Carbohydrate Polymers 83 (2011) 1558–1564

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Preparation and characterization of branched chitosans

Kristoffer Tømmeraas*, Sabina P. Strand, Bjørn E. Christensen, Olav Smidsrød, Kjell M. Vårum

Norwegian Biopolymer Laboratory (NOBIPOL), Dept. of Biotechnology, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway

ARTICLE INFO

Article history:
Received 16 August 2010
Received in revised form 6 October 2010
Accepted 6 October 2010
Available online 12 October 2010

Keywords:
Branching
Chitosan
^{1}H NMR spectroscopy
SEC-MALLS-RI-visc
Reductive *N*-alkylation

ABSTRACT

Fully de-*N*-acetylated chito-oligomers were prepared by de-amination of chitosan using nitrous acid (HNO_2). The dimer to hexamer fractions of chitosan oligomers were fractionated with preparative size-exclusion chromatography (SEC) and characterized using ^{1}H NMR spectroscopy. The purified oligomers were polymerized by reductive *N*-alkylation by the action of $NaCNBH_3$, in a reaction between the terminal 2,5-anhydro-D-mannofuranose (**M**-unit) and the primary amines of the repeating units (1→4) bound β-D-glucosamine (**D**-unit) resulting in a covalent bond and formation of secondary amines. The polymerized products were studied by SEC-RI and SEC-MALLS-RI and were confirmed to be branched reaching molecular weights of up to 10 000 g/mol. Analysis by ^{1}H NMR spectroscopy confirmed that the chitosan oligomers had been branched by formation of secondary amines.

Fully de-*N*-acetylated chitosan (F_A < 0.001) was degraded to various extents using nitrous acid resulting in degrees of scission (α_s) in the range 0.01–0.10. The resulting chitosans, with the reactive **M**-unit on the reducing end, were branched by reductive *N*-alkylation using $NaCNBH_3$. Analysis by SEC-MALLS-RI-visc showed that the weight-average molecular weight (M_w) and polydispersity (M_w/M_n) had increased, with a degree of branching in the range of 1.2–2.1. The structure in solution had become more compact compared to linear chitosan of the same molecular weight as demonstrated by a significant reduction of the radius of gyration and the intrinsic viscosity, with a corresponding change in the calculated viscosity contraction factor (g') and the geometric contraction factor (g), respectively.

1. Introduction

Chitosan is a linear polysaccharide composed of (1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose (GlcN, **D**-unit) and 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc, **A**-unit) where the *N*-acetylated units are randomly distributed and given as fraction of *N*-acetylated units, F_A (Roberts, 1992).

Several studies on the covalent attachment of aldehydes to chitosan by the use of reductive *N*-alkylation have been reported. Yalpani and Hall (1984) performed an extensive study on the branching of chitosan with monosaccharides, oligosaccharides and dextrans. Sugimoto, Morimoto, Sashiwa, Saimoto, and Shigemasa (1998) prepared water-soluble chitin and chitosan derivatives by grafting chitosan with variable length PEG aldehydes and subsequent *N*-acetylation. Similarly prepared cross-linked re-acetylated chitosans were observed by Dal Pozzo et al. (2000) to give biocompatible insoluble aggregates that only dissociated by the action of papain and lipase. Kurita, Amemiya, Mori, and Nishiyama (1999) prepared comb-shaped chitosan derivatives having oligo-(ethylene glycol) side chains showing higher affinity for organic solvents as well as water, in addition to improved adsorption capacity towards metal ions. Holme and Hall (1991) observed temperature-induced gelation when they *N*-alkylated chitosan with a variable length alkyl-glycoside. Capitani, De Angelis, Crescenzi, Masci, and Segre (2001) cross-linked chitosan by reductive alkylation with 1,1,3,3-tetramethoxypropane resulting in a hydrophilic hydro-gel.

We have previously reported on the preparation and characterization of oligosaccharides with a reactive 2,5-anhydro-D-mannofuranose (**M**) unit at the position corresponding to the reducing end (Tømmeraas, Vårum, Christensen, & Smidsrød, 2001). It was found that the **M**-units of de-*N*-acetylated oligomers easily decomposed to hydroxy-methyl furfural (HMF) by β-eliminations catalyzed by the formations of Schiff bases between the primary amines and the free aldehyde of the reducing end. In this study we report on the preparation of stable de-*N*-acetylated chitosan oligomers of the type **D**-[**D**]$_n$-**M** and their subsequent polymerization by reductive *N*-alkylation. These were characterized by ^{1}H NMR spectroscopy and SEC-MALLS-RI. Further, three fully de-*N*-acetylated chitosans of varying degree of polymerization (DP) with terminal **M**-units were prepared by HNO_2 degradation and subsequently branched by reductive alkylation.

Chitosans are of interest in e.g. gene delivery systems, because of its biocompatibility and non-toxicity (VandeVord et al., 2002). Several workers have already reported the use of native, unmodified chitosans for this purpose (Köping-Höggård et al., 2001;

* Corresponding author. Current address: Novozymes A/S, Krogshøjvej 36, DK-2880 Bagsværd, Denmark. Tel.: +45 44461038; fax: +45 44 46 71 72.
E-mail address: ktmm@novozymes.com (K. Tømmeraas).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.10.008

MacLaughlin et al., 1998; Richardson, Kolbe, & Duncan, 1999). In addition, preparation of chitosan-based dendrimers (Sashiwa, Shigemasa, & Roy, 2002) and quaternized chitosans (Thanou, Florea, Geldof, Juninger, & Borchard, 2002) for potential applications in gene delivery systems have been reported. The branched chitosans described in this work have already been shown to have promising applications in non-viral gene-delivery due to a less compact structure when polyplexes are prepared with plasmid DNA (Issa et al., 2006; Strand, Issa, Christensen, Vårum, & Artursson, 2008).

