Sulfated hyaluronic acid as heparin-like material: physicochemical and biological characterization

R. BARBUCCI, M. BENVENUTI, M. CASOLARO, S. LAMPONI, A. MAGNANI
C.R.I.S.M.A. - Università di Siena Via Ettore Bastianini, 12-53100 Siena-Italy
Dipartimento di Chimica-Università di Siena Piano dei Mantellini, 44-53100 Siena-Italy

Hyaluronic acid was partially sulfated and a polymer containing a precise number of sulfate groups for each repeating unit was prepared. The properties of this macromolecule in aqueous solution were studied at different pH levels by using potentiometric, viscosimetric techniques and were compared with that of the native hyaluronic acid, partially esterified hyaluronic acid and heparin. The influence of sulfate groups on the macromolecule is evident in the protonation constant and in the conformation of the polymer. This material exhibits an antithrombotic effect as evidenced by the lengthening of both the thrombin time and the whole blood clotting time. Moreover, the absence of hemolysis and the growth and shape of endothelial cells put in contact with the sulfated hyaluronic acid indicate that this polymer is a promising heparin-like compound.

1. Introduction

Heparin (Hep) is the most biologically reactive member of the family of sulfated glycosaminoglycans. It is well known for its antithrombotic and anticoagulant properties; in fact it is extensively used in the management of cardiovascular diseases and contributes enormously to the success of open heart surgery. Nevertheless, the structure of heparin is not simple and, due to the number of variations, is not entirely known. Commercial heparins consist of a spectrum of 21 heparins [1] ranging in molecular weights from 3000 to 37 500 in varying anticoagulant activities. Usually the structure of heparin is represented in terms of its prevalent repeating disaccharide sequences.

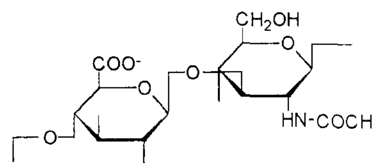
The blood anticoagulant activity of heparin is attributed to structural features, e.g. degree of sulfation, degree of dissociation, particular sequences of COO^- and SO_3^- groups, as well as to molecular shape and size. These factors appear to be related to biological activity by virtue of their importance in the ion binding capacity of heparin [2]. Heparin by virtue of its high negative charges has a strong affinity for cations, and a pH dependence is observed.

Most of the readily available natural polysaccharides have been sulfated in an attempt to obtain heparin analogues [3], and recently sulfate, carboxylic, and sulfonate groups are attached to some synthetic polymers such as polystyrene [4], and PU [5]. The anticoagulant activities of these materials were much lower than heparin and were dependent on the type and binding of the substituents, the degree of substitution and sequences. Our approach to studying the structural properties associated with the anticoagulant property was first to choose polymers possessing well-defined chemical groups consisting of regular repeating units, and secondly to modify their chemical structure in order to render them more similar to the heparin molecule.

Therefore the macromolecule must satisfy these requirements:

(1) Regular sequences of monomeric units.
(2) Chemically modifiable without destroying its structure.

Hyaluronic acid (Hyal), the major component of mammalian extracellular matrix, consisting of alternating units of N-acetylglucosammine and glucoronic acid residues, seems a suitable macromolecule.



Structural unit of hyaluronic acid

The present study is concerned with the modification of hyaluronic acid by the introduction of sulfated groups, the pH dependent behaviour of this macromolecule in aqueous solution, and its antithrombotic activity.

2. Materials and methods

$HyalSO_3$ was synthesized by using a modification of a procedure previously described [6]. The number of $-OSO_3$ groups per repeating unit of Hyal was obtained by C, H, N, S elemental analysis and NMR spectra. Hyal was obtained as a gift from Fidia Advanced Biopolymers (Abano Terme, Padova, Italy). Heparin was purchased from Roche.

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2.1. Viscosimetric and potentiometric titrations

The apparatus and the method utilized have been widely described [7].

2.2. Clotting assay: thrombin time

Venus blood from healthy donors was placed in plastic tubes containing sodium citrate (9:1, v/v). A pool of human plasma was obtained as previously reported [8]. The biological activity of hyaluronic acid was evaluated by the thrombin time (TT) as already described [8].

2.3. Human whole blood clotting time

Non-anticoagulated whole blood (5 ml) was withdrawn from human volunteers into a plastic tube containing 25 mg of $HyalSO_3$. The control contained whole blood only. The tubes were gently mixed until thrombus was formed. At this point, the coagulation time was recorded.

2.4. Hemolysis test

25 mg of $HyalSO_3$ were dissolved in 0.5 ml of sodium citrate, the assay tube was then filled with 5 ml of fresh human blood. The control contained whole citrated blood only. The hemolysis test was carried out as already reported [8].

2.5. Endothelial cell cultures

(HUVEC) were isolated from umbilical cords by collagenase digestion following a standard protocol. The cells were maintained in a 5% CO_2 atmosphere at 37 °C in Medium 199 (GIBCO Laboratories) with 20% fetal calf serum, L-glutamine and gentamicin.

