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Cu^{2+}- and Ag^{+}-complexes with a hyaluronane-based hydrogel

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Copper(II) and silver(I) hyaluronane-based hydrogel complexes were synthesised. The amount of metal ion uptaken by hydrogel was determined by atomic absorption and the hydrogel–metal ion complexes were characterised by water-uptake measurements, SEM-EDAX and FT-IR analysis. The coordination sites were identified for both metal ions and the stability at various pH levels was determined. The cytotoxicity of hydrogel metal ions was evaluated by using fibroblast (3T3) cells. The copper(II) complex was "*in vivo*" tested and showed proangiogenic activity, stimulating the growth of new vessels without inducing an inflammatory reaction.

The antibacterial activity of a silver complex was determined, showing that the presence of silver ions drastically reduced the adhesion and proliferation of *Staphylococcus epidermidis*.

Introduction

It is widely known that polysaccharides are capable of coordinating several metal ions, assuming new biological properties on the basis of their activities.[1–3] One of the most largely analysed elements is copper. Among the eight biologically essential metals (Ca, Mg, Mn, Fe, Cu, Zn, Co and Mo), it can indeed be considered as the most versatile one. It plays a key role in several physiological processes, such as electron transfer reactions, oxygen transport, enzyme catalysis. Furthermore, copper deficiency can cause dangerous effects, for example, anemia.[4]

On the basis of this wide spectrum of activities, several copper complexes were studied and tested for use in different applications. Tabata *et al.* showed that an amilopectin based biodegradable hydrogel matrix, which was enriched with copper ions, favours the capture of bFGF (basic fibroblast growth factor) and regulates its release. They demonstrated that the presence of copper decreased the rate of bFGF release.[5] Hyaluronane, a linear polysaccharide present in many physiological fluids, shows a remarkable capability to coordinate copper(II) and chemotactically mobilise endothelial cells, inducing an angiogenic response.[6]

In this work, we want to verify whether hydrogels based on hyaluronic acid maintain the ability to capture both Cu(II) ions and to show angiogenic properties. The development of new vessels is a physiological process which can appear in several phases of human life, but it also represents a crucial event in several diseases such as diabetes, cancer or rheumatoid arthritis.[7–9] Consequently, a material which autonomously has the capability of stimulating the growth of new vessels can be used in several biomedical fields, for example to develop vascularisation after burns, surgical operations.[10] So a hyaluronic acid 50% based hydrogel was synthesised and enriched with copper ions. It was characterised in terms of the amount of copper ions taken up, the water-uptake, the chemical groups involved in the coordination, citotoxicity, and angiogenic activity.

Moreover, in the development of new materials for making devices, one of the crucial aspects is the infection that can be favoured after implantation. Generally, bacteria adhere to biomedical device surfaces through different and several interactions. This phenomenon, or bacterial adherence to biomaterials, is only the initial event. It is followed by colonisation and the formation of an adherent biofilm, and it is this biofilm in which bacteria are embedded on the device surface that renders antibiotic treatments and host defence mechanisms ineffective. In addition, the biofilm acts as a continuous infectious focus.[11] There are several strategies that may decrease bacterial adherence, such as improved surface treatment processes, coating, or the incorporation of antimicrobial compounds within them. Of these, silver treatment is of particular interest.[12] It is a well-known antimicrobial agent and different formulations have been found effective in the treatment of burns, urinary tract infections, and dentistry. Thus, and in addition, a hydrogel–silver complex has now been studied and its antimicrobial activity evaluated. A complete physicochemical characterisation, consisting of FTIR-ATR, SEM-EDAX analysis, and water-uptake measurements, was researched to unequivocally identify their biological properties.

Experimental

Materials

The sodium salt of hyaluronic acid (HA) ($\cong$150–200 kDa) was supplied by Biophil Italia S.p.A. *N*,*N*′-Dimethylformamide (DMF), 2-chloro-1-methylpyridinium iodide (CMP-J), tetrabutylammonium hydroxide (TBA), 1.3-diaminopropane, triethylamine, ethanol, silver nitrate, copper sulfate, and all the other reagents were purchased from Fluka Chemie AG (Switzerland).

Methods

a) Hydrogel synthesis and characterisation. The synthesis reaction, described elsewhere,[13] is a multi-step process which

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has varied applicability. Briefly, the polysaccharide was transformed into a TBA salt on an exchange column and dissolved in dimethylformamide (DMF) under stirring and nitrogen flow. Maintaining the solution at about 0 °C, stoichiometric amounts of 2-chloro-1-methylpyridine iodide (CMP-J), required to activate a prefixed percentage (50%) of the carboxylate groups, was added. An excess of the cross-linking agent, 1.3 diaminopropane, was then added together with a small amount of triethylamine as catalyst. The gel formed immediately, but the process was completed only by keeping the mixture under stirring at room temperature for three to four hours. The gel was washed several times with EtOH and water and dried in a vacuum oven at 40 °C for 24 hours. The potentiometric titration performed on the swollen hydrogel, carried out as previously reported,[14] confirmed the degree of cross-linking .

b) Ninhydrin test. The ninhydrin test was carried out to verify the presence of unreacted NH_2 arms of the 1,3-diaminopropane cross-linker.[15] The test was conducted following the previously reported procedure.[16]

c) Complex formation. Established amounts of dried hydrogels were immersed for 24 hours in a metal ion solution (10^{-1} M $CuSO_4$, and 10^{-1} M and 10^{-3} M $AgNO_3$). Then they were washed and the washing solutions were continuously checked to detect the presence of metal ions. To check for the permanence of the metal ion in the hydrogels the samples were enclosed in small bags made of hydrophobic water-permeable net (Nylon) and maintained in a PBS (phospate-buffered saline) solution (50 mL), under stirring, for 7 days. Every 24 hours a small amount of solution (1 mL) was taken out and analysed by atomic absorption to detect the metal ion release.

