Carbohydrate Polymers 130 (2015) 69–76

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Stoichiometric C6-oxidation of hyaluronic acid by oxoammonium salt $TEMPO^+Cl^-$ in an aqueous alkaline medium

Irina Yu Ponedel'kina*, Elvira A. Khaibrakhmanova, Tatyana V. Tyumkina, Irina V. Romadova, Victor N. Odinokov

Institute of Petrochemistry and Catalysis, Russian Academy of Sciences, Ufa 450075, Russian Federation

ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form 8 April 2015
Accepted 21 April 2015
Available online 1 May 2015

Keywords:
Hyaluronic acid
Oxidation
2,2,6,6-Tetramethylpiperidine-1-oxoammonium
chloride
Hydroxylamine TEMPOH
Radical TEMPO

Chemical compounds studied in this article:
Hyaluronic acid (CID 3084049)
2,2,6,6-Tetramethylpiperidine-1-oxyl (CID 2724126)

ABSTRACT

This paper reports the selective oxidation of hyaluronic acid (HA) by stoichiometric quantity of 2,2,6,6-tetramethylpiperidine-1-oxoammonium chloride ($TEMPO^+$) in aqueous alkaline medium. High efficiency of the HA oxidation and quantitative yield of carboxy-HA per starting $TEMPO^+$, as well as unusual behavior of the oxidation system generating an oxygen upon alkali-induced oxoammonium chloride decomposition are demonstrated. The scheme for HA oxidation involving both $TEMPO^+$ and oxygen produced upon the $TEMPO^+Cl^-$ decomposition and/or air oxygen is proposed. For comparison, the data on stoichiometric oxidation of such substrates as dermatan sulfate, water-soluble potato starch, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and ethanol are presented.

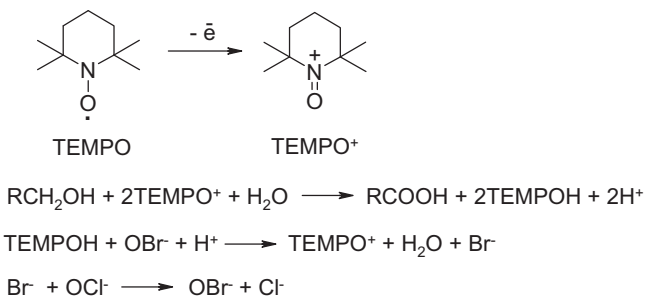
1. Introduction

Hyaluronic acid (HA) is a natural heteropolysaccharide of a linear structure belonging to the class of glycosaminoglycans and consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine units. The biocompatibility, water-retaining and reparative and regenerative properties, and the ability to form high-viscosity aqueous solutions (hydrogels) are valuable properties of HA demanded in medicine and cosmetics. By derivatization at the carboxyl, hydroxyl, or acetamido groups, it is possible to modify the physicochemical and biological properties of HA and to develop new products for biomedicine and cosmetics (Kogan, Šoltés, Stern, & Gemeiner, 2007; Mero & Campisi, 2014; Prestwich, 2011; Schanté, Zuber, Herlin, & Vandamme, 2011). For example, oxidation of the C6 primary hydroxyl groups of the *N*-acetyl-D-glucosamine unit of HA affords polyuronic acid, i.e. carboxy-HA (or percarboxylated HA) (Bellini, Crescenzi, & Francescangeli, 2002; Ponedel'kina, Odinokov, Saitgalina, & Dzhemilev, 2007), which appreciably differs in properties from natural HA. Carboxy-HA has a better solubility in water (Crescenzi, Francescangeli, Renier, & Bellini, 2001) and an unusually high stability to the action of testicular hyaluronuridase (Ponedel'kina et al., 2007), which is important for the development of prolonged action drugs. Being a promising biomaterial and a carrier for other biomolecules, carboxy-HA is an attractive object for conjugation with physiologically active amines (Bellini et al., 2002; Ponedel'kina, Lukina, Khaibrakhmanova, Sal'nikova, & Odinokov, 2011).

The C6-oxidation of HA is usually performed with an oxidative system comprising sodium hypochlorite as a stoichiometric oxidant, the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) nitroxyl radical as a catalyst, and sodium bromide as a co-catalyst (Scheme 1). Higher conversion of the primary alcohol groups of HA to carboxy groups (80–100%) is achieved by oxidation in an alkaline medium (pH 10), at a temperature of ~0 °C, and a reaction time of 70–80 min (Crescenzi et al., 2001; Jiang, Drouet, Milas, & Rinaudo, 2000). On the other hand, upon oxidation of HA with sodium hypochlorite in a concentrated aqueous solution of $NaHCO_3$ or with trichlorocyanuric acid in aqueous DMF, aldehyde hydrate groups (5–18%) can be introduced into the macromolecules (Buffa, Kettou, Pospišilová, Berková, & Velebný, 2011). Aldehyde hydrates

* Corresponding author. Tel.: +7 347 284 2750; fax: +7 347 284 2750.
E-mail address: ponedelkina@rambler.ru (I.Y. Ponedel'kina).

http://dx.doi.org/10.1016/j.carbpol.2015.04.054
0144-8617/



Scheme 1. Oxidation of primary hydroxyl groups of polysaccharides by the TEMPO/NaOCl/NaBr system in water.

and aldehydes are known to be intermediates in the TEMPO oxidation of alcohols to carboxylic acids (Bragd, van Bekkum, & Besemer, 2004; Ponedel'kina, Khaibrakhmanova, & Odinokov, 2010).

According to the generally accepted scheme of C6-oxidation of polysaccharides with the NaOCl–NaBr–TEMPO system in aqueous alkali, the active oxidant is the $TEMPO^+$ oxoammonium cation formed *in situ* from TEMPO on treatment with hypohalite. As the hydroxymethyl group is oxidized to the carboxy group, $TEMPO^+$ is reduced to the hydroxylamine TEMPOH. Then TEMPOH is oxidized to $TEMPO^+$ by NaOBr formed from NaBr under the action of hypochlorite (Bragd et al., 2004; Jiang et al., 2000).

Oxidation of polysaccharides, including HA, by a stoichiometric amount of oxoammonium salts, has not been performed previously. Meanwhile, this method has long been used for selective oxidation of low-molecular-weight primary and secondary alcohols to aldehydes (or carboxylic acids) and ketones both in organic and aqueous medium (Bobbitt, Bartelson, Bailey, Hamlin, & Kelly, 2014; De Nooy, Besemer, & van Bekkum, 1996; Golubev, Rozantsev, & Neiman, 1965; Luderer et al., 2011; Qiu, Pradhan, Blanck, Bobbitt, & Bailey, 2011). This paper reports the first application of this effective method for the oxidation of HA in order to prepare carboxy-HA. As the stoichiometric oxidant, we have chosen 2,2,6,6-tetramethylpiperidin-1-oxoammonium chloride ($TEMPO^+Cl^-$) as it is easy to prepare and readily soluble in water.

The mechanisms of both catalytic and stoichiometric oxidation of alcohols with oxoammonium cation under basic conditions have not been ultimately established. The mechanism considering the formation of the intermediate complex of alkoxide anion and the oxoammonium cation followed by intramolecular hydrogen atom transfer resulting in the aldehyde (ketone) and TEMPOH as the key step is regarded as most likely (Scheme 2) (Bailey, Bobbitt, & Wiberg, 2007; Semmelhack, Schmid, & Cortés, 1986). The main reasons in favor of this mechanism are the quantitative yields of target products and the absence of by-products.