2. Experimental

2.1. Materials

Chitosan with fraction of acetylated units (F_A) < 0.001 and intrinsic viscosity ([η]) of 283 mL/g was prepared by heterogeneous de-*N*-acetylation as earlier described (Sannan, Kurita, & Iwakura, 1976; Tømmeraas et al., 2001; Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991).

2.2. Preparation of de-N-acetylated oligomers

Fully de-*N*-acetylated chitosan oligomers (dimer to hexamer) of the type **D**-[**D**]$_n$-**M** (n = 0, 1, 2, 3, 4) were prepared by nitrous acid depolymerization of chitosan (Allan & Peyron, 1989) (F_A < 0.001 and [η] = 283 mL/g) and separated by size exclusion chromatography (SEC) as described by Tømmeraas et al. (2001). The oligomers were then stored in aqueous 0.15 M ammonium acetate solution at pH 4.5 until the time of the polymerization reaction.

2.3. Polymerization of chito-oligomers by reductive N-alkylation

The de-*N*-acetylated dimer to hexamer fractions (**D**-[**D**]$_n$-**M**, n = 0, 1, 2, 3, 4) were each polymerized (self-branched) by reductive *N*-alkylation (Borch, Bernstein, & Durst, 1971) of a 1% solution at pH 5.5 in 0.15 M ammonium acetate and 0.1 M NaCl. $NaCNBH_3$ was added in excess (approx. 50 mg) twice, after 30 min and 24 h. The reaction was stopped after 4 days on stirring in room temperature by adding concentrated HCl (pH < 2) to remove $NaCNBH_3$. The products were then lyophilized after adjusting the pH < 4 to obtain the derivatives in their HCl form.

2.4. Preparative chromatography of chitosan oligomers

The chitosan oligomers **D**-[**D**]$_n$-**M** (n = 0, 1, 2, 3, 4) were separated by SEC on two 2.5 cm × 100 cm columns connected in series and packed with Superdex 30, eluted with 0.15 M ammonium acetate at pH 4.5, before and after their self-branching. A flow rate of 0.8 mL/min was used. The relative amounts of oligomer fractions were monitored by means of an on-line refraction index (RI) detector (Shimadzu RID-6A). This procedure was used to both study the distribution of the resulting branched chitosan oligomers and to remove remains of borate salts that might interfere with the subsequent studies by ^{1}H NMR spectroscopy.

2.5. ^{1}H NMR spectroscopy

All samples were dissolved in D_2O, and transferred to 5 mm NMR tubes. The measurements were performed on a Dpx 400 Bruker Avance spectrometer. All chemical shifts were determined relative to internal TSP (sodium 3-(trimethylsilyl)-propionate-d_4) from Aldrich Chem. Co., 5 μL added from a 1% stock solution (Wishart et al., 1995). Typical conditions for acquisition of ^{1}H NMR spectra: 400.13 MHz, 25 °C, size of spectral window: 8220 Hz, centre of the ^{1}H NMR spectra: 1880 Hz, acquisition time: 3 s, actual pulse repetition time: 4 s, number of scans: 64 and a 30° excitation pulse-angle was used, data size: 32 K. The pH* (value obtained when measuring with a pH-meter, correction for isotopic effect (Nilges, Habazettl, Brünger, & Holak, 1991): pD = pH* + 0.4) were measured by use of a Mettler Toledo pH electrode. The pH* value was measured in all NMR samples.

2.6. ^{1}H NMR titration studies of polymerized chitosan oligomers

The branched tetramer (**D**-**D**-**D**-**M**) was dissolved (10 mg in 0.7 mL D_2O, 0.1 M NaCl) and transferred to a 5 mm NMR tube. Starting at a low pD value (2.5), adjusted by 0.1 M DCl, a standard ^{1}H NMR spectrum was acquired. The pD was increased in a step-wise fashion using 0.1 M NaOD. After each adjustment, a new ^{1}H NMR spectrum was acquired. This process was continued until the sample pD was above 10.

2.7. Depolymerization of fully de-N-acetylated chitosan

Chitosan (F_A < 0.001, [η] = 283 mL/g, 300 mg, HCl form) was dissolved and degraded by nitrous acid (Tømmeraas et al., 2001) to degrees of scission (α_s): 0.01, 0.025, 0.05 and 0.10 by addition of 0.015, 0.0375, 0.075 and 0.15 mmol $NaNO_2$, respectively. The chitosans prepared were dialyzed under acidic conditions to remove oligomers under DP 6 and excessive salts. SEC-MALLS-RI was used to determine the obtained molecular weights (M).

2.8. Branching of chitosans by reductive N-alkylation

Fully de-*N*-acetylated chitosans with **M**-units at the reducing end (α_s of 0.01, 0.025, 0.05 and 0.1) were branched by reductive *N*-alkylation (1% solution) at pH 5.5 in 0.1 M ammonium acetate and 0.1 M NaCl. $NaCNBH_3$ was added in excess (approx. 50 mg) twice, after 30 min and 24 h. The reaction was stopped after 4 days on stirring in room temperature by adding concentrated HCl (pH < 2) to eliminate remaining $NaCNBH_3$. Borate ions were removed by dialysis (0.2 M NaCl followed by de-ionized water). The products were then lyophilized after adjusting the pH < 4 to obtain the derivatives in their HCl form.

2.9. Polymer characterization with SEC-MALLS-visc (SMV)

SEC with refractive index (RI) and multi-angle laser light scattering detectors (MALLS), and in som cases an additional viscosity detector, was performed as described previously (Christensen, Vold, & Vårum, 2008).

