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Preparation and characterisation of copper(II) hyaluronate

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Abstract

Amorphous copper complexes of the general composition $Cu(C_{14}H_{20}O_{11}N)_2{\cdot}xH_2O$ have been prepared with high- and low-molecular-weight hyaluronic acid (HA). Optimal conditions for preparation are obtained at pH values from 5.0 to 5.5, with a molar ratio of HA versus Cu^{2+} of 1:1, and at a mass concentration of 5 and 10 mg/mL for high- ($M_w = 1.8 \times 10^6$ Da) and low-molecular-weight sodium hyaluronate ($M_w = 2 \times 10^5$ Da), respectively. The coordination polyhedron of the copper ion has been elucidated by EXAFS and XANES spectroscopy. Copper atoms are octahedrally coordinated in both cases with four equatorial Cu–O bond lengths of 1.95 Å, and two axial Cu–O bonds of 2.46 Å. Visible spectra of acidic aqueous solution suggest that substitution of axial oxygens by NH groups occurs at pH 6.5 or higher. If the pH value of the copper(II) hyaluronate solution increases above 6.5, the coordination of copper(II) changes. It is very likely that the N atom coming from the acetamido group enters into the coordination sphere of the copper(II) ion.

Keywords: Copper(II) hyaluronate; Complex; UV–Vis; EXAFS

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1. Introduction

Hyaluronic acid (HA) is a charged macromolecule, known for more than 50 years. It is structurally the simplest and the most frequent among naturally occurring glycosaminoglycans. HA consists of repeating disaccharide units composed of 2-acetamido-2-deoxy-D-glucose and D-glucuronic acid, linked by (1 → 3) and (1 → 4) glycosidic bonds. The molecular weight of HA is between 10^4 and 10^7 Da [1].

HA occurs in the intracellular matrix of most vertebrates, usually as the sodium salt [1–5]. Its roles, for example, in the musculoskeletal system, have been well established [6]. The activity of HA is a function of its rheological properties, which depend on physiological concentration and on HA molecular weight [7]. HA is also used clinically because it is able to modulate a number of cellular functions. The pharmacological effects are ascribed either to direct binding on cellular receptors, or to indirect action, e.g., through binding of inflammatory mediators [1]. The efficiency of both mechanisms depends on the molecular weight of HA.

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Some metal salts of HA are also of medical interest, especially in ophthalmology [8]. Besides the molecular weight, additional factors have to be considered when evaluating the composition and properties of metal hyaluronate complexes. There are many potential binding sites for metal ions, e.g., carboxylate and acetamide groups. Their availability is strongly pH dependent. The composition of coordination compounds also changes with the molar ratio of the metal ion to HA.

The interaction of HA with copper ions is influenced by the oxidation state of the metal. Cu^{+} depolymerises HA in an oxidative–reductive process. Cu^{2+} forms complexes without depolymerisation; gels can be produced [9,10]. Even though the conformation of HA chains depends on pH and ionic strength, the presence of the metal ion has the largest effect [1,5,11–13].

An important point, which is usually neglected when studying these systems, is the purity of the substance. HA often contains considerable amounts of proteins that also bind metal ions and influence the composition of the product [14].

The complex formation between copper ion and HA has been investigated by UV–Vis [3,4,15], ESR and NMR spectrometry [16], circular dichroism [3], potentiometry, and polarography [10]. A species of composition CuL_2, L being a disaccharide unit of HA, with a stability constant $\log K$ of 3.47 has been reported [1]. Polarographic and potentiometric investigations indicate that the first step of the reaction between Cu^{2+} and L involves the formation of a water-soluble complex CuL. Transformation to CuL_2 is a slow process, which possibly involves drastic conformational changes and results in a neutral, water-insoluble substance. This process can be accelerated by shearing or stirring or by precipitation with methanol [10]. Nagy et al. have studied the coordination of copper and zinc bound to HA [17]. They found distorted octahedra and tetrahedra, respectively [17].

Gura et al. [18] have reported that the majority of bivalent cations do not affect the rheological behaviour significantly. This is in direct contradiction with earlier data [10,19], where a significant drop in viscosity with increasing copper(II) concentration was found. This has been ascribed to the formation of CuL and CuL_2 — the stepwise deprotonation of the copper(II) hyaluronate solutions results in gelation of the system due to rearrangement of bonds between copper(II) and the polymeric chain [19].