However, it has been found not long ago that the selectivity of oxidation by oxoammonium salts and the composition of products can depend substantially on the alcohol structure and oxidation conditions. For instance, upon oxidation with 4-acetylamino-2,2,6,6-tetramethyl-1-oxopiperidinium tetrafluoroborate (Bobbitt's salt) in the presence of pyridine bases (in CH_2Cl_2), primary alcohols containing an oxygen atom (of the ether group) in the β-position to the alcohol group are over-oxidized to dimeric esters, and their yield depends on the nature of the pyridine base (Bobbitt et al., 2014; Merbouh, Bobbitt, & Brückner, 2004). The mechanism of this over-oxidation of alcohols containing β oxygen has not yet been elucidated.

N-Acetyl-D-glucosamine unit in HA also contains an oxygen atom (pyranosyl) in the β-position to the hydroxymethyl group, and under basic conditions, this can affect the selectivity of oxidation and the structure of oxidized products. Meanwhile, the known ability of cation $TEMPO^+$ to decay in aqueous alkalis (Endo, Miyazawa, Shiihashi, & Okawara, 1984; Golubev et al., 1965; Golubev & Sen', 2011) or to react with hydroxylamine TEMPOH (Eq. (1)) (Israeli et al., 2005) can also affect the efficiency of HA oxidation.

$$TEMPO^+ + TEMPOH \rightarrow 2TEMPO + H^+ \quad (1)$$

Therefore, we studied both the products of HA oxidation and the transformations of the $TEMPO^+$ cation. We believe this study could be helpful for better understanding of the mechanism of both stoichiometric and catalytic oxidation of alcohols with participation of TEMPO derivatives.

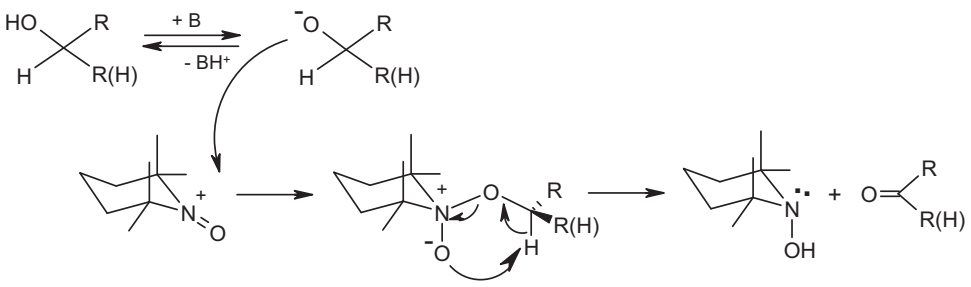
2. Experimental

2.1. Materials

In the oxidation experiments two samples of sodium hyaluronate were used: HMW HA from bacterial source ($M = 1.5 \times 10^6$, supplier Leko Style, St.-Petersburg) and LMW HA from human umbilical cord ($M = 4–6 \times 10^4$), which was isolated as described elsewhere (Ponedel'kina et al., 2005). Other polysaccharides and low-molecular-weight compounds used in the oxidation were: dermatan sulfate (DS) from pig skin, which was prepared as elsewhere (Ponedel'kina, Khaibrahmanova, Odinokov, Khalilov, & Dzhemilev, 2010), starch (Sigma–Aldrich), ethanol and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (MeNAG, for preparation see Section 2.3). TEMPO, 2-acetamido-2-deoxy-D-glucopyranoside (D-GlcNAc), sodium dithionite $Na_2S_2O_4$ and cation exchanger Dowex 50WX4 were purchased from Acros Organics; NaOD and DCl were purchased from Sigma–Aldrich; D_2O was bought from Eurisotop. Molar extinctions of TEMPO in water were determined as 1960 and 320 at 245 and 290 nm, respectively (the ratio $\varepsilon_{245}/\varepsilon_{290} \sim 6$). Other chemicals were of analytical reagent grade.

2.2. Preparation of $TEMPO^+Cl^-$ and TEMPOH

Oxoammonium salt $TEMPO^+Cl^-$ was prepared by reacting of TEMPO radical with Cl_2 according to the procedure described in the literature (Golubev, Zhdanov, & Rozantsev, 1970). TEMPO (200 mg,



Scheme 2. Mechanism of alcohol oxidation by oxoammonium cation in basic media.

1.28 mmol) was dissolved in 3 ml CCl_4 after then 3 ml of 1.5% Cl_2 solution in CCl_4 was added. The yellow precipitate was separated, washed with CCl_4, dried (yield 100%) and stored not more than 3 days. Molar extinctions of $TEMPO^+Cl^-$ were determined as $\varepsilon_{245} = 1730$, $\varepsilon_{290} = 860$ (the ratio $\varepsilon_{245}/\varepsilon_{290} \sim 2$), and $\varepsilon_{470} = 19$.

Hydroxylamine TEMPOH was synthesized by the TEMPO reduction with sodium dithionite according to literature (Ozinskas & Bobst, 1980). Briefly, to a water–acetone (1:1) solution of TEMPO (200 mg, mmol) excess $Na_2S_2O_4$ was added at room temperature and under flowing Ar. The suspension was stirred ~10 min after then acetone was removed under vacuum. The product was extracted from aqueous layer with Et_2O (3 × 3 ml). After Et_2O evaporation, 160 mg TEMPOH (80%) was obtained. ^1H NMR (400 MHz; D_2O) δ 1.58 (br. s, 6H), 1.14 (s, 12H); ^{13}C NMR (125 MHz; D_2O) δ 63.9 (C-2,6), 38.0 (C-3,5), 29.4, 18.9 (CH_3), 16.0 (C-4).

It should be noted, that under storing in air atmosphere the slow transformation of colorless TEMPOH crystals to red-orange TEMPO was observed.

2.3. Preparation of MeNAG

MeNAG was synthesized from D-GlcNAc (Scheme 1S) according to the work (Cai, Ling, & Bundle, 2005; Mack, Basabe, & Brossmer, 1988). To a suspension of D-GlcNAc (2 g, 9 mmol) in 40 ml dry acetone $BF_3{\cdot}Et_2O$ (4 g, 2.8 mmol) was added. The mixture was stirred and boiled under reflux for 12 min with the exclusion of moisture, then cooled in ice, treated with 13 ml triethylamine, and added dropwise to cold solution of sodium carbonate (14.5 g in 100 ml water). Acetone and triethylamine were removed under diminished pressure at <30 °C. The obtained mixture was multiple extracted with ether, and the combined extracts were dried with $MgSO_4$ and concentrated. The crude product was dissolved in 80 ml dry methanol, and *p*-toluenesulfonic acid (PTSA) (0.5 g, 3 mmol) was added. After 3 h the reaction mixture was neutralized with triethylamine, and methanol was removed in vacuum. The residue was multiple washed with CH_2Cl_2 in order to remove PTSA salt and then was purified by column chromatography (methanol:chloroform, 1:1) on silica gel to give MeNAG (1 g, 4.5 mmol) as white powder. Yield 50%.

^1H NMR (400 MHz; D_2O) δ 4.45 (d, H-1, $J_{1,2} = 8.4$), 3.95 (d, H-6, $J_{6,6'} = 12.1$), 3.44–3.78 (H-2–5, H-6′), 3.52 (s, CH_3O), 2.05 (s, CH_3CON), cf. Perkins, Johnson, Phillips, and Dwek (1977) (Fig. 9S).

