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Twenty Years of Chitosan Research in Norway

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Abstract

This short review discusses some of the basic and applied research carried out at NOBIPOL in the last two decades on the chemistry, physical chemistry, biological and technical properties of chitosan. The linear polysaccharide chitin is composed of (1-4) linked 2-acetamido-2deoxy-β-D-glucopyranose units (GlcNAc ; **A**-unit). Chitosans can be considered as a family of water-soluble binary heteropolysaccharides, where the A-units in chitin have been de-*N*-acetylated to varying extents (0-60% **A**-units) thereby introducing positively charged glucosamine units (**D**-unit) in the polymer. Chitosan can thus be regarded as an amphiphilic polymer composed of the charged **D**-units and the more hydrophobic and neutral **A**-units with different chemical, physical and biological properties.

Knowledge of the chemical composition and sequence of chitosans, as determined by NMR-spectroscopy has formed the basis for the study of solution properties, and stability of the molecules. Studies of the effects of the enzyme lysozyme on solutions of chitosans, revealed a large dependence of the degradation rate on the degree of acetylation, F_A. Some structure-function relationships in cell-flocculation, emulsion braking, in drug delivery and gene transfection is also discussed. Very different functionalities of the diverse chitosans were found in each application. Such information is needed for the use of chitosan in established and potential application areas.

In conclusion it is suggested that only a careful and systematic search for optical performance in each potential application can develop the field to its full potential.

Introduction

Some 20 years ago the Norwegian alginate producing company PROTAN (now a part of the USA based FMC Biopolymer) decided to invest into the production of chitosans from Norwegian shrimp and crab shells. Instead of developing the needed technology in-house, PROTAN acquired a chitosan producing plant in Redmond, Washington, which is now owned by an Icelandic Company, Primex. The research group in Trondheim with long experience in working with seaweed polysaccharides like alginates and carrageenans consulted with PROTAN and The Norwegian Research Council to build up Norwegian competence in the field of chitin and chitosans. Since chitosans, like alginate and carrageenans were water soluble binary heteropolysaccharides we saw similar scientific challenges concerning composition, sequence, structure and physical and biological properties to that we had dealt with previously inside the families of seaweed polysaccharides. In 1985 we were asked by PROTAN to arrange the 4th International Conference on Chitin and Chitosan in Trondheim in 1988, and the PhD-student Kjell Morten started on some work that were to be presented as a poster at the Trondheim Conference, (Vårum *et al.*, 1989). Since then, more than 70 international papers on chitin or chitosan have been published from our Institute alone, or in collaboration with international colleges. In addition, 7 PhD-students have so far finished their works on chitosan, as shown in Figure 1. The late Professor Hirano is the first author on two of the international papers, written after a stay at our Institute in the summer of 1997. A long an friendly relationship between us and Professor Hirano was started already in 1988 in Trondheim where he gave an invited plenary lecture, "Production and Application of Chitin and Chitosan in Japan", (Hirano, 1988), and lasted to our last pleasant meeting in Montreal, Canada in August 2003.

1994 **Anthonsen, M.W.** Chitosan, chemical structure and physical properties.

1996 **Hjerde, N.R.J.** Biodegradation of chitosans.

1997 **Ottøy, M.H.** Chemical and physical characterization of chitosans.

1998 **Gåserød, O.** Microcapsules of alginate-chitosan: A study of capsule formation and functional properties.

1999 **Kristiansen, A.** Specific interactions of chitosans with lysozyme and wheat germ agglutinin (WGA).

2001 **Strand, S.P.** Interactions between chitosans and bacteria: flocculation and adhesion.

2002 **Fredheim, G.E.** Macromolecular characterisation of lignosulfonates and their interactions with chitosans.

Figure 1. PhD Theses on Chitosan at NOBIPOL,
NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY,
TRONDHEIM, NORWAY
All Theses are available from Department of Biotechnology, NTNU, 7491 Trondheim, Norway
E-mail: Anne.Bremnes@biotech.ntnu.no

The strategic concept for the research in NOBIPOL has since 1986 been "Polysaccharide Engineering" as visualized in Figure 2.