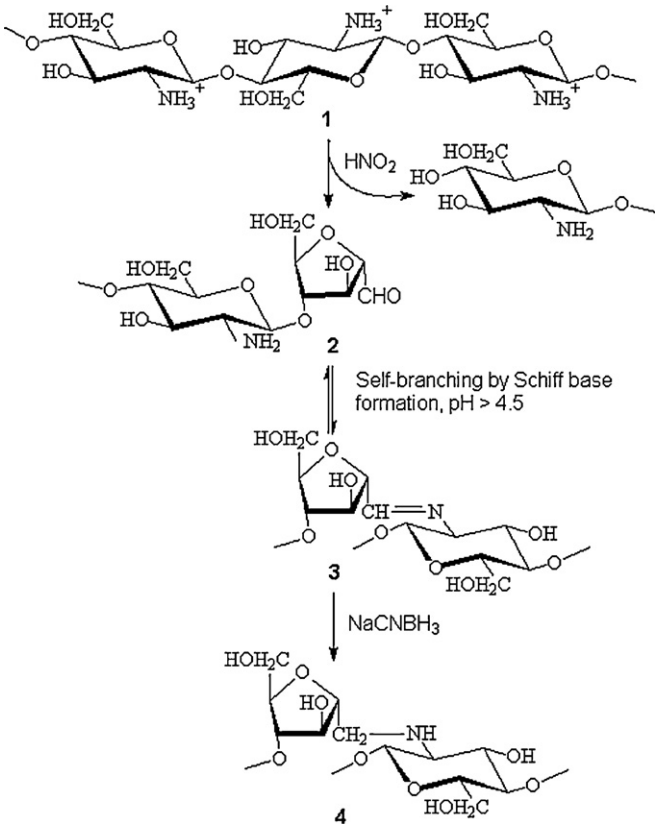
2.10. Intrinsic viscosity measurements

Intrinsic viscosities were measured at 20 °C by the use of an Ubbelohde Viscosimeter (Type Schott 53101/0a) at constant ionic strength ([I] = 0.1 M NaCl), as previously described (Draget, Vårum, Moen, Gynnild, & Smidsrød, 1992).

3. Results and discussion

3.1. Polymerization of chitosan oligomers by reductive N-alkylation

Chitosan oligomers of the type (**D**-[**D**]$_n$-**M**, n = 0, 1, 2, 3, 4) prepared by nitrous acid depolymerization (Allan & Peyron, 1989; Tømmeraas et al., 2001) and preparative gel filtration, were subjected to reductive *N*-alkylation ($NaCNBH_3$) (Borch et al., 1971) to obtain polymerized (or self-branched) chitosans (Scheme 1).



Scheme 1. Nitrous acid degradation and subsequent reductive *N*-alkylation produce branched chitosans (based on Tømmeraas, 2002).

Reversible Schiff bases are formed between reducing ends (**M**-units) and primary amines (**D**-units) that subsequently are reduced to secondary amines. It was shown by SEC that distinct components of higher molecular weight were formed (see Fig. 1), indicating that a polymerization had indeed occurred. In the chromatograms of all the branched samples, a new peak was observed at a slightly higher

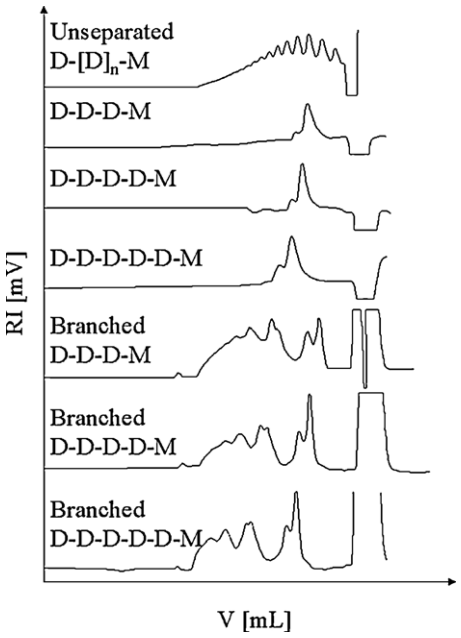


Fig. 1. Chromatograms of de-*N*-acetylated chitosan oligomers before the first separation, and before and after polymerization by reductive *N*-alkylation (data shown for tetra-, penta- and hexamer).

retention time than the starting chito-oligosaccharide (i.e. lower molecular weight). This was attributed to un-reacted oligomers that have lost their reducing **M**-units during the acidic conditions used to remove surplus $NaCNBH_3$ (Tømmeraas et al., 2001). At the branching point, secondary amines were formed. The observation of an H-2 resonance corresponding to **D**-units with *N*-branches in the ^{1}H NMR spectra of the polymerized oligomers, further substantiated that branching had occurred (see Fig. 2). Further, it was observed no signs of covalently bound 5-hydroxy-2-methylfurfural (HMF), which showed the oligomers to have been stable until the decomposition of remaining reduction compound at the end of the branching reaction.

