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Periodate oxidation of chitosans with different chemical compositions

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Abstract—Periodate oxidation of chitosans with different chemical compositions were investigated by determining the consumption of periodate consumed, and the amount of ammonia and formaldehyde liberated during the reaction. Oxidised chitosans were further characterised by size-exclusion chromatography with online multi-angle light scattering (SEC-MALLS) to obtain the molecular weight distributions, and by elemental analysis to obtain the N/C ratio. Chitosans became only partially oxidised by periodate, reaching degrees of oxidation around 0.5, when oxidising with excess periodate. Overconsumption of periodate is attributed to the extensive depolymerisation, which occurs concomitantly with the oxidation, thereby exposing novel reducing and non-reducing ends which consume additional periodate. Both the rate and extent of overoxidation, and the rate of depolymerisation decreased with increasing F_A. A chitosan-specific degradation mechanism is probably involved in the depolymerisation in addition to the general free-radical-mediated degradation.

Keywords: SEC-MALLS; Free radicals; Depolymerisation; Dialdehyde

1. Introduction

Periodate oxidation has been widely used as a routine method for elucidation of structures in complex carbohydrates, and its earliest applications helped to interpret fundamental structures in many polysaccharides such as cellulose, starch, glycogen and xylan.[1] The periodate ion, IO_4^-, attacks vicinal diols to cleave the carbon–carbon bond by an oxidation reaction, leading to the formation of a dialdehyde.[1] In addition to the vicinal diols, other 1,2-dioxygenated groups and 1,2-amino alcohols[2] are also oxidatively cleaved by the periodate. *N*-acetylation of the amino group, however, prevents cleavage.[3] The depolymerisation commonly occurs concomitantly with the periodate oxidation, partly because of the peeling reaction which may take place from the reducing end due to overoxidation,[4] Secondly, internal linkages may be cleaved due to random attack by hydroxyl radicals formed by the spontaneous decomposition of periodate in the solution.[5,6] Scavengers for hydroxyl radicals such as 1-propanol,[7] may inhibit the depolymerisation and the ensuing overoxidation.[8–10]

Periodate oxidation may also be used to obtain novel functionalities, as demonstrated by 'dialdehyde starch',[11] scleraldehyde[12] or carboxylated scleroglucan.[13] Opening of the pyranosidic rings may also result in increased chain flexibilities, and thereby reduced chain extensions, as demonstrated for alginates.[14,15]

Chitosans consist of two different monomers, 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN), respectively (Fig. 1). The monomer composition of the chitosan is conveniently characterised by the fraction of acetylated units, F_A, since the two residues are randomly distributed along the chain. Periodate oxidation of chitosan has been relatively little explored. It was used to verify the proposed β-1,4 structure in chitin and chitosan, but chitosans with intermediate F_A were not studied.[16] Moore and Roberts compared periodate oxidation and infrared spectrometry in order to determine the F_A in

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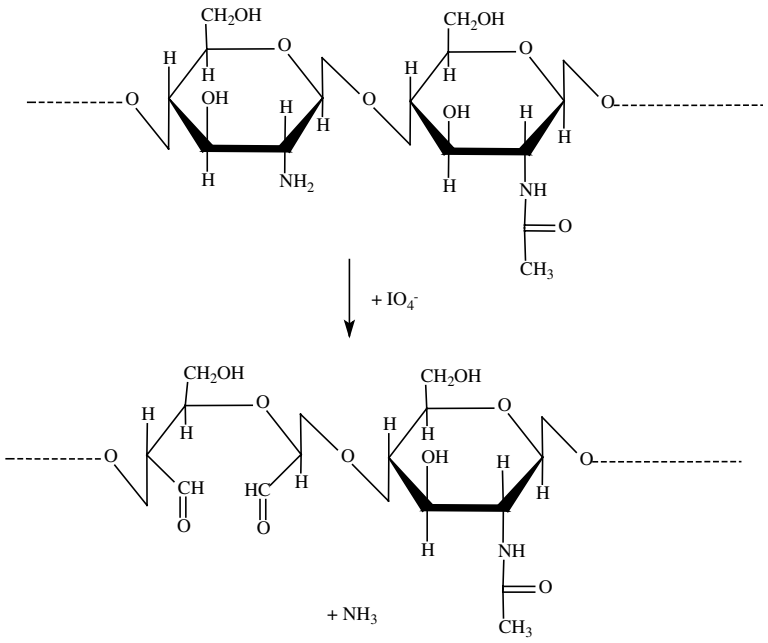

