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Periodate oxidised chitosans: structure and solution properties

Doctoral thesis
for the degree of doktor ingeniør

Trondheim, September 2004

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Faculty of Natural Sciences and Technology
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NTNU

ISBN 82-471-6476-0
ISBN 82-471-6475-2 (electronic)

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor, Professor Bjørn E. Christensen, for excellent guidance and inspiration during this work. I also sincerely want to thank my co-supervisors Professor Olav Smidsrød and Professor Kjell M. Vårum for their interest and guidance during these years.

Ann-Sissel Ulset and Mildrid Myhr are thanked for technical assistance and for helping out with the many practical problems in the lab.

I would also like to thank all my colleagues in the lab and at the institute for all help, and for making these years enjoyable. My roommates Ingvild and Synnøve are thanked for friendship, help and support during all up and down periods. Catherine and Ingvild are thanked for commenting on this manuscript.

Encouragement and concern from my family and friends have been most valuable to me.

Finally, I want to thank my husband, Knut Arild, for his love and support, and for always being there for me.

PREFACE

This thesis is submitted in partial fulfilment of the degree *Doktor ingeniør* at the Norwegian University of Science and Technology. The work has been carried out at NTNU, Department of Biotechnology with Professor Bjørn E. Christensen, Professor Olav Smidsrød and Professor Kjell M. Vårum as supervisors. The work has been financed by The Research Council of Norway.

The thesis consists of 4 papers in an appendix and an extended summary. Some results not presented in the papers relevant to the presented work are also included in the summary. Chapter 1 gives a general introduction to the work presented, including the motivation for the work and the scope of the thesis. Chapter 2 sums up the study of the periodate oxidation of chitosans of different chemical composition (paper I). Some results on the periodate oxidation of alginates, not presented in the appendix papers, are also included. In chapter 3 the chain stiffness and water-solubility of oxidised and unoxidised chitosans (paper II) and the chain stiffness of oxidised alginates (not included in the appendix papers) are discussed. Chapter 4 sums up a preliminary study of the chemical structure of the periodate oxidised chitosans (paper III), while chapter 5 deals with the selective ion binding properties of chitosans (paper IV). Finally, the main findings are discussed on a general basis, and concluding remarks are given in chapter 6.

SUMMARY

Chitosans are considered promising for a wide range of applications, and especially in the biomedical and pharmaceutical field numerous possible applications are being investigated. However, some of the properties of chitosans are not ideal for biomedical applications. Most high molecular weight chitosans are insoluble near and above physiological pH, limiting its uses. Moreover, the relatively high stiffness of chitosan chains restricts their electrostatic interactions with polyanions. To overcome this problem, chemical modification is an important approach.

The main objective of this work has been to obtain well-characterised modified chitosans with increased solubility and chain flexibility suitable for potential use in biomedical applications, with periodate oxidation being the chosen method of chemically modification.

In order 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. Chitosans became only partially oxidised during the oxidation with periodate, reaching degrees of oxidation around 0.5, when oxidised with excess periodate. Chitosans were severely depolymerised during the oxidation, and the large overconsumption of periodate observed, can probably be explained by the consumption of periodate at the increasing number of end groups. Free radical mediated depolymerisation seems not to be sufficient to explain the depolymerisation, and a chitosan specific degradation mechanism is probably involved in the depolymerisation. The reaction rates, overconsumption of periodate, and depolymerisation all increased with decreasing F_A. No methods to considerably reduce the depolymerisation were found.

The resulting periodate oxidised chitosans were characterised by investigating the chain stiffness, water-solubility and chemical structure. The chain stiffness was measured using size-exclusion chromatography combined with light-scattering and viscosity

detectors (SEC-MALLS-VISC), and it was found that the persistent length, q, decreased with increasing degrees of oxidation. The water-solubility of periodate oxidised chitosans with F_A = 0.01 and F_A = 0.16 increased with increasing degrees of oxidation, and were completely soluble at F_{ox} = 0.10. Chitosans and periodate oxidised chitosans with F_A = 0.52 were completely soluble at all pH-values investigated. Thus, periodate oxidised chitosans with increased solubility and chain flexibility were obtained, but the molecular weight could not be retained. The chemical structure was investigated by NMR spectroscopy, but more work remains before any conclusions can be drawn.

The chain stiffness of chitosans with different chemical composition was investigated. Persistence lengths and exponents from double logarithmic plots of [η]-M and R_G-M indicate that the chain stiffness is independent of F_A, with average q-values of 7 nm, a-values of 0.78 and a'-values 0.56.

To support the methods used and to get a standard of reference the periodate oxidation of alginates, which are more studied than chitosans, was also undertaken and the resulting products characterised. The reaction rates, dP_t/dt, in the periodate oxidation of alginates were highly dependent on the type of alginate used and the concentration of the polymer. The chain stiffness was investigated by SEC-MALLS-VISC, and clearly showed that the persistence length decreased with increasing degrees of oxidation.

For use in biomedical applications chitosans have to be pure and well characterised with documented safety profiles. Impurities like metal ion contamination must be strictly controlled. An additional objective of this work was to gain a better understanding of the binding of ions to chitosans, and to develop a method to measure the selective ion-binding properties. A method was successfully developed. No selectivity in the binding of chloride and nitrate ions to chitosan was observed. Molybdate anions were selectively bound to chitosans in the presence of excess nitrate or chloride ions, with selectivity coefficients around 100. Cu^{2+} ions were bound selectively to chitosan in the presence of Ni^{2+}, Zn^{2+} or Cd^{2+} ions, with selectivity coefficients in the range 10 to 1000. Chitosans with low and high F_A did not show any difference in their selectivity towards

ions. Binding of metal cations increased with pH indicating that only the deprotonised amino groups can bind the ions.

CONTENTS

LIST OF APPENDIX PAPERS

I Vold, I.M.N.; Christensen, B.E. (2004). Periodate oxidation of chitosans of different chemical composition. Submitted to *Carbohydr. Res.*

II Vold, I.M.N.; Vårum, K.M.; Christensen, B.E. (2004). A study on the chain stiffness and water-solubility of chitosans and periodate oxidised chitosans. Manuscript in preparation.

III Vold, I.M.N; Christensen, B.E. (2004). Preliminary study on the chemical structure of periodate oxidised chitosans. Manuscript in preparation.

IV Vold, I.M.N., Vårum, K.M., Guibal, E., Smidsrød, O. (2003). Binding of ions to chitosan – selectivity studies. *Carbohydr. Polym.* **54**, 471-477.

Related manuscript in preparation, not included:

Christensen, B.E.; Vold, I.M.N. (2004). A study of the chain stiffness in epimerised and periodate-oxidised alginates using size-exclusion chromatography combined with light scattering and viscosity detectors.

LIST OF SYMBOLS AND ABBREVIATIONS

$[\eta]$	intrinsic viscosity
a	exponent in MHKS-equation
DP_n	number-average degree of polymerisation
F_A	fraction of acetylated units
FA_t	moles of formaldehyde liberated per mole GlcN
F_{ox}	fraction of oxidised units
GlcN	2-amino-2-deoxy-β-D-glucopyranose
GlcNAc	2-acetamido-2-deoxy-β-D-glucopyranose
k_{Cd}^{Cu}	selectivity coefficient
K	constant in MHKS-equation
MALLS	multi angle laser light scattering
M_w	weight-average molecular weight
N_t	moles of ammonia liberated per mole GlcN
P_0	moles of periodate added per mole GlcN
P_t	moles of periodate consumed per mole GlcN
q	persistence length
R_G	radius of gyration
SEC	size exclusion chromatography
M_L	molecular weight per unit contour length

1. INTRODUCTION

1.1 Motivation

Chitosans are polysaccharides prepared by deacetylation of chitin, a by-product of the seafood industry. Due to its poly-cationic properties, which are unique among abundant polysaccharides and natural polymers in general, and other advantageous properties such as biodegradability and biocompatibility, chitosans are considered promising for a wide range of applications. Especially in the biomedical and pharmaceutical field numerous possible applications are being investigated. However, some of the properties of chitosans are not ideal for biomedical application. Most high molecular weight chitosans are insoluble near and above physiological pH, limiting its uses. Moreover, the relatively high stiffness of chitosan chains restricts their electrostatic interactions with polyanions. To overcome this problem, chemical modification is an important approach.

The main objective of this work has been to obtain well-characterised modified chitosans with increased solubility and chain flexibility suitable for potential use in biomedical applications, with periodate oxidation being the chosen method of chemically modification.

Characterisation of the physical properties of the modified chitosans is crucial in order to find the chitosan best suited for a given application. For many applications, and especially in the biomedical and pharmaceutical field, key parameters like the fraction of acetylated units, F_A, molecular weight and molecular weight distribution must be specified. Impurities like metal ion contamination must also be strictly controlled. Knowledge of the selective ion binding properties of chitosans is of considerable importance.

1.2 Chitosans

1.2.1 Structure and properties

Chitosans are linear binary heteropolysaccharides composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; **A**-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; **D**-unit) in varying composition and sequence. The structure is schematically illustrated in figure 1.1.

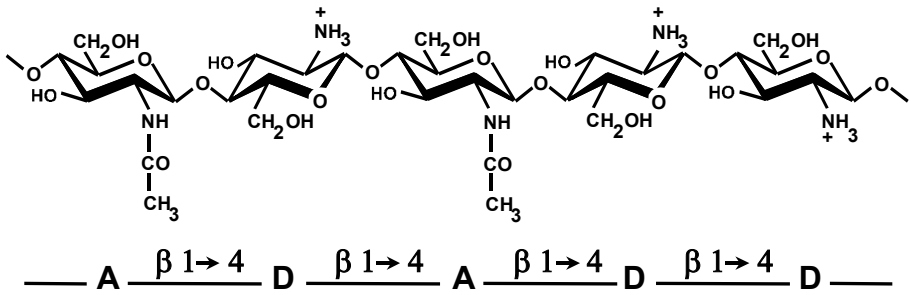


Figure 1.1 Chemical structure of a partially de-*N*-acetylated chitosan.

Chitosans are produced from chitin by complete or partial de-*N*-acetylation. They are also found in nature, to a lesser extent than chitin, in the cell walls of fungi (Roberts, 1992). Chitin is one of the most abundant biopolymers in nature, occurring mainly as a structural polysaccharide in the exoskeleton of animals with an outer backbone (crustaceans and insects). Chitin is also found in microorganisms such as certain species of fungi, yeast and green algae. There is no distinct border between chitin and chitosan. It has been proposed that chitin and chitosan can be distinguished by their insolubility or solubility in dilute aqueous acid solutions (Roberts, 1992). The main resources for

commercial utilisation of chitin are from crab and shrimp shell waste, with an annual world wide chitin production around 10,000 metric tons (Sandford, 2002).

It has been shown that A- and D-units in the chitosan chains are randomly distributed (Vårum *et al.*, 1991; Ottøy *et al*, 1996a), and the chemical composition of chitosans can be described by the molar fraction of acetylated units, F_A. The amino group in chitosan has a pK_a-value ranging from 6.2 to 7, depending on the type of chitosan and conditions of measurement (Domard, 1987; Anthonsen and Smidsrød, 1995; Sorlier *et al.*, 2001; Strand *et al.*, 2001), and will therefore be positively charged in neutral and acidic solutions. F_A and the molecular weight average are main factors determining the properties of chitosans, and thereby also suitable applications.

1.2.2 Biomedical applications

There are numerous potential commercial uses of chitosan, and the great commercial interest in chitosan is reflected by the high number of patent applications (Sandford, 2002). The potential use of chitosans and chitosan derivatives includes such diverse fields as agricultural, cosmetic, water-treatment, textile and food applications (Peter, 1995; Shahidi *et al.*, 1999; Kumar, 2000; Guibal, 2004).

Some of the most promising applications of chitosans are in the biomedical and pharmaceutical field, where chitosans have been investigated for a considerable number of applications (Illum, 1998; Skaugrud *et al.*, 1999; Singla and Chawla, 2001, Sinha *et al.*, 2004). Chitosans are biodegradable, biocompatible and have low toxicity. Due to their cationic properties chitosans are bioadhesive and able to interact strongly with epithelial cells and the overlying mucus layer, and hence lead to prolonged contact time between the drug formulation and the adsorptive sites. Combined with the fact that chitosans have the ability to open tight junctions in epithelial cells (Dodane *et al.*, 1999; Smith *et al.*, 2004), thereby further enhancing the absorption of drugs, this makes the use of chitosans in peroral, nasal and ocular drug-delivery applications highly promising. Nasal drug-delivery has recently gained attention for the delivery of

challenging drugs that are not easily administered via other routes than by injection, or where a rapid onset of action is required. With the use of bioadhesive delivery systems containing e.g. chitosan the delivery of small polar molecules, peptides and proteins and even the large proteins and polysaccharides used in vaccines could be delivered through the nasal route (Illum *et al.*, 2003). Chitosan has shown very promising results in this field (Illum, 2001).

Chitosans form complexes with anionic drugs and polyanions such as alginates, xanthan collagen and DNA, and such complexes have been studied for potential use in drug-delivery systems (Taravel and Domard, 1996; Gåserød *et al.*, 1998 a,b); Borchard, 2001; Köping-Höggard *et al.*, 2001). Microcapsules formed from these polyelectrolyte complexes or by other techniques can exhibit pH- and ion-sensitive swelling behaviour, and controlled drug release systems can be designed (Berger, 2004). Chitosans can be used for coating of e.g. alginate microcapsules to enhance the bioadhesive properties of the capsules, performing the action in drug-delivery systems (Gåserød *et al.*, 1998b) In gene therapy chitosans are of current interest, due to the need for non-viral, non-toxic and biodegradable gene delivery vectors (Mansouri, 2004).

Chitosans also promote wound healing (Ueno *et al.*, 2001), and have been shown to exhibit antimicrobial effects (Rabea *et al.*, 2003). They are also promising as cholesterol lowering agents (Ormrod *et al.*, 1998), and have been suggested as weight reducing agents (Ernst and Pittler, 1998).

1.3 Alginates

Alginates are linear copolymers consisting of (1→4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) of widely varying composition and sequence. The alginate structure is shown in figure 1.2. The alginate molecule is a block copolymer composed of three types of blocks; homopolymeric regions of M and G, termed M- and G-blocks, respectively, and regions with alternating residues (MG-blocks).

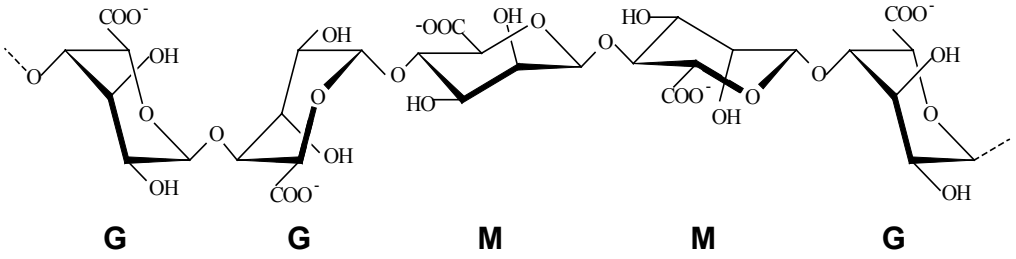


Figure 1.2 Structure of alginate

Alginates are quite abundant in nature, occurring as the main structural component in marine brown algae (*Phaeophyceae*), comprising up to 40 % of the dry matter, and as capsular polysaccharides in soil bacteria such as *Azotobacter vinelandii* and several species of *Pseudomonas.* All commercial alginates are still extracted from algal sources. The industrial applications of alginates are linked to their ability to retain water, and their gelling, viscosifying, and stabilising properties. Upcoming biotechnological applications, on the other hand, are based either on specific biological effects of the alginate molecule itself or on its unique sol/gel transition in the presence of multivalent cations (Draget *et al.*, 2002).

1.4 Periodate oxidation

The splitting of α-glycol groups by periodate was discovered by Malaprade in 1928, and the reaction has since found wide application (Guthrie, 1961). The mild conditions of the reaction are especially well adapted for application to the sensitive carbohydrate structures. 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 (Perlin, 1980).

Polysaccharides oxidised with periodate have novel functionalities. Dialdehyde starch is an example where periodate oxidation is used to obtain new properties in industrial polysaccharides (Veelaert, 1997a). However, the industrial applications of periodate

oxidised polysaccharides are limited due to the high cost of the oxidant. In order to reduce the production costs, processes have been designed which combine the oxidation of starch with electrochemical regeneration of periodate. An economically feasible process has not been found up to now (Veelaert, 1994).

1.5 Scope of the thesis

The main objective of this work has been to obtain well-characterised modified chitosans with increased solubility and chain flexibility suitable for potential use in biomedical applications, with periodate oxidation being the chosen method of chemically modification.

In order 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, have been investigated. The resulting periodate oxidised chitosans have been characterised by investigation of both their physical and chemical properties. To support the methods used and to get a standard of reference the periodate oxidation of alginates, which are more studied than chitosans, was also undertaken and the resulting products characterised.

An additional objective of this work has been to gain a better understanding of the binding of ions to chitosans, and to develop a method to measure the selective ion-binding properties.

2. PERIODATE OXIDATION OF CHITOSANS AND ALGINATES

2.1 Chitosans

Periodate oxidation of chitosan has previously been relatively little explored, with only a few studies on the periodate oxidation reaction and products formed (Jeanloz and Forchielli, 1950; Krysteva *et al.*, 1994; Ohya *et al.*, 1996; Matsumura *et al.*, 1997). A schematical presentation of the periodate oxidation of chitosan is shown in figure 2.1.

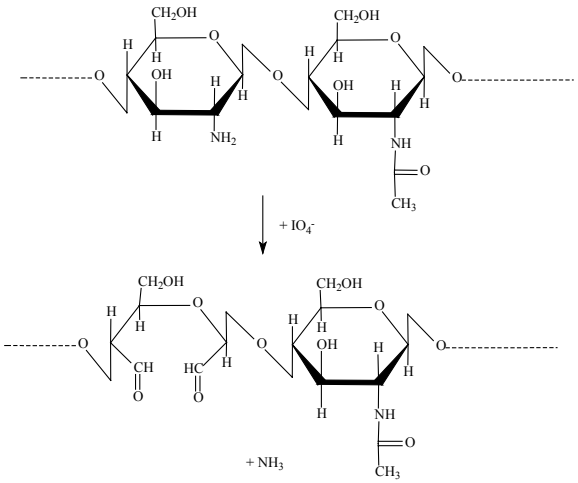


Figure 2.1. Periodate oxidation of a partially de-*N*-acetylated chitosan.

The figure implies that at the internal GlcN units in the chitosan chain, one molecule of periodate will be consumed, resulting in the liberation of ammonia and the formation of a dialdehyde. Periodate oxidation of the non-reducing end group will lead to the consumption of 2 molecules of periodate and the liberation of formic acid. The oxidation of the reducing end is more complex, and a schematic illustration is shown in figure 2.2 Up to 6 molecules of periodate can be consumed, depending on the stability of the formyl ester formed after cleavage of the C1-C2 and C2-C3 bond and the following overoxidation (Perlin, 1980).

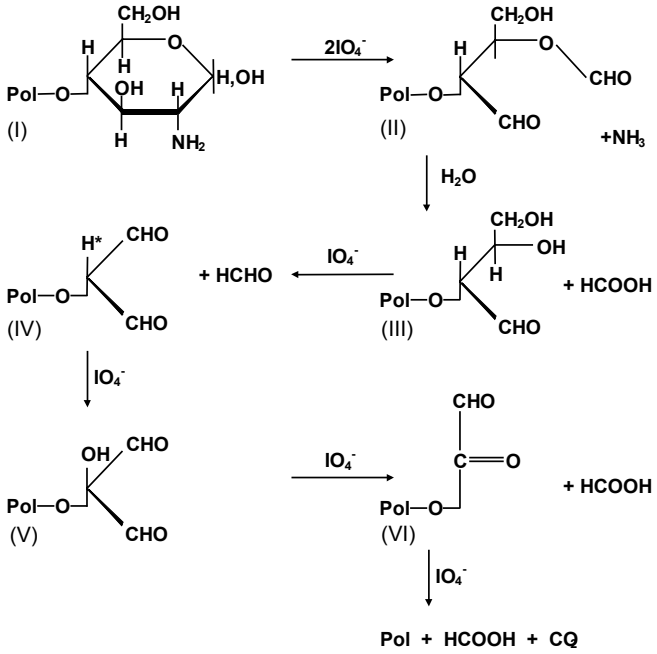
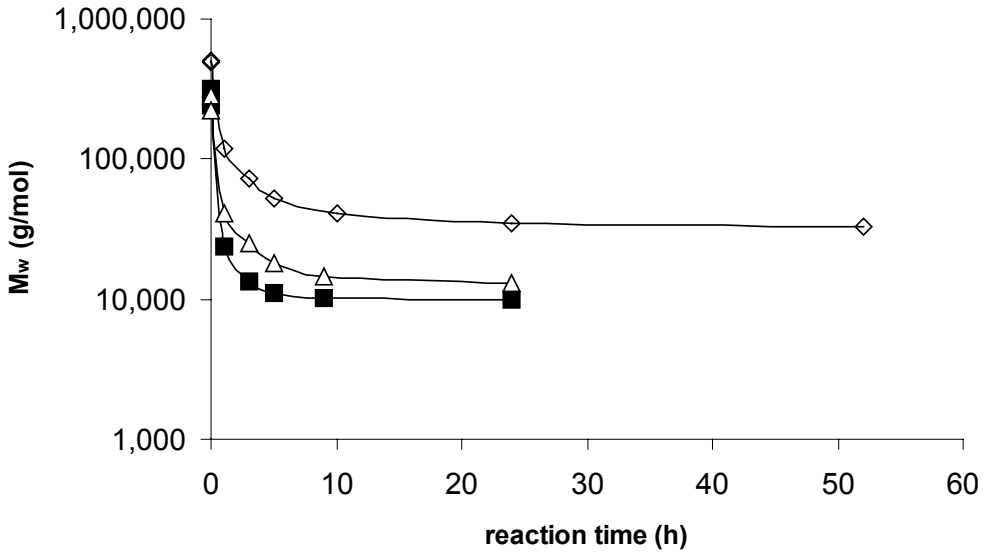


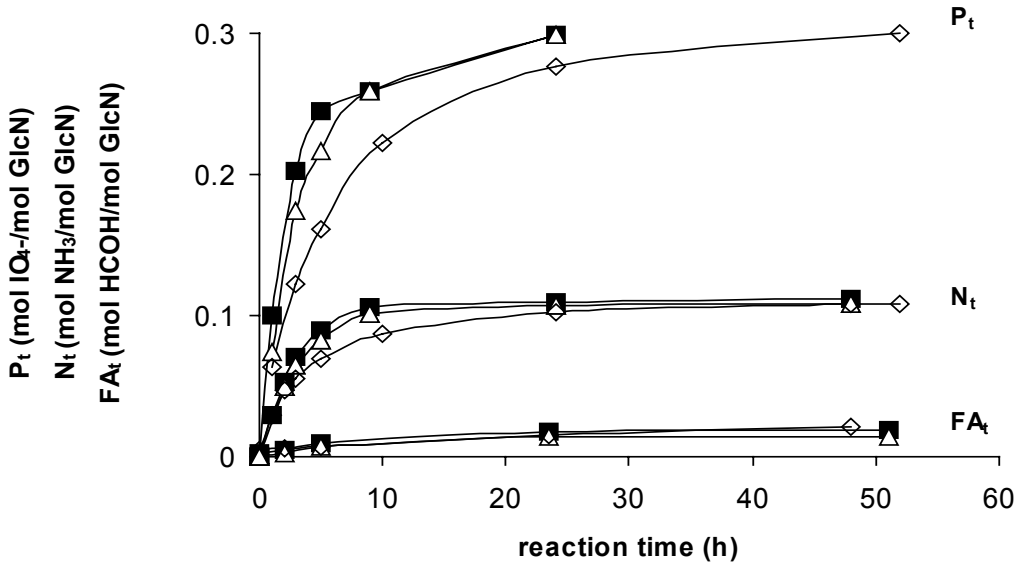
Figure 2.2 Oxidation of the reducing end in chitosan (modified from Dryhurst, 1970)

In most polysaccharides depolymerisation occurs concomitantly with periodate oxidation. 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. This work is presented in paper I, and is summarised below.

The periodate oxidation reaction is in this work described by the molar proportion of periodate consumed (P_t) and ammonia (N_t) and formaldehyde (FA_t) released during the oxidation, together with weight average molecular weights (M_w). Results obtained for partial oxidation of chitosans (P_0 = 0.30; moles of periodate added per mole GlcN) as a function of time are shown in figure 2.3 (a-b). Figure 2.3 shows that the depolymerisation was considerable during the oxidation. Further on, far more periodate was consumed as compared to ammonia liberated. This large overconsumption of periodate can mainly be explained by the amount periodate consumed at the reducing and non-reducing ends, which increase greatly upon depolymerisation, and a higher



(a)



(b)

Figure 2.3 M_w (a) and P_t, N_t and FA_t (b) during the partial periodate oxidation (P_0 = 0.3) of chitosans with F_A = 0.01 (■), F_A = 0.16 (Δ), F_A = 0.49 (◇). The concentration of GlcN was 2.5 mM. The reactions were performed at pH 4,5, 4˚C, and 10 % n-propanol was included.

rate of oxidation at the end groups as compared to the internal residues (Tiziani *et al.*, 2003). Changes in the experimental conditions such as pH and temperature, and the inclusion of different free radical scavengers (propanol, TEMPO), did not considerably reduce the degree of depolymerisation.

Another approach to diminish the depolymerisation was to perform the periodate oxidation in dimethyl sulfoxide (DMSO) to obtain a water-free solution. This could lead to less depolymerisation if water was involved in the degradation mechanism, and in addition DMSO is known to be a free radical scavenger. Chitosans were made soluble in DMSO by transferring them to the p-toluenesulfonic acid (pTsOH) salts (Sashiwa *et al.*, 2000). The methods used for the determination of N_t and P_t in the water solutions were not optimal for the DMSO solutions. N_t values could not be determined, but P_t values suggested that no, or only very limited, oxidation took place. This approach was therefore abandoned.

Figure 2.3 further shows that the degree of acetylation, F_A, of the chitosans is important for the course of the reaction. The reaction rates, overconsumption of periodate, and extent of depolymerisation increased with decreasing F_A. It is therefore easier to retain high molecular weight in chitosans with high F_A. This 'protective' effect of N-acetylated residues, which themselves are resistant towards oxidation, remains unexplained. Taken together with the comparatively greater resistance towards depolymerisation of other periodate oxidised polysaccharides, for example alginates, it may be speculated that depolymerisation must involve unreacted amino groups. 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. Free radical mediated depolymerisation, proposed as the mechanism responsible for the depolymerisation in other periodate oxidised polysaccharides (Painter and Larsen, 1970a; Painter, 1988), seems insufficient to explain the severe depolymerisation, and a chitosan specific degradation mechanism is probably involved in the depolymerisation. To be able to suppress the depolymerisation, this degradation mechanism should be

solved. One approach could be to explore the structure of the periodate oxidised chitosans.

When the oxidation was performed with excess periodate, chitosans became only partially oxidised, reaching degrees of oxidation around 0.5. Severe depolymerisation during the first hour of reaction was observed, together with the ensuing large overconsumption of periodate. This suggests that chitosans have an oxidation limit near 0.5. The reason for this oxidation limit remains to be explained. The protection of neighbouring residues by the formation of internal hemiacetals, as observed in a number of other polysaccharides (Painter and Larsen, 1970a-c; Ishak and Painter, 1971), could be one possible explanation.

2.2 Alginates

Both alginates and chitosans are 1→4 bound polysaccharides. Structural similarities together with the fact that the periodate oxidation of alginates has been thoroughly studied, make alginates a good reference for the studies on chitosans. Due to intramolecular hemiacetal formation between the oxidised unit and neighbouring unoxidised units, an oxidation limit of about 0.44 occurs in alginates (Painter and Larsen, 1970a). Subsequent reduction of the hemiacetals allows further oxidation. The C2-C3 cleavage results in a dramatic increase in the flexibility of the oxidised residues because of the ring opening (Andresen *et al.*, 1977; Smidsrød and Painter, 1973).

Mannuronan and an alginate isolated from the leaf of *L. hyperborea* were subjected to oxidation with periodate. Figure 2.4 shows P_t as a function of time for both alginates when oxidised in an excess of periodate ($P_0 = 1.2$) and in a periodate limited situation ($P_0 = 0.20$). Mannuronan became oxidised more slowly than the leaf alginate. For $P_0 = 0.20$, both alginates consume all the periodate, whereas for $P_0 = 1.2$ P_t levels off at about 0.5-0.6. Thus, both alginates exhibit an oxidation limit.

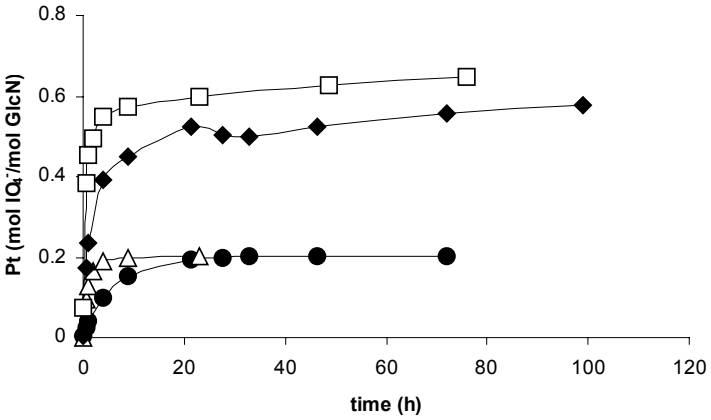


Figure 2.4. P_t during the periodate oxidation of *L. hyperborea* (□) and mannuronan (♦) using excess periodate (P_0 = 1.24) and during the partial periodate oxidation (P_0 = 0.20) of alginate B13 (Δ) and mannuronan (●). The concentration of alginate was 4 mg/ml, and the reactions were performed at 20˚C with 10 % (v/v) n-propanol included.

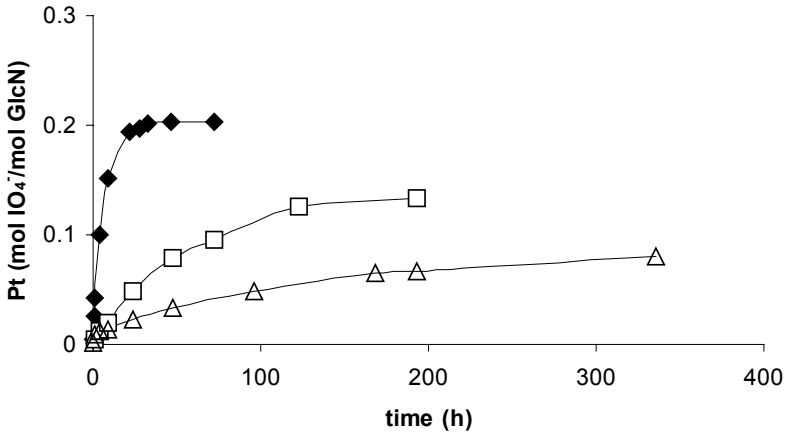


Figure 2.5. P_t during the partial periodate oxidation (P_0 = 0.20) of mannuronan of different concentration; 4 mg/ml (♦), 1 mg/ml (□) and 0.4 mg/ml (Δ).

P_t as a function of reaction time is shown in figure 2.5 for the partial oxidation (P_0 = 0.2) of mannuronan of three different concentrations. The figure clearly shows the concentration of mannuronan influence the reaction rate, dP_t/dt. Using the lowest concentrations, the expected degree of oxidation is not reached within reasonable time. This is important to take into consideration when preparing periodate oxidised alginates, to make sure that the wanted degree of oxidation is obtained.

