New Hyaluronan Chemical Derivatives. Regioselectively C(6) Oxidized Products

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ABSTRACT: Hyaluronan (HA) has been regioselectively oxidized so to transform nearly quantitatively the primary alcoholic functions of D-*N*-acetylglucosamine residues in carboxylate groups. The resulting product (HAOX) has been characterized by means of ^{1}H NMR, viscosity, and spectroscopic measurements in aqueous media. A comparative analysis of Zn(II) uptake and of methylene blue binding by HA and HAOX in aqueous solution has been also carried out (FTIR, UV absorption, and CD experiments). HAOX exhibits polyelectrolytic features traceable to a doubling of chains charge density with respect to native HA and to the disruption of the intrachain hydrogen bonds prevailing in the latter.

Introduction

Hyaluronan (HA)[1,2] having one carboxylate group per disaccharide repeating unit can be considered a weakly charged polyelectrolyte, the average distance between fixed charges (carboxylate groups) along HA chains being ca. 1 nm which corresponds to a "charge density" parameter ξ below the monovalent counterions condensation threshold.[3]

Counterions (including divalent metal ions) electrostatic binding properties of HA are therefore rather limited, and this contributes to limit the performances of HA as a carrier of biologically active ionic species such as Zn(II) ions.

Upgrading HA from the standpoint of charge density-related features—limiting attention to carboxylate groups exclusively—would obviate this drawback. For example, for HA samples in which all primary alcoholic functions have been converted to carboxylate groups, one has a doubling of ξ, as a first approximation, and the theoretically expected fraction of monovalent or divalente counterions bound increases markedly. Of like importance, such charge density increase allows to obtain novel, more densely functionalized HA carboxylate esters and amides.[4]

Quite naturally an ordered, controlled increase in the number of carboxylate groups along HA chains requires a process that must be at the same time regioselective, nearly quantitative, and with limited chain degradation.

TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl)-mediated oxidation of carbohydrate polymers appears the process of choice whose C(6) regioselectivity, high yields, and limited chains scission, provided certain experimental precautions are taken, is well documented in the recent literature[5] also in the case of HA.[6]

In our laboratories, TEMPO-mediated oxidation of HA has been applied to obtain from partially to totally C(6) oxidized HA samples. Dilute aqueous solutions of the latter (HAOX, Figure 1) have been characterized, in a comparative fashion, in terms of ionic strength dependence of viscosity, and of Zn(II) ions and methylene blue binding (UV and circular dichroism data), respectively. The results are reported herein.

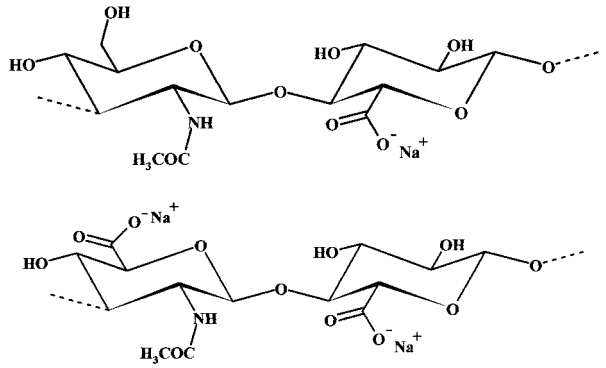

Figure 1. Structure of hyaluronic acid (above) and hyaluronic acid oxidized (below) repeating unit.

The synthesis and characterization of new water-insoluble esters[4] of the HYAFF series[7] with potential in a number of biomedical applications will be illustrated in detail in a forthcoming paper.

Materials and Methods

A hyaluronic acid (HA) sample, a Fidia Advanced Biopolymers (FAB Srl, Abano Terme Padua, Italy) product, $M_\eta = 160$ kDa, has been used throughout. All other chemicals were reagent grade and have been used without further purification.

HA Oxidized Samples. Regioselective C(6) oxidation of HA has been carried out following closely the procedure already reported in the literature[5,6] aiming at the obtainment of fully oxidized products (HAOX) (Figure 1).