Figure 1. Chemical structure of a fragment of a partially de-*N*-acetylated chitosan, and the mechanism of periodate oxidation of GlcN residues.

chitosans.[17] Periodate-oxidised chitosan has been described as a component for achieving biocompatible solid surfaces.[18] It has further been reacted with urea and formaldehyde for subsequent enzyme immobilisation,[19] or oxidised to the corresponding dicarboxylate.[20] Extensive depolymerisation during the oxidation of chitosans was observed, whereas 6-*O*-glycol chitosan appeared to be somewhat less susceptible to degradation.[21]

Most high molecular weight chitosans are insoluble near and above physiological pH.[22] Moreover, the relatively high chain stiffness of chitosans restrict their electrostatic interactions with polyanions, for instance in the coating of alginate beads, where polycations such as poly(L-lysine) are commonly used.[23] Periodate oxidation of chitosan is expected to increase the chain flexibility and solubility. Different methods for determining the degree of oxidation (F_{ox}) or the dialdehyde content, in periodate oxidised polysaccharides have been described.[15,24,25] However, most of these are not appropriate at an analytical scale, or produce questionable results. For instance, the method of Lee et al.[15] fails to identify the well-documented oxidation limit in alginates, and may possibly overestimate F_{ox}.

The aim of the present study is to obtain an improved basis for producing high molecular weight, periodate-oxidised chitosans. The kinetics and stoichiometry of the reaction, and the effect of the experimental conditions on the reaction product, were investigated. We particularly focus on the role of F_A, and further evaluate the release of ammonia (N_t) and the measurements of the nitrogen/carbon (N/C) ratio to estimate the dialdehyde content or F_{ox}.

2. Experimental

Chitosan with $F_A = 0.01$ was prepared by further deacetylation of a commercial chitosan. Chitosan with $F_A = 0.16$ was provided by FMC Biopolymer (Drammen, Norway). Chitosan with $F_A = 0.49$ or 0.52 was prepared by homogeneous deacetylation. F_A was determined by ^{1}H NMR spectroscopy.[26]

The chitosans were dissolved overnight, and thereafter mixed with a given amount of periodate, and the proper amount of deionised water/buffer. The final concentration of GlcN was 2.5 mM. Prior to mixing the solutions was degassed with N_2 and adjusted to the desired temperature. The periodate was analysed by titration with sodium thiosulfate, with starch as an indicator.[27] Ammonia was analysed by a Cuvette Test LCK 303 (Dr. Bruno Lange GMBH & CO. KG, Germany). To eliminate the influence of the amino groups in chitosan on the analysis, blank corrections of chitosan always were performed. Formaldehyde was analysed by a Cuvette Test LCK 325 (Dr. Bruno Lange GMBH & CO. KG, Germany) after eliminating excess periodate by adding myo-inositol. To measure the molecular weight, ethylene glycol was first added to eliminate the unreacted periodate, and the samples were subsequently analysed by size-exclusion chromatography combined with multi-angle laser light scattering (SEC-MALLS).[28]

When preparing periodate-oxidised chitosans the reaction was ended after 48 h by adding ethylene glycol. (C_{GlcN} = 12.5 mM, 0.2 M acetate buffer, pH 4.5, T = 4 °C, 10% (v/v)) 1-propanol. The solution was dialysed against 0.2 M NaCl (adjusted to pH 4.5) to convert the chitosans to the hydrochloride salts, and

thereafter against deionised water adjusted to pH 4.5. After the dialysis, the pH was adjusted to 4.5 and a part of the chitosan was freeze dried. The remaining oxidised chitosan was reduced with sodium borohydride (2 g $NaBH_4$/g chitosan), dialysed (first 0.2 M NaCl and thereafter deionised water) and freeze dried. The content of carbon and nitrogen was determined with a Carlo Erba Elemental Analyzer NA 1500.

