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Study on TEMPO-mediated selective oxidation of hyaluronan and the effects of salt on the reaction kinetics

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Abstract

2,2,6,6-Tetramethyl-1-piperidinyloxy radical (TEMPO)-mediated oxidation of hyaluronan was studied at pH 10.2 and temperature of 0 °C with NaOCl as the primary oxidant. As with other polysaccharides, a high selectivity of oxidation was observed. The degradation of the polymer was essentially caused by the oxidation process. The primary oxidant and the pH of the reaction mixture did not alter the molecular weight of hyaluronan during oxidation. The kinetics of the oxidation process was investigated at different concentrations of reactants and the inorganic salts, NaBr, NaCl, and Na_2SO_4. An increase in the salt concentration in the mixture causes a major decrease in the rate of the oxidation, and this decrease is independent of the nature of the salt.

Keywords: Hyaluronan; TEMPO; Oxidation kinetics; IR spectroscopy

1. Introduction

Relatively few oxidation procedures are available for the selective transformation of primary alcohol groups in the presence of secondary ones, which is the situation with many polysaccharides. The early work of Ganem [1] and Semmelhack et al. [2] introduced a promising method, using the nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy radical (TEMPO), for catalyzing the oxidation of alcohols and it has been widely used in organic synthesis [3–6]. This mild and selective method usually oxidizes primary alcohols with high selectivity while leaving secondary alcohol groups unaffected. The actual oxidant in this reaction is the nitroxyl radical in the form of a nitrosonium cation, which is continuously regenerated by another oxidant present in the reaction mixture. The oxidation can thus proceed to a high yield with only a catalytic amount of TEMPO [6–8].

Since the introduction of this method in carbohydrate chemistry for the selective modification of primary alcohol groups, numerous publications have described TEMPO-mediated oxidation of polysaccharides. De Nooy et al. [9] studied the oxidation of glucans and the effects of several parameters, such as the concentrations of TEMPO and sodium bromide. Chang and Robyt [10,11] used this method to modify several different polysaccharides and

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observed improved water solubility of the oxidized polysaccharides. The TEMPO-catalyzed transformations of pullulan [12], chitin [13], and galactomannans [14] have also been reported. However, the mechanism of TEMPO-mediated oxidation is still not well established.

Hyaluronan is a linear polysaccharide of high molecular weight whose disaccharide repeating-unit has *N*-acetyl-D-glucosamine β-(1→4)-linked to D-glucuronic acid, and the repeating units are β-(1→3)-linked to form the hyaluronan chain [15,16]. This biopolymer has found wide applications in the cosmetic industry and in drug delivery system because of its high water retention capacity and good biodegradability. Chemical modifications to improve its performance have targeted essentially alkylation of the hydroxyl and carboxylate groups of hyaluronan [17,18]. There has been no report on the oxidation of this polysaccharide, probably because carboxylate groups already exist in the hyaluronan chain. However, the introduction of additional carboxylate groups along the polymer chain is expected to alter the properties of this polysaccharide to afford, for example, improved water solubility for the modified polysaccharide, as in hyaluronan partially esterified on the D-glucuronic unit. However, the presence of more carboxylate groups in the polymer chain may also have repercussion on the TEMPO-mediated oxidation process. Bearing all of this in mind, the present work was intended to study the TEMPO-mediated oxidation of hyaluronan and to examine factors influencing the oxidation process.

2. Experimental

Materials.—Hyaluronan (M_W 1.4×10^6) was obtained from Soliance Cy, Pomacle France. TEMPO was purchased from Aldrich. All other chemicals were commercial products of analytical grade and were used without further purification.

Oxidation procedure.—A typical oxidation experiment was carried out as follows.

Hyaluronan (1 g, 2.5 mmol) was dissolved in 200 mL distilled water and TEMPO (0.027 mmol) and a determined quantity of sodium bromide or other salt (see Table 1) were added. The mixture was stirred at about 400 rpm and cooled to 0 ± 1 °C, and N_2 gas was passed through the reactor. The pH of the solution was brought to ~9 with NaOH solution. At time zero, 4 mL (or 8 mL) of a 13% sodium hypochlorite solution, whose pH had previously been adjusted to 10, was added to the mixture. The reaction rate was then monitored by the consumption of a solution of NaOH (0.19 M). The pH of the mixture, controlled with a pH-meter, was maintained at 10.2.

