Metal-ion Complexes in the Angiogenetic Effect

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SUMMARY: The complex formation between two polysaccharides, hyaluronic acid (Hyal) and its sulphated derivative (HyalS), and two metal ions, Cu^{2+} and Zn^{2+}, was investigated in aqueous solution by thermodynamic and spectroscopic techniques. A stoichiometry for the complex species in solution was obtained. The bioactivity of the metal-polysaccharide complexes was then evaluated in terms of their influence on endothelial cell migration and adhesion. The biological response of the complex species was found to be dependent of both the polysaccharide (Hyal or HyalS) and the metal ion (Cu^{2+} or Zn^{2+}).

INTRODUCTION

Angiogenesis is a morphogenetic process which leads to the development of new blood vessels from pre-existing ones. Therefore, it is essentially for the growth of tissues and organs. Angiogenesis is a very complex process, requiring the sprouting and migration of endothelial cells, their proliferation and their differentiation into tube-like structures and the production of a basement membrane matrix around the vessel.[1]

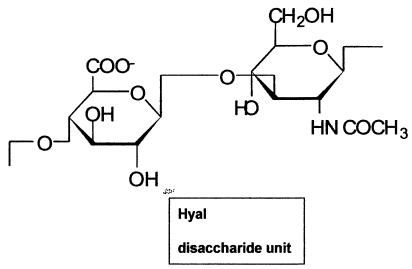
Among the several angiogenetic effectors, the heparin-copper(II) system has been particularly studied because of its capacity to induce angiogenesis in vivo (neovascularisation) and mobilisation of endothelium in vitro.[2] Zinc is another element which modulates some endothelial cells functions related to angiogenesis. It stimulates the proliferation of endothelial cells[3] and promotes the repair of wounded monolayers of this cell type.[4] However, it is unknown whether zinc is an angiogenic inducer. The similar electronic structure of Cu(II) and Zn(II) may not let to think that they have a similar behaviour also in aqueous solution and in the biological environments.

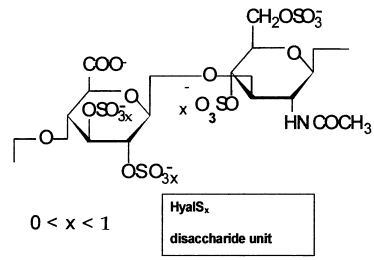
Heparin, and more in general proteoglycans play an important role in angiogenesis being low affinity receptors for angiogenic inducers such as basic fibroblast growth factor, vascular endothelial growth factor-A and trasforming growth factor β[5] and modulating endothelial cell adhesion and migration.[6,7] However, heparin is not entirely known by the chemical point of view, and due to its variety, a definition of the stoichiometry of the metal ion-complex is yet hard.

 CCC 1022-1360/00/$ 17.50+.50/0

As a matter of fact some polysaccharides have been sulphated to create compounds with a heparin like activity.[8]

A heparin-like molecule has been obtained by us through the sulphation of a particular glycosaminoglycan: the hyaluronic acid (Hyal), consisting in alternating units of N-acetyl-glucosamine and D-glucuronic acid residues.[9] Samples with different sulphation degree ($HyalS_x$) have been obtained and their heparin-like activity evaluated.[10]





The presence of the sulphate groups affected the polyelectrolyte behaviour of the hyaluronic acid, providing the sulphated polysaccharide with a more rigid and stretched conformation in aqueous solution.[11]

The present work deals with the study of the complexes formation of the two polysaccharides (Hyal and HyalS) with two metal ions (Cu^{2+} and Zn^{2+}) and with the biological evaluation of the complexes in terms of their influence on endothelial cell behaviour.