The degree of oxidation of the latter has been controlled by means of ^{1}H NMR measurements and of potentiometric titrations. In the NMR spectra of Figure 2 one observes that while in the case of native HA the ratio of non anomeric protons (signals at 3.2−4.1 ppm) to anomeric protons (signals at 4.4−4.9 ppm) is 5, as expected, in the case of HAOX the ratio comes out 4, indicating that only eight non anomeric protons are left per repeating unit and therefore that the C(6) oxidation has been close to 100%. This is in agreement with the fact that in the NMR spectrum of HAOX there are only two resonances in the anomeric protons region (in fact, a third peak in the anomeric region is barely visible) while, quite naturally, partial oxidation would have given rise to three distinct resonances.

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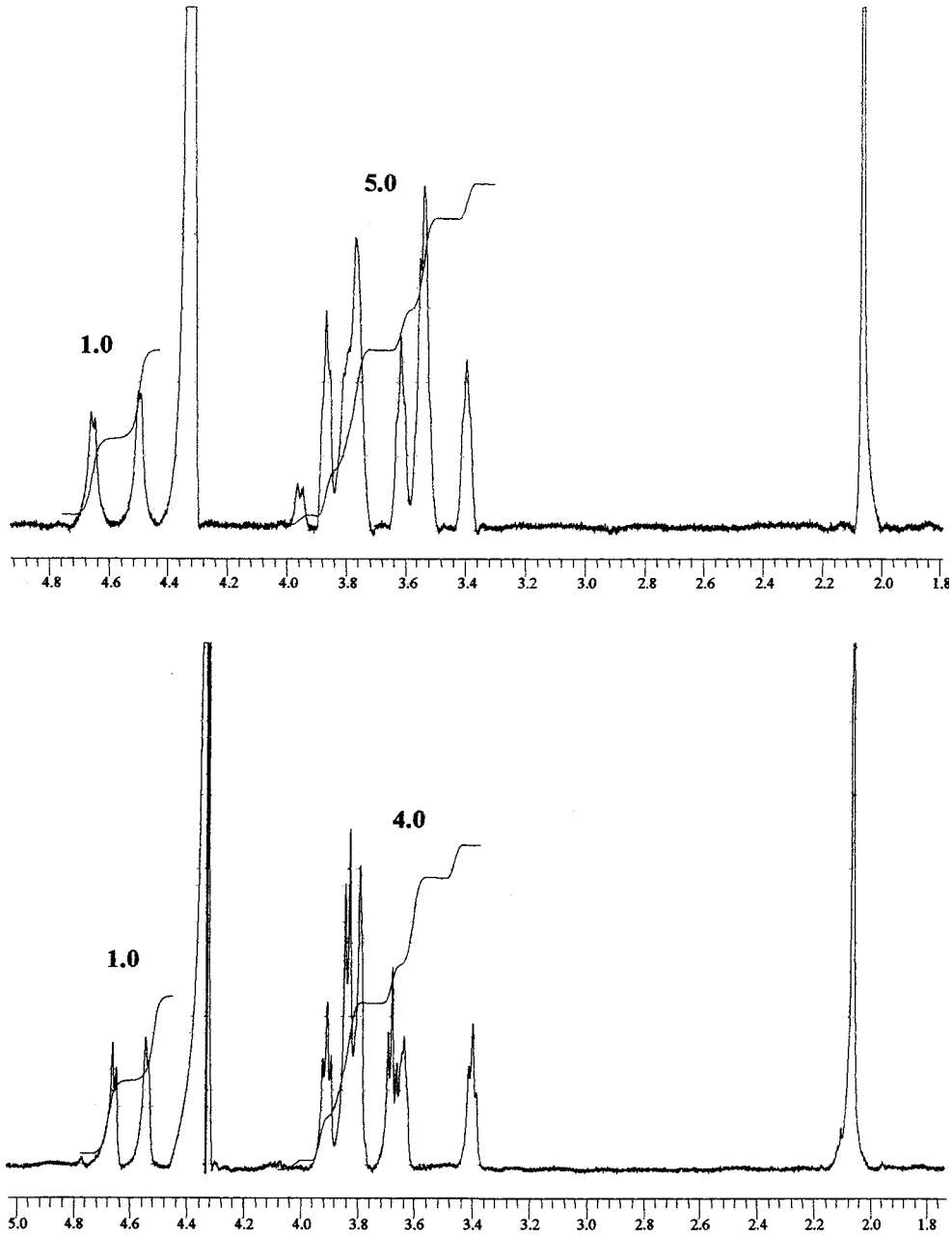

Figure 2. ^{1}H NMR spectra of hyaluronic acid (HA) (above) and of oxidized HA (HAOX) (below). Spectra were recorded at 600 MHz with a Bruker AMX600 spectrometer.