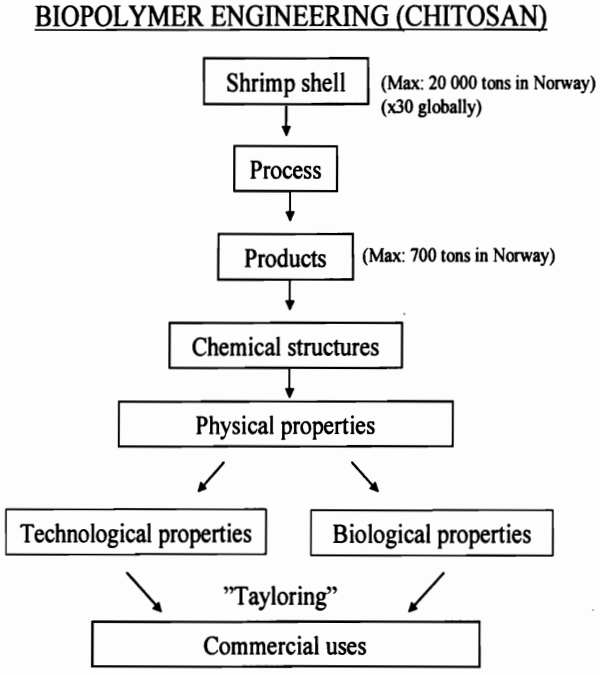

Figure 2. The concept "Biopolymer Engineering" as applied on the chitosan system.

The first task then was to use high-field NMR-spectroscopy, (Vårum *et al.*, 1991a; and Vårum *et al.*, 1991b) to study the composition and sequence of the two monomer residues in chitosans. It was found that for water-soluble chitosans the experimentally determined diad and triad frequencies agreed well with those calculated from the monad fractions assuming a random (Bernoullian) distribution of acetylated (A) and deacetylated (D) units along the chains. Some insoluble material in heterogeneously deacetylated chitosan was later shown to consist of almost pure chitin, (Vårum *et al.*, 1994; and Ottøy *et al.*, 1996), while the soluble material had a compositional distribution consistent with a random distribution of the units along the chains, (Vårum *et al.*, 1994; and Ottøy *et al.*, 1996).

Much data have been presented on the solution properties of chitosans. For a review see, (Berth and Dautzenberg, 2002; Vårum and Smidsrød, 2004). The chitosans seem best to be described as partly free-drained, wormlike chains (extended coils), with an extension depending both on the ionic strength and the degree of acetylation as illustrated in Figure 3.

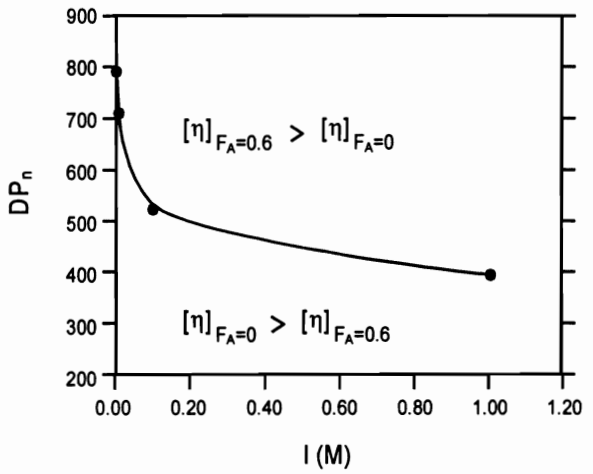

Figure 3. Number average degrees of polymerisation, DPn, at which [η] is equal for chitosans with $F_A = 0.6$ and 0 versus ionic strength.

The enzymatic degradation of chitosans is of great interest for uses in e.g. food, neutraceutical, biomedicine and agriculture. The rate and mechanisms of the degradation of chitosans by lysozyme is of particular interest because of the presence of lysozyme in most animal body fluid. We have shown in a series of papers, (Nordtveit *et al.*, 1994; Nordtveit *et al.*, 1996; Vårum *et al.*, 1997; Hjerde *et al.*, 1997; and Kristiansen *et al.*, 1998), that for soluble chitosans

the initial degradation rate as measured by a viscosimetric assay is proportional to the degree of acetylation, F_A, in approximately the fourth power (as seen in figure 4). The simple reason for this rate dependence is that four acetylated units contained in the active site active cleft of the enzyme is needed to give the highest catalytic efficiency. Knowledge of this specificity is important for understanding of the oligomer composition in a given degradation experiments as visualized in figure 5.

Relative lysozyme degradation rates of chitosans with different F_A

F_A	Relative rate of degradation, r	
0.04	(0.033)	
0.12	1	
0.17	2	
0.27	12	
0.42	44	$\Rightarrow r \propto F_A^4$
0.47	53	
0.51	125	
0.53	169	
0.59	280	
0.60	359	

Figure 4. Relative lysozyme degradation rates of chitosans with different F_A in dilute solutions of substrate.