2.4. Typical oxidation procedure

HA (50 mg, 0.125 mmol of primary hydroxyl groups) was dissolved in 10 or 20 ml of distilled water, then the temperature and pH of HA solution were adjusted to required values (Table 1). When an inert atmosphere was required argon was passed through the solution for 15 min and above the solution for all reaction time. The required quantity of $TEMPO^+$ (0.24–48 mg, 0.00125–0.25 mmol, Table 1) in 1 ml H_2O was added to HA solution under vigorous stirring. After $TEMPO^+$ addition, 0.4 M NaOH was immediately added to neutralize the acidic reaction products and maintain the reaction pH at the necessary level. Reaction was stopped at 15 min by adding 2 ml methanol, and then the solution was neutralized. Samples of oxidized HMW HA were precipitated with three volumes of MeOH, and LMW HA solutions were concentrated to 2–3 ml under vacuum (temperature of the bath ~60 °C) and were also treated with MeOH. The resulting precipitates were centrifuged, washed with MeOH and diethyl ether and dried in vacuum at 60 °C for 2 h. Oxidized samples (44–48 mg) were obtained as white water-soluble powders or fibers.

Carboxy-HA. ^1H NMR (500 MHz; D_2O) δ 4.55 (H-1″), 4.48 (H-1), 3.87 (H-2″), 3.78 (H-5″), 3.77 (H-3″), 3.70 (H-5), 3.70 (H-4), 3.61 (H-4″), 3.59 (H-3), 3.34 (H-2), 2.03 (3H, CH_3CON); ^{13}C NMR (125 MHz; D_2O) δ 176.4 (C-6), 176.1 (C-6″), 175.3 (CH_3CON), 104.2 (C-1), 101.7 (C-1″), 82.7 (C-3″), 81.7 (C-4), 77.6 (C-5), 77.1 (C-5″), 74.8 (C-3), 73.6 (C-2), 72.0 (C-4″), 55.5 (C-2″), 23.7 (CH_3CON).

Table 1
Experimental conditions for HA oxidation by $TEMPO^+$.

Entry	[HA], mmol/ml, HA:$TEMPO^+$	pH	DO, %		[TEMPO][a]/[$TEMPO^+$]$_0$, %	
			HMW	LMW	HMW	LMW
1	0.0125, 1:1	8.5	42[b]	60	46	29
2	0.0125, 1:1	10.2	43[b]	50	30	22
3	0.0125, 1:1	11.5	45[b]	72	33	19
4	0.0125, 1:2	10.2	90	100	67	41
5	0.0125, 1:0.5	10.2	26[b]	–	20	–
6	0.0125, 1:0.25	10.2	8[b]	–	35	–
7	0.0063, 1:1	10.2	50	56	27	43
8[c]	0.0063, 1:1	10.2	50	55	18	26
9	0.0063, 1:2	10.2	100	100	42	41
10[d]	0.0125, 1:1	10.2	51[b]	55	56	56

[a] Quantity of TEMPO after oxidation was determined from UV spectra at 245 nm. TEMPOH solutions at the wave length do not absorb.
[b] Determined by IR.
[c] Reactions were carried out in Ar.
[d] Reactions were carried out at ~20 °C.

For FTIR analysis, the samples were converted in their acidic forms with a cation exchange resin Dowex 50WX4 in H^+ form and were prepared in the form of films.

2.4.1. Oxidation in D_2O for the NMR analysis

Oxidation of LMW HA, DS, starch, ethanol and MeNAG (0.0125 mmol/3.3 ml D_2O) for the NMR analysis was carried out analogously to the typical procedure (HA:$TEMPO^+$ = 1:2, 2 ± 2 °C, pH 10.2). To regulate the reaction pH, NaOD and DCl were used. Reaction solution was placed onto NMR tube and NMR spectra were monitored (Fig. 3 and Figs. 6S, 8S, 10S–13S).

For oxidized DS DO_{total} was found from the ratio of intensities of signals at 5.03 ppm (H-4 in oxidized galactosamine) and 2.10 ppm (CH_3CON, as reference); the content of units with aldehyde and aldehyde hydrate groups was found using signals at 9.22 and 5.19 ppm respectively; carboxyl groups content was calculated as the difference between DO_{total} and content of units with aldehyde + aldehyde hydrate groups (Fig. 6S) (Ponedel'kina, Khaibrahmanova, Odinokov, et al., 2010). For oxidized starch DO_{total} was determined from the ratio of intensities of signals at 4.09 ppm (H-5 in oxidized unit) and 5.43–5.62 ppm (anomeric region, as reference); the content of aldehyde hydrate units was found using signals at 5.31–5.38 ppm; the content of units with carboxyl groups was calculated as the difference between DO_{total} and aldehyde hydrate content (Fig. 8S) (Ponedel'kina, Araslanova, Tyumkina, Lukina, & Odinokov, 2014). For oxidized MeNAG the DO, i.e. the content of methyl 2-acetamido-2-deoxy-*β*-D-glucopyranosyluronic acid, was found as 100%-unreacted MeNAG. The last was calculated from the ratio of signals at 3.95 ppm (H-6) and 4.45 ppm (H-1, as reference) (Figs. 10S and 11S). In the case of oxidized ethanol, NMR signals for methyl protons at 2.26 (aldehyde), 1.93 (acid) and 1.20 ppm (unoxidized ethanol) were used (Figs. 12S and 13S).

2.5. Effect of basic media on the $TEMPO^+Cl^-$ stability

$TEMPO^+Cl^-$ (0.24 or 2.4 mg) in 1 ml H_2O was added to water (10 ml) cooled to 2 ± 2 °C. The pH of solutions with finishing $TEMPO^+Cl^-$ concentrations of 0.022 (0.00011) or 0.22 (0.0011) mg (mmol)/ml was adjusted to 10.2, and UV spectra were recorded at various timepoints between 1 and 15 min (Table 2).

Table 2
Absorption of $TEMPO^+Cl^-$ solutions depending on the reaction time.

[$TEMPO^+$], mg/ml	A_{245}/A_{290}			
	0 min[a]	1 min	5 min	15 min
0.22	0.96/0.49[b]	0.56/0.11[b]	0.56/0.11[b]	0.57/0.11[b]
0.022	0.11/0.06	0.06/0.01	0.06/0.01	0.06/0.01

[a] Measurements were made at neutral pH.
[b] Absorptions are given for solutions diluted twofold.

2.5.1. Oxygen evolution measurement in the $TEMPO^+Cl^-$ solution

Water (30 ml) was cooled to 2 ± 2 °C and adjusted to pH 10.2, after then dissolved oxygen was removed by passing Ar for 15 min up to concentration of 0.00 mg/l, accordingly to oximeter reading. $TEMPO^+Cl^-$ (72 mg, 0.375 mmol) was added to water under Ar atmosphere and the concentration of the resulting oxygen was monitored for 15 min. Then the $TEMPO^+Cl^-$ solution was kept for 3 h under Ar for further oxygen evolution monitoring.

2.5.2. TEMPOH stability under reaction conditions

TEMPOH (19 mg, 0.125 mmol) was added to an aerated solution of HMW HA (50 mg/11 ml H_2O) prepared as for the oxidation procedure (2 ± 2 °C, pH 10.2) and mixture was stirred for 15 min. At various timepoints between 1 and 15 min UV spectra were monitored for the TEMPO detection. Under these conditions the UV spectra of TEMPOH remained unchanged. The TEMPOH/HA solution was then kept for a week at room temperature but the TEMPO formation was not detected.

