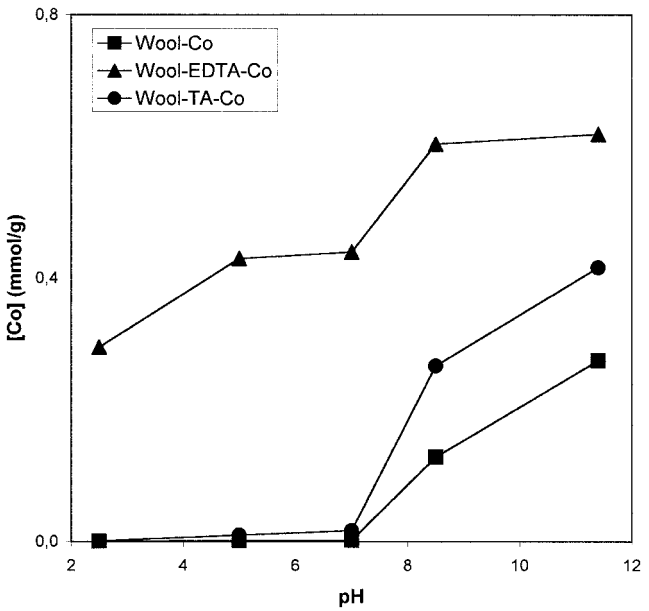

Figure 7 Absorption of Co^{2+} by wool fibers chemically modified with TA (8.2% w.g.) or EDTA-dianhydride (20.2% w.g.) as a function of solution pH.

this ion to oxidize easily at alkaline pH. Although this aspect has not been addressed in the present study, it is of chief importance to consider that changes in the oxidation state of Co ions may influence the properties of the resulting metal complexes with protein fibers. This subject will be considered in future studies.

Reversibility of metal ion absorption

The stability of the various metal complexes prepared in this study was evaluated by measuring the amount of metal released at acidic pH (Table III). It is well known that the absorption of metal ions by protein fibers is a reversible process, and that hydrogen ions may compete for the same binding sites.[1] Therefore, the determination of the extent of metal release is a measure of the durability of the fiber-metal complex.

The data of metal desorption showed a noticeable variability, depending on the kind of fibrous substrate, metal ion, and chelating agent bound to the polypeptide chains, confirming the previously reported results.[11,12] Untreated protein fibers usually showed the highest values of metal release, with only few exceptions, such as the *B. mori* silk-Co complex. Fibers treated with TA were the most effective in retaining the metal ions (with the exception of the *B. mori*-TA-Co complex), while those acylated with EDTA-dianhydride ranked in the middle as far as the stability of the metal complex is concerned. The desorption data are an important parameter for evaluating the performance of the metal complexes formed with protein fibers and for addressing their application.

Antimicrobial activity

The antimicrobial activity of metal ions is attributed to their ability to adsorb to the negatively charged cell surface and diffuse toward the cytoplasmic membranes, which are the target of their action. Disruption of these membranes and release of the internal constituents lead to the death of the cell.[19] Bacitracin, a natural antibiotic peptide, requires the formation of complexes with divalent transition metal ions to express its biological activity, and it has been demonstrated that the antimicrobial activity of the metal-peptide complex correlates directly to the metal binding mode.[20]

Ag^{+}, Cu^{2+}, and Co^{2+} are known to be effective biocides.[19] The antimicrobial activity of different silk and wool complexes with Ag^{+} and Cu^{2+} was reported in previous studies.[11,12] In this work we focused on the characterization of the antimicrobial activity of the Co-complexes by using the agar-based test, and by measuring the size of the zone of inhibition of microbial growth.[21] As shown in Table IV, Co^{2+} in form of a nitrate salt or bound to various kinds of organic ligands exhibited noticeable antimicrobial activity against the plant pathogen *Cornebacterium*. Also *B. mori* silk-Co complexes were effective biocides and their performance was slightly improved by light exposition. Because the size of the zone of growth inhibition on the agar matrix is dependent on the extent of metal ion diffusion from the fiber into the agar, it follows that metal release occurred also under the neutral pH conditions used for the test. Wool-Co complexes, either untreated or modified with the chelating agents, displayed antimicrobial activity, but a persistent opacity of the zone of growth inhibition ham-