Potentiometric titrations of samples of HAOX in the H^+ form (freeze-dried after passage through excess H^+ Dowex ion-exchange resin) with standard NaOH solution (0.107 M) lead to an average equivalent weight (average of three titrations) corresponding nearly, within an estimated experimental error of about 5%, to two carboxylate groups per repeating unit. In conclusion, we can assume that our HAOX sample has a degree of oxidation of at least 95%.

HA and HAOX in the Zn(II) Salt Form. HA and HAOX samples in the Zn(II) salt form have been prepared by prolonged dialysis (3 weeks, to ensure complete ion exchange) of the sodium salt form against frequently changed $Zn(OCOCH_3)_2$-saturated solution followed by dialysis against distilled water (10 days) and freeze-drying of the products. An obviously faster procedure consists of preparing the tetrabutylammonium salt of HA and then in trasforming it into the Zn(II) salt by zinc chloride addition. However, in our experience, the final purity of the required product is higher with the slower dialysis procedure.

By means of atomic absorption measurements the following % (w/W) of bound Zn(II) have been found: HA, 5.2, and HAOX, 12.8, assuming a 10% w/W water content for both samples. The calculated stoichiometric values are respectively 7.9 and 14.3. In any event, it is clear that the Zn(II) content of the HAOX sample is well beyond the value (ca. 10% w/W) considered the threshold for pharmacological relevant antibacterial activity.

FTIR spectra of the Zn(II) salt forms of HA and HAOX have been recorded using a Mattson 5020 FTIR spectrophotometer with a Golden Gate Hellma ATR cells system (P/N10500 series).

Viscosity, Circular Dichroism, and UV Spectral Measurements. The viscosity (25 °C) of dilute HA and HAOX (sodium salts) aqueous solutions as a function of ionic strength (NaCl) has been studied using a Schotte-Geraete automatic dilution viscometer.

Circular dichroism (CD) measurements were taken with a Jasco J715-A dichrograph. The study has dealt with a comparative analysis of the CD spectra of aqueous HA and HAOX as well as of the extrinsic CD spectra of methylene blue (MB) ($[MB]_0 = 1.3 \times 10^{-5}$ M) in the presence of varying concentrations of HA and HAOX, respectively.

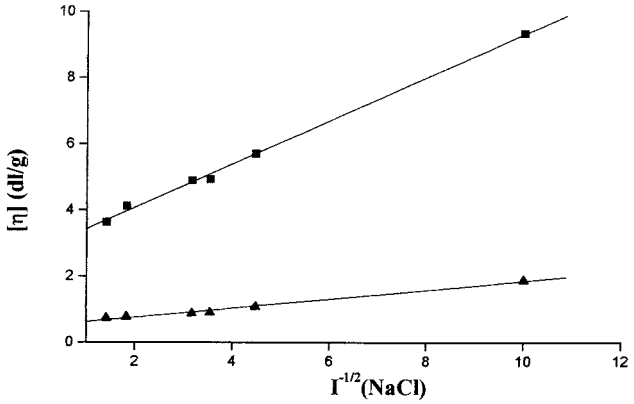

Figure 3. Dependence on ionic strength (*I*, NaCl) at 25 °C of the intrinsic viscosity of HA (■) and HAOX (▲).

Absorbance spectra were recorded with a HP 8452A diode array spectrophotometer using a 1 cm quartz cell. Absorbance measurements as a function of the molar polymer/dye concentration ratio, P/D, were carried out by adding directly into the cell to a known amount (3 mL) of a dye solution (1.3×10^{-5} M) suitable amounts of a concentrated polymer solution (1.5×10^{-2} equiv/L) to obtain P/D values ranging from 0 to about 100. After mixing, the solutions were equilibrated for 1 h: afterward, no change in the spectra could be recorded even after several hours (always at 25 °C).

Absorbance spectra as a function of NaCl concentration were recorded adding the proper amount of a concentrated salt solution to a polymer−dye mixture at a given P/D ratio, 15 for HA and 7.5 for HAOX. These are close to the values for which the dye absorbance at 664 nm (free, monomeric dye band) attains a minimum in the two cases.