Table 1
Experimental conditions of TEMPO-mediated oxidation of hyaluronan [a]

	Run	NaOCl (mmol)	NaBr (mmol)	NaCl (mmol)	Na_2SO_4 (mmol)	Oxidation rate, linear region (μmol/min)
A	OA-1	7	0.0	/	/	43.3
	OA-2	7	0.5	/	/	41.7
	OA-2a [b]	7	0.5	/	/	44.4
	OA-3	7	3.5	/	/	42.3
	OA-4	7	/	3.5	/	18.2
B	OB-1	14	0.0	/	/	25.1
	OB-2	14	1.0	/	/	29.7
	OB-3	14	3.5	/	/	38.8
	OB-4	14	/	3.5	/	24.0
C	OC-1	7	/	/	3.5	17.8
	OC-2	7	/	/	1.8	18.3
	CT [c]	7	0.5	/	/	/

[a] General conditions: pH 10.2, $T = 0$ °C, TEMPO = 0.027 mmol, hyaluronan = 2.5 mmol in 200 mL of water.
[b] Oxidation OA-2a was stopped at an earlier stage than OA-2.
[c] CT, control test without TEMPO.

At the end of the oxidation, 0.1 g of $NaBH_4$ and 5 mL of EtOH were added to the mixture, which was stirred for ~1 h more. The solution was then adjusted to pH 8 and 12 g of NaCl was added. After complete dissolution of the NaCl, the oxidized hyaluronan was precipitated with EtOH and washed successively with 100 mL of EtOH–water (4:1, 9:1, 19:1 and 100:0). The products were then dried under diminished pressure at room temperature.

IR analysis.—Dried samples of initial and oxidized hyaluronans were dissolved in water at ~2 g/L and 1-mL aliquots of these solutions were freeze-dried. The dried samples (1 mg) were then dispersed in 100 mg of dried KBr and pressed to make the test samples for IR analysis. The IR spectra were recorded on a Perkin–Elmer FT-IR 1720X instrument.

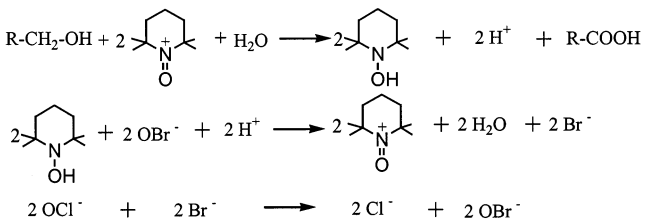
To obtain the IR spectra of hyaluronans in their acidic forms, 4 mL of hyaluronan solution (2 g/L) was treated with ~2 g of the ion exchanger Dowex 50W-X8 (H^+ form), which transformed the polysaccharides in their carboxylic acid forms. The filtered solutions were then used to prepare the test samples according to the preceding procedure.

^{13}C NMR spectra.—In order to obtain a solution of sufficiently high concentration for ^{13}C NMR analysis, samples of initial and oxidized hyaluronans were first hydrolyzed in a solution of 1 M HCl at 50 °C for ~40 min, and then isolated and purified. This treatment decreased the molecular weight of hyaluronan to about 60,000 without any modification of the chemical structure. Solutions of the hydrolyzed samples at a concentration of 25 g/L in D_2O were prepared, and ^{13}C NMR spectra were obtained with a Bruker AC-300 instrument at 70 °C.

Gel permeation chromatography.—The molecular weights of several oxidized hyaluronans were determined by GPC. The hyaluronan samples were dissolved in distilled water at a concentration of 1 g/L. The eluent was 0.1 M aqueous $NaNO_3$. A Waters-150C apparatus was used with two columns (Shodex B-804 and Shodex B-805) in series. The apparatus was equipped with a multiangle laser light scattering detection system DAWN DSP-F from Wyatt Technology, USA, which permitted the determination of the absolute molecular weights of the polymers.

3. Results and discussion

The TEMPO-mediated oxidation of alcohols is a complex reaction. When TEMPO is added in a catalytic amount, the oxidation process involves several reaction steps, as shown in Scheme 1.



