Periodate oxidation and macromolecular compaction of hyaluronan.

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Abstract: Partial periodate oxidation of hyaluronan leads to ring opening of the 4-linked D-glucuronate (GlcA) residues, providing a particularly flexible element within otherwise semi-flexible chains. This leads to compaction of the chains as demonstrated by a pronounced decrease in the intrinsic persistence length, which was determined on the basis of the molecular weight dependence of radius of gyration and the intrinsic viscosity. These parameters were readily obtained using size-exclusion chromatography with an online multi-angle laser light scattering detector, a viscosity detector, and a concentration sensitive detector. The electrostatic contribution to the total persistence length increased with increasing degree of oxidation. Compared to alginates and chitosans hyaluronan becomes less degraded during the oxidation, which is attributed to a protective effect of periodate-resistant N-acetyl-D-glucosamine (GlcNAc) residues adjacent to periodate-sensitive GlcA residues in hyaluronan.

INTRODUCTION

Polysaccharides such as hyaluronan (HA) and alginates (Figure 1) are much used as biomaterials because they may form solutions or gels mimicking mammalian tissues or parts thereof such as the extracellular matrix. Both are water-soluble at pH 7, primarily because of the presence of carboxylate groups and the associated counter-ions (normally sodium). HA may form gels by covalent cross-linking or by forming polyelectrolyte complexes with polycations such as chitosans or poly-L-lysine. Alginates form gels with calcium salts, the mechanism being chain dimerization involving homopolymeric blocks of L-guluronic acid (G).

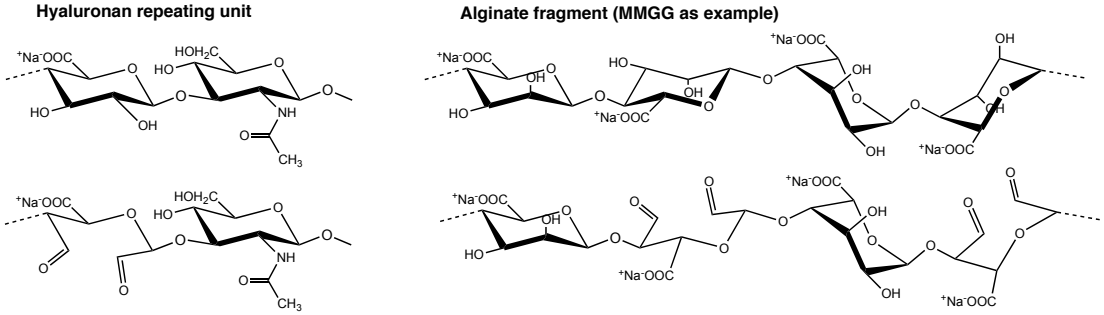

Fig. 1. Repeating units of hyaluronan (top left) and alginate (top right), and the corresponding periodate oxidized derivatives (bottom left and right, respectively). Note all residues in alginates may be oxidized, whereas the 3-linked GlcNAc residue in hyaluronan is periodate-resistant. The amount of dialdehydes or the degree of oxidation (D_{ox}) may be controlled either by adjusting the periodate/polysaccharide ratio or the reaction time. The ring opening leads to high local chain flexibility, allowing compaction of otherwise semi-flexible chains. In the present work the dialdehydes were reduced with $NaBH_4$ to the corresponding alcohols prior to further analyses.

In recent years we have explored partially periodate oxidised alginates as components of alginate based materials [1, 2]. The influences of a relatively low degree of oxidation (2-8%) are multiple. First, the introduction of dialdehydes renders the chains more labile to β-elimination, even at physiological conditions [2]. Secondly, the ring opening results in significant chain compaction due to the large

flexibility of oxidised residues. The compaction (Figure 2) is demonstrated by a large decrease in the radius of gyration (R_G) and intrinsic viscosity at constant molecular weight [3]. The oxidation also influences the ability of alginate chains to interact with calcium ions and form gels, as the average length of G-blocks decreases, resulting in softer calcium alginate gels [1]. If the periodate oxidation is applied to homopolymeric mannuronan the action of C5-epimerases is restricted since oxidised residues prevent the processive action of the epimerases [1]. Finally, limited periodate oxidation of polysaccharides leads to non-specific depolymerization [4, 5]. It has been proposed the degradation is a free radical induced side reaction due to spontaneous decomposition of periodate [6]. On the other hand, the protecting influence of periodate resistant GlcNAc residues in chitosans [5] suggests that the chemistry of the polysaccharide itself plays a major role in determining the susceptibility to degradation during oxidation. HA may in this case serve as an interesting model substance since every periodate-sensitive GlcA residue is flanked by periodate-resistant (1→3)-linked GlcNAc residues.