Dominant degradation products

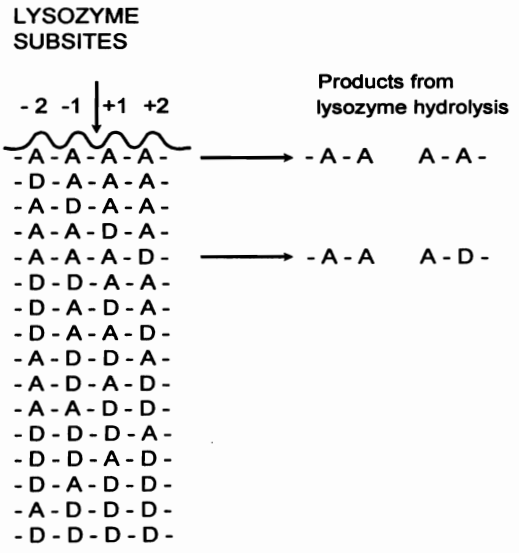

Figure 5. Schematic illustration of the specificity of hydrolysis of lysozyme towards the 16 different tetrade sequences in chitosan. The tetrade sequences are positioned in the active site of lysozyme in such a way that the tetrades are hydrolyzed at the arrow (between subsite -1(D) and +1(E)).

NOBIPOL is still in an early stage in exploring the functionality of various chitosans in the established and potential applications of chitosans, but some work have been done in the area of flocculation and drug and gene delivery to establish structure-function relationships.

As a positively charged amphiphilic polymer chitosans represent an environmental friendly alternative to synthetic polycations for flocculation of harmful solid substances in e.g. drinking water, and harvesting of biological material for further use, such as cell cultures in a fermentation process. (Strand *et al.*, 2001) studied the flocculation of several strains of E. coli as a function of molecular weight and F_A for a series of chitosans. Whereas variations in molecular weight had small effect in a rather large range of molecular weight, the degree of acetylation, and therefore the hydrophobisity of the chitosans had a large impact on their efficiency as a flocculation agent as shown in figure. 6. It is seen that for obtaining a given flocculation efficiency the needed chitosan concentration is more than one order of magnitude lower for a chitosan with F_A = 0.6 than a chitosan with F_A = 0.0. Similar differences have been found for the breaking of oil in water emulsions (Strand, Vårum & Smidsrød, unpublished results).

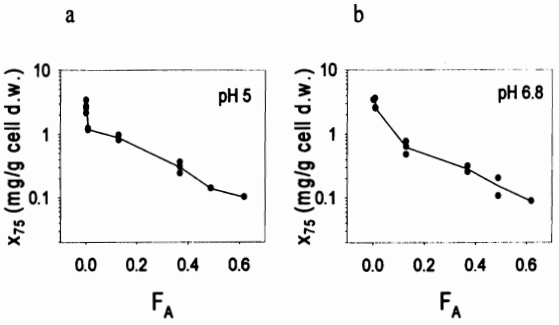

Figure 6. Chitosan concentration at 75% flocculation (x_{75}) of *E.coli* as a function of F_A after 24 hours of sedimentation at pH 5 (a) and 6.8 (b). (From Strand *et al.*, 2001)

Chitosans properties such as bioadhesity, biodegradability and low toxicity are important in drug delivery applications, especially for nasal delivery and delivery to the mucosal layer in the stomach, (Gåserød *et al.*, 1998). Alginate microcapsules covered with chitosan have been shown to be a potential vehicle for such drug delivery.

In vitro studies of the effect of the chemical composition of the chitosan (F_A) and

molecular weight on the uptake of mannitol, as a model of poorly absorbable drugs, using monolayers of human epithelial Caco-2 cells, have been reported, (Schipper *et al.*, 1996), and some results are given in figure 7). The most effective absorption enhancers were chitosans with a low F_A, with low and high molecular weight, as seen in Figure 7a. A chitosan with F_A of 0.35 and relatively high molecular weight was found to have properties that were especially advantageous, but the chitosans were also shown to display a clear dose-dependent toxicity, although at concentrations well below those needed to improve mannitol transport, with lowest toxicity of the chitosans with the highest F_A-values, and little effect of the molecular weight (figure 7b). By choosing the chitosan with F_A of 0.35, effective absorption combined with a low toxicity could be achieved.