The degree of salt-induced metachromasy decrease (α) is defined as

$$\alpha = \frac{[MB]_s - [MB]}{[MB]_0 - [MB]} \times 100 \qquad (1)$$

where $[MB]_s$, [MB], and $[MB]_0$ are the concentration of free monomeric methylene blue in the presence of salt, in the absence of salt, and in pure water, respectively ($[MB]_0 = 1.3 \times 10^{-5}$ M).

$[MB]_s$ and [MB] have been estimated by normalizing the relevant absorbance readings for the molar extinction coefficient value of the 72 000 L/(mol cm) at 664 nm, assumed constant.

Results and Discussion

Viscosity Data. Intrinsic viscosity data for HA and HAOX (sodium salt forms) in dilute NaCl aqueous solutions at 25 °C have been determined for each ionic strength (*I*) by extrapolation of double Huggins−Kramer plots and are plotted in Figure 3 as [η] against $I^{-1/2}$.

The lower intrinsic viscosity of HAOX relative to that of the initial HA sample is due to the partial chain depolymerization that accompanies the TEMPO-mediated C(6) oxidation of polysaccharides.[5,6]

As is known,[8,9] simple elaboration of a plot such as that of Figure 3 permits to derive the so-called "chains flexibility parameter", *B*, which should be independent of sample molecular weight. The origin and meaning of such a parameter can be briefly schematized as follows. The dependence of a polyelectrolyte intrinsic viscosity on ionic strength can be expressed by the Pals and Hermans[10] equation:

$$[\eta] = [\eta]_\infty + SI^{-1/2} \qquad (2)$$

where $[\eta]_\infty$ is the intrinsic viscosity at infinite *I*. According to Fixman,[11] the coefficient *S* can be assumed to depend linearly on *M* as a first approximation. Then, with the Mark−Houwink equation written for a reference ionic strength of 0.1 M, one arrives at the expression[8]

$$S = B[\eta]_{0.1}^{\nu} \qquad \nu = 1.2-1.4 \qquad (3)$$

The *B* parameter—which depends on the parameters of the Mark−Houwink equation, in addition to *S*, and which should be independent of sample molecular weight—can therefore be taken as a measure of the propensity of a polyelectrolyte chain to adapt its average dimensions to a change in ionic strength, i.e., a qualitative indicator of macroions flexibility. Stiff chains have low *B* values, e.g., 0.0055 for DNA, while flexible chains (e.g., polyacrylates) have *B* values around 0.25.[8]

In our case, taking $\nu = 1.3$, we estimate that $B = 0.083$ and $B = 0.152$ for HA and HAOX, respectively. These results indicate that there is a difference in chain rigidity between native HA and HAOX, the latter being distinctly less stiff.

At first sight, a doubling of charge density that brings about an increase in macroions flexibility seems paradoxical. We believe that the explanation of such an apparent paradox lies in the widely accepted model for the native HA chains due to Scott[12] (Figure 4) in which an extented array of specific intrachain hydrogen bonds shields the polysaccharide backbone from attack of chemical agents (e.g., periodate) and from the influence of physical factors (e.g., ionic strength). Oxidation at C(6) of *N*-acetyl-D-glucosamine residues along HA chains would break down such shielding.[13]

Finally, if notwithstanding this evidence, we assume that the *K* and α parameters of the Mark−Houwinks equation are the same for HA and HAOX in 0.5 M NaCl (i.e., $K = 31.8 \times 10^{-5}$ dL/g and $\alpha = 0.78$); we calculate $M_\eta = 20$ kDa for HAOX. This represents a substantial reduction in molecular weight with respect to the native sample ($M_\eta = 160$ kDa): however, the M_η figure evaluated for HAOX is certainly underestimated in view of the difference in *B* values mentioned above (which should reflect a reduction in both *K* and α values for the more randomly coiled HAOX). In any event, it is