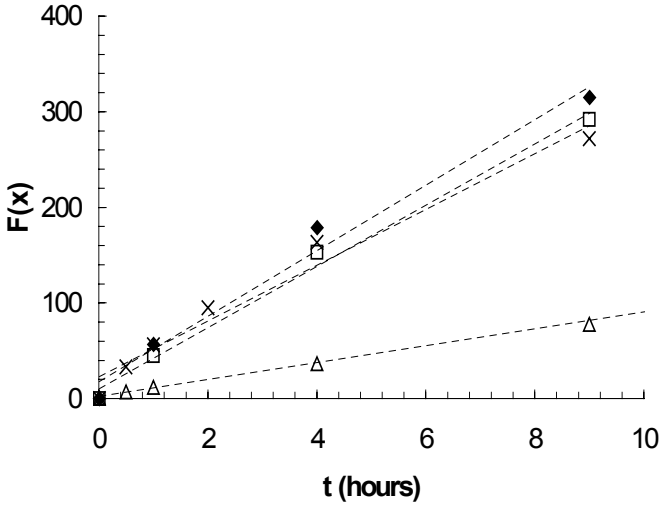
In the initial phase, the periodate oxidation of polysaccharides can be described as a second-order reaction, with the added complication that the intermediates formed may exist either in a reactive, acyclic form, or an unreactive, cyclic hemiacetal form (Aalmo and Painter, 1981; Veelaert *et al.*, 1994).

The stoichiometry in the initial reaction between alginate (A) and periodate (B) corresponds to A + B $\rightarrow$ C. According to second-order kinetics the integrated form of the rate equation may be written (Frost and Pearson, 1961; Laidler, 1987):

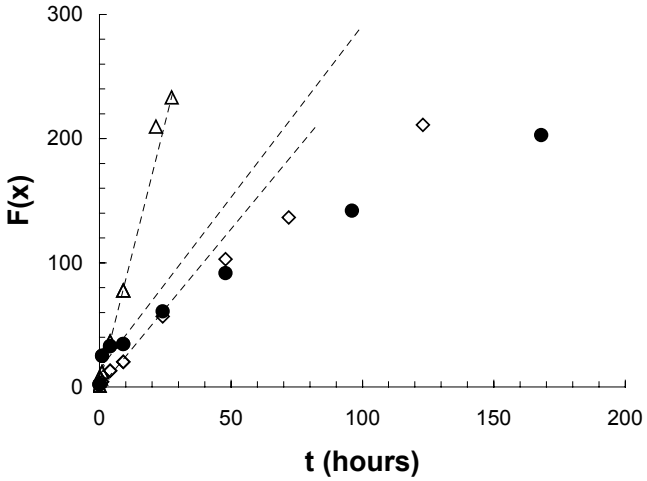
$$F(x) = \frac{1}{a_0 - b_0} \ln\left[\frac{b_0(a_0 - x)}{a_0(b_0 - x)}\right] = k_A t \qquad (2\text{-}1)$$

where a_0 and b_0 is the initial concentrations of A and B, and the variable x represents the decrease in concentration of a reactant in a given time.

F(x) is plotted as a function of time in figure 2.6 (a-b) for the partial oxidation of alginates (P_0 = 0.20), and the rate constants can be determined from the slope of the curves obtained. The calculated rate constants are given in table 2.1.



(a)



(b)

Figure 2.6 Second order kinetic analysis. L.digitata 4 mg/ml (◆), 1 mg/ml (□). L. hyperborea (X). Mannuronan; 4 mg/ml (Δ), 1 mg/ml (◇) and 0.4 mg/ml (●). The regression lines are based on the first 9 hours of the reaction.

Table 2.1 Rate constants for the partial periodate oxidation of alginates during the first 9 h at 20°C

Alginate	Concentration (mg/ml)	k_A (l mol^{-1} h^{-1})	R^2
L. digitata	4.0	34	0.98
	1.0	32	0.99
L. hyperborea	4.0	39	0.99
Mannuronan	4.0	8.4	1.00
	1.0	2.6	0.89
	0.4	2.8	0.57

The figures show that the periodate oxidation of most alginates gives linear curves of F(x) versus time, and thus that the reaction follows second order reaction kinetics. The periodate oxidation of mannuronan (1mg/ml and 0.4 mg/ml), however, deviates more from linearity, and after an extended period the slope of the curves decrease and the curves flatten off. During oxidation with excess periodate (results not shown), the reaction deviates from second order kinetics after a short period of time, probably due to the higher degrees of oxidation obtained, resulting in a considerable amount of internal hemiacetals in the alginate chain. This analysis further illustrates the fact that the oxidation of mannuronan is slower than the oxidation of the other alginates.

The study of the periodate oxidation of chitosans and alginates has formed an extended basis for the production of periodate oxidised products. Knowledge of physical properties such as chain stiffness and water-solubility is important both from a basic and an applied point of view. The physical properties of both modified and unmodified polymers have therefore been investigated.

3. CHAIN FLEXIBILITY AND SOLUBILITY

3.1 Chain conformation

3.1.1 General

Hydrodynamic properties such as the intrinsic viscosity, are closely related to polymer chain conformations. The relation between the intrinsic viscosity, [η], and molecular weight provides information about the conformation and extension of polymer chains expressed by the Mark-Houwink-Kuhn-Sakurada (MHKS) equation:

$$[\eta] = KM^a \tag{3-1}$$

where K and a are empirical constants that are valid for a specific polymer-solvent pair. The exponent, a, depends upon the conformation of a polymer. For the idealised conformations compact sphere, random coil and rigid rod, the a-values are 0, 0.5-0.8 (solvent dependent) and 1.8, respectively. Non-drained Gaussian chains (the equivalent sphere model of Flory) obtain an a-value of 0.5. Stiff and expanded molecules obtain a-values higher than 0.5 due to partial free drainage and deviation from Gaussian segment distribution due to inherently stiff chain backbone, or chain expansion due to solvent or polyelectrolyte effects.

Analysis of the conformational properties of stiff chain macromolecules has often been made on the basis of a continuous wormlike-chain model (Yamakawa and Fujii, 1974). From experimentally determined intrinsic viscosities and molecular weights parameters characterising the cylinder model, i.e. the contour length (L), the effective hydrodynamic diameter (d), and the persistence length (q), can be estimated. One limit of the model ($L\rightarrow 0$, $d\rightarrow 0$) is the rod, and the other ($L\rightarrow\infty$) is the random coil. Bohdanecky (1983) developed a simplified method to treat the intrinsic viscosity data for stiff-chain polymers, and showed that the Yamakawa-Fujii theory for wormlike chains can be approximated with the equation:

$$\left(\frac{M^2}{[\eta]}\right)^{1/3} = A_\eta + B_\eta M^{1/2} \qquad (3\text{-}2)$$

where

$$A_\eta = A_0 \cdot M_L \cdot \Phi_{0,\infty}^{-1/3} \qquad (3\text{-}3)$$

$$B_\eta = B_0 \cdot \Phi_{0,\infty}^{-1/3} \cdot (2q/M_L) \qquad (3\text{-}4)$$

$\Phi_{0,\infty}$ is the limiting value of the Flory viscosity constant, and equals $2.86 \cdot 10^{23}$. A_0 and B_0 are known functions of the reduced hydrodynamic diameter, d_r, and B_0 can be replaced by the mean value (=1.05). q is the persistence length and M_L is the molar mass per unit of contour length.

In this work M_L is estimated by two different methods:

I) Predetermined from structural data, $M_L = M_0/b_0$. M_0 is the monomer weight and b_0 is the monomer length. In alginates b_0-values of 0.515 nm were used for M-units and 0.435 nm for G-units (Smidsrød *et al.*, 1973). In chitosans a b_0 values of 0.515 nm were used. For oxidised units b_0 is calculated to 0.424 nm using the Kirkwood-Riseman freely rotating chain model (Kirkwood and Riseman, 1948).

II) M_L estimated from experimental data assuming that the hydrodynamic volume occupied by 1 g of the wormlike cylinder is equal to the partial specific volume; $\nu = (\pi N_A/4)(d^2/M_L)$ and assuming that ν is 0.57 cm^3/g for chitosans (Errington *et al.*,1993; Cölfen *et al.*, 2002) and 0.49 for alginates (Wedlock *et al.*, 1986).

The total persistence length of wormlike polyelectrolyte chains can be considered to be composed of an intrinsic part (q_0) and an electrostatic part (q_e). The electrostatic wormlike chain model (Odijk model) allows estimation of the effect of electrostatic chain repulsion on the dimensions of a polyelectrolyte by explicit calculation of the electrostatic persistence length, q_e, and the expansion factor, α_s as done by Higashimura *et al*., 2000).

Calculations of q_e for chitosans used in this work at ionic strengths of 0.2 M give q_e values ranging from 0.04 to 0.16 nm dependent on charge density. This shows that the electrostatic contribution to the total persistence length is minor.

The relation between the radius of gyration, R_G, and molecular weight can also give information on the conformation of the molecules, and is expressed by:

$$R_G = K'M^{a'} \qquad (3\text{-}5)$$

For a flexible chain a' obtained is around 0.5-0.6, whereas for a rod it is 1.
For a monodisperse system of unperturbed wormlike chains, the persistence length is related to R_G, and the intrinsic persistence length, q_0 can be calculated as described by Berth *et al*. (2002).

Chitosans and alginates, and polysaccharides in general, are stiff molecules. This stiffness leads to highly extended chains in solution. Both the acetylated and deacetylated units in chitosan exist in the $^4C_1$ ring conformation, resulting in diequatorial glycosidic linkages between the monomers. In alginate M-units exist in the $^4C_1$ ring conformation, while G units are in the $^1C_4$ conformation (Atkins *et al.*, 1970). Alginate therefore contains all the four possible glycosidic linkages: diequatorial (MM), diaxial (GG), equatorial-axial (MG) and axial-equatorial (GM). Probably, the diaxial linkages in G-blocks result in a significantly hindered rotation around the glycosidic linkages, and may account for the stiff and extended nature of the alginate chain. Rigid six-member sugar rings and restricted rotation around glycosidic bonds lead to the high chain stiffness in chitosans. Chitosans and alginates are polyelectrolytes, and an ionic strength dependent electrostatic repulsion between the charged groups on the polymer chain will increase the chain extension.

3.1.2 Alginates

A well-established method for introducing flexibility in alginates is by periodate oxidation (Smidsrød and Painter, 1973). This makes the characterisation of periodate oxidised alginates a good model to support the method used to evaluate the chain stiffness. The C2-C3 cleavage results in a dramatic increase in the flexibility of the oxidised residues because of the ring opening. In consequence, the alginate becomes more flexible, as demonstrated experimentally by a large increase in the B-parameter (Smidsrød and Painter, 1973) and a decrease in the persistence length (Lee *et al.,* 2002).

In this work an alginate from *L. hyperborea*, $F_G = 0.55$ and mannuronan were studied. The chain stiffness in periodate oxidised alginates, subsequently reduced with $NaBH_4$, was studied using size-exclusion chromatography combined with light-scattering and viscosity detectors providing M - [η] relationships subject to further analysis. A significant advantage of this method is that it gives M and [η] directly for each elution slice, where each slice can be considered monodisperse. In this way no correction for polydispersity is necessary, and the relation between M and [η], and between R_G and M, can be obtained from one single polydisperse sample. SEC combined with light scattering and viscosity detectors has previously been used to study solution properties of polysaccharides such as pectins (Fishman *et al.*, 2003), cereal β-glucans (Gomez *et al.*, 1997 a-b), hyaluronan (Mendichi *et al.*, 2003) and viilinan (Higashimura *et al.*, 2000), and the method is well established.

From the results obtained it is noted that the oxidation process and subsequent reduction is accompanied by depolymerisation. This phenomenon is well known and is ascribed to side-reactions involving free radicals produced by decomposition of periodate (Painter and Larsen, 1970a; Painter, 1988). Because of the depolymerisation the chains become too short for the determination of R_G, and stiffness analysis relied only on the [η]-M relationship shown in figure 3.1. All the plots, except the unoxidised sample, indicate relatively straight lines, and the exponent, a, in the MHKS relation can be determined. The reason for the poor quality of the unoxidised sample is probably due to a too high

amount alginate injected and elution near the void volume with poor separation. The values determined for the unoxidised sample are therefore highly uncertain. Calculated a-values, given in table 3.1, show a progressive decrease with increasing degrees of oxidation. The a-values are in the area between 0.65 and 0.9, suggesting flexible to stiff chains. The figure shows a shift in the linear regions of the curves towards lower [η] at a given M_w with increasing degrees of oxidation. This further suggests that the chain stiffness decreases upon oxidation.

Table 3.1: Alginate from *L. hyperborea*, $F_G = 0.55$. Weight-average molecular weight, M_w, intrinsic viscosity, $[\eta]_w$, the exponent a derived from the MHKS-plot, persistence lengths,q, calculated with M_L (structurally determined)^{I} and M_L (experimentally estimated)^{II}.

F_{ox}	M_w (g/mol)	$[\eta]_w$ (ml/g)	a	q^{I} (nm)	$M_L{}^{I}$ (nm^{-1})	q^{II} (nm)	$M_L{}^{II}$ (nm^{-1})
0	260,000	1,411	0.62	11.7	420	4.5	158
0.05	189,000	925	0.90	13.7	423	1.8	494
0.10	137,000	553	0.83	9.9	425	10.2	436
0.20	91,000	297	0.80	6.8	430	5.4	345
0.30	66,000	182	0.73	5.3	435	3.5	284
0.44	48,000	107	0.65	3.8	442	1.8	204

The data were further analysed in terms of the wormlike chain model. From the molecular weight and intrinsic viscosity data, the persistence length, q, can be calculated using the Bohdanecky procedure (1983). A Bohdanecky plot of partially oxidised alginates is shown in figure 3.2. Except for the unoxidised sample all samples displayed linear regions from which the persistence length could be estimated, and the calculated persistent lengths and corresponding M_L values are given in table 3.1. The persistence lengths versus the degree of oxidation are shown in figure 3.3 together with persistence lengths of oxidised mannuronan. The results indicate that periodate oxidation leads to a profound increase in the chain flexibility.

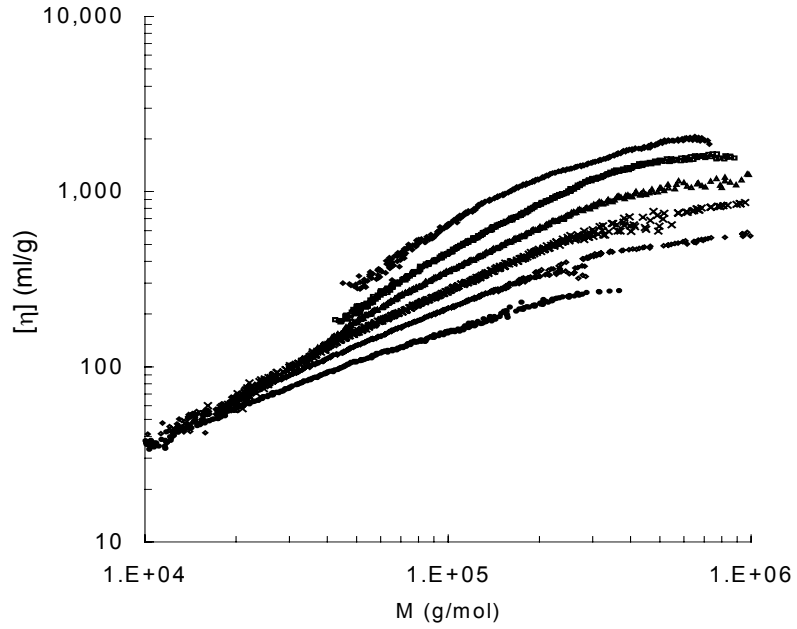


Figure 3.1 MHKS plots (log-log plots of the intrinsic viscosity versus weight-average molecular weight) of partially oxidised alginates with degree of oxdiation 0 (upper curve), 0.05, 0.10, 0.20, 0.30 and 0.44 (lower curve) at 0.17 M ionic strength.

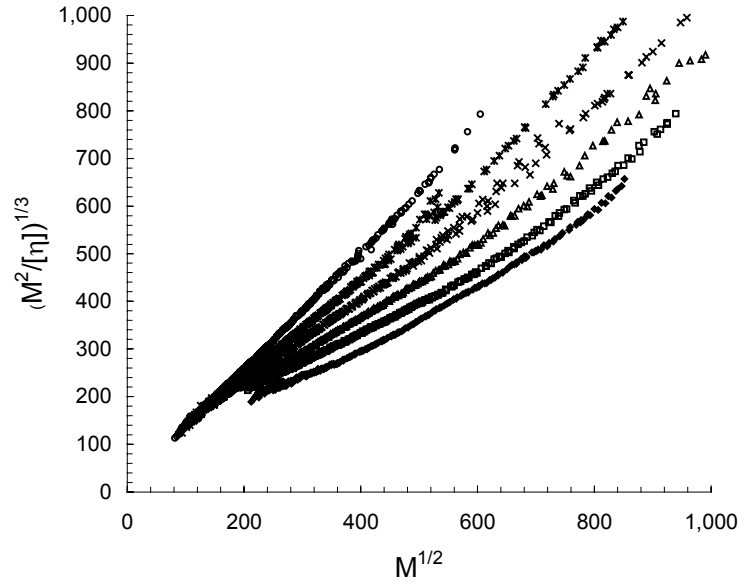


Figure 3.2 Bohdanecky plot of partially oxidised alginates with degree of oxidation 0 (lower curve), 0.05, 0.10, 0.20, 0.30 and 0.44 (upper curve) at 0.17 M ionic strength.

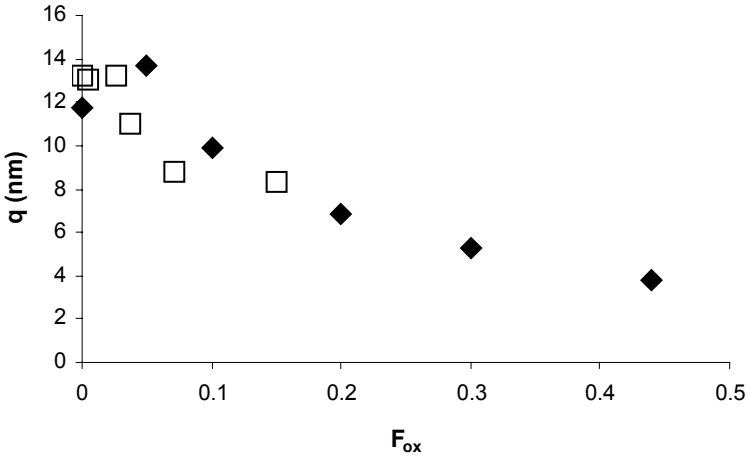


Figure 3.3 Persistence lengths, q,of a *L. hyperborea* alginate (♦)and mannuronan (□) versus F_{ox}. (M_L values structurally determined.)

3.1.3 Chitosans

The chain stiffness and the effect of F_A on the conformation of chitosans in solution have been investigated by several research groups. Some research groups have reported that the chain stiffness increases with increasing F_A (Anthonsen *et al.*, 1993; Wang *et al.*, 1991; Mazeau, 2000). Berth and Dautzenberg (2002), however, concluded from the results of several works (Terbojevich *et al.*, 1991; Errington *et al.*, 1993; Rinaudo *et al.*, 1993; Ottøy *et al.*, 1996b; Berth *et al*, 1998; Cölfen *et al.*, 2001; Berth and Dautzenberg, 2002) that all the samples of chitosan behaved consistently with a model of a nearly free-draining wormlike chain (extended coil), and that differences between samples were less than what had been claimed by the different authors. They reported an a-value of 0.92, and an a'-value of 0.55. The discrepancy between the works reported is recently discussed by Vårum and Smidsrød (2004), and they stressed the importance of considering both chemical composition, molecular weight and ionic strength before comparison is made at the molecular level of chain conformation. The effect of the bulky hydrophobic acetyl group and the hydrophilic, charged amino group seems to affect chain extension and hydration in such a way that the macroscopic solution properties are relatively little dependent on F_A at moderately high ionic strengths in the range of intrinsic viscosity/molecular weights commonly used (Vårum and Smidsrød, 2004). More work remains to get any clear conclusions concerning the effect of F_A on the chain conformation.

In this work the chain stiffness of chitosans of different F_A and M_w was studied using size-exclusion chromatography combined with light-scattering and viscosity detectors providing M - [η] and R_G - M relationships subject to further analysis. The exponent a in the MHKS equation was determined from double logarithmic plots of [η] versus M_w. An example of plots for chitosans with different F_A are given in figure 3.4. All the plots indicated straight lines, and calculated a-values are given in table 3.2. The data were also in excellent agreement with independent measurements by capillary viscometry (unpublished results and results form Ottøy et al., 1996b). The curves for chitosans with different F_A coincide, and a-values are independent of F_A with an average value of 0.78.

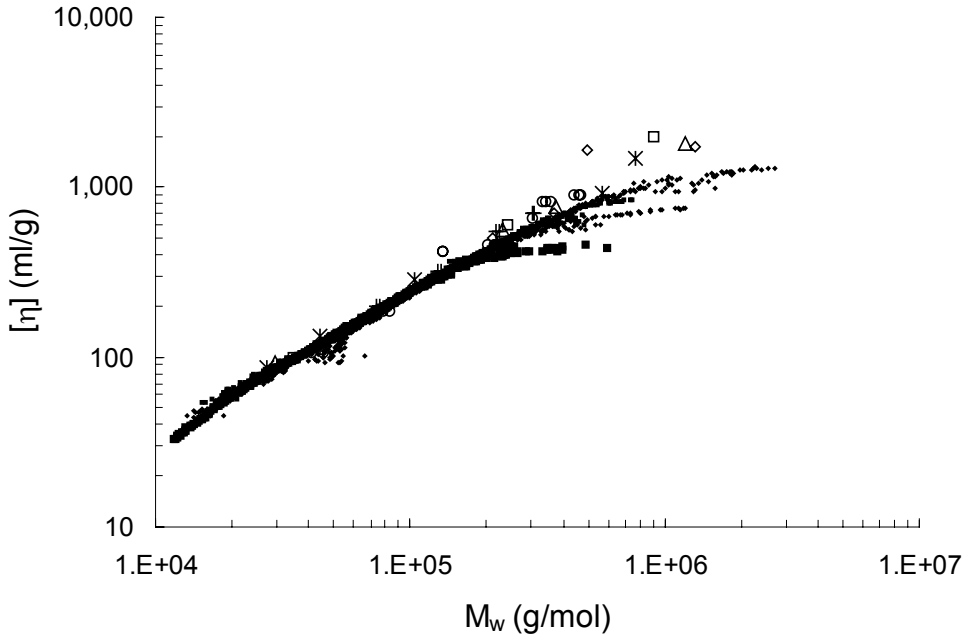


Figure 3.4 MHKS plot of chitosans with F_A = 0.05, F_A = 0.16, F_A = 0.44 and F_A = 0.64 as obtained by SEC-MALLS-VISC (filled points). Results obtained from individual measurements of [η] by capillary viscometry and M_w by SEC-MALLS are included in the figure (unpublished results and results from Ottøy *et al*., 1996b). F_A = 0.06 (◇), F_A = 0.10 (+), F_A = 0.16 (□), F_A = 0.34 (Δ), F_A = 0.44 (×), F_A = 0.52 (-), F_A = 0.64 (✶).

Exponents, a', from the double logarithmic plots of R_G versus M_w are also included in table 1, with an average value of 0.56. The determined exponents correspond to values previously reported (Berth and Dautzenberg, 2002). The determined exponents indicate flexible to stiff chains, and the results suggest that the chain conformation in chitosans is independent of F_A.

The Bohdanecky plots of all samples displayed linear regions from which the persistence length could be estimated. The persistence lengths and M_L values obtained for the different chitosans are included in table 3.2. The calculated persistence lengths show that at the conditions used the chain stiffness of chitosans is about 7 nm irrespective of F_A.

The use of the electrostatic wormlike chain model as outlined by Higashimura *et al*. (2000) allows estimation of the effect of electrostatic repulsion, and hence the calculation of the intrinsic persistence length, q_0, from the R_G –M data. Determined q_0-values varied between 3 and 5 nm, with a possible small increase in the intrinsic persistence length with F_A.

Table 3.2: Chitosans. Weight-averages of molecular weight, M_w, intrinsic viscosity, [η], and $(R_G)_w$, exponents a and a' derived from the [η]-M and R_G-M relations, persistence lengths, q, calculated with M_L (structurally determined)[I] and M_L (experimentally estimated)[II].

F_A	M_w (g/mol)	$[\eta]_w$ (ml/g)	$R_{G,w}$ (nm)	a	a'	q^I (nm)	$M_L{}^I$ (nm^{-1})	q^{II} (nm)	$M_L{}^{II}$ (nm^{-1})
0.05	270,000	535	44	0.73	0.56	7.4	427	6.3	363
	40,000	103		0.83		6.5	427	6.2	407
0.16	290,000	539	45	0.72	0.54	7.5	424	7.5	423
	250,000	533	42	0.70	0.59	7.4	424	6.2	356
	170,000	353	29	0.70	0.56	7.3	424	6.9	405
	110,000	261	23	0.76	0.56	6.8	424	5.7	355
	90,000	210	24	0.81	0.56	7.5	424	8.4	473
	40,000	98		0.88		6.9	424	7.4	455
0.44	220,000	414	38	0.75	0.55	7.2	414	8.0	459
	30,000	74		0.83		5.6	414	5.1	376
0.64	450,000	813	64	0.70	0.56	7.5	407	8.0	434
	110,000	257	26	0.79	0.55	6.8	407	6.9	413
	30,000	77		0.88		6.4	407	6.6	418
avg.				0.78	0.56	7.0		6.8	

Furthermore, the chain stiffness of partially periodate oxidised chitosans with different F_A and varying degrees of oxidation, subsequently reduced with $NaBH_4$, was studied using SEC-MALLS-VISC.

MHKS plots of oxidised chitosans with $F_A = 0.01$ are given in figure 3.5.

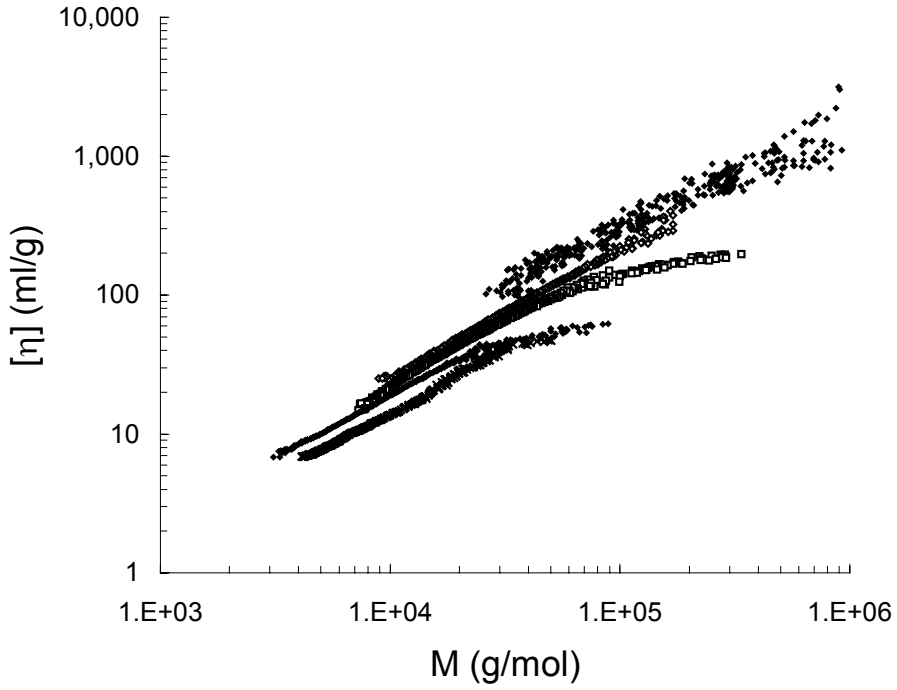


Figure 3.5 MHKS plots (log-log plots of the intrinsic viscosity versus the weight-average molecular weight) of partially oxidised chitosans with degree of oxidation 0 (upper curve), 0.02, 0.04, 0.09 and 0.16 (lower curve) at 0.2 M ionic strength.

All the plots indicate relatively straight lines, and a and K values can be determined for the oxidised chitosans. The calculated a-values are given in table 3.3 together with characterisations of the chitosans used. The a-values vary mainly between 0.8 and 0.9, suggesting flexible to stiff chains. The figure shows a shift in the linear regions of the curves towards lower [η] at a given M_w with increasing degrees of oxidation. This suggests that the chain stiffness decreases upon oxidation. Similar MHKS-plots were obtained with oxidised chitosans with F_A = 0.16 and F_A = 0.52, and a- values are included in table 3.3.

Table 3.3:Periodate oxidised chitosans. Weight-average molecular weight, M_w, intrinsic viscosity, [η], a derived from the MHKS-plot, persistence lengths,q, calculated with M_L (structurally determined)^{I} and M_L (experimentally estimated)^{II}.

F_A	F_{ox}	M_w (g/mol)	$[\eta]_w$ (ml/g)	a	q^{I} (nm)	$M_L{}^{I}$ (nm^{-1})	q^{II} (nm)	$M_L{}^{II}$ (nm^{-1})
0.01	0	189,000	840	0.77	8.2	429	6.2	329
	0.02	33,000	108	0.92	6.1	428	5.9	416
	0.04	21,000	58	0.91	4.8	428	4.2	380
	0.09	8,000	18	0.87	3.4	425	2.9	360
	0.16	7,000	9	0.87	2.4	423	2.3	403
0.16	0.00	363,000	779	0.79	7.3	424	7.4	424
	0.03	50,000	156	0.87	6.3	423	6.5	432
	0.06	31,000	84	0.87	5.2	422	5.0	401
	0.13	12,000	31	0.97	4.6	420	3.8	351
0.52	0.00	330,000	746	0.75	7.5	411	8.2	447
	0.04	99,000	259	0.73	6.3	410	6.3	412
	0.07	79,000	166	0.78	5.1	410	5.1	409
	0.14	33,000	63	0.87	4.2	408	4.1	401
	0.40	9,000	17	0.82	2.2	403	2.0	358

A Bohdanecky plot of partially oxidised chitosans with $F_A = 0.01$ is shown in figure 3.6. The calculated persistence lengths as a function of the degree of oxidation are given in figure 3.7. Persistence lengths and M_L-values obtained from both structural data and from experimental estimations are included in table 3.3.

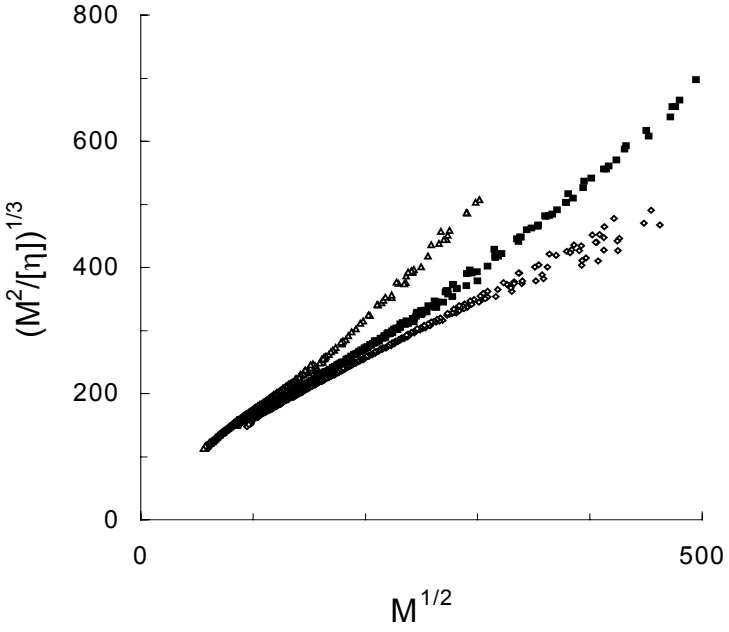


Figure 3.6 Bohdanecky plot for chitosans with $F_A = 0.01$, $F_{ox} = 0.02$ (◇), $F_{ox} = 0.04$ (●) and $F_{ox} = 0.09$ (Δ).