The washing process was continued until no detectable metal ion concentration was revealed. The amount of metal ion captured by the hydrogels was determined using a Perkin Elmer Analyst 100 HGA-800 atomic absorption spectrometer, following the usual procedure.[17]

Particular attention must be given to the realisation of the silver complex. To prevent the reduction of Ag^+ the Ag(I)–HA complex was protected from direct exposure to light.

d) FTIR-ATR analysis. ATR spectra of the samples in dry form were recorded on a Bio-rad FTS6000 between 4000 and 750 cm^{-1}, as previously reported.[18,19] An MCT detector was used and the apparatus was purged with nitrogen. Typically, 50 scans at a resolution of 1.0 cm^{-1} were averaged. The frequency scale was internally calibrated with a helium–neon reference laser to an accuracy of 0.01 cm^{-1}. A deconvolution method was applied to the 1800–1500 cm^{-1} spectral regions, the most representative for this system.

e) Water-uptake measurements. Established amounts of dried hydrogels, with or without metal ions, were enclosed in small bags of hydrophobic water-permable net (Nylon) and immersed for 24 hours at room temperature in 50 mL of 0.9 NaCl solution (for copper complexes) and 0.1 M KNO_3 solution (for silver complexes). At regular intervals the bag was removed from the solvent, its surface was pressed gently with tissue paper to remove the excess solvent on the surface, weighed, and then returned to the medium. This process of water-uptake and weighing was continued until the gels attained a constant final weight.

$$\text{Water-uptake} = (W_s - W_d/W_d) \times 100$$

where W_s is the weight of the swollen hydrogel and W_d is the weight of dry hydrogel.

f) Scanning electron microscopy and X-ray microanalysis. Scanning electron microscopy (SEM) and X-ray microanalysis (EDAX) of cooled and dried gels were performed in order to analyse gel morphology, gel structure and metal ion distribution. Water swollen gels (2.5 mg) were put in cryotubes and cooled by liquid nitrogen. After cooling, gels were lyophilised, mounted on SEM stubs and gold-sputtered with an automatic sputter coater (BAL-TEC SCD 050, Balzer). The morphology and structure of the gels were viewed using an XL 20 SEM (Philips). The distribution of metal ions in the hydrogel (dot-map) was carried out by using EDAX (Energy Dispersive X-ray Analyzer, Philips).

g) *In vitro* tests. *Cytotoxicity of Cu(II)–HA and Ag(I)–HA complexes.* In order to assess the citoxicity of the metal ion–HA hydrogels, Cu(II)–HA and Ag(I)–HA, hydrogel complexes with different amounts of metal ion were prepared. The Cu(II)–HA complexes were obtained using Cu(II) solutions with the following ion concentrations: 10^{-1} and 10^{-2} M. The Ag(I)–HA complexes were obtained using Ag(I) solutions with ion concentrations of: 10^{-1} M, 10^{-2} M and 10^{-3} M. The test was performed with mouse fibroblasts (cell line 3T3). Fibroblasts 3T3 were maintained in culture in DMEM, supplemented with 10% fetal calf serum (FCS), 1.2% L-glutamine and 1% penicillin–streptomycin (Sigma, Germany) on polystyrene flasks. The fibroblast cultures were incubated at 37 °C in an atmosphere of 5% CO_2 until cells were harvested with the aid of trypsin and suspended in fresh medium.

The cytotoxicity of metal ion–hydrogel complexes towards mouse fibroblasts 3T3 was evaluated by the direct contact method. Briefly, 3T3 cells (4000 cells mL^{-1}) were suspended in DMEM, containing 10% fetal calf serum and placed on the bottom of each well of a multi-well plate (24-well). The multi-well was then incubated in a humidified atmosphere containing 5% CO_2 at 37° C until the cells reached confluence (24 hours). Afterwards, 5 mg of each ethanol sterilised sample, swollen for 24 hours at 37 °C in 500 μL of DMEM (Dulbecco's modification of Eagle's minimum essential medium) were added to each fibroblast monolayer and incubated at 37 °C for 24 hours. The samples were then removed and the cells were fixed with glutaraldehyde, stained with trypan blue and counted by direct observation with an optical microscopy (BX40, Olympus). After determining the real amount of metal ion uptaken by hydrogels (through atomic absorption), solutions containing the same quantity of free metal ion were prepared with the aim of evaluating the effect of coordination on the cytotoxicity of these metals. The bottom of the polystyrene wells was used as a negative control. Four samples of each different material were tested.

Bacterial adhesion on HA and Ag(I)–HA complexes. The antimicrobial activity of silver complexes was evaluated by two different methods, a quantitative technique, for the evaluation of bacterial adhesion onto the materials[20] and a qualitative one based on the formation of an inhibition circle.

Staphylococcus epidermidis was the Christensen RP62A original slime-forming strain (obtained from Dr L. Baldassarri, Istituto Superiore di Sanità, Rome, Italy) maintained in our laboratory with frequent adherence selection.

Five mg of HA and Ag(I)–HA samples were sterilised in 95% ethanol for 3 min and then swollen in sterile saline solutions. The samples were placed in 12-well plates with 2 mL of trypticase soy broth (TSB) and incubated for 24 h with and without *S. epidermidis* RP62A seeded in each well to a final concentration of 10^6 colony-forming units (CFU) per mL. After incubation, the samples were washed five times with saline and finally incubated with a fresh sterile culture medium (TSB) at 37 °C for 24 h. After incubation, the broth rich in bacteria was drained and examined in a turbidimeter (Ratio-XR Turbidimeter, Hach). Quantitative analysis of *staphylococci* adhered to the materials was carried out by evaluating the broth bacterial

concentration in nephelometric turbidity units (NTU). Polystyrene was used as a positive control.

The second technique consists of a mixture of 1 mL of bacterial suspension with 40 mL of TSA (Tryptic Soy Agar) maintained at a solidification temperature. After solidification the samples were collocated on the plates. After 24 hours of incubation at 37 °C, the final observation was made. If the bacterial growth has been blocked on the plates an inhibition circle will appear. The dimension of the halo may be correlated to the antimicrobial activity. The pictures were taken using a digital Nikon Optiphot Coolpix 950.

h) *In vivo* test. *Angiogenic activity of Cu(II)–HA.* Samples of HA hydrogel and their complexes with Cu(II) ion were sterilised and inserted into sterile cylindrical steel net cages (diameter of 1.5 cm, height of 3.5 cm). The biocompatibility was evaluated by using three, five month-old male Wistar rats, weighing 300 g. Two cages, one containing the test sample and the other a control polymer, were implanted in the subscapular panniculus adiposus of each animal and left for 21 days. Then the animals were sacrificed and the cages recovered. The samples in the cages were washed with sterile buffer solution and prepared for SEM according to the following procedure. They were incubated in 2.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate for 30 minutes, washed in 100 mM buffer cacodylate for 30 seconds, rinsed with distilled water and left standing in a dehydration solution (70% v/v ethanol in H_2O) for 15 minutes. They were then transferred to a second dehydration solution (90% v/v ethanol in H_2O) for 15 minutes and subsequently, to absolute ethanol for 15 minutes for total cell dehydration. Finally, the samples were critical point dehydrated (Emitech K850, UK), and coated with gold in an automatic sputter coater (BAL-TEC SCD 050, Balzers, Germany). Scanning electron microscopy (SEM, XL 20 Philips, The Netherlands) at a 10 kV accelerating voltage was used to observe the samples.