Scheme 1. TEMPO-mediated oxidation process.

Oxidation of 1 mol of primary alcohol to the carboxylic acid requires 2 mol of the primary oxidant NaOCl. The OBr^- anion is a more reactive oxidant than OCl^- and the reaction rate can be greatly enhanced by the addition of NaBr [9,10]. Nevertheless, when there is no NaBr present in the mixture, NaOCl can serve for regenerating the nitrosonium cation from hydroxylamine. It is worthy noting that some authors [19] proposed that regeneration of the nitrosonium cation could also be accomplished by using equimolar proportions of nitrosonium cation and hydroxylamine, producing two molecules of TEMPO radical, which are then oxidized by the primary oxidant NaOCl to give the nitrosonium cations. However, where the concentration of TEMPO is very low, as in our experiments in the range of 10^{-5} M, the direct oxidation of hydroxylamine by the primary oxidant has a much greater chance of taking place.

In order to examine factors influencing the process of TEMPO-mediated oxidation, several oxidation experiments were conducted under different conditions. The concentrations of reactants in the experiments are listed in Table 1.

To eliminate the effect of possible direct oxidation of hyaluronan by NaOCl, a control

experiment was performed where all of the reactants were added except TEMPO. After 90 min, consumption of NaOH was negligible proving that no direct oxidation of hyaluronan by NaOCl occurred during the process.

The molecular weight of hyaluronan and selected samples of oxidized hyaluronan were determined by GPC (Table 2). The sample for the control run showed almost no change in M_W, while all oxidized samples had molecular weights clearly lower than that of initial hyaluronan. When the concentration of NaOCl was doubled, the decrease in M_W remained roughly the same (OA-2 and OB-2), while if the reaction was stopped at an earlier stage (experiment OA-2a), a less pronounced decrease in M_W was observed. This indicates that the polymer was not degraded by contact with NaOCl, but the oxidation process itself was the essential cause of the degradation of the polymer.

IR and ^{13}C NMR spectra of initial and oxidized hyaluronans were recorded to evaluate possible changes in structure after oxidation. The IR spectra of hyaluronan samples in their acidic forms were used to determine the degree of oxidation (τ). The carboxylic acid ($-CO_2H$) band at 1728 cm^{-1} is well separated from the amide group (–CONH–) bands at 1645 and 1555 cm^{-1} (Fig. 1(c, d)). Using the band at 1555 cm^{-1} as the reference and setting the baseline and splitting of the bands as shown in Fig. 1(c), the areas of the bands at 1728 and 1555 cm^{-1} of the absorbance spectra of initial and oxidized samples permitted calculation of the values of τ (Table 2). Good agreement was observed between the values of τ obtained from IR and from NaOH consumption. It is emphasized that the value of nearly zero found for the degree of oxidation of the control test sample from IR spectroscopy, shows unambiguously that there is no oxidation of hyaluronan when TEMPO is absent from the reaction mixture. In the IR spectra of neutralized hyaluronan samples (Fig. 1(a, b)), the $-CO_2Na$ group gives rise to a band that is shifted to longer wavelength (1550–1650 cm^{-1}). The absorption bands of the $-CO_2Na$ and –CONH– groups are not resolved well enough to allow good quantitative evaluation of the values of degree of oxidation. However, no absorption band was observed in the 1730 cm^{-1} region for the oxidized hyaluronan (Fig. 1(b)), indicating that this sample contained no carbonyl group and providing good evidence that only the primary alcohol of hyaluronan was oxidized to carboxylate by the TEMPO-mediated oxidation.

Fig. 2 shows ^{13}C NMR spectra. Similar ^{13}C NMR spectra of hyaluronan have been reported previously and the signals assigned [20]. The resonance at 61.8 ppm corresponds to C-6 of the *N*-acetyl-D-glucosamine unit. The carboxylate and acetamido carbons resonate at 175 and 173 ppm, respectively. Several new resonances in the spectrum of the oxidized sample result from formation of a new sugar unit in the polymer chain, but no resonance in the 198–205 ppm region was observed, indicating no ketone group formation during the oxidation. The spectra of oxidized hyaluronan shows a decrease in intensity of the resonance at 61.8 ppm for the primary alcohol group and an increase in the resonance at 175 ppm for the carboxylate group. The resonance at 55.3 ppm for C-2 of the *N*-acetyl-D-glucosamine residue was not al-