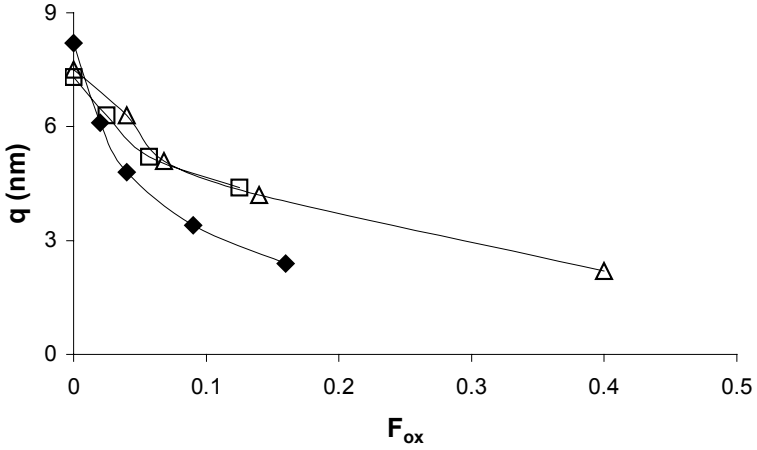


Figure 3.7 The persistent lengths of periodate oxidised chitosans with $F_A = 0.01$ (◆), $F_A = 0.16$ (□) and $F_A = 0.52$ (Δ) as a function of the degree of oxidation at an ionic strength, I = 0.2 M.

The figure clearly shows the decrease in the chain stiffness upon oxidation. The cleavage of C2-C3 linkage in (1→4)-linked polysaccharides is expected to increase the chain flexibility due to the introduction of extra degrees of freedom on each oxidised residue; each oxidised pyranose ring acting as a flexible joint in an otherwise relatively rigid polymer backbone (Smidsrød *et al.*, 1973). For chitosans, it is likely that the loss of amino groups during oxidation, leading to less electrostatic repulsion, would also lead to a decrease in the chain stiffness. This effect is, however, decreased by the relative high ionic strength used in these experiments (0.2 M).

The chain flexibility of both chitosans and alginates increases upon oxidation. Both polymers have persistence lengths, indicating stiff chains, and alginates are somewhat stiffer than chitosans.

3.2 Water-solubility of oxidised chitosans

The solubility of chitosans is to a large extent governed by the fact that the driving force to dissolution is the entropy of mixing, caused by the release of counter ions. In general, there are three essential parameters determining and limiting the solubility of chitosans in water. The pH of the solvent is important because it will determine the presence of electrostatic charges on the amino groups. Total ionic strength in the solution also plays an important role (salting-out effects, reduction of the entropy of mixing of the counter ion with water). Furthermore, the content of ions in the solvent that specifically interact with chitosans (e.g. Cu and multivalent negative ions such as molybdate) may also limit the solubility of chitosans.

All chitosans are soluble at pH values below 6. In the pH range from 6 to 8, commercial chitosans will precipitate upon increase of the pH as a consequence of the neutralisation of the positively charged amino groups. The precipitation range depends on the molecular weight of the chitosans and F_A. Chitosans with F_A around 0.5 are fully water-soluble at all pH-values. The presence of homopolymeric regions, decreasing with increasing amount of acetylated units, seems to favour precipitation by the formation of crystalline regions with high possibility of aligning the polymer chains. This could explain the increase in solubility with increasing F_A as observed by Vårum *et al.* (1994) and the neutral solubility of chitosans with intermediate F_A-values (Sannan, 1976).

The solubility of chitosans is important for a significant range of applications. It is important for biological properties such as the accessibility of chitosans to enzymes, interactions with negatively charged mucin layers and transportation in the body. Considerable efforts have been made in synthesising derivatives of chitin and chitosan (Roberts, 1992; Kurita, 2004), and some derivatives, such as N-carboxymethyl chitosan and chitosan phosphates, have been prepared in order to extend the water-solubility of chitosans to neutral pH.

In this work the water-solubility of periodate oxidised chitosans was investigated. During the oxidation chitosans also became depolymerised. Since the solubility is dependent on the molecular weight (Vårum *et al.*, 1994), unoxidised chitosans of comparable M_w were prepared by nitrous acid degradation. The solubility of the oxidised and unoxidised chitosans as a function of pH was investigated by mixing a solution of the chitosan with dilute sodium hydroxide. The amount of soluble chitosan was determined at increasing pH-values. Figure 3.8 and 3.9 show the results obtained for chitosans with $F_A = 0.01$ and $F_A = 0.16$, respectively.

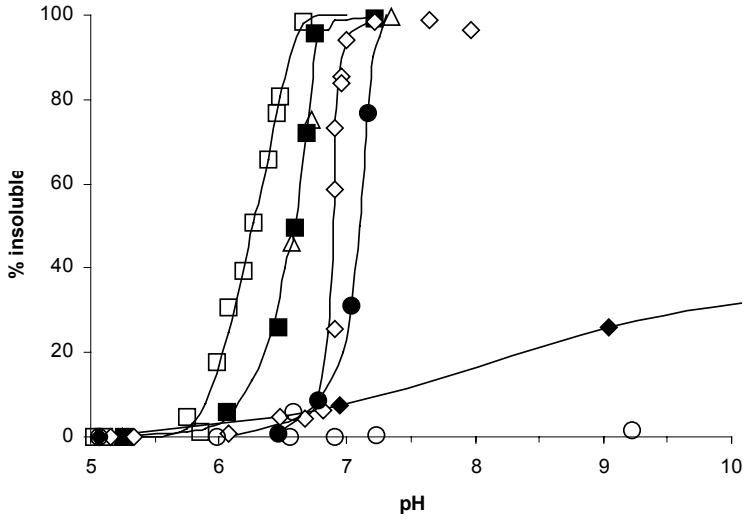


Figure 3.8 Precipitation of chitosans and periodate oxidised chitosans with $F_A = 0.01$ with sodium hydroxide. M_w = 300,000 (□), M_w = 60,000 (■),M_w = 16,000 (Δ), F_{ox} = 0.02, M_w = 50,000 (◇), F_{ox} = 0.04, M_w = 20,000 (●), F_{ox} = 0.09 reduced with $NaBH_4$, M_w =8,000 (◆) and F_{ox} = 0.09, M_w = 11,000 (o).

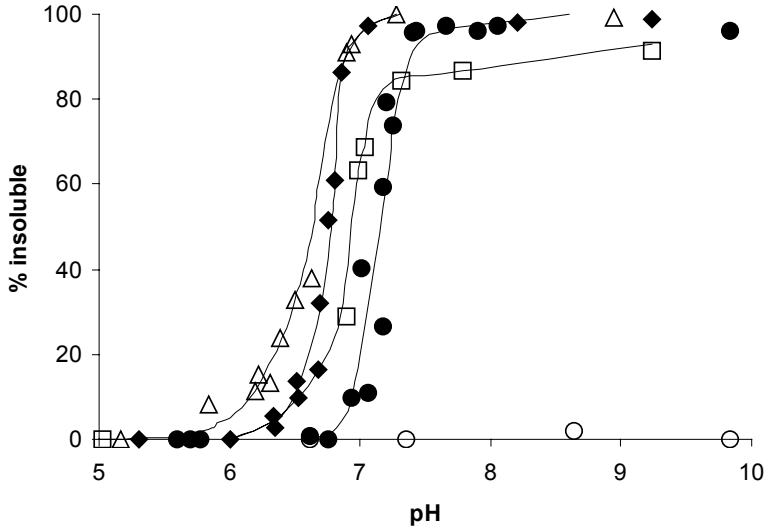


Figure 3.9 Precipitation of chitosans and periodate oxidised chitosans with $F_A = 0.16$ with sodium hydroxide. M_w = 300,000 (Δ), M_w = 110,000 (◆), M_w = 20,000 (□), $F_{ox} = 0.03$, M_w = 80,000 (●), $F_{ox} = 0.10$, M_w = 14,000 (o).

Figure 3.8 and 3.9 clearly show that the solubility of chitosans with approximately constant molecular weight increases with increasing degree of oxidation. In fact, chitosans with a degree of oxidation, $F_{ox} = 0.10$, seem to be completely soluble at all pH-values. Reduction of oxidised chitosans with $NaBH_4$ seems to lead to a slight decrease in the solubility. However, at pH around 9 a considerable fraction (~ 75%) of chitosans ($F_{ox} = 0.10$) was still soluble. The curves obtained for the undegraded chitosan are in agreement with the curves obtained by Vårum *et al.* (1994) in similar experiments. The solubility at higher pH-values increases with decreasing molecular weights of unoxidised chitosans, but it is clear that the solubility increases more upon periodate oxidation. Chitosans and oxidised chitosans ($P_0 = 0.05$, $P_0 = 0.30$) with F_A = 0.52 were completely soluble at all pH-values.

Periodate oxidised chitosan contain fewer amino groups than unoxidised chitosans, which should contribute to a lower solubility of periodate oxidised chitosans. At the same time, the introduction of unoxidised units in the chain will decrease the content of homopolymeric regions in the chitosan chains, thereby decreasing the possibility for

formation of crystalline regions, and hence increasing the solubility of oxidised chitosans. The increased solubility of the oxidised chitosans may also be partly caused by an increased chain flexibility, leading to a higher value of ΔS_m (difference in entropy between the crystalline and the dissolved states). This would lead to a lower melting temperature, T_m (= $\Delta H_m/\Delta S_m$), and hence increased solubility (Smidsrød and Christensen, 1991). The fact that reduced oxidised chitosans are less soluble than the unreduced oxidised chitosans is contradictory to results obtained in other periodate oxidised polysaccharides (Casu *et al*., 1985; Rahn and Heinze, 1998), and remains to be explained.

The physical characterisation of the periodate oxidised chitosans shows that both the chain flexibility and water-solubility increase with increasing degrees of oxidation. This makes oxidised chitosans promising for use in many applications. In addition to the characterisation of the physical properties of the oxidised chitosans, the chemical structure should be investigated in order to obtain well-characterised products.

4. CHEMICAL STRUCTURE

The periodate oxidation of chitosan obviously leads to changes in the chemical structure. It is desirable to characterise the chemical structure of the oxidised chitosans in order to get a better understanding of the periodate oxidation of chitosans. A characterisation of the reaction products could also potentially suggest approaches for reducing the severe depolymerisation that accompanies the oxidation.

The cleavage of C2-C3 in GlcN units leads to the formation of a dialdehyde. These dialdehydes are capable of existing in a variety of forms, and in solution, equilibrium occur between the various forms. In water they may exist as hydrated, acyclic aldehydes, hemiacetals or hemialdals or as combinations of these. In polysaccharides these links may be intermolecular as well as intramolecular (Guthrie, 1961; Perlin, 1980). The oxidation of the reducing end is quite complex, as shown in figure 2.2. This results in numerous possible structures, especially after partial oxidation where the amount of periodate is limited.

NMR spectroscopy is widely used to investigate chemical structure in polysaccharides, and has also been used to study periodate oxidised polysaccharides (Hirano *et al.*, 1974; Drobchenko *et al*., 1996; Maekawa, 1991; Kim *et al.*, 2000). In this work NMR-spectroscopy has been used to investigate the structure of periodate oxidised chitosans, and to evaluate the possibility of using NMR to determine the degree of oxidation of the oxidised chitosans.

^{1}H NMR spectroscopy and ^{13}C NMR spectra of chitosans with F_A = 0.01, F_{ox} = 0.10 (reduced and non-reduced) did not show significant deviations from the spectra of unoxidised chitosans. No peaks were observed at chemical shifts characteristic for aldehydes (around 10 ppm). This suggests that all the aldehydes present are hydrated or exist as hemiacetals or hemialdals, as observed previously by various techniques in other periodate oxidised polysaccharides (Hirano *et al.*, 1974; Drobchenko *et al*., 1996; Kim *et al.*, 2000). ^{13}C NMR spectroscopy has been suggested as an alternative way for

determination of the degree of oxidation in oxidised celluloses, where signals due to unoxidised D-glucose residues are easily identified (Casu, 1985; Painter, 1988). It is unclear why the spectra of oxidised chitosan, as distinct from cellulose spectra, do not deviate from the ordinary chitosan spectra.

To further investigate the chemical structure, periodate oxidised chitosans were fractionated by size exclusion chromatography (SEC). The chromatogram from SEC of chitosan F_A = 0.01 with four different F_{ox} showed the large depolymerisation that is accompanied with the oxidation. The separation between the oligomers was not complete, and the oligomers isolated contained mixtures of different structures as bimodal peaks were observed and no baseline separation was obtained.

Selected collected oligomers (F_{ox} 0.16) were studied by ^{1}H NMR spectroscopy. A spectrum of the dimer is shown in figure 4.1, and illustrates the very complex spectra that are obtained. The spectra of oligomers from trimer up to decamer diverged to some extent from the dimer spectrum, but were apparently similar to each other. It is very difficult to assign the peaks observed in these complex spectra. To get information about the structure of the oligomers, they should be further fractionated by e.g. other chromatographic techniques such as ion exchange chromatography. 2D NMR-techniques, elemental analysis and mass spectrometry could also give additional information. However, even after further separation the interpretation of the data would be a difficult task due to the many possible structures, especially at the reducing end.

Some observations in the spectra should, however, be mentioned. The dimer spectrum shows a peak around 9.7 ppm. In this region aldehydes are the most probable structures, and suggesting that free aldehydes exist in the dimer. This peak is not present in the spectra of larger oligomers. Peaks at around 8.2 ppm, are also present. This could suggest the presence of formyl esters (Perlin, 1964; Mackie and Perlin, 1965), suggesting that not all the formyl esters at the reducing ends are hydrolysed, as also suggested by the low FA_t values obtained in the study of the reaction (figure 2.3). Formic acid also appears around 8.25 ppm (Hirano *et al.*, 1974). Formic acid could possibly be released upon heating of the solution. Veelaert *et al.* (1997b), however,

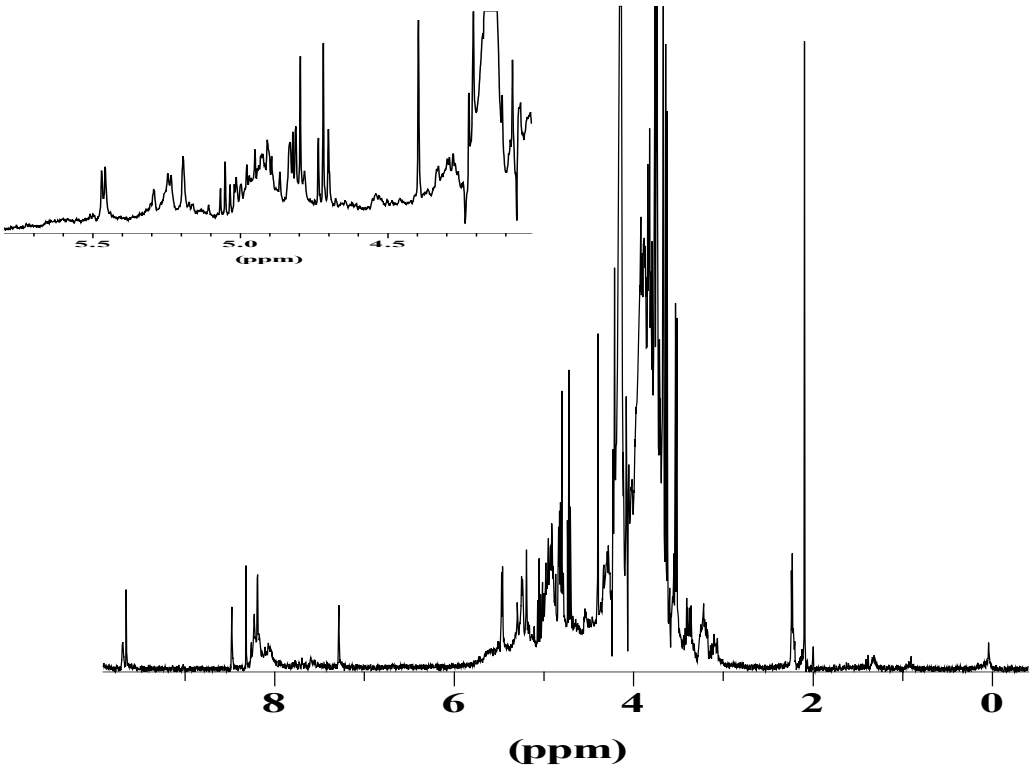


Figure 4.1 ^{1}H-NMR spectra of the "dimer" fraction of a periodate oxidised chitosan with F_A = 0.01 and F_{ox} = 0.16

reported that the dialdehyde starch structure was preserved when heated at low pH (3-4) and 90°C.

For use in biomedical applications chitosans have to be pure and well characterised with documented safety profiles. Impurities like metal ion contamination must be strictly controlled. It is of considerable importance to gain a better understanding of the selective ion-binding properties of chitosans.

5. ION BINDING

It is well known that chitosans may complex with certain metal ions, particularly transition metal and post-transition ions (Roberts, 1992). Possible applications of this metal binding property include decontamination of effluents, recovery of valuable metals, and development of new materials or new processes involving metal-loaded chitosan. Despite a large number of studies on the use of chitosan for metal ion recovery for the last twenty years, this research area fails to find practical application at industrial scale (Guibal, 2004). This could probably be explained by the relative high costs of chitosans as compared to synthetic polymers and resins combined with aspects such as variation in characterisation of chitosans, stability of the polymer and the production level. New applications of the ion binding properties of chitosans that are more financially viable than environmental applications are however possible. The loading of the polymer matrix with metal can give the support interesting complementary properties for the sorption of other organic or inorganic materials, for catalytic applications and for the manufacturing of new optical and electronic devices (Guibal, 2004).

The binding mechanism of metal ions to chitosan is not yet fully understood. It is, however, generally accepted that the amino groups in chitosan are the main reactive group for the binding of metal ions. The nitrogen atoms contain free electron doublets that can react with metal cations (through the d-orbitals) by a chelation mechanism. At pH below the pK_a value, the amino group will be protonated, and metal anions (e.g. molybdate) can be bound to chitosan by electrostatic attraction. The type of interaction depends on the metal ion, its chemistry and the pH of the solution (Guibal *et al*., 2000; Inoue *et al*., 1993).

In many applications of chitosans, for example in food, biomedical and pharmaceutical applications, knowledge of the metal ion binding properties of chitosan and the selectivity in the binding is of considerable importance. A well-documented application of chitosan is as a cholesterol-lowering agent (Ormrod *et al*., 1998), and also the much

more controversial use as a weight reducing agent (Ernst and Pittler, 1998). Knowledge of the competitive binding of essential metal ions to chitosan is important in order to assess the potential harm to humans that could arise from consuming chitosan.

The aim of the majority of the studies in this field has been to determine whether or not chitosan will complex with a given ion, to determine the amount of ion that can be bound, or to understand the process involved (Roberts, 1992). Not many studies have involved determination of the selectivity of binding of different metal ions to chitosan.

The competitive binding of two ions, e.g. Cu^{2+} and Ni^{2+}, at pH-values around the pK_a-value of chitosan may formally be expressed by the equations:

$$-NH_3^+ + Cu^{2+} \leftrightarrow -NH_2Cu^{2+} + H^+ \quad (5\text{-}1)$$

$$-NH_3^+ + Ni^{2+} \leftrightarrow -NH_2Ni^{2+} + H^+ \quad (5\text{-}2)$$

$$(5.1)\text{-}(5.2) = \quad Cu^{2+} + -NH_2Ni^{2+} \leftrightarrow Ni^{2+} + -NH_2Cu^{2+} \quad (5\text{-}3)$$

It can be seen that any selective binding of metal cations should be independent of pH according to equation (5-3) in the reaction scheme above. The reaction schemes indicate that the formation of a metal complex at pH-values around the pK_a-value of chitosan must be accompanied by the release of protons, as discussed previously (Piron and Domard, 1998; Tsezos, 1983; Domard, 1987; Koshijima *et al*., 1973).

To get a method for quantifying the binding of ions to chitosans from binary ion-mixtures, a selectivity coefficient, k_A^B , was defined in the ion-exchange equilibrium

$$\text{Chitosan-A} + B \leftrightarrow \text{Chitosan-B} + A \quad (5\text{-}4)$$

by the equation:

$$k_A^B = \frac{X_B \cdot C_A}{X_A \cdot C_B} \quad (5\text{-}5)$$

where A and B represent the two ions, X_A and X_B is the mole fraction of ions bound to the chitosan ($X_A+X_B=1$), and C_A and C_B are the molar concentrations of ions in solution at equilibrium. This definition of the selectivity coefficient has been widely used in ion binding studies on alginate (Smidsrød and Haug, 1968; Haug and Smidsrød, 1970; Smidsrød and Haug, 1972). In systems concerning metal cations and chitosan, the sorption probably occurs by complexation rather than ion-exchange. The definition of the selectivity coefficient given above will nevertheless be a measure of the selectivity in the system, regardless of the sorption mechanism. The experiments were performed using equilibrium dialysis as previously described to determine selectivity coefficients for alginates (Smidsrød and Haug, 1968).

Binary mixtures of chloride and nitrate:

Many pharmaceutical and biomedical applications of chitosans require the use of chitosan salts instead of the free amine form (ref.). It is practical to prepare chitosan salts (e.g. chitosan chloride and chitosan nitrate), as these salts, as opposed to chitosan in its free amine form, are directly soluble in water. Chitosan chlorides are also widely used commercially, and we initially tested the selectivity of chitosan towards chloride and nitrate. The determined selectivity coefficients for chitosans with $F_A=0.01$ and $F_A=0.49$ were close to 1, meaning that chitosans showed no selectivity towards chloride or nitrate ions.

Binary mixtures of molybdate/chloride and molybdate/nitrate

Molybdate anions were selectively bound to chitosans in the presence of excess nitrate or chloride ions, with selectivity coefficients around 100. The selectivity coefficients increased considerably when the system changed from chitosan in solution to precipitated chitosan (from the point $X_{Mo}\approx0.5$ to $X_{Mo}=1$), suggesting some sort of inter-chain binding as in the binding of Ca^{2+} to alginate (Smidsrød and Haug, 1968). The chitosans with $F_A = 0.01$ and $F_A = 0.49$ showed no differences in the value of the selectivity coefficients. This supports the hypothesis that the increased gel strength of

chitosan molybdate gels formed with chitosans of higher F_A is caused by interactions between the acetyl groups (Draget, 1996). The molar ratio between amino groups and molybdenum has been determined. At high metal concentration this ratio exceed 1 (between 1.3 and 1.9). This confirms that molybdate is adsorbed in the form of polynuclear species, and is consistent with previous results (Draget *et al.*, 1992).

Binary mixtures of Cu^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}:

The selectivity coefficients of chitosans (F_A=0.01 and F_A=0.49) towards the metal ions Cu^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} were investigated by studying the selectivity coefficients in binary systems as a function of the ionic composition of the metal solution, as shown in figure 5.1 (a-f). Cu^{2+} ions were found to be bound selectively to the chitosans in the presence of Ni^{2+}, Zn^{2+} and Cd^{2+} ions. The highest selectivity coefficients were determined for Cu/Cd at pH 6, with k_{Cd}^{Cu} - values between 100 and 1000 with $X_{Cu} < 0.7$. The selectivity of chitosans for Cu^{2+} was confirmed by the fact that the total uptake capacity of chitosan increased when the fraction of Cu^{2+} in the solution increased. The increasing uptake capacity was most pronounced in the Cu-Cd and Cu-Ni systems (about 5 times), and less pronounced for the Cu-Zn system. Chitosans have no selectivity in the binding of ions in the binary systems Ni^{2+}/Cd^{2+}, Zn^{2+}/Cd^{2+} and Zn^{2+}/Ni^{2+}.

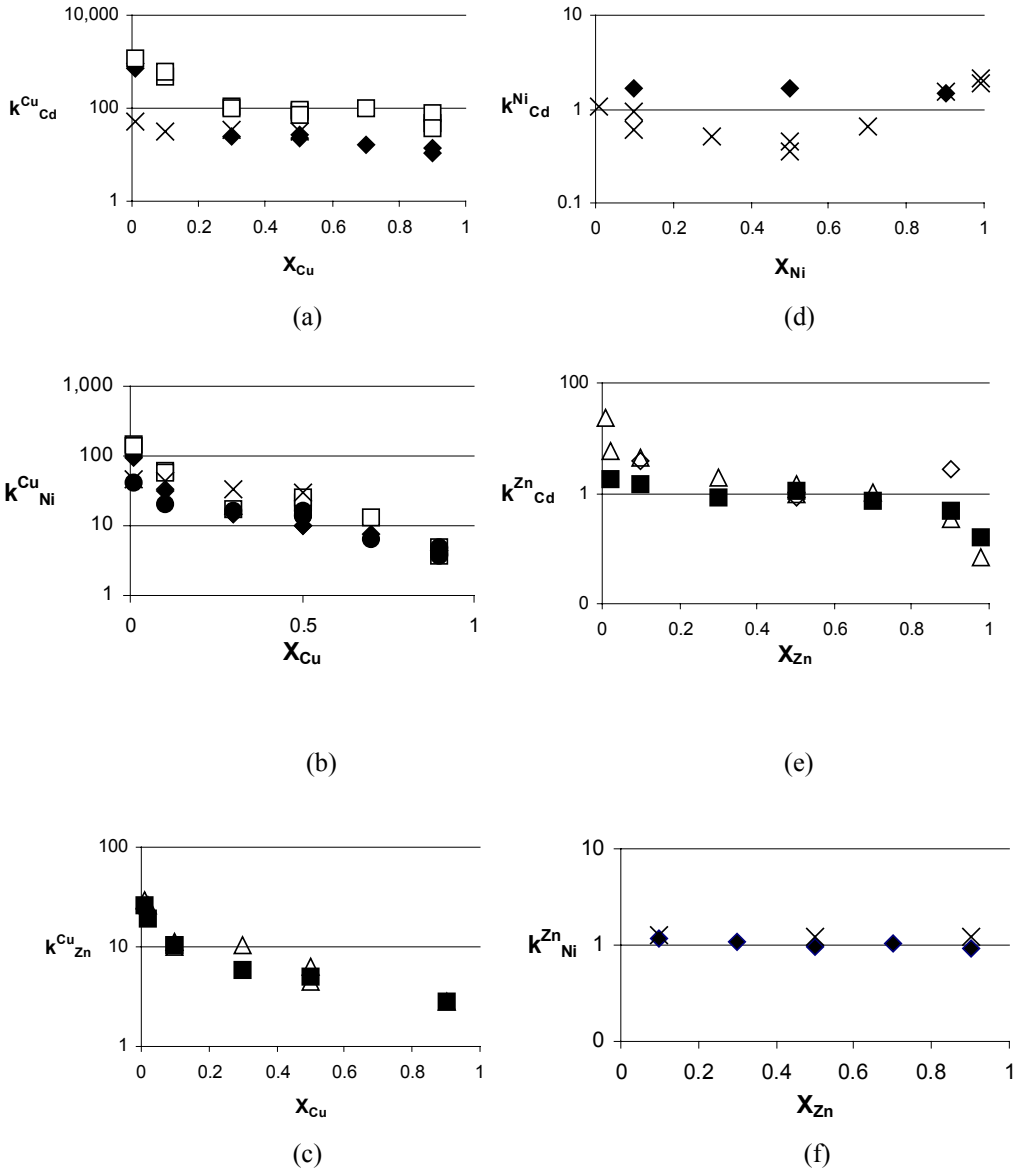


Figure 5.1: Selectivity coefficients for chitosans in the exchange reaction between (a) Cu^{2+} and Cd^{2+}, (b) Cu^{2+} and Ni^{2+}, (c) Cu^{2+} and Zn^{2+}, (d) Ni^{2+} and Cd^{2+}, (e) Zn^{2+} and Cd^{2+}, (f) Zn^{2+} and Ni^{2+}, as a function of the ionic composition in the exchange solution. Sulphate salts at pH 5: F_A 0.01(◆), F_A 0.49 (X); Sulphate salts at pH 6: F_A 0.01(□);Chloride salts at pH 5: F_A 0.01(Δ), F_A 0.49 (■), Chloride salts at pH 6: F_A 0.01(●);Nitrate salts at pH 5: F_A 0.01(◊).

The effect of pH and ionic strength on the selectivity coefficients and the total amount of ion bound to chitosans were also investigated. The selectivity coefficient increased slightly with increasing pH (figure 3, paper IV). According to equation (5-4), the selectivity should be independent of pH. The slight increase in the selectivity coefficients could be due to some kind of cooperativity or interchain binding. Also the total amount of metal ions bound to the chitosan increased with increasing pH, in agreement with previous results (Koshijima *et al.*, 1973; Becker *et al*., 2000; Dzul Erosa *et al*., 2001; Juang and Shao, 2002). The increase in the capacity of the chitosan at higher pH-values can be explained by the fact that at low pH the metal ions compete with protons for binding sites, as the reaction schemes in the introduction indicate. Moreover, the increased protonation of the amine groups at lower pH-values also induces long-range electrostatic repulsion of metal ions. Both these effects will favour binding of metal ions at high pH-values, far above the pK_a-value of the amino group of chitosan. Conversely binding will be impossible at pH-values far below the pK_a-value of the amino groups.

The selectivity coefficient decreased slightly with increasing ionic strength (figure 4, paper IV). However, no dramatic changes in the selectivity coefficient or the amount of metal ion bound was found up to 0.5 M ionic strength, and even at an 1.7 M ionic strength the selectivity coefficient remained high. At low ionic strength a small increase in the ionic strength seems to lead to an increasing amount of ions bound to the chitosan. This could be caused by screening of the long-range electrostatic repulsion between the positively charged metal ions and the positively charged chitosan. Figure 4, paper IV, shows that when the ionic strength is further increased, the amount of ions bound to chitosan decreases.

Neither the speciation of the metal salt (5.1b) nor the chemical composition of the chitosan, F_A, (5.1a,b,c,e,f) influenced the selectivity coefficients. Metal sulphates did, however, bind to a greater extent to chitosan than chloride salts. Increasing F_A-values increased the total amount of ions bound pr mol GlcN slightly.

6. GENERAL DISCUSSION AND CONCLUDING REMARKS

6.1 Main findings

Chitosans become only partially oxidised during the oxidation with periodate, reaching degrees of oxidation around 0.5, when oxidised with excess periodate. Chitosans are severely depolymerised during the oxidation, and the large overconsumption of periodate observed, can probably be explained by the consumption of periodate at the increasing number of end groups. Free radical mediated depolymerisation seems not to be sufficient to explain the depolymerisation, and a chitosan specific degradation mechanism is probably involved in the depolymerisation. The reaction rates, overconsumption of periodate, and depolymerisation all increase with decreasing F_A.

No methods to considerably reduce the depolymerisation have been found, and the preparation of high molecular weight oxidised chitosans with high degrees of oxidation is not possible at present. Partial oxidised chitosans with F_{ox} around 0.02 have M_w from around 30,000 to 100,000 depending on F_A, whereas chitosans with F_{ox} around 0.10 retains M_w around 10,000 to 30,000. This fact limits the potential use of the oxidised chitosans to applications were high molecular weight is not a demand.

Periodate oxidised chitosans with $F_A = 0.01$ and $F_A = 0.16$ are more water-soluble as compared to unoxidised chitosans of comparable molecular weight. Even low degrees of oxidation leads to significant changes in the solubility. Chitosans with F_{ox} of 0.02 show slightly increased solubility and chitosans with F_{ox} around 0.10 are completely soluble at least up to pH 10. The chain stiffness of chitosans decreases with increasing degrees of oxidation, with approximately 50 % reduction of the persistence length for chitosans with F_{ox} around 0.10.

