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Cu(II) and Zn(II) complexes with hyaluronic acid and its sulphated derivative
Effect on the motility of vascular endothelial cells

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Abstract

With the aim of improving the compatibility of biomaterials to be used for the construction of cardiovascular prosthesis, we have designed bioactive macromolecules resulting from chemical modifications of hyaluronic acid (Hyal). The stability constants of Cu(II) and Zn(II) complexes with the sulphated derivative of hyaluronic acid ($HyalS_{3.5}$) were evaluated. Two different complexes have been found for each metal ion, CuL, $Cu(OH)_2L$ and ZnL, $Zn(OH)_2L$ (L means the disaccharide unit of the ligands) in aqueous solution at 37°C. The dihydroxo Cu(II) complex was present in high percentage at pH=7.4. On the contrary, the Zn(II) ion was present with a relatively low percentage of both complexes. The ability to stimulate endothelial cell adhesion and migration was evaluated for Hyal, $HyalS_{3.5}$ and their complexes with Cu(II) and Zn(II) ions. The results revealed that Hyal and $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ induced cell adhesion, while $[ZnHyalS_{3.5}]^{(2.5)-}$ and $[Zn(OH)_2HyalS_{3.5}]^{(4.5)-}$ inhibited the process. The chemotactic activity of increasing concentrations of the above complexes was also evaluated, demonstrating that $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ complex at 1 μM concentration was the most active in inducing cell migration. These results have been also strengthened by analysing adherent cell migration in agarose. In conclusion, sulphated hyaluronic acid coordinated to Cu(II) seems to be a promising matrix molecule for the construction of cardiovascular prosthesis.

Keywords: Metal ion complexes; Sulphated hyaluronic acid; Cu(II) and Zn(II) complexes; Endothelial cells; Adhesion; Migration; Chemotaxis

1. Introduction

In recent years a big effort has been made to improve the cellular compatibility of biomaterials to be used for the construction of cardiovascular, orthopedic, plastic and reconstructive surgery prosthesis. Recently our group has actively been involved in the demonstration that some chemical modifications of polysaccharides are able to change their biological activity. Sulphation of hyaluronic acid (Hyal) [1] allows for the acquisition of a biological activity (inhibition of platelet aggregation, anticoagulant activity and increased endothelial cell proliferation rate) in comparison with the native molecule, leading to a potential endothelium-dependent action [2–4]. Moreover sulphation confers a major stability to hyaluronic acid, leading to an increased half-life [3]. The design of new anticoagulant molecules which can replace heparin is of extreme importance to avoid the complications due to continuous adjuvant therapy and to produce novel blood compatible materials to be linked to the surface of medical devices.

One of the events relevant for the reduction of xenograft rejection in humans is the cellular biocompatibility of the matrix. Specifically, in the case of cardiovascular prosthesis the adhesion, attachment and spreading of vascular endothelial cells on the biomaterial are necessary, along with the absence of procoagulant activity. Many are the growth and adhesion factors able to stimulate endothelial cell adhesion, movement and proliferation. A great number of vascular endothelial growth factors are polypeptides (i.e. vascular endothelial growth factors, fibroblast growth factors, angiopoietins, interleukin-8, transforming growth

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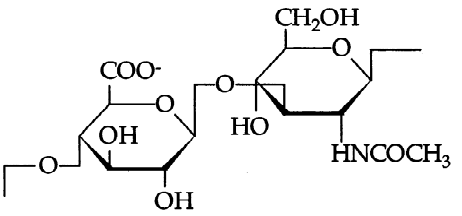
factor, tumor necrosis factor), or to a less extent lipid molecules (prostaglandin E1, platelet-activating factor) [5–7]. A pivotal role is played by extracellular matrix components such as collagen, fibronectin and heparan sulphates. Heparin, and generally proteoglycans, play an important role in angiogenesis and vascularization, being low affinity receptors for angiogenic inducers such as basic fibroblast growth factor, vascular endothelial growth factor–A and transforming growth factor-beta [8–11] and modulating endothelial cell adhesion and migration [12,13].

Other components important for endothelial cell functions are oligoelements and metals such as copper and zinc. They are essential constituents of macromolecules as enzymes, soluble proteins and matrix components. They play an important role both for the conformation and the catalytic activity of the macromolecules. Micromolar concentrations of copper induce endothelial cell migration [14] and proliferation [15] by unknown mechanisms that could require the ion binding to fibroblast growth factor receptors [16] or to angiogenin [17]. It has been also observed that the accumulation of copper preceded the rabbit cornea vascularization induced by implanted tumor cells [18] and that chelation of copper ions inhibits the angiogenesis associated with the growth of a experimental gliosarcoma in rats [19]. Furthermore, copper as salt [20] or coordinated with some biological molecules, such as ceruloplasmin, is an angiogenic inducer [14,21,22]. Actually, heparin-copper (II) complex is an efficient angiogenic factor in rabbit cornea and induces the in vitro migration of capillary endothelial cells [23,14,18]. Zinc is another element which modulates some endothelial cell functions related to angiogenesis. It is an essential component of metalloproteinases, key enzymes involved in endothelial cell invasiveness during the early stages of angiogenesis. When used as soluble form, zinc stimulates the proliferation of endothelial cells [24] and promotes the repair of wounded monolayers of this cell type [25].