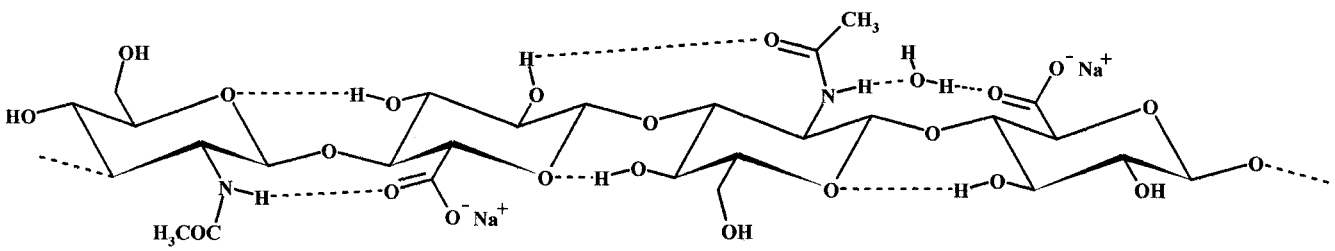

Figure 4. Secondary structure of HA in water.

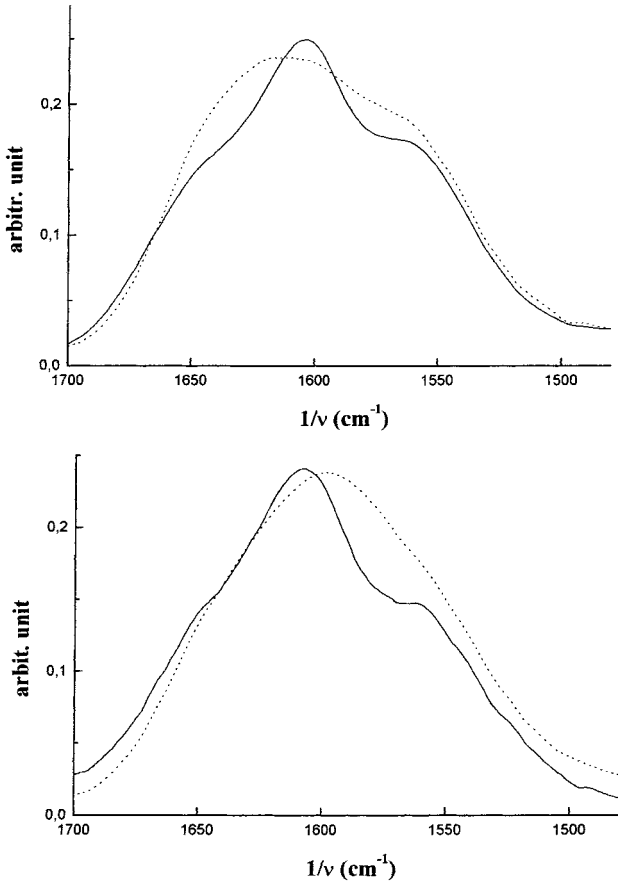

Figure 5. FTIR-ATR spectra of HA (—), HA−Zn (- - -) (above) and HAOX (—), HAOX−Zn (- - -) (below).

clear that the C(6) selective oxidation procedure adopted remains a depolymerizing process despite various attempts we have made at curbing such drawback by "optimizing" main working parameters (basically: pH, temperature, amount of NaClO).

Spectral Data. *(a) FTIR Spectra of HA, HAOX, and of Their Zn(II) Salt Forms.* Portions of the IR spectra of hyaluronan and of C(6)-oxidized hyaluronan in the sodium salt form (HA, HAOX) and in the Zn(II) salt form (HA−Zn, HAOX−Zn) are reported in Figure 5. In both HA and HAOX spectra a band typical of (carboxyl) carbonyl stretching (at about 1600 cm^{-1}) as well as the amide I (at about 1560 cm^{-1}) and amide II (a shoulder at about 1650 cm^{-1}) bands are visible.[14] In the spectra of the Zn(II) salt forms said bands are broadened probably because both carboxyl groups and amide groups participate in the coordination of the divalent counterions. However, the set of spectra show distinctive features, i.e.:

(1) In the HA−Zn spectrum the characteristic signals of HA are still discernible (relatively low amount of Zn(II) ions bound), and the maximum of the (carboxyl) carbonyl band is shifted of about 10 cm^{-1} to higher wavenumbers. The underlying slight increase in force constant of the C═O bond may be due to a Zn(II)-induced loosening of intrachain hydrogen bonds engaging many carboxyl groups in native HA.