The reaction rates, dP_t/dt, during the periodate oxidation of alginates are highly dependent on the type of alginate used and the concentration of the polymer. Characterisation of periodate oxidised alginates confirms the decrease in chain stiffness with degree of oxidation observed by Smidsrød *et al.* (1973).

A method to measure the selective ion-binding properties of chitosans has been developed. No selectivity in the binding of chloride- and nitrate ions to chitosan can be determined. Molybdate anions are selectively bound to chitosans in the presence of excess nitrate or chloride ions, with selectivity coefficients around 100. Cu^{2+} ions are bound selectively to chitosan in the presence of Ni^{2+}, Zn^{2+} or Cd^{2+} ions, with selectivity coefficients in the range 10 to 1000. Chitosans with low and high F_A do not show any difference in their selectivity towards ions. Binding of metal ions increases with pH indicating that only the deprotonised amino groups can bind the ions.

The main objective of this work was to obtain well-characterised modified chitosans with increased solubility and chain flexibility for use in biomedical applications. The increased solubility and flexibility have been achieved, but concerning the chemical characterisation of the oxidised chitosans, further work is required to gain a complete characterisation.

6.2 Biomedical applications

For use in biomedical applications the periodate oxidised polysaccharides have to be pure and well characterised and with documented safety profiles. Detailed knowledge of the metabolic fate of the polymers in the human body, and thus an understanding of its biodegradation is essential. Physical and chemical properties such as the chemical composition, molecular weight and molecular weigh distribution are also of major importance to be able to optimise the effect of chitosans in a given application.

The oxidised chitosans most suitable for biomedical applications seem to be the partial oxidised chitosans with quite low degrees of oxidation. In these polymers large parts of

the molecule retains the advantageous properties of chitosan, but due to oxidation the solubility and chain flexibility are considerably increased. Increased solubility is desirable for instance to increase absorption enhancement at neutral pH values such as those found in the intestinal tract (Thanou, 2001). For many applications chitosans of high molecular weights are desirable, and the problems with retaining high molecular weight restricts the potential of the oxidised chitosans. However, these modified chitosans still show promise for many applications. In gene delivery studies it has been found that chitosan with DP_n of 24 was as efficient as high molecular weight chitosans in forming physically stable DNA complexes, suggesting that low molecular weight chitosans may potentially be used for gene transfer (Köping-Höggård *et al.*, 2001, 2003). In the coating of alginate microcapsules with chitosans Gåserød *et al.* (1998a) showed that the binding of chitosan to alginate capsules increased when using low molecular weight chitosans.

6.3 Future studies

The chemical structure of the periodate oxidised chitosans should be further explored to further characterise the product obtained, and possibly to find a way to decrease the depolymerisation. Further on, the oxidised chitosans should be tested in biomedical applications such as DNA compaction and coating of alginate microcapsules.

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Paper I

Periodate oxidation of chitosans with different chemical compositions

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Abstract

The kinetics and stoichiometry in periodate oxidation of chitosans of different chemical compositions were investigated by determining the amount of periodate consumed and the amount 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 was observed, which is attributed to the extensive depolymerisation which occurs concomitantly with the oxidation, thereby exposing novel reducing and non-reducing ends which consume additional periodate. Increasing oxidation rates, increasing consumption of periodate, and increasing depolymerisation with decreasing F_A were observed. Decreasing the temperature, varying the pH or the addition of free radical scavengers did not reduce the extent of depolymerisation. Free radical mediated depolymerisation is not sufficient to explain the severe depolymerisation, and a chitosan specific degradation mechanism is probably involved in the depolymerisation reaction.

Keywords: chitosan, periodate oxidation

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 vicinal diols, other 1,2-dioxygenated groups and 1,2-amino alcohols[2] are also oxidatively cleaved by periodate. *N*-acetylation of the amino group, however, prevents cleavage.[3]

Polysaccharides oxidised with periodate have novel functionalities. Periodate oxidised starch is an example where periodate oxidation is used to obtain new properties in industrial polysaccharides.[4] In comparison to native alginates, periodate oxidised alginates have increased chain flexibilities, and thereby reduced chain extensions, due to the opening of the pyranosic rings.[5,6]

Alginate from *Laminaria digitata* has an oxidation limit of 44 %, which is attributed to the formation of intramolecular hemiacetals between the oxidised unit and unoxidised neighbour units.[7] Formation of intramolecular hemiacetals occurs also when oxidising other polysaccharides, e.g. dextran, xylan, amylose, and guaran[8-11], and it seems reasonable to conclude that inter-residue hemiacetals formation is a general phenomenon in the periodate oxidation of polysaccharides.[12]

In most polysaccharides depolymerisation occurs concomitantly with periodate oxidation, and it is therefore essential to increase the rate of Malapradian oxidation (glycol cleavage) relative to the rate of non-specific oxidation. There are mainly two side-reactions leading to depolymerisation. The first is the well-known erosion ("overoxidation") of the chains from their reducing end.[13] Optimised experimental conditions like low pH, low temperature will minimise the over-oxidation. The second is the scission of internal linkages in the chains, caused by hydroxyl radicals formed in

the spontaneous decomposition of periodate in solution.[14,15] This reaction is strongly catalysed by light, but it also occurs very slowly in the dark. Direct evidence that hydroxyl radicals also cause depolymerisation under the conditions of periodate oxidation has been obtained by showing that 1-propanol, which is a good scavenger for hydroxyl radicals,[16] inhibits depolymerisation and the ensuing, enhanced rate of over-oxidation, in the periodate oxidation of alginate and glycogens.[7,17,18]

Chitosans are polysaccharides consisting of two different monomers, 2-acetamido-2-deoxy-ß-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-ß-D-glucopyranose (GlcN), respectively (Figure 1). The chemical composition of the chitosan is given by the fraction of acetylated units, F_A. Chitosan is produced by complete or partial de-N-acetylation of chitin, one of the most abundant biopolymers in nature.

Periodate oxidation of chitosan has been relatively little explored. Jeanloz and Forchielli used periodate oxidation to verify the proposed ß-1,4 structure in chitin and chitosan, but chitosans with intermediate F_A were not studied.[19] They also found that the consumption of IO_4^- depended strongly on pH and temperature. At pH 4.1, T = 5˚C, they obtained P_t-values (moles of consumed periodate per mole of GlcN) around 1.4-1.5. They did also measure the ammonia liberated during the reaction and obtained N_t-values (moles of liberated ammonia per mole of GlcN) around 0.6-0.7 at pH 4.1-6.1, T = 25˚C, in agreement with corresponding results for glucosamine and α-methyl glucosaminide.[20]

Moore and Roberts compared periodate oxidation and infrared spectrometry in order to determine F_A in chitosans.[21] They concluded that due to depolymerisation and overconsumption of periodate, periodate oxidation was not a suitable method.

Periodate oxidised chitosans have found industrial applications only in a limited number of cases. Inventions containing periodate oxidised chitosan to achieve biocompatible solid surfaces are described.[22] Chitosan has been modified by periodate oxidation followed by reaction with urea and formaldehyde in order of covalent enzyme immobilisation.[23] Water-soluble polyelectrolyte derivatives have been prepared on the

basis of periodate oxidised chitosan, but only low molecular weight degradation products were obtained. After periodate oxidation of 6-O-glycolchitosan somewhat higher molecular weight derivatives were obtained.[24] Dicarboxylated chitosans have been prepared, via the partial oxidation of chitosan by periodate followed by subsequent oxidation of aldehydes to carboxylic acids. The chitosans became depolymerised, with M_n ranging from 4100 to 6200 in the final product, as well as low yields.[25]

Chitosans have long been considered promising biopolymers for use in biomedical applications. Chitosan is biocompatible, biodegradable and due to its positive charge it is bioadhesive. Chitosan also have the ability to open epithelial tight junctions, thereby enhancing the absorption of various compounds across the mucosal barrier.[26] These properties make chitosan well suited in applications like nasal drug-delivery, DNA-transfection, and encapsulation of cells and drugs.[27,28] However, most high molecular weight chitosans are insoluble near and above physiological pH.[29] Moreover, the relatively high stiffness of chitosan chains restrict their electrostatic interactions with polyanions, for instance in the coating of alginate beads, where polycations such as poly(L-lysine) are commonly used.[30] Periodate oxidation of chitosan is expected to increase the chain flexibility and also to increase the solubility, thereby improving the performance in biomedical applications. 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. In the present work we particularly study the role of F_A on the kinetics and stoichiometry of the periodate oxidation.

We define the degree of oxidation (F_{ox}) as the fraction of GlcN residues that react with periodate to give the expected dialdehyde (Figure 1). Different methods for determining the dialdehyde content in periodate oxidised polysaccharides have been described in the literature.[6,31,32] However, most of these are not appropriate at an analytical scale, or produce questionable results. For instance, the method of Lee et al.[6] fails to identify the well documented oxidation limit in alginates, and may possibly overestimate F_{ox}. Given the accepted mechanism for periodate oxidation of chitosans, the amount of ammonia liberated per mol GlcN (N_t) should equal 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:

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

This equation does not take into account oxidation taking place at the chain termini, and is therefore only valid for sufficiently long chains.

A number of abbreviations used throughout the paper are defined below: P_0 (mol periodate added/mol GlcN), P_t (mol IO_4^- consumed/mol GlcN), N_t (mol NH_3 liberated/mol GlcN), FA_t (mol HCOH liberated/mol GlcN), M_w (measured molecular weight average from SEC-MALLS), F_{ox}, fraction of oxidised GlcN units (mol oxidised GlcN units/mol GlcN total).

2. Experimental

Chitosans:

F_A=0.01, [η] = 800 ml/g, prepared by further deacetylation of a commercial chitosan F_A=0.16, [η] = 458 ml/g, F_A=0.49, F_A = 0.52, [η] = 826 ml/g, F_A = 0.60. The chitosans were provided by FMC Biopolymer (Drammen, Norway). The chitosan with F_A=0.49 and F_A 0.52 was prepared by homogenous deacetylation.

The fraction of acetylated units (F_A) was determined by ^{1}H NMR spectroscopy[33] and intrinsic viscosities were measured according to Draget et al.[34].

Oxidation:

The chitosans were dissolved in acetate buffer or HCl (pH ranging from 3-4.5) (free amines) or deionised water of Milli-Q grade (chlorides) over night (final concentration

of GlcN was 2.5 mM). The chitosan solution was mixed with periodate (P_0 = 5 or 0.30), and the proper amount of deionised water/buffer. Prior to mixing the solutions was adjusted to the desired temperature (-3˚C to 4˚C) and degassed with N_2. In some of the experiments n-propanol (10 % (v/v)) or other free radical scavengers (propionate buffer (0.2 M, pH 4), trolox, 2,2,6,6,-tetramethyl-1-piperidinyloxy radical (TEMPO)) were added to the solution. Periodate containing solutions were protected from light by covering the flasks with aluminium foil, and all weighing, pipetting and mixing operations were performed in subdued light. During the reaction period samples were collected to determine concentrations of periodate, ammonia, formaldehyde and formic acid.

In one experiment buffers/acid in the pH range from 0 – 9.1 were used (phosphate buffer at pH = 5.8, 7.4, 9.1, acetate buffer at pH 4.5, HCl at pH < 4.5). The concentration of GlcN was 24.8 mM (F_A 0.01) or 10 mM (F_A 0.52). After 24 h the reaction was ended by adding ethylene glycol.

Preparation of partially oxidised chitosans:

The preparation of chitosans oxidised to different degrees of oxidation was performed as described above (C_{GlcN} = 12.5 mM, 0.2 M acetate buffer, pH 4.5, T=.4˚C, (10 % (v/v)) n-propanol). After 48 h in the dark the reaction was ended by adding ethylene glycol, and the solution was dialysed against 0.2 M NaCl (adjusted to pH 4.5 by adding HCl) 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 chitosan was reduced conventionally with sodium borohydride (2g $NaBH_4$/g chitosan), dialysed (first 0.2M NaCl and thereafter deionised water) and freeze dried.

Methods:

Periodate was analysed by titration with sodium thiosulphate, with starch as indicator.[35] 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. The content of carbon and nitrogen was determined with a Carlo Erba Elemental Analyzer NA 1500. 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 multiangle laser light-scattering (SEC-MALLS).[36]

3. Results

3.1 Oxidation with excess periodate (P_0 = 5)

Consumption of periodate and release of ammonia:

The molar proportion of periodate consumed (P_t) and ammonia released (N_t) obtained during the periodate oxidation of four different chitosans (F_A=0.01, 0.16, 0.52 and 0.60) are given in Figure 2a and 2b, 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 shows that also the rate of ammonia release increases with decreasing F_A. N_t apparently approaches a 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.

Molecular weight

The molecular weight of the oxidised chitosans were monitored by SEC-MALLS. Results for the weight average molecular weight (M_w) (Figure 2c) shows that the chitosan with F_A=0.16 becomes extensively depolymerised even during the first minutes

of the reaction. Similar results were obtained for a chitosan with F_A = 0.01 (data not shown).

Release of formaldehyde and formic acid:

Formaldehyde was gradually liberated during the oxidation, reaching FA_t-values in the range of 0.05-0.08 after 24 h (Figure 2b). The data suggest that the extent of formaldehyde liberated increases slightly with decreasing F_A. However, after standing for 2 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.[37] However, this method gave inconsistent results, and no reliable data were obtained.

3.2 Partial oxidation (P_0 = 0.3)

Consumption of periodate and the release of ammonia and formaldehyde :

Figures 3a-b show P_t, N_t, and FA_t obtained during partial oxidation of three different chitosans (F_A = 0.01, 0.16 and 0.49). As with excess periodate the reaction rates decrease with increasing F_A. All the 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 48h (Figure 3b).

Molecular weight

The molecular weight of partially oxidised chitosans was monitored by SEC-MALLS, and the results (M_w) are shown in figure 3c. The results again demonstrate that the oxidation in all cases is associated with extensive depolymerisation. The results were, however, dependent on F_A, with the most acetylated chitosans being less susceptible to

depolymerisation. The depolymerisation appears to be most predominant during the first hours of oxidation.

The chromatograms from the SEC-MALLS analysis of oxidised chitosan with F_A=0.49 are given in figure 4. The chromatograms are shifted towards higher elution volumes as the oxidation time increases as a consequence of the shift towards lower molecular weights, as also calculated from the accompanying light scattering data (Figure 3c). However, the figure also reveals the presence of smaller peaks eluting in front of the main peak. These peaks exhibited strong light scattering, corresponding to molecular weights 1-2 orders of magnitude higher than the main peak, whereas the estimate of the radius of gyration (R_G) was in the range of 40-60 nm. Taken together, this suggests that the pre-peak contains small amounts of compact structures with high particle molecular weight, suggesting some kind of aggregation in the system. Following ultracentrifugation, these structures could to a large extent be removed. Chromatograms from analysis of oxidised chitosans with three different F_A are given in figure 5. The figure shows that the pre-peak is smaller in chitosans with low F_A, and oxidation of chitosan with F_A = 0.01 resulted in essentially no high molecular weight aggregates, except at high concentrations (results not shown). This may suggest that the association is concentration dependent, a phenomenon which sometimes occurs in unoxidised chitosan as well.[38]

Determination of the fraction of oxidised units, F_{ox}:

Three different chitosans were oxidised (P_0 = 0.025–2) for 48 hours, and F_{ox} (given by N_t) and F_{ox} (calculated from N/C) were determined separately (the latter after dialysis to remove low molecular weight fragments). Results are shown in figure 6, demonstrating that F_{ox} (N_t) ≈ F_{ox} (N/C) for all F_A as long as $P_0 < 1$. Figure 7 further shows a plot of F_{ox} (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, whereafter some downward curvature is observed.

Intramolecular hemiacetal formation:

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

aldehyde reduction.[7] However, because of the large depolymerisation accompanying the oxidation, no oxidised material could isolated after dialysis, and the experiment could not be completed

3.3 Effect of temperature, pH, solvent/buffer and free radical scavengers

To investigate the dependence of reaction conditions on P_t and N_t, and possibly identify conditions with minimum depolymerisation and overconsumption of periodate during the reaction, different experimental conditions were additionally tested. P_t and N_t measured during the oxidation suggests that the changes in experimental condition did not lead to significantly less overconsumption of periodate (results not shown). Neither 1-propanol nor TEMPO had significant influence on the rate of depolymerisation (figure 3c).

The pH dependence of N_t and M_w in partial oxidation (P_0=0.05 and P_0=0.30, t = 48h) of chitosans with F_A=0.01 and F_A=0.52, are given in figure 8. 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.[39] 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.

SEC-MALLS chromatograms after partial oxidation (P_0 = 0.30) of the chitosan with F_A=0.52 at different pH values (from 4.5 to 9.1) are given in figure 9. As pH increases the oxidised chitosans tend to give bimodal peaks, with increasing amounts of a high molecular weight fraction. It was further found that the relative content of material in this fraction increased upon storage prior to analysis (chromatograms not shown). R_G ranges between 50 and 100 nm for this fraction, suggesting that the material has a relatively compact structure. Even at pH 4.5 bimodal peaks were observed when the sample was stored for a few days before analysis. A chitosan solutions with P_0=0.30,

showed clouding upon storage when oxidised at pH 7.4 and pH 9.1. This may possibly be caused by inter-chain Schiff base formation.

4. Discussion

Dependence of F_A:

Periodate oxidation of chitosans differs in many respects to oxidation of other polysaccharides because of the involvement of the amino group. It is well known that chitosans are susceptible to overoxidation[20] and that oxidation is associated with depolymerisation[25]. We here show that the behavior - not unexpectedly – depends strongly on the degree of N-acetylation. First, the rate of oxidation increases with decreasing F_A (keeping the concentration of oxidation-sensitive GlcN residues constant). This could possibly be ascribed to the higher charge density in highly deacetylated chitosans, leading to a stronger electrostatic attraction between the positively charged chitosan and the negatively charged periodate ion, thereby increasing the effective concentration of periodate near the polymer chain. This effect might in principle be reduced by performing the oxidation at higher ionic strength. However, this may affect the solubility of the chitosan and the approach was abandoned.

Also the extent of depolymerisation and overconsumption of periodate decreases as F_A increases. This 'protective' effect of N-acetylated residues, which themselves are resistant towards oxidation, remains unexplained. Taken together with the comparatively larger resistance towards depolymerisation of other periodate oxidised polysaccharides, for example as alginates, it may be speculated that depolymerisation must involve unreacted amino groups. 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.

Oxidation limits:

Despite the high consumption of periodate, chitosans are only partially oxidised even with an excess of periodate ($P_0 > 1$). Measured amounts of liberated ammonia (Nt) indicate that chitosans have an oxidation limit near 0.5. Also partially oxidised chitosans ($P_0 < 1$) become incompletely oxidised since $N_t < P_0$. One possibility which comes in addition to overconsumption of periodate due to depolymerisation and subsequent exposure of chain termini, is the formation of intramolecular hemiacetals. An alginate from *L. digitata* had an oxidation limit of 44 % due to the formation of intramolecular hemiacetals between the oxidised unit and unoxidised neighbour units.[8] The formation of inter-residue hemiacetals is probably a general phenomenon in the periodate oxidation of polysaccharides.[13] However, the method devised by Painter and Larsen[8], involving several oxidation/reduction cycles, could not be applied to chitosans because of the depolymerisation, and the question remains unresolved.

Determination of the fraction of oxidised units, F_{ox}:

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 ($P_0 < 0.2$) (Figure 7), 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. One possibility for the underestimation of N_t and hence, F_{ox}, could be that ammonia is removed from the solution by reaction with other reagents, such as liberated formaldehyde or the aldehyde groups in chitosan. The reaction between ammonium salts and formaldehyde is well known[40,41], and was verified in a separate experiment using ammonium chloride and formaldehyde (results not shown). According to these results the amount formaldehyde released during the oxidation would only lead to a 2 % decrease of the N_t-value, and is therefore considered insignificant. A source of systematic underestimation 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. However, we estimate on the basis of molecular weights measurements (M_n obtained from SEC-MALLS) and the assumption that 4 carbon atoms are lost from the end groups upon oxidation, that the underestimation of F_{ox} based on N/C analysis is maximum 9 % for a chitosan with F_A=0.01 at P_0 = 2. Thus, additional and so far unidentified factors contribute to the observed discrepancy between the two estimates of $F_{ox.}$

Depolymerisation during the oxidation:

Figure 10 gives DP_n (the number-average degree of polymerisation) as a function of F_{ox} (P_0 = 0.30) for three different chitosans. The figure clearly shows that the chitosan with F_A = 0.49 becomes much less degraded. Hence, N-acetylated residues protects against depolymerisation. Figure 10 further form a basis for estimating the relative number of chain scissions per oxidised GlcN residues (α/F_{ox}). Assuming M_w/M_n = 2 (random degradation) we obtain the degree of chain scission, α, defined as the number of broken linkages divided by the number of linkages originally present, given by $1/DP_n$. Figure 11 shows α/F_{ox} as a function of F_{ox}. The relative number of chain scissions per oxidised GlcN residues decreases from around 0.4 (F_A = 0.01) to around 0.1 (F_A = 0.49), clearly demonstrating that the presence of N-acetylated units decrease the depolymerisation during the oxidation.

The depolymerisation of many polysaccharides during periodate oxidation has earlier been shown to be free radical mediated.[42] In chitosan oxidation the addition of 1-propanol, a free radical scavenger, did not lead to a reduction in the consumption of periodate when oxidising with excess periodate, and neither addition of 1-propanol nor TEMPO did reduce the depolymerisation considerably during partial oxidation. It seems also clear that chitosan become more depolymerised than e.g. alginate (results not shown) and cellulose[42] during periodate oxidation. Comparison of the oxidative-reductive degradation (ORD) of chitosan and alginate with H_2O_2 in the presence of Fe^{3+} suggest that the degradation caused by hydroxyl radicals is actually slower for chitosan than alginate.[16,43,44] This suggests that some other chitosan specific degradation mechanism connected to the oxidation is involved.

Overconsumption of periodate during the oxidation

The major reason for the overconsumption of periodate is obviously the extensive depolymerisation that occurs concomitantly with the oxidation. Depolymerisation will lead to more end groups, which consume more periodate than the internal monomers in the chitosan chains. The reducing ends could also be overoxidised. Overoxidation is especially a problem in polysaccharides when malonaldehyde-derived structures are a product of the oxidation of reducing residues.[1] Because the other residues are oxidised relatively slowly, overoxidation can proceed during the oxidation, exposing a succession of new, reducing residues as the degradation proceeds along the chain. The overoxidation should however be at a minimum at the experimental conditions in these experiments.[13,39]

The amount of formaldehyde liberated provides a good index of the extent of overoxidation as the latter is derived from the primary alcohol group of degraded, reducing residues. At the same time the molecular weights measurements provide an independent estimate of the degree of scission, α. Assuming random degradation, an oxidised chitosan has $\alpha \cdot F_A$ GlcNAc-end residues and $\alpha \cdot (1\text{-}F_A)$ GlcN-end residues.

The fraction of reducing ends overoxidised will be given by the amount of formaldehyde released per monomer divided by the degree of scission, $FA_t \cdot (1\text{-}F_A)/\alpha$. The non-reducing ends will consume 2 moles of periodate, whereas the reducing ends will consume 2 moles of periodate if no formaldehyde is released. 6 moles of periodate will be consumed when the reducing end is completely overoxidised, and further 2 moles consumed at the newly exposed reducing end group. Assuming that the reducing ends that release formaldehyde is completely overoxidised, P_t should thus be given by the equation:

$$P_t = \frac{N_t \cdot (1 - F_A) + \alpha \cdot 1 + \frac{FA_t \cdot (1 - F_A)}{\alpha} \cdot \alpha \cdot 7 + (1 - \frac{FA_t \cdot (1 - F_A)}{\alpha}) \cdot \alpha \cdot 1}{1 - F_A}$$

Calculated values of P_t are compared to experimentally measured values in table 1. This show that the overconsumption of periodate most likely can be explained by the

increased uptake of periodate at the reducing and non-reducing ends for the partial oxidised chitosans. The low N_t-values obtained in partial oxidation could possibly be due to a faster reaction at the reducing and non-reducing ends as compared to the internal units.

5. Conclusion

Chitosans become only partially oxidised during the oxidation with periodate, reaching degrees of oxidation around 0.5, when oxidising with excess periodate. A large overconsumption of periodate is observed, and this could probably be explained by the severe depolymerisation during the reaction. The reaction rates, overconsumption of periodate, and depolymerisation increase with decreasing F_A. Free radical mediated depolymerisation is not sufficient to explain the severe depolymerisation, and a chitosan specific degradation mechanism is probably involved in the depolymerisation.

Acknowledgements

This work was financially supported by The Research Council of Norway.
Ann-Sissel Ulset is thanked for technical assistance.

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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.

Figure 2a-c. 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 (Δ), F_A = 0.52 (◇) and F_A 0.60 (●).The concentration of GlcN was 2.5 mM. The reactions were performed at pH 4.5, T = 4˚C, excess periodate (P_0 = 5).

Figure 3a-c. 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 (Δ), F_A = 0.49 (◇). The concentration of GlcN was 2.5 mM. The reactions were performed at pH 4.5, 4˚C, and 10 % n-propanol was included. M_w measured in corresponding experiments with no propanol added (F_A = 0.16) (X) and TEMPO added (F_A = 0.01) (□) are included in figure 3c.

Figure 4. SEC-MALLS elution profiles and plots of log M versus volume (calibration curves) (a) and log R_G versus volume (b) for chitosan with F_A = 0.49 during periodate oxidation (t=1, 3 and 52h), P_0 = 0.30.

Figure 5. SEC-MALLS elution curves and calibration plots for chitosans with F_A = 0.01, F_A = 0.16, and F_A = 0.49 after 1 hour of oxidation, P_0 0.3.

Figure 6 The degree of oxidation, F_{ox}, of the oxidised chitosans with F_A = 0.01 (a), F_A = 0.16 (b), F_A = 0.49 (c) as determined by N_t-measurements (□) and elemental analysis (♦).

Figure 7. F_{ox} determined by amount of 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 (Δ), and F_A = 0.49 (◇).

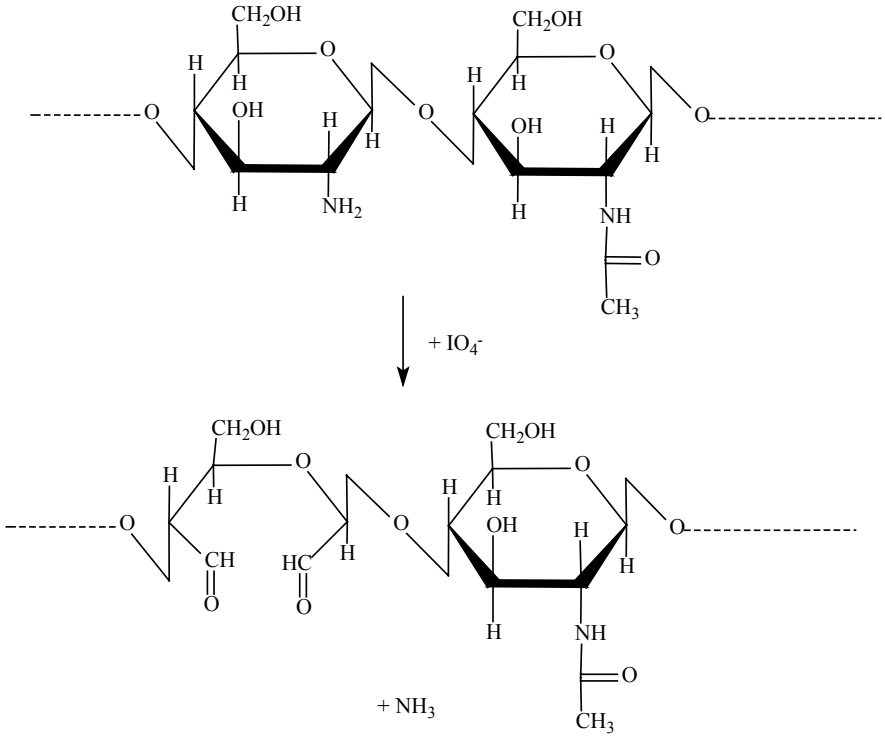
Figure 8. Molecular weight, M_w, (a) and N_t (b) obtained after 24 h when oxidising chitosan $F_A = 0.01$ ($P_0 = 0.05$ (□), $P_0 = 0.30$ (■) and $F_A = 0.52$ ($P_0 = 0.05$ (▲), $P_0 = 0.30$ (Δ)) at different pH-values. T = 4˚C, 10 % (v/v) 1-propanol, 4 mg/ml chitosan.

Figure 9. SEC-MALLS elution curves and calibration plots for chitosan. The effect of pH on the degradation of chitosan with $F_A = 0.52$ after 24 hours of oxidation, $P_0 = 0.30$.

Figure 10. DP_n as a function of F_{ox} for chitosans with $F_A = 0.01$ (■), $F_A = 0.16$ (Δ), $F_A = 0.49$ (◇).

Figure 11. α/F_{ox} as a function of F_{ox} for chitosans with $F_A = 0.01$ (■), $F_A = 0.16$ (Δ), $F_A = 0.49$ (◇).