The present work is based on the fact that the molecular weight of HA and the mode of copper coordination to HA play an important role in defining its properties. The aim is to evaluate experimental conditions for reproducible preparation of well-defined complexes, and to characterise isolated complexes with regard to the molecular weight. The emphasis is on the differences in coordination geometry around metal ions, since these influence the behaviour of the whole macromolecule.

2. Materials and methods

High-molecular-weight sodium hyaluronate (HW NaHA) samples of highest purity (protein content $< 0.1\%$) were obtained from Genzyme (USA), with a molecular weight of approximately 1.8×10^6 Da. Low-molecular-weight sodium hyaluronate (LW NaHA) samples of highest purity were obtained from ARD (France), with a molecular weight less than 2×10^5 Da. Water content was determined by thermogravimetry: 8.9 and 7.5% for HW and LW NaHA, respectively.

Analytical grade $CuCl_2 \cdot 6H_2O$ was used without further purification. A solution of $CuCl_2$ (0.10 M) was prepared and the exact concentration was determined by titration with EDTA. All solutions were prepared with bidistilled water. pH measurements were made using an Iskra MA5740 pH meter. UV–Vis spectra were measured on Perkin–Elmer Lambda 19 UV–Vis spectrometer. A Hellma cuvette with a path length of 10.00 mm was used. IR spectra were measured on a Digilab Division spectrophotometer in KBr. Elemental analyses were performed on a Perkin–Elmer 2400 CHN microanalyser. Atomic absorption spectrometry (AAS) was performed on a Perkin–Elmer 2280 Atomic, using a mixture of acetylene–air, and a wavelength $\lambda = 324.8$ nm. Thermogravimetric analysis (TGA) was carried out on Mettler TA 3000, with a platinum crucible, at a heating rate of 5 K/min over the temperature range 25–600 °C and a dynamic air atmosphere with a flow rate of 50 mL/min.

Copper was titrated with EDTA in the presence of PAN as indicator and glacial acetic acid (pH ~ 3). Magnetic susceptibility was measured on an MSB-Sherwood Scientific Ltd. magnetic balance (Evans method). The balance was calibrated with $HgCo(SCN)_4$ (mass magnetic susceptibility was 16.44×10^{-6} c.g.s. at 20 °C).

X-ray absorption spectra of LW and HW $Cu(HA)_2$ at Cu K edge were measured in transmission mode at the HASYLAB X1.1 beamline of DESY, Hamburg, using synchrotron radiation from the DORIS storage ring running at 4.44 GeV and 80 mA. The beamline was equipped with a Si(311) double crystal monochromator with 1.5 eV resolution at 9 keV. A harmonic-free beam was prepared by a slight detuning of the monochromator crystals, keeping the intensity at 60% of the rocking curve with the beam stabilisation feedback control. The intensity of the incident and the transmitted beams was measured with nitrogen-filled ionisation cells. Near-edge (XANES) and wide-range (EXAFS) spectra were recorded in separate runs, each with a respective reference spectrum without any sample. Precise energy calibration of the spectra was established with a simultaneous absorption measurement on a Cu metal foil. For X-ray absorption measurements, fine powders of LW and HW $Cu(HA)_2$ samples were sprinkled uniformly onto adhesive tape. Several layers were stacked to obtain unit attenuation and improve the homogeneity. The standard stepping progression within the -250 to 1000 eV region of the K edge was adopted for EXAFS spectra with an integration time of 1 s/step.

Preparation of copper high- and low-molecular weight hyaluronate complexes.—HW NaHA (0.25 or LW NaHA (0.50 g)) corresponding to 0.285 mmol was added to a 50 mL volumetric flask, distilled water (30 mL) was added and left to swell for several hours. After addition of a solution of $CuCl_2$ (0.100 M, 5.7 mL), the mixture was left to swell overnight, while reaching a pH of 5.5. Cold, absolute ethanol (150 mL) was added under vigorous stirring. A blue, threaded precipitate appeared, due to the sudden reduction of solubility. The product was filtered using a glass filter, washed twice with ethanol (50 mL) and once with methanol (50 mL), and dried in desiccator under silica gel for 24 h.