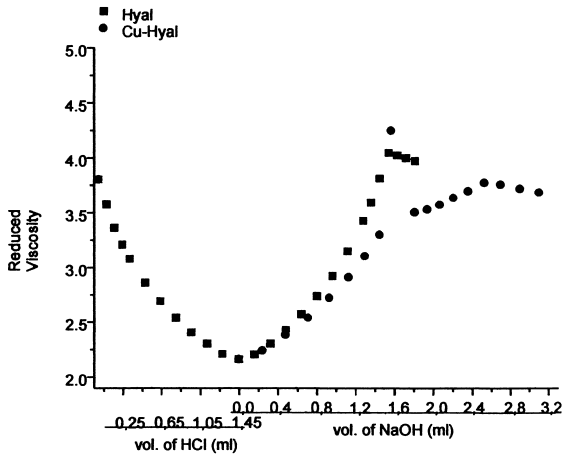
RESULTS AND DISCUSSION

Complex formation

The potentiometric and viscosimetric titrations of the metal-polysaccharide systems reveal that both Hyal and HyalS are able to form complexes with Cu^{2+} and Zn^{2+}. In Figures 1, 2, 3 and 4 the potentiometric and viscosimetric data are compared. Table 1 summarises the stability constant values for the complex species and the parameters of the visible spectra for the Cu(II) complexes.

As it can be seen from the potentiometric data, Hyal forms only one complex species with both Cu^{2+} and Zn^{2+}, with the same stoichiometry $M(OH)_2Hyal$. $HyalS_4$ forms two complex species with Cu^{2+}, i.e. $[CuHyalS_4]^{3-}$, and $[Cu(OH)_2HyalS_4]^{5-}$, and only one complex species with Zn^{2+}, $[Zn(OH)_2HyalS_4]^{5-}$. In particular, at pH<6, no complex species were formed in the

case of Hyal with either Cu^{2+} or Zn^{2+}. In the case of $HyalS_4$, the simple complex CuL is present but always at a very low concentration (see Figure 2).



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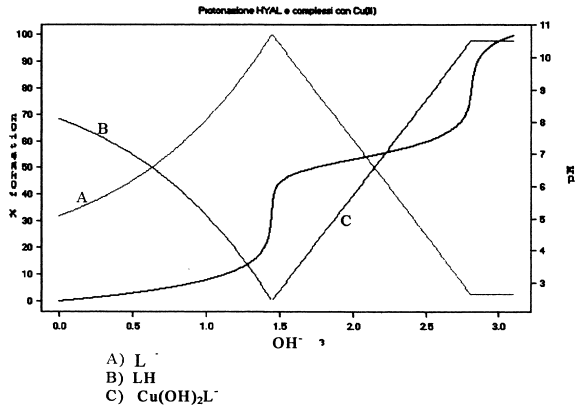


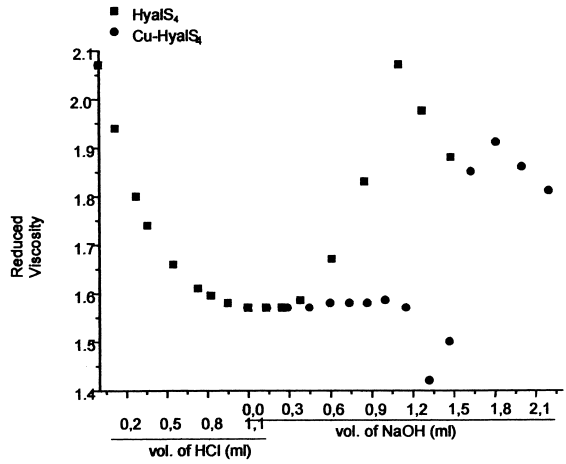
L^- = $Hyal^-$

(b)

Figure 1. a) Viscosimetric Titrations and **b)** Potentiometric Titration and Species Distribution Curves for Cu-Hyal Complex Formation

At pH>6, a release of two moles of H^+ ions per mole of metal ion is observed. This means that two hydroxyl groups are involved in the coordination process. The stoichiometry for the

complexes at pH>6 is the same, $M(OH)_2L$ (where M = metal ion, L = ligand), independent of both the metal ion (Cu^{2+} or Zn^{2+}) and the polysaccharide (Hyal or $HyalS_4$).