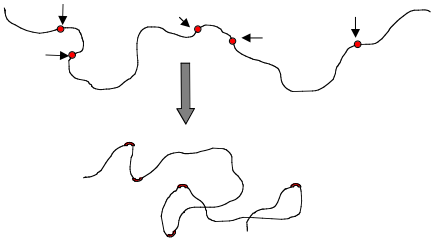

Fig. 2. Macromolecular compaction following partial periodate oxidation and ring opening (arrows)

Here we present novel data on partially oxidised HA. First, the rate of oxidation was determined, enabling control of the degree of oxidation (D_{ox}) by controlling the reaction time. Secondly, the partially oxidized HA samples were analysed by size-exclusion chromatography (SEC) with online multi-angle laser light scattering (MALLS) detector, viscosity detector, and concentration sensitive detector (refractive index (RI)). The method provides in a single experiment both the chain length distribution, the distribution in radius of gyration (R_G) (often just referred to as r.m.s. radius), and the distribution of intrinsic viscosity. From these distributions the appropriate averages, e.g. number and weight average molecular weight (M_n and M_w), the weight average R_G ($R_{G,w}$), and the weight average intrinsic viscosity ($[\eta]_w$), the latter corresponding to the average obtained by conventional off-line viscometry. Further, analysis of the R_G-M and [η]-M data for each chromatographic slice provides basis for showing and quantifying the extent of chain compaction, where estimates of the persistence length (q) on basis of the wormlike chain model are obtained. Results are then compared to those obtained previously for alginates.

RESULTS AND DISCUSSION

Oxidation kinetics of hyaluronan: Comparison to mannuronan, alginate and pullulan.

Classical studies have shown that hyaluronan is oxidised extremely slowly by periodate [7]. Moreover, the oxidation of soluble polysaccharides is first order with respect to periodate [8]. Hence, partial oxidation by low periodate/HA ratios to obtain low degrees of oxidation is time consuming. We therefore performed partial oxidation using an excess of periodate (2 moles per GlcA residue) combined with control of the reaction time to obtain a desired degree of oxidation (D_{ox}), defined as moles of periodate consumed per mol of GlcA. Results are shown in Figure 3. The linear curve up of 0.60 allows accurate determination of D_{ox} by controlling the oxidation time.

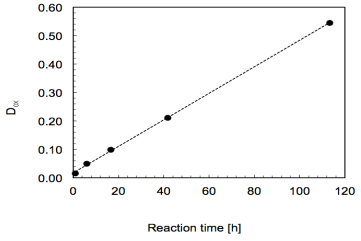

Fig. 3. Oxidation of hyaluronan at 4°C by an excess (2-fold) of periodate. D_{ox} refers to moles of

periodate consumed per mole of GlcA.

Multi-detector SEC analysis of partially oxidised hyaluronan: Molecular weight distributions

Partially oxidised hyaluronan (0-80%) was prepared as described above. Residual periodate was quenched by an excess ethylene glycol. The samples were further treated with $NaBH_4$ to reduce the dialdehydes to the corresponding alcohols. The samples were further analysed by SEC-MALLS including an online viscosity detector. Figure 4 shows the primary results (concentration profiles and slice values for M, R_G and intrinsic viscosity). Figure 5 shows the weight average molecular weight (M_w) as a function of the degree of oxidation. Plots of the $1/M_w$ versus D_{ox} (Figure 5, right axis) were linear, suggesting the degradation can be analysed (or approximated) in terms of the model of random depolymerisation of polymers [9], with the difference that the degradation time (t) is replaced by D_{ox}. The slope of the plot then provides a *pseudo* first order rate constant (k): Slope = $kD_{ox}/2M_0$, where M_0 is the 'monomer' weight of 410 Da [10] for HA (one oxidation site per disaccharide repeating unit). A rate constant of 5.4E-4 is about one order of magnitude lower that that obtained for alginate (data taken from ref. [2]).

This shows that the depolymerisation occurring during periodate oxidation certainly depends much on the chemical structure of the oxidised polysaccharide. The faster degradation of alginates could, for example, be tentatively ascribed to a slightly enhanced preference for oxidation 'doublets' compared to random oxidation, provided the 'double dialdehydes' are especially unstable.