The threaded, blue products in the form of a cake were obtained for both HW and LW NaHA. AAS measurements of Na^+ and Cu^{2+} in the two filtrates indicated that after precipitation, exactly half of the amount of copper added and all Na^+ remained in the solution, which confirms the stoichiometry of the reaction as:

$$NaHA + CuCl_2 \\ \rightarrow 0.5\ Cu(HA)_2 + NaCl + 0.5\ CuCl_2$$

The coiled products contain neither chloride nor sodium ions. They are amorphous, soluble in water, insoluble in methanol, ethanol, ether, dimethylsulfoxide, *N*,*N*-dimethylformamide, and acetone.

The C, H, N content was determined by elemental analysis. The copper content was determined by AAS, thermogravimetry and complexometry. The formula of each copper hyaluronate is given in terms of disaccharide units. Anal. Calcd for HW CuHA, $(C_{14}H_{20}O_{11}N)_2Cu \cdot 6H_2O$: C, 36.23; H, 5.65; N, 3.02; Cu, 6.85. Found: C, 36.10; H, 5.61; N, 2.99; Cu, 6.80 (6.71 by TG). Anal. Calcd for LW CuHA, $(C_{14}H_{20}O_{11}N)_2Cu \cdot 5H_2O$: C, 36.95; H, 5.54; N, 3.08; Cu, 6.98. Found: C, 36.74; H, 5.55; N, 3.10; Cu, 7.02 (7.12 by TGA).

When $CuCl_2$, $CuSO_4$ or $Cu(ClO_4)_2$ were used as copper precursors, the pH values of the copper hyaluronate solutions before precipitation were found in the range 5.0–5.5. Increasing the pH of the solution with dilute sodium hydroxide above 6.0 led to the formation of a gel, judging by the increased viscosity, and precipitation was impossible. When cupric acetate was used in the preparation the initial pH value was 6.5, and the solutions started to gel, even without addition of sodium hydroxide.

The best conditions for precipitation were obtained at pH values of the solution of 5.0–

5.5, and a molar ratio between the copper ion and hyaluronate of 1:1, with copper in excess, as mentioned above. The optimal concentrations of the LW and HW NaHA were 10 and 5 mg/mL, respectively. At lower concentrations of hyaluronate, precipitation yields decrease. With hyaluronate in excess, the preparation reproducibility is lost owing to the varying composition of the precipitated product.

It should be noted that the calculated composition of copper hyaluronate is different from the results obtained by Nagy et al. [17]. They reported the following EA results for $CuL_2 \cdot 2H_2O$: C, 37.37; H, 4.94; N, 3.02. The composition of L is not given.

3. Results and discussion

Electronic absorption spectra of LW $Cu(HA)_2$ at different pH values in the range 600–1000 nm are compared in Fig. 1 with that of $Cu(H_2O)_6^{2+}$. The absorption maximum of $Cu(HA)_2$ is 783 nm in solution with a pH value of 5.0, whereas in $Cu(H_2O)_6$ it appears at about 800 nm (Table 1). At that pH, carboxylate groups coordinate to copper ions resulting in a stronger ligand field, which shifts the absorption band to lower wavelengths, and this is in agreement with circular dichroism studies between pH 3 and 6 [2]. Increasing the pH values of the $Cu(HA)_2$ solution results in additional hypsochromic shifts, as other binding sites become available, especially the acetamide group. The coordinating properties of the amide bond were comprehensively and critically summarised by Siegel and Martin [20]. In acidic media the complexation of the amide group occurs at the oxygen atom. In alkaline media, where the amide is anionic, the complexation on nitrogen is favoured. It is also well known that the substitution of oxygen by nitrogen donor atoms moves the absorption maximum to shorter wavelengths due to a stronger ligand field effect [21]. In our case, the effect of increasing pH value could be attributed to participation of the acetamide nitrogen in the coordination sphere of the copper ion. Additionally, a low-intensity absorption band near 260 nm appears at pH 7, which can be attributed to NH–Cu charge transfer [15]. The bonding of the NH group to a copper atom at higher pH values has also been proposed from titration curves [19].