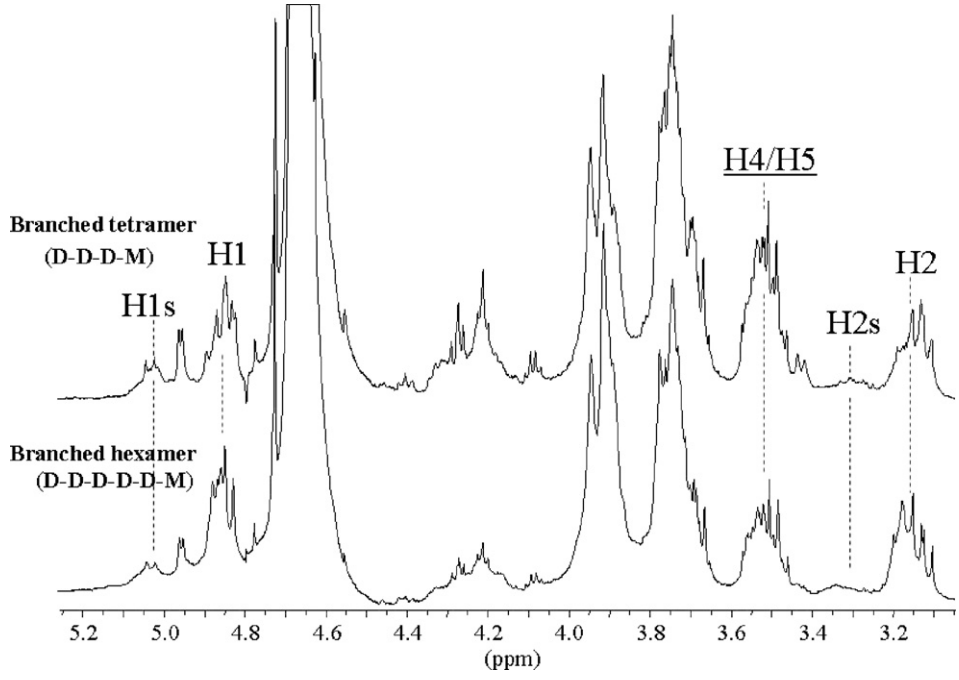


Fig. 2. ^{1}H NMR spectra of branched tetramer (**D-D-D-M**) and hexamer (**D-[D]$_4$-M**), respectively.

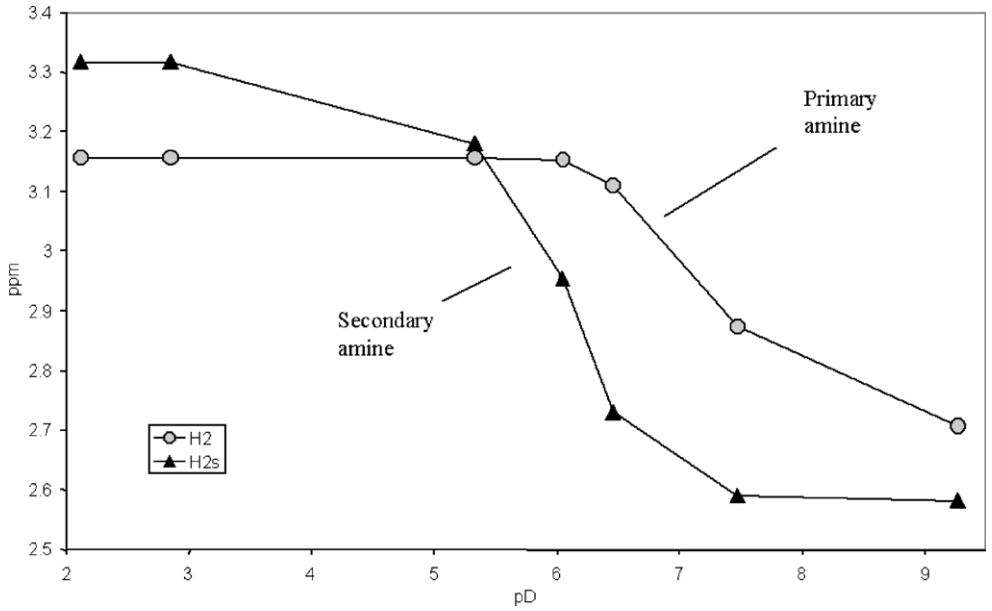


Fig. 3. Determination of pK_a^* values of the primary (un-reacted) and secondary (reacted) amines by ^{1}H NMR titration of H-2 resonances of **D**-units of the branched tetramer (**D**-**D**-**D**-**M**).

To evaluate the pK_a of the introduced secondary amines, a ^{1}H NMR titration experiment was made for the branched tetramer (**D**-**D**-**D**-**M**), and the titration curve is presented in Fig. 3. It was found that the primary and secondary amines had pK_a^* values (in 90% D_2O) of 6.8 and 5.8, respectively. This difference in acidity is in agreement with the results of Tømmeraas et al. (2001), that found that secondary amines in chitosans branched by carbohydrates have a significantly lower pK_a value than the primary un-substituted amines.

The branched chito-oligomers were studied by SEC-MALLS and the molecular weight distributions and concentration profiles are presented in Fig. 4. Although much of the polymerized dimer (**D**-**M**) was eluted in the salt peak, a significant amount was polymerized to an extent that a partial molecular weight distribution could be obtained. All the five separate polymerized samples reached molecular weights up to 10 000 g/mol, proving that a substantial polymerization had occurred. Further, all the branched products have broad molecular weight distributions. Probably, there are also variations in where along the chains the branching points are introduced giving variations in retention time for oligomers of identical mass. The only exception from this should be the polymerized dimer fraction, where only one possible reaction point (primary amine) exists.

3.2. Preparation of branched chitosans

Fully de-*N*-acetylated chitosan was partially degraded with nitrous acid to degrees of scissions of 0.1, 0.025, 0.05 and 0.01, respectively, resulting in chitosans of various sizes with terminal **M**-units. Dialysis removed the lower molecular weight fractions (approx. DP < 6) and excessive salts. Subsequently, the degraded chitosans were self-branched by reductive *N*-alkylation under sim-