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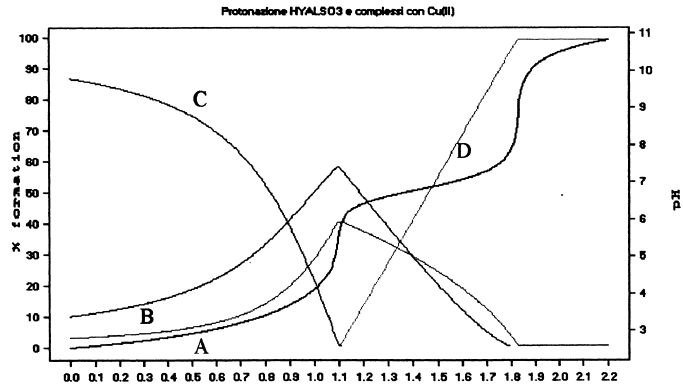


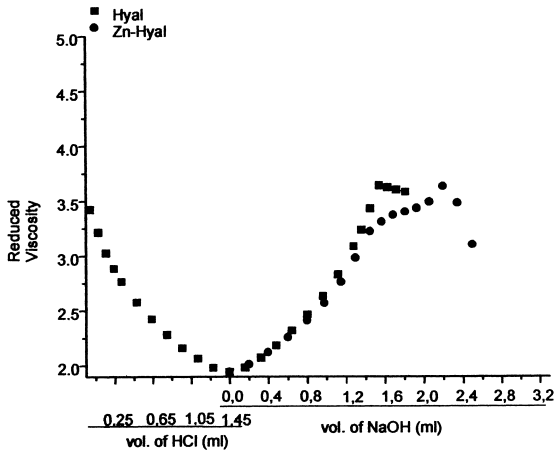
OH^-

A) L^{5-}
B) CuL^{3-}
C) LH^{4-}
D) $Cu(OH)_2\,L^{5-}$

$L^{5-} = HyalS_4{}^{5-}$

(b)

Figure 2. a) Viscosimetric Titrations and b) Potentiometric Titration and Species Distribution Curves for Cu-$HyalS_4$ Complex Formation



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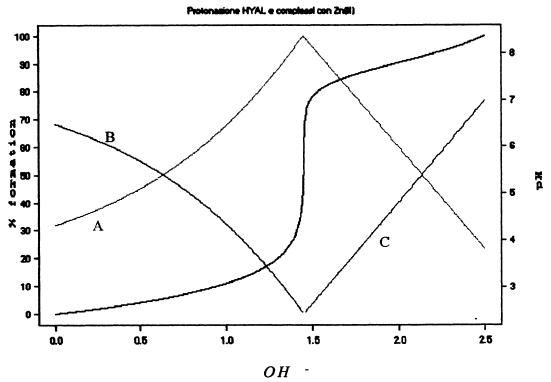


A) L^-
B) LH
C) $Zn(OH)_2L^-$

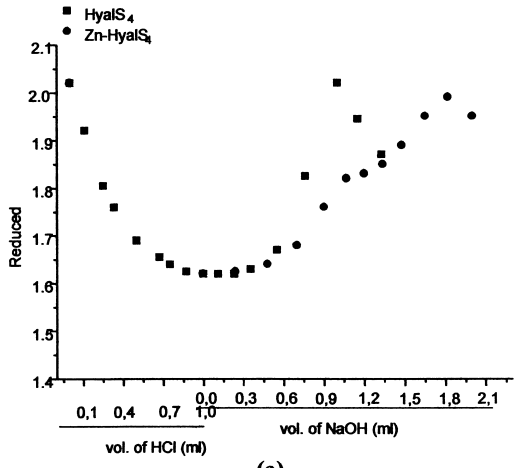
L^- = Hyal$^-$

(b)

Figure 3. a) Viscosimetric Titrations and **b)** Potentiometric Titration and Species Distribution Curves for Zn-Hyal Complex Formation

The formation of a complex between a ligand and a metal ion generally affects the conformation of the ligand polymer chains and thus the viscosity of the solution. When the viscosimetric titration curves of the metal-polysaccharide systems are compared to those of

the corresponding free ligands (Figures 1, 2, 3, 4) it can be seen that the formation of the complex species always corresponds to a decrease of the reduced viscosity of the solution, indicating that some charged groups are involved in the coordination site, thus decreasing the inter-chain electrostatic repulsions and favouring a more compact conformation of the coordinated polysaccharide.



