Thang Trung Khong

Vietnamese chitin raw material, the chitin de-*N*-acetylation reaction, and a new chitosan-alginate gelling concept

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
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Preface

This thesis is submitted to the Norwegian University of Science and Technology (NTNU) for partial fulfilment of the requirements for the degree of philosophiae doctor.

This doctoral work has been performed at the Department of Biotechnology, NTNU, Trondheim, Norway and at the Institute for Biotechnology and Environment, Nha Trang University (NTU), Nha Trang, Vietnam with Professor Kjell Morten Vårum (NTNU) as main supervisor and with co-supervisor, Associate Professor Trang Si Trung (NTU). The work are mainly financed by the Component 3 of the SRV 2701 Project, entitled "*Improving training and research capacity of the Nha Trang University, Vietnam – Phase 2*", funded by the Royal Norwegian Government. The work also receives the financial support from the Norwegian Research Council (NFR) via KMB Project (182695/I40).

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Finally, I would like to express my gratitude to my family, especially my wife and two children for their support. Without their encouragement, I would not have had the chance to come to Trondheim and finish this work.

Thang Trung Khong

Nha Trang, March 2013

Contents

Summary

Chitin is a linear biopolymer composed of 2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylglucosamine or GlcNAc, **A**-unit) linked by β – (1-4) glycosidic linkages. Chitin occurs as a structural polysaccharide in animals with an outer skeleton (Arthropoda), and in the cell wall of certain fungi. In the cuticle of crustaceans and insects, chitin exists in close association with proteins, minerals and pigments. In Vietnam, a country endowed with favorable conditions for aquaculture, the annual shrimp production from aquaculture is approximately 450 000 metric tons (2010), and one third of this is by-products, including head and shell. The two major species are white shrimp (*Penaeus vannamei*) and black tiger shrimp (*Penaeus monodon*). These shrimp by-products are a large resource not only for chitin but also for other valuable components as proteins and pigments.

The chemical composition of heads and shells of the black tiger and the white shrimp was analysed. The amounts of the three main components, i.e. proteins, chitin, and minerals, were found to be similar in the by-products from the two shrimp species. The protein contents of the heads were 44.39 ± 0.50 % and 48.56 ± 1.33 % of the dry weight in the white shrimp and black tiger shrimp, respectively, which were about 50% higher than in the shells. In the shells, the chitin content were 27.37 ± 1.82 % and 29.29 ± 1.78% of the dry weight in the white shrimp and black tiger shrimp, respectively, which were more than 2.5 times higher than in the heads. These large differences in the chemical composition of the heads and the shells had consequences for the optimal extraction conditions in order to isolate a pure and high molecular weight chitin from isolated heads and shells. The amino acid composition of the proteins were similar for the two species, both for heads and shells, and with a profile that was suitable as a source for fish feed.

Chitin is insoluble in aqueous solvents, which limits its applications. However, by partly removing chitin's acetyl groups and thereby introducing amino groups that can be protonated and positively charged (**D**-units), the water-soluble polysaccharide chitosan can be prepared. This is performed by chemical de-*N*-acetylation of chitin at highly alkaline conditions and high temperature. The de-*N*-acetylation reaction was studied in

detail with the chitin disaccharide (GlcNAc-GlcNAc or **AA**) as a model substrate. The resonances in ^{1}H NMR spectrum of the chitin disaccharide in 2.77 M NaOD were assigned. The β-anomeric protons of the four different disaccharides, i.e. **AA**, **DD**, **AD**, and **DA**, are well separated and can be monitored during the de-*N*-acetylation. Thus, the rate of de-*N*-acetylation of the reducing end was found to be twice the rate of the non-reducing ends. The total rate of de-*N*-acetylation of chitin disaccharide was for the first time determined to be second order with respect to sodium hydroxide concentration. This contributes to explain the differences between the homogeneous and heterogeneous de-*N*-acetylation reaction. The activation energy for the reaction was determined to 114.4 and 98.6 kJ/mol in 2.77 M and 5.5 M NaOD, respectively.