Results and discussion

Physico-chemical characterisation

The hyaluronane-based hydrogel was synthesised with a cross-linking degree of 50%, as confirmed by potentiometric titration (48 ± 2%) (Fig. 1). The diamine cross-linker fully reacted and no free NH_2 groups were detected in the hydrogel, as determined by the ninhydrin test. The linear non-cross-linked polysaccharide is capable of coordinating the Cu^{2+} ion through the amidic and carboxylate moieties, as previously reported[21] and the 50% hydrogel retains this coordinating ability towards the Cu^{2+} and Ag^+ ions. The amount of coordinated Cu^{2+} and Ag^+ ions, as ascertained by atomic spectroscopy and starting from a 0.1 M concentration of each metal ion, was 0.024 mg Cu^{2+} per mg gel and 0.046 mg Ag^+ per mg gel.

The permanence of the metal ion in the hydrogel was determined by atomic absorption. No release of Ag^+ was detected even after 7 days of washing, whereas the copper complex showed a small release of Cu^{2+}, valued at 10%, in the first 24 hours of washings.

The amounts of Cu^{2+} and Ag^+ uptaken, expressed in molar quantities, are not dissimilar from each other considering the different MW's of the two metal ions. The infrared spectra of the HA hydrogel and the HA hydrogels coordinated to Cu(II) and Ag(I) were recorded at three different pH's (2.0, 5.5 and 7.0)' The spectra of the metal coordinated hydrogels were compared with those of the bare 50% hydrogel at the same pH's in order to examine the functional groups of the network involved in the coordination of metal ions and the influence of pH on the coordinating ability of the gel.[22]

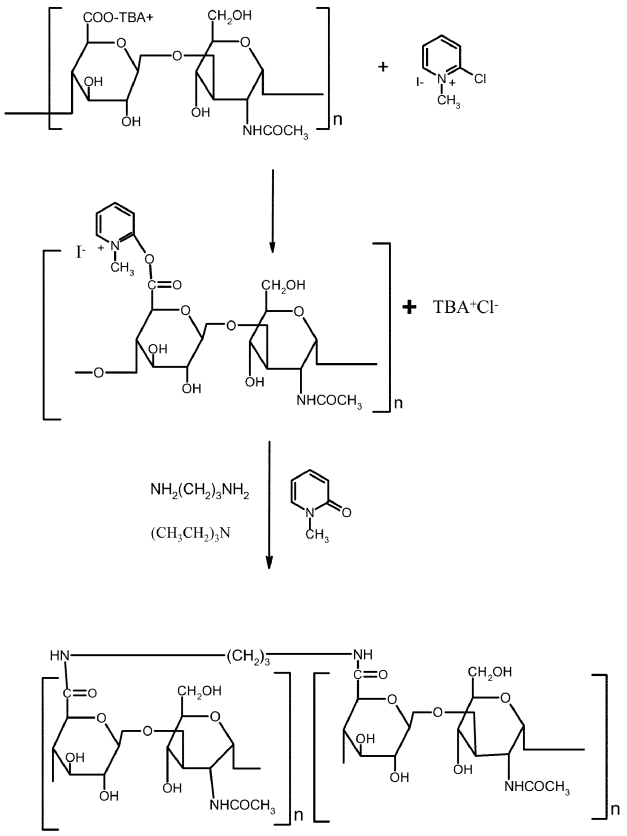


Fig. 1 Cross-linking reaction of HA using CMP-J as the activating agent.

Cu(II)–HA hydrogel complex. Figs. 2–4 show the ATR-FTIR spectra of the Cu(II)–HA complexes at pH 2.0, 5.5 and 7.0 respectively. The diagnostic absorption bands lie in the 1800–1300 cm^{-1} spectral region, so only this range is reported (Figs. 2–4), together with that of the HA hydrogel. Table 1 summarises the main wavenumbers observed in the 1800–1300 cm^{-1} spectral region together with their assignments.

pH = 2.0. The spectrum of the Cu(II)–HA hydrogel complex at pH = 2.0 is almost the same as that of the bare HA hydrogel at the same pH value. The wavenumbers of the main functional groups remain unaffected by the presence of copper(II) ion (see Table 1), demonstrating that no coordination occurs at this pH (Fig. 2).

pH = 5.5. When the pH is increased to 5.5, the infrared spectrum of the Cu(II)–HA hydrogel system is no longer superimposable on that of the HA hydrogel at the same pH.

As we can see from Fig. 3 and Table 1, the spectrum of the HA hydrogel still shows the COOH absorption centered at

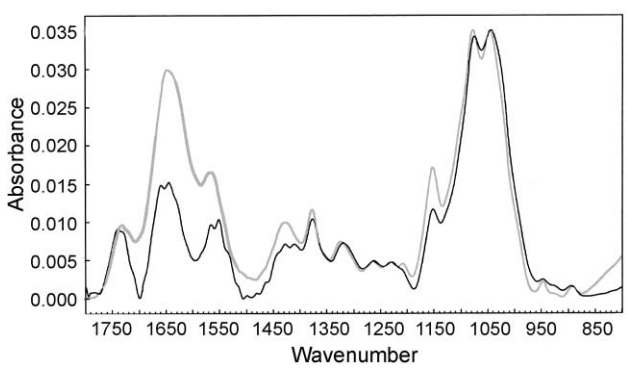


Fig. 2 IR spectra of HA (black) and Cu(II)–HA (grey) recorded at pH 2.0.

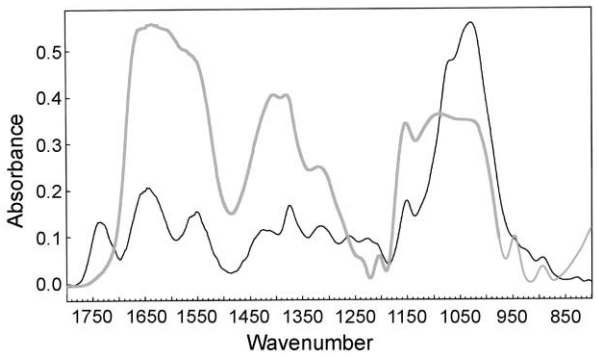


Fig. 3 IR spectra of HA (black) and Cu(II)–HA (grey) recorded at pH 5.5.