The endothelial cells were identified as such by their polygonal morphology. For proliferation experiments cells were used when cultures had reached confluence. Hyaluronic acid was dissolved in Medium 199 until a concentration of 5 mg/ml was obtained. The assay was planned in order to allow contact periods of 24, 48 and 72 h between the material and the cells. Every 24 h the medium was removed from the wells and sterile PBS solution was rinsed over the film to remove the unattached cells. The cells were analyzed with an inverted microscope (DIAPHOT TMD Nikon) and pictures taken with a Nikon camera. The cells were then detached with trypsin and counted in a Bürker chamber. Trypan Blue was used to distinguish between dead and live cells.

3. Protonation equilibria in solution

3.1. Viscosimetric titration

The viscosimetric titration of hyaluronate with H^+ and OH^- is reported in Fig. 1.

The reduced viscosity (η/c) is decreased by adding H^+ to hyaluronate, reaching a minimum when the macromolecule is fully neutralized. Further, by adding OH^- to this solution, η/c rapidily increases reaching

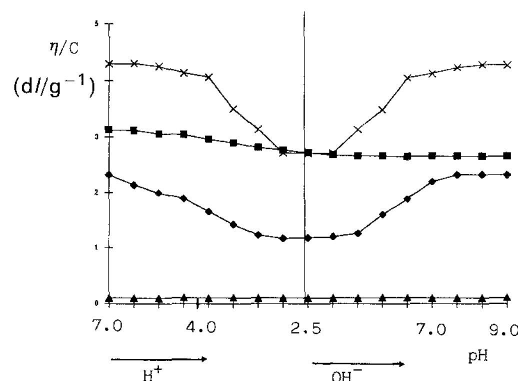

Figure 1 Viscosimetric titration plot for the different polysaccharides. (◆) Hyal, (■) $HyalSO_3$, (×) Hyaff 11 p25 and (▲) Heparin.

the same value as the starting solution of ialuronate. This behaviour is typical of a macromolecule which contracts or expands as a result of electrostatic repulsion of the dissociating groups. In this case, the unique groups bearing charges in this polymer are the carboxylate ions.

When some of the ionizable groups are esterified, as in Hyaff 11p25 (a 25% benzilic esterified derivative of hyaluronic acid), a trend similar to Hyal is observed, but more marked. The decreased number of charges in the partially esterified material and the presence of phenyl groups, which can interact with each other intramolecularly, renders the macromolecule more sensitive to pH variations (i.e. to the uncoiling–coiling process).

In contrast, by inserting other, not easily protonable groups like sulfate groups ($HyalSO_3$), in the Hyal backbone, a different trend is observed (Fig. 1). A slight decrease of η/c occurs by adding H^+, then it remains constant. The presence of non-neutralizable negative charges (SO_3^- groups) hinders the coiling of the macromolecule when the COO^-s are neutralized, rendering the $HyalSO_3$ macromolecule more rigid and stretched.

The viscosity of heparin by neutralizing the COO^- groups shows a smoother trend than that of $HyalSO_3$: η/c slightly decreases when the pH solution reaches the value of 4.0 where the carboxyl groups are beginning to be neutralized [9].

3.2. Protonation constants

In Table I the log Ks relative to the protonation reaction $-COO^- + H^+ \rightleftharpoons -COOH$ of the carb-

TABLE I Protonation constants of polysaccharides in 0.1 M NaCl at 25 °C

	log K	n	Range of the protonation degree
$HyalSO_3$	3.40	1.53	0.1–0.6
Hyal	3.02	1.18	0.1–0.6
Hyaff 11p25	2.92	0.88	0.1–0.7
Hep	~ 4.1	–	–

oxylate group present in the structural unit of polysaccharides are reported. In the same table the n value and the degree of protonation (α) range are also reported. The reaction follows a linear pattern corresponding to the modified Henderson–Hasselbalch equation [$\log K = \log K^{\circ} + (n - 1)\log(1 - \alpha)/\alpha$] for a wide range of α. K° is intrinsic protonation constant.

The protonation constant of Hyaff 11p25 increases slightly with the degree of protonation, while for Hyal and $HyalSO_3$ the opposite trend occurs (log K decreases with increasing α). The trend observed for Hyal is consistent with the fact that neutralization of more and more carboxylate groups reduces the electrostatic attraction towards H^+ ions by the remaining COO^-s.

The n value for Hyaff 11p25 is < 1; this means that the approach of the incoming H^+ ion to the COO^- groups becomes easier and easier as the degree of protonation of the whole macromolecule increases. An n value less than 1 was suggested to be characteristic of a compact conformation which uncoils during protonation [7]. This could not be the case because, even if the presence of benzilic groups renders the macromolecule partially hydrophobic, the trend of the η/c versus H^+ plot indicates the polymer assumes a tighter conformation upon neutralization. The hydrophobic moieties interact with each other, pushing out the hydrophilic COO^- groups which, once protonated, form hydrogen bonds with the amino groups and bury themselves in the hydrophobic microphase. The increasing size of the hydrophobic microphase pushes the remaining COO^- groups to the surface, making further protonation easier.