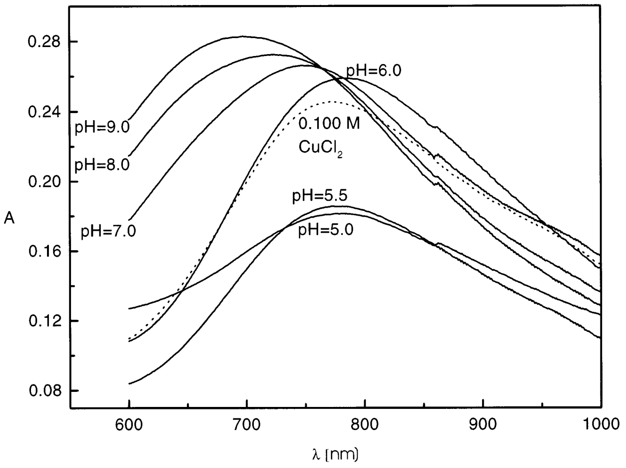

Fig. 1. Visible spectra for solutions of LW Cu–hyaluronate at different pH values.

Table 1
Wavelengths and absorbances of absorption maxima for LW $Cu(HA)_2$ ($\gamma = 4.55$ mg/mL) at different pH values [a]

pH	λ (nm)	A
5.0	783.2	0.1817
5.5	772.8	0.1862
6.0	772.8	0.2458
7.0	746.4	0.2665
8.0	720.0	0.2730
9.0	696.0	0.2830

[a] Absorption maximum of 0.100 M solution of $CuCl_2$ is at 810.4 nm, pH 5.0 (see dotted line in Fig. 1).

FTIR spectra of LW and HW $Cu(HA)_2$ and corresponding starting materials NaHA were measured. The spectra of $Cu(HA)_2$ are comparable (see Table 2). The spectra of NaHA agree well with those obtained by Gilli et al. [22], who made assignments. When replacing Na^+ with Cu^{2+} (Fig. 2), the only significant shifts observed are for the carboxyl bands (1739 and 1419 cm^{-1}), indicating the bonding of copper ions to carboxyl oxygen.

The effective magnetic moment of HW $Cu(HA)_2$ is 2.21 μ_B and that one of LW $Cu(HA)_2$ 2.11 μ_B. Experimental values for octahedral Cu(II) complexes containing a single magnetic centre range from 1.7 to 2.2 μ_B at room temperature [23]. In binuclear Cu(II) compounds the effective magnetic moment is

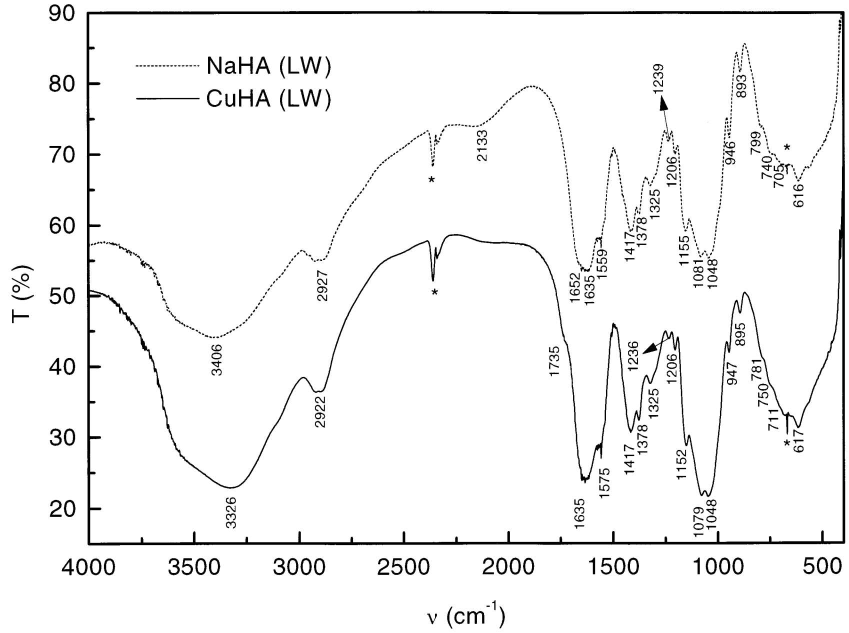

Fig. 2. IR spectra of copper hyaluronate (LW) and sodium hyaluronate (LW).

found at about 1.4 μ_B/Cu atom due to a weak antiferromagnetic coupling of the unpaired electrons [24]. The copper ions in both compounds $Cu(HA)_2$ are therefore sufficiently separated to exhibit no magnetic interaction.