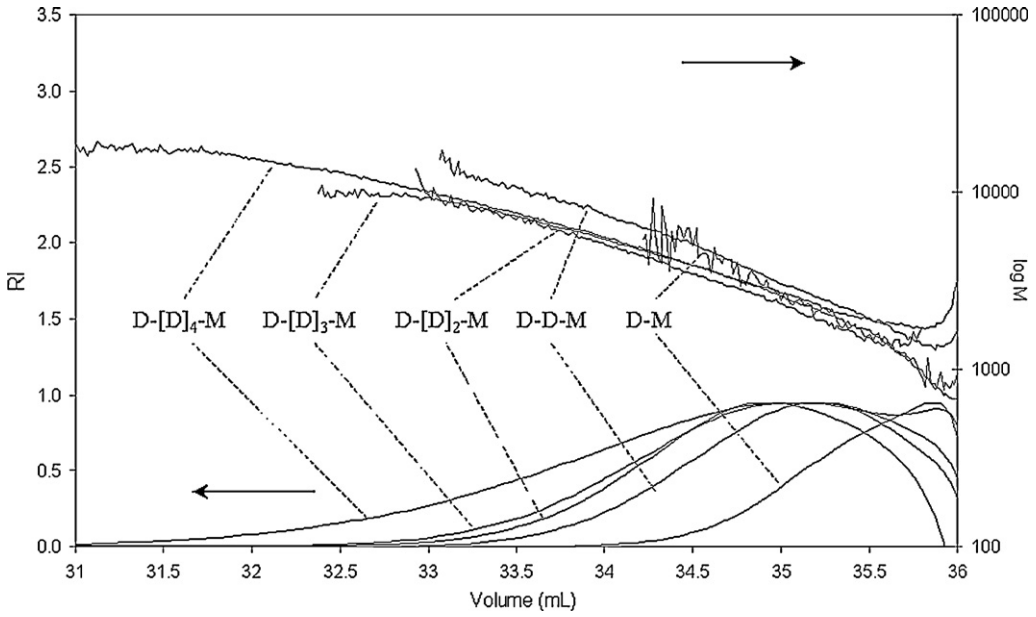


Fig. 4. Molecular weight (*M*) distribution and concentration profile (RI-response) of polymerized chitosan oligomers, dimer (**D**-**M**) to hexamer (**D**-[**D**]$_4$-**M**).

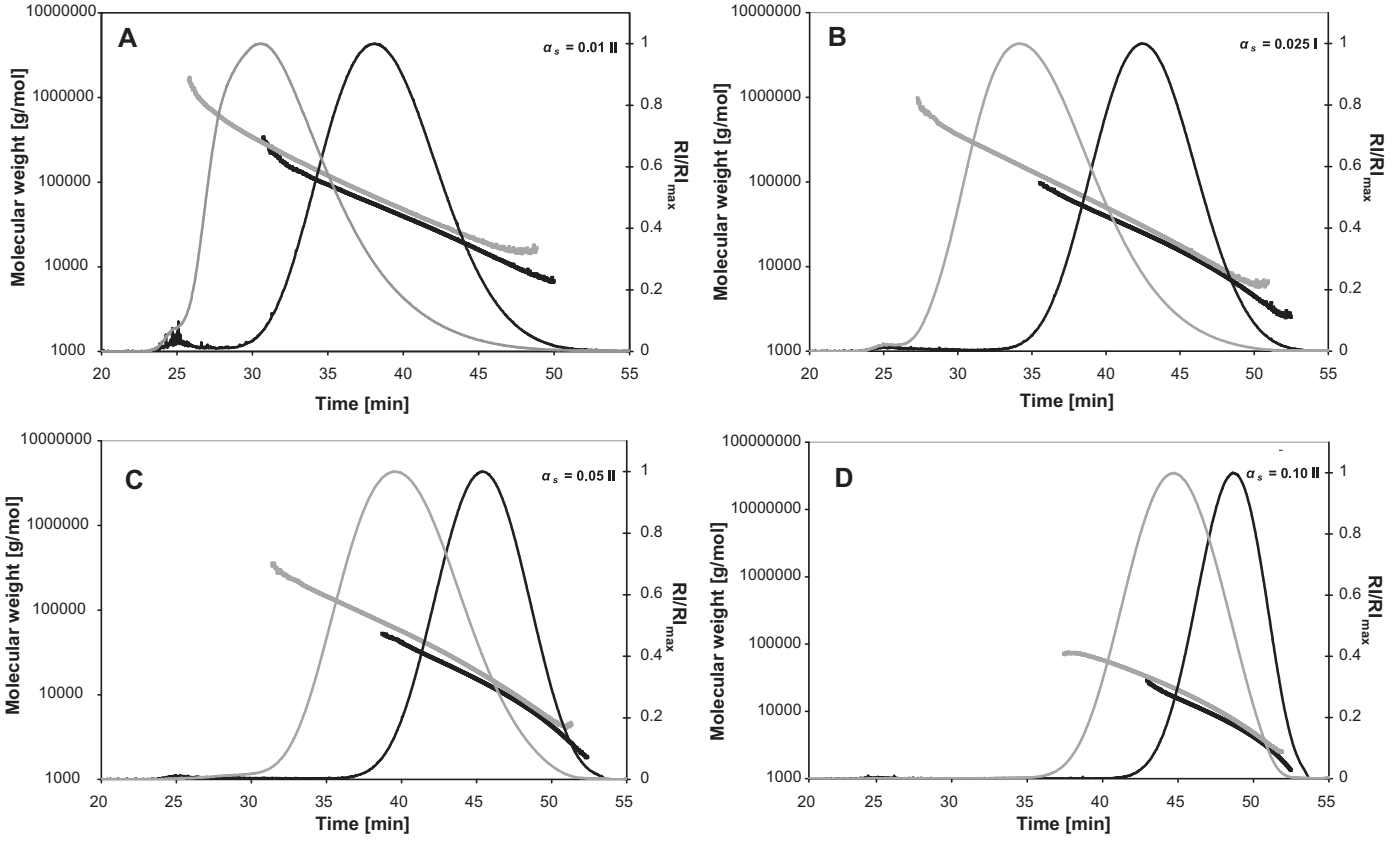


Fig. 5. Molecular weight (M) and relative concentration (RI/RI$_{max}$), as determined by SEC-MALLS-RI-visc, for degraded and branched chitosans with degree of scission (α_s), 0.01, 0.025, 0.05 and 0.10 are given in (A), (B), (C) and (D), respectively. Degraded chitosans are plotted in black and resulting branched products in grey.

ilar conditions as for the low molecular weight chito-oligomers described above. The introduction of branches was confirmed by ^{1}H NMR spectroscopy (data not shown) for the two low molecular weight chitosans (α_s 0.05 I and 0.10 I). Fig. 5 shows the molecular weight and concentration profile obtained by SEC-MALLS for four of the degraded chitosans before and after the branching reaction. The weight-average molecular weights (M_w) and polydispersities (M_w/M_n) are presented in Table 1.