Hydrogels of biopolymers have attracted much attention for their applications in e.g. tissue engineering, immobilization of cells and controlled drug release. A new gelling system of chitosan – alginate, or their corresponding oligomers, is described. The gelling system was studied by combining either poly-mannuronate and chitosan oligomers, or polymeric chitosan and mannuronate oligomers. The two components were mixed at a pH well above the pK_a-values of the amino-groups, where the chitosan/chitosan oligomers are almost uncharged, allowing mixing with the negatively charged poly-mannuronate/mannuronate oligomers without the precipitation that would otherwise occur upon mixing a polyanion with a polycation. Then the pH was lowered by adding D-glucono-δ-lactone (GDL), a proton donating substance with the ability to release protons in a controlled way, so that the amino groups of chitosan/chitosan oligomers were protonated and thereby positively charged, resulting in the formation of a hydrogel. The neutral-solubility of the polymeric chitosan is achieved by selecting a polymeric chitosan with a degree of acetylation of 40%, while the neutral-solubility of the (fully de-*N*-acetylated) chitosan oligomers is obtained by selecting oligomers with a chain length below 10. The kinetics of gelation was fast in both gelling systems, with a sol-gel transition within the time for the first measurements. Initial rates of gelation and gel strengths (measured as storage modulus, G') increased with increasing concentration of oligomers. The gel strength (G') of both gelling systems increased with increasing GDL concentration (and thereby the final pH of the gel) from neutral pH down to pH 4, and decreased with increasing ionic strength, indicating that ionic hydrogels are formed.

The importance of the nearly perfect match in distance between the negative charges on the same side of poly-mannuronate/mannuronate oligomers and the positive charges on the same side of chitosan/chitosan oligomers is crucial for these gelling systems, as demonstrated by the very different gel strengths of two alginates with extreme composition, i.e. a poly-mannuronate and a poly-guluronate, where poly-mannuronate formed relatively strong gels with chitosan oligomers while poly-guluronate formed gels of very limited mechanical strength.

Abbreviations

BT	Black tiger shrimp
CO	Chitosan oligosaccharide
DA	Degree of acetylation
DD	Degree of de-*N*-acetylation
DM	Demineralization
DP	Degree of polymerization
DP_n	Number-average degree of polymerization
DPr	Deproteinization
G	Guluronic acid unit
GDL	D-glucono-δ-lactone
GlcN or **D**	D-Glucosamine
GlcNAc or **A**	N-acetyl-D-Glucosamine
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
M	Mannuronic acid unit
MO	Mannuronan oligosaccharide
MW	Molecular weight
NMR	Nuclear Magnetic Resonance
Poly guluronate	Poly-G
Poly mannuronate	Poly-M
ppm	Part per million
SEC-MALLS	Size Exclusion Chromatography Multi-Angle Laser Light Scatter
WS	White shrimp

List of papers

1. T.T. Khong, H.D.T. Ngo, N.D. Ngo, T.S. Trang, K.M. Vårum (2012). Chemical composition and quality of chitin from white shrimp and black tiger shrimp. In: Advances in Chitin Science, Proceedings of the 6th Iberoamerican Symposium and the 12th International Conference on Chitin and Chitosan, Fortaleza, Brazil.

2. Khong, T. T., Aachmann, F. L., & Vårum, K. M. (2012). Kinetics of de-*N*-acetylation of the chitin disaccharide in aqueous sodium hydroxide solution. *Carbohydrate Research, 352*, 82-87.

3. Thang Trung Khong, Olav A. Aarstad, Gudmund Skjåk-Bræk, Kurt I. Draget and Kjell M. Vårum. A new gelling concept combining chitosan and alginate – Proof of principle. Manuscript

Introduction

1. Shrimp aquaculture in Vietnam

Vietnam, a country in South-East Asia, is endowed with a very long coastal line of over 3,200 km and a complex system of rivers and estuaries that are ideal for fisheries and aquaculture. With those advantages, the fisheries sector has become one of the key industries of Vietnam, especially aquaculture of shrimp and catfish (*Pangasius*).

Vietnamese shrimp production by means of aquaculture has increased steadily over the last ten years, tripling from around 150 thousand tons in 2001 to 450 thousand tons in 2010 (Figure 1.1) (MOIT, 2012). The main region for shrimp aquaculture is the Mekong Delta in the south of Vietnam, which accounts for over 75% of the country's shrimp production.