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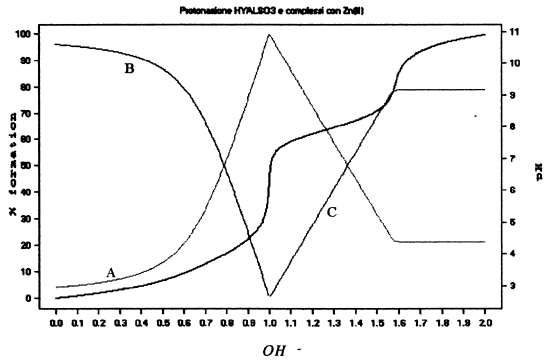


A) L^{5-}
B) LH^{4-}
C) $Zn(OH)_2L^{5-}$
L^{5-} = HyalS$_4$ $^{5-}$

(b)

Figure 4. a) Viscosimetric Titrations and **b)** Potentiometric Titration and Species Distribution Curves for Zn-HyalS$_4$ Complex Formation

Table 1. Stability constants and parameters of the visible spectra of the polysaccharide-metal complexes

Reaction	log $\beta^{(*)}$	λ_{max} (nm)	ε ($dm^3mol^{-1}cm^{-1}$)
$Cu^{2+} + Hyal^- + 2OH^- \leftrightarrow Cu(OH)_2Hyal^-$	16.39 (8)	696	19
$Cu^{2+} + HyalS_4^{5-} \leftrightarrow CuHyalS_4^{3-}$	4.11 (9)	622	17
$Cu^{2+} + HyalS_4^{5-} + 2OH^- \leftrightarrow Cu(OH)_2HyalS_4^{5-}$	17.29 (9)	720	20
$Zn^{2+} + Hyal^- + 2OH^- \leftrightarrow Zn(OH)_2Hyal^-$	14.54 (3)	—	—
$Zn^{2+} + HyalS_4^{5-} + 2OH^- \leftrightarrow Zn(OH)_2HyalS_4^{5-}$	14.63 (3)	—	—

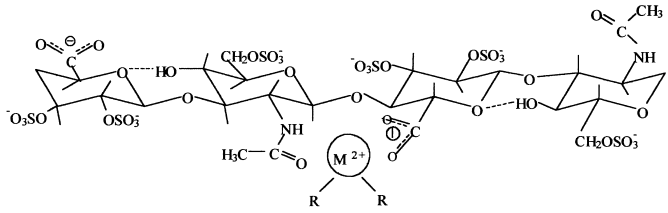
(*) in parenthesis are standard deviations

$Hyal^-$ refers to disaccharide unit containing only one carboxylate moiety.
$HyalS_4^{5-}$ refers to disaccharide unit containing one carboxylate and four sulphate groups.

The visible spectra of Cu(II)-polysaccharide complex species provide the evidence that the Cu^{2+} is coordinated by oxygen atoms, infact the λ_{max} values correspond to a CuO_x chromophore[10].

The infrared analysis of the metal-polysaccharide systems[9] confirmed the data of the visible spectra showing that the metal ion is coordinated by oxygen atoms. Infact the spectra of the metal-polysaccharide systems in aqueous solution showed a drop of the acetyl C=O and carboxyl (COO^-) vibrations, suggesting that only these groups are involved in the coordination site. The sulphate groups instead seem not to be involved in the coordination site since their vibration wavenumbers were not affected by the presence of the metal ions.[9]

On the basis of the thermodynamic and spectroscopic data the following stoichiometry for the metal-polysaccharide complexes is obtained.