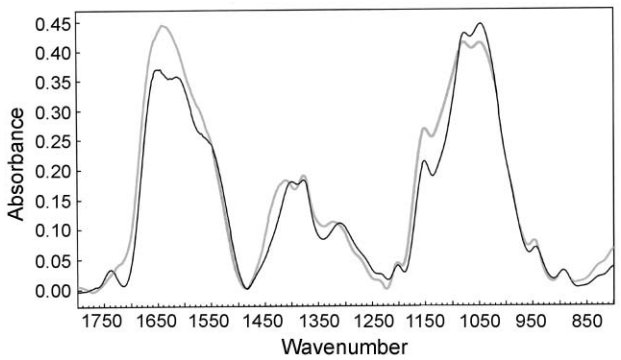


Fig. 4 IR spectra of HA (black) and Cu(II)–HA (grey) recorded at pH 7.0.

around 1730 cm^{-1} and 1738 cm^{-1}, whereas this band completely disappeared in the spectrum of the complex, emphasising that the carboxylic group is no longer present in the network, but is converted to carboxylate (COO^-), as revealed by the absorptions at 1603 (asym. stretch.) and 1406 (sym. stretch.) cm^{-1}. These data clearly suggest the involvement of the carboxylate groups in the coordination of copper(II).

By deconvoluting the 1800–1500 cm^{-1} spectral region, which is characterised by a very broad band, we also observe more than one type of amidic C=O absorption (indicating the presence of differently bonded carbonyl groups). In particular, the 1635 cm^{-1} component can be correlated with a C=O group involved in the coordination. However, no variation in the amidic N–H frequency is observed.

All these data clearly showed the 50% hydrogel was coordinated to Cu(II) through both the carboxylate and C=O amidic groups (Fig. 3).

pH = 7.0. By looking now at the spectrum collected at pH = 7.0, we may still observe the absorptions of the coordinated amidic C=O and COO^- groups in the hydrogel–Cu(II) ion complex (Fig.4).

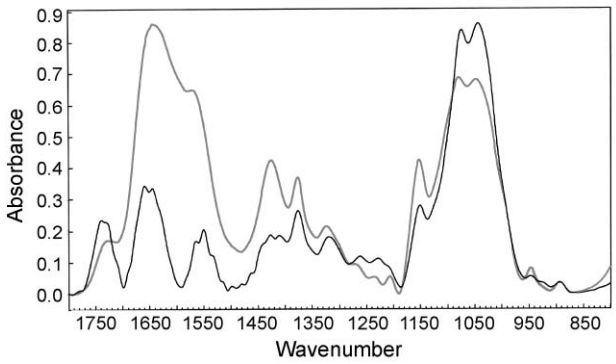


Fig. 5 IR spectra of HA (black) and Ag(I)–HA (grey) recorded at pH 2.0.

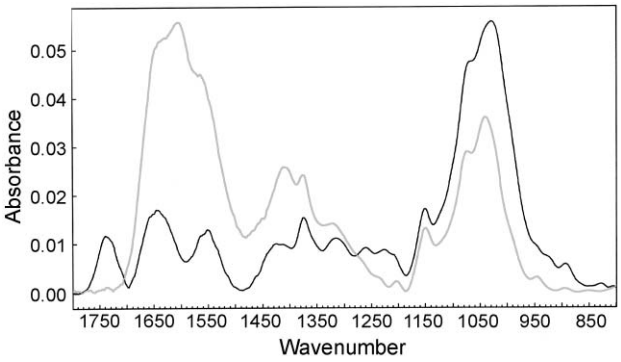


Fig. 6 IR spectra of HA (black) and Ag(I)–HA (grey) recorded at pH 5.5.

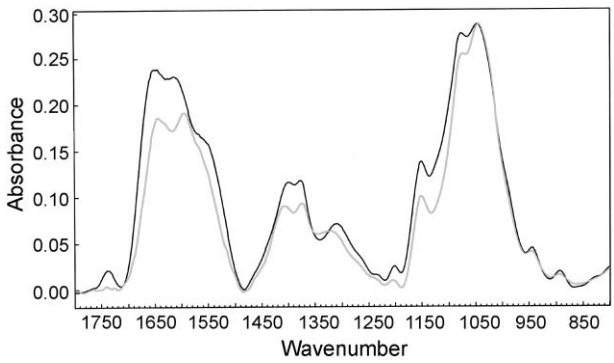


Fig. 7 IR spectra of HA (black) and Ag(I)–HA (grey) recorded at pH 7.0.

Ag(I)–HA hydrogel complex. Figs. 5–7 show the infrared spectra of the Ag(I)–HA hydrogel systems at pH = 2.0, 5.5 and 7.0 respectively, together with those of the free HA hydrogel at the same pH. Table 1 summarises the main wavenumbers observed in the 1800–1300 cm^{-1} spectral range of these spectra together with their assignments.

Table 1 Main wavenumbers observed in the IR spectra of HA, Ag(I)–HA and Cu(II)–HA and their assignments

HA			Cu(II)–HA			Ag(I)–HA			
pH 2.0	pH 5.5	pH 7.0	pH 2.0	pH 5.5	pH 7.0	pH 2.0	pH 5.5	pH 7.0	Assignments
1738	1738	1738				1735sh[a]			COOH free
1730	1730		1731						COOH H-bonded
1659	1659	1650	1650	1664					C=O free
1644	1640		1640			1640	1649	1643	C=O H-bonded
				1635	1638				C=O coordinated
	1610	1610							COO^- free
				1603	1603	1605	1605	1595	COO^- coordinated (asym. stretch.)
1566	1568	1568	1566	1565	1568	1566	1561	1560	NH free
1551	1550	1549	1551	1548	1548				NH H-bonded
		1403		1406	1409		1407	1410	COO^- coordinated (sym. stretch.)

[a]sh = shoulder.

pH = 2.0. The presence of Ag(I) within the HA network is reflected by a decrease in the band intensity of the COOH vibration centered at 1735 cm^{-1}. At the same time the absorption of COO^- appears as a shoulder at 1605 cm^{-1}. Thus, at pH = 2.0, Ag^+ competes with H^+ in forming bonds with the HA carboxylate. However, the amidic C=O and N–H vibrations remained unaffected by the presence of the metal ion (Fig. 5).

pH = 5.5. The infrared spectrum at pH = 5.5 only differs in the greater intensity of the band at 1605 cm^{-1} than at pH = 2.0, suggesting that the amount of complex species increases with increasing pH, with COO^- remaining the only coordinating group (Fig. 6).

pH = 7.0. When the pH is raised to 7.0, the carboxylic absorption of the asymmetrically coordinated COO^- shifts to 1595 cm^{-1}, indicating a strong bond between this group and the Ag(I) metal ion (Fig. 7).