On this basis, the development of macromolecules coordinated to metal ions seems promising for the development of matrix for improving endothelial cell biocompatibility and reducing blood clotting in vivo. However, heparin is not entirely understood from the chemical point of view, and due to its variety, it is still difficult to define the stoichiometry of the metal ion complex. Some authors have found the *N*-sulphate, *O*-sulphate and the carboxyl groups are involved in the Cu(II) binding process [26,27], whereas our group ascertained the presence of only carboxyl and acetyl groups in Cu(II) binding [1]. By using low molecular weight saccharide molecules, it has been reported that the heparin cell-modulating activities depend primarily on a minimum intramolecular density of neighbouring anionic groups (sulphate) [28]. All these activities are associated with the multi-ionic complex formation between the clusters of anionic and cationic sites on the complexing molecules.



Scheme 1. Disaccharide unit of hyaluronic acid.

We have recently shown that hyaluronic acid can easily be sulphated and molecules with a certain number of sulphates per repeating unit (disaccharide unit) can be obtained ranging from 1 to 4 OSO_3^- groups. The most sulphated terms of the series, i.e. $HyalS_{3.5}$ and $HyalS_{4.0}$, show quite good anticoagulant activities [2]. The precise structure of hyaluronic acid allowed us to study the protonation behaviour in aqueous solution by a thermodynamic point of view, as well as the ability to form complexes with Cu(II) and Zn(II) ions (Scheme 1).

The aim of the present study was to evaluate the stability and the biological activity of hyaluronic acid complexes with Cu(II) and Zn(II) ions and its sulphated derivative and to assess the effect of Cu(II)-$HyalS_{3.5}$ and Zn(II)-$HyalS_{3.5}$ complexes present at physiological pH on cultured endothelial cells. The studies were integrated with analogous tests performed on Cu(II), Zn(II) complexes with non sulphated Hyal. The protonation and Cu(II), Zn(II) stability constants were determined at 37°C to identify the stoichiometry of the complexes and their percentage at body temperature. Endothelial cell fuctions relevant for the biocompatibility of the materials under study (i.e. adhesion and migration) were studied.

2. Materials and methods

2.1. Reagents

Sulphated hyaluronic acid samples, with 3.5 sulphate groups per disaccharide unit ($HyalS_{3.5}$), were obtained as previously reported [1,29] and determined by C, H, N, S, elemental analysis. Hyaluronic acid (M.W.=180 000) was kindly provided by FAB (Fidia Advanced Biopolymers-Padova, Italy).

2.2. Potentiometric measurements

2.2.1. Protonation studies

Potentiometric titrations were performed according to a previously described procedure [30]. A Crison MicropH-2002 potentiometer, equipped with a combined electrode (Crison mod. 6.0204.000) was used together with an automatic Crison microburette (mod. 2031) connected to a PC 386 DX 40 MHz. For each titration experiment, the cell was filled with ca. 20–25 ml of 0.1 M NaCl solution

in which a known amount of solid polymer was dissolved by magnetic stirring under nitrogen stream. The titration data were automatically stored on a floppy disk for further processing. The basicity constants were computed by the HYPERQUAD package program [31] on the 386 DX 40 MHZ computer, once it was ascertained that the basicity constant of the COO^- of the repeating unit does not depend on the protonation degree of the whole macromolecules [32].

2.2.2. Zn(II) and Cu(II) complex-formation

The potentiometric titrations were carried out at 37°C by the same potentiometric apparatus described above. The stability constants of the Cu(II)-polymer and Zn(II)-polymer complexes were determined by adding standard sodium hydroxide solution to a solution containing the polysaccharide and the metal ion. Computation of the stability constants was performed by the HYPERQUAD program [31].

The experimental details for the potentiometric titrations are summarised in Table 1.

2.3. Cell culture

H.end.FB cell line was obtained from heart cells taken from a mouse fetus DBA/2Xc57b1/6 at the 13th day of gestation and immortalised by infection with N-TKmT retroviral vector containing mT antigen of polyoma virus [33–35]. This cell line was characterised as endothelial on the basis of CD31, VE-cadherin and factor VIII expression, uptake of low density acetylated lipoproteins, synthesis of platelet activating factor and monocyte chemotactic peptide-1 after stimulation with interleukin-1 and tumor necrosis factor [36,37]. H.end.FB cells were kept in DMEM (Dulbecco's Modified Eagle Medium, Gibco-Europe Paisley, Scotland) supplemented wih 10% fetal bovine serum (FCS) (Sigma Chemical Co., St. Louis, MO).

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins by collagenase digestion following a standard protocol [33]. Cells were maintained in a 5% CO_2 atmosphere at 37°C in medium 199 (Sigma, Italy) with 20% FCS, L-glutamine and gentamicin. Cells were identified as endothelial by their polygonal morphology. For migration experiments cells were used when cultures had reached confluence.