R = OH^- or H_2O

Hypothesised structure of the M(II)(OH)-$HyalS_4$ complexes in aqueous solution. In the case of M(II)$(OH)_2$Hyal complexes, OH groups replace the OSO_3^- groups in the polysaccharide chain

Stability constants at 37°C

Since the biological activity of these complexes is evaluated at 37°C, potentiometric measurements at this temperature were performed. Table 2 summarises the values of the stability constants for the metal-$HyalS_{3.5}$ complex species together with the % of the different species at physiological pH. The dihydroxo Cu(II) complex is present in very high percentage (90%); thus this compound can be considered responsible of any biological effect. On the contrary, Zn(II) ion at pH=7.4 is present with a relatively low percentage of both complexes, 33% as $[ZnHyalS_{3.5}]^{(2.5)-}$ and 22% as $[Zn(OH)_2HyalS_{3.5}]^{(4.5)-}$.[12]

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

Reaction	Log β*	% species at 37°C and pH=7.4 #		
		$ML^{(2.5)-}$	$M(OH)_2L^{(4.5)-}$	$L^{(4.5)-}$
$Cu^{2+} + HyalS_{3.5}^{(4-5)-} \leftrightarrow [Cu\ HyalS_{3.5}]^{(2.5)-}$	3.58(1)			
$Cu^{2+} + HyalS_{3.5}^{(4-5)-} + 2OH^- \leftrightarrow [Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$	17.52(2)	0	90	10
$Zn^{2+} + HyalS_{3.5}^{(4-5)-} \leftrightarrow [Zn\ HyalS_{3.5}]^{(2.5)-}$	3.37(2)			
$Zn^{2+} + HyalS_{3.5}^{(4-5)-} + 2OH^- \leftrightarrow [Zn(OH)_2\ HyalS_{3.5}]^{(4.5)-}$	15.17(2)	33	22	45

(*) values in parenthesis are standard deviations
(#) $L = HyalS_{3.5}^{(4.5)-}$; $M = Cu^{2+}$ or Zn^{2+}
$LogK\ HyalS_{3.5}^{(4.5)-} + H^+ \leftrightarrow HyalS_{3.5}H^{(3.5)-} = 3.99(1)$

Effect of Hyal, $HyalS_{3.5}$ and their complexes with Cu^{2+} and Zn^{2+} to the endothelial cells adhesion

Two-way ANOVA was applied to the number of adhered cells after 30 minutes of contact with the different compounds at 1 μM concentration. ANOVA showed significant differences among the basal and Hyal, Cu(II)-$HyalS_{3.5}$ and Zn(II)-$HyalS_{3.5}$ (Fig. 5). In particular, the most effective compounds inducing adhesion are Hyal ($p < 0.05$) and $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ ($p < 0.05$). The Zn(II)-$HyalS_{3.5}$ complexes are instead the most effective to inhibit cell adhesion ($p < 0.05$). All the other compounds stimulate cell adhesion as well as the control (medium) without any significant differences. Furthermore, after 1 h a complete adhesion of endothelial cells was observed in all the wells.[12]

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

Figure 6a shows the endothelial cell migration as a function of 3 different concentrations (0.1, 1 and 10 μM) of Hyal and $HyalS_{3.5}$ after 6 hours of incubation at 37°C. While Hyal does not affect endothelial cell migration at any tested concentration, $HyalS_{3.5}$ significantly ($p < 0.05$) increases cell chemotaxis with comparison to Hyal both at 1 and 10 μM concentration.

The effect is concentration-dependent with maximal activity at 1 μM. A plateau phase is 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 it is evident from Figure 6c, $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ complex, even at the lowest tested concentration (0.1 μM) ($p < 0.05$), strongly induces cell migration. This effect is higher increasing its concentration up to 1 μM ($p < 0.05$), which represents the maximal effective concentration. Also at 10 μM cell migration induced by $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ complex is significantly different with respect to control ($p < 0.05$). We then assessed the chemotactic activity of different concentrations of Zn(II)-$HyalS_{3.5}$ and $ZnCl_2$ in comparison with $HyalS_{3.5}$. Figure 6b reveals that whilst $ZnCl_2$ at all the three tested concentrations shows to be devoid of any chemotactic effect, Zn(II)-$HyalS_{3.5}$ significantly induces cell migration ($p<0.05$). Maximal effect is observed at 1 and 10 μM concentration ($p<0.05$). As desumed from the potentiometric data at 37°C, two complexes are contemporaneously present in solution at pH=7.4 (Table 2), thus we can not discriminate which species induces cell migration. Besides, as shown in Figure 6b, the extent of cell migration in presence of Zn(II)-$HyalS_{3.5}$ might approximately considered the sum of that induced by the $HyalS_{3.5}$ and by Zn(II) ion. At last, data relative to the chemotactic ability of Cu(II)-Hyal and Zn(II)-Hyal are reported in Table 3. None of the two species seems to be able to stimulate cell migration at the concentrations tested, as demonstrated by statistical analysis ($p < 0.05$ for all the tested species).[12]