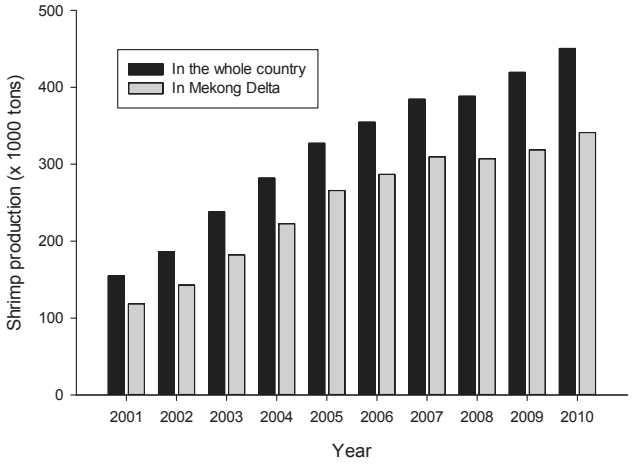


Figure 1.1. Shrimp production in Vietnam and the Mekong delta over the last 10 years

In seafood processing factories, approximately 70% of the mass of each crustacean is converted into products for human consumption. The remaining mass is discarded as waste and includes the heads, thoraxes, claws, and shells (Figure 1.2). Crustacean by-

products are very perishable because of their high protein content. If these by-products are dumped in the sea, they pollute the sea water and cause environmental problems. However, if they are landfilled, they provide sustenance for pathogens and spoilage organisms, causing environmental and public health issues (Bruck *et al.*, 2009). In addition to protein, crustacean by-products contain two other main components, i.e. chitin and minerals, while pigments and lipids are present as minor components. Because the components of crustacean by-products are potentially valuable, considerable effort has been invested into developing methods for their recovery.

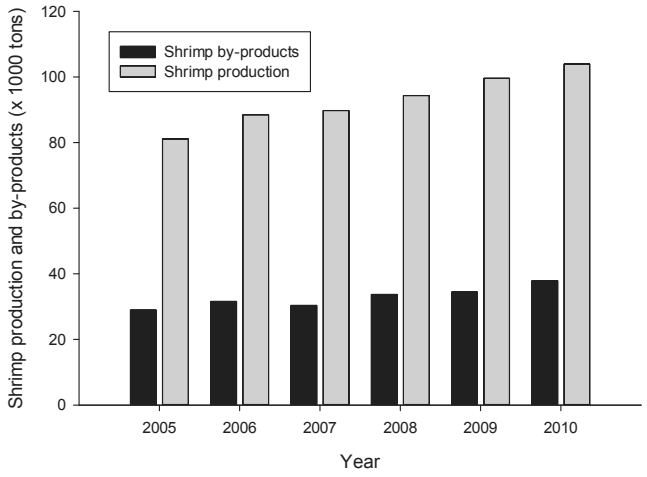


Figure 1.2. Shrimp and shrimp by-product output in Camau province (the southernmost province of Vietnam)

2. Chitin

2.1. Chemical structure

Chitin is a linear biopolymer composed of β (1-4) linked 2-acetamido 2-deoxy-D-Glucose. Although chitin is a linear chain, every single sugar unit is in the $^4C_1$ conformation and is rotated by 180^0 relative to the units on either side. Thus, chitobiose is the repeating unit in the chitin chain. In chitin molecules, the monomers are linked by

glycosidic bonds, with a distance of 0.516 nm between successive bonds. An intramolecular hydrogen bond, C(3)-OH···O-C(5), stiffens the chitin chain and makes it more rigid: only the C(6)-OH and NH-CO-CH_3 groups can rotate (Minke & Blackwell, 1978).

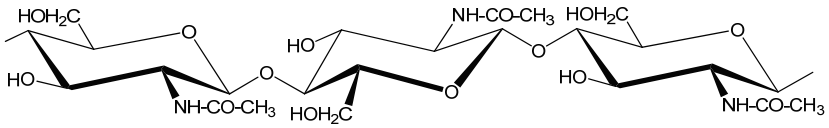


Figure 2.1 Chemical structure of chitin

In nature, chitin exists in crystalline form with two main allomorphs: α and β chitin. In both cases, chitin chains with the same directionality are linked by intra-sheet hydrogen bonds to form sheets of chains. These bonds are formed between the following functional groups in adjacent chains: C=O···H-N, C(6)-OH···O=C and C(6)-OH···OH-C(6). In α chitin, the sheets are arranged in an anti-parallel fashion, while β chitin has a parallel chain arrangement (Kameda *et al.*, 2005; Minke & Blackwell, 1978; Rinaudo, 2006; Sikorski *et al.*, 2009). The sheets of chains are held together by C(6)-OH···OH-C(6) inter-sheet hydrogen bonds, which are not present in β chitin.