2.4. Adhesion assay

To assess the ability of test substances to interfere with cell adhesion, endothelial cells were let to adhere to polystyrene plastic wells in the presence of test substances. Elisa 96-multiwell plates were coated with 10 μg/ml fibronectin. Cells were detached from confluent cultures and suspended at the density of 5×10^4/ml. 100 μl of cell suspension in the presence of test substances were put in each well and incubated at 37°C for 30 min^{-1} h. Cells were washed with PBS and then fixed in methanol and stained in Diff-Quick (Harleco, Gibbstown, NJ, USA). Adherent cells were counted at 100× magnification with the aid of an ocular grid (21 mm^2) [38]. Data are expressed as total cell number counted/well.

2.5. Motility assays

2.5.1. Chemotaxis assay

Chemotaxis experiments were performed with the Boyden chamber technique (48-well microchemotaxis chamber) using polycarbonate filters (5 μm pore size, polyvinylpyrrolidone-free, Nucleopore Costar Italia, Milano,Italy) [16–18]. Three different concentrations of Hyal, Hyal$S_{3.5}$, Cu(II)-Hyal, Zn(II)-Hyal, Cu(II)-Hyal$S_{3.5}$, Zn(II)-Hyal$S_{3.5}$, $CuCl_2$ and $ZnCl_2$ (0.1, 1 and 10 μM) in 2% FCS-DMEM were placed in the lower compartment of the chamber, and 1×10^5 cells suspended in DMEM containing 2% FCS were put in the upper compartments. After 6 h of incubation at 37°C, the upper surface of the filter was scratched in order to remove non-migrated cells.

Table 1
Experimental details for the protonation and complex-formation potentiometric measurements at 37°C in NaCl 0.1 M

Compound	System	T_L, mmol[a]	T_M, mmol[a]	added HCl mmol	C_T, mM[a]	Range of pH
Hyal$S_{3.5}^{(4.5)-}$	H^++L	0.03552	–	0.09110	0.0993	2.5–10.8
		0.03547	–	0.08733	0.0993	2.5–10.5
	Cu^{2+}+L	0.03134	0.02846	0.09534	0.1000	2.5–10.8
		0.03066	0.00605	0.08364	0.1000	2.7–10.9
		0.03152	0.00608	0.08419	0.1000	2.6–11.3
		0.03115	0.00616	0.08096	0.1000	2.6–11.3
	Zn^{2+}+$L^{4.5-}$	0.02660	0.01330	0.12769	0.0998	2.4−10.6
	Zn^{2+}+L	0.02491	0.01245	0.11306	0.0998	2.4−10.8
		0.02660	0.00532	0.13103	0.0998	2.4−11.0
		0.02696	0.00539	0.13089	0.0998	2.4−10.9
		0.02600	0.02340	0.12388	0.0998	2.4−10.8
		0.02708	0.02708	0.12273	0.0998	2.4−10.8

[a] T_L: initial amount of the ligand; T_M: initial amount of metal ion; C_T: concentration of titrating solution (NaOH).

Filters were fixed and stained with Diff-Quick. The number of cells present in five oil immersion fields/well was counted at 100× magnification. DMEM containing 2% FCS was used as negative control and complete M199 containing 10% FCS as positive control.

2.5.2. Migration assay

The migration tests were performed on agarose gels as previously described [23]. Briefly, tissue culture dishes were coated with gelatin and agarose solution was poured on the gelatin and allowed to harden at room temperature. Then, three wells were cut with a suitable template. Endothelial cells were seeded into the central well and dishes were incubated for 5 h (37°C, 5% CO_2) to allow attachment. The sample test solution was put into one well while the sample control solution in the controlateral well. The samples containing Hyal/Cu(II) and Cu(II)-Hyal$S_{3.5}$ were dissolved in phosphate buffered saline (PBS) solution. The sample containing $CuCl_2 2H_2O$ salt was dissolved in Tris solution. The molar ratio of Cu(II)-Hyal and Hyal$S_{3.5}$/Cu(II) was nearly 1. All the solutions were sterilised by filtering with 0.2 μm filters. Incubation (37°C, 5% CO_2) followed for 12 h. Cells were then fixed and stained with Diff-Quick. Cell migration toward the stimuli was quantified microscopically at a magnification of 40× in a blind manner by two independent operators. Cell migration was quantified by using the following score: 0, no directional movement; 1, at least 10% of the cells directed toward one side; 2, at least 50% of the cells directed toward one side; 3, 95–100% of the cells directed toward one side.

2.6. Statistical analysis

Analysis of variance (ANOVA) was applied to the data for cell adhesion and motility; P values <0.05 were considered statistically different. Post hoc comparisons between groups (95% confidence for mean) were carried out by a least squares difference test (LSD test) when appropriate.