From all these data we may conclude that Cu(II) was coordinated by HA hydrogel at pH $\geqslant$ 5.5, by the amidic C=O group and carboxylic COO^- group.

In the case of Ag(I), the complex formed at a pH as low as pH = 2.0 and the only coordinating group is the carboxylic COO^- moiety.

Water-uptake and morphology. The water-uptake values of the HA hydrogel by itself and once coordinated with Cu(II) and Ag(I) are shown in Fig. 8.

The coordination of Cu(II) reduced the water-uptake of the polysaccharide hydrogel with water-uptake decreasing with increasing amounts of Cu^{2+} ion coordinated to the polysaccharide network. These findings are in agreement with the more compact morphology shown by the Cu(II)–hydrogel complex with respect to the HA hydrogel (Fig. 9a–c). The HA hydrogel shows a lamellar morphology, composed of wide, stretched out and superimposed sheets. Among the sheets, hollows of varying dimensions are present. Metal coordination alters the morphology of the hydrogel with loss of the lamellar structure. The Cu(II)–HA hydrogel assumes an irregular, indented structure characterised by the presence of small pellets which become thicker with increasing amounts of Cu^{2+} ions, providing the gel with a more and more compact morphology. EDAX analysis showed a consistent presence of copper corresponding to the pellets (Fig. 10a). It is hypothesised, on the basis of the tendency of copper (II) to form a structure in which it is coordinated by more than one COO^- group,[22,23] that the decrease in the water up-take is due to charge neutralisation following complex formation. In fact, as represented by the scheme shown below, the neutralisation of the two COO^-

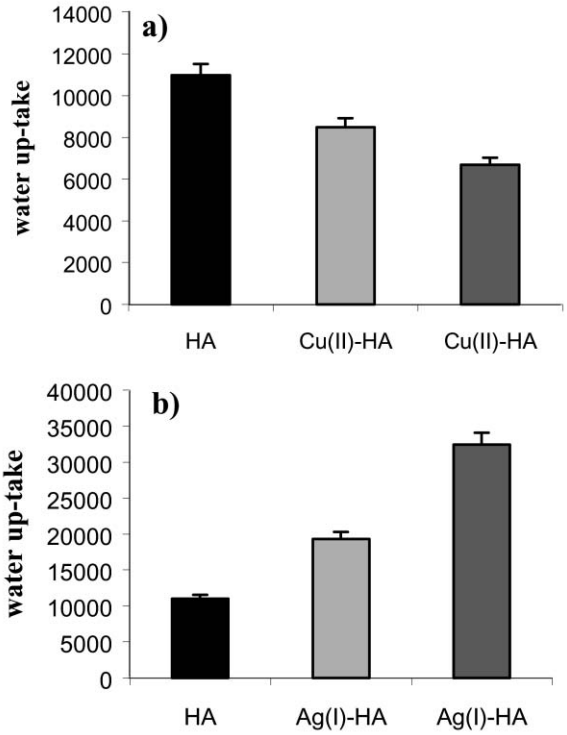
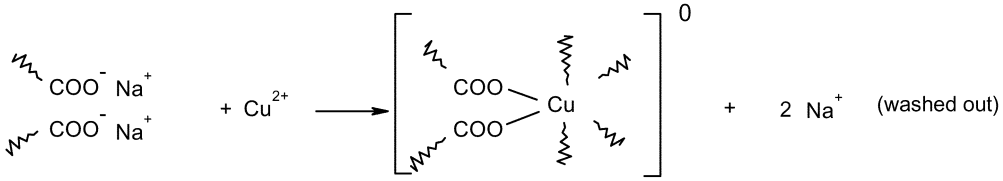


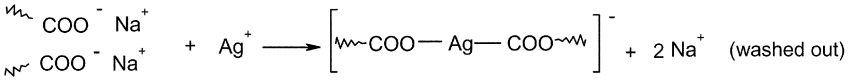
Fig. 8 Water up-take behaviour. a) Water up-take of HA (black) and Cu(II)–HA complexes (grey) containing incremental amounts of metal ion, respectively 0.018 mg_{Cu}/mg_{dryHA} and 0.024 mg_{Cu}/mg_{dryHA}, in a physiological solution (NaCl 0.9%). The presence of copper ions provokes a decrease in the amount of absorbed water. The last histogram for the complex containing 0.024 mg of metal ions per mg of dried hydrogel shows the lowest amount of water up-take. b) Water-uptake of HA (black) and Ag(I)–HA complexes (grey), containing different amounts of metal ion, respectively 0.035 mg_{Ag}/mg_{dryHA} and 0.046 mg_{Ag}/mg_{dryHA} in 0.1 M KNO_3 solution. An opposite trend is recorded for silver ions; their presence provokes a large increase in water-uptake proportional to the increase in silver ion. The last histogram for the Ag(I)–HA complex containing 0.046 mg of metal ions per mg of dried hydrogel shows the highest amount of absorbed water.

Ag(I)–hyaluronan hydrogel increases by a factor of 3.5 with respect to that of the bare HA hydrogel. When silver(I) ions are coordinated by the HA gel, the large lamellar morphology is broken into thinner and thinner lamellae, until a scaling process occurs so that a more open morphological structure of the gel is obtained (Fig. 11). EDAX analysis showed a diffuse homogeneous distribution of the metal ion (Fig. 10b).