2.6. Analyses

One- (^1H and ^{13}C) and two-dimensional (HSQC) NMR spectra were recorded on a Bruker Avance III 500 MHz (500.17 MHz for ^1H and 125.78 MHz for ^{13}C) and on a Bruker Avance II 400 MHz (400.13 MHz for ^1H and 100.62 MHz for ^{13}C) spectrometers. Samples were analyzed as solutions in D_2O (15 mg/ml) at room temperature using acetone as internal standard (δ 2.22 ppm for ^1H and 31.45 ppm for ^{13}C). For HMW HA samples the pulse conditions were as follows: for the ^1H NMR spectrum, 90° pulse flip angle, acquisition time (AQ) = 2.05 s, high-power pulse (P1) = 8.00 s, spectral width (SW) = 4000 Hz, data points (TD) = 16 384, number of scans (NS) = 32; for the ^{13}C NMR spectrum, 30° pulse flip angle, P1 = 4.7 s, AQ = 0.9 s, dummy scans (DS) = 2, Relaxation Delay (RD) = 1.0 s, SW = 18 116 Hz, TD = 32 768; for the HSQC spectrum, AQ = 0.32 s, RD = 0.84 s, SW(F1) = 7043.1 Hz, SW(F2) = 1600.5 Hz, TD = 1024 × 128.

Dissolved oxygen concentration (mg/l) was measured with an oximeter Mark 303 T. Absorbance spectra were obtained in 1 cm cell using a Perkin Elmer Lambda 750 UV/VIS spectrometer. FTIR spectra were recorded using a Bruker Vertex 70 spectrometer. The reaction pH was controlled with pH meter HANNA 2210.

3. Results and discussions

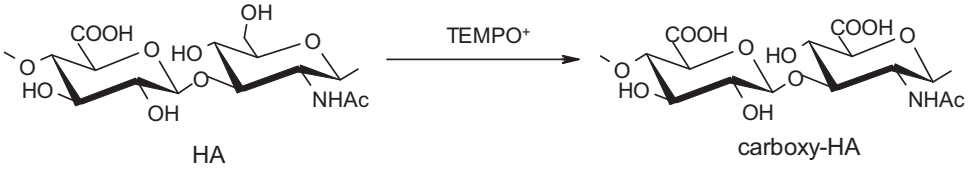
The oxidation of HMW and LMW HA with the oxoammonium salt $TEMPO^+Cl^-$ (below $TEMPO^+$) (Scheme 3) was performed in aqueous solutions at various pH and temperatures, variable reactant concentrations and ratio, either with free access of air oxygen or under Ar (Table 1).

$TEMPO^+$ was used in no more than twofold excess and added to the HA solution as one portion for the following reasons. According to the equation $RCH_2OH + 2TEMPO^+ + H_2O \rightarrow RCOOH + 2TEMPOH + 2H^+$ (see Scheme 1), during oxidation of the alcohol to the carboxylic acid, the $TEMPO^+$ cation is converted to the hydroxylamine TEMPOH. In an alkaline medium, TEMPOH and $TEMPO^+$ can undergo comproportionation (Eq. (1)) to give the TEMPO radical ($k = 10^4\ M^{-1}\ s^{-1}$) (Israeli et al., 2005).

In addition, $TEMPO^+$ is unstable to the action of aqueous alkalis, which was demonstrated previously in relation to 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxo chloride (4-hydroxy-$TEMPO^+Cl^-$) (Golubev et al., 1965), 4-methoxy-2,2,6,6-tetramethylpiperidin-1-oxo bromide (4-methoxy-$TEMPO^+Br^-$) (Endo et al., 1984) and 2,2,6,6-tetramethyl-4-oxopiperidin-1-oxo perchlorate (4-oxo-$TEMPO^+ClO_4^-$) (Golubev & Sen', 2011). The major product formed upon decomposition of these salts is the corresponding radical. Besides radical, other byproducts can be formed at alkaline pH (see Footnote below). When $TEMPO^+$ is used in a more than twofold excess or when $TEMPO^+$ is gradually added to a solution with an alkaline pH, the probability of side reactions can increase, while the degree of oxidation (DO) of HA can hence decrease. To prevent this, the pH 10.2 was chosen. It is also optimal for the catalytic oxidation (Jiang et al., 2000). The pH 8.5 was difficult to control due to rapid formation of acidic reaction products, which moved the pH toward more acidic values. At pH 7 and lower, HA oxidation did not occur.

The stoichiometric HA oxidation with the $TEMPO^+$ cation under basic conditions proceeded very rapidly, and already after 1–2 min the peak at 470 nm for $TEMPO^+$ in the vision spectrum was completely disappeared indicating the end of the oxidation (Fig. 2). As was shown earlier, catalytic TEMPO oxidation of HA was much longer (Jiang et al., 2000).

As expected, the HA oxidation with the $TEMPO^+$ cation was accompanied by the formation of the hydroxylamine TEMPOH (Fig. 3, the reaction conditions as in entry 4). Its signals were observed at 1.14 (s, 12H) and 1.58 (br. s, 6H) ppm in ^1H NMR spectrum. However, the intensities ratio of these signals (>2) and the presence of additional unknown signals in the near area of the spectrum gave evidence for the formation of some quantity of products of the oxoammonium chloride decomposition. Apart from TEMPOH, a considerable amount of the TEMPO radical was detected (Fig. 2), although attempts were made to avoid its formation. The yield of TEMPO reached a maximum also after 1–2 min, being equal to 18–67% of the $TEMPO^+$ cation taken in the reaction (Table 1), and it was higher in an aerated solution (entry 7) than under Ar (entry 8). A decrease in the $TEMPO^+$ concentration down to catalytic also resulted in a higher yield of the radical. For HA to $TEMPO^+$ ratios of 1:0.1, 1:0.025, and 1:0.01 (other conditions as in entry 2), the percentages of TEMPO were 27, 38, and 58%, respectively. It is clear that the more TEMPO is formed upon HA oxidation, the lower the yield of TEMPOH (approximately $TEMPO^+$–TEMPO); however, due to side reactions, there was no sense to determine its yield more accurately. For example, in entry 4 (DO of LWM HA 100%) the yield



Scheme 3. Oxidation of hyaluronic acid by $TEMPO^+$.

of TEMPOH calculated as $TEMPO^+$–TEMPO (100–41) was 59%, while the same found from ^1H NMR spectrum (Fig. 3) was 48% per starting $TEMPO^+$.

The ^{13}C NMR spectra of all of the oxidized HA samples exhibited no signal for the aldehyde group. The minor signal at ~5.20 ppm corresponding to the methine proton in the $CH(OH)_2$ group (Bubb, 2003), present in the ^1H NMR spectra of some samples, was indicative of a slight content of aldehyde hydrate units; see Fig. S1 (Supplementary Data). The hydroxamic test for ester groups was negative. Evidently because of aqueous alkaline conditions, the pyranosyl oxygen located in the β position to the alcohol group of *N*-acetyl-D-glucosamine did not affect the selectivity of HA oxidation and the oxidation of HA hydroxyl groups afforded exclusively carboxyl groups. The content of these groups (i.e. DO) in HA was found from the IR spectra (Fig. 1) according to reported method (Jiang et al., 2000) and using 2D HSQC experiment (Fig. 4).

The DO of HA depended mainly on the reactant ratio. Under identical oxidation conditions, the DO of almost all HWM HA samples was somewhat lower than in the case of LWM HA (Table 1). Apparently, due to the high viscosity of HWM HA, the reactant miscibility was insufficiently high and $TEMPO^+$ was partially consumed for side reactions rather than for HA oxidation. In less concentrated solutions and at room temperature where the viscosity was lower, the HWM HA oxidation was more effective (cf. entries 4 and 9, 2 and 10).