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



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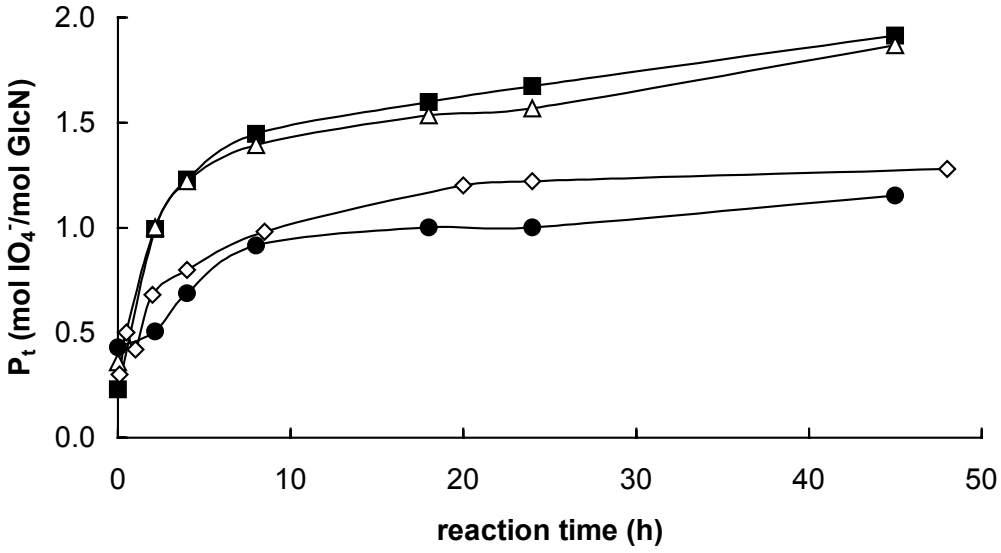

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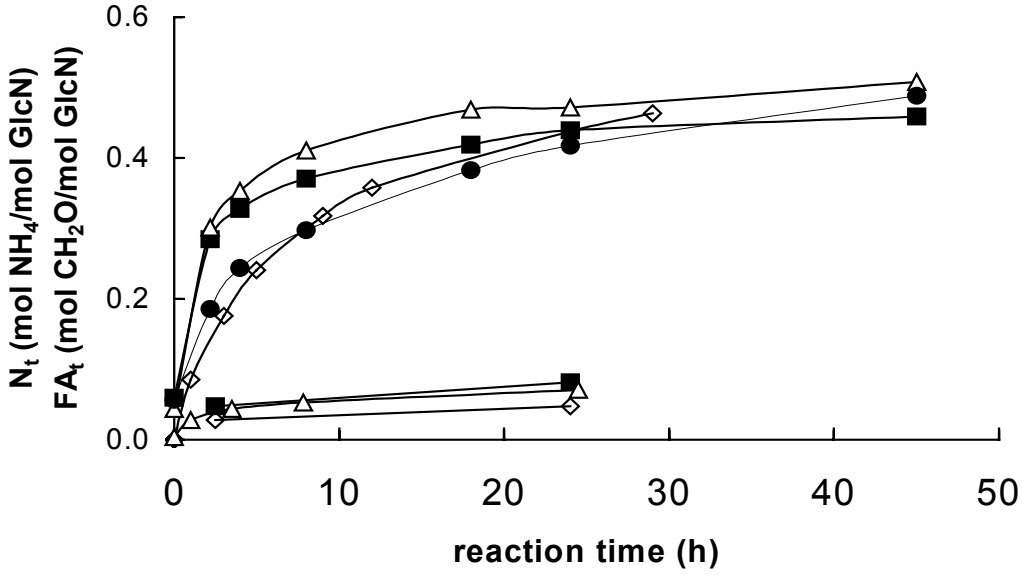

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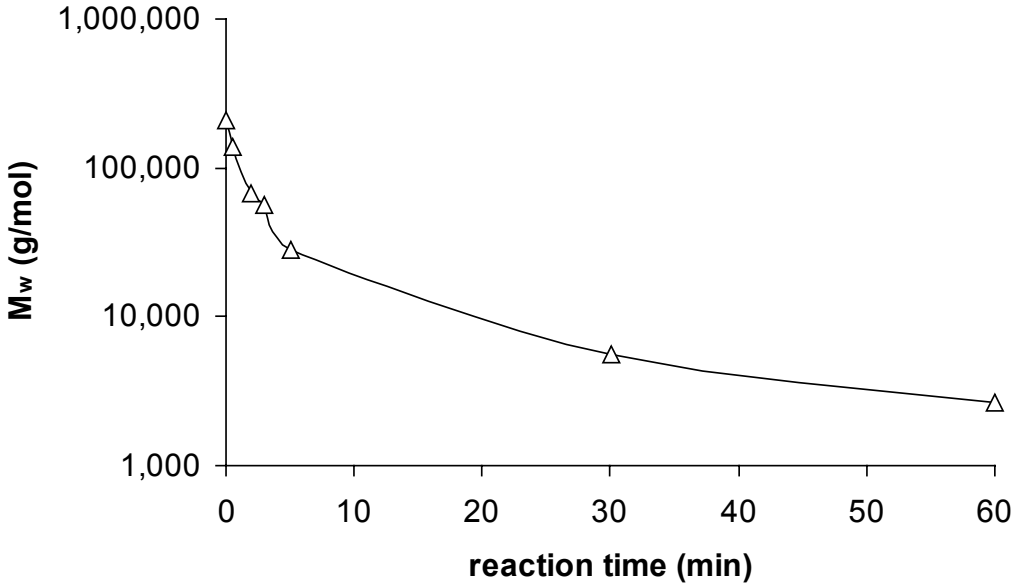

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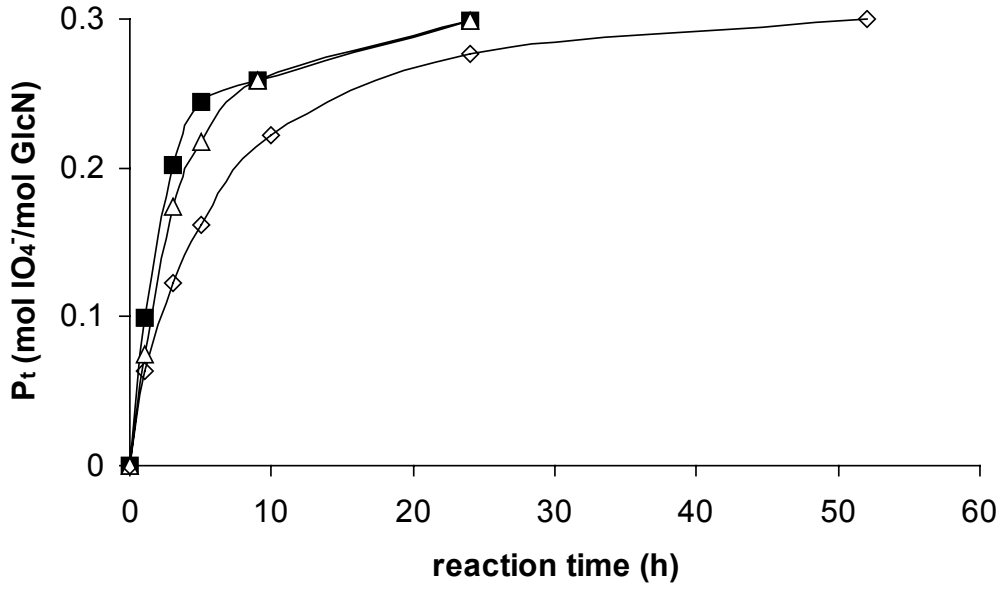

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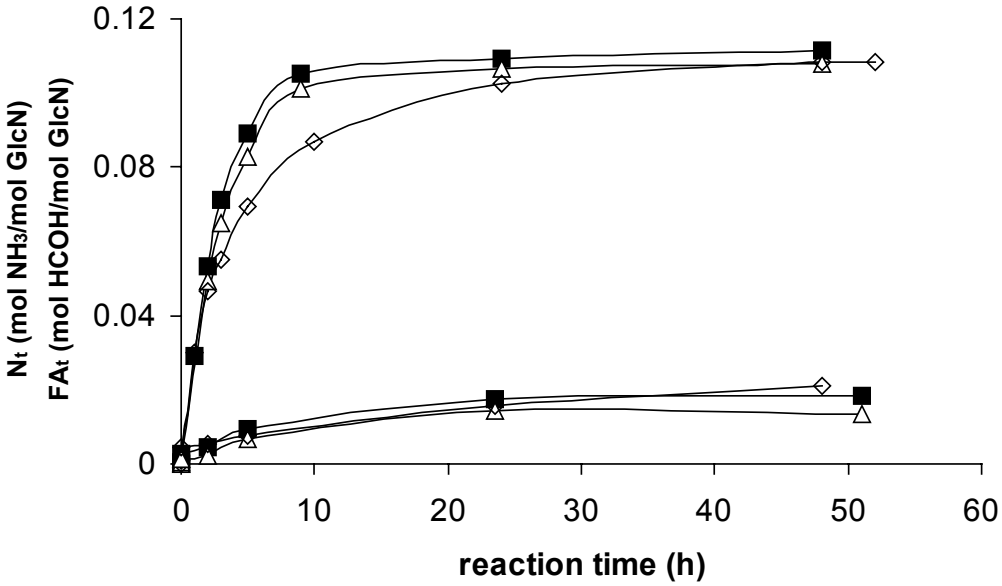

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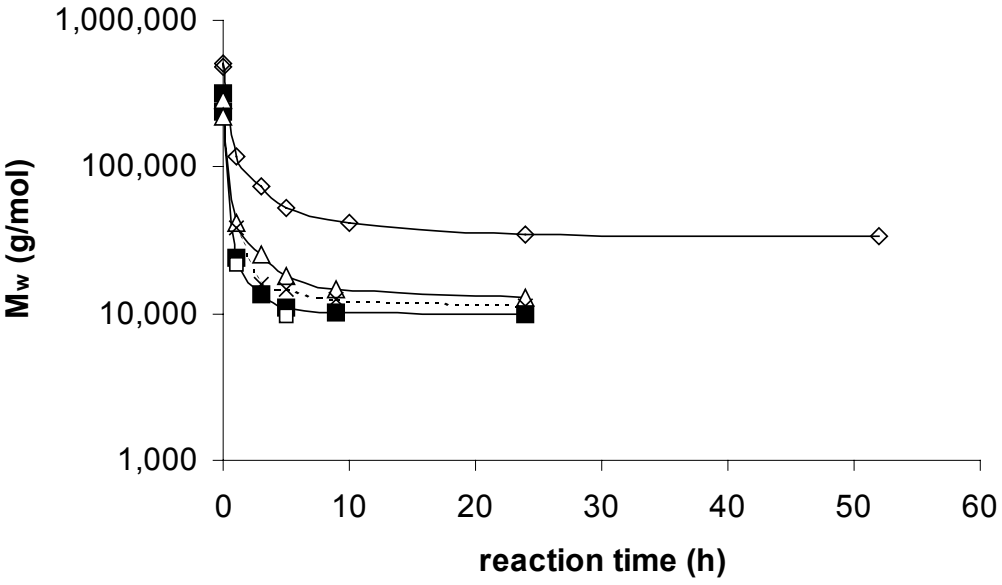

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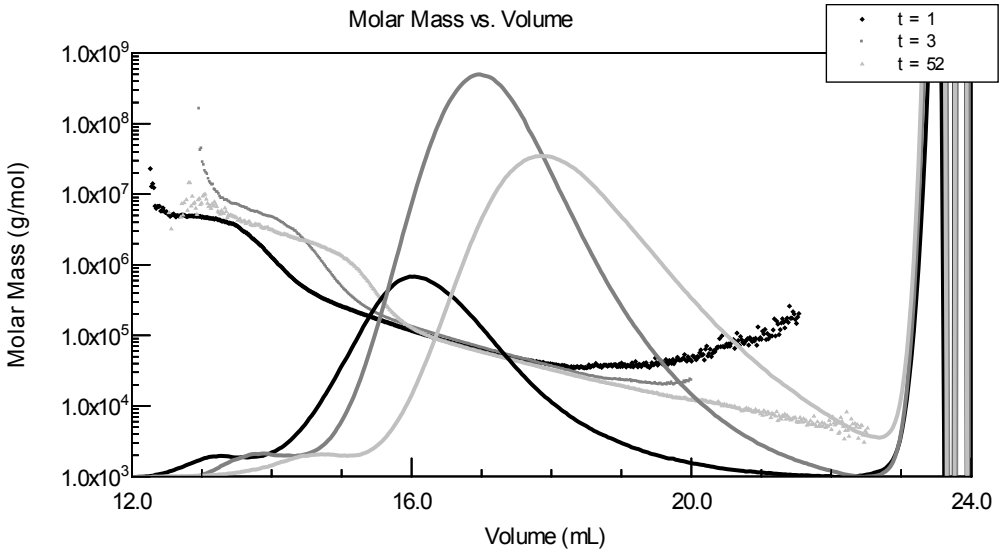

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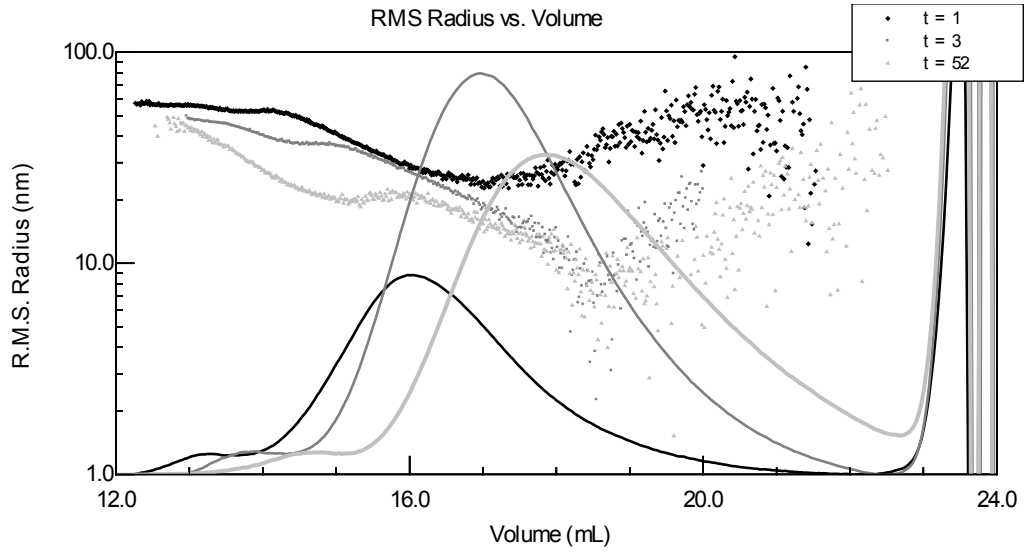

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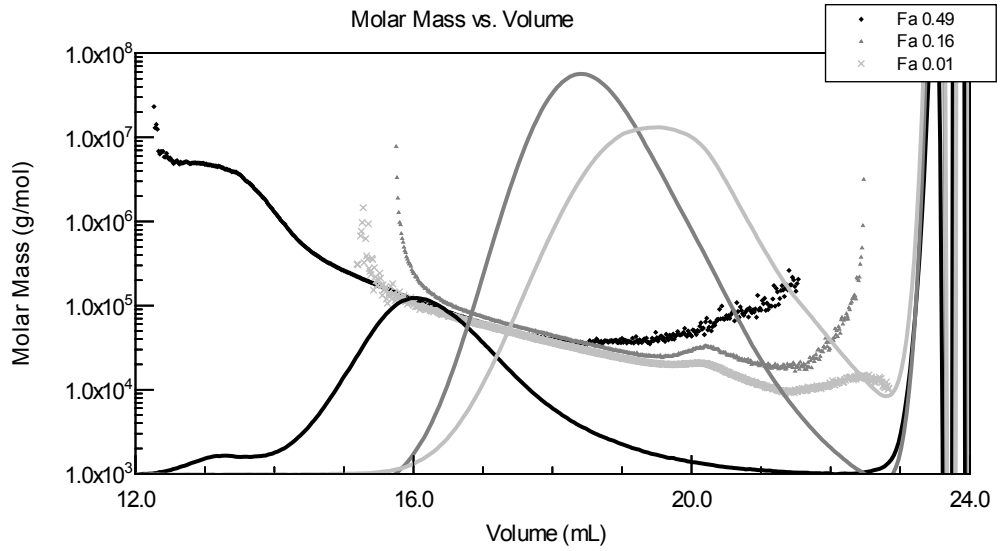
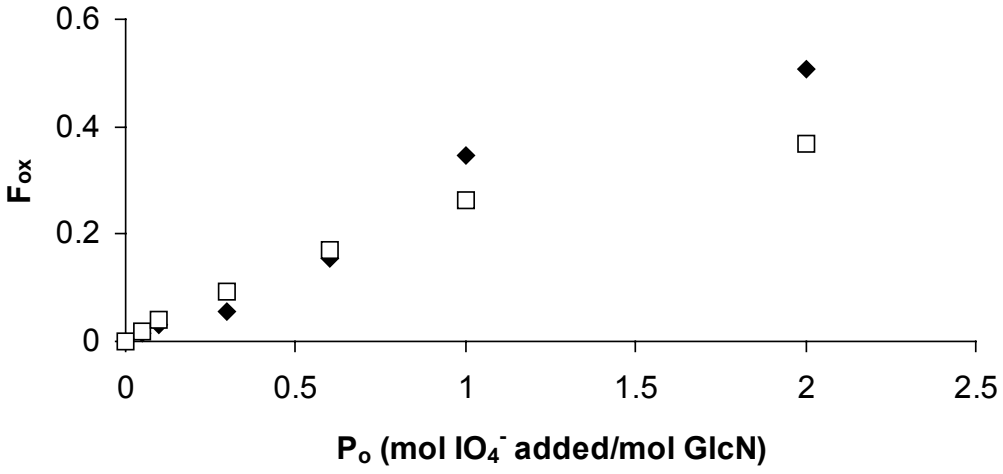


Figure 5



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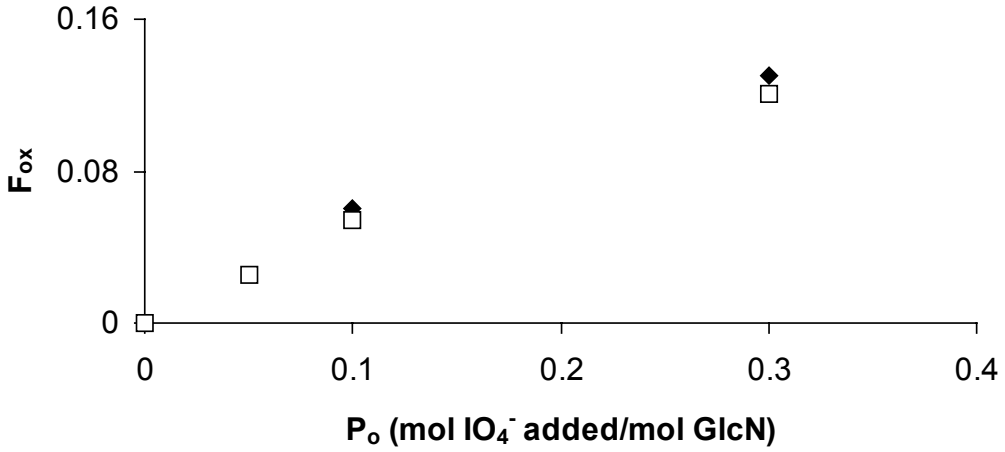

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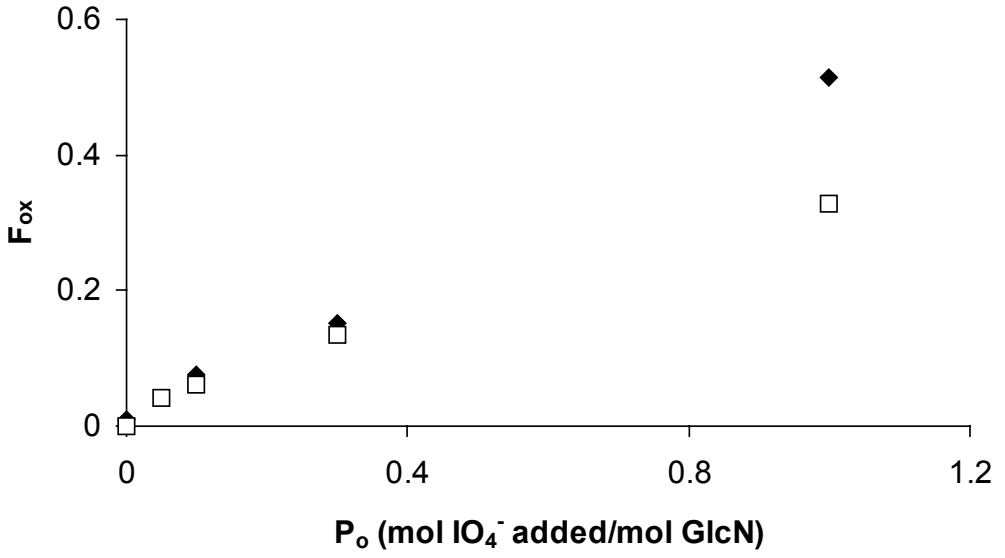

Figure 6 (c)

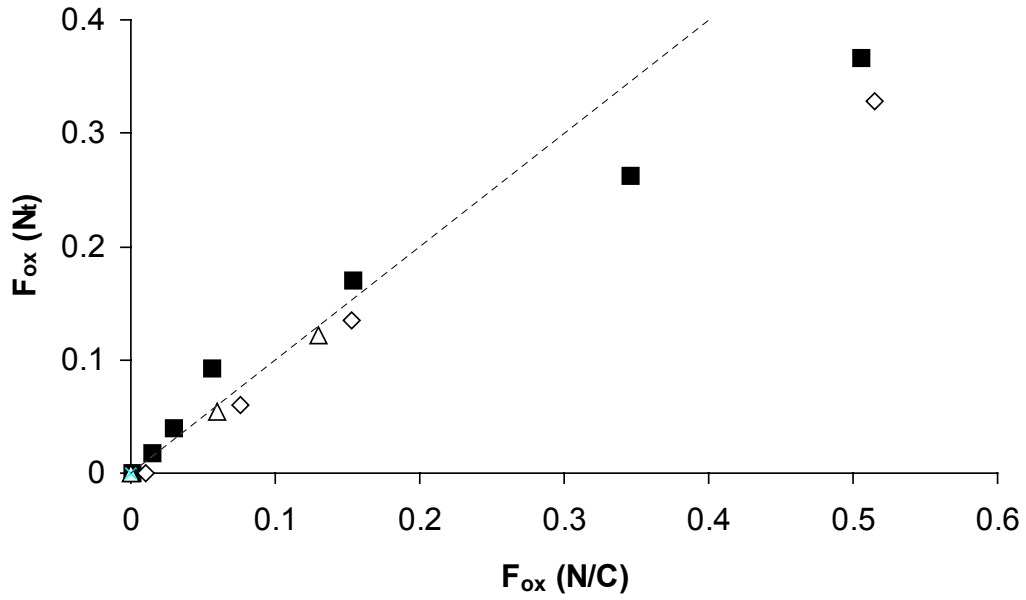

Figure 7

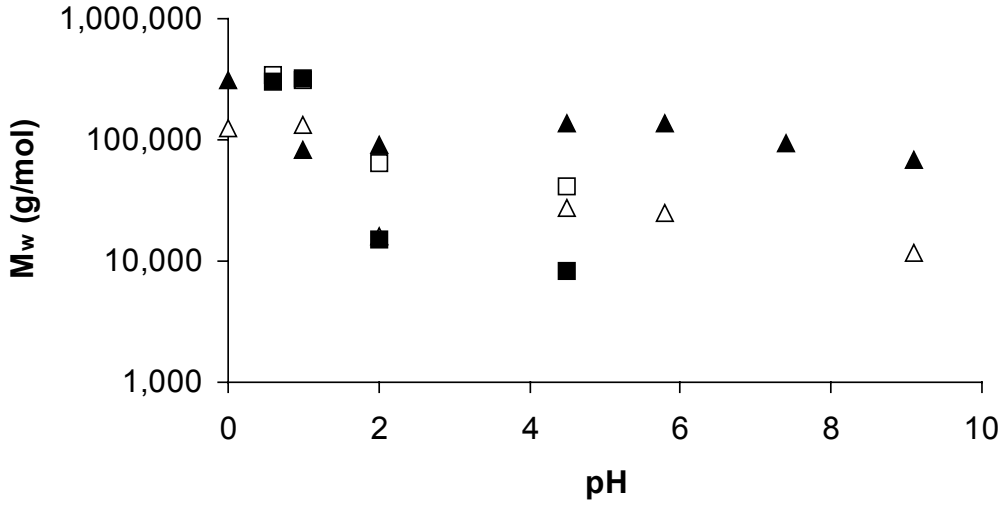

Figure 8 (a)

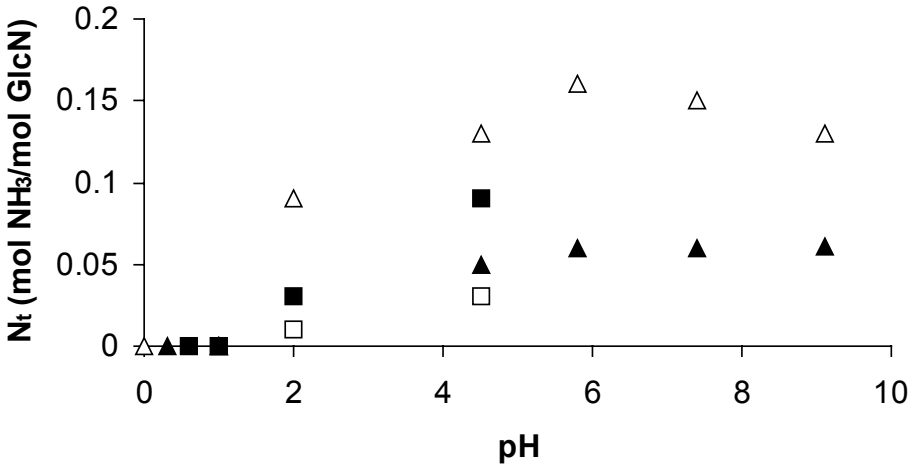

Figure 8 (b)

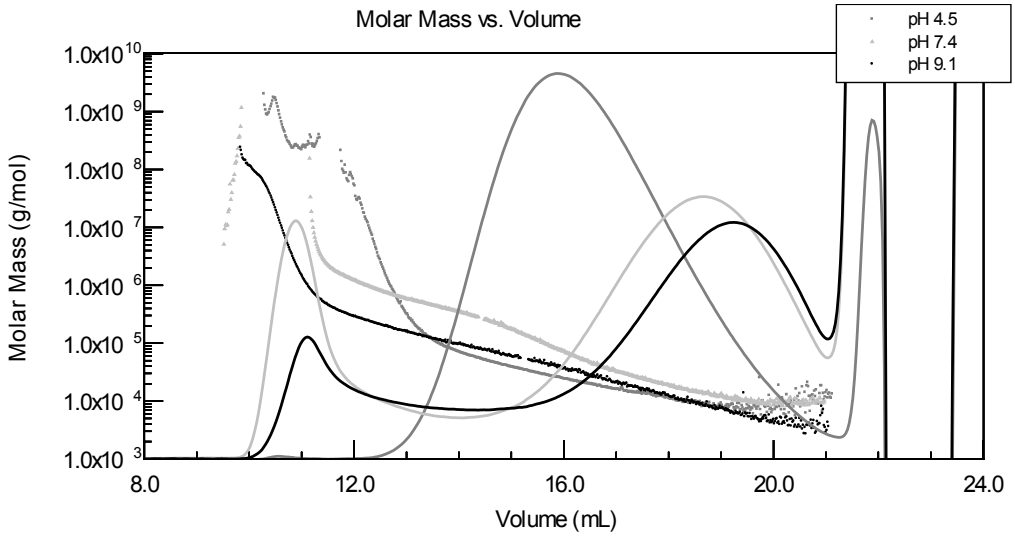

Figure 9 (a)

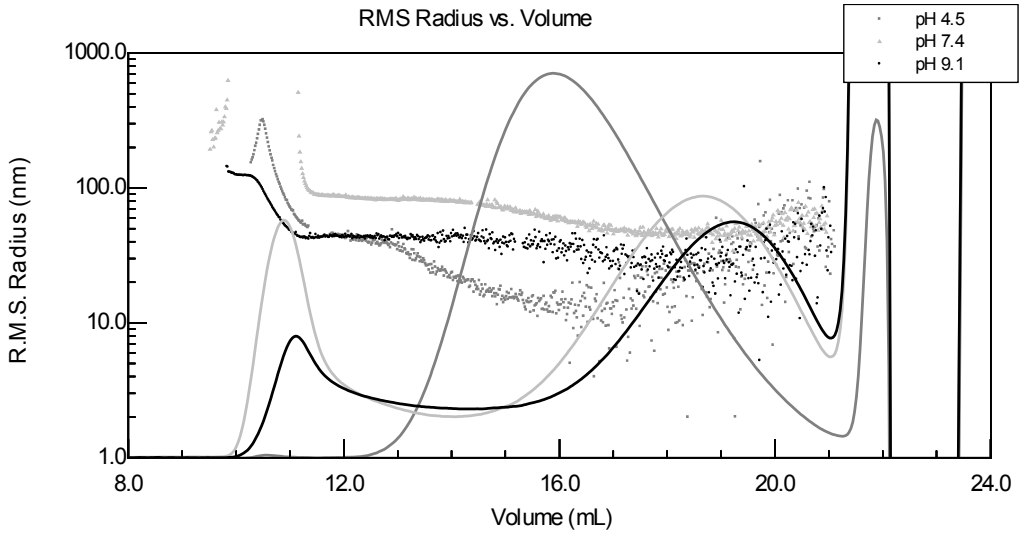
RMS Radius vs. Volume
pH 4.5
pH 7.4
pH 9.1
1000.0
100.0
10.0
1.0
R.M.S. Radius (nm)
8.0
12.0
16.0
20.0
24.0
Volume (mL)

Figure 9 (b)

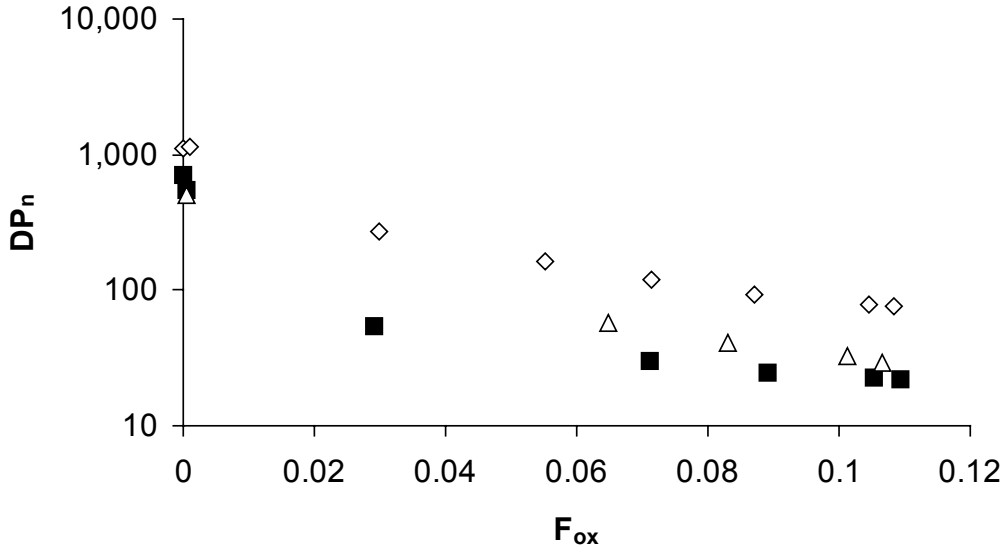

Figure 10

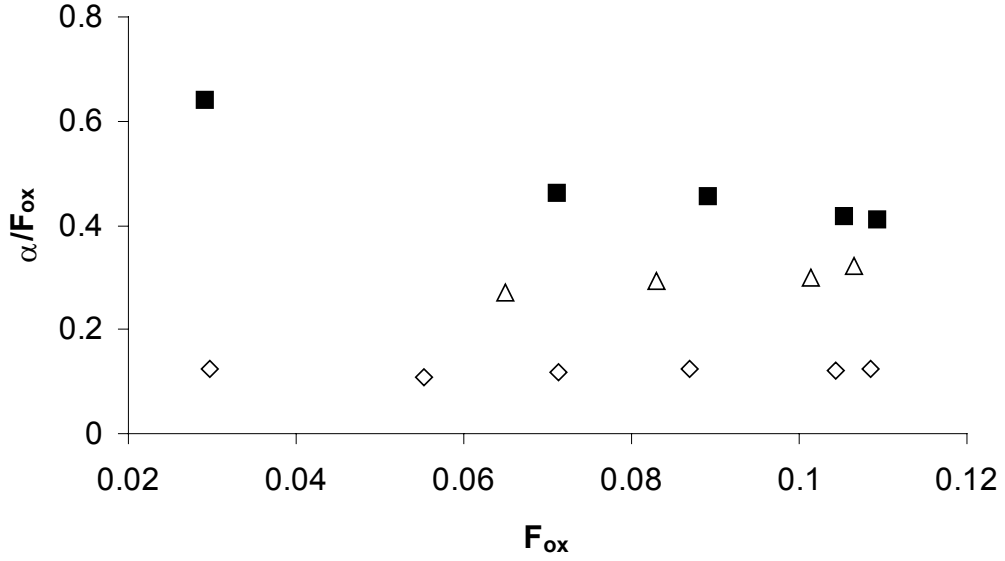

Figure 11

Table 1

F_A	Time (h)	P_t (measured)	P_t (calculated)
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

Paper II

A study of the chain stiffness and water-solubility of chitosans and periodate oxidised chitosans

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Abstract

Chitosans of different chemical composition, F_A ranging from 0.05 to 0.64, were depolymerised to different extents using $NaNO_3$ or acid hydrolysis, and thereafter characterised using size-exclusion chromatography combined with light-scattering and viscosity detectors (SEC-MALLS-VISC). At the conditions used (0.2 M NH_4Ac, pH 4.5) the persistence length of chitosans are about 7 nm irrespective of F_A.