3. Results

3.1. Protonation constant and Cu(II) and Zn(II) stability constants

The protonation constant relative to the COO^- group of the disaccharide unit of hyaluronic acid derivative Hyal$S_{3.5}$ at 37°C (log K 3.99) was very close to the analogous constant determined at 25°C (log R=3.94) [39]. Thus, the protonation reaction of the COO^- group in this molecule was almost athermic, not being influenced by the temperature. The Cu(II) complex species found at 37°C were the same ones previously seen at 25°C for HyalS_4^{5-} [27], i.e. the simple $[CuHyalS_{3.5}]^{(2.5)-}$ complex and the dihydroxo $[Cu(OH)_2 HyalS_{3.5}]^{(4.5)-}$ complex (Fig. 1). Zn(II) ion with

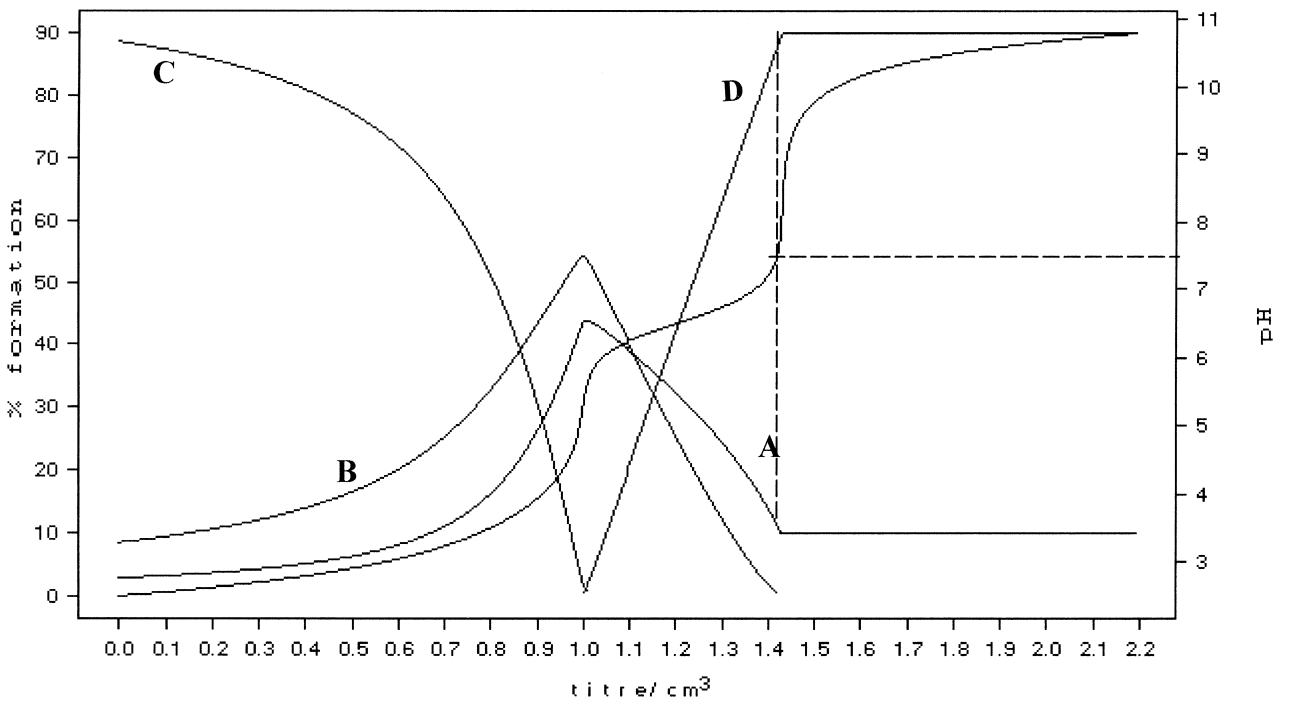


Fig. 1. Cu(II)-Hyal$S_{3.5}$ complex: Potentiometric titration curve and distribution curves of the species in solution at different pH's at 37°C in 0.1 M NaCl; A=$L^{4.5-}$; B=$CuL^{2.5-}$; C=$LH^{3.5-}$; D=$Cu(OH)_2 L^{4.5-}$; [L=Hyal$S_{3.5}^{4.5-}$].