Table 2
Assignments of IR spectra of NaHA, HW $Cu(HA)_2$ and LW $Cu(HA)_2$ after Gilli et al. [22]

NaHA	HW $Cu(HA)_2$	LW $Cu(HA)_2$	Assignation
	1739 (m.sh)	1739 (m, sh)	ν asym. (C–O)
1652 (s.sh)	1653 (s.sh)	1653 (s.sh)	amide I [CO related]
1617 (s)	1619 (s.sh)	1619 (s.sh)	ν_{as} (CO) [COO$^-$]
1560 m)	1560 (s)	1560 (s)	amide II [NH related]
1410 (m)	1419 (s)	1419 (m)	ν_s(CO) [COO$^-$]
1378 (m)	1378 (m)	1378 (m)	δ(O–H) [CH$_2$]. δ(O–H)
1322 (w)	1323 (m)	1325 (w)	amide III
1151 (m)	1153 (s)	1151 (m)	ν_{as}(C–O–C) [O bridge] ν(CO) [COO$^-$]
1079 (s)	1079 (s)	1079 (s)	ν(CO)
1045 (s)	1045 (s)	1044 (s)	ν(C–OH)
946 (w)	948 (w)	947 (w)	ring as.
894 (w)	894 (w)	894 (w)	δ(C1–H)
791 (w)	784 (w)	786 (w)	
613 (m)	611 (m)	614 (m)	ν type [H$_2$O]

In XANES spectra the detailed shape of the absorption edge and the pre-edge region is used as a fingerprint for identification of the local symmetry of the site of the investigated atom. The normalised Cu XANES spectra of the $Cu(HA)_2$ (HW and LW) samples are shown in Fig. 3. By convention, the K-shell

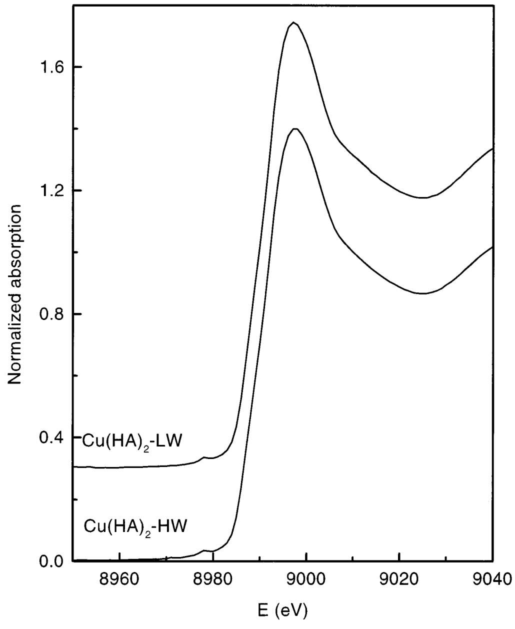

Fig. 3. Cu K-edge XANES spectra of HW and LW $Cu(HA)_2$ samples displaced vertically for clarity.

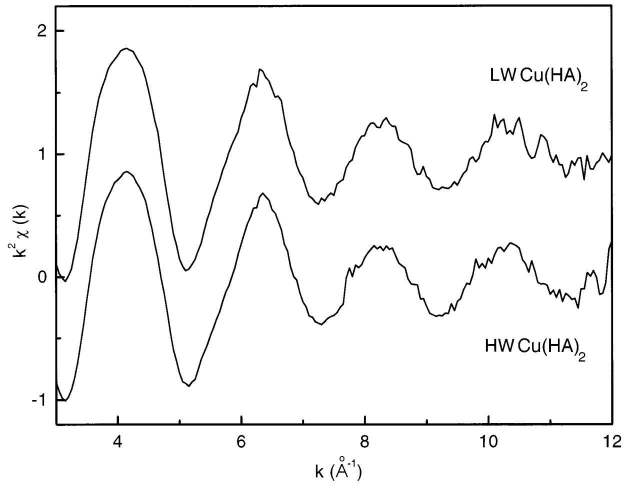

Fig. 4. The k^2-weighted Cu K-edge EXAFS spectra of HW and LW Cu(HA)$_2$ samples. The LW Cu(HA)$_2$ spectrum is shifted vertically for clarity.