3. Results

Values for P_t (moles of consumed periodate per mole of GlcN) and N_t (moles of released ammonia per mole of GlcN) obtained with an excess of periodate (P_0 = moles of periodate added per mole of GlcN = 5) for four different chitosans (F_A = 0.01, 0.16, 0.52 and 0.60) are given in Figure 2a and b, respectively. Following an initial rapid increase, P_t increases asymptotically towards an apparently linear region, and no plateau seems to be reached within 48 h. The initial reaction rates, (dP_t/dt), are clearly dependent on F_A, with increasing rates for chitosans with decreasing degree of acetylation. The final consumption (48 h) of periodate is also dependent on F_A, as chitosans with low F_A consume more periodate than highly acetylated chitosans. P_t generally exceeds the theoretical maximum value of 1. Figure 2b also shows that dN_t/dt increases with decreasing F_A. N_t approaches an apparent maximum value of 0.5, independent of F_A. These observations indicate that chitosans become only partially oxidised to form GlcN dialdehydes, and that most of the periodate must be consumed in secondary reactions without the release of ammonia.

The oxidised chitosans were further analysed by SEC-MALLS. Results for the weight average molecular weight (M_w) (Fig. 2c) show that the chitosan with F_A = 0.16 becomes extensively depolymerised even during the first minute of the reaction. Similar results were obtained for a chitosan with F_A = 0.01 (data not shown). It was further noted (Fig. 2c) that the addition of the free radical scavengers TEMPO and 1-propanol, respectively, had marginal effects on the rate and extent of the depolymerisation.

Formaldehyde was gradually liberated during the oxidation, reaching values of FA_t (moles of formaldehyde released per mole of GlcN) 0.05–0.08 after 24 h (Fig. 2b). The data suggest that the extent of formaldehyde liberated increases slightly with decreasing F_A. However, after standing for two months at room temperature the amount of formaldehyde had increased to 0.51, clearly demonstrating that the oxidation can proceed until the chitosan has been almost completely degraded. Attempts were made to determine the release of formic acid using the 2-thiobarbituric acid method.[29] However, this method gave inconsistent results, and no reliable data were obtained.

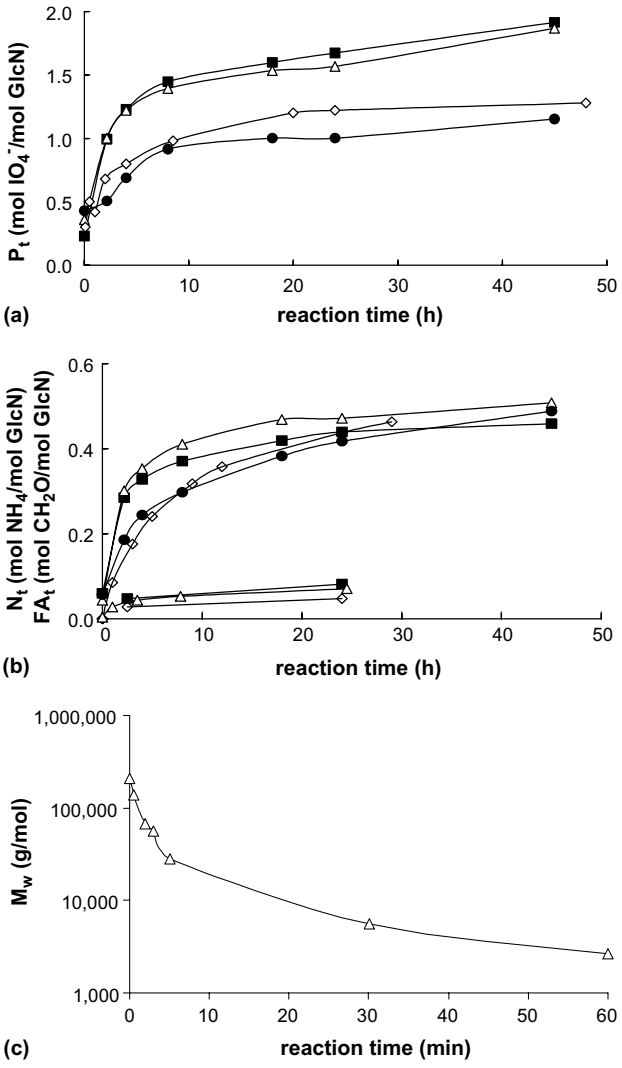

Figure 2. P_t (a), N_t and FA_t (b) and M_w (c) during the periodate oxidation of chitosans with F_A = 0.01 (■), F_A = 0.16 (△), F_A = 0.52 (◇) and F_A = 0.60 (●). The concentration of GlcN was 2.5 mM. The reactions were performed using acetate buffer (0.2 M, pH 4.5), T = 4 °C, excess periodate (P_0 = 5).