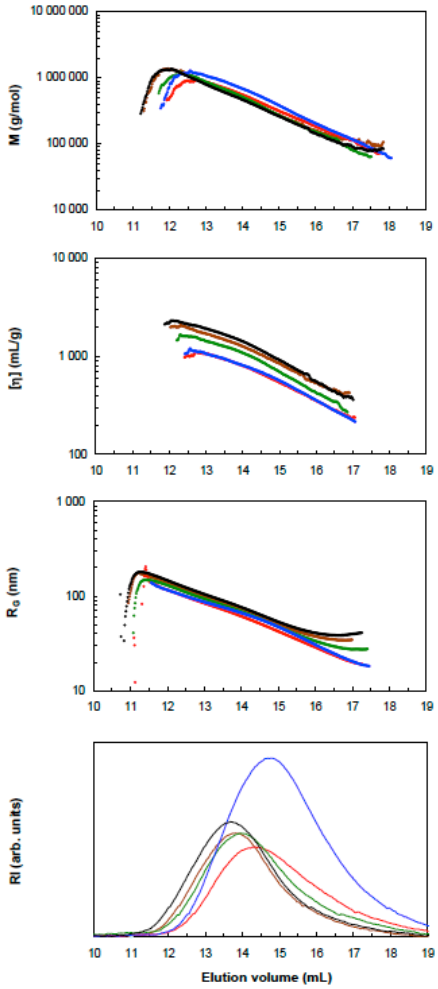

Fig. 4. SEC-MALLS profiles for partially oxidized HA. Top-down: Molecular weights (M),

Intrinsic viscosities ([η]), Radii of gyration (R_G), Concentrations (RI detector response).

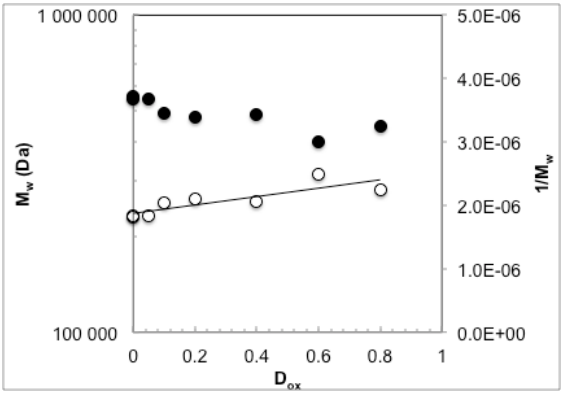

Fig. 5. Dependency of M_w (left axis) of periodate oxidised hyaluronan (filled circles) on the degree of periodate oxidation (D_{ox}) (left axis). Open symbols refer to $1/M_w$ (right axis).

Chain stiffness analysis based on molecular weight dependence of the radius of gyration (R_G).

Figure 6 shows the classical R_G-M plots for the partially oxidized hyaluronan samples (elution slice data). The plots are linear with a slope (0.60 +/- 0.037) essentially independent of D_{ox}, suggesting that the samples all behave as random coils. However, a progressive shift to lower R_G values is observed for increasing degrees of oxidation, which is ascribed to the compaction. Although partially oxidized hyaluronan is inhomogeneous with respect to chain flexibility along the chain due to the assumed random positioning of flexible 'hinges' resulting from oxidation, the wormlike chain model can be applied to calculate an 'apparent' persistence length. The Benoit-Doty model has been previously applied for semi-flexible polysaccharides [11, 12], including their partially periodate oxidized derivatives [3]. The model provides the following analytical expression for the relationship between R_G and M:

$$R_G^2 = \frac{qM}{3M_L} - q^2 + \frac{2q^3M_L}{M}\left[1 - \frac{qM_L}{M}\left(1 - e^{-\left(\frac{M}{qM_L}\right)}\right)\right]$$

Here, q denotes the persistence length, M the molecular weight, and M_L the mass per unit length.

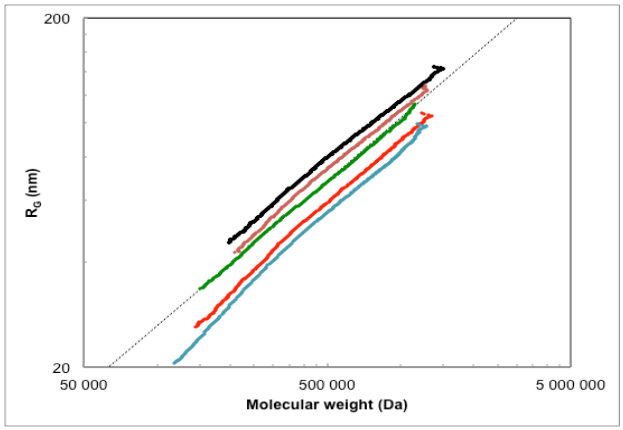

Fig. 6. Molecular weight dependence of the radius of gyration (R_G) for periodate oxidized HA (Top-down: D_{ox} = 0, 0.1, 0.2, 0.4 and 0.8). The data were obtained by SEC-MALLS. The dotted line corresponds to literature values for HA in 0.15 M NaCl [11].