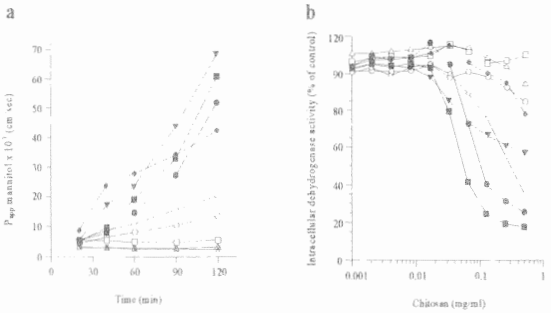

Figure 7. Effects of chitosans of varying F_A on the permeability of mannitol in Caco-2 cell monolayer (a). Effects on intra-cellular dehydrogenase activity (b). ■ F_A = 0.01; M_n = 31 000, ● F_A =0.01; M_n = 170 000, F_A = 0.15; M_n = 4 700, ▼ F_A = 0.15, M_n = 190 000, ▲ F_A = 0.35; M_n = 12 000, ◆ F_A = 0.35; M_n = 17 000, ○ F_A = 0.49; M_n 22 000, ◇ F_A = 0.49; M_n = 98 000. (From Schipper *et al.*, 1996)

Gene transfer carriers of non-viral origin have recently attracted much attention as a method to condense and deliver DNA for expression of the protein in gene therapy. Synthetic polycations, cationic lipids, polylysines, and, recently, chitosans have been successfully used as effective condensation agents for the negatively charged plasmid DNA in gene delivery, (Vårum and Smidsrød, in print). Recently, (Köping-Höggård *et al.*, 2001), studied the ability of chitosans with varying F_A to form physically stable DNA-complexes, and it was found that only chitosans with F_A lower than 0.35 form stable complexes with DNA as shown in Figure 8a.

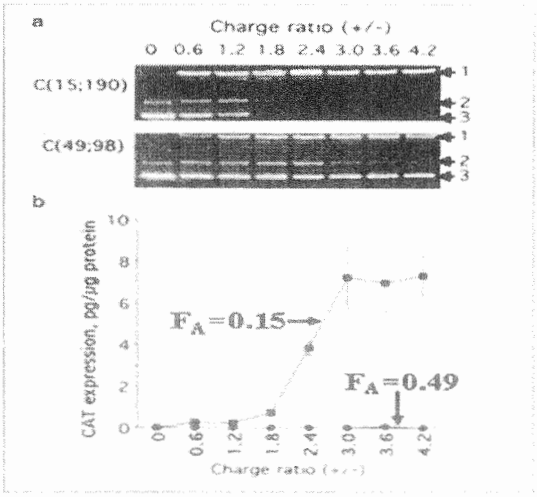

Figure 8. The effect of F_A on (a) the stability of complexes between DNA and chitosan as tested in the agarose gel retardation assay at increasing charge ratios and (B) in vitro transfection efficiency in 293 cells at increasing charge ratios. Arrows indicate (1) loading position, (2) open circle, and (3) supercoiled form of pDNA. C(15;190) is a chitosan with F_A = 0.15 and a molecular weight of 190 000, while C(49;98) is a chitosan with F_A = 0.49 and a molecular weight of 98 000. (From Köping-Höggård *et al.*, 2003)

The ability to form stable complexes was correlated to the *in vitro* transfection efficiency in 293 cells (Figure 8b). Fully deacetylated monodisperse oligomers have also been tested for their efficiency as gene delivery systems, (Köping-Höggård *et al.*, 2003), and it was found that smaller oligomers up to DP14 were ineffective whereas a chitosan with number average degree of polymerization 24 was found to be highly efficient, suggesting that highly deacetylated, low molecular weight chitosans may potentially be used for gene transfection. Moreover, the molecular weight distribution of the fully deacetylated chitosan oligomers were found important for their *in vivo* transfection efficiencies (Köping-Höggård *et al.*, 2004), and it seems that the stability and structure of the chitosan-DNA polyplexes are important parameters to increase transfection efficiencies.

As a conclusion in this short review one could comment that compared to the established commercial application of other marine polysaccharides, such as alginates, carrageenans and agar, the commercial use of chitosans in The Western Countries is still in its infancy, despite an enormous number of suggested applications in published papers. The present authors believe that the potential for the use of chitosans, especially in the biomedical field, is at least comparable with that of the other polysaccharide of marine origin. However, due to the diverse physical and biological properties of chitosans, and their potent

interaction with biological tissues, only a careful and systematic search for optimum performance in each potential can develop the field to its full potential.

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