The coordination of Ag(I) occurs only through the COO^- groups of HA, allowing Ag(I) to undergo linear coordination



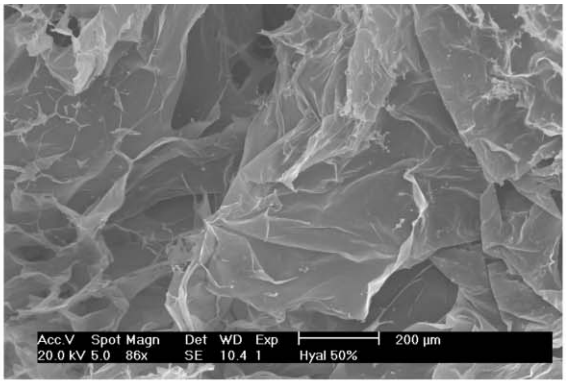
charges occurs in Cu^{2+} complex formation reaction, but it does not occur with the ionically bound Na^+ ions . The 4+2 [COO–Ag–COO]$^-$, as generally assumed by this metal ion.[24]This structure does not alter the overall charge and



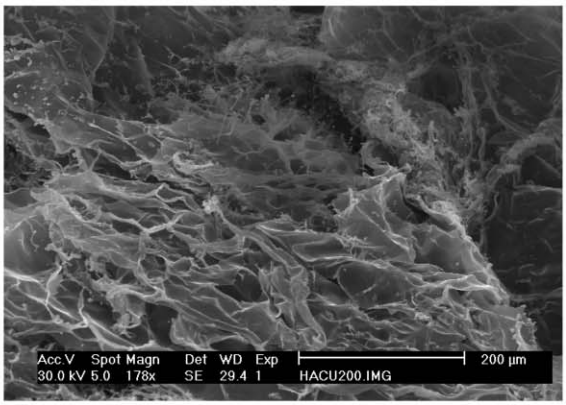
structure of Cu^{2+} favours the shrinkage of the polymeric chains around the metal ion. Furthermore, the involvement of the amidic moiety in the coordination sphere means that there is a hydrophilic group that is unable to interact with water, thus reducing the water-uptake ability of the hydrogel.

On the other hand, an opposite trend is observed in the case of coordinated Ag(I) (Fig. 8b.). The water-uptake of the leads to a repulsion of the polysaccharide chains which favours their hydration.

A further contribution to the understanding of the water up-take behaviour of Cu(II)–HA and Ag(I)–HA complexes can be obtained by considering the different ion radius of the three metal ions (Cu^{2+} 72 pm; Na^+ 102 pm; Ag^+ 113 pm). The Cu^{2+} ions, with a smaller radius than Na^+ ions, occupy a smaller



a)



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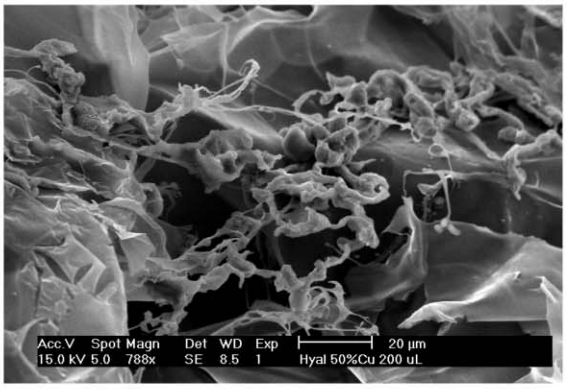


c)

Fig. 9 Electronic micrographs of a) HA which shows a lamellar morphology; b) Cu(II)–HA. The presence of copper ions drastically affects the hydrogel morphology which becomes more and more compact; c) pellets present in great number in Cu(II)–HA hydrogel complex due to an accumulation of metal ion.

"space". Consequently, the replacement of Na^+ by Cu^{2+} provokes a decrease in the distance between the polymeric chains, or a more compact structure, and a subsequent decrease in the water up-take.

In contrast, the presence of bigger ions, such as Ag^+, causes an increase in the distance between polymer chains leading to the formation of microcavities and favouring the up-take of water.

Biological characterisation

In vitro tests. *Cytotoxicity of Cu(II)–HA and Ag(I)–HA complexes.* Previous studies showed that the 50% HA hydrogels are not cytotoxic.[25]

To determine whether our complexes were cytotoxic and to analyse the influence of both metal ion and hydrogel on the response of cells, experiments with 3T3 fibroblast cells were carried out on both the gels coordinated to Ag(I) and Cu(II) ions and on each of the metal ions alone. Hydrogels with different amounts of metal ions were tested. Starting from the maximum uptake of metal ion, *i.e.* Cu^{2+} 0.024 mg/mg gel and Ag^+0.046 mg/mg gel, the quantity of metal ion was decreased until a non-cytotoxic sample was found. The same quantity of the free metal ion in solution was also biologically tested.

With the maximum amount of Cu^{2+} in the gel (0.024 mg/mg gel), the cells adhered on the bottom of the wells with a flat shape. Similar behaviour was observed for the cells in contact with a negative control (PS = polystyrene), also maintaining the same number of alive cells. The same experiment carried out with only metal ion in solution showed the quantity of the Cu^{2+} ion was non-cytotoxic. With a higher quantity of Cu^{2+}, a few cells were alive and showed a cell-suffering round shape.

In contrast, the maximum amount of Ag(I) uptaken by the gel (0.046 mg/mg gel) was cytotoxic, as was the same amount of metal ion in solution. On decreasing the amount of Ag^+ ions in the hydrogel, the quantity 0.0042 mg/mg gel turned out to be non-cytotoxic. A solution containing the same amount of metal ion up-taken by the hydrogel was tested and its non-cytotoxicity proven. Table 2 summarises the metal ion–HA complexes formed and characterised.

Bacterial adhesion on HA hydrogel and Ag(I)–HA hydrogel complexes. Table 3 shows the results of the adhesion of the RP62A strain of *S. epidermidis* to polystyrene (PS, negative control), the HA hydrogel and the Ag(I)–HA complex containing 4.6×10^{-2} mg_{Ag}/mg_{dryHA}. The hydrogel without the metal ion showed a large bacterial adhesion, larger than that found on PS. In contrast, adhesion to the Ag(I)–HA complex was strongly inhibited in comparison to both PS and HA. The anti-adhesive effect of the Ag(I)–HA complex underlines the efficacy of Ag(I) complexes as antibacterial treatments.

Furthermore, the silver–hydrogel complex with a smaller amount of metal ion (4.2×10^{-3} mg_{Ag}/mg_{dryHA}) showed good capability with regard to inhibiting bacterial proliferation. The pictures shown in Fig. 12 clearly show an inhibition halo, due to the reduction of *Staphylococcus epidermidis* growth. In bare HA the halo was absent.

***In vivo* tests.** *Angiogenic activity of the Cu(II)–HA complex.* To evaluate, unequivocally, the angiogenic activity of the Cu(II)–HA *in vivo* experiments were executed by implanting the hydrogel in rats and the exudate was analysed to evaluate inflammatory response.