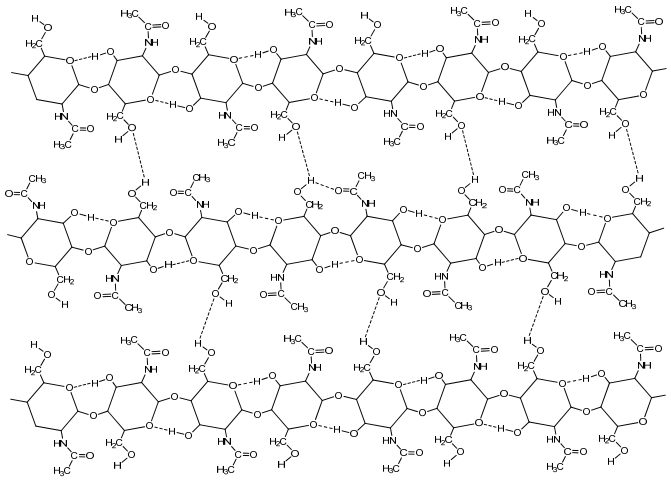


Figure 2.2 Intramolecular and inter-sheet hydrogen bonds in α chitin crystals

In addition to different chain arrangements, the α and β chitin allomorphs also differ in the dimensions of their unit cells and the number of chains per unit cell. Because β chitin lacks inter-sheet hydrogen bonds, its crystal structure can be penetrated by polar guest molecules such as water, alcohols and amines without disrupting its sheet organization and crystallinity. Thus, β chitin can rapidly swell in water and some alcohol and amines, and this swelling is reversible. Conversely, α chitin has extensive inter-sheet hydrogen bonding and so does not swell when exposed to water or alcohols. However, certain diamines can penetrate into the α chitin crystalline lattice (Rinaudo, 2006).

2.2. Occurrence

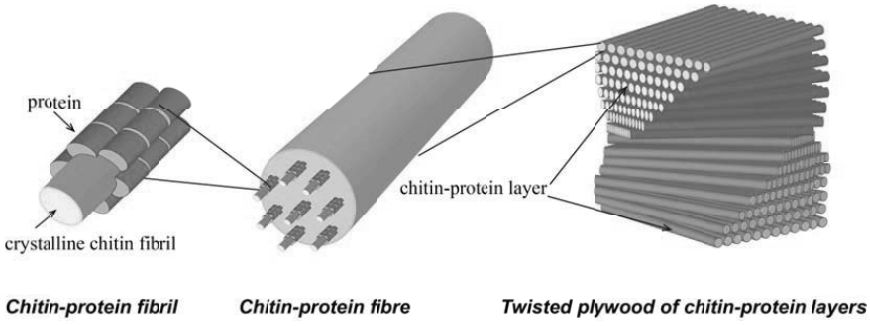


Figure 2.3 Hierarchical levels in the chitin-protein matrix in arthropod cuticle

Chitin is one of the most abundant polysaccharides on earth. It primarily occurs as the α allomorph and is found in the exoskeletons of arthropods such as insects and crustaceans, as well as the cell walls of fungi, algae and yeasts. The rarer β chitin can be found in association with proteins in squid pens. The main function of chitin is analogous to that of cellulose in plants: it serves to increase the mechanical strength of arthropod exoskeletons and fungal cell walls.

Chitin is synthesized by the membrane-integral enzyme chitin synthase, a processive enzyme that binds to the polymer and catalyzes several successive polymerization steps, each of which adds a single GlcNAc unit to the non-reducing end of the growing polymer. The process requires UDP-N-Acetylglucosamine as the substrate and divalent

metal cations such as Mg^{+2} or Mn^{+2} as co-factors (Merzendorfer, 2006). UDP-N-Acetylglucosamine is the end product of a cascade of cytoplasmic biochemical transformations (Cohen, 1987). After being synthesized in the cytoplasmic site, the nascent chitin polymer is translocated across the membrane and released into the extracellular space where the single polymers spontaneously assemble into crystalline nanofibrils. Finally, the nanofibrils are bound to other sugars, proteins, glycoproteins and proteoglycans to form fungal septa and cell walls, arthropod cuticles, or peritrophic matrices (Merzendorfer, 2006).