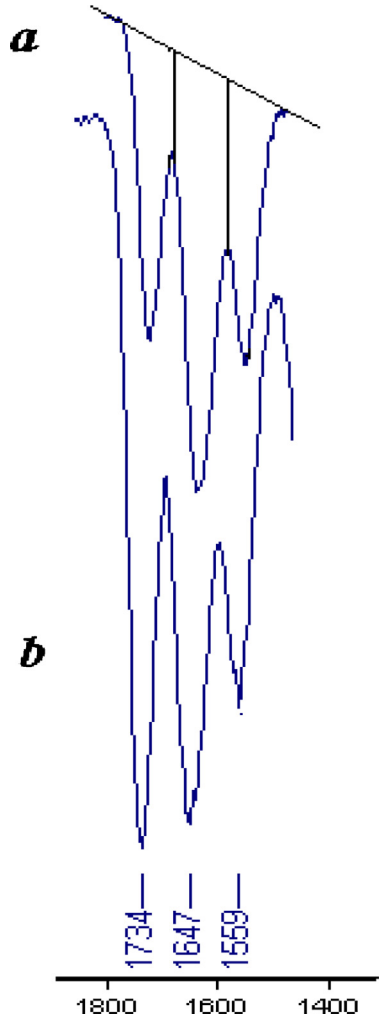
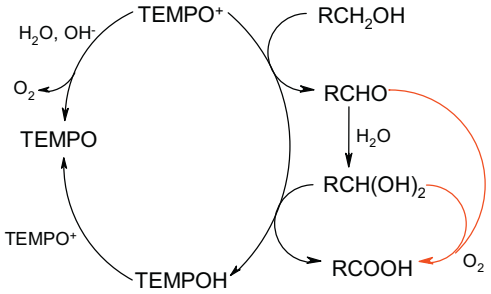


Fig. 1. Fragments of FTIR spectra of (a) intact HMW HA and (b) partially oxidized HMW HA (DO 45%) in their H^+ form. The degree of HA oxidation was calculated using the areas of bands at 1728 cm^{-1} (CO_2H) and at 1555 cm^{-1} (NH, as the reference).



Scheme 4. Oxidation of hyaluronic acid.

In most cases, in both aerated solutions and an inert medium, the DO of HA nearly corresponded to $TEMPO^+$ consumed in the reaction except for LWM HA samples oxidized at pH 8.5 (entry 1) and 11.5 (entry 3). Their DO values were 60 and 72%, respectively, instead of the expected 50%. In all cases, the DO of HA was higher than it followed from the amount of hydroxylamine TEMPOH formed.

Most likely, the low yield of TEMPOH was caused by the redox reactions typical of the $TEMPO^+$ cation to give the TEMPO radical. However, in these reactions, $TEMPO^+$ is spent not for HA oxidation; hence, the deficient $TEMPO^+$ should be counterbalanced by another oxidant. In aerated solutions, the role of the second oxidant can be performed by air oxygen. In addition, O_2 can be generated in the reaction medium, possibly, upon decomposition of oxoammonium chloride. Indeed, when we removed the dissolved oxygen from water by Ar purging (pH 10.2) and then added the $TEMPO^+$ salt in a concentration of 0.013 mmol/ml, then the formation of O_2 was detected by an oximeter. During 15 min, the oxygen concentration increased from 0 to 0.53 mg/l, and another 3 h later it reached 4.5 mg/l. Sodium hydroxide was required to maintain the pH of this reaction at 10.2. Apparently, apart from TEMPO and O_2, decomposition of the salt afforded acidic products.[1] At high concentration of the $TEMPO^+$ salt (0.013 mmol/ml), it is difficult to detect the formation of the TEMPO radical, especially in the first minutes of the reaction. However, at lower $TEMPO^+$ concentrations (0.0011 and 0.00011 mmol/ml), when the influence of the alkaline medium increased, fast conversion of the $TEMPO^+$ cation to the TEMPO radical was observed (yield ~50%) (Table 2).

Thus, the obtained data suggest that along with $TEMPO^+$, molecular oxygen is involved in HA oxidation. The HA oxidation process can be described by the following chart (Scheme 4). The alcohol groups of HA are first oxidized to the aldehyde groups, this is accompanied by consumption of an equimolar amount of $TEMPO^+$ and gives an equivalent amount of TEMPOH. Some of the remaining $TEMPO^+$ undergoes comproportionation reaction with an equimolar amount of TEMPOH to give the TEMPO radical (see Eq. (1)).

[1] For the reaction and decomposition products of 4-hydroxy-$TEMPO^+Cl^-$ (Golubev et al., 1965), 4-methoxy-$TEMPO^+Br^-$ (Endo et al., 1984), and 4-oxo-$TEMPO^+ClO_4^-$ (Golubev & Sen', 2011) under alkaline conditions, see the relevant references. Only 4-oxo-$TEMPO^+ClO_4^-$ was studied in detail. Among the nine identified products of decomposition, the yield of the 4-oxo-TEMPO radical was 60%, and no oxygen was found. On the other hand, at pH 9–11 and room temperature, 4-methoxy-$TEMPO^+Br^-$ was quantitatively converted to 4-methoxy-TEMPO, the other product being H_2O_2 (~90%). If decomposition of $TEMPO^+Cl^-$ also gives H_2O_2, the formation of O_2 that we detected can be due to its reaction with the $TEMPO^+$ cation: $TEMPO^+ + H_2O_2 \rightarrow TEMPO + O_2 + 2H^+$ (Sen, Golubev, Kulyk, & Rozantsev, 1976). Hence, it should be noted that decomposition of oxoammonium salts (including $TEMPO^+Cl^-$) has not been systematically studied as a function of substituent in the piperidinium ring, the counter-ion type, and the reaction conditions. All the listed factors can have a very pronounced influence on the redox properties of oxoammonium salts both in the decomposition and in oxidation of alcohols. For example, it was found that 4-methoxy-$TEMPO^+Br$ is an order of magnitude less reactive in alcohol oxidation than 4-methoxy-$TEMPO^+Cl^-$ (Miyazawa, Endo, Shiihashi, & Okawara, 1985).

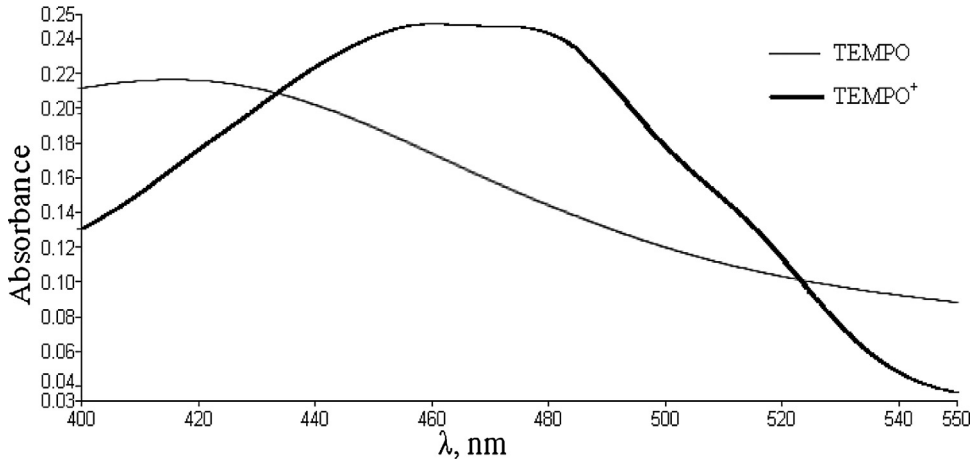


Fig. 2. UV spectra of 0.0125 M $TEMPO^+Cl^-$ prior to reaction and TEMPO formed after 1–2 min oxidation (conditions as in entry 2).