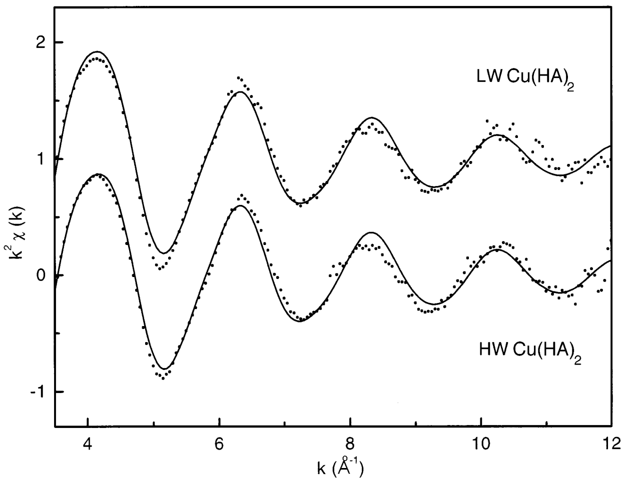

Fig. 6. The distorted octahedron model: the data of Fig. 4 (dots) compared with the FEFF model function (solid line).

contribution is obtained by removing the extrapolated pre-edge (− 250 to − 50 eV) trend [25]. Both spectra show identical Cu K-edge structure, with a weak pre-edge resonance at about 8979 eV, which can be attributed to the 1s → 3d transition [26–28]. The observed edge structure points to a distorted octahedral Cu site. Such sites are characteristic of copper(II) complexes [26,28].

EXAFS spectra are quantitatively analysed for the coordination number, distance and Debye–Waller factors of successive shells of neighbouring atoms. The analysis is routinely performed with the University of Washington's UWXAFS package using FEFF6 code [29,30], in which photoelectron scattering paths are calculated ab initio from a presumed distribution of neighbouring atoms. The simulated interference pattern, based on a self-consistent calculation of phase shifts and scattering factors, is compared with the measured spectrum. The comparison is preferentially made in the Fourier-transform space, where successive shells of neighbouring atoms can be distinguished as successive peaks in the transformed spectra.

The original spectra in the standard k^2 weight (Fig. 4) exhibit no significant difference

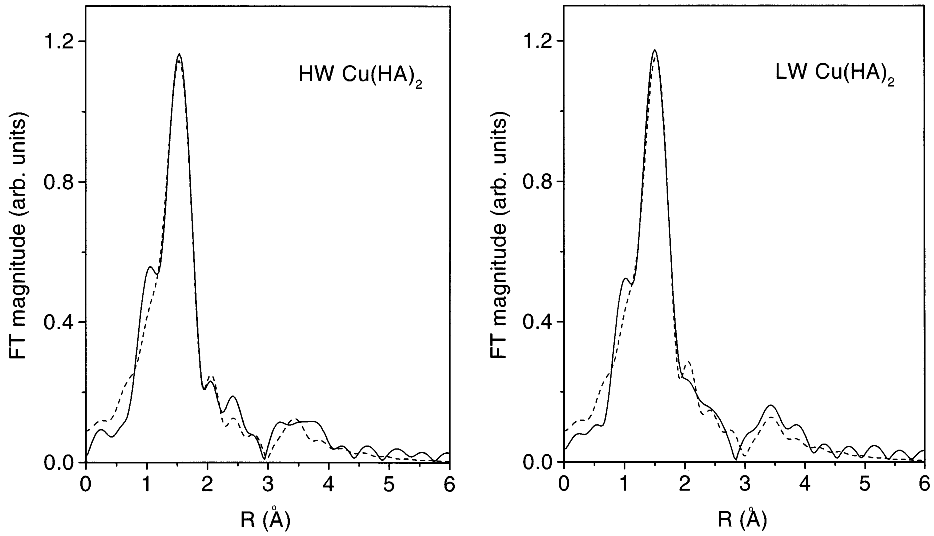

Fig. 5. Fourier transforms of k^2-weighted EXAFS spectra of HW and LW Cu(HA)$_2$ samples. Experiment, solid line; best-fit model function, dashed line.