The average number of branches per molecule (DB$_n$), was calculated as:

$$\mathrm{DB}_n = \frac{M_n\,(\text{branched})}{M_n\,(\text{unreacted})} - 1 \qquad (1)$$

Results are given in Table 1. In general, values in the range of 1.2–2.1 were obtained, i.e. the chitosans contain 1.2–2.1 branches per molecule after reaction. If the reaction is close to random (all glucosamine residues are equally reactive, and all M residues are equally reactive, respectively) then such DB values imply that the reaction products are mixtures of branched and linear chains.

The intrinsic viscosities ([η]) of three branched chitosans were determined and are compared to the intrinsic viscosities expected for linear de-*N*-acetylated chitosans of the same molecular weights (see Table 2). These data were calculated by the use of the Kuhn–Mark–Houwink–Sakurada (KMHS) equation:

$$[\eta] = K \times M^a \qquad (2)$$

Table 1
Comparison of weight–average molecular weight (M_w), polydispersity (M_w/M_n) and Z-average radius of gyration ($R_{g,z}$) for randomly degraded, fully de-*N*-acetylated chitosans (F_A < 0.001) of various degrees of scission (α_s) before and after branching by reductive *N*-alkylation. Further, the degree of branching (DB$_n$) calculated from DB$_n$ = [M_n (branched)/M_n (unreacted)] − 1, is presented.

α_s	M_w degr. $\times 10^3$ g/mol	M_w bran. $\times 10^3$ g/mol	M_w/M_n degr.	M_w/M_n bran.	$R_{g,z}$ degr. nm	$R_{g,z}$ bran. nm	DB
0[a]	130	n/a	1.9	n/a	41	n/a	n/a
0.01 I	40	180	1.7	3.1	18	40	1.5
0.01 II	39	140	1.8	2.5	13	32	1.6
0.01 III	34	150	1.6	2.4	17	34	1.9
0.025 I	19	89	1.6	2.5	9	21	2.0
0.025 II	19	82	1.6	2.4	11	34	1.9
0.05 I	14	31	1.6	1.6	11	10	1.2
0.05 II	11	40	1.6	2.1	–[b]	13	1.8
0.05 III	11	38	1.6	2.1	–[b]	21	1.6
0.10 I	6.0	26	1.4	2.7	–[b]	11	1.2
0.10 II	5.1	17	1.6	1.7	–[b]	–[b]	2.1
0.10 III	5.3	16	1.5	1.7	–[b]	8	1.7

[a] Starting material (linear chitosan).
[b] Size of molecule is too small to give a meaningful value by SEC-MALLS-RI-visc.

Table 2
Intrinsic viscosities ([η]) for three randomly degraded, fully de-*N*-acetylated chitosans (F_A < 0.001) (degree of scission (α_s), 0.10, 0.05, 0.025 and 0.01) after branching by reductive *N*-alkylation. Intrinsic viscosity values have been estimated for linear chitosan of same M_w and F_A. Further, the viscosity contraction factor (g') is presented.

α_s	[η][a] branched mL/g	[η][b,c] linear mL/g	g'
0.01 I	270	414	0.65
0.01 II	215	312	0.69
0.01 III	207	337	0.62
0.025 I	111	202	0.55
0.025 II	110	190	0.58
0.05 I	54	60	0.90
0.05 II	52	106	0.49
0.05 III	50	85	0.59
0.10 I	33	71	0.46
0.10 II	22	38	0.58
0.10 III	23	37	0.61

[a] Experimental results (0.1 M NaCl).
[b] Calculations based on experimental data.
[c] Calculated for M_w of branched samples.

where M is the molecular weight and K and a are constants specific for a given polymer/solvent system. We used $K = 0.559$ and $a = 0.58$ as determined by Anthonsen, Vårum, and Smidsrød (1993) for fully de-*N*-acetylated chitosan ($F_A = 0$) in aqueous 0.1 M NaCl. When comparing the intrinsic viscosities it is apparent that the more branched the chitosans are, the bigger the deviation from the expected value for a linear chitosan of the same chemical composition. An important quantity for characterization of branching is g, which stands for the ratio between the mean square radiuses of gyration ($\langle R_\theta^2 \rangle$) of branched molecules to that of linear molecules of the same molecular weight in the unperturbed state:

$$g = \frac{\langle R_\theta^2 \rangle_{br}}{\langle R_\theta^2 \rangle_{lin}} \tag{3}$$