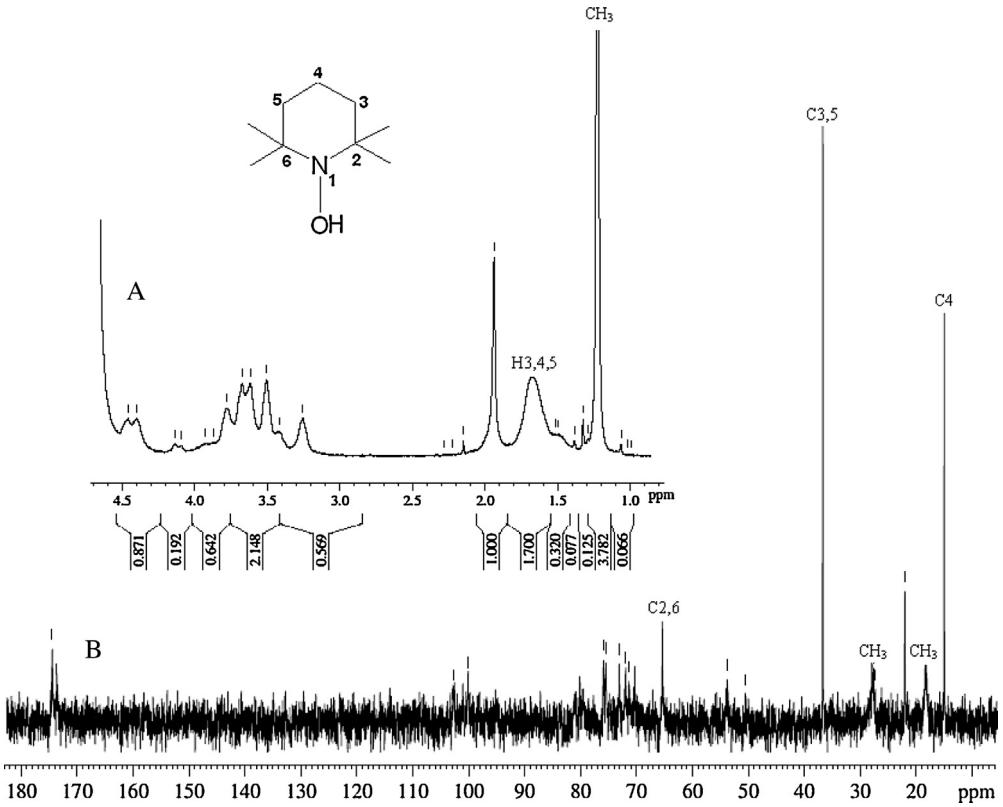


Fig. 3. ^1H (A) and ^{13}C NMR (B) spectra of mixture of carboxy-HA and TEMPOH in reaction solution after LMW HA oxidation in D_2O (15 mg/3.3 ml D_2O, HA:$TEMPO^+Cl^-$ = 1:2, 2 ± 2 °C, pH 10.2). Unlabeled signals are attributed to carboxy-HA and given in experimental part.

The rest of $TEMPO^+$ is spent for oxidation of some of the aldehyde groups of HA (apparently, as aldehyde hydrate groups; Qiu et al., 2011) to carboxyl groups being thus reduced to TEMPOH or it partly decomposes under the action of alkali to give TEMPO, O_2 and other products. The air oxygen and/or oxygen generated upon $TEMPO^+$ decomposition oxidizes another part of the aldehyde groups of HA, thus counterbalancing $TEMPO^+$ spent for side reactions. It is difficult to estimate the contribution of each of the side reactions. It is quite possible that decomposition in which $TEMPO^+$ is consumed for the formation of O_2 prevails over comproportionation in which $TEMPO^+$ is consumed for the formation of TEMPO. Otherwise, the DO of HA would be lower in an inert atmosphere than it is observed in the experiment (entry 8).

Apart from the above side reactions, the low yield of TEMPOH and, hence, the high yield of the TEMPO radical in the oxidation of HA may be due to the known re-oxidation reaction of the hydroxylamine TEMPOH by air oxygen (Eq. (2)) (Ozinskas & Bobst, 1980):

$$TEMPOH + (1/2)O_2 \rightarrow TEMPO + (1/2)H_2O \quad (2)$$

However, TEMPOH that we synthesized proved to be stable and did not react with oxygen under the process conditions. Therefore, this pathway to the TEMPO radical was ruled out.

The proposed scheme of HA oxidation involving oxygen accounts not only for the formation of TEMPO but also for the fact that the DO of some LWM HA is higher than the expected DO (50%) by 10–22% (entries 1 and 3).

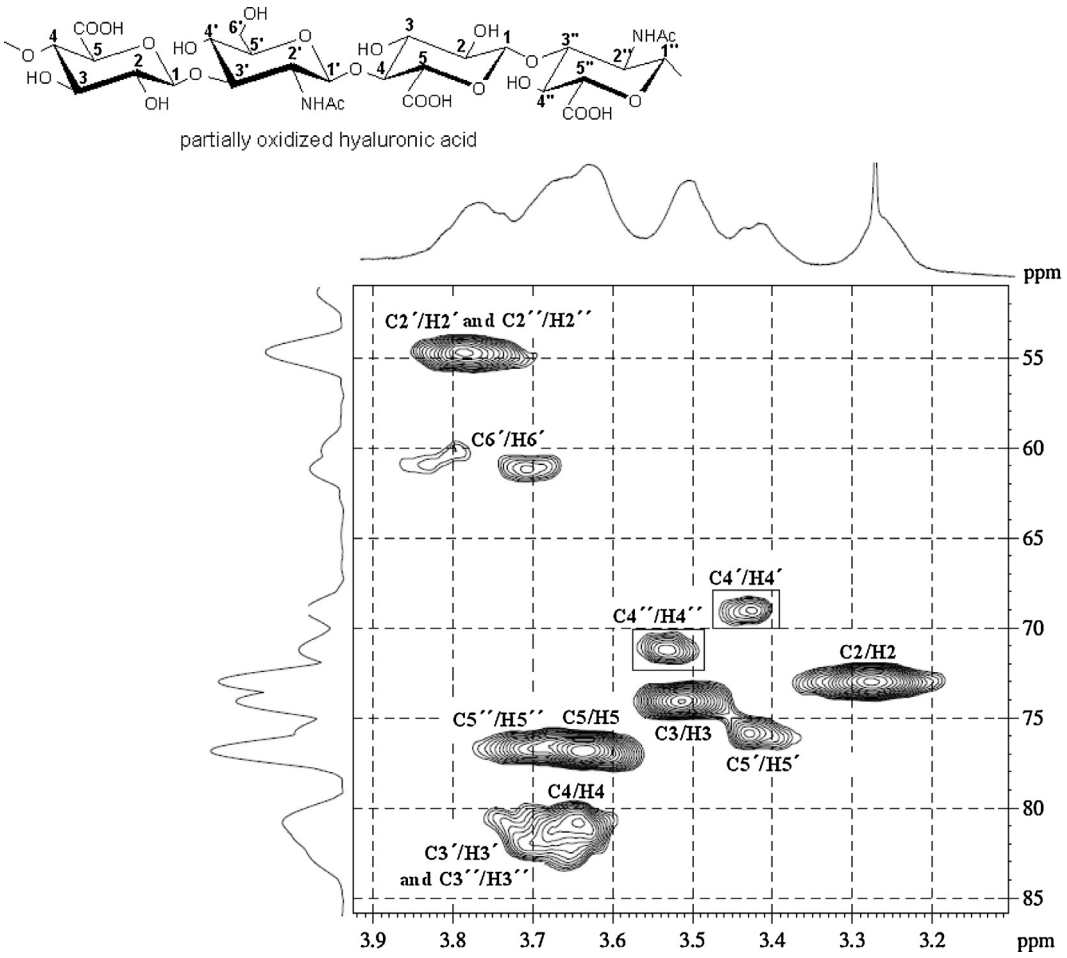


Fig. 4. Fragment of HSQC spectrum of partially oxidized LMW HA sample. DO of HA hydroxymethyl groups was found as follows. Intensities of marked C4/H4 cross-peaks for oxidized (72.0/3.61 м.д, I_{ox}) and non-oxidized (69.9/3.51 м.д, $I_{non\text{-}ox}$) glucosamine units were determined by contour integration and DO was calculated by formula $DO = I_{ox}/(I_{ox} + I_{non\text{-}ox})$.