Table 3. Cell migration studied by Boyden's chamber technique induced by three different concentrations of Zn(II)-Hyal e Cu(II)-Hyal complexes after 6 h of incubation at 37°C.

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

All these data taken together demonstrate the marked chemotactic activity of $[Cu(OH)_2HyalS_{3.5}]^{(4.5)-}$ complex which shows to be the most effective. This effect is specific

for the complex present in solution since $CuCl_2$ was not able to induce cell migration.

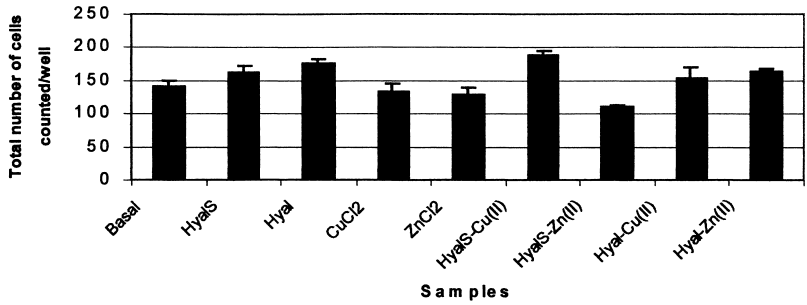


Figure 5. Murine endothelial cells adhesion.

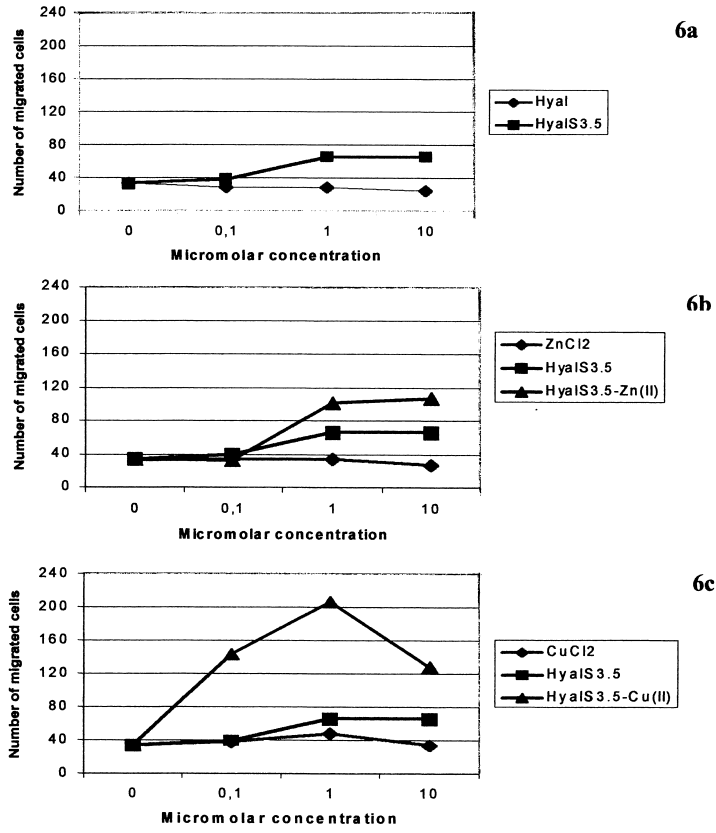


Figure 6. Murine endothelial cells migration.