Table 2
Evaluation of degree of oxidation and effect of oxidation on the molecular weight of hyaluronan [a]

	HA	CT	OA-1	OA-2	OA-2a	OB-1	OB-2	OC-2
Reaction time (min)	/	90	70	66	26	90	80	106
τ (NaOH)	/	0.00	0.71	0.69	0.31	0.61	0.51	0.55
τ (IR)	/	0.06	0.67	0.78	0.30	0.51	0.58	0.57
M_W (10^5)	13.5	12.4	6.9	5.9	7.8	5.1	5.7	6.5

[a] τ is the degree of oxidation which is equal to the number of moles of oxidized primary alcohol group per repeat unit. τ (NaOH) are the values of τ calculated from the total amount of NaOH consumed. τ(IR) are the values of τ determined by IR spectroscopy.

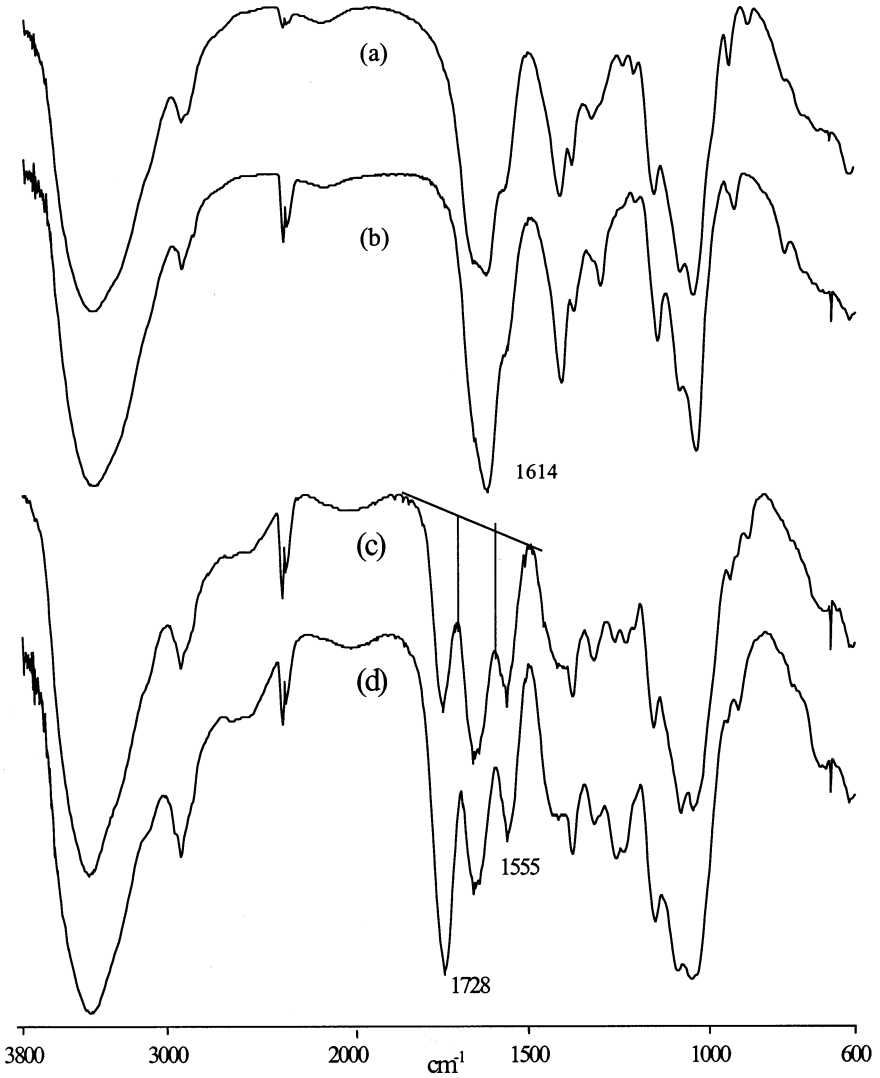


Fig. 1. IR spectra of hyaluronan. (a) Hyaluronan sodium salt, (b) oxidized hyaluronan sodium salt, (c) hyaluronan in acidic form, (d) oxidized hyaluronan in acidic form.

tered by the oxidation. Integrations of the resonances for C-2 and C-6 (61.8 ppm) of the *N*-acetyl-D-glucosamine residue gave the values of 0.97 and 0.86 for hyaluronan and 0.98 and 0.20 for oxidized hyaluronan (Sample OA-2), respectively. The integrations permitted calculation of the degree of oxidation, and a value of 0.78 was found for the sample OA-2, in agreement with the values of 0.69 and 0.78 obtained from the consumption of NaOH and IR spectroscopy, respectively.