The influence of electrostatics may be incorporated into the model thanks to the model of Odijk [13] by

treating the persistence length as a sum of an intrinsic persistence length (q_0), which reflects the chain extension in the absence of electrostatic forces, and an electrostatic term (q_{el}), which depends primarily on the linear charge density and the ionic strength. Analytical expressions are found in the articles of Mendichi et al. [11] and Higashimura et al.[12]. Using the theoretical values for M_L (410 nm^{-1}) [11] the results shown in Table 1 (column 2 and 3) were obtained.

Table 1. The total persistence length (q) and intrinsic persistence length (q_0) of periodate oxidised hyaluronan obtained from R_G-M data (Odijk model) and [η]-M data (columns 4-7) (Bohdanecky model).

	R_G-M data (Odijk model)		[η]-M data (Bohdanecky model with fixed M_L)		[η]-M data (Bohdanecky model with calculated M_L).	
D_{ox}	q (nm)	q_0 (nm)	M_L	q (nm)	M_L	q (nm)
0	16	8.6	410	11.8	431	12.4
0.05	16	8.5	410	10.8	437	11.1
0.10	14	6.4	410	10.4	430	11.4
0.20	12	4.9	410	8.4	387	7.9
0.40	9	2.7	410	7.1	402	7.0
0.60	7	2.3	410	7.1	540	9.4
0.80	8	2.1	410	6.2	459	6.9

It may be noted the fit of the R_G-M data to the model was not perfect, especially for D_{ox} above 0.10, so data should be treated as 'apparent' or relative values. Nevertheless, both approaches clearly demonstrate the compaction of HA chains upon oxidation.

An interesting feature of Table 1 is the difference in the estimates of q and q_0, respectively. Although it can be argued against the application of the wormlike chain model to the highly flexible chains, it appears the extension of the most oxidized chains is governed by electrostatic repulsion (between carboxyl groups). This implies a high sensitivity of the chain extension (or compaction) to ionic strength, in line with earlier findings for periodate oxidized alginates [3, 14].

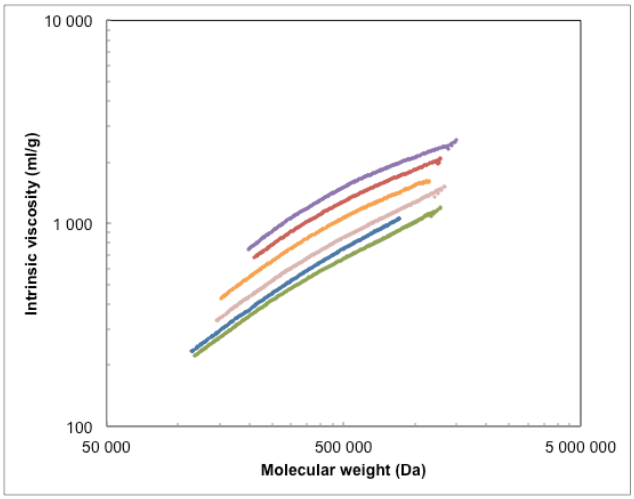

Fig. 7. Molecular weight dependence of the intrinsic viscosity ([η]) for periodate oxidized HA (Top-down: D_{ox} = 0, 0.1, 0.2, 0.4, 0.6 and 0.8). The data were obtained by SEC-MALLS (including an online viscosity detector)

Chain stiffness analysis based on molecular weight dependence of the intrinsic viscosity.

Figure 7 shows the classical MHS plots (intrinsic viscosity as a function of molecular weight) for elution slice data in Figure 4. As observed earlier for periodate oxidized alginates [3, 14] and chitosans [5], periodate oxidation of hyaluronan is accompanied by a reduction of the intrinsic viscosity (when compared at constant molecular weight). The plots in Figure 7 show qualitatively the same features as for R_G-M, i.e. a shift to lower values with increasing degree of oxidation. However, the curves are not

linear as predicted by the MHS equation, but show curvature. According to Mendichi et al. [11] this can be ascribed to '..the "partial draining" nature of charged chains changes with their length, that is, their hydrodynamic volume changes with M..'.