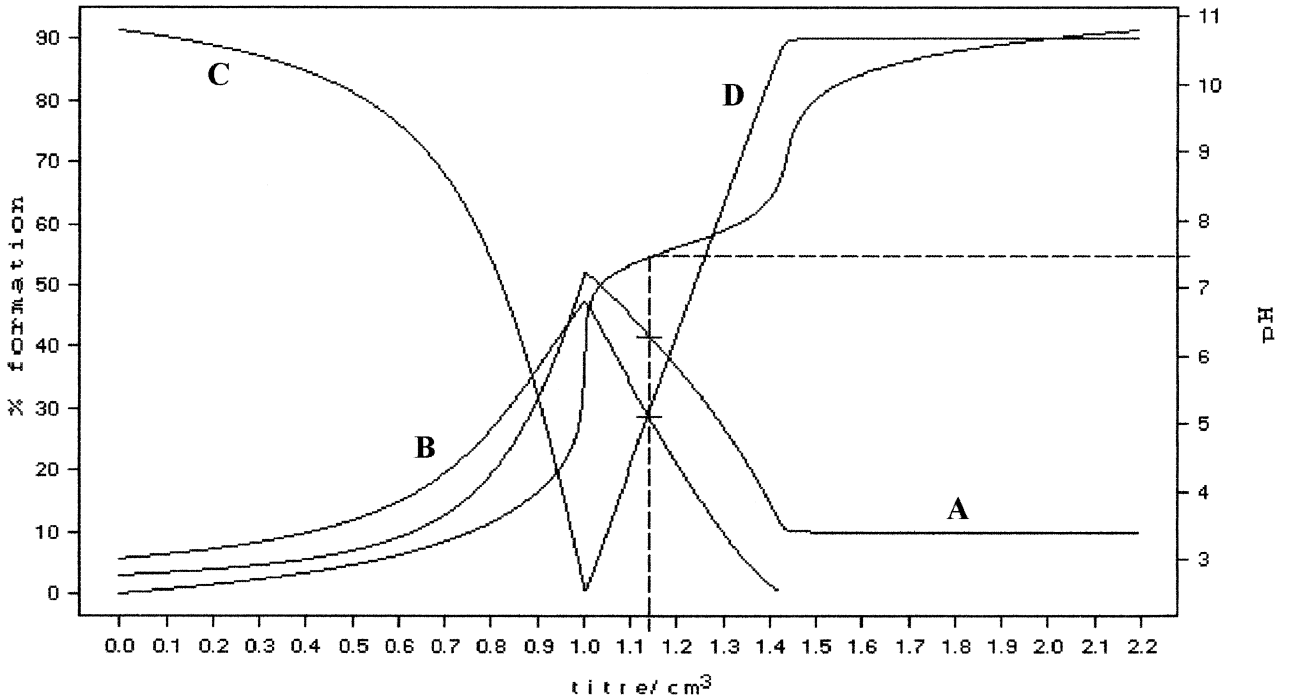


Fig. 2. Zn(II)-Hyal$S_{3.5}$ complex: Potentiometric titration curve and distribution curves of the species in solution at different pH's at 37°C in 0.1 M NaCl; A=$L^{4.5-}$; B=$ZnL^{2.5-}$; C=$LH^{3.5-}$; D=$Zn(OH)_2L^{4.5-}$; [L=Hyal$S_{3.5}^{4.5-}$].

Hyal$S_{3.5}$ formed two complexes [ZnHyal$S_{3.5}$]$^{(2.5)-}$ and [$Zn(OH)_2$Hyal$S_{3.5}$]$^{(4.5)-}$ at 37°C (Fig. 2), the same species found with Cu(II), but not exactly the same found with Zn(II) ion and HyalS_4^{5-} at 25°C, where only the dihydroxo complex was present [39].

Knowing the stability constants at 37°C, the percentage of the complex species at physiological pH (7.4) could be calculated (Table 2). The dihydroxo Cu(II) complex was present in a very high percentage (90%); thus this compound could be considered responsible for any of the biological effects. On the contrary, Zn(II) ion at pH=7.4 was present with a relatively low percentage of both complexes, 33% [ZnHyal$S_{3.5}$]$^{(2.5)-}$ and 26% as [$Zn(OH)_2$Hyal$S_{3.5}$]$^{(4.5)-}$.

3.2. Adhesion of endothelial cells in the presence of Hyal and Hyal$S_{3.5}$, and their complexes with Cu(II) and Zn(II) ions

Cellular adhesion is one of the early events during the attachment of endothelial cells to the extracellular matrix. The number of cells adhered to polystyrene plates coated with fibronectin was evaluated after 30 min of stimulation of endothelial cells in suspension with the different compounds at 1 μM concentration. Two-way ANOVA showed significant differences among the basal and Hyal, Cu(II)-Hyal$S_{3.5}$ and Zn(II)-Hyal$S_{3.5}$ (Fig. 3). Specifically, the compounds most effective in inducing endothelial cell adhesion were Hyal ($P<0.05$) and

Table 2
Stability constants of the sulphated hyaluronic acid (Hyal$S_{3.5}^{(4.5)-}$) with Cu^{2+} and Zn^{2+} at 37°C in 0.1 M NaCl[a]

Reaction	Log β*	% species at 37°C and pH=7.4[b]		
		$ML^{(2.5)-}$	$M(OH)_2L^{(4.5)-}$	$L^{(4.5)-}$
$Cu^{2+}+Hyal S_{3.5}^{(4-5)-} \rightleftharpoons [Cu\ Hyal S_{3.5}]^{(2.5)-}$	3.58(1)	0	90	10
$Cu^{2+}+Hyal S_{3.5}^{(4-5)-}+2OH^- \rightleftharpoons [Cu(OH)_2 Hyal S_{3.5}]^{(4.5)-}$	17.52(2)			
$Zn^{2+}+Hyal S_{3.5}^{(4-5)-} \rightleftharpoons [Zn\ Hyal S_{3.5}]^{(2.5)-}$	3.37(2)	31	26	41
$Zn^{2+}+Hyal S_{3.5}^{(4-5)-}+2OH^- \rightleftharpoons [Zn(OH)_2\ Hyal S_{3.5}]^{(4.5)-}$	15.17(2)			

[a] The potentiometric titration were carried out at 37°C. The stability constants of the Cu^{2+} polymer and Zn^{2+}polymer complexes were determined by adding standard sodium hydroxide solution to solution containing the polysaccharide and the metal ion at pH=7.4.The values of the stability constants are expressed as mean+S.D. *Values in parentheses are standard deviation.

