Carbohydrate Polymers 79 (2010) 660–664

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Preparation of low molecular weight alginate by hydrogen peroxide depolymerization for tissue engineering

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ARTICLE INFO

Article history:
Received 1 September 2009
Accepted 15 September 2009
Available online 20 September 2009

Keywords:
Alginate
Oxidation
Hydrogen peroxide
Scaffold

ABSTRACT

Oxidation reactions of alginate solution with H_2O_2 were performed under different conditions to prepare low molecular weight alginate, and its potential application for tissue engineering was investigated. H_2O_2 oxidation is an effective method for alginate depolymerization with the rate depending on reaction time, temperature, solution pH and H_2O_2 concentration. The structure analyses by FT-IR, elemental analysis and ^{13}C NMR indicated that, the rupture of glucoside bonds in alginate chains into low molecular weight polymers is the basic process during H_2O_2 depolymerization. Oxidation of alginate under weak oxidation conditions (H_2O_2 0.6% (w/v), 50 °C, 1 h) did not significantly interfere in the formation of ionic junctions with calcium ions. The cross-linked alginate scaffold prepared from the obtained alginate under these conditions showed faster degradation rate than that from the unmodified alginate, due to the lower molecular weight of oxidized alginate and the formed aldehyde groups susceptible to hydrolysis.

1. Introduction

Alginate, a natural anionic polysaccharide obtained by extraction from brown algae, is composed of (1–4)-linked β-D-mannuronic acid and α-L-guluronic acid blocks (Rowley, Madlambayan, & Mooney, 1999). Due to their gelling ability in the presence of divalent cations such as calcium and barium, stabilizing properties and high viscosity in aqueous solutions, alginate and its derivatives have been extensively utilized in biomedical applications of cell transplantation, drug delivery and bulk agent gels (Augst, Kong, & Mooney, 2006; George & Abraham, 2006; Simmons, Alsberg, Hsiong, Kim, & Mooney, 2004; Wang et al., 2006). However, as no hydrolytic or enzymatic chain breakages occur within alginate chains, high molecular weight alginate polymers cannot be easily degraded and may be very slow to clear from the body (Al-Shamkhani & Duncan, 1995).

Recently, two approaches are typically used to obtain degradable alginate: one is to introduce degradable cross-linkers into the non-degradable alginate hydrogels (Lee, Bouhadir, & Mooney, 2000, 2004), and the other is to make the backbone of alginate degradable by acid hydrolysis, enzymatic degradation, gamma-irradiation and some oxidation technologies. For example, Ashton, Banerjee, Punyani, Schaffer, and Kane (2007) created degradable alginate hydrogels by incorporating alginate lyase in the hydrogels. Gamma-irradiation increased the degradation rate of alginate in vivo by decreasing the size of alginate chains, and improved the new bone formation (Alsberg et al., 2003). Periodate oxidation has also been developed to promote the hydrolysis of alginate in aqueous solution by cleaving the C—C bond and altering the chain conformation (Bouhadir et al., 2001).

Hydrogen peroxide is an effective and environmentally friendly oxidant, and has been used to oxidize many polysaccharides such as chitosan (Kabal'nova et al., 2001; Qin, Du, & Xiao, 2002), starch (Harmon et al., 1971; Parovuori, Hamunen, Forssell, Autio, & Poutanen, 1995), cellulose (Zeronian & Inglesby, 1995), and dextran (Ahrgren & de Belder, 1975). The oxidation method can not only depolymerize the polysaccharides, but also change the structure of the main chain. During cellulose bleaching, the oxidation formed high content of ketone groups at high pH (Zeronian & Inglesby, 1995). Starch was also oxidized by hydrogen peroxide and the reaction introduced carboxyl and carbonyl groups at the C-6 groups (Harmon et al., 1971). In the degradation of chitosan, hydrogen peroxide oxidized functional groups only under tough conditions, resulting in ring-opening reaction, formation of carboxyl groups and deamination (Kabal'nova et al., 2001). In contrast, there have been very few reports on the treatment of alginate by H_2O_2. Only Yang, Li, and Guan (2004) utilized H_2O_2 to treat polymannuronates from acid hydrolysis products of alginate; but their interest was to develop biological active oligosaccharides and they did not evaluate the degradation behavior of the oxidized products.