High efficiency of stoichiometric oxidation and unusual behavior of the oxidation system in whole are interesting in application to other substrates with primary alcohol groups. Therefore, such polysaccharides as glycosaminoglycan DS and water-soluble potato starch were involved in oxidation. Ethanol was used as a simplest low-weight-molecular alcohol, and MeNAG was chosen as nearest analog of *N*-acetyl-β-D-glucosamine unit (Table 3). The oxidation conditions were as in entry 4 (Table 1): RCH_2OH:$TEMPO^+$ = 1:2, pH 10.2, 2 ± 2 °C. In these conditions the reaction rate was extremely rapid as in the case of HA, and cation $TEMPO^+$ was consumed during 1–2 min. However, in contrast to HA the DO_{total} of all substrates was lower than 100%, and oxidized products, except oxidized MeNAG, contained both acids and aldehydes (aldehyde hydrates). Their composition depended on the structure of used alcohol but practically did not depend on atmosphere (air or Ar), in which the oxidation was performed. The $TEMPO^+$ consumed only in oxidation and calculated based on the stoichiometry of the oxidation of alcohol to acid and aldehyde was 31–56% (Table 3). Therefore, it is not necessary to discuss the deficiency of oxidizer, as in the case of HA oxidation. The remaining oxoammonium salt was consumed in side reactions of decomposition and/or comproportionation. Among products of these reactions, the radical TEMPO (Table 3) and hydroxylamine TEMPOH prevailed (Figs. 6S, 8S, 10S–13S). Other unknown products of $TEMPO^+$ decomposition were more diversified and were formed with higher yield than upon HA oxidation; their quantity and composition depended on the nature of oxidized substrate more than alkali pH (cf. Fig. 3 and Figs. 6S, 8S, 10S–13S).

As for the scheme proposed for HA oxidation (Scheme 4), the question of its applicability to other substrates such as DS, starch, ethanol or MeNAG remains open. The reaction ability of alcohols strongly depends on their structure and, presumably, the HA behavior in oxidation with $TEMPO^+$ is unique.

Table 3
Oxidation of DS, starch, MeNAG and ethanol by $TEMPO^+Cl^-$.

substrate	DO_{total}, %[a]	Oxidized products, mol%			$TEMPO^+$ consumed[b]	[TEMPO]/$[TEMPO^+]_0$, %
		Aldehyde	Aldehyde hydrate	Acid		
DS	70	4	24	42	56	81
Starch	42	0	23	19	31	50
MeNAG	54	0	0	54	54	70
	45[c]	0	0	45	45	71
Ethanol	44	4	0	40	44	52
	53[c]	5	0	48	50	50

[a] DO was found from ^1H NMR (see Section 2 and Figs. 6S, 8S, 10S–13S).
[b] $TEMPO^+$ consumed was calculated based on the oxidation stoichiometry of alcohol to aldehyde/aldehyde hydrate and acid.
[c] Reactions were carried out in Ar.

4. Conclusion

The oxidation of HA (HMW and LMW) by 0.25–2 equivalents of oxoammonium salt TEMPO$^+$Cl$^-$ occurs rapidly and selectively in water–alkaline medium and leads to formation of carboxy-HA with nearly quantitative yield of carboxyl groups per the starting TEMPO$^+$. Among products of the cation TEMPO$^+$ reduction, apart from expected hydroxylamine TEMPOH the radical TEMPO was found in the considerable amount (18–67% per the starting TEMPO$^+$). We assumed that TEMPO is formed upon the interaction of TEMPO$^+$ with TEMPOH (i.e. comproportionation reaction) and/or upon alkali-induced TEMPO$^+$ decomposition. Dioxygen evolution was observed in the decomposition reaction. The side reactions account for the deficiency of TEMPO$^+$ as the oxidant and this deficiency is counterbalanced by participation of air oxygen and/or oxygen generated upon TEMPO$^+$ decomposition. A new expanded scheme for HA oxidation with combined participation of both TEMPO$^+$ and molecular oxygen is proposed. Stoichiometric oxidation of other substrates such as DS, starch, MeNAG or ethanol was less effective in compared with HA, and these results do not allow us to make a conclusion about general character of the scheme proposed for HA.

Acknowledgments

This study was supported by the Russian Foundation for Basic Research ("My first grant" number 14-03-31131).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2015.04.054

References

Bailey, W. F., Bobbitt, J. M., & Wiberg, K. B. (2007). Mechanism of the oxidation of alcohols by oxoammonium cations. *Journal of Organic Chemistry*, *72*(12), 4504–4509. http://dx.doi.org/10.1021/jo0704614

Bellini, D., Crescenzi, V., & Francescangeli, A. (2002). *WO 2002018448*.

Bobbitt, J. M., Bartelson, A. L., Bailey, W. F., Hamlin, T. A., & Kelly, C. B. (2014). Oxoammonium salt oxidations of alcohols in the presence of pyridine bases. *Journal of Organic Chemistry*, *79*(3), 1055–1067. http://dx.doi.org/10.1021/jo402519m

Bragd, P. L., van Bekkum, H., & Besemer, A. C. (2004). TEMPO-mediated oxidation of polysaccharides: Survey of methods and applications. *Topics in Catalysis*, *27*(1–4), 49–66. http://dx.doi.org/10.1023/b:toca.0000013540.69309.46

Bubb, W. A. (2003). NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. *Concepts in Magnetic Resonance Part A*, *19A*(1), 1–19. http://dx.doi.org/10.1002/cmr.a.10080

Buffa, R., Kettou, S., Pospišilová, L., Berková, M., & Velebný, V. (2011). *WO 2011069474*.

Cai, Y., Ling, C.-C., & Bundle, D. R. (2005). Facile approach to 2-acetamido-2-deoxy-β-D-glucopyranosides via a furanosyl oxazoline. *Organic Letters*, *7*(18), 4021–4024. http://dx.doi.org/10.1021/ol051523k

Crescenzi, V., Francescangeli, A., Renier, D., & Bellini, D. (2001). New hyaluronan chemical derivatives. Regioselectively C(6) oxidized products. *Macromolecules*, *34*(18), 6367–6372. http://dx.doi.org/10.1021/ma0102363

De Nooy, A. E. J., Besemer, A. C., & van Bekkum, H. (1996). On the use of stable organic nitroxyl radicals for the oxidation of primary and secondary alcohols. *Synthesis*, *1996*(10), 1153–1176. http://dx.doi.org/10.1055/s-1996-4369

Endo, T., Miyazawa, T., Shiihashi, S., & Okawara, M. (1984). Oxidation of hydroxide ion by immonium oxide. *Journal of the American Chemical Society*, *106*(13), 3877–3878. http://dx.doi.org/10.1021/ja00325a038

Golubev, V. A., Rozantsev, É. G., & Neiman, M. B. (1965). Some reactions of free iminoxyl radicals with the participation of the unpaired electron. *Bulletin of the Academy of Sciences of the USSR, Division of Chemical Science*, *14*(11), 1898–1904. http://dx.doi.org/10.1007/bf00845878