(2) In the IR spectrum of HAOX−Zn all signals are smeared out and the maximum of the carboxyl carbonyl band is shifted of about 10 cm^{-1} to lower wavenumbers. This, we believe, is because of the relatively large number of coordinated Zn(II) counterions which would weaken the force constant of the C═O bonds.

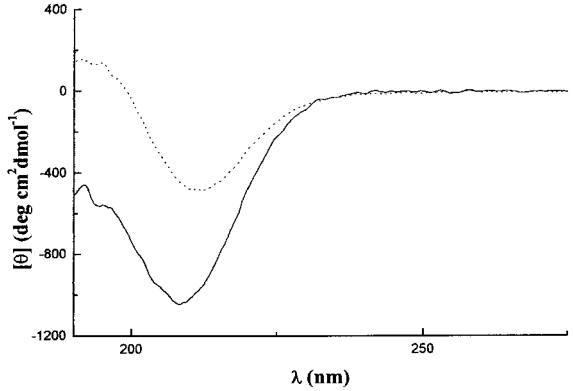

Figure 6. CD spectra of HA (—) and HAOX (- - -) in water at 25 °C.

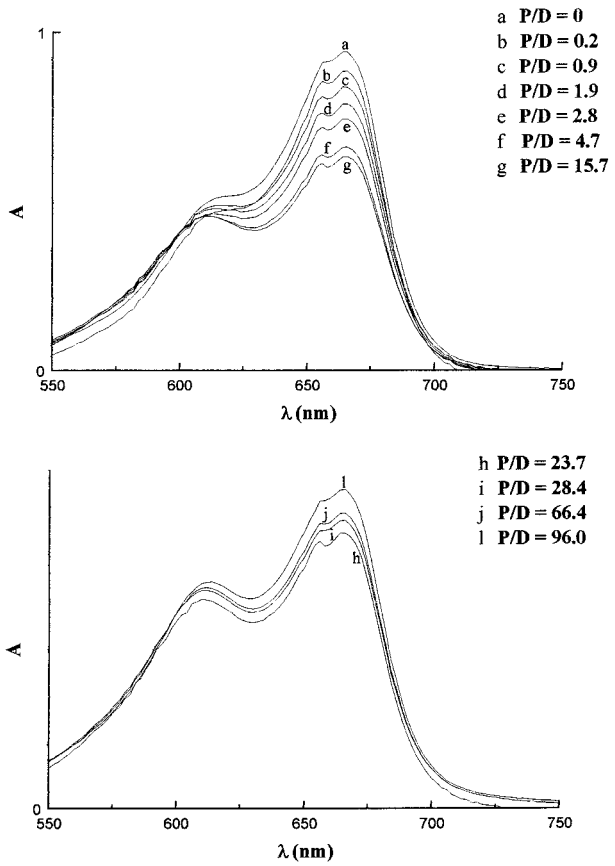

Figure 7. MB absorption spectra at different HA concentrations at 25 °C ($[MB]_0 = 1.3 \times 10^{-5}$ M).

(b) CD Spectral Data of HA and HAOX in Water. Circular dichroism spectra of HA and HAOX in water (Figure 6; polymer concentration around 0.07 mg/mL) show that the ellipticity of the two polycarboxylates (instrumental readings normalized by the number of moles of carboxylate groups in solution calculated assuming 400 and 213 as equivalent weights for HA and for HAOX, respectively) are different in the range of wavelengths investigated.

In fact, the spectrum of HAOX has a positive contribution around and below 190 nm, likely to be ascribed to the C(6) oxidized *N*-acetylglucosamine chromophores, which brings about a red shift and an apparent weakening of the glucuronic acid residues band having a typical negative through at about 210 nm.

(c) Comparative Analysis of UV and CD Spectra of Dilute Aqueous Solutions of HA and HAOX in the Presence of Methylene Blue (MB). Polyelectrolyte−dye