Table 3
Parameters of the nearest coordination shells around Cu atoms in the $Cu(HA)_2$ samples [a]

Sample	Cu neighbour	N	R (Å)	σ^2 (Å^2)	R factor
HW $Cu(HA)_2$	O_{xy}	4.0(3)	1.952(4)	0.0043(5)	0.0021
	O_z	2.0(5)	2.46(5)	0.027(9)	
LW $Cu(HA)_2$	O_{xy}	4.0(3)	1.952(4)	0.0053(5)	0.0030
	O_z	2.0(5)	2.46(6)	0.030(9)	

[a] N is the number of oxygen atoms in a coordination shell, R is the Cu–O distance and σ^2 is the Debye–Waller factor. The uncertainty of the last digit is given in parentheses. The R factor [29,31] is used as a measure of goodness-of-fit and is given in the last column. The best fit is obtained with the energy shift $\Delta E_0 = -1.8(2)$ eV for both samples. The constant amplitude reduction factor $S_0^2 = 0.70$ [31] is kept fixed during the fit.

between LW and HW samples beyond the noise level, showing that the Cu site is identically organised. In Fig. 5, the Fourier transforms (k range 3.2–12.0 Å^{-1}; k^2 weighted; Hanning window) are compared with the FEFF model, based on the distorted octahedron geometry as suggested by XANES analysis. The single prominent peak in the FT spectra suggests that only the immediate neighbourhood of the Cu atom can be discerned. Consequently, four equivalent oxygen atoms in the xy plane (O_{xy}) and two in the perpendicular direction (O_z) at a different distance are inserted into the model and all single and multiple photoelectron scattering paths up to the total length of 2 × 3.9 Å are used in the simulation of the EXAFS spectra. A very good fit is found for the FT interval of 1.2–2.3 Å (Figs. 5 and 6), with best-fit parameters given in Table 3. The non-structural peak at about 1 Å is a residual of the atomic absorption background, which cannot be completely eliminated using the standard spline ansatz in the background removal procedure [32].

The results of the coordination are definite and strongly confirm the distorted octahedron model. The distances are in good agreement with previously published values for copper hyaluronate complexes [28], where the Cu ion is surrounded by similar ligand groups. The agreement extends to the Debye–Waller factor of the planar bonds. An essential difference, however, is found in the Debye–Waller factor for the O_z atoms. The large value in our samples is an indication of a large spread of the bond distance, caused either by a static disorder or by a stronger effect of the thermal motion due to a weak bond. In any case, the nature of the Cu–O_z bond in $Cu(HA)_2$ (LW, HW) is different from that in copper hyaluronate complex obtained by Nagy et al. [28].

It should be noted that, in view of a limited sensitivity of the EXAFS signal, neighbouring atoms with close atomic numbers cannot always be completely distinguished. In our case, the signal of the axial neighbours is so weak that the exchange of the two oxygens by two nitrogens in the FEFF model results in only a small increase in the R factor (from 0.0021 to 0.0025 for the HW sample, in comparison with $R = 0.0045$ for the model including only the four planar oxygens).

4. Conclusions

The composition of copper hyaluronate is very dependent on preparation conditions. An excess of copper ions is required in order to achieve a constant composition. pH values in the range 5–6 are optimal for the synthesis and give reproducible results. Elemental composition of the product does not depend on molecular weight. Acetate groups are protonated on the HA molecule at lower pH (~ 2) and binding of copper ions is hindered. At higher pH values (between 6 and 7), gels are formed. Cupric chloride is a convenient copper precursor. Cupric acetate is completely unsuitable for the synthesis, as it raises pH and causes gelation.

The coordination polyhedron of the copper ion is a distorted octahedron and it is the same for both HW and LW CuHA. The distortion is ascribed to the Jahn–Teller effect. The four equatorial sites are occupied by oxygen atoms. For the two axial sites at a larger distance, oxygen atoms are preferentially indicated, although nitrogen cannot be excluded. However, visible spectra recorded as a function of pH suggest that the two additional ligand atoms at pH 5 are oxygens.

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