[b] L=Hyal$S_{3.5}^{(4.5)-}$; M=Cu^{2+} or Zn^{2+}; Log K (Hyal$S_{3.5}^{(4.5)-}+H^+$ Hyal$S_{3.5}H^{(3.5)-}$)=3.99(1).

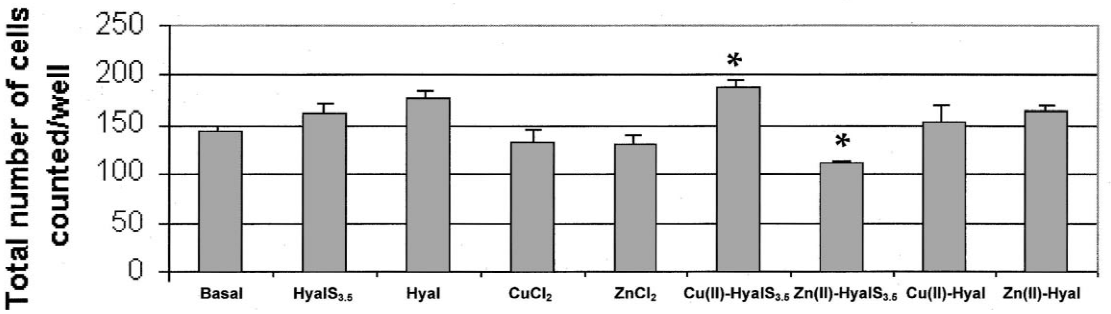


Fig. 3. Adhesion of endothelial cells in the presence of 1 μM $CuCl_2$, $ZnCl_2$, Hyal, $HyalS_{3.5}$, Cu(II)-Hyal, Zn(II)-Hyal, Cu(II)-$HyalS_{3.5}$ and Zn(II)-$HyalS_{3.5}$. H.end.FB cells were let to adhere to polystyrene plates coated with 10 μg/ml fibronectin in the presence of test substances for 30 min. Data are expressed as adherent cells counted in six random fields/well. Numbers represent mean±S.E. (n=3); *P<0.05 vs. basal condition.

$[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ (P<0.05). On the contrary, the Zn(II)-$HyalS_{3.5}$ complexes were the most effective in inhibiting cell adhesion (P<0.05). All the other compounds stimulated cell adhesion without any significant differences when compared to basal condition.

Furthermore, after 1 h a complete adhesion of endothelial cells was observed in all the wells with no difference among the tested compounds (data not shown).

3.3. Chemotactic activity by Hyal and $HyalS_{3.5}$ complexes with Cu(II) and Zn(II) ions

Cell migration is another early function of endothelial cells during vascularization. Chemotaxis of endothelial cells in suspension toward a gradient of stimuli can be studied in vitro by the Boyden Chamber procedure. Increasing concentrations of test substances (0.1, 1 and 10 μM) were assayed. In Fig. 4a endothelial cell migration in response to Hyal and $HyalS_{3.5}$ is reported. While Hyal did not affect endothelial cell migration at any tested concentration, $HyalS_{3.5}$ significantly (P<0.05) increased cell chemotaxis in comparison to Hyal both at 1 and 10 μM concentration. The effect was concentration-dependent with maximal activity at 1 μM. A plateau phase was observed with higher concentration (10 μM).

Endothelial cell migration was then evaluated in response to different concentrations of Cu(II)-$HyalS_{3.5}$ and $CuCl_2$. As evident from Fig. 4b, $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ complex, even at the lowest tested concentration (0.1 μM) (P<0.05), strongly induced cell migration. This effect was stronger with a higher concentration (up to 1 μM, P< 0.05), which represented the maximal effective concentration. Also at 10 μM cell migration induced by $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ complex was significantly different with respect to control (P<0.05).

We then assessed the chemotactic activity of increasing concentrations of Zn(II)-$HyalS_{3.5}$ and $ZnCl_2$ in comparison with $HyalS_{3.5}$. While $ZnCl_2$ at all the three tested concentrations was devoid of any chemotactic effect, Zn(II)-$HyalS_{3.5}$ significantly induced cell migration (P<