The aim of the present work is to prepare low molecular weight alginate by H_2O_2 treatment and explore its potential application for tissue engineering. We investigated the oxidation of alginate

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0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.09.020

with H_2O_2 under different conditions, and studied the changes of composition and structure of oxidized alginate. Calcium alginate scaffold was further prepared from oxidized alginate by cross-linking, and their degradation behavior was monitored by measuring the changes of dry weight, molecular weight, and the amount of soluble reducing sugars in solution.

2. Experimental

2.1. Depolymerization reaction of alginate

Sodium alginate was purchased from Zhejiang Jingyan Biomedical Co. (Zhejiang, China) and purified in our lab (G/M ratio was 0.501 determined by ^{1}H NMR). The oxidation reaction was carried out in a water bath. After alginate was completely dissolved in distilled water to a final concentration of 1.5% (w/v), the solution was pre-heated to the desired temperature, then hydrogen peroxide (30%) was added. After reaction, the solution was removed from the water bath and cooled to chill the reaction. The solution pH was adjusted by HCl or NaOH. The depolymerization rate was studied by measuring the apparent viscosity with a Brookfield DV-II+-Pro viscosimeter at 20 °C. Weight average molecular weights of selected samples were also determined.

The oxidized alginate sample with a high oxidation degree used for characterization was prepared by freeze-drying after reaction of 5 h at 50 °C with a high H_2O_2 concentration of 1.5% (w/v), while the sample with a low oxidation degree was prepared by reaction of 1 h at 50 °C with 0.6% (w/v) H_2O_2. The sample used for preparation of calcium alginate scaffold was also obtained under the same weak conditions.

2.2. Characterization of alginate

Molecular weight (M_w) was determined by gel permeation chromatography (GPC) on a Waters instrument with two columns in series of G5000PW$_{XL}$ and G3000PW$_{XL}$. The mobile phase was 0.1 M $NaNO_3$ maintained at 0.7 mL/min, and the sample concentration was adjusted to 0.5% (w/v). Pullulan standards (Shodex Standard P-82, Japan) were used for a calibration curve. The elemental analysis was carried out with an Elementar Analysensystem GmbH VarioEL. FT-IR spectra were recorded on a Bruker Vector 22 FT-IR spectrometer and ^{13}C NMR spectra on a spectrometer Bruker AM 500 at 80 °C in D_2O.

2.3. Formation and degradation of calcium alginate scaffolds

Aqueous solutions of oxidized alginate (3% w/v) were injected into 24-well plates followed by freeze-drying; the dry products were then immersed in a 0.1 M $CaCl_2$ solution for 5 min, washed with distilled water, and freeze-dried again to obtain calcium alginate scaffolds. To assess its degradation rate, the weighted scaffolds were immersed in a 0.9% NaCl solution at 37 °C with a rotation rate of 120 rpm. Three samples of each matrix type were collected at fixed time intervals, and their dry weight loss was measured after washing and freeze-drying. The solution was also centrifuged for 10 min for analysis of soluble reducing sugars by the modified Schales method (Imoto & Yagishita, 1971) and molecular weights by GPC.

3. Results and discussion

3.1. Depolymerization of alginate by H_2O_2

The depolymerization of 1.5% (w/v) solutions of alginate was firstly studied by viscometry under different reaction conditions. As shown in Fig. 1, the apparent viscosity (η) of the solution

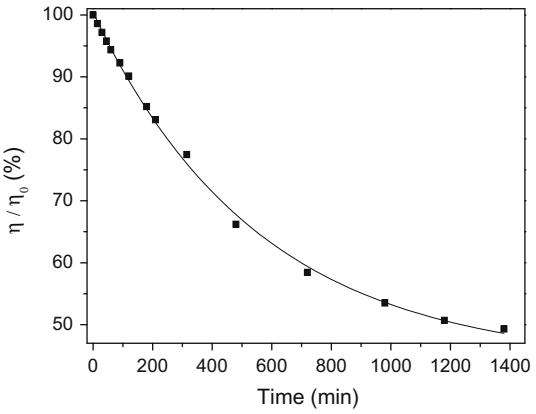

Fig. 1. Change of apparent viscosity of the alginate solution as a function of reaction time (H_2O_2 = 0.25% (w/v), T = 35 °C, pH 7.12).