Fig. 3 shows a typical kinetic plot of TEMPO-mediated oxidation of hyaluronan as monitored by the consumption of 0.19 M NaOH. Three phases were observed: a latent phase, a linear region in which oxidation proceeds with a constant rate, and finally an asymptotic region corresponding to a decrease in the rate of carboxylic acid production. The same behavior was observed in all of the oxidation experiments performed in this work. The existence of latent and asymptotic phases is probably due to the generation of nitrosonium cations at the beginning of the reaction, diffusion of reactants, background oxidation and perhaps availability of some reactants, but these problems are not examined here. The interesting value from the kinetic study is the slope of the linear region, which gives a measure of the rate of oxidation. These values are listed in Table 1, where the rate of oxidation is defined as the μmol of NaOH consumed per min by the reaction mixture in order to maintain the pH at 10.2.

Comparing the oxidation experiments performed with and without NaBr, it is surprising to find that the reaction rate was nearly independent of the concentration of NaBr (Fig. 3). To investigate further the effect of NaBr and NaOCl, several oxidation experiments were performed with a higher concentration of NaOCl and at different NaBr concentrations. The kinetics of the reactions are given in Fig. 4, and the rates of oxidation in the linear region are listed in Table 1. An acceleration of the oxidation was then observed, and the rate of reaction increased with increase of the amount of NaBr added to the mixture, in accord with previous results [9,10]. However the different behavior of NaBr on the rate of TEMPO-mediated oxidation at lower and higher concentrations of NaOCl seems to be a contradiction.

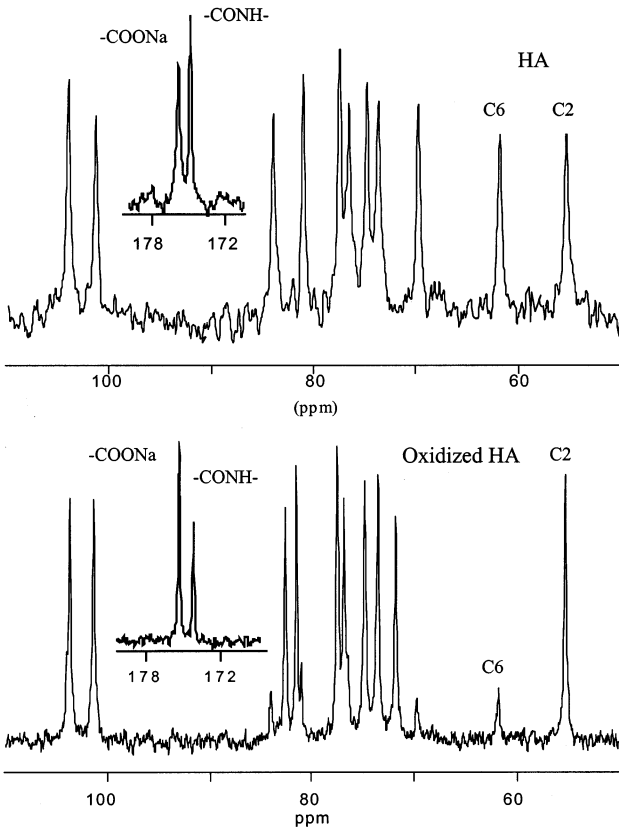


Fig. 2. ^{13}C NMR of hyaluronan and oxidized hyaluronan.