The explanted samples of the Cu(II)–HA gel from the rats appeared greatly hydrated and were tightly adhered to the cage walls. By macroscopic analysis of the tissues surrounding the implant area a remarkable neo-vascularisation was noted in the regions where the Cu(II)–HA samples passed through the steel cage. In contrast, where the complex was absent, no neo-vascularisation was observed. Moreover, the Cu(II)–HA complexes appeared to have plunged in exudates devoid of blood cells (monocytes, macrophages) and fibroblasts, whose presence is an example of inflammatory processes. This feature is very important as it means that neo-angiogenesis is really due to the effect of the Cu(II)–HA hydrogel complex and not to inflammation. In fact, it is well known that inflammatory processes are able to stimulate the generation of new blood vessels.

From the microscopic analysis of the Cu(II)–HA complex inside the cage we noted the presence of a fibrin net surrounding the completely swollen hydrogel (Fig. 13a). Inside the hydrogel a new blood vessel is visible (Fig. 13b)

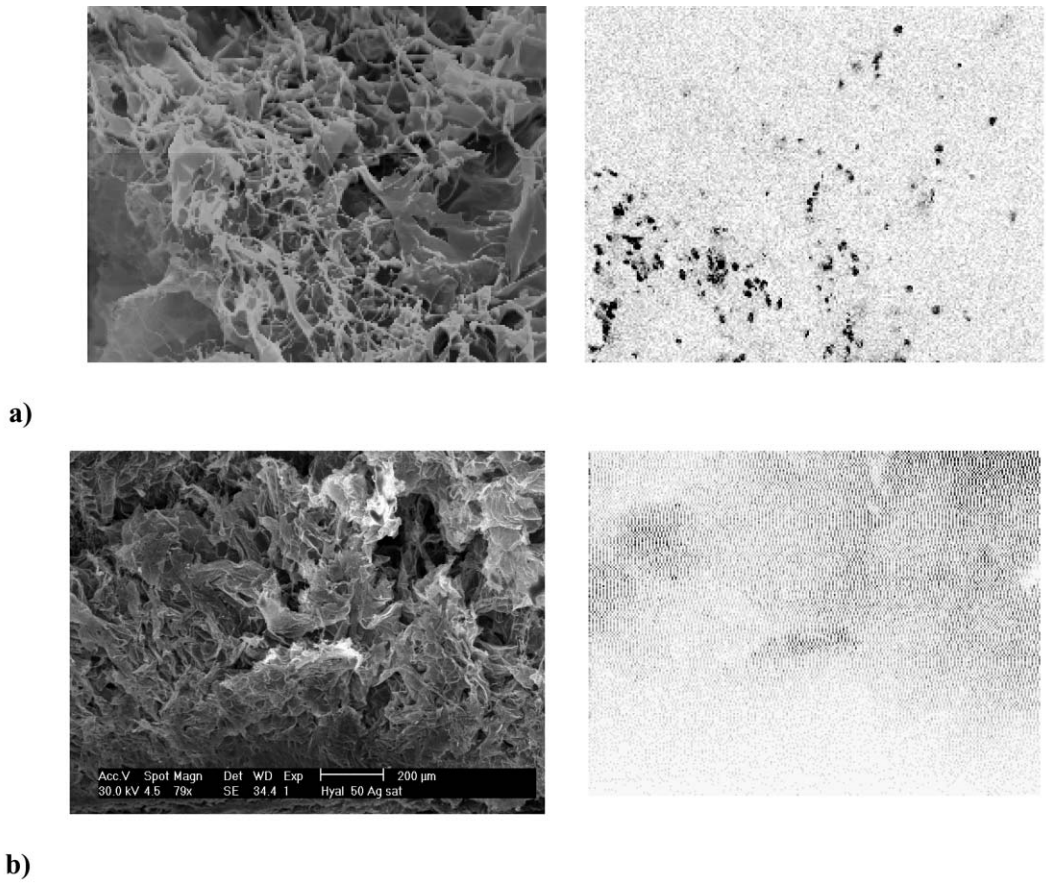


Fig. 10 EDAX analysis: from the dot-map it appears evident that a) fibers and pellets contain an accumulation of copper ions (increase in colour tone); b) a homogeneous distribution of silver ion is present on the hyaluronane–Ag(I) hydrogel.

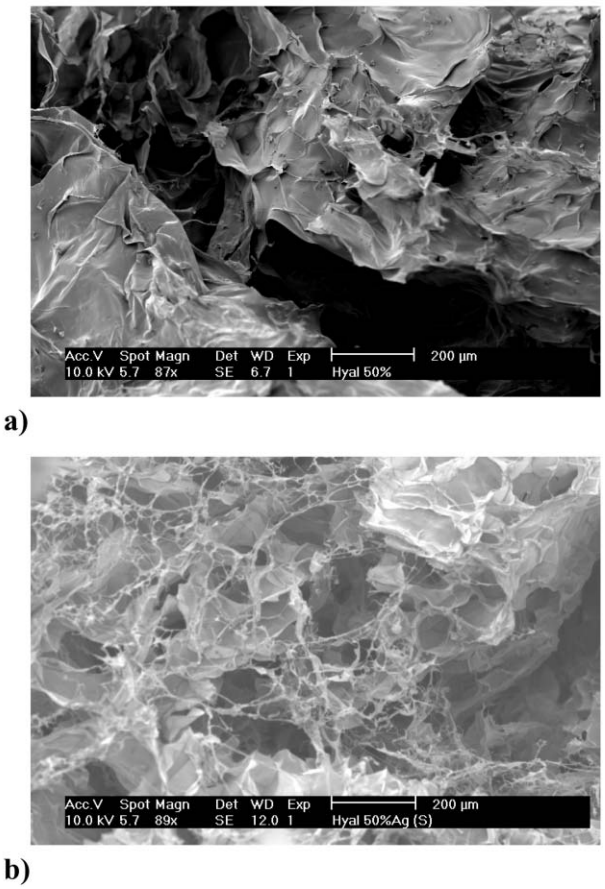


Fig. 11 Electronic micrographs of a) bare HA which show a lamellar morphology; b) the wide and soft structure of Ag(I)–HA.

demonstrating the pro-angiogenic activity of the Cu(II)–HA hydrogel complex.

Conclusions

A hyaluronan-based hydrogel was able to bind to metal ions such as copper(II) and silver(I).