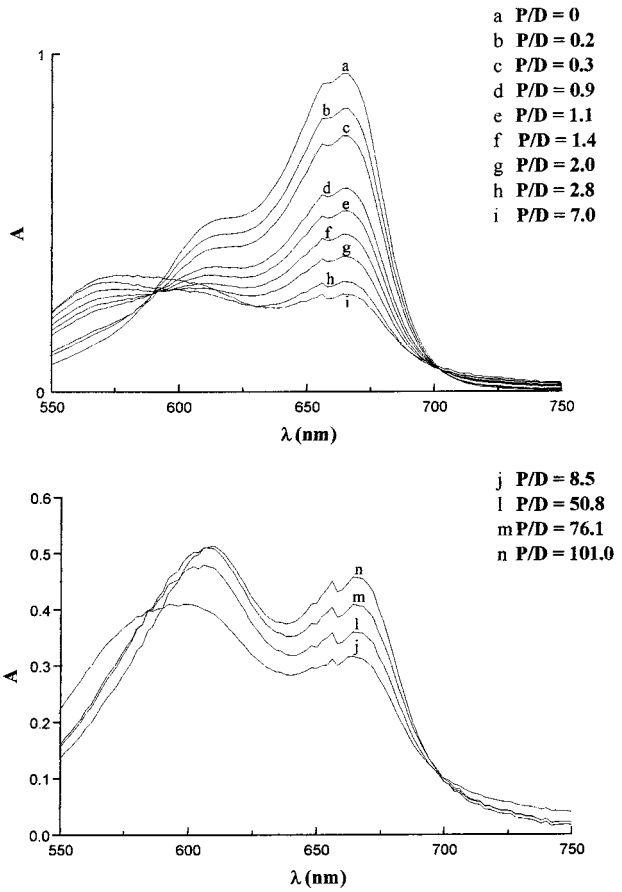

Figure 8. MB absorption spectra at different HAOX concentrations at 25 °C ($[MB]_0 = 1.3 \times 10^{-5}$ M).

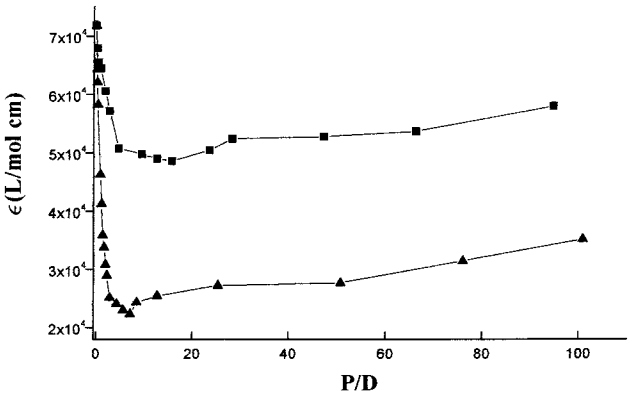

Figure 9. Effect of polymer−dye ratio (P/D) on the molar absorption coefficient (ϵ) at 664 nm of MB at 25 °C ($[MB]_0 = 1.3 \times 10^{-5}$ M) for HA (■) and HAOX (▲).

binding studies have been frequently carried out in order to shed light on the extent and steric features of the interactions between macroions and bulky, partially hydrophobic counterions in dilute aqueous solution.[15] In the present case, UV and CD spectroscopies have been used for a comparative analysis of the interaction of MB (used at a fixed concentration $[MB]_0 = 1.3 \times 10^{-5}$ M) with HA and with HAOX for different polymer concentrations (P) in water (25 °C).

UV data (recorded as explained in the Experimental part) collected in Figures 7 and 8 clearly show that HAOX has a much more marked influence on the methylene blue spectrum than HA. With the latter, increasing the P/D molar concentration ratio brings about a decrease in the monomeric dye band (peak at

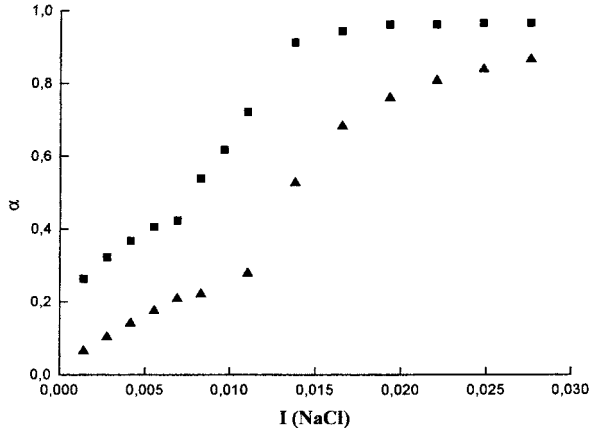

Figure 10. Effect of NaCl concentration on the metachromasy of MB induced by HA (■) and HAOX (▲) at 25 °C (total, constant MB concentration is 1.3×10^{-5} M) See eq 1 (Experimental part) and the text.