Figure 3a and b shows P_t, N_t and FA_t for partial oxidation (P_0 = 0.3) of three different chitosans (F_A = 0.01, 0.16 and 0.49). As with excess periodate the oxidation rates decrease with increasing F_A. All periodate is apparently consumed after 48 h (hereafter denoted as 'final'). However, N_t levels off at values around 0.1, indicating that only about one third of the periodate is used for oxidation leading to the liberation of ammonia. The final N_t value is apparently independent of F_A. At the same time, FA_t values around 0.01–0.02 were obtained after 48 h (Fig. 3b). The oxidation is also associated with extensive depolymerisation (Fig. 3c). The results are, however, dependent on F_A, with the most acetylated chitosans being clearly less susceptible to depolymerisation. The depolymerisation appears

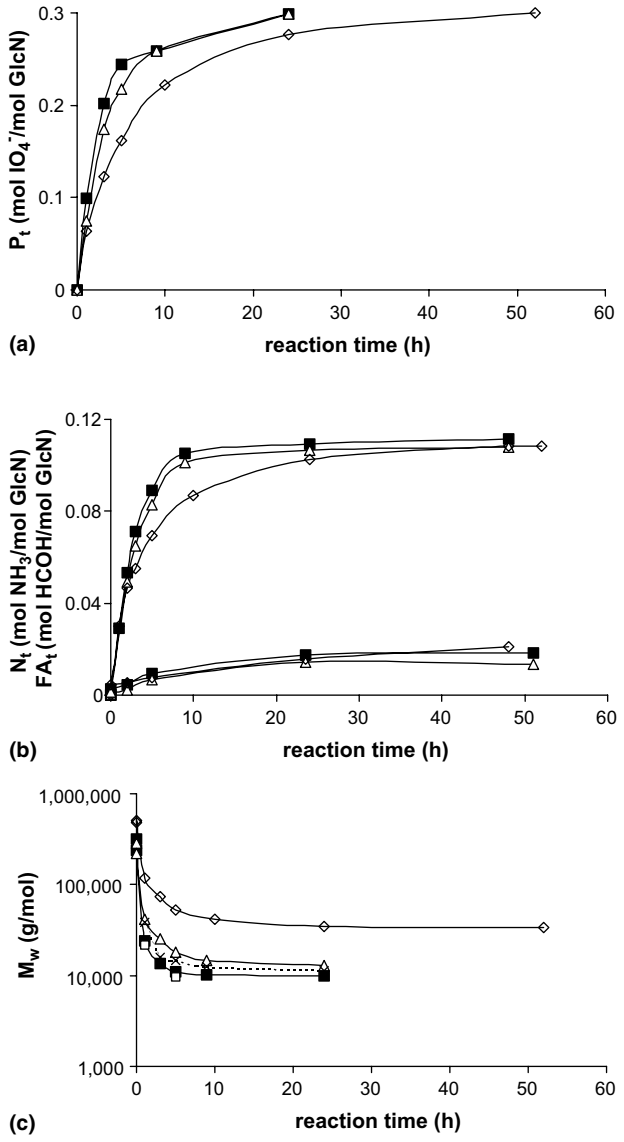

Figure 3. P_t (a), N_t and FA_t (b) and M_w (c) during the partial periodate oxidation ($P_0 = 0.30$) of chitosans with $F_A = 0.01$ (■), $F_A = 0.16$ (△), $F_A = 0.49$ (◇). The concentration of GlcN was 2.5 mM. The reactions were performed using acetate buffer (0.2 M, pH 4.5), $T = 4$ °C, and 10% *n*-propanol was included. M_w measured in corresponding experiments with no propanol added ($F_A = 0.16$) (×) and TEMPO added ($F_A = 0.01$) (□) are included in Figure 3c.

to be most predominant during the first hours of oxidation.