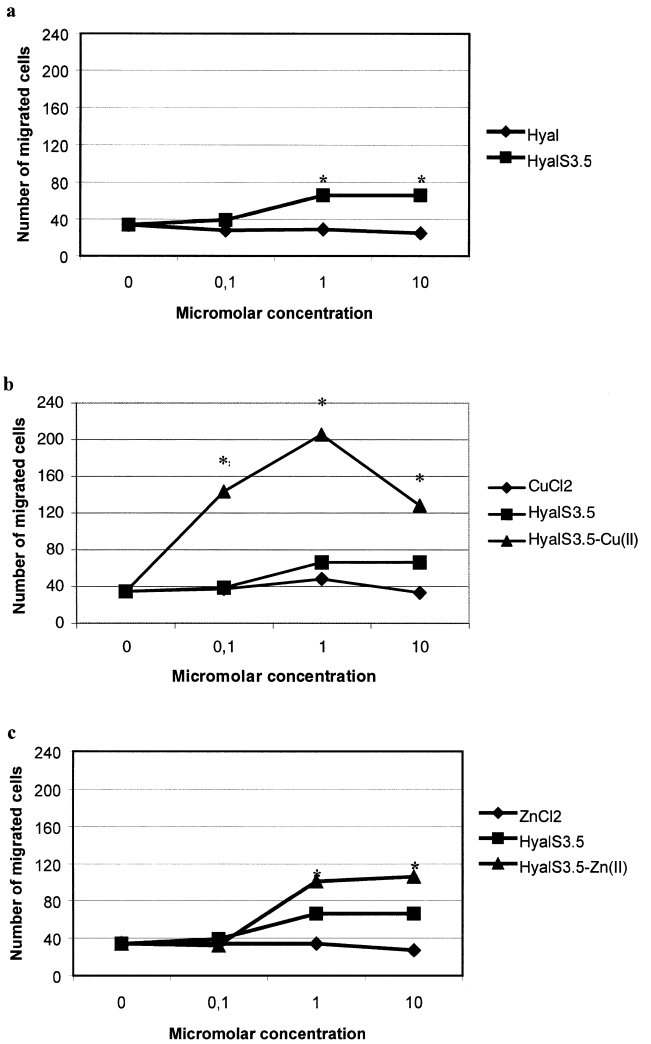


Fig. 4. Endothelial cell migration in the presence of (a) Hyal and $HyalS_{3.5}$, (b) $HyalS_{3.5}$,Cu(II)-$HyalS_{3.5}$ and $CuCl_2$ and (c) $HyalS_{3.5}$,Zn(II)-$HyalS_{3.5}$ and $ZnCl_2$. Test substances were assayed at increasing concentrations (0.1, 1 and 10 μM). Migration of H.end.FB cells was evaluated by the modified Boyden chamber technique after 6 h of incubation. The number of migrated cells is expressed as mean±S.E. (n=5); *P<0.05 vs. the control.

0.05). Maximal effect was observed at 1 and 10 μM concentration ($P<0.05$). As shown in the distribution curves at 37°C, two complexes were contemporaneously present in solution at pH=7.4 (Table 2), thus we were unable to determine which species induced cell migration. Besides, as shown in Fig. 4c, the extent of cell migration in the presence of Zn(II)-Hyal$S_{3.5}$ might be considered approximately the sum of that induced by the Hyal$S_{3.5}$ and by Zn(II) ion (Fig. 4c).

Data related to the chemotactic ability of Cu(II)-Hyal and Zn(II)-Hyal demonstrated that neither of the two species seemed to be able to stimulate cell migration at the concentrations tested (Table 3).

All these data taken together demonstrate the marked chemotactic activity of $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ complex which proves to be the most effective in inducing endothelial cell chemotaxis. This effect was specific for the complex present in solution, since $CuCl_2$ was not able to induce cell migration.

3.4. Migration of endothelial cells toward Hyal and Hyal$S_{3.5}$ complexes with Cu(II) and Zn(II) metal ions

Cell migration and spreading on extracellular matrix can be studied on endothelial cells by using the agarose assay. This test differs from the chemotaxis assay for three reasons: (i) adherent cells are used; (ii) the effect of two different compounds is compared in the same plate; (iii) the result of the test is the general tendency of cells to migrate towards a gradient of the compound which diffuses through the gel. Based on chemotaxis results, in this assay the motogenic effect of $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ was compared to the activity elicited by Hyal$S_{3.5}$ and Cu(II)-Hyal complex. The results obtained showed that $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ was more powerful than Hyal$S_{3.5}$ and Cu(II)-Hyal complex in inducing cell migration, whereas Cu(II) was devoid of any migratory activity (Table 4). These observations confirm the results obtained in the chemotaxis assay, underlining the high ability of $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ in stimulating cell migration.

Table 3
Endothelial cell migration in the presence of Zn(II)-Hyal and Cu(II)-Hyal[a]

Samples	Number of migrated cells
Control	30.3±3.4
Zn(II)-Hyal 0.1 μM	31.0±2.8
Zn(II)-Hyal 1 μM	33.7±3.1
Zn(II)-Hyal 10 μM	31.7±4.2
Cu(II)-Hyal 0.1 μM	29.7±1.9
Cu(II)-Hyal 1 μM	32.7±3.3
Cu(II)-Hyal 10 μM	28.3±2.1

[a] Test substances were assayed at increasing concentrations (0.1, 1 and 10 μM). Migration of H.end.FB cells was evaluated by the modified Boyden chamber technique after 6 h of incubation. The number of migrated cells is expressed as mean±S.E. ($n=5$).