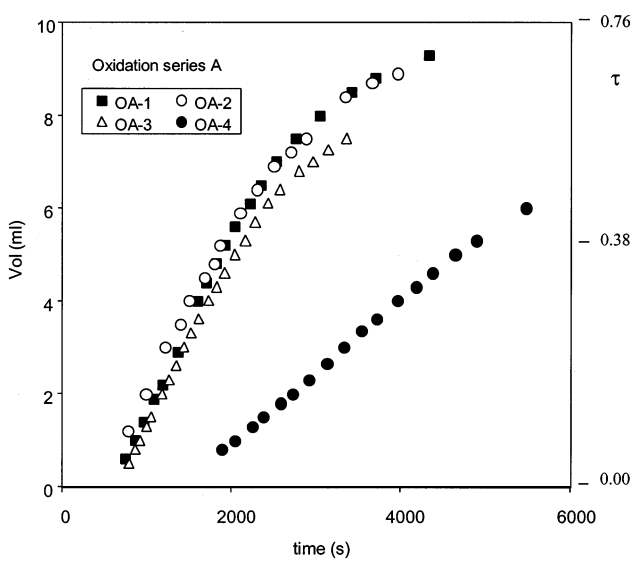


Fig. 3. The kinetic course of TEMPO-mediated oxidation of hyaluronan.

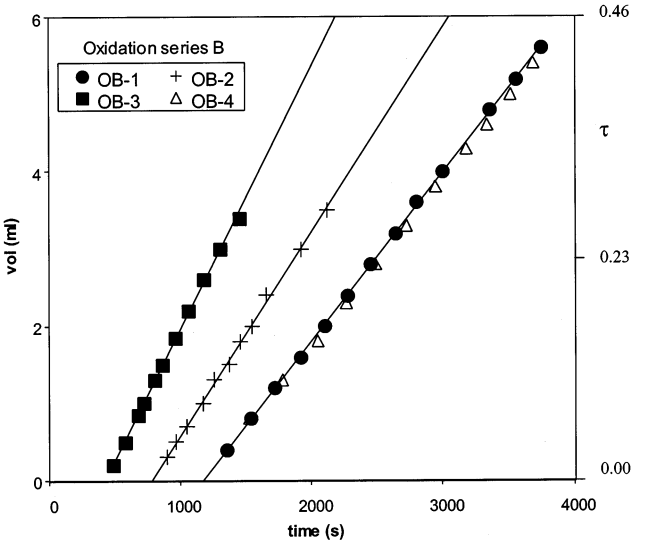


Fig. 4. The kinetics of TEMPO-mediated oxidation of hyaluronan at higher concentration of NaOCl: effect of NaBr on the oxidation rate.

To reconcile these observations, it is necessary to consider the ionic nature of the oxidant and the hyaluronan chain. The most probable cause of this apparently contradictory kinetic behavior may be attributable to the effect of ionic strength in the reaction mixture. The actual oxidant is the nitrosonium cation and the hyaluronan is a polyanion, and so when the ionic strength increases in the reaction mixture by the addition of salt, the coulombic attraction of the nitrosonium cation by the anionic polymer chain is screened. The local concentration of the nitrosonium cations along the hyaluronan chains becomes lower, leading to a decrease in the rate of oxidation. This screening effect reaches an upper limit and further additions of salt have no more adverse effect on the rate of oxidation. The fact that an upper limit exists for this salt effect is quite reasonable in view of the very low concentration of TEMPO in the reaction mixture.

Thus, in the experiments shown in Fig. 3, as the concentration of NaOCl was relatively low, upon addition of NaBr, the oxidation rate was on the one hand accelerated by Br^-. On the other hand, the addition of NaBr resulted in an increase in ionic strength of the reaction mixture, contributing to a decrease of the rate of the oxidation reaction, as already discussed. The accelerating effect of NaBr as a more reactive oxidant was compensated by the decelerating effect due to an increase in ionic strength, and so no apparent acceleration of the rate of oxidation is observed.

In another series of experiments (Fig. 4), the concentration of NaOCl was doubled and, as NaOCl is also a salt, the ionic strength in the reaction mixture was raised and could now be near its upper limit, so that further addition of salt would have no more adverse effect on the kinetics of the process. With the addition of NaBr, only the accelerating effect of the NaBr salt was apparent, and this therefore resulted in a clear acceleration of the oxidation rate. If NaCl was added instead of NaBr, at low concentration of NaOCl (OA-4), a sharp decrease in the oxidation rate was observed, while at high concentration of NaOCl (OB-4), neither acceleration nor deceleration was observed, indicating that the upper limit of salt concentration was reached in the latter case.