decreased as a function of reaction time and the kinetic traces exhibited typical first-order kinetics. After reaction of 23 h at 35 °C with 0.25% (w/v) H_2O_2, the ratio of η/η_0 decreased to 50%. Although the viscosity change of alginate was very slow under the conditions, the rise of reaction temperature remarkably accelerated the process. As examples, the ratio of η/η_0 fast decreased to 50% after 2 h at 50 °C with 0.25% (w/v) H_2O_2, and decreased to 5.5% at 80 °C. The thermal degradation had little influence on the rate of alginate depolymerization when compared to the H_2O_2 degradation at the same temperature. The change of M_w after H_2O_2 treatment was also obtained by GPC measurement. After reaction of 2 h at 50 °C and 80 °C, the M_w of alginate decreased to 230 kDa and 143 kDa from the initial M_w of 254.5 kDa, respectively, indicating that low molecular weight alginate can be prepared by H_2O_2 treatment.

Fig. 2 shows the effect of H_2O_2 concentration on the extent of alginate depolymerization. There was nearly no change of apparent viscosity observed in the absence of H_2O_2, and the use of a small quantity of peroxide led to a fast decrease of viscosity. When the H_2O_2 concentration was more than 1%, the increase of the depolymerization rate became slow. Fig. 3 shows the effect of solution pH within the range of 5.3–9.5 on the extent of alginate depolymerization, in which the rate of depolymerization decreased proportionally with pH increasing.

The depolymerization of alginate by H_2O_2 is considered to be caused predominantly by free radicals. As measurable traces of metal ions exist in alginate samples (Davis, Volesky, & Mucci, 2003), the highly reactive radicals may be mainly formed from catalytic decomposition of H_2O_2 by the metal ions. When EDTA was added

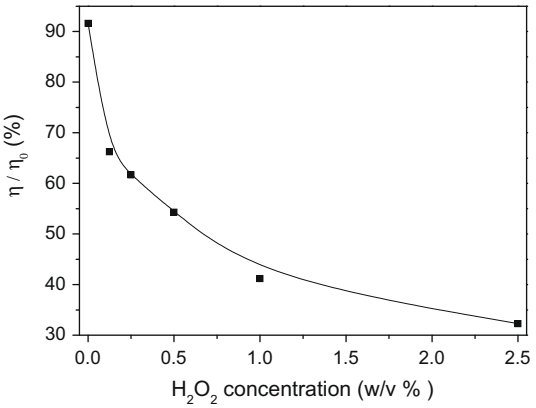

Fig. 2. Effect of H_2O_2 concentration on the change of apparent viscosity of alginate solution (T = 35°C, pH 7.12, Time = 2 h).

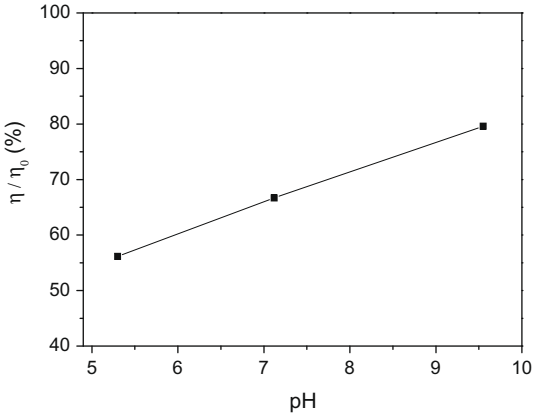

Fig. 3. Effect of pH on the change of apparent viscosity of alginate solution (H_2O_2 = 0.25% (w/v), T = 35°C, Time = 2 h).