Given the accepted mechanism for periodate oxidation of chitosans, the amount of ammonia liberated per mol GlcN (N_t) should equal the relative dialdehyde content (F_{ox}). To test this hypothesis we chose to compare N_t with the N/C ratio, since the latter represents an independent approach, and should directly reflect F_{ox} through the following equation, which simply reflects the chemical changes depicted in Figure 1:

$$\frac{N}{C} = \frac{F_A + (1F_A)(1F_{ox})}{2F_A + 6} \quad (1)$$

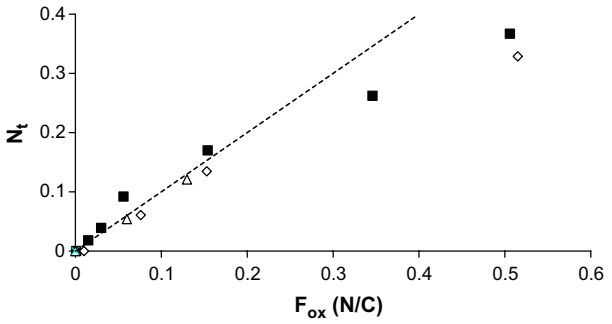

Figure 4. Ammonia liberated (N_t) compared to the degree of F_{ox} determined by elemental analysis for chitosans with $F_A = 0.01$ (■), $F_A = 0.16$ (△), and $F_A = 0.49$ (◇).

This equation does not take into account oxidation taking place at the chain termini, and is therefore only valid for sufficiently long chains. Three different chitosans were oxidised ($P_0 = 0.025$–2) for 48 h, and the N/C ratio was determined by elemental analysis (after dialysis to remove low molecular weight fragments). In general, we found F_{ox} (from N/C) $\approx N_t$ for all F_A as long as $P_0 < 0.3$. In Figure 4 is shown a plot of N_t as a function of F_{ox} (N/C). All data seems to fall on the theoretical 1:1 line for F_{ox} up to 0.2, thereafter some downward curvature is observed.

The formation of possible intramolecular hemiacetals in chitosans was investigated by the procedure used for alginates, involving several cycles of oxidation, each

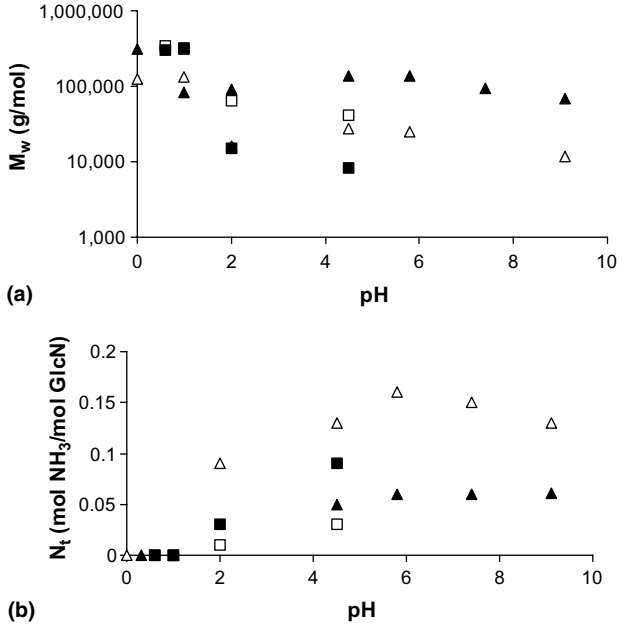

Figure 5. Molecular weight, M_w, (a) and N_t (b) obtained after 24 h when oxidising chitosan $F_A = 0.01$ $P_0 = 0.05$ (□), $P_0 = 0.30$ (■) and $F_A = 0.52$ ($P_0 = 0.05$ (▲), $P_0 = 0.30$ (△)) at different pH values. Phosphate buffers were used at pH = 5.8, 7.4, 9.1, acetate buffer at pH 4.5, HCl at pH < 4.5. $T = 4$ °C, 10% (v/v) 1-propanol, 4 mg/mL chitosan.

followed by aldehyde reduction.[8] However, because of the extensive depolymerisation accompanying the oxidation, no oxidised material could be recovered after dialysis, and the experiment could not be completed.

The pH dependence of the oxidation was investigated ($P_0 = 0.05$ and 0.30, $t = 48$ h) for two chitosans with $F_A = 0.01$ and 0.52, respectively. Results (N_t and M_w) are given in Figure 5a and b. At low pH no ammonia is liberated and chitosan retains a high molecular weight, suggesting that no oxidation has taken place. This is reasonable, since protonated amino groups are protected from periodate oxidation.[30] The extent of depolymerisation appears to be lowest at pH 4.5, but this can partially be ascribed to the fact that the chitosan is also less oxidised at this pH as compared to, for instance, pH 5.8. Solutions of partially oxidised chitosans were unstable, with the gradual emergence at pH 4.5–9.1 of material with high M_w (>10^6) but relatively low R_G (50–100 nm), suggesting rather compact structures. At higher pH (7.4–9.1) solutions also showed slight precipitation.