Table 4
Endothelial cell migration in agarose gel[a]

System	Score	System
PBS	←0→	Cu(II)
Cu(II)-Hyal$S_{3.5}$	←0.5	Cu(II)-Hyal
Cu(II)-Hyal$S_{3.5}$	←2.5	Hyal$S_{3.5}$

[a] HUVEC were seeded in the central well while test substances (at 1 μM concentration) were seeded in the lateral wells. Following 12 h of incubation, cells were fixed and stained. Cell migration was quantified as reported in the Material and method section. The arrow indicates the direction of the cells toward the indicated stimulus. Data are representative of three plates with similar results.

4. Discussion

In this study we demonstrate, for the first time, that sulphated hyaluronic acid derivatives coordinated with Cu(II), beside their anticoagulant and antithrombotic activity, facilitate endothelial cell functions relevant for vascularization as adhesion, migration and spreading. These characteristics are fundamental for the potential use of these compounds in the construction of medical devices to be used in cardiovascular surgery. Until now the major problem of cardiovascular prosthesis, once implanted, has been blood clotting also due to the absence of a whole and functioning endothelial cell monolayer in the inner surface of the artificial graft. The criteria for the design of an artificial basement membrane for vascular graft must include: (1) Structural matrix, (2) enhanced adhesion and growth of endothelial cells and (3) ensured antithrombogenicity. Our results indicate that these criteria have all been satisfied by the Cu(II)-coordinated to suphated hyaluronic acid.

The use of hyaluronic acid in reconstructive surgery has already been reported. Non-modified hyaluronic acid has been used for the development of artificial skin material and has been demonstrated to accelerate granulation tissue ingrowth and to increase the number of capillaries present in the matrix [40].

Copper and zinc are two metal ions which activate in vitro a pro-angiogenic program in vascular endothelial cells (i.e. migration and proliferation) [7–15,24,25]. Copper as salt or coordinated to proteins or proteoglycans is also angiogenic in vivo [7,20–22]. The results presented here identify another class of compounds, i.e. the sulphated hyalurane derivatives which, once coordinated to Cu(II) ion, are able to induce cell migration. The first chemically defined complex species between Hyal$S_{3.5}$ and Cu(II), i.e. $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$, is able to activate adhesion of endothelial cells to fibronectin as well as their migration. Furthermore we have found evidence that the system Zn(II) and Hyal$S_{3.5}$ slacken the adhesion and do not promote the migration of endothelial cells. In solution at pH=7.4 Zn(II) is present with two complexes: $[ZnHyalS_{3.5}]^{(2.5)-}$ and $[Zn(OH)_2HyalS_{3.5}]^{(4.5)-}$. Thus we are unsure which complex species is responsible for the

biological effect found. Furthermore, while the stability constant of the dihydroxo complex species is high enough to presume that it is present in solution even with the contemporaneous presence of other competitive ligands such as aminoacids, proteins etc. of the medium, the simple complex shows a very low stability which does not assure its presence in the medium. In conclusion, with Zn(II) we also attribute the biological effects to the dihydroxo complex species even if present in low percentage in solution. $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$, which presents a precise structure in solution [29], is the first defined coordination complex between copper and a polysaccharide eliciting a biological response on endothelial cells.

It is possible to speculate on the mechanisms responsible for the facilitation of endothelial cell adhesion, migration and proliferation. The presence of numerous sulphate groups may provide an anion group rich surface which allows for the electrostatic interaction and attachment of the endothelial cell membrane on the matrix. It is widely known that surface charge on endothelial cells is critical for their interaction with soluble factors, the extracellular matrix and circulating blood cells. Concerning the degree of sulphatation of oligosaccharides, it has been demonstrated that non- or low-sulphated saccharides have anti-angiogenic activity compared to highly sulphated molecules [41]. We can speculate that the same surface charge on the macromolecule could be responsible for the correct refolding and ''presentation'' of the complex to the cells. Another hypothesized mechanism might be the following: endothelial cells incorporate the sulphated complex on their surface (pericellular coating) and then interact with the extracellular matrix components. All the experiments were performed by using sulphated Hyal complexes in solution, and thus this latter mechanism cannot be excluded.

There have been many different attempts in the literature to develop functioning artificial vascular grafts which could facilitate endothelization: impregnation of basic fibroblast growth factor and heparin [42], coating with single or mixed extracellular matrix components [43]. Even if these grafts were demostrated to be endothelized both in vitro and in vivo and to be non-thrombogenic surfaces, no information is provided on the stability of coating substances. Our previous results indicate the stability of sulphated hyaluronic acid derivatives to the enzymatic digestion by hyaluronidase and chondroitinase [3].

Since $HyalS_x$ compounds are not subject to enzymatic degradation, they may be used in the preparation of blood-compatible materials. Different chemical routes have been developed to immobilize $HyalS_x$ and Cu(II)-$HyalS_x$ complexes [unpublished results] on various materials, including adsorption on positively charged polymeric films [44], grafting by photoimmobilization [45] and glow discharge treatment [46] and the preparation of networks with commercial polymers by cross-linking agents [47]. However, a complete assessment of biocompatibility of these biomaterials on vascular endothelial cells both in vitro and in vivo is still absent.

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