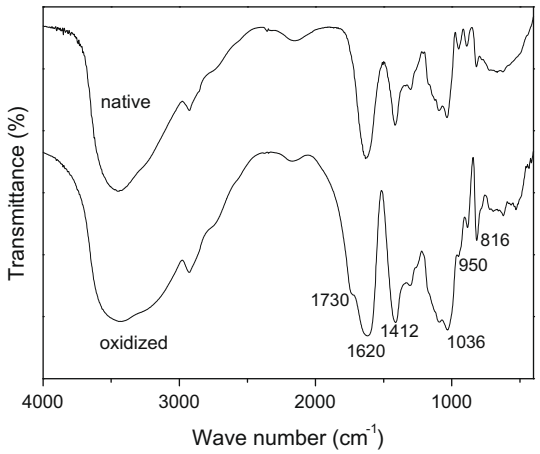

Fig. 4. FT-IR spectra of alginate before and after H_2O_2 oxidation.

into alginate solution, the slightly decrease of the extent of viscosity reduction was observed, suggesting the catalysis of metal ions for H_2O_2 decomposition (Chang, Tai, & Cheng, 2001). The rate of depolymerization decreased with increasing pH also confirmed the presumption, as the combination of metal ions and H_2O_2 is only effective at acidic conditions (Martinez et al., 2008); if not, the activity of H_2O_2 oxidation should be accelerated by base treatment (Uchiyama, Kiritoshi, Watanabe, & Ishihara, 2003). The resulting radicals are powerful oxidants and capable of abstracting hydrogen atoms from the glycosidic bonds of alginate, rearranging the structure of the molecular and breaking glycosidic bonds (Yang et al., 2004).

3.2. Characterization of oxidized alginate

Different techniques were used to analyze the changes of composition and structure of alginate after H_2O_2 treatment. The main properties of native and oxidized alginate are shown in Table 1. Elemental composition of alginate did not change significantly after reaction with a high oxidation degree, but the mass ratio of C/H slightly increased. As no residual H_2O_2 in oxidized alginate sample after freeze-drying was detected by addition of KI and starch, the result can be attributed to the increase of oxygen content, that is to say, the chemical structure of alginate was modified by H_2O_2 treatment. In addition, a large decrease in M_w and solution pH was observed, mainly due to the scission of the main chain and the production of carboxylic acid during alginate oxidation, respectively.

FT-IR spectra of alginate before and after depolymerization are shown in Fig. 4. As can be seen, the native alginate showed the characteristic absorption bands of polysaccharide structure around 1298 cm^{-1} (C–C–H and O–C–H stretching), 1130 cm^{-1} (C–O stretching), 1094 cm^{-1} (C–O and C–C stretching of pyranose rings) and 1034 cm^{-1} (C–O stretching). The asymmetric and symmetric stretching of carboxylate vibrations appeared at 1620 and 1415 cm^{-1}, respectively (Leal, Matsuhiro, Rossi, & Caruso, 2008). The spectrum of oxidized alginate exhibited most of the characteristic adsorption peaks of native alginate but with some differences. For instance, the peaks at 1620 cm^{-1} and 1094 cm^{-1} became broader and moved to lower wave numbers. The spectrum also showed the new bands appeared at 1730 cm^{-1} and 948.5 cm^{-1}; the former indicated the formation of carboxyl groups and the latter can be assigned to the C–O stretching vibration of uronic acid residues (Leal et al., 2008). These analyses are in agreement with the date given in Table 1, suggesting that H_2O_2 treatment under extreme conditions broke the glucoside bonds with the change of the structure of reducing end residue and formation of –COOH groups.

Table 1
Main properties of alginate before and after H_2O_2 oxidation.