For simple analysis according to the wormlike chain model the approach introduced by Bohdanecky [15] was applied, where data are transformed (linearized) and presented as plots of $(M^2/[\eta])^{1/3}$ as a function of $M^{1/2}$. In theory, such plots should be linear, providing the mass per unit length (M_L) and the persistence length (q) from a linear fit. The approach is identical to that applied earlier to oxidized alginates [3] and chitosans [5], as well as unoxidized hyaluronan [11]. The reader is referred to the latter articles for detailed equations and their validities. In practise, the plots were not strictly linear but tended to end upwards for the highest molecular weights. Therefore, only the linear part corresponding to lower molecular weights were used and presented (Figure 8). In this way excluded volume effects are minimised, which is a prerequisite for applying the procedure of Bohdanecky. The results from the calculations are included in Table 1. By keeping M_L fixed to the theoretical value of 410 nm^{-1} we observe a steady decrease in persistence length from 11.8 to 6.2 nm. The initial value (D_{ox} = 0) is somewhat larger than that obtained by Mendichi et al. [11] using 0.15 M NaCl as solvent, but closer to the values (9-15 nm) given by Gamini et al. [10]. Using the 'original' method of Bohdanecky, where both M_L and persistence length is calculated from the fitted data, we obtain only small changes. The mass per unit length will increase slightly as a result of the oxidation since the open (oxidised) residue will have an average distance between the glycosidic oxygen atoms of 0.424 nm [3]. The intact GlcA residue has a distance of 0.51 nm. The theoretical (average) M_L should then equal $410(1\text{-}D_{ox}) + 448D_{ox}$. We are not able to detect a systematic increase in M_L corresponding to the theoretical increase with increasing D_{ox}, but data are at least in the range expected from theory. Hence, the decrease in persistence length upon periodate oxidation is well documented.

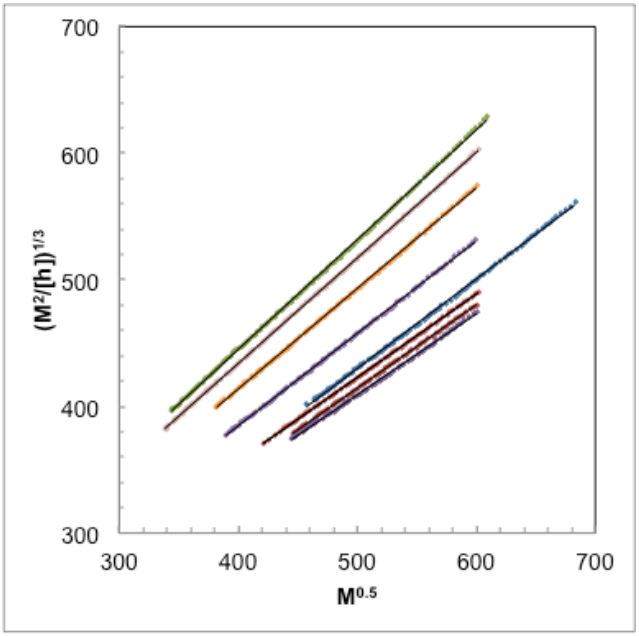

Fig. 8. Bohdanecky plots (linear part only) for periodate oxidized HA (Down-top: D_{ox} = 0 (unoxidised), 0 (oxidised and immediately quenched), 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8).

Conclusions

Partial periodate oxidation of hyaluronan provides highly flexible dialdehydes only for the GlcA residues, preventing an influence from adjacent oxidized residues. Compared to alginates hyaluronan undergoes less degradation during oxidation. Retention of high molecular weight and high R_G in HA during oxidation allows chain stiffness analysis based in the R_G-M data. In contrast, the more rapid degradation of alginates prevents such analysis, as accurate R_G data can only be obtained by light scattering when R_G exceeds $\lambda_0/20$ (approximately).

The introduction of flexible residues leads to compaction of both hyaluronan and alginates, as shown experimentally by obtaining R_G-M and [η]-M data using multidetector SEC. Application of the

wormlike chain model demonstrated a progressive decrease in total and intrinsic persistence length with increasing degree of oxidation. Due to the polyelectrolytic character of hyaluronan, a relatively large electrostatic contribution to the persistence length was observed. In the absence of electrostatic repulsion (i.e. high ionic strength) highly oxidized hyaluronan approaches flexible polysaccharides such as pullulan, which have high flexibility due to 1,6-linkages.

Acknowledgements

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