The parameter g has been calculated for several structures, e.g. star- and comb-shaped molecules as well as randomly branched molecules (Yamakawa, 1971; Zimm & Stockmayer, 1949). Light scattering measurements under θ-conditions should in principle allow the determination of g, but usually the parameter g' is used instead:

$$g' = \frac{[\eta]_{br,\theta}}{[\eta]_{lin,\theta}} \tag{4}$$

where

$$g' = g^{\varepsilon} \tag{5}$$

It is much more difficult to calculate [η] for different branching structures than it is for $\langle R_\theta^2 \rangle$. Values for ε between 1/2 and 3/2 for different structures in θ-solvents have been proposed (Burchard, 1999; Zimm & Kilb, 1959). Viscosity measurements are, however, normally performed in good solvents, i.e. the polymer molecules are much more expanded than in θ-solvents, and the degree of expansion is generally different for branched and linear molecules (Burchard, 1999; Hjertberg, Kulin, & Sörvik, 1983; Yamakawa, 1971). The influence of non-ideality can be expressed by an extra exponent in g. For measurements in good solvents the following expression is used:

$$g' = g^{b} \tag{6}$$

where b includes the effects of all unknown factors. Different values of b have been found experimentally, e.g. $b = 0.5$ for 4-star polystyrene under θ-conditions (Roovers & Bywater, 1972), $b = 0.8$–1.0 for randomly branched polyethylene in good solvents (Hert & Strazielle, 1983) and $b = 0.71$ for low molar mass hyperbranched dextrans in water (Ioan, Aberle, & Burchard, 2001). Because chitosans in water in general are much stiffer chains than dextrans, we choose to use $b = 0.8$ in our estimations of g using Eq. (6) (see Table 2). It is apparent that the contraction factors (g and g') decrease as the chitosans become more densely branched.

Fig. 6 shows the contraction factor (g) calculated for the same four branched chitosans relative the native chitosan precursor using Eq. (3). As can be seen, as more branches are introduced, the structure obtained becomes more compact which is also confirmed by that the intrinsic viscosities determined for the branched chitosans are much lower than expected for a linear chitosan on the same composition and molecular weight (Table 2).

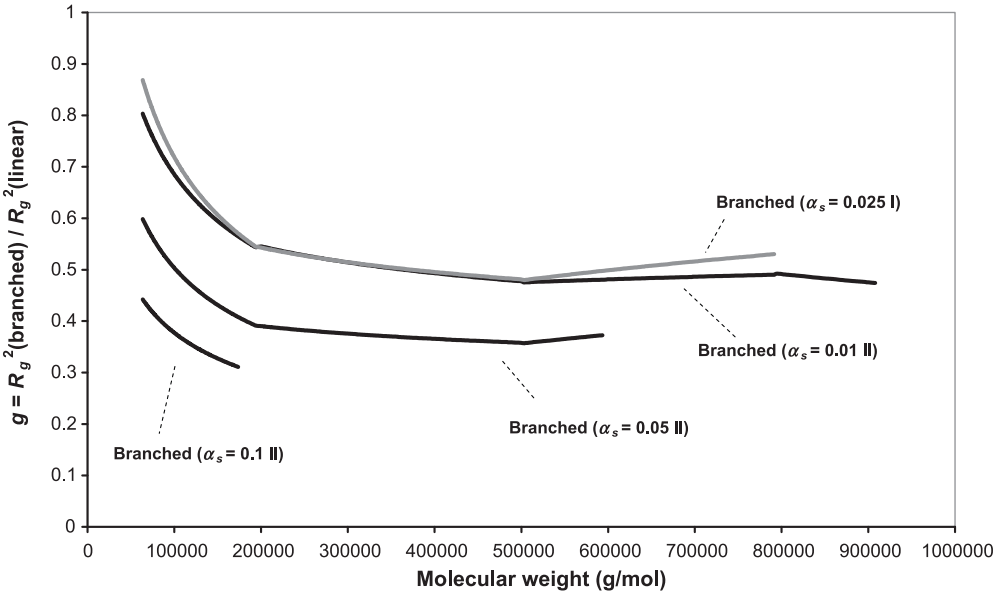


Fig. 6. Plot of the geometric contraction factor (g as function of molecular weight for four branched chitosans relative a native linear chitosan).

4. Conclusion

Fully de-*N*-acetylated chitosan oligomers, and higher molecular weight distributions of chitosans, were polymerized by reductive *N*-alkylation. Analysis by ^{1}H NMR spectroscopy showed that secondary amines (branch points) had been generated. It was further shown by SEC-MALLS-RI-visc that the branched chitosans had increased molecular weight and a more compact structure compared to their precursor molecules.

Acknowledgements

Drs. *H. Grasdalen* (Dept. of Biotechnology, NTNU) and *T. Hjertberg* (Dept. of Polymer Science, Chalmers Technical University) are thanked for their interest and helpful discussions. *A.-S. Ulset* is acknowledged for her skilful assistance in SEC-MALLS-RI-visc analysis. The late *M. Myhr* is thanked for her skilful assistance in doing the intrinsic viscosity measurements. This work was supported by a grant from the Research Council of Norway (Grant No. 121887/112).

References

Allan, G. G., & Peyron, M. (1989). The kinetics of the depolymerization of chitosan by nitrous acid. In G. Skjåk-Bræk, T. Anthonsen, & P. Sandford (Eds.), *Chitin and chitosan* (pp. 443–466). London: Elsevier Applied Science.

Anthonsen, M. W., Vårum, K. M., & Smidsrød, O. (1993). Solution properties of chitosans: Conformation and chain stiffness of chitosans with different degrees of N-acetylation. *Carbohydrate Polymers*, *22*, 193–201.

Borch, R. F., Bernstein, M. D., & Durst, H. D. (1971). The cyanohydridoborate anion as a selective reducing agent. *Journal of the American Chemical Society*, *93*, 2897–2904.

Burchard, W. (1999). Solution properties of branched macromolecules. *Advances in Polymer Science*, *143*, 113–194.

Capitani, D., De Angelis, A. A., Crescenzi, V., Masci, G., & Segre, A. L. (2001). NMR study of a novel chitosan-based hydrogel. *Carbohydrate Polymers*, *45*, 245–252.

Christensen, B. E., Vold, I. M. N., & Vårum, K. M. (2008). Chain stiffness and extension of chitosans and periodate oxidised chitosans studied by size-exclusion chromatography combined with light scattering and viscosity detectors. *Carbohydrate Polymers*, *74*, 559–565.