In order to try to increase the chain flexibility and solubility chitosans were partially oxidised by periodate. The resulting oxidised chitosans was characterised using SEC-MALLS-VISC and investigation of solubility as a function of pH. $[\eta]$-M_w plots suggests that the chain stiffness of chitosans decrease with increasing degree of oxidation. Persistent lengths calculated using the Bohdanecky plot for wormlike chains, suggest a decrease in q of approximately 50 % for chitosans with F_{ox} around 0.10. The water-solubility of periodate oxidised chitosans with F_A = 0.01 and F_A = 0.16 increases significantly with increasing degrees of oxidation. Chitosans with F_{ox} = 0.10 is apparently completely soluble up to pH 10. After reduction of the oxidised chitosans the solubility decreases. However, at pH 9 a considerable fraction (~ 75%) of chitosans (F_{ox} = 0.10) is still soluble. Chitosans and periodate oxidised chitosans with F_A = 0.52 was soluble at all pH-values examined.

Keywords: chitosans, periodate oxidation, solubility, chain stiffness, wormlike chain, light scattering

1. Introduction

Chitosans are linear binary heteropolysaccharides composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN). The amount of GlcNAc, expressed as the fraction of acetylated units (F_A), and the molecular weight average are main factors determining the properties of chitosans, and thereby also applications.

Chitosans have long been considered promising biopolymers for use in pharmaceutical and biomedical applications. Chitosan is biocompatible, biodegradable and due to its positive charge it is bioadhesive. Chitosans have also the ability to open epithelial tight junctions, thereby enhancing the absorption of various compounds across the mucosal barrier (Dodane *et al.*, 1999). These properties make chitosan well suited in applications like nasal drug-delivery, DNA-transfection, and encapsulation of cells and drugs (Illum, 1998; Illum *et al.*, 2001; Köping-Höggård *et al.*, 2001). However, most high molecular weight chitosans are insoluble near and above physiological pH (Vårum *et al.*, 1994). Moreover, the relatively high stiffness of chitosan chains restrict their electrostatic interactions with polyanions, for instance in the coating of alginate beads, where polycations such as poly(L-lysine) are commonly used (Gåserød *et al.*, 1998).

Knowledge of solution properties like chain stiffness and solubility of chitosans is important both from a basic and an applied point of view. The physical properties of chitosans are dependent on the chemistry of the polymer (F_A), the molecular weight, the pH, and the ionic strength of the solution. For many applications, and especially in the pharmaceutical industry, parameters like F_A, molecular weight and molecular weight distribution must be strictly controlled. This was clearly demonstrated by Schipper *et al.* (1996) who showed that the absorption enhancement of chitosans varied considerably with F_A and molecular weight. Knowledge about the physical and biological properties is crucial in order to find the chitosan best suited for a given application.

Chitosans, and polysaccharides in general, are stiff molecules. This stiffness leads to highly extended chains in solution. Rigid six-member sugar rings and restricted rotation

around glycosidic bonds contributes to the high chain stiffness in chitosans. In addition the electrostatic repulsion between the charges groups on the polymer chain leads to an added ionic-strength-dependent extension.

The chain stiffness and the effect of F_A on the conformation of chitosans in solution have been investigated by several research groups. Some research groups have reported that the chain stiffness increases with increasing F_A (Anthonsen *et al.*, 1993; Wang *et al.*, 1991; Mazeau, 2000). Berth and Dautzenberg (2002), however, concluded from the results of several works (Terbojevich *et al.*, 1991; Errington *et al.*, 1993; Rinaudo *et al.*, 1993; Ottøy *et al.*, 1996; Berth *et al*, 1998; Cölfen *et al.*, 2001; Berth and Dautzenberg, 2002) that all the samples of chitosan behaved consistently with a model of a nearly free-draining wormlike chain (extended coil), and that differences between samples were less than what often had been claimed by the different authors. They reported an a-value of 0.92, and an a'-value of 0.55. The discrepancy between the works reported is recently discussed by Vårum and Smidsrød (2004), and they stressed the importance of considering both chemical composition, molecular weight and ionic strength before comparison is made at the molecular level of chain conformation. Two contradictory effects have to be considered concerning F_A. A high content of bulky acetyl groups may increase the chain stiffness for steric reasons. On the other hand, at low F_A the chains will expand due to electrostatic repulsion of positively charged amino groups. High ionic strength will suppress the electrostatic repulsion, and the electrostatic contribution to the chain stiffness becomes small. It is therefore desirable to perform the characterisation of polyelectrolytes at high ionic strength to approach unperturbed dimensions. More work remains to get any clear conclusions concerning the effect of F_A on the chain conformation.

The solubility of chitosans is to a large extent governed by the fact that the driving force to dissolution is the entropy of mixing, caused by the release of counter ions. In general, there are three essential parameters determining and limiting the solubility of chitosans in water. The pH of the solvent is important because it will determine the presence of electrostatic charges on the amino groups. Total ionic strength in the solution also plays an important role (salting-out effects, reduction of the entropy of mixing of the counter

ion with water). Furthermore, the content of ions in the solvent that specifically interact with chitosans (e.g. Cu and multivalent negative ions such as molybdate) may also limit the solubility of chitosans.

All chitosans are soluble at pH values below 6. In the pH range from 6 to 8, commercial chitosans will precipitate upon increase of the pH as a consequence of the neutralisation of the positively charged amino groups. The precipitation range depends on the molecular weight of the chitosans and F_A. Chitosans with F_A around 0.5 are fully water-soluble at all pH-values. The presence of homopolymeric regions, decreasing with increasing amount of acetylated units, seems to favour precipitation by the formation of crystalline regions with high possibility of aligning the polymer chains. This could explain the increase in solubility with increasing F_A as observed by Vårum *et al.* (1994) and the neutral solubility of chitosans with intermediate F_A-values (Sannan, 1976).

Periodate cleaves the C2-C3 linkages in the GlcN units forming the corresponding dialdehyde. Chitosans become only partially oxidised during oxidation with periodate, reaching degrees of oxidation around 0.5 when oxidised with excess periodate. Chitosans become severely depolymerised during the oxidation, and the large overconsumption of periodate can probably be explained by the consumption of periodate at the increasing amount of end groups (Vold *et al.*, 2004). The depolymerisation decreases with increasing F_A. Polysaccharides oxidised with periodate have earlier been shown to have novel functionalities. Periodate oxidised starch is an example where periodate oxidation is used to obtain new properties in industrial polysaccharides (Veelaert *et al.*, 1997). Periodate oxidation of alginates has been shown to increase the chain flexibility (Smidsrød and Painter, 1973; Lee *et al.*, 2002) and the oxidation is also expected to modify the solubility.

In this work periodate oxidised chitosans of different chemical compositions and different degrees of oxidation have been produced, and the water-solubility as a function of pH has been explored. The chain stiffness of the oxidised and unoxidised chitosans of different chemical compositions have been investigated using size-

exclusion chromatography combined with multi angle laser light-scattering and viscosity detectors, SEC-MALLS-VISC.

The combination of SEC with multiple detectors has several advantages over classical approaches such as 'stand-alone' light scattering or viscometry. In addition to the experimental simplicity allowing multiple, automated analyses, it combines fractionation and analysis. 'Slice-by-slice' processing of polydisperse samples provides both the R_G-M relationship (provided R_G is sufficiently large, generally > $\lambda/20$) and the [η]-M relationships. Another advantage is that particles, aggregates or other inhomogeneities may be excluded from the data analysis provided they elute in a confined region. If the data are to be compared with results from static measurements some additional criteria must be met. First, the columns should separate effectively across the entire chain length distribution, and avoid material eluting in the void volume or overlapping with the salt peak, allowing complete sample recovery. For linear polymers such as chitosans it is possible to adjust the molecular weight distribution by partial depolymerisation to fit a selected column set. Secondly, sample loadings must be appropriately determined to avoid overloading effects (viscous tunnelling) but allowing the best possible signal-to-noise ratio, which may be low in the low molecular weight tail. Interdetector volumes must be accurately determined, and band-broadening arising from multiple detectors should be corrected for, although these effects are usually only important for samples of low polydispersity.

SEC combined with light scattering and viscosity detectors has previously been used to study solution properties of polysaccharides such as pectins (Fishman *et al.*, 2003), cereal β-glucans (Gomez et al, 1997a-b), hyaluronan (Mendichi *et al.*, 2003), viilinan (Higashimura *et al.*, 2000), and the method is therefore well established.

2. Experimental

Chitosans:

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). The chitosan with F_A = 0.52 was prepared by homogenous deacetylation. Chitosans with F_A = 0.05, F_A = 0.16, F_A = 0.44 were prepared by heterogeneous deacetylation, whereas the chitosans with F_A = 0.52 and F_A = 0.64 were prepared by homogeneous deacetylation. The fraction of acetylated units (F_A) was determined by ^{1}H NMR spectroscopy (Vårum *et al.*, 1991) and intrinsic viscosities were measured according to Draget *et al.* (1992).

Chitosans with a specific molecular weight were prepared by nitrous acid depolymerisation and subsequent reduction with $NaBH_4$ (Vårum *et al.*, 1994) or by acid hydrolysis (Vårum *et al.*, 2001).

Preparation of oxidised chitosans:

The oxidised chitosans were prepared as descried previously (Vold and Christensen, 2004)

Determination of fraction of oxidised units, F_{ox}:

The degree of oxidation was determined by measuring the amount of ammonia liberated and from the content of carbon and nitrogen in the periodate oxidised chitosans as determined with a Carlo Erba Elemental Analyzer NA 1500, as described by Vold and Christensen (2004).

SEC-MALLS-VISC:

SEC-MALLS was performed as described by Fredheim *et al.* (2003).

The order of the detectors in the SEC-MALLS.VISC system was first MALLS, then RI and finally the viscometer (Viscotek model 301).

Solubility:

Equal volumes of a chitosan solution (10 mg/ml) in 0.1 M NaCl and a solution containing a certain amount of NaOH were mixed. The pH-value obtained was determined after leaving the solution for 30 min, and thereafter it was centrifuged. The concentration of dissolved chitosan was determined by the ninhydrin method (Prochazkova *et al.*, 1999).

3. Results and discussion

Chain stiffness of chitosans

The chain stiffness of chitosans with different F_A and M_w was studied using size-exclusion chromatography combined with light-scattering and viscosity detectors providing M-[η] and M–R_G relationships subject to further analysis. A typical chromatogram, including the elution profile, log M, log [η] and log R_G versus volume is given in figure 1. A significant advantage of this method is that it gives M and [η] directly for each elution slice, where each slice can be considered monodisperse. In this way no correction for polydispersity is necessary, and the relation between M and [η], and between R_G and M, can be obtained from one single polydisperse sample.

The exponent a in the MHKS equation was determined from double logarithmic plots of [η] versus M_w. Some of the plots for chitosans with different F_A ranging from $F_A = 0.05$ to $F_A = 0.64$ are given in figure 2. All the plots indicated straight lines, and the curves for chitosans with different F_A coinside. Calculated a-values are given in table 1, and shows that at the conditions used chitosans have a-values of about 0.78. Exponents, a', from the double logarithmic plots of R_G versus M_w are also included in table 1, with an average value of 0.56. The determined exponents correspond to values previously reported (Berth and Dautzenberg, 2002). The determined exponents indicate flexible to

stiff chains, and the results suggest that the chain conformation in chitosans is independent of F_A.

From the molecular weight and intrinsic viscosity data, the persistence length, q, can be calculated using the Bohdanecky procedure (1983). Bohdanecky developed a simplified method to treat the intrinsic viscosity data for stiff-chain polymers, and showed that the Yamakawa-Fujii theory for wormlike chains can be approximated with the equation:

$$\left(\frac{M^2}{[\eta]}\right)^{1/3} = A_\eta + B_\eta M^{1/2} \qquad (1)$$

where

$$A_\eta = A_0 \cdot M_L \cdot \Phi_{0,\infty}^{-1/3} \qquad (2)$$

$$B_\eta = B_0 \cdot \Phi_{0,\infty}^{-1/3} \cdot (2q/M_L) \qquad (3)$$

$\Phi_{0,\infty}$ is the limiting value of the Flory viscosity constant, and equals $2.86 \cdot 10^{23}$. A_0 and B_0 are known functions of the reduced hydrodynamic diameter, d_r, and B_0 can be replaced by the mean value (=1.05). q is the persistence length and M_L is the molar mass per unit of contour length.

In this work M_L is estimated by two different methods:

I) Predetermined from structural data, $M_L = M_0/b_0$. M_0 is the monomer weight and b_0 is the monomer length. It is assumed that oxidised units have b_0-values of 0.515 nm (Smidsrød *et al.*, 1973), and for oxidised units b_0 is calculated to 0.424 nm using the Kirkwood-Riseman freely rotating chain model (Kirkwood and Riseman, 1948).

II) M_L estimated from experimental data assuming that the hydrodynamic volume occupied by 1 g of the wormlike cylinder is equal to the partial specific volume; $\nu = (\pi N_A/4)(d^2/M_L)$ and assuming that ν is 0.57 cm^3/g for chitosans (Errington *et al.*,1993; Cölfen *et al.*, 2002).

The Bohdanecky plots of all samples displayed linear regions from which the persistence length could be estimated. The persistence lengths and M_L values obtained for the different chitosans are shown in table 1. The calculated persistence lengths show that at the conditions used the chain stiffness of chitosans is about 7 nm irrespective of F_A.

Chain stiffness of oxidised chitosans

The chain stiffness of periodate oxidised chitosans, subsequently reduced with $NaBH_4$ was also studied using SEC-MALLS-VISC. MHKS plots of oxidised chitosans with F_A = 0.01 are given in figure 3. All the plots indicate relatively straight lines, and a and K values can be determined for the oxidised chitosans. The calculated a-values are given in table 2. The a-values vary between 0.8 and 0.9, suggesting flexible to stiff chains. The figure shows a shift in the linear regions of the curves towards lower [η] at a given M_w with increasing degrees of oxidation. This suggests that the chain stiffness decreases upon oxidation. Similar MHKS-plots were obtained with oxidised chitosans with F_A = 0.16 and F_A = 0.52, and a- values are included in table 2.

Figure 4 shows the Bohdanecky plot of the samples, F_A = 0.01 (P_0 = 0.05, P_0 = 0.10, P_0 = 0.30). The persistence lengths obtained for the different oxidised chitosans are given in figure 5, and are included in table 2 together with M_L values. The figure clearly shows that the persistence lengths decrease with increasing degrees of oxidation, and hence that the chain flexibility increases upon oxidation.

Water-solubility

Most chitosans are insoluble in water at neutral and basic pH-values. Oxidation of chitosan is expected to modify the solubility, due to the cleavage of the C2-C3 linkage in GlcN units. During the oxidation chitosans become depolymerised (Vold and Christensen, 2004). Since the solubility is dependent on the molecular weight (Vårum *et*

al., 1994), unoxidised chitosans of comparable M_w were prepared by nitrous acid degradation.

The solubility of the chitosans and periodate oxidised chitosans as a function of pH is shown in figure 6 ($F_A = 0.01$) and figure 7 ($F_A = 0.16$). The figures clearly show that the solubility of chitosans with approximately constant molecular weight increases with increasing degree of oxidation. In fact, chitosans with a degree of oxidation, $F_{ox} = 0.10$, seems to be completely soluble at all pH-values. Reduction of oxidised chitosans with $NaBH_4$ seems to lead to a slightly decrease in the solubility. However, at pH around 9 a considerable fraction (~ 75%) of chitosans ($F_{ox} = 0.10$) was still soluble. The curves obtained for the undegraded chitosan are in agreement with the curves obtained by Vårum *et al.* (1994) in similar experiments. The solubility at higher pH-values increases with decreasing molecular weights of unoxidised chitosans, but it is clear that the solubility increases more upon periodate oxidation. Chitosans and oxidised chitosans ($P_0 = 0.05$, $P_0 = 0.30$) with F_A 0.52 were completely soluble at all pH-values (results not shown).

It was noted that if the samples with the highest molecular weight were left for longer times (up to 48 h) before centrifugation, much sharper precipitation curves were obtained. On the other hand, if samples with lower chitosan concentration (1 mg/ml) were used, the apparent precipitation curves could be shifted to higher pH-values (≈ 0.5 pH-values). After 48 hours, however, the precipitation curves were similar for the two concentrations and sharper than the curves after 30 minutes. Equilibrium is probably not reached after 30 minutes for the high molecular weight chitosans, and the precipitation seems to be slower as the chitosan concentration decreases. It should be noted that for the periodate oxidised chitosans and the other chitosans the dependence on concentration was not that pronounced, if observed at all.

Periodate oxidised chitosan contains fewer amino groups than unoxidised chitosans, which should suggest a slightly lower solubility of periodate oxidised chitosans. The introduction of unoxidised units in the chain will, however, decrease the presence of homopolymeric regions in the chitosan chains, thereby decreasing the possibility for

formation of crystalline regions, and hence increasing the solubility of oxidised chitosans. This is also suggested to be the reason for the neutral solubility of chitosans with F_A around 0.5 (Sannan *et al.*, 1976). The increased solubility of the oxidised chitosans may also be partly caused by an increased chain flexibility, leading to a higher value of ΔS_m (difference in entropy between the crystalline and the dissolved states). This would lead to a lower melting temperature, T_m (= $\Delta H_m/\Delta S_m$), and hence increased solubility (Smidsrød and Christensen, 1991).

The fact that the reduced oxidised chitosans are less soluble than the unreduced oxidised chitosans are difficult to explain. Sloan *et al.* (1954) reported that dialdehyde dextrans were more difficult to dissolve in water than the original dextrans. Also periodate oxidised starches showed decreasing solubility in solvents commonly used for starches (Sloan *et al*., 1956). Veelaert *et al.* (1994), however showed that the water absorption of periodate oxidised starches increased with increasing degree of oxidation, reaching a maximum at a degree of oxidation of 0.4-0.5, followed by a decrease in the water absorption. This decrease is attributed to the introduction of hydrophobic aldehyde groups with inherent hemiacetal formation and eventually crosslinks. Periodate oxidised celluloses are insoluble in water, but becomes more/partially water-soluble after reduction of its aldehyde groups to primary alcohols (Casu *et al.*, 1985; Rahn and Heinze, 1998). The insolubility has been ascribed to hemiacetal formation of aldehyde with hydroxyl groups in the cellulose chain. Any regularity in the sequence of monomers that leads to stronger interactions between chains (increasing ΔH_m) will increase the melting temperature, T_m (=$\Delta H_m/\Delta S_m$), and hence reduce solubility (Smidsrød and Christensen, 1991). This should apparantly also lead to an increased solubility of reduced oxidised chitosans as compared to oxidised chitosans. Smidsrød *et al.* (1973) showed that there were no significant differences in the chain stiffness of reduced and non-reduced oxidised alginates. However, figure 1 clearly shows that the reduced oxidised chitosans were less soluble than the reduced samples. The reason for this remains to be explained.

4. Conclusion

The persistence length of chitosans in 0.2 M NH_4Ac, pH 4.5, was determined to be about 7 nm, irrespective of F_A.

The persistence length, and hence the chain stiffness, of chitosans decreases with increasing degree of oxidation.

The water-solubility of periodate oxidised chitosans with F_A = 0.01 and F_A = 0.16 increases significantly with increasing degrees of oxidation. Chitosans with F_{ox} = 0.10 is apparently completely soluble up to pH 10. After reduction of the oxidised chitosans the solubility decreases. However, at pH 9 a considerable fraction (~ 75%) of reduced oxidised chitosans (F_{ox} = 0.10) is still soluble. Chitosans and periodate oxidised chitosans with F_A = 0.52 was soluble at all pH –values.

Acknowledgements

This work was financially supported by The Research Council of Norway.
Ann-Sissel Ulset and Mildrid Myhr are thanked for skilful technical assistance.

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Figure captions:

Figure 1. Typical size-exclusion chromatogram (SEC) of chitosan including the elution profile, log M, log [η] and log R_G versus volume.

Figure 2. MHKS plot of chitosans with F_A 0.05, F_A 0.16, F_A 0.44 and F_A 0.64 at ionic strength of 0.2 M.

Figure 3. MHKS plots (log-log plots of the intrinsic viscosity versus the weight-average molecular weight) of partially oxidised chitosans with degree of oxidation 0 (upper curve), 0.02, 0.04, 0.09 and 0.16 (lower curve) at 0.2 M ionic strength.

Figure 4. Bohdanecky plot for chitosans with F_A = 0.01, F_{ox} = 0.02 (◇), F_{ox} = 0.04 (●) and F_{ox} = 0.09 (Δ).

Figure 5. The persistent lengths of periodate oxidised chitosans with F_A = 0.01 (◆), F_A = 0.16 (□) and F_A = 0.52 (Δ) as a function of the degree of oxidation at an ionic strength, I = 0.2 M.

Figure 6. Precipitation of chitosans and periodate oxidised chitosans with F_A = 0.01 with sodium hydroxide. M_w = 300,000 (□), M_w = 60,000 (■),M_w = 16,000 (Δ), F_{ox} = 0.02, M_w = 50,000 (◇), F_{ox} = 0.04, M_w = 20,000 (●), F_{ox} = 0.09 reduced with $NaBH_4$, M_w =8,000 (◆) and F_{ox} = 0.09, M_w = 11,000 (o).

Figure 7. Figure 3.7 Precipitation of chitosans and periodate oxidised chitosans with F_A = 0.16 with sodium hydroxide. M_w = 300,000 (Δ), M_w = 110,000 (◆), M_w = 20,000 (□), F_{ox} = 0.03, M_w = 80,000 (●), F_{ox} = 0.10, M_w = 14,000 (o).

Table 1. Weight-averages of molecular weight, M_w, intrinsic viscosity, [η], and R_G, exponents a and a' derived from the [η]-M and R_G-M relations, persistence lengths,q, calculated with M_L (structurally determined)^{I} and M_L (experimentally estimated)^{II} at an ionic strength of 0.2 M.

Table 2. Weight-average molecular weight, M_w, intrinsic viscosity, [η], a derived from the MHKS-plot, persistence lengths,q, calculated with M_L (structurally determined)^{I} and M_L (experimentally estimated)^{II} at an ionic strength of 0.2 M for oxidised chitosans.

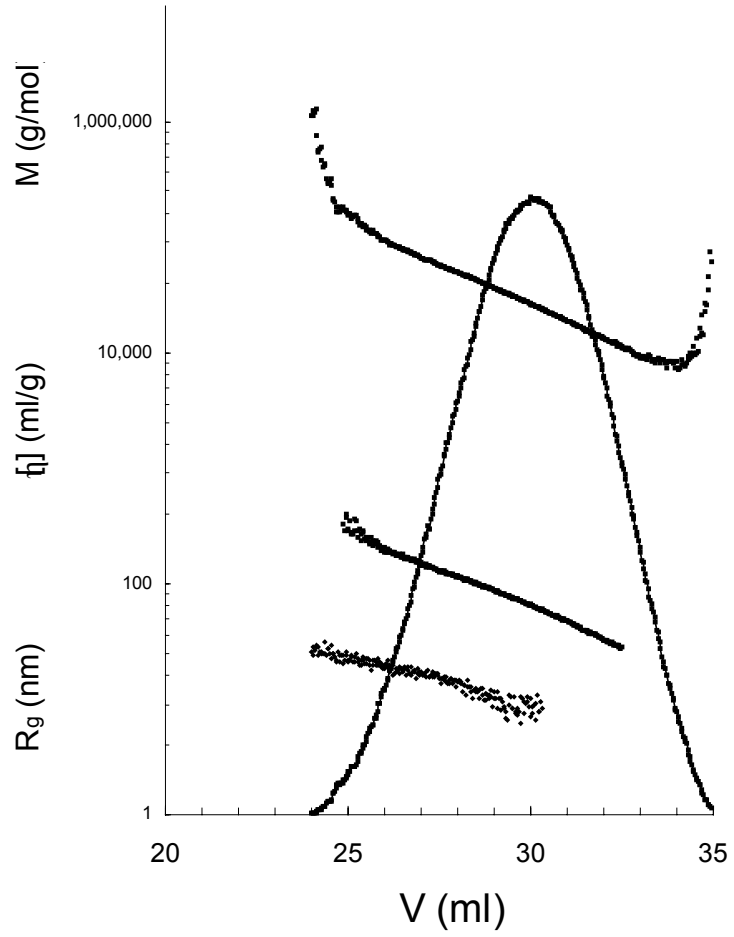

Figure 1

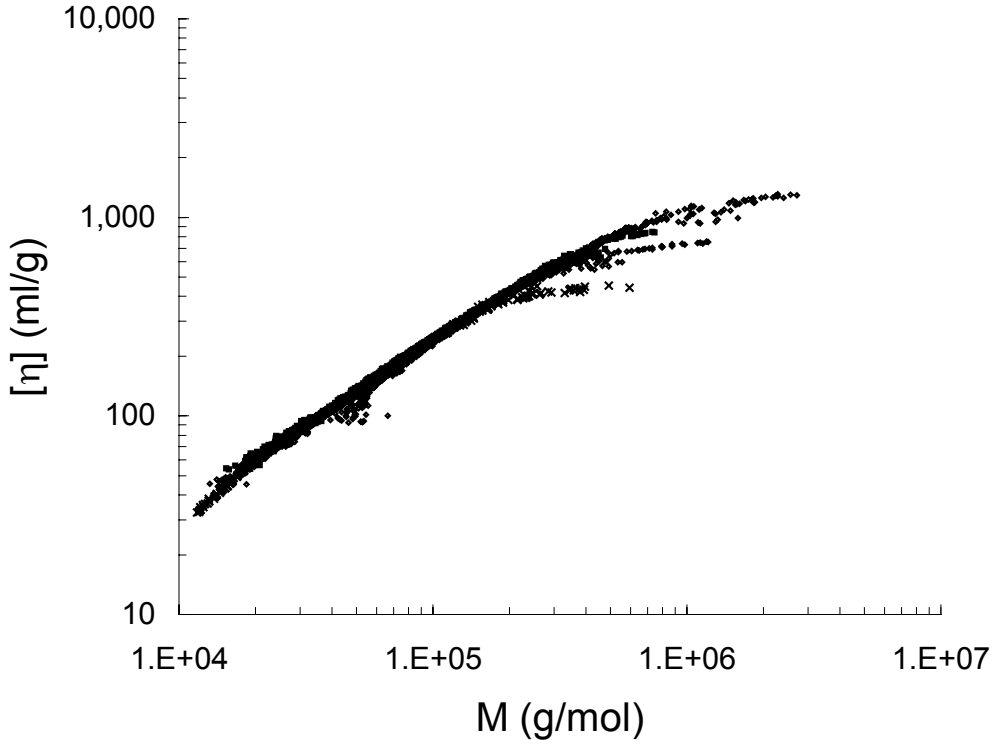

Figure 2

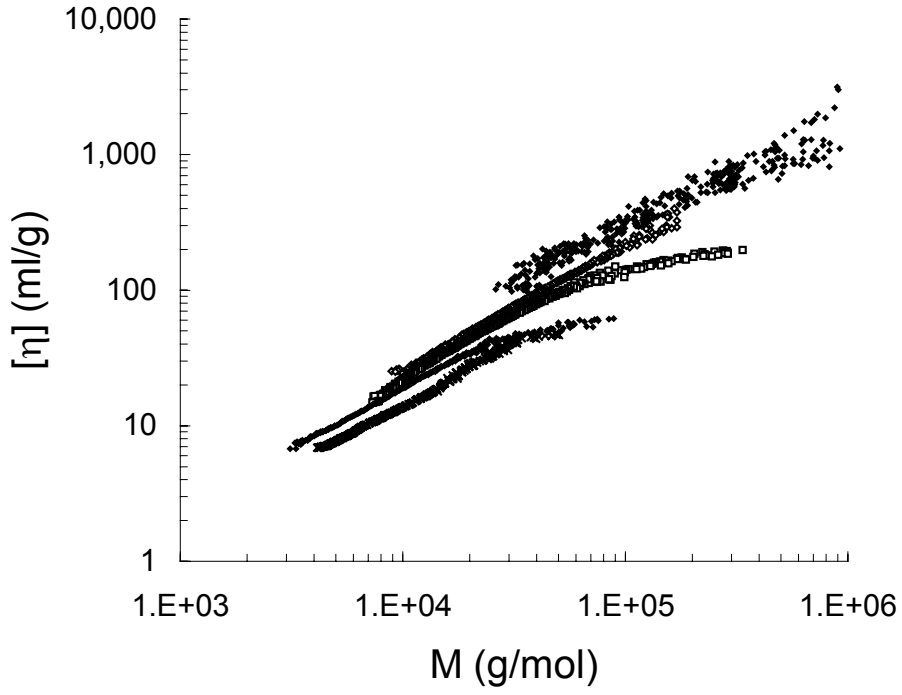

Figure 3

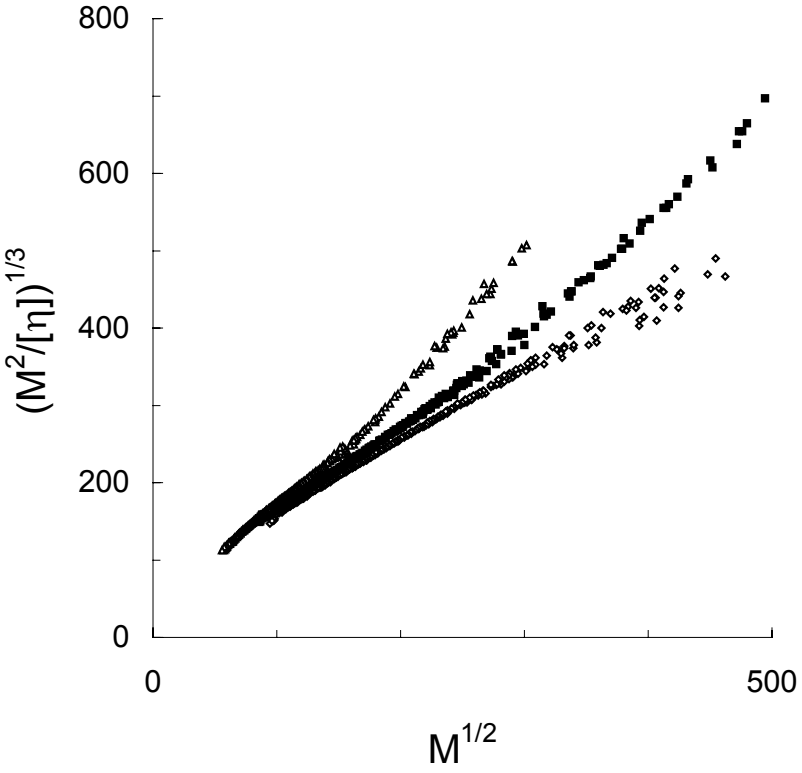

Figure 4

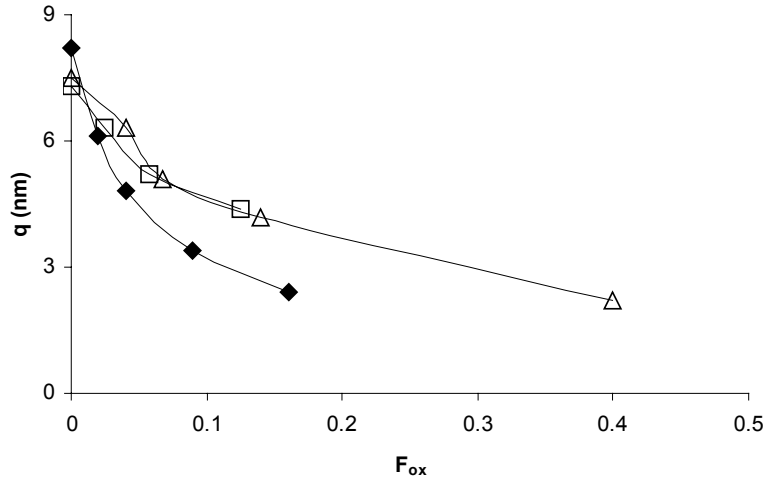

Figure 5

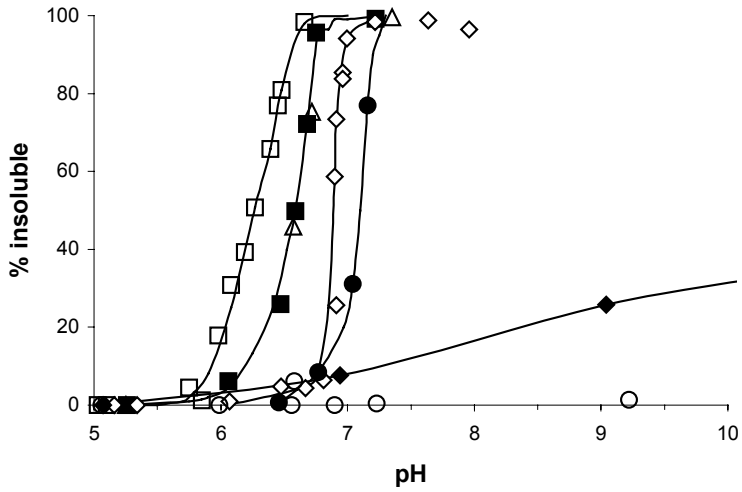

Figure 6

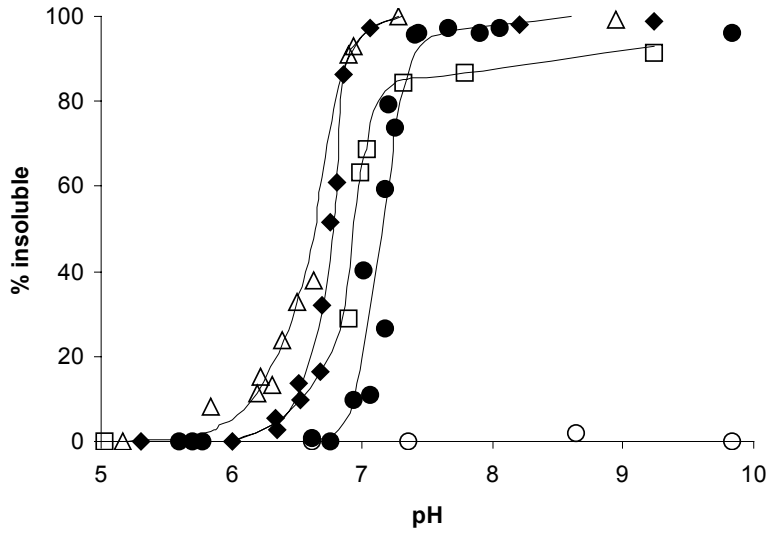

Figure 7

Table 1

F_A	M_w (g/mol)	$[\eta]_w$ (ml/g)	$(R_G)_w$ (nm)	a	a'	q^I (nm)	$M_L{}^I$ (nm^{-1})	q^{II} (nm)	$M_L{}^{II}$ (nm^{-1})
0.05	270,000	535	44	0.73	0.56	7.4	427	6.3	363
	40,000	103		0.83		6.5	427	6.2	407
0.16	290,000	539	45	0.72	0.54	7.5	424	7.5	423
	250,000	533	42	0.70	0.59	7.4	424	6.2	356
	170,000	353	29	0.70	0.56	7.3	424	6.9	405
	110,000	261	23	0.76	0.56	6.8	424	5.7	355
	90,000	210	24	0.81	0.56	7.5	424	8.4	473
	40,000	98		0.88		6.9	424	7.4	455
0.44	220,000	414	38	0.75	0.55	7.2	414	8.0	459
	30,000	74		0.83		5.6	414	5.1	376
0.64	450,000	813	64	0.70	0.56	7.5	407	8.0	434
	110,000	257	26	0.79	0.55	6.8	407	6.9	413
	30,000	77		0.88		6.4	407	6.6	418
avg.				0.78	0.56	7.0		6.8	