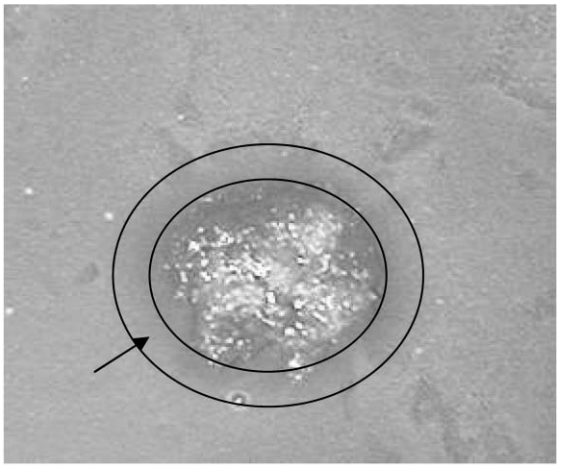
The complexes formed at different pH values, depending on the bound metal ion: the Ag(I)–HA hydrogel complex formed at pH $\geqslant$ 2.0, whereas the Cu(II)- hydrogel complexes started

Table 2 Amount of metal ion uptaken by HA hydrogel obtained starting from different volumes of the metal ion solution (10^{-1} M $CuSO_4$; 10^{-1} M and 10^{-3} M $AgNO_3$)

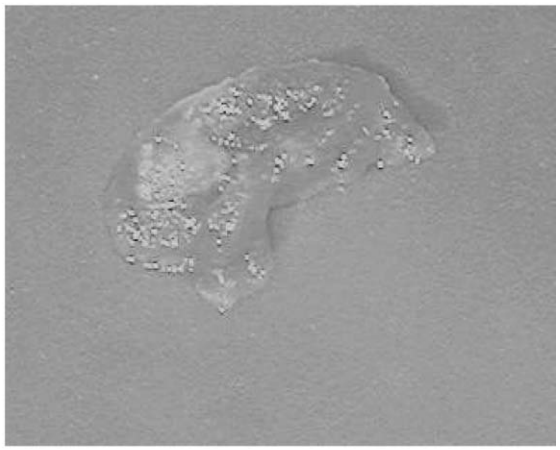
System	M^{n+} solution	Amount of metal ion uptaken by the gel (expressed as mg of metal ion per mg of dry hydrogel)
Cu(II)–HA hydrogel	100 µL of 10^{-1} M $CuSO_4$	0.018 mg_{Cu}/mg_{dryHA}
	5 mL of 10^{-1} M $CuSO_4$	0.024 mg_{Cu}/mg_{dryHA}
Ag(I)–HA hydrogel	5 mL of 10^{-3} M $AgNO_3$	0.0042mg_{Ag}/mg_{dryHA}
	100 µL of 10^{-1} M $AgNO_3$	0.035 mg_{Ag}/mg_{dryHA}
	5 mL of 10^{-1} M $AgNO_3$	0.046 mg_{Ag}/mg_{dryHA}

Table 3 Bacterial adhesion on HA and Ag(I)–HA hydrogels expressed as NTU $\pm$ SD

Samples	Bacterial adhesion (NTU $\pm$ SD)
Polystyrene	10.4 $\pm$ 1.2
HA	25.3 $\pm$ 2.9
Ag(I)–HA	1.3 $\pm$ 0.3



a)



b)

Fig. 12 a) The presence of silver ions inhibits the bacterial proliferation as shown by the inhibition halo alone; b) plate control (HA).

to form only when the pH reaches the value of 5.5.This can be ascribed to the different stability of the two complexes, with Ag(I) more stable than Cu(II).

Different functional moieties of the HA hydrogel were involved in the coordination process depending on the metal ion: Cu(II) was coordinated by the amidic C=O and carboxylate groups, whereas only the carboxylate groups were involved in binding Ag(I). Different local structures were thus created within the coordinated HA network depending on the bound metal ion.

Metal ion coordination affected both the morphology and the water-uptake behaviour of the HA hydrogel: Cu(II) coordination created a more compact structure of the HA network and decreased its water-uptake, whereas Ag(I) binding was reflected in a more open morphology of the HA gel with a consequent increase in its water content.

All these findings were explained in terms of:

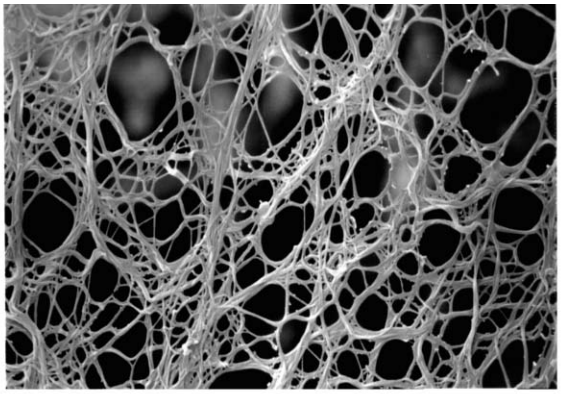
(1) HA charge neutralisation by the coordinated metal ion,

(2) local structural organisation of the HA ligand around the coordinated metal ion,

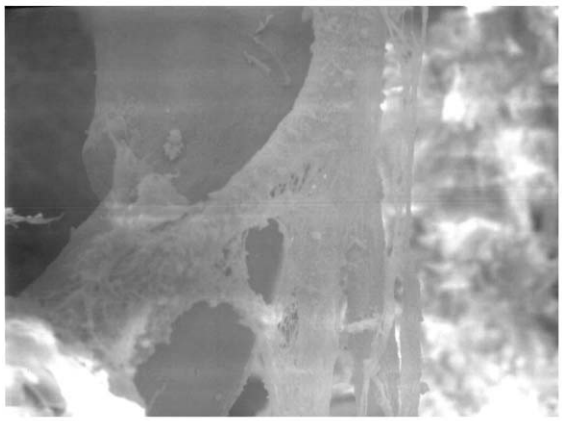
(3) involvement of the hydrophilic amidic moiety in the coordination site.

The formation of new capillary vessels was demonstrated to be activated by the Cu(II)–HA hydrogel complex. This behaviour coincides with the mobilisation of endothelial cells, a phenomenon previously observed for the linear polysaccharide Cu(II)–HA complex. The possibility of utilising this material for this purpose, even as a hydrogel, widens its scope of application.

The stability of the Ag(I)–HA hydrogel complex is much higher than that of the corresponding Cu(II) complex, existing at pH 2.0 as well as at higher pH values. This compound shows antibacterial properties even at concentrations of Ag(I) ions that are low enough to be non-cytotoxic, both in solution and in the hydrogel. This finding, together with the previously observed possibility for the HA to be linked to various material surfaces,[26] allows the utilisation of the Ag(I)–HA hydrogel to coat medical device surfaces for rendering them bacteria resistant.



a)



b)

Fig. 13 a) Fibrin net surrounding the completely swollen hydrogel (white mass); b) a new blood vessel inside hydrogel mass.

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