The exoskeletons (or cuticles) of arthropods are non-cellular coatings secreted by the epidermal cells. They consist of three main layers: the epicuticle, the exocuticle and the endocuticle. The exocuticle and endocuticle layers are made of hard mineralized fibrous chitin and protein, and have a strict hierarchical organization with various structural levels. The lowest hierarchical level is the crystalline chitin nanofibrils, each of which consists of 18-25 chitin chains and is 2-5 nm in diameter with a length of around 300 nm. The chitin nanofibrils are individually wrapped with proteins to form chitin-protein fibrils. In the next hierarchical level, chitin-protein fibrils are assembled into chitin-protein fibres with a diameter of 50-300 nm. The chitin-protein fibres are then arranged into a planar woven and periodically branched network, forming chitin-protein layers. In these layers, the chitin-protein fibres are embedded in proteins and also micro- and nano-scale biominerals. The most abundant of these minerals is the crystalline form of $CaCO_3$, but the amorphous form also contributes in some species and at certain stages of the molt cycle. The chitin-protein layers are stacked and twisted in a helicoidal fashion. A stack of layers with a complete 180° rotation is referred to as having a twisted plywood or Bouligand pattern. The Bouligand layers of the exocuticle in crustaceans are made up of chitin-protein layers with a greater stacking density than those of the endocuticle. Consequently, the exocuticle is stronger and harder than the endocuticle (Chen *et al.*, 2008; Gartner *et al.*, 2010; Nikolov *et al.*, 2011; Raabe *et al.*, 2005).

As discussed above, chitin is closely associated with protein in the chitin-protein fibrils and fibres of arthropods. While around half of the cuticular proteins have been

sequenced, the nature of the interactions between these proteins and chitin is not clearly understood (Hamodrakas *et al.*, 2002; Iconomidou *et al.*, 2005; Rebers & Willis, 2001). However, these interactions are very strong and it is difficult to completely separate the protein from the chitin. Hackman (1960) prepared six chitin samples from insect and crustacean cuticles by repeated extraction with hot (100 °C) aqueous alkali. All of the resulting samples were found to contain small amounts of aspartic acid and histidine. Brine (1981) isolated chitin from four crustaceans and horseshoe crabs (*Limulus polyphemus*) by using various treatments to disrupt the chitin-protein interactions. However, even after vigorous treatment with hot alkali (1M NaOH, 100 °C, 48h) the remaining chitin still contained some amino acids, including aspartic acid, serine and glycine. The persistence of aspartic acid in this case was particularly striking.

The chitin and proteins found in crustacean cuticles are conjugated with carotenoids, giving them a blue color (Cianci *et al.*, 2001; Tharanathan & Kittur, 2003). The keto groups of these carotenoids may react with the amino groups of chitin or proteins to form imines (also known as Schiff's bases). For instance, in the carapace of the red kelp crab, the carotenoid-chitin complex seems to be shielded by a calcarious matrix. Consequently, extraction with common organic solvents (e.g. ethanol and acetone) and protein denaturants (e.g. with hot aqueous alkali) failed to yield significant quantities of pigment in the absence of prior decalcification. However, extraction with warm aqueous acetic acid readily decalcified the shell and extracted all its pigment (Fox, 1973).

2.3. Isolation

Chitin was first isolated from mushrooms by Henry Braconnot in 1811, 30 years before the identification of cellulose. Despite this, the properties of chitin have not been extensively studied, whereas there has been a very large body of work focused on cellulose (Kurita, 2006). However, in the 1970s it was realized that chitin is an abundant source of the unique natural cationic aminopolysaccharide chitosan, prompting a resurgence of interest in this biopolymer (Kumar *et al.*, 2004). While chitin is widely distributed on earth, waste from seafood production (especially shrimp and crab processing) is the main source of commercial chitin.