Oxidation experiments were also performed by adding Na_2SO_4 (OC-1, OC-2). As expected, a decrease in the oxidation rate was observed, which proves that the decrease in the rate of oxidation depends only on the total concentration of salt in the mixture, and not on the nature of the salt. Higher concentration of Na_2SO_4 caused no more decrease in the oxidation rate, which is again an indication of the existence of an upper limit of the concentration of salt in the reaction mixture beyond which no more adverse effect of the salt is observed.

Conclusions

The TEMPO-mediated oxidation of primary alcohol groups of polysaccharides is a highly selective but rather complex process. The molecular weight of the polymer is not altered by the primary oxidant (NaOCl) and the oxidation process itself is responsible for the degradation of the polymer. The kinetics of the oxidation depends on several factors, among which the attraction of the nitrosonium cation by the ionic hyaluronan chains is important. The experiments clearly showed that the rate of TEMPO-mediated oxidation decreased with increase in the ionic strength of the reaction mixture. This may be explained by a decrease in the local concentration of oxidant species around the polyelectrolyte chains.

References

[1] B. Ganem, *J. Org. Chem.*, 40 (1975) 1998–1999.
[2] M.F. Semmelhack, C.S. Chou, D.A. Cortes, *J. Am. Chem. Soc.*, 105 (1983) 4492–4494.
[3] P.L. Anelli, C. Biffi, F. Montanari, S. Quici, *J. Org. Chem.*, 52 (1987) 2559–2562.
[4] M. Yamaguchi, T. Takana, T. Endo, *J. Org. Chem.*, 55 (1990) 1490–1492.
[5] A.E.J. de Nooy, A.C. Besemer, H. Van Bekkum, *Synthesis*, (1996), 1153–1174.
[6] M. Zhao, J. Li, E. Mano, Z. Song, D.M. Tschaen, E.J.J Grabowski, P.J. Reider, *J. Org. Chem.*, 64 (1999) 2564–2566.
[7] S. Renata, S. Jacek, M. Jacek, *Tetrahedron Lett.*, 31 (1990) 2177–2180.
[8] N.J. Davis, S.L. Flitsch, *Tetrahedron Lett.*, 34 (1993) 1181–1184.
[9] A.E.J. de Nooy, A.C. Besemer, H. Van Bekkum, *Carbohydr. Res.*, 269 (1995) 89–98.
[10] P.S. Chang, J.F. Robyt, *J. Carbohydr. Chem.*, 15 (1996) 819–830.
[11] P.S. Chang, J.F. Robyt, *Carbohydr. Lett.*, 3 (1998) 31–38.
[12] A.E.J. de Nooy, A.C. Besemer, H. Van Bekkum, J.A.P.P. Van Dijk, J.A.M. Smit, *Macromolecules*, 29 (1996) 6541–6547.
[13] R.A.A. Muzzarelli, A. Cosani, M. Terbojevich, *Carbohydr. Polym.*, (1999), in press.
[14] M.R. Sierakowski, M. Milas, J. Desbrieres, M. Rinaudo, *Carbohydr. Polym.*, 42 (2000) 51–57.
[15] L. Lapcik, J.L. Lapcik, S.D. Smedt, J. Demeester, P. Chabrecek, *Chem. Rev.*, 98 (1998) 2663–2684.
[16] M.B. Brown, C. Marriott, G.P. Martin, *Int. J. Tiss. React.*, 17 (1995) 133–140.
[17] T. Pouyani, J. Kuo, G.S. Harbison, G.D. Prestwich, *J. Am. Chem. Soc.*, 114 (1992) 5972–5976.
[18] D. Campaccia, P. Doherty, M. Radice, P. Brun, G. Abatangelo, D.F. Williams, *Biomaterials*, 19 (1998) 2101–2127.
[19] H. Van Bekkum, A.C. Besemer, *Carbohydr. Eur.*, 13 (1995) 16–21.
[20] M.K. Cowman, D.M. Hittner, J. Feder-Davis, *Macromolecules*, 29 (1996) 2894–2902.