Dal Pozzo, A., Vanini, L., Fagnoni, M., Guerrini, M., De Benedittis, A., & Muzzarelli, R. A. A. (2000). Preparation and characterization of poly(ethylene glycol)-crosslinked reacetylated chitosans. *Carbohydrate Polymers*, *42*, 201–206.

Draget, K. I., Vårum, K. M., Moen, E., Gynnild, H., & Smidsrød, O. (1992). Chitosan cross-linked with Mo(VI) polyoxyanions: A new gelling system. *Biomaterials*, *13*, 635–638.

Hert, M., & Strazielle, C. (1983). Etude de la structure ramifiée du polyéthylène basse densité par viscosité et diffusion de la lumière de fractions issues de la chromatographie d'exclusion gpc. *Makromolecular Chemie*, *184*, 135–145.

Hjertberg, T., Kulin, L.-I., & Sörvik, E. (1983). Laser light scattering as GPC detector. *Polymer Testing*, *3*, 267–289.

Holme, K. R., & Hall, L. D. (1991). Chitosan derivatives bearing C10-alkyl glycoside branches: A temperature-induced gelling polysaccharide. *Macromolecules*, *24*, 3828–3833.

Ioan, C. E., Aberle, T., & Burchard, W. (2001). Structure properties of dextran. 3. Shrinking factors of individual clusters. *Macromolecules*, *34*, 3765–3771.

Issa, M. M., Köping-Höggård, M., Tømmeraas, K., Vårum, K. M., Christensen, B. E., Strand, S. P., et al. (2006). Targeted gene delivery with trisaccharide-substituted chitosan oligomers in vitro and after lung administration in vivo. *Journal of Controlled Release*, *115*, 103–112.

Kurita, K., Amemiya, J., Mori, T., & Nishiyama, Y. (1999). Comb-shaped chitosan derivatives having oligo(ethylene glycol) side chains. *Polymer Bulletin*, *42*, 387–393.

Köping-Höggård, M., Tubulekas, I., Guan, H., Edwards, K., Nilsson, M., Vårum, K. M., et al. (2001). Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Therapy*, *8*, 1108–1121.

MacLaughlin, F. C., Mumper, R. J., Wang, J., Tagliaferri, J. M., Gill, I., Hinchcliffe, M., et al. (1998). Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *Journal of Controlled Release*, *56*, 259–272.

Nilges, M., Habazettl, J., Brünger, A. T., & Holak, T. A. (1991). Relaxation matrix refinement of the solution structure of squash trypsin inhibitor. *Journal of Molecular Biology*, *219*, 499–510.

Richardson, S. C. W., Kolbe, H. V. J., & Duncan, R. (1999). Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. *International Journal of Pharmaceutics*, *178*, 231–243.

Roberts, G. A. F. (1992). *Chitin chemistry*. Hong Kong: Macmillian.

Roovers, J. E. L., & Bywater, S. (1972). Preparation and characterization of four-branched star polystyrene. *Macromolecules*, *5*, 384–388.

Sannan, T., Kurita, K., & Iwakura, Y. (1976). Studies on chitin 2: Effect of deacetylation on solubility. *Makromolecular Chemie*, *177*, 3589–3600.

Sashiwa, H., Shigemasa, Y., & Roy, R. (2002). Chemical modification of chitosan 8: Preparation of chitosan-dendrimer hybrids via short spacer. *Carbohydrate Polymers*, *47*, 191–199.

Strand, S. P., Issa, M. M., Christensen, B. E., Vårum, K. M., & Artursson, P. (2008). Tailoring of chitosans for gene delivery: Novel self-branched glycosylated chitosan oligomers with improved functional properties. *Biomacromolecules*, *9*, 3268–3276.

Sugimoto, M., Morimoto, M., Sashiwa, H., Saimoto, H., & Shigemasa, Y. (1998). Preparation and characterization of water-soluble chitin and chitosan derivatives. *Carbohydrate Polymers*, *36*, 49–59.

Thanou, M., Florea, B. I., Geldof, M., Junginger, H. E., & Borchard, G. (2002). Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. *Biomaterials*, *23*, 153–159.

Tømmeraas, K. (2002). *Preparation and characterization of branched chitosans*. Ph.D. Thesis. Department of Biotechnology, NOBIPOL, Norwegian University of Science and Technology, Trondheim.

Tømmeraas, K., Vårum, K. M., Christensen, B. E., & Smidsrød, O. (2001). Preparation and characterisation of oligosaccharides produced by nitrous acid depolymerisation of chitosans. *Carbohydrate Research*, *333*, 173–144.

VandeVord, P. J., Matthew, H. W., DeSilva, S. P., Mayton, L., Wu, B., & Wooley, P. H. (2002). Evaluation of the biocompatibility of a chitosan scaffold in mice. *Journal of Biomedical Materials Research*, *59*, 585–590.

Vårum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991). Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field NMR spectroscopy. *Carbohydrate Research*, *211*, 17–23.

Wishart, D. S., Bigam, C. G., Yao, J., Abildgaard, F., Dyson, H. J., Oldfield, E., et al. (1995). 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *Journal of Biomedical NMR*, *277*, 135–140.

Yamakawa, H. (1971). *Modern theory of polymer solutions*. NY, Harper & Row: Evanstone.

Yalpani, M., & Hall, L. D. (1984). Some chemical and analytical aspects of polysaccharide modifications: 3. Formation of branched-chain, soluble chitosan derivatives. *Macromolecules*, *17*, 272–281.

Zimm, B. H., & Kilb, R. W. (1959). Dynamics of branched polymer molecules in dilute solution. *Journal of Polymer Science*, *37*, 19–42.

Zimm, B. H., & Stockmayer, W. H. (1949). The dimensions of chain molecules containing branches and rings. *Journal of Chemical Physics*, *17*, 1301–1314.