4. Discussion

Periodate oxidation of chitosans differs in many respects to oxidation of other polysaccharides because of the involvement of the amino group. We here show that the behaviour—not unexpectedly—depends strongly on the degree of *N*-acetylation. First, the rate of oxidation decreases with increasing F_A. This may in part be ascribed to the decreasing charge density with increasing F_A, leading to a weaker electrostatic attraction between the positively charged chitosan and the negatively charged periodate ion, thereby increasing the effective concentration of periodate near the polymer chain.

Also the rate of depolymerisation and overconsumption of periodate decreased as F_A increases. This 'protective' effect of *N*-acetylated residues, which are resistant towards oxidation, remains unexplained.

Despite the high consumption of periodate we observe an oxidation limit (N_t limit) near 0.5, even with an excess of periodate. Also partially oxidised chitosans became incompletely oxidised ($N_t < P_0$). Oxidation limits due to the formation of intramolecular hemiacetals has been observed in alginates,[8] and is probably a general phenomenon in the periodate oxidation of polysaccharides.[4] However, the method devised by Painter and Larsen,[8] involving several oxidation/reduction cycles, could not be applied to chitosans because of the extensive depolymerisation, and the question remains unresolved.

In the absence of appropriate methods for direct determination of the dialdehyde content in periodate-oxidised chitosans, indirect measurements were considered. P_t is clearly not a measure of the F_{ox}, because of the overconsumption of periodate. N_t could, however, be a measure for the degree of oxidation, provided that ammonium is released from all the oxidised monomers. As an alternative method, determination of the N/C ratio, was investigated. Excellent agreement between these two methods were obtained for low degrees of oxidation ($P_0 < 0.2$) (Fig. 4), indicating that the oxidation up to this point proceeds as expected from general theory, and that both N_t and the N/C ratio can be used for determining F_{ox}. At $N_t > 0.2$, F_{ox} estimated from N/C measurements becomes significantly higher than N_t. A source of systematic underestimation (up to 9%) of F_{ox} (N/C) is the selective loss of formaldehyde and formic acid due to oxidation at the reducing and non-reducing ends, respectively, since the samples were dialysed prior to analysis.

Chitosan become more extensively depolymerised during periodate oxidation than both alginate[9] and cellulose.[31] In contrast, degradation caused by hydroxyl radicals is actually slower for chitosan than alginate.[7,32,33] Moreover, the rate of depolymerisation decreased with increasing F_A. Taken together this suggests that some other, and so far unidentified, chitosan-specific degradation mechanism connected to the free amino groups must be involved. One possibility that remains to be further investigated is a possible intramolecular reaction between an oxidised GlcN residue and a neighbouring, unoxidised GlcN residue, which by subsequent reactions finally leads to cleavage of the polymer chain.

The major reason for the overconsumption of periodate is obviously the extensive depolymerisation, which leads to the exposure of novel periodate consuming end groups. This may be verified by comparing the amount of formaldehyde with the degree of chain scission $\alpha = DP_n^{-1}$, where DP_n is the number average chain length obtained by SEC-MALLS. P_t should thus be given by the equation

$$P_t = \frac{N_t(1-F_A) + \alpha + \left(\frac{FA_t(1-F_A)}{\alpha}\right)7\alpha + \left(1 - \frac{FA_t(1-F_A)}{\alpha}\right)\alpha}{1-F_A} \quad (2)$$

The calculated values agree reasonably well with the experimentally measured values (Table 1), confirming

Table 1. Experimental P_t values compared to theoretically calculated P_t values for chitosans with $F_A = 0.01$, 0.16 and 0.49

F_A	Time (h)	P_t (measured)	P_t (Eq. 2)
0.01	5	0.24	0.23
	24	0.30	0.30
0.16	5	0.22	0.18
	24	0.30	0.27
0.49	5	0.16	0.15
	24	0.28	0.25
	52	0.30	0.29

that the overconsumption of periodate is directly a result of secondary oxidation at the reducing and non-reducing ends formed due to the depolymerisation.

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