Sample	$M_w \times 10^3$	Solution pH	C%	H%	C/H
Native alginate	254.5	6.93	29.85	4.69	6.36
Oxidized alginate	12.2	4.91	31.97	4.00	7.99

To further confirm the structural changes during alginate depolymerization, the native and oxidized alginates were analyzed by ^{13}C NMR spectroscopy. From the ^{13}C NMR spectra shown in Fig. 5, it can be seen that the presented signals for native alginate after acid hydrolysis appeared at about 178, 103, 81, 79, 74 and 72 ppm, which can be assigned to C-6, C-1, C-4, C-5, C-3, C-2, respectively, and were consistent to the reported results in some literatures (Heyraud et al., 1996; Holtan, Zhang, Strand, & Skjåk-Bræk, 2006; Leal et al., 2008). Additionally, the peak at 94.1 ppm can be attributed to hemiacetalic carbons corresponding to aldehyde groups generated by reducing end of C-1 of the polymer (Gomez, Rinaudo, & Villar, 2007; Holtan et al., 2006). The spectrum of the oxidized alginate was almost the same as that of the acid hydrolyzed product, indicating the formation of hemiacetalic carbons after H_2O_2 treatment. The same conclusion was obtained when the alginate was heated with potassium ferricyanide, as more reducing sugars were measured on the oxidized than that on the native.

For the alginate sample with a low oxidation degree prepared under weak oxidation conditions, its M_w decreased to 140.9 kDa from 254.5 kDa due to the rupture of glucoside bonds just like that of strong oxidized alginate. But no obvious differences were observed in the spectra of FT-IR in comparison with that of native alginate, indicating its chemical structures of the main chain remaining unchanged after slight oxidation. During degradation of other polysaccharides with H_2O_2, carboxyl groups were only formed under tough conditions (Kabal'nova et al., 2001). The spectrum of ^{13}C NMR also indicated the formation of hemiacetalic carbons after H_2O_2 treatment.

By comparison of the above results and related literature reports, it can be concluded that the rupture of glucoside bonds in alginate chains into low molecular weight polymers with the formation of aldehyde groups by reducing end of C-1 is the basic process during H_2O_2 oxidation. Under more extreme reaction conditions, carboxyl groups associated with oligomers were also formed.

3.3. Degradation of oxidized alginate scaffold

After treatment by H_2O_2 (0.6% w/v) at 50 °C for 1 h, the lower M_w of alginate (140.9 kDa) was obtained, but the oxidation of alginate under these conditions did not significantly interfere in the

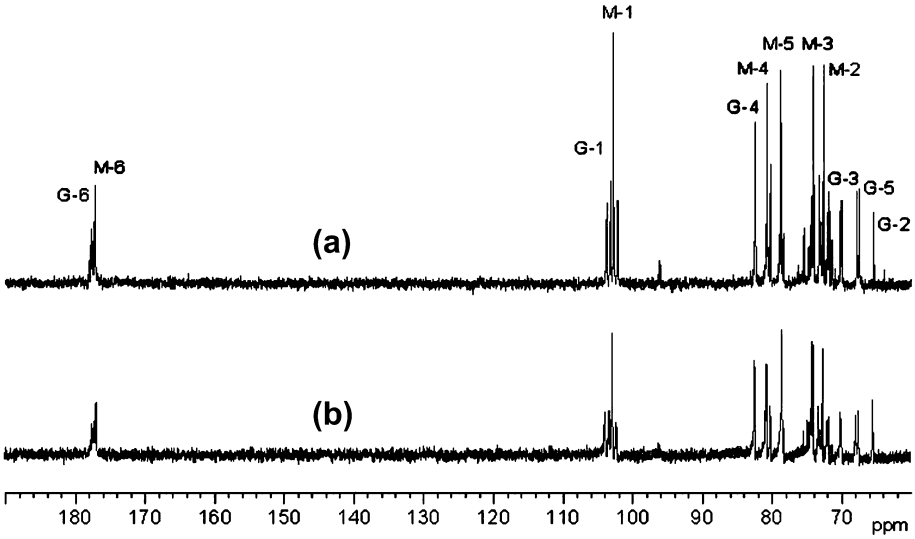

Fig. 5. ^{13}C NMR spectra of oxidized alginate (a) and alginate hydrolyzed in 0.1 M HCl at 100°C for 2 h and freeze-dried (Grasdalen, Larsen, & Smidsrød, 1979) (b).

formation of ionic junctions with calcium ions, which is one of the important features of alginate. However, under more extreme conditions such as the higher H_2O_2 concentration or the longer reaction time, the obtained alginate cannot be cross-linked by calcium any more. Thus, the above weak reaction conditions were selected, and the obtained oxidized alginate were used to prepare the calcium alginate scaffolds. The scaffolds were then incubated in NaCl solution to assess their degradation behavior, as the controllable biodegradation is a critical requirement for polymeric matrices in tissue engineering applications.