Anyway, all the n values are close enough to 1 demonstrating that the protonation reaction of one carboxylate group belonging to a repeating unit is scarcely influenced by the state of protonation of the other carboxylate groups in the other units. This behaviour occurs when the basic groups are far enough apart on the skeleton, or some cumbersome chemical groups shield interactions among the groups to be protonated.

In this case both of these factors seem to participate in rendering the behaviour of these macromolecules more similar to the protonation of small molecules exhibiting "real" equilibrium constants.

The protonation constants follow a trend connected to the number of negative charges present in each repeating unit. In fact the sulfation of some –OH groups of the structural unit of hyaluronic acid renders the $-COO^-$ of $HyalSO_3$ more basic than that of Hyal, allowing a stronger attraction towards H^+ ion. The importance of the negative charges on the basicity constant appears evident when observing that log K of Hyal is higher than that of Hyaff 11p25 a partially (25%) esterified Hyal. The lower quantity of $-COO^-$ groups, even if they are randomly spread along the whole macromolecule, lowers the basicity constant.

The $\log K$ of heparin is the highest of the series showing that this macromolecule bears the highest number of negative charges. The different MWs of heparin and the Hyal derivatives do not seem to influence this trend.

4. Biological tests

4.1. TT test and human whole blood clotting time

The antithrombotic activity has been determined by measuring thrombin times of $HyalSO_3$ in plasma. For the sake of comparison, results obtained with Hyal and Hyaff 11p25 are reported in Table II. It was shown that TT times are not lengthened in the presence of Hyal or Hyaff 11p25. On the contrary, $HyalSO_3$ exhibits a lengthening of TT corresponding to 0.4 UI or 0.0026 mg/ml of heparin per mg of product.

Furthermore the clotting time of human non-anticoagulated blood was measured to evaluate the enhancing effect of hemostatic agents on both plasmatic and cellular activation of the coagulation cascade. The coagulation time was longer than 2 h in the presence of $HyalSO_3$, while for the whole blood control it was 15 min. Moreover, after 45 min, blood in the presence of Hyal was completely coagulated, and a network of small coagules was observed on the wall of the tube containing blood and Hyaff 11p25.

These data show that $HyalSO_3$ exhibits an anticoagulant activity via a heparin-like mechanism.

4.2. Interaction with erythrocytes (hemolysis test)

The hemolysis assay measures the direct interaction of substances with the plasma membrane of erythrocytes.

The results obtained with sulfated hyaluronic acid shows that this material does not have any hemolitic activity. In fact the control curve and the $HyalSO_3$ curve are superimposed.

4.3. Cultured human endothelial cells

Fig. 2 shows the human umbilical vein endothelial cells (HUVEC) growth curves.

The number of endothelial cells in medium containing $HyalSO_3$ increased with time and better growth is shown than in medium containing Hyal or in a pure medium control.

The morphology of endothelial cells was examined using inverted microscopy. Endothelial cells in medium containing $HyalSO_3$ were well spread with no morphological alteration and without structural changes in cell organization.

The same morphology was noted for the endothelial cells in the presence of Hyal and for the control. The only remarkable difference was in the cell proliferation. In fact, after 1 day the cells in the medium containing $HyalSO_3$ were almost a confluent monolayer, while the cells in medium containing Hyal or pure medium reached confluency only after 3 days.

TABLE II Coagulation assay

Materials	Thrombin time (s)
Control	10.5
Hyaff 11p25	9.5
Hyal	9.4
$HyalSO_3$	30.0 ± 1

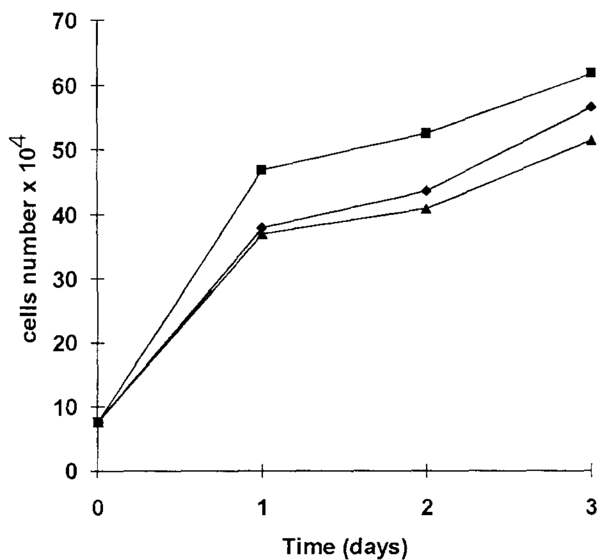

Figure 2 Growth of HUVEC in control (◆), $HyalSO_3$ (■) and Hyal (▲).

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