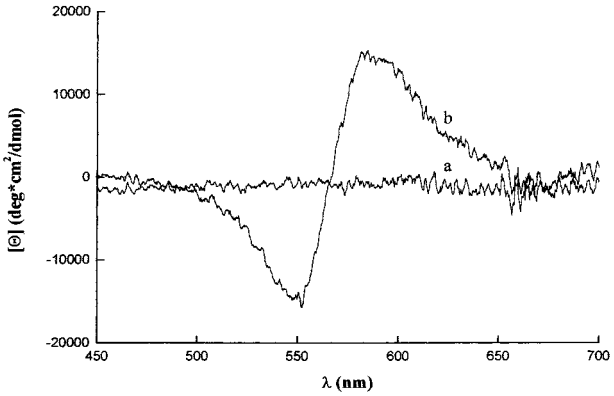

Figure 11. CD spectra of MB in the presence of HA (a) and HAOX (b) at a P/D ratio equal to 15 and 7.5, respectively (see also Figure 7) ($[MB]_0 = 1.3 \times 10^{-5}$ M). The CD signal was normalized by the bound dye concentration (the different between the total, initial dye concentration, 1.3×10^{-5} M, and the free dye concentration, 0.4×10^{-5} M, calculated from the absorbance at 664 nm).

about 664 nm with a molar extinction coefficient $\epsilon =$ 72 000 L/(mol cm)) and a concomitant modest increase in the absorption around 620 nm, which is normally attributed to the dimeric form of the dye.

With HAOX, to the contrary, under similar conditions the intensity of the monomeric dye band is drastically reduced, and at the same time a broad band first develops around 570 nm up to a P/D value of about 7 and then decreases, yielding to the dimeric dye band. The latter finally prevails for high P/D values. We conclude that HA binds a relatively low amount of dye molecules and almost exclusively in the dimeric form while HAOX exhibits a quite higher binding capacity with the formation of dye aggregates (band at around 570 nm) onto the polymer chains. For what concerns the monomeric dye band, data plotted in Figure 9 clearly show the distinctly higher ability of HAOX with respect to HA to suppress it with a maximum capacity around P/D = 7. For higher P/D values there is an increase in the apparent ϵ (664 nm) values; i.e., the dye molecules redistribute themselves onto the polymer chains being bound basically in either monomeric (HA) or dimeric form (HAOX).

The higher affinity of MB for HAOX is also deducible by observing the trend of the α values (see Experimental part, eq 1) plotted in Figure 10: in fact, a higher NaCl

concentration is required in order to reduce dye binding and, eventually, to restore the free dye spectrum in the case of HAOX.

Finally, of particular interest are the CD data reported in Figure 11 which demonstrate that only in the case of HAOX bound dye molecules can form aggregates which can acquire chirality, giving rise to a strong CD spectrum in the 500−650 nm region.

Data for HA do not show any symptom of methylene blue-induced optical activity.

Concluding Remarks

Regioselective, quantitative C(6) oxidation of the *N*-acetyl-D-glucosamine residues along the chains of hyaluronic acid leads to a new, semiartificial uronan (HAOX) with a "stoichiometric" charge density twice that of the native biopolymer. This shows up in a distinctly higher binding capacity of HAOX toward Zn(II) ions, which is relevant for antibacterial formulations, and methylene blue cationic molecules in dilute aqueous solution. Such a difference between HA and HAOX is, in our opinion, traceable at least in part also to the intramolecular H-bonds in HA[12,16] which reduce the "activity" of carboxylate groups in interacting with their counterions.[13]

In addition, for appropriate dye to polymer (HAOX) concentration ratios, bound dye aggregates exhibit a strong ellipticity with a peak at ca. 580 nm and through at 550 nm.

The increase in flexibility going from HA to HAOX, estimated according to viscosity data, may seem to contradict the expectation of a stiffening of the chains with an increase in fixed charges density. On the other hand, introduction of a carboxyl group on every *N*-acetyl-D-glucosamine moiety (HAOX) would modify the local geometry and break down the delicate balance of interactions, allowing for the above-mentioned intrachain H-bonds in native HA, with a concomitant overall increase in available conformational space.

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