Golubev, V. A., & Sen', V. D. (2011). Mechanism of autoreduction of 2,2,6,6-tetramethyl-1,4-dioxopiperidinium cation in alkaline medium. *Russian Journal of Organic Chemistry*, *47*(6), 869–876. http://dx.doi.org/10.1134/s1070428011060066

Golubev, V. A., Zhdanov, R. I., & Rozantsev, É. G. (1970). Reaction of iminoxyl radicals with chlorine. *Bulletin of the Academy of Sciences of the USSR, Division of chemical science*, *19*(1), 186–187. http://dx.doi.org/10.1007/bf00913958

Israeli, A., Patt, M., Oron, M., Samuni, A., Kohen, R., & Goldstein, S. (2005). Kinetics and mechanism of the comproportionation reaction between oxoammonium cation and hydroxylamine derived from cyclic nitroxides. *Free Radical Biology and Medicine*, *38*(3), 317–324. http://dx.doi.org/10.1016/j.freeradbiomed.2004.09.037

Jiang, B., Drouet, E., Milas, M., & Rinaudo, M. (2000). Study on TEMPO-mediated selective oxidation of hyaluronan and the effects of salt on the reaction kinetics. *Carbohydrate Research*, *327*(4), 455–461. http://dx.doi.org/10.1016/S0008-6215(00)00059-8

Kogan, G., Šoltés, L., Stern, R., & Gemeiner, P. (2007). Hyaluronic acid: A natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology Letters*, *29*(1), 17–25. http://dx.doi.org/10.1007/s10529-006-9219-z

Luderer, M. R., Mamros, A. N., Sharrow, P. R., Weller, W. E., Luderer, M. R., Fair, J. D., et al. (2011). Oxidation of primary and secondary alcohols by 4-acetylamino-2,2,6,6-tetramethylpiperidine-1-oxoammonium tetrafluoroborate in aqueous media. *ARKIVOC: Archive for Organic Chemistry*, *1*(5), 23–33.

Mack, H., Basabe, J. V., & Brossmer, R. (1988). 2-Acetamido-2-deoxy-D-gluco- and -D-manno-furanose: A simple preparation of 2-acetamido-2-deoxy-d-mannose. *Carbohydrate Research*, *175*(2), 311–316. http://dx.doi.org/10.1016/0008-6215(88)84154-5

Merbouh, N., Bobbitt, J. M., & Brückner, C. (2004). Oxoammonium salts. 9. Oxidative dimerization of polyfunctional primary alcohols to esters. An interesting β oxygen effect. *Journal of Organic Chemistry*, *69*(15), 5116–5119. http://dx.doi.org/10.1021/jo049461j

Mero, A., & Campisi, M. (2014). Hyaluronic acid bioconjugates for the delivery of bioactive molecules. *Polymers*, *6*(2), 346–369.

Miyazawa, T., Endo, T., Shiihashi, S., & Okawara, M. (1985). Selective oxidation of alcohols by oxoaminium salts ($R_2N{=}O^+X^-$). *Journal of Organic Chemistry*, *50*(8), 1332–1334. http://dx.doi.org/10.1021/jo00208a047

Ozinskas, A. J., & Bobst, A. M. (1980). Formation of *N*-hydroxy-amines of spin labeled nucleosides for ^{1}H-NMR analysis. *Helvetica Chimica Acta*, *63*(6), 1407–1411. http://dx.doi.org/10.1002/hlca.19800630609

Perkins, S. J., Johnson, L. N., Phillips, D. C., & Dwek, R. A. (1977). High resolution ^{1}H- and ^{13}C-N.M.R spectra of D-glucopyranose, 2-acetamido-2-deoxy-D-glucopyranose, and related compounds in aqueous media. *Carbohydrate Research*, *59*(1), 19–34. http://dx.doi.org/10.1016/S0008-6215(00)83289-9

Ponedel'kina, I. Y., Araslanova, D. I., Tyumkina, T. V., Lukina, E. S., & Odinokov, V. N. (2014). Partially oxidized potato starches from bromide-free TEMPO-mediated reaction: Characterization of monosaccharide composition. *Starch/Stärke*, *66*(5–6), 444–449. http://dx.doi.org/10.1002/star.201300130

Ponedel'kina, I. Y., Khaibrakhmanova, E. A., & Odinokov, V. N. (2010). Nitroxide-catalyzed selective oxidation of alcohols and polysaccharides. *Russian Chemical Reviews*, *79*(1), 63.

Ponedel'kina, I. Y., Khaibrahmanova, E. A., Odinokov, V. N., Khalilov, L. M., & Dzhemilev, U. M. (2010). Oxidation of dermatan sulfate with a NaOCl–NaBr–2,2,6,6-tetramethylpiperidine-1-oxyl reagent in an aqueous medium. *Russian Journal of Bioorganic Chemistry*, *36*(3), 354–358. http://dx.doi.org/10.1134/s1068162010030106

Ponedel'kina, I. Y., Lukina, E. S., Khaibrakhmanova, E. A., Sal'nikova, E. V., & Odinokov, V. N. (2011). Conjugates of hyaluronic and carboxy hyaluronic acids and their in vitro biodegradability upon the action of testicular hyaluronidase. *Russian Chemical Bulletin*, *60*(4), 754–759. http://dx.doi.org/10.1007/s11172-011-0116-9

Ponedel'kina, I. Y., Odinokov, V. N., Saitgalina, E. A., & Dzhemilev, U. M. (2007). The *in vitro* resistance of oxidized hyaluronic acid to testicular hyaluronidase. *Doklady Biochemistry and Biophysics*, *417*(1), 341–342. http://dx.doi.org/10.1134/s1607672907060142

Ponedel'kina, I. Y., Odinokov, V. N., Vakhrusheva, E. S., Golikova, M. T., Khalilov, L. M., & Dzhemilev, U. M. (2005). Modification of hyaluronic acid with aromatic amino acids. *Russian Journal of Bioorganic Chemistry*, *31*(1), 82–86. http://dx.doi.org/10.1007/s11171-005-0011-y

Prestwich, G. D. (2011). Hyaluronic acid-based clinical biomaterials derived for cell and molecule delivery in regenerative medicine. *Journal of Controlled Release*, *155*(2), 193–199. http://dx.doi.org/10.1016/j.jconrel.2011.04.007

Qiu, J. C., Pradhan, P. P., Blanck, N. B., Bobbitt, J. M., & Bailey, W. F. (2011). Selective oxoammonium salt oxidations of alcohols to aldehydes and aldehydes to carboxylic acids. *Organic Letters*, *14*(1), 350–353. http://dx.doi.org/10.1021/ol203096f

Schanté, C. E., Zuber, G., Herlin, C., & Vandamme, T. F. (2011). Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications. *Carbohydrate Polymers*, *85*(3), 469–489. http://dx.doi.org/10.1016/j.carbpol.2011.03.019

Semmelhack, M. F., Schmid, C. R., & Cortés, D. A. (1986). Mechanism of the oxidation of alcohols by 2,2,6,6-tetramethylpiperidine nitrosonium cation. *Tetrahedron Letters*, *27*(10), 1119–1122. http://dx.doi.org/10.1016/S0040-4039(00)84193-3

Sen, V. D., Golubev, V. A., Kulyk, I. V., & Rozantsev, E. G. (1976). Mechanism of the reaction of hydrogen peroxide with oxopiperidine salts and piperidinoxyl radicals. *Bulletin of the Academy of Sciences of the USSR, Division of Chemical Science*, *25*(8), 1647–1654. http://dx.doi.org/10.1007/bf00921418