The relative abundance of the different components of crustacean by-products varies depending on the species from which they are derived, the body parts comprising the waste, the season, and the organisms' diet (Synowiecki & Al-Khateeb, 2003). The composition of some crustacean species is shown in Table 2.1.

Table 2.1 The composition of selected marine crustacean species (% dry weight)

Species	Protein	Chitin	Ash	Lipid	Reference
Crab					
Collinectes sapidus	25.1	13.5	58.6	2.1	(Synowiecki & Al-Khateeb, 2003)
Chinoecetes opilio	29.2	26.6	40.6	1.3	(Synowiecki & Al-Khateeb, 2003)
Shrimp					
Penaeus monodon	49.6	13.5	21.9	6.3	(Ngoan *et al.*, 2000)
Penaeus semisulcatus	49.8	14.1	21.6	7.4	(Ngoan *et al.*, 2000)
Metapenaeus affinis	44.0	18.1	22.8	7.3	(Ngoan *et al.*, 2000)
Penaeus aztecus (shell)	29.50	21.53	48.97	-	(Abdou *et al.*, 2008)
Penaeus durarum (shell)	34.02	23.72	42.26	-	(Abdou *et al.*, 2008)
Xiphopenaeus kroyeri	39.42	19.92	31.98	3.79	(De Holanda & Netto, 2006)
Crangon crangon	40.6	17.8	27.5	9.95	(Synowiecki & Al-Khateeb, 2000)
Pandalus borealis	33-40	17-20	32-38	0.3-0.5	(Rodde *et al.*, 2008)
Solenocera melantho	16.4	23.3	42.4	8.4	(Chang & Tsai, 1997)
Squilla (*S. empusa*)	21.2	36.0	42.8	-	(Rao *et al.*, 2007)
Crayfish (*Procambarus clarkii*)	17.8	25.7	51.8	0.7	(Bautista *et al.*, 2001)

The precise composition of crustacean biowaste arising from individual species will depend on the growth stage and gender of the organisms, as well as their diet and environmental conditions. However, some of the observed variation is due to the

analytical methods used to determine the composition of the waste. The existence of chitin-protein complexes makes it very challenging to precisely determine the material's protein content. Díaz-Rojas (2006) developed an improved method for simultaneously determining a sample's chitin and protein content based on its total nitrogen and a set of stoichiometric equations, which is reliable provided that the other components of the waste (i.e. ash, moisture, and lipids) have been precisely determined. Percot et al. (2003b) hydrolyzed shrimp shells in 6 M HCl and analyzed the released amino acids using an amino acid analyzer. This enabled them to determine the protein content of the shells based on the total mass of the isolated amino acids. Rao et al. (2007) and Lertsutthiwong et al. (2002) used the Biuret method to determine the protein content of crustacean biowaste. According to their protocol, protein is extracted from the raw material using an alkaline solution and determined using calorimetric methods with the Biuret reagent and bovine serum albumin (BSA) as the standard protein. However, if an excessively long extraction time is used, the proteins will be partially hydrolyzed and the material's protein content will be underestimated.

In general, chitin is isolated by solubilizing all of the other components of the exoskeleton. The isolation of chitin from crustacean waste usually involves three main steps: demineralization (DM), deproteinization (DPr), and decolorization (DC), in which DPr can be done either chemically or biologically. The order in which the first two steps are performed can affect the properties of the resulting chitin (Lertsutthiwong *et al.*, 2002).

Chemical method for chitin isolation

In chemical isolation, demineralization is performed by using acids to solubilize minerals in the cuticle according to the following stoichiometric equations:

$$CaCO_3 \rightleftharpoons Ca^{2+} + CO_3^{2-} \xrightarrow{H^+} HCO_3^- \xrightarrow{H^+} H_2CO_3 \longrightarrow H_2O + CO_2\uparrow$$

The dissolution of minerals consumes H^+ and releases carbon dioxide. Therefore, the pH of the acid solution increases as the reaction proceeds and the process can be monitored by observing the evolution of gas bubbles. Various mineral acids have been used for this purpose, including HCl, HNO_3, H_2SO_4, and H_2SO_3. Of these, HCl is the

most widely used (Charoenvuttitham *et al.*, 2006). DM is often performed using acid concentrations ranging from 0.25 M to 2 M for 1-48 h at temperatures of 0 to 100 °C (Percot *et al.*, 2003b). The acid