Table 2

F_A	F_{ox}	M_w (g/mol)	$[\eta]_w$ (ml/g)	a	q^I (nm)	$M_L{}^I$ (nm^{-1})	q^{II} (nm)	$M_L{}^{II}$ (nm^{-1})
0.01	0	189,000	840	0.77	8.2	429	6.2	329
	0.02	33,000	108	0.92	6.1	428	5.9	416
	0.04	21,000	58	0.91	4.8	428	4.2	380
	0.09	8,000	18	0.87	3.4	425	2.9	360
	0.16	7,000	9	0.87	2.4	423	2.3	403
0.16	0.00	363,000	779	0.79	7.3	424	7.4	424
	0.03	50,000	156	0.87	6.3	423	6.5	432
	0.06	31,000	84	0.87	5.2	422	5.0	401
	0.13	12,000	31	0.97	4.6	420	3.8	351
0.52	0.00	330,000	746	0.75	7.5	411	8.2	447
	0.04	99,000	259	0.73	6.3	410	6.3	412
	0.07	79,000	166	0.78	5.1	410	5.1	409
	0.14	33,000	63	0.87	4.2	408	4.1	401
	0.40	9,000	17	0.82	2.2	403	2.0	358

Paper III

Preliminary study on the chemical structure of periodate oxidised chitosans

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Abstract

The structure of periodate oxidised chitosans have been investigated by NMR-spectroscopy techniques. The ^{1}H NMR spectra and ^{13}C NMR spectra of chitosans with F_{ox} = 0.10, show insignificant deviations from the NMR-spectra of ordinary chitosans. ^{1}H NMR spectra were also obtained from oligomers collected after separation of periodate oxidised chitosan by size exclusion chromatography. The spectrum of the dimer diverge to some extent from the spectra of trimer to decamer, which are virtually similar to each other. However, all the spectra are very complex, and assigning the peaks are difficult. To get specific information concerning the structure of the oligomers, the isolated fractions should be further separated, and complementary techniques like 2D NMR, elemental analysis and mass spectrometry should be used.

Introduction

Chitosans are linear binary heteropolysaccharides composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN). Periodate oxidation of chitosan have been relatively little explored, with only a few studies on the periodate oxidation reaction and products formed (Jeanloz and Forchielli, 1950; Krysteva *et al.*, 1994; Ohya *et al.*, 1996; Matsamura *et al.*, 1997). A schematical presentation of the periodate oxidation of chitosan is shown in figure 1. Recently, however, Vold and Christensen (2004a-b) studied the periodate oxidation and the physical characterisation of oxidised chitosans more in detail. Chitosans become only partially oxidised when excess periodate is used. It is also shown that the chitosans become severally depolymerised during the oxidation. The depolymerisation increases with decreasing F_A, and it is suggested that a chitosan specific degradation mechanism is involved (Vold and Christensen, 2004a).

The periodate oxidation of chitosan obviously leads to changes in the chemical structure. It is desirable to characterise the chemical structure of the oxidised chitosans in order to get a better understanding of the periodate oxidation of chitosans. A characterisation of the reaction products could also potentially suggest approaches for reducing the severe depolymerisation that accompanies the oxidation.

The cleavage of C2-C3 in GlcN units leads to the formation of a dialdehyde. These dialdehydes are capable of existing in a variety of forms, and in solution, equilibrium occur between the various forms. In water they may exist as a hydrated, acyclic aldehyde, hemiacetals or hemialdals or as combinations of these. In polysaccharides these links may be intermolecular as well as intramolecular (Guthrie, 1961; Perlin, 1980). The oxidation of the reducing end is quite complex, as shown in figure 2. This results in numerous possible structures, especially after partial oxidation where the amount of periodate is limited.

Experimental

Chitosans:

F_A=0.01, [η] = 800 ml/g, prepared by further deacetylation of a commercial chitosan provided by FMC Biopolymer (Drammen, Norway). The fraction of acetylated units (F_A) was determined by ^{1}H NMR spectroscopy (Vårum *et al.*, 1991) and intrinsic viscosities were measured according to Draget *et al.*(1992).

The fraction of oxidised units, F_{ox}, was determined by measuring the amount of ammonia liberated per unit GlcN, N_t, as described by Vold and Christensen (2004a).

Size-exclusion chromatography (SEC):

Periodate oxidised chitosans were separated on three columns in series, packed with Superdex^{TM}30, from Amersham Pharmacia Biotech (overall dimensions 2.60 x 180 cm). The column was eluted with 0.15 M ammonium acetate, pH 4.5 at a flow rate of 0.8 ml/min. The effluent was monitored with an online refractive index (RI) detector (Shimadzu RID 6A), coupled to a datalogger.

NMR:

The samples were dissolved in D_2O and the pD was adjusted to 3 - 4. The deuterium resonance was used as a field-frequency lock. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance DPX 300 or 400 spectrometer at 90˚C.

Results and Discussion

NMR spectroscopy is extensively used to investigate chemical structure in polysaccharides, and has also been used in studies of periodate oxidised polysaccharides (Hirano *et al.*, 1974; Drobchenko *et al*., 1996; Maekawa, 1991; Kim *et al.*, 2000). In this work NMR-spectroscopy has been used to investigate the structure of periodate oxidised chitosans, and to explore the possibility of determining the degree of oxidation from NMR data.

^{1}H NMR spectroscopy and ^{13}C NMR spectra of chitosans with F_A = 0.01, F_{ox} = 0.10 (reduced and non-reduced) did not show significant deviations from the spectra of unoxidised chitosans. ^{1}H NMR spectrum of the reduced sample is shown in figure 3. No peaks were observed at chemical shifts characteristic for aldehydes (around 10 ppm). This suggests that all the aldehydes present are hydrated or exist as hemiacetals or hemialdals, as observed previously by various techniques in other periodate oxidised polysaccharides (Hirano *et al.*, 1974; Drobchenko *et al*., 1996; Kim *et al.*, 2000). ^{13}C NMR spectroscopy has been suggested as an alternative way for determination of the degree of oxidation in oxidised celluloses, where signals due to unoxidised D-glucose residues are easily identified (Casu, 1985; Painter, 1988). A ^{13}C NMR spectrum of the reduced sample is shown in figure 4. It is unclear why the spectra of oxidised chitosan, as distinct from cellulose spectra, show no deviation from the spectrum of an unoxidised chitosan.

To further investigate the chemical structure, periodate oxidised chitosans were fractionated by size exclusion chromatography (SEC). The chromatogram from SEC of chitosan F_A = 0.01 with four different F_{ox} are given in figure 5. GlcN standards (monomer, dimer, trimer, tetramer and hexamer) are included in the figure. The SEC chromatogram further demonstrates the large depolymerisation that is accompanied with the oxidation (Vold and Christensen, 2004a). The separation between the oligomers was not complete, and the oligomers isolated contained mixtures of different structures as bimodal peaks were observed and no baseline separation was obtained.

Selected collected oligomers (F_{ox} = 0.16) were studied by ^{1}H NMR spectroscopy. A spectrum of the dimer is shown in figure 6. The figure illustrates the very complex spectra that are obtained. The spectra of oligomers from trimer up to decamer diverged to some extent from the dimer spectrum, but were apparently similar to each other. The spectrum of the trimer is shown in figure 7. It is very difficult to assign the peaks observed in these complex spectra. To get information about the structure of the oligomers, they should be further fractionated by e.g. other chromatographic techniques such as ion exchange chromatography. 2D NMR-techniques, elemental analysis and mass spectrometry could also give additional information. However, even after further separation the interpretation of the data would be a difficult task due to the many possible structures, especially at the reducing end.

Some observations in the spectra should, however, be mentioned. The dimer spectrum shows a peak around 9.7 ppm. In this region aldehydes are the most probable structures, and this suggests that free aldehydes exist in the dimer. This peak is not present in the spectra of larger oligomers. Peaks at around 8.2 ppm, are also present. This could suggest the presence of formyl esters (Perlin, 1964; Mackie and Perlin, 1965), suggesting that not all the formylesters at the reducing ends are hydrolysed, as also suggested by the low FA_t values (moles formaldehyde released/mole GlcN) obtained in previous experiments (Vold and Christensen, 2004a). Formic acid also appears around 8.25 ppm (Hirano, 1974). Formic acid could possibly be released upon heating of the solution. Veelaert *et al.* (1997), however, reported that the dialdehyde starch structure was preserved when heated at low pH (3-4) and 90°C.

Acknowledgements

This work was financially supported by The Research Council of Norway.
Audun Sørbotten is thanked for technical assistance.

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Figure captions

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

Figure 2. Periodate oxidation of the reducing end (modified figure from Dryhurst, 1970).

Figure 3. ^{1}H NMR spectrum (400 MHz) of a periodate oxidised chitosan with $F_A = 0.01$ and $F_{ox} = 0.10$, reduced with $NaBH_4$

Figure 4. ^{13}C NMR spectrum (100 MHz) of a periodate oxidised chitosan with $F_A = 0.01$ and $F_{ox} = 0.10$, reduced with $NaBH_4$

Figure 5. SEC-separation of partially periodate oxidised chitosan; $F_{ox} = 0.07$ (lowest dimer peak), $F_{ox} = 0.16$ (second lowest dimer peak), $F_{ox} = 0.30$ (second highest dimer peak) and $F_{ox} = 0.40$ (highest dimer peak). GlcN standards (monomer, dimer, trimer, tetramer and hexamer) are included in the figure.

Figure 6. ^{1}H NMR spectrum (300 MHz) of a dimer collected from a sample of a periodate oxidised chitosan with $F_A = 0.01$ and $F_{ox} = 0.16$.

Figure 7. ^{1}H NMR spectrum of a trimer (300 MHz) collected from a sample of a periodate oxidised chitosan with $F_A = 0.01$ and $F_{ox} = 0.16$.

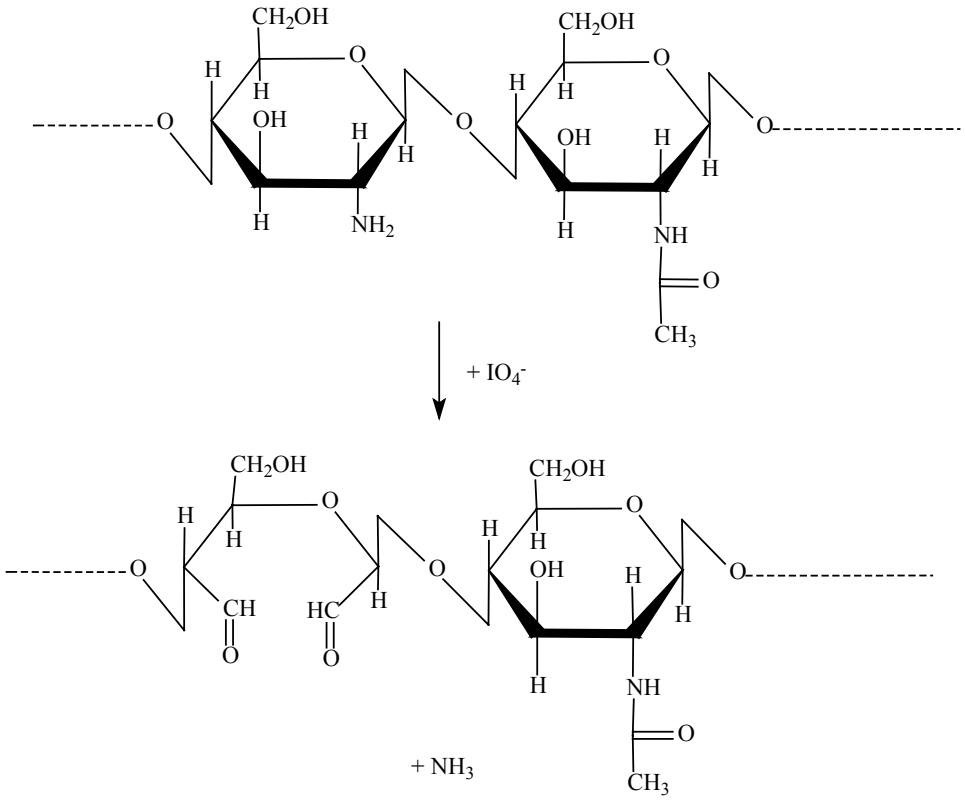
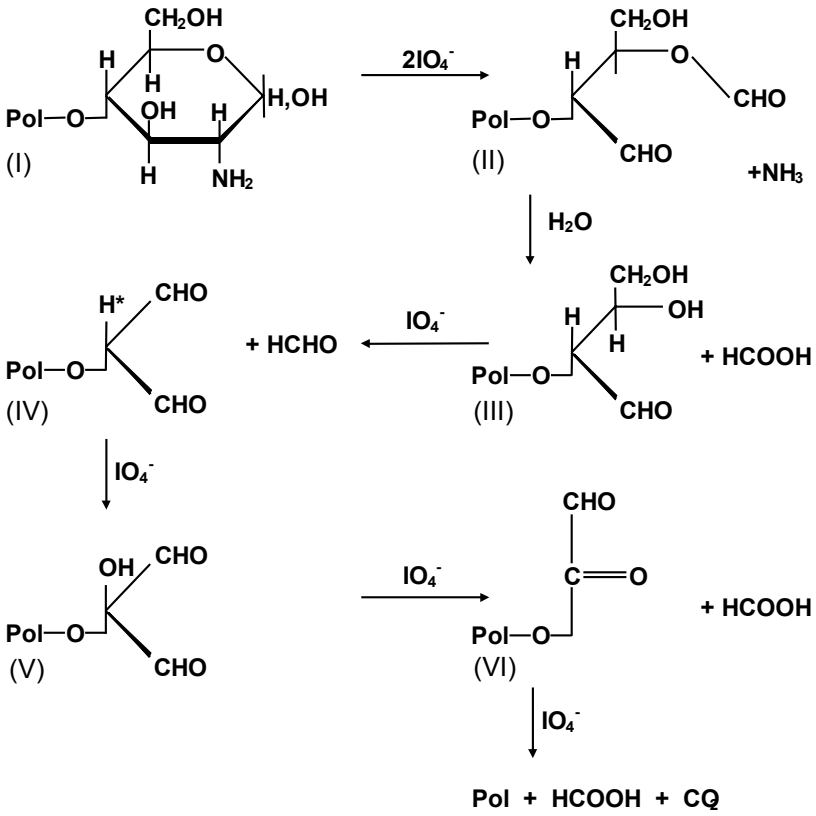


Figure 1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(II)
+NH3
H2O
CH2OH
OH
H
H
Pol—O
CHO
(III)
IO4-
+ HCHO
+ HCOOH
H*
CHO
Pol—O
CHO
(IV)
IO4-
OH
CHO
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Pol + HCOOH + CO2

Figure 2

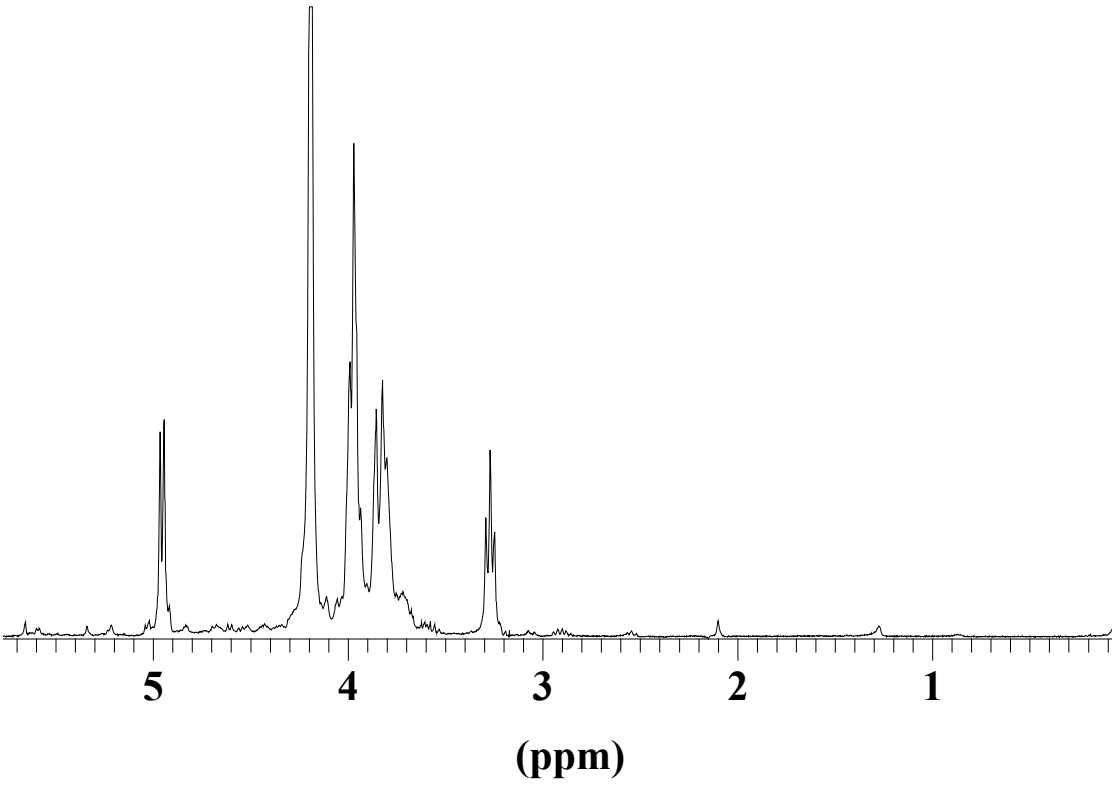

Figure 3

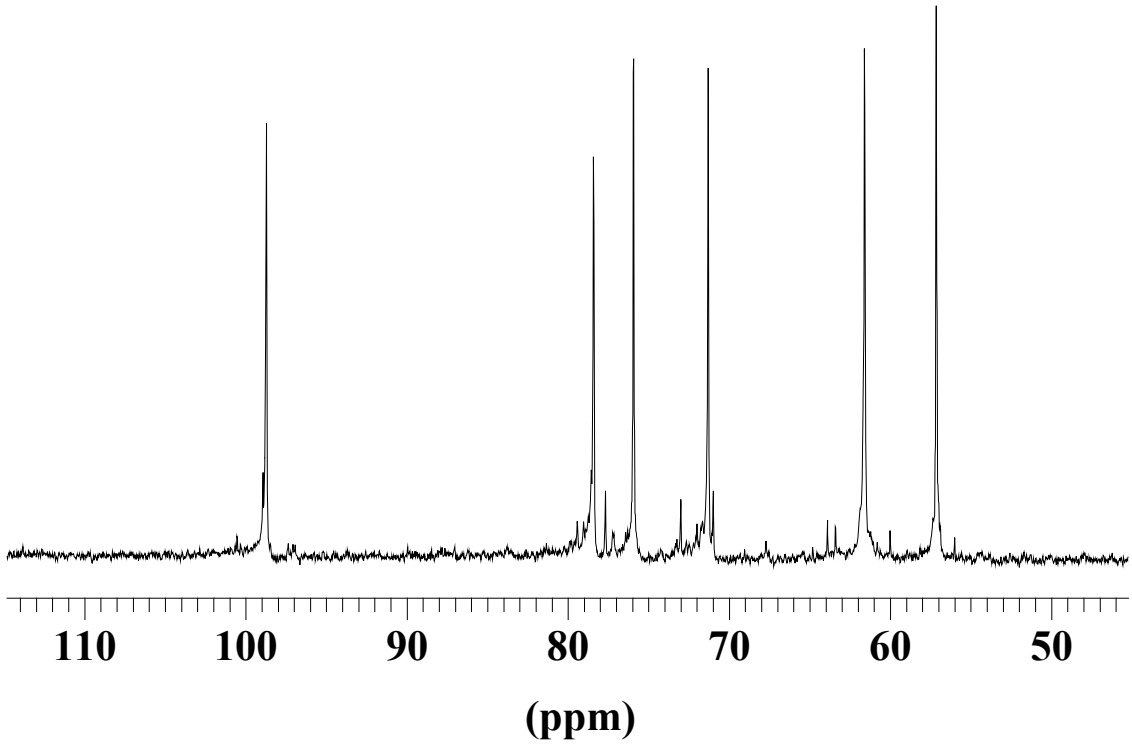

Figure 4

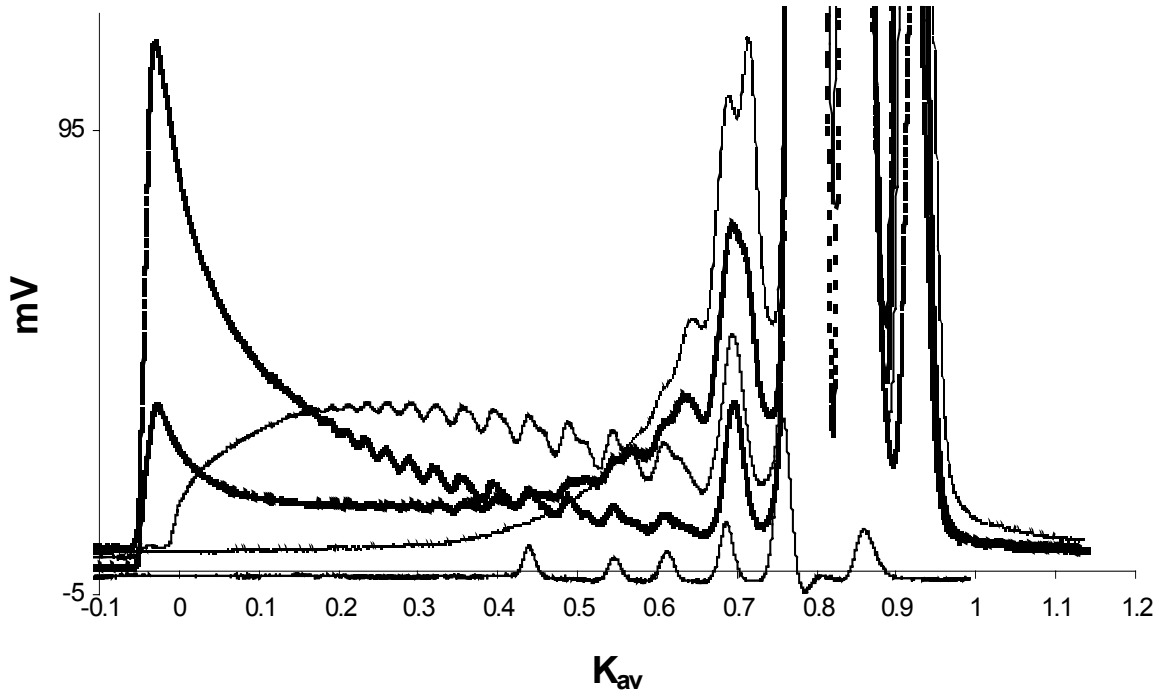

Figure 5

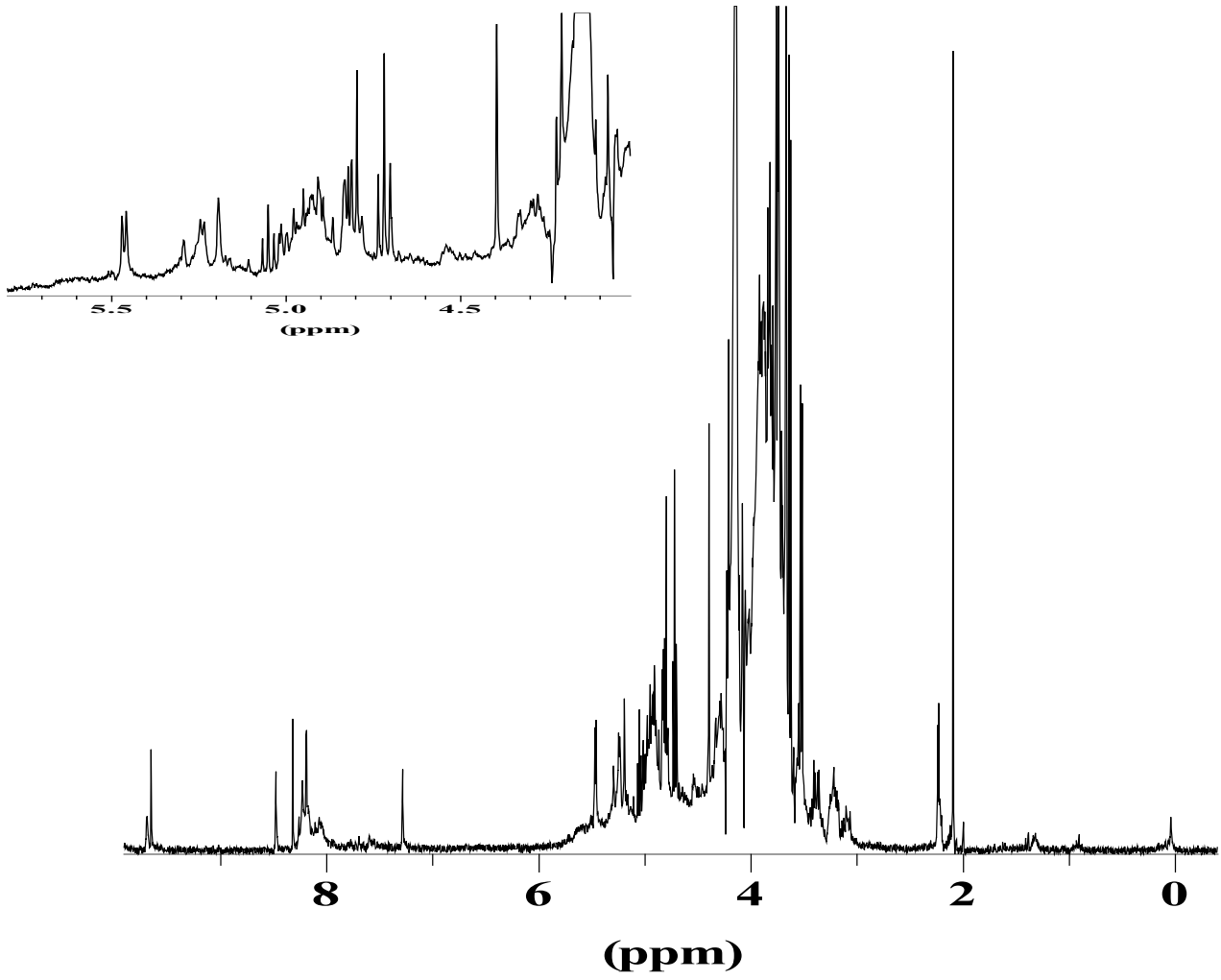

Figure 6

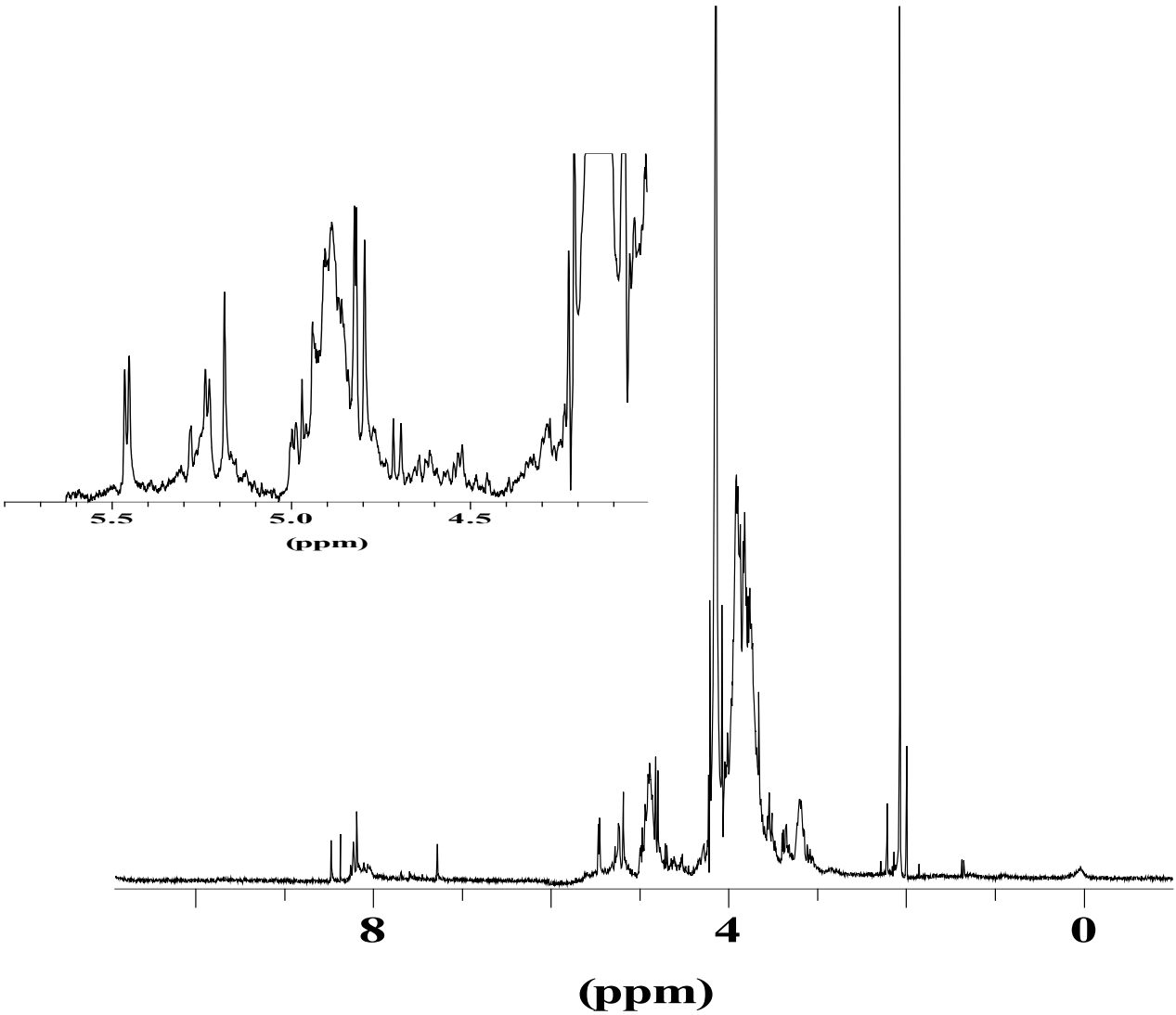

Figure 7

Paper IV

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Carbohydrate Polymers 54 (2003) 471–477
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Binding of ions to chitosan—selectivity studies

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Received 13 March 2003; revised 14 July 2003; accepted 19 July 2003

Abstract

Selectivity coefficients for binding of negative and positive ions to chitosans of different chemical composition have been determined by equilibrium dialysis. Chitosans with different fraction of acetylated units (F_A of 0.01 and 0.49) generally behaved similarly in their selectivity towards both negative and positive ions. No selectivity was found in the binding of chloride and nitrate ions, while chitosan showed a strong selectivity towards molybdate polyoxyanions, with selectivity coefficients around 100. Chitosan showed a strong selectivity towards copper (Cu^{2+}) compared to the metal ions zinc (Zn^{2+}), cadmium (Cd^{2+}) and nickel (Ni^{2+}), with selectivity coefficients from 10 to 1000, while little or no selectivity could be detected with the other metal ions. Ionic strength and pH did not influence the selectivity coefficients of the chitosans towards the metal ions.