As shown in Fig. 6, the dry weight for oxidized alginate decreased approximately by 10% at the first day and got loss gradually to about 23% of the initial dry mass with physically break down in the post-incubation. On the contrary, the native scaffold displayed a relatively low mass loss without change of physical integrity. This result clearly demonstrated that the scaffolds formed from oxidized alginate degraded over time more easily. This fact was likely attributed to the lower M_w of oxidized alginate; in addition, the formed aldehyde groups in oxidized alginate are susceptible to hydrolysis (Bouhadir et al., 2001). For the hydrolysis of alginate can produce new reducing residues and can be detected by potassium ferricyanide, the concentration of reducing sugars was then analyzed. As shown in Fig. 7, the oxidized alginate exhibited an initial burst and subsequent slower rate of reducing sugars release from the scaffold, likely due to the loss of hydrolyzed low molecular alginate from the scaffold. Meanwhile, there were so few soluble reducing sugars released from the native alginate scaffold in the process, indicating its slower degradation rate. The results were consistent with the change of dry weight.

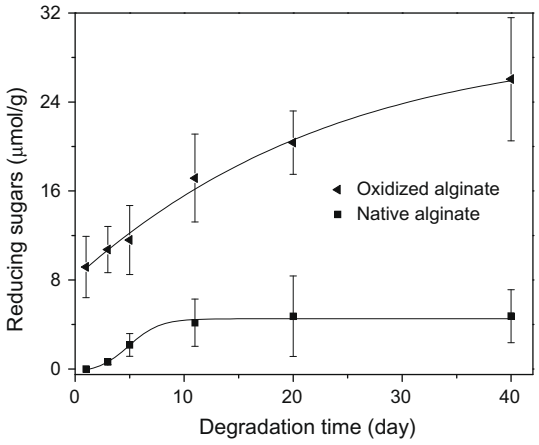

Fig. 7. Reducing sugars produced by degradation of alginate scaffold.

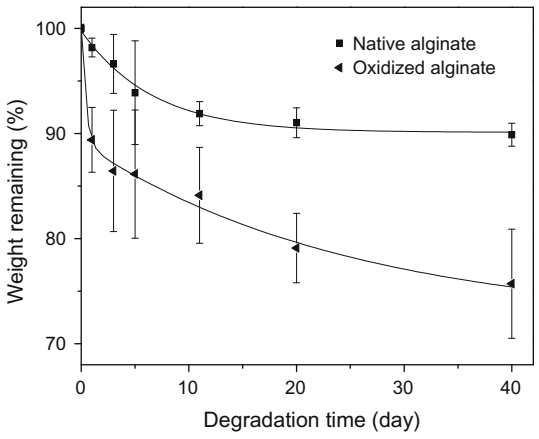

Fig. 6. Dry weight change of alginate scaffold during degradation.

4. Conclusions

Low molecular weight alginate can be effectively prepared by H_2O_2 treatment and the rate of depolymerization was dependent on the reaction time, temperature, solution pH and H_2O_2 concentration. The treatment of alginate by H_2O_2 led to a decrease of the chain length and the formation of aldehyde groups by reducing the end of C-1. But under more extreme reaction conditions, carboxyl groups associated with oligomers can be formed. The calcium cross-linked scaffold formed from oxidized alginate showed faster degradation than the unmodified alginate. These results suggest that hydrogen peroxide oxidized alginate can be used in biodegradable tissue engineering and drug delivery.

Acknowledgements

The authors acknowledge the financial support from the National Natural Science Foundation of China (Grant Nos. 20736006, 20806080).

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