CONCLUSIONS

From the data obtained the following conclusions can be drawn:

- Hyaluronic acid (Hyal) and sulphated hyaluronic acid (HyalS) are able to bind Cu(II) and Zn(II) ions.
- The stoichiometry of the polysaccharide-metal complexes is independent of both the polysaccharide (Hyal or HyalS) and metal ion (Cu^{2+} or Zn^{2+}).
- Only the HyalS-metal complexes are able to influence the endothelial cell behaviour. In particular: the $[Cu(OH)_2HyalS_{3.5}]^{4.5-}$ species explicates the greatest bioactivity in terms of inducing the migration and enhancing the adhesion of the endothelial cells, whereas the $[Zn\text{-}HyalS_{3.5}]^{2.5-}$ and $[Zn(OH)_2HyalS_{3.5}]^{4.5-}$ species seem to slow their adhesion without showing a great chemotactic activity.

EXPERIMENTAL SECTION

Materials

Hyaluronic acid (Hyal) (M.W. = 180000) was kindly provided by FAB (Fidia Advanced Biopolymers - Padova, Italy). Sulphated Hyaluronic acid ($HyalS_{3.5}$ and HyalS4) samples were synthesised as previously reported[10].

Metal complex formation studies

a) Potentiometric analysis

The potentiometric titrations were carried out in a glass cell kept at a constant temperature of 25°C or 37°C under a constant ionic strength of 0.1 M NaCl. 0.1 M HCl and NaOH standardised solutions were used. A Crison MicropH-2002 potentiometer equipped with a combined electrode (mod. 6.0204.000) was used together with an automatic Crison microburette (mod. 2031) connected to a PC 386 DX 40 MHz.[11]

b) Viscosimetric analysis

The viscosimetric titration data were obtained at 25°C in 0.1 M aqueous NaCl solution with Schott Geraete N.53113 and N.53101 viscometers. The titrating solution was added with a Metrohm Multidosimat burette.[11]

c) UV- visible spectrophotometric analysis

Visible absorption spectra of the polysaccharide-Cu(II) systems were recorded at room temperature in 0.1 M aqueous NaCl solution at two different pHs with a Biochrom 4060-Pharmacia LKB UV-Visible spectrophotometer, equipped with a 386 DX 40 MHz, using a 1 cm^3 silica cell.[11]

d) 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 stimuli. Elisa 96-multiwell plates were coated with 10 μg/ml fibronectin. Cells were detatched from confluent cultures and suspended at the density of $5x10^4$/ml. 100 μl of cell suspension were put in each well in the presence of test substances, and incubated at 37°C for 30 min-1 hour. Cells were washed with PBS and then fixed in methanol and stained in Diff Quick. Adherent cells were counted at 100X magnification with the aid of an ocular grid (21 mm^2). Data are expressed as total cell number counted/well.[12]

e) Motility assays

Chemotaxis experiments were performed by 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, $HyalS_{3.5}$,Cu(II)-Hyal, Zn(II)-Hyal, Cu(II)-$HyalS_{3.5}$, Zn(II)-$HyalS_{3.5}$, $CuCl_2$ and $ZnCl_2$ (0.1, 1 and 10 μM) in 2% FCS-DMEM were placed in the lower compartment of the chamber, and 1x105 cells suspended in DMEM containing 2% FCS were put in the upper compartments. After 6 hours of incubation at 37°C, the upper surface of the filter was scratched in order to remove non-migrated cells. Filters were fixed and stained in Diff-Quick (Harleco, Gibbstown, NJ, USA), and for each sample cells present in 5 oil immersion fields/well were counted. DMEM containing 2% FCS was used as negative control and complete M199 containing 10% FCS as positive control.[12]

f) Statistical analysis

Analysis of variance (ANOVA) was applied to the data for cell adhesion and motility. Post hoc comparison between groups (95% confidence for mean) were carried out when appropriate by a least squares difference test (LSD test).[12]

ACKNOWLEDGEMENT

This work has been supported by the "Progetto Finalizzato Materiali Speciali per Tecnologie Avanzate II" of the Italian C.N.R. (National Council of Research).

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