Keywords: Chitosan; Metal ion; Selectivity coefficient

1. Introduction

Chitosan may be considered as a family of linear binary copolymers of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN). It is produced from chitin, one of the most abundant biopolymers in nature. Chitosans have a very diverse range of established and potential applications. The great potential of chitosans is more or less related to its poly-cationic properties, which are unique among abundant polysaccharides and natural polymers in general.

It is well known that chitosans may complex with certain metal ions (Muzzarelli, 1977). Possible applications of the metal binding property are waste water treatment for heavy metal and radio isotope removal with valuable metal recovery, and potable water purification for reduction of unwanted metals (Onsøyen & Skaugrud, 1990). Another well-documented application of chitosan is as a cholesterol-lowering agent (Ormrod, Holmes, & Miller, 1998), and the much more controversial use as a weight reducing agent (Ernst & Pittler, 1998). It is important to investigate the competitive binding of essential metal ions to chitosan in order to judge if chitosan could be harmful to humans consuming chitosan.

The binding mechanism of metal ions to chitosan is not yet fully understood. Various processes such as adsorption, ion exchange, and chelation are discussed as the mechanisms responsible for complex formation between chitosan and metal ions. The type of interaction depends on the metal ion, its chemistry and the pH of the solution (Guibal, Milot, & Roussy, 2000; Inoue, Baba, & Yoshizuka, 1993). Metal anions (e.g. molybdate) can be bound to chitosan by electrostatic attraction. It is likely that the chitosan–metal cation complex formation occurs primarily through the amine groups functioning as ligands (Roberts, 1992). The competitive binding of two ions, e.g. Cu^{2+} and Ni^{2+}, at pH-values around the pK_a-value of chitosan may formally be expressed by the equations:

$$-NH_3^+ + Cu^{2+} \leftrightarrow -NH_2Cu^{2+} + H^+ \quad (1)$$

$$-NH_3^+ + Ni^{2+} \leftrightarrow -NH_2Ni^{2+} + H^+ \quad (2)$$

(1) − (2) gives:

$$Cu^{2+} + -NH_2Ni^{2+} \leftrightarrow Ni^{2+} + -NH_2Cu^{2+} \quad (3)$$

It is seen that any selectively binding of metal cations is independent of pH according to Eq. (3) in the reaction

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doi:10.1016/j.carbpol.2003.07.001

scheme above. The reaction schemes indicate that the formation of a metal complex at pH-values around the pK_a-value of chitosan must be accompanied by the release of protons, as also pointed out earlier (Domard, 1987; Koshijima, Tanaka, Muraki, Yamada, & Yaku, 1973; Piron & Domard, 1998; Tsezos, 1983).

Different theories exist concerning the structure of the chitosan–metal ion complex. Some experiments support the theory that two or more amino groups from one chain bind to the same metal ion; the bridge model (Blair & Ho, 1980; Focher, Massoli, Torri, Gervasine, & Morazzoni, 1986). Other studies indicate that only one amino group is involved in the binding, and that the metal ion is bound to the amino group as a pendant; the pendant model (Domard, 1987; Piron & Domard, 1998). Domard (1987) found that only one type of complex was made, and suggested the structure to be $[CuNH_2(OH)_2]$. It seems that the most probable structure is the one where the metal ion is bound to the amino group in the chitosan as a pendant (Roberts, 1992). Nevertheless, the variety of the mechanisms of chelation and the possibility of ion exchange makes the situation even more complex. Recently Rhazi et al. (2002a) suggested that two different complexes exist, dependent on pH. ($[Cu(-NH_2)]^{2+}$, $2OH^-$, H_2O) is the more stable for pH-values between 5 and 5.8, and ($[Cu(-NH_2)_2]^{2+}$, $2OH^-$) is more stable at pH-values larger than 5.8.

Many parameters will affect chitosans ability to complex metal ions and the stability of the metal–chitosan complex. One of the major parameter in the complexation seems to be the fraction of acetyled units, F_A (Guibal, Dambies, Milot, & Roussy, 1999; Kurita, Sannan, & Iwakura, 1979). The chain length is also an important parameter; Rhazi et al. (2002a) have demonstrated that a degree of polymerisation of six appears as the threshold value for an efficient complexation of copper ions by chitosan oligomers. Various ways of mixing and the physical state of the chitosan will also influence the metal ion capacity of chitosan (Rhazi et al., 2002a). In addition external factors as pH and ionic strength will affect the complexation of metal ions to chitosan.

The aim of most of the studies in this field has been to determine whether or not chitosan will complex with a given ion, determine the amount of ion that can be bound, or to understand the process involved. Not many studies have involved determination of the selectivity of binding of different metal ions to chitosan, however, this aspect has recently been studied. It is of great importance to determine the selectivity of chitosans towards metal ions. Rhazi et al. (2002b) determined the following order in the selectivity from mixtures of ions; $Cu(II) \geq Hg(II) > Zn(II) > Cd(II) > Ni(II) > Co(II)$,Ca(II), using potentiometric and spectrophotometric methods, and showed that the selectivity was independent of the physical form of chitosan.

The aim of this study has been to develop methods to quantify the binding of different ions to chitosan through the determination of selectivity coefficients, and to determine the influence of pH, ionic strength and F_A on the selectivity coefficients.

2. Experimental

The following chitosan samples were used in this study: $F_A = 0.01$, $[\eta] = 800$ ml/g ($M_n = 250,000$), $F_A = 0.49$, $[\eta] = 1270$ ml/g ($M_n = 290,000$). The chitosan with $F_A = 0.01$ was prepared by further deacetylation of a commercial chitosan provided by Pronova Biopolymer (Drammen, Norway), while the chitosan with $F_A = 0.49$ was prepared by homogeneous deacetylation. The fraction of acetylated units (F_A) was determined by ^{1}H NMR spectroscopy (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991) and intrinsic viscosities were measured according to Dragnet, Vårum, moen, Gynnild, & Smidsrød (1992a). The number-average molecular weights (M_n) were calculated from the Mark–Houwink–Sakurada (MHS) equation using a calibration against chitosan samples with known M_n (from osmometry) and intrinsic viscosity. The coefficients K and a in the MHS equation used in the calibration were calculated from the relationships given by Anthonsen, Vårum, and Smidsrød (1993).

The selectivity of chitosan in different binary mixtures of ions was determined as described below. The chitosans were dissolved in deionised water of Milli-Q grade (1% (w/v)), and adjusted to the actual pH.

2.1. Chloride/nitrate

Three millilitre (or 6 ml) of the chitosan solution was enclosed in small dialysis-bags (Inf Dia 18/32″–14.3 mm, MWCO 12–14,000 Da), and dialysed towards 3×50 ml^2 solutions of the mixed anions at pH 4.5, using $NaNO_3$ and NaCl in a total concentration of 0.2 M. The ratio of the two competitive anions was varied. The solutions were exchanged three times, to ensure that the ion concentrations at equilibrium in the dialysate were the same as the predetermined ratio. The chitosan solutions were then dialysed exhaustively against deionised water adjusted to pH 4.5 to remove all unbound salts. Then the chitosan solutions were dialysed three times against 0.2 M NaOH to displace the bound ions, and the resultant final three dialysates were combined and analysed. The chloride ions were analysed by complex titration with mercury(II)nitrate using diphenylcarbazone as indicator (Clarke, 1950; Førland, 1989; Meites, 1963). Nitrate was analysed by UV spectroscopy after reduction of nitrate to nitrite followed by a diazotiation reaction (Hellebust & Craigie, 1978; Strickland & Parsons, 1972).

2.2. Molybdate/chloride and molybdate/nitrate

Six millilitre of the chitosan solutions were enclosed in small dialysis-bags, and dialysed towards 3×50 ml^2

solutions of the mixed anions adjusted to pH 5, using MoO_3 and NaCl or $NaNO_3$ in a total concentration of 0.2 M. The experiments were performed as described above. Molybdate ions were analysed by atomic absorption spectroscopy using a Varian SpectrAA 400. Chloride and nitrate ions were analysed as described above.

2.3. Cu^{2+}/Cd^{2+}, Cu^{2+}/Ni^{2+}, Cu^{2+}/Zn^{2+}, Ni^{2+}/Cd^{2+}, Zn^{2+}/Cd^{2+}, Zn^{2+}/Ni^{2+}

Three millilitre of the chitosan solutions were enclosed in small dialysis-bags, and dialysed towards 3×50 ml[2] solutions of the mixed metal ions at a total concentration of 0.1 M (or towards 3×100 ml solutions at a total concentration of 0.05 M) adjusted to the actual pH. Metal sulphates, metal chlorides or metal nitrates were used. The ratio of the two competitive ions was varied. The metal ion solutions were kept at a pH lower than 4.7 to avoid copper hydroxide precipitation. The solutions were exchanged three times, to ensure that the ion concentrations at equilibrium in the solution phase were according to the predetermined ratio. The chitosan solutions were then dialysed exhaustively against deionised water to remove all unbound salts. Then the chitosan solutions were dialysed three times against 0.2 M hydrochloric acid to displace the bound ions, and the resultant final three dialysates were combined and analysed. The ionic strength of the metal ion solutions was varied by addition of sodium sulphate. Copper, cadmium, nickel, and zinc were analysed by atomic absorption spectroscopy using a Perkin–Elmer 560 Atomic Absorption Spectrophotometer.

3. Results and discussion

To quantify the binding of anions to chitosan, a selectivity coefficient, k_A^B, was defined in the ion-exchange equilibrium.

$$\text{Chitosan-A} + \text{B} \leftrightarrow \text{Chitosan-B} + \text{A}$$

by the equation:

$$k_A^B = \frac{X_B \cdot C_A}{X_A \cdot C_B}$$

where A and B represent the two anions, X_A and X_B is the mole fraction of ions bound to the chitosan ($X_A + X_B = 1$), and C_A and C_B are the molar concentrations of ions in solution at equilibrium. This definition of the selectivity coefficient has been widely used in ion binding studies on alginate (Haug & Smidsrød, 1970; Smidsrød & Haug, 1968, 1972). In systems concerning metal cations and chitosan, the sorption probably occurs by complexation rather than ion-exchange. The definition of the selectivity coefficient given above will nevertheless be a measure of the selectivity in the system, regardless of the sorption mechanism, and this definition will be used throughout the paper.

3.1. Selectivity of chitosan for anions

3.1.1. Binary mixtures of chloride and nitrate

It is practical to prepare chitosan salts (e.g. chitosan chloride and chitosan nitrate), as these salts, as opposed to chitosan in its free amine form, are directly soluble in water. Chitosan chlorides are widely used also commercially, and we initially tested the selectivity of chitosan towards chloride and nitrate. The experiments were performed using equilibrium dialysis as previously described to determine selectivity coefficients for alginates (Smidsrød & Haug, 1968). Starting with a 1% chitosan chloride solution in the dialysis bag, the chitosan was dialysed against solutions with varying amounts of chloride to nitrate (sodium salts) to obtain the same concentration of the anions inside the dialysis bag as on the outside. The chitosan was thereafter dialysed extensively against deionised water, and then against 0.2 M NaOH to remove the ions bound to chitosan. The amounts of chloride and nitrate were determined in the final combined dialysates. The selectivity of chitosans towards nitrate and chloride ions was investigated for two different chitosans of different chemical composition ($F_A = 0.01$ and 0.49) in binary salt solutions of constant ionic strength, but varying composition of the two ions. Fig. 1 shows the calculated selectivity coefficients in the exchange reaction between chloride and nitrate at different ionic composition for the two chitosans. The selectivity coefficients are around one, meaning that chitosans show no selectivity towards chloride or nitrate ions. In the experiment the total amount of ions bound to the chitosans was nearly constant at the different ionic composition, with around 0.8 mol ion bound per mol GlcN. The pH in the dialysate increased during the dialysis against the sodium salts. During dialysis with deionised water the pH in the dialysate decreased while the pH inside the dialysis bag increased, probably caused by the Donnan equilibrium.

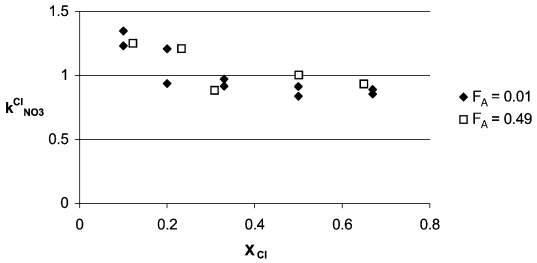

Fig. 1. Selectivity coefficients of chitosans ($F_A = 0.01$ and 0.49) in the exchange reaction between chloride and nitrate as a function of the ionic composition in the bound anion fraction at pH 4.5. The fraction of chloride in the exchange solution varies from 0.1 to 0.7.

3.1.2. Binary mixtures of molybdate/chloride and molybdate/nitrate

In earlier experiments (Draget et al., 1992a; Draget, Vårum, & Smidsrød, 1992b) cross-linking of chitosan with Mo(VI) polyoxyanions has been used to prepare chitosan gels. The selectivity in binary systems of molybdate and nitrate ions, and molybdate and chloride ions was investigated for two chitosans of different chemical composition ($F_A = 0.01$ and 0.49) in salt solutions with constant ionic strength, but different ionic composition in the binary salt solution. Fig. 2 shows the selectivity coefficients for chitosans in the binding of molybdate and chloride, and molybdate and nitrate, and it is clearly seen that both chitosans show strong selectivity for binding of molybdate, with selectivity coefficients around 100. However, the evaluation of the results is complicated by the fact that the molybdate solution is very complex with different composition of polyoxyanions at different pH and molybdate concentrations (Tytko, Baethe, Hirschfeld, Mehmke, & Stellhorn, 1983). In addition the analysis of chloride and nitrate ions at high molybdate concentrations were complicated by the fact that the molybdate interfered with the chloride and nitrate analysis. It is, however, obvious that chitosans can bind molybdate ions in the presence of a large excess of chloride or nitrate ions. The selectivity coefficients increased considerably when the system changed from chitosan in solution to precipitated chitosan (from the point $X_{Mo} \approx 0.5$ to $X_{Mo} = 1$), suggesting some inter-chain binding as in the binding of Ca^{2+} to alginate (Smidsrød & Haug, 1968). The chitosans with F_A of 0.01 and 0.49 show no differences in the value of the selectivity coefficients. This supports the hypothesis that the increased gel strength of chitosan molybdate gels formed with chitosans of higher F_A is caused by interactions between the acetyl groups (Draget, 1996). The molar ratio between amino groups and molybdenum has been determined. At high metal concentration, this ratio exceeds one (between 1.3 and 1.9). This confirms that molybdate is adsorbed in the form of polynuclear species. It is consistent with previous results (Draget et al., 1992a).

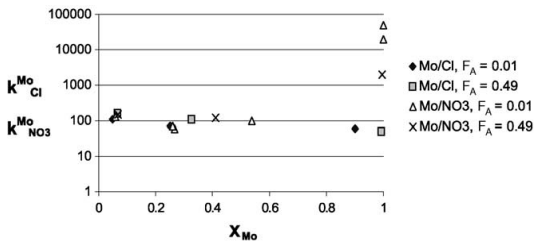

Fig. 2. Selectivity coefficients of chitosans ($F_A = 0.01$ and 0.49) in the exchange reaction between molybdate and chloride, and molybdate and nitrate as a function of the ionic composition in the bound ion fraction at pH 5.0. The fraction of molybdate in the exchange solution varies from 0.001 to 0.1.

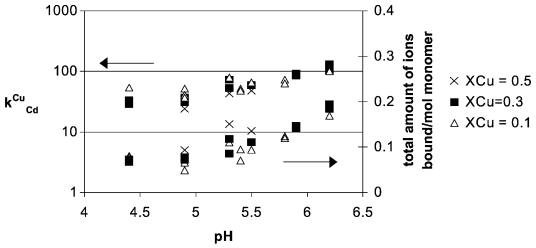

Fig. 3. The influence of pH on the selectivity coefficient in the Cu^{2+}/Cd^{2+} exchange reaction (upper points) and on the total amount Cu^{2+} and Cd^{2+} ions bound to the chitosan per mol GlcN (lower points). The selectivity experiments are carried out at three different ionic compositions of Cu^{2+} and Cd^{2+}.

Guibal et al. (2000) also found that molybdate in the form of heptamolybdate species was sorbed at pH 3, reaching sorption capacities as high as 7–8 mmol Mo/g.

3.2. Selectivity in the binding of metal cations to chitosan

It has previously been shown that pH is important in the binding of metal ions to chitosan, and we have tested the influence of pH and ionic strength on the chitosan selectivity coefficient between Cu^{2+} and Cd^{2+}, k^{Cu}_{Cd}. Also, the total amount of ions bound to the chitosan was determined as a function of pH and ionic strength. When the dialysis bags containing dissolved chitosan chloride were immersed in solutions of Cu/Cd sulphate the interaction of the ion with chitosan was accompanied by the appearance of a blue colour and precipitation of the chitosan. The pH in the dialysis bags was found to decrease during dialysis towards metal solution, indicating that the formation of the complex was accompanied by the release of protons.

Figs. 3 and 4 show the influence of pH and ionic strength, respectively, on the selectivity coefficient of chitosan ($F_A = 0.01$) towards Cu^{2+} and Cd^{2+}, k^{Cu}_{Cd} (upper points)

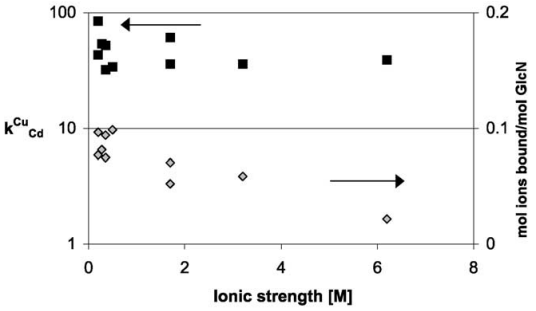

Fig. 4. The influence of ionic strength on the selectivity coefficient in the Cu^{2+}/Cd^{2+} exchange reaction (upper points) and on the total amount Cu^{2+} and Cd^{2+} ions bound to the chitosan per mol GlcN (lower points). The selectivity experiments are carried out at pH 5.3 with a fraction of copper, $X_{Cu} = 0.1$, in the exchange solution.

and on the total amount Cu^{2+} and Cd^{2+} ions bound to the chitosan per mol GlcN (lower points).

Fig. 3 suggests that the selectivity coefficient increases slightly with increasing pH. According to Eq. (3), the selectivity should be independent of pH. The slight increase in the selectivity coefficients could be due to some kind of cooperativity or interchain binding. Results from Juang and Shao (2002) on binary systems of Cu^{2+}/Ni^{2+} and Cu^{2+}/Zn^{2+} using cross-linked chitosan indicate on the other hand that the selectivity coefficient increased up to a pH range maximum and then decreased. The optimum pH-range was determined to 5.1–5.3 for binary mixtures of Cu^{2+} and Ni^{2+}, and 4.5–4.9 for binary mixtures of Cu^{2+} and Zn^{2+}. The discrepancy between their result and the present data is not understood. Fig. 3 also shows that the total amount of metal ions bound to the chitosan increased with increasing pH, in agreement with previous results (Becker, Schlaak, & Strasdeit, 2000; Dzul Erosa, Saucedo Medina, Navarro Mendoza, Avila Rodriguez, & Guibal, 2001; Juang and Shao, 2002; Koshijima et al., 1973). The increase in the capacity of the chitosan at higher pH-values can be explained by the fact that at low pH the metal ions compete with protons for binding sites, as the reaction schemes in the introduction indicate. Moreover, the increased protonation of the amine groups at lower pH-values also induce long-range electrostatic repulsion of metal ions. Both these effects will favour binding of metal ions at high pH-values, far above the pK_a-value of the amino group of chitosan. Conversely binding will be impossible at pH-values far below the pK_a-value of the amino groups.

Fig. 4 suggests that the selectivity coefficient decrease slightly with increasing ionic strength. However, no dramatic changes in the selectivity coefficient or the amount of metal ion bound was found up to 0.5 M ionic strength, and even at an 1.7 M ionic strength the selectivity coefficient remained high. At low ionic strength a small increase in the ionic strength seems to lead to an increasing amount of ions bound to the chitosan. This is consistent with the findings of Mitani, Nakajima, Sungkono, and Ishii (1995), who found that the removal efficiencies of cobalt and nickel by swollen chitosan beads increased with increasing ionic strength from 5 to 25 mM. This could be

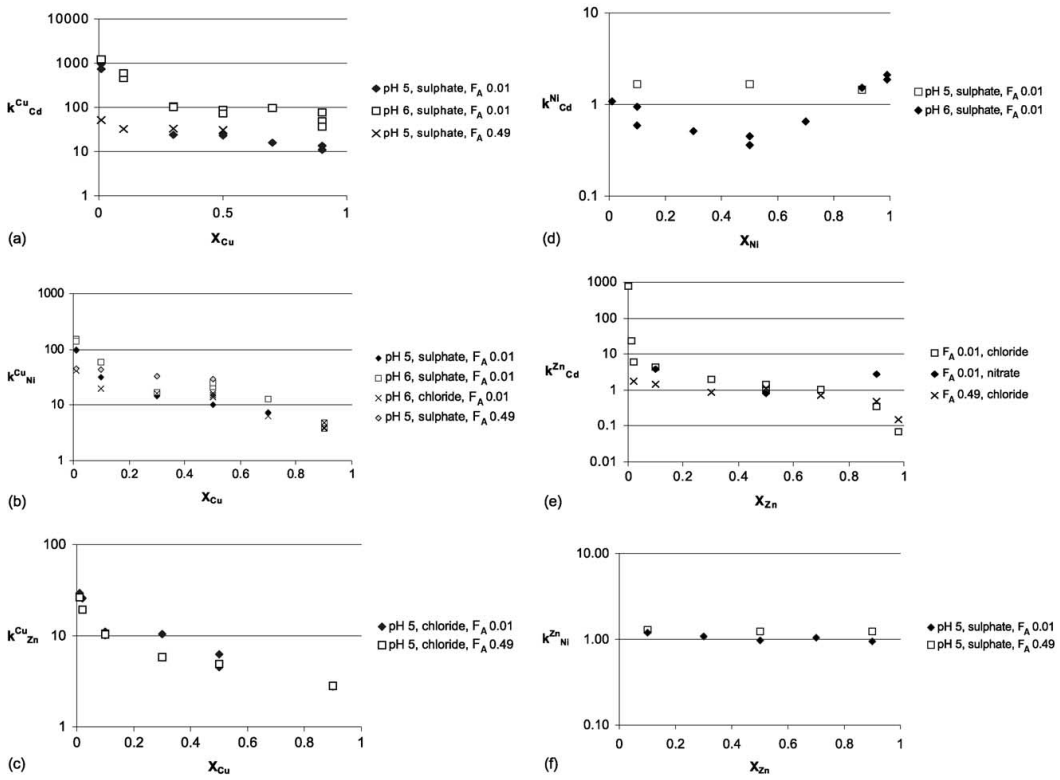

Fig. 5. Selectivity coefficients in the exchange reaction between (a) Cu^{2+} and Cd^{2+} as sulphate salts at pH 5 and pH 6; (b) Cu^{2+} and Ni^{2+} as sulphate and chloride salts at pH 5 and pH 6; (c) Cu^{2+} and Zn^{2+} as chloride salts at pH 5; (d) Ni^{2+} and Cd^{2+} as sulphate salts at pH 5 and pH 6; (e) Zn^{2+} and Cd^{2+} as chloride and nitrate salts at pH 5; (f) Zn^{2+} and Ni^{2+} as sulphate salts at pH 5, as a function of the ionic composition in the exchange solution for chitosans with $F_A = 0.01$ and 0.49.

caused by screening of the long-range electrostatic repulsion between the positively charged metal ions and the positively charged chitosan. Fig. 4 shows that when the ionic strength is further increased, the amount of ions bound to chitosan decreases.

The selectivity coefficients of chitosans ($F_A = 0.01$ and $F_A = 0.49$) towards the metal ions Cu^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+} were investigated by studying the selectivity coefficients in binary systems: (a) Cu^{2+} and Cd^{2+}, (b) Cu^{2+} and Ni^{2+}, (c) Cu^{2+} and Zn^{2+}, (d) Ni^{2+} and Cd^{2+}, (e) Zn^{2+} and Cd^{2+}, (f) Zn^{2+} and Ni^{2+} as a function of the ionic composition of the metal solution, as shown in Fig. 5(a)–(f).

Cu^{2+} ions were found to bind selectively to the chitosans in the presence of Ni^{2+}, Zn^{2+}, and Cd^{2+} ions. The highest selectivity coefficients were determined for Cu/Cd at pH 6, with $k_{Cd}^{Cu}-$ values between 100 and 1000 with $X_{Cu} < 0.7$. The selectivity of chitosans for Cu^{2+} was confirmed by the fact that the total uptake capacity of chitosan increased when the fraction of Cu(II) in the solution increased. This is shown in Fig. 6(a) for the Cu–Cd system. The increasing uptake capacity was most pronounced in the Cu–Cd and Cu–Ni systems (about five times), and less pronounced for the Cu–Zn system. This is in agreement with other results (Bassi & Prasher, 2000; Juang & Shao, 2002; Rhazi et al., 2002b), and it is clear that chitosan selectively adsorb Cu^{2+} from such multi-component solutions.

Fig. 5(d)–(f) indicate that chitosans have no selectivity in the binding of ions in the binary systems Ni^{2+}/Cd^{2+}, Zn^{2+}/Cd^{2+}, and Zn^{2+}/Ni^{2+}. This is in accordance with results from Becker et al. (2000) who investigated the selectivities between chlorides, nitrates and sulphate salts of Cd^{2+}, Ni^{2+} and Zn^{2+}, and found that the metal ion selectivities of chitosan derivatives (including a chitosan derivative made by about 20% cross-linking with glutardialdehyde and subsequent reduction) were low. Our results indicate that in the Zn/Cd and Zn/Ni system the total uptake of ions are independent of the ionic composition in the solution (shown in Fig. 6(b) for the Zn–Ni system), whereas in the Ni/Cd system nickel has a depreciating effect on the sorption capacity.

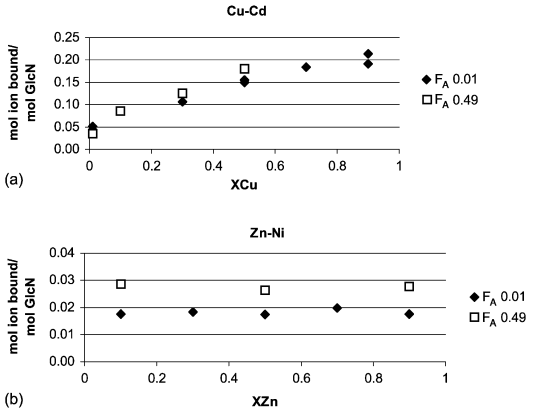

Fig. 6. The total amount of ions bound to the chitosans per mol GlcN in the binary systems (a) $CuSO_4/CdSO_4$, pH 5 and (b) $ZnSO_4/NiSO_4$, pH 5, as a function of the ionic composition in the exchange solution for chitosans with $F_A = 0.01$ and 0.49.

It seems that neither the anion of the metal salt (Fig. 5(b)) nor the chemical composition of the chitosan, F_A, (Fig. 5(a–c,e, f)) influence the selectivity coefficient. The anion in the metal salt does, however, influence the total amount of ions bound to chitosan. Results from experiments with Cu/Cd at pH 4.3 and 5.3 (data not shown) show that sulphate salts bind to a greater extent to chitosan than chloride salts. This result is in agreement with results from other research groups (Becker et al., 2000; McKay, Blair, & Grant, 1987; Mitani, Fukumuro, Yoshimoto, & Ishii, 1991; Mitani et al., 1995). In most cases the total amount of ions bound pr mol GlcN was slightly higher in experiments with chitosan of the high degree of acetylation, $F_A = 0.49$, as compared to chitosan with a very low degree of acetylation, $F_A = 0.01$ (shown for the systems Cu–Cd and Zn–Ni in Fig. 6).

4. Conclusion

No selectivity in the binding of chloride and nitrate ions to chitosan can be determined. Molybdate anions are selectively bound to chitosans in the presence of excess nitrate or chloride ions, with selectivity coefficients around 100. Cu^{2+} ions are bound selectively to chitosan in the presence of Ni^{2+}, Zn^{2+} or Cd^{2+} ions, with selectivity coefficients in the range 10–1000. Chitosans with low and high F_A do not show any difference in their selectivity towards ions. Binding of metal ions increases with pH indicating that only the deprotonised amino groups can bind the ions.

Acknowledgements

This work was financially supported by The Research Council of Norway.

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