TABLE III
Release of Metal Ions by Treatment of Metal-Containing Protein Fibers

Sample	Ion content (mmol/g)		Metal released (%)
	Before	After	
Tussah-Ag	0.115	0.058	49.1
Tussah-EDTA-Ag	0.184	0.117	36.3
Tussah-TA-Ag	1.685	1.607	4.6
Tussah-Cu	0.485	0.254	47.6
Tussah-EDTA-Cu	0.576	0.349	39.4
Tussah-TA-Cu	0.817	0.701	14.3
Tussah-Co	0.620	0.332	46.4
Tussah-EDTA-Co	0.648	0.379	41.4
Tussah-TA-Co	0.644	0.588	8.7
B. mori-Co	0.483	0.269	44.2
B. mori-EDTA-Co	0.580	0.190	67.2
B. mori-TA-Co	0.651	0.153	76.5
Wool-Co	0.275	0.104	62.3
Wool-EDTA-Co	0.618	0.411	33.6
Wool-TA-Co	0.353	0.281	20.4

Treatment in aqueous solution at pH 3.8 for 120 h.

TABLE IV
Antimicrobial Activity of Metal Complexes Between Co-Ions and Organic Ligands or Protein Fibers

Sample	Size of the inhibition zone (mm)
$Co(NO_3)_2$	18.5
Co(II)acetylacetonate	20
Bis(salicylaldehyde)Co(II)diahydrate	12
[1,2-Bis(diphenylphosphino)ethane]dichloroCo(II)	11
Bis(salicylideniminato-3-propyl)methylaminoCo(II)	3
Silk-Co	19 (13)[a]
Silk-EDTA(10%)-Co	19 (6)
Silk-EDTA(16%)-Co	17 (2.5)
Wool-Co	0
Wool-EDTA-Co	6 (3)

[a] Values in parenthesis were obtained under dark conditions.

pered the precise determination of its size. The antimicrobial activity against *E. coli* was very low or nonexistent, in agreement with previously reported results.[12]

CONCLUSIONS

Chemical modification of *B. mori*, tussah, and wool fibers with chelating agents, obtained by loading with TA or by acylation with EDTA-dianhydride, effectively promoted the absorption and binding of metal ions. In particular, the acylation technique seemed effective because metal absorption occurred over a wide range of pH conditions and was significantly high at acidic and neutral pHs, where untreated or TA-treated protein fibers absorbed only negligible amounts of metal ions. This feature can be exploited for the development of safer metal treatments of protein fibers, because the harmful alkaline conditions usually adopted to promote metal uptake can be avoided.

Acylated silk and wool fibers containing different metal ions exhibited good performance as antimicrobial textiles. Acylation with EDTA-dianhydride, followed by absorption and binding of metal ions from aqueous solution, is a novel approach for developing an antimicrobial finishing treatment for protein fibers. Durability of the metal coordinated within the fiber and absence of toxic effects to the user are important prerequisites that must be met when clothing applications are considered. In this respect, desorption data seem to suggest other possible fields of use for the metal-containing protein fibers. In fact, the controlled migration of the metal from the textile to the environment could become a key factor for the development of devices with enhanced barrier-effect against microorganisms, able to prevent diffusion of pathogens. Additionally, another application can be envisaged, that is, the manufacture of a new range of ion exchangers able to support repeated metal absorption-desorption cycles, to be applied as sorbents for removing and recycling metal ions. The latter option appears attractive for the metal complexes prepared in this study, especially those based on protein fibers acylated with EDTA-dianhydride, owing to their effectiveness as sorbents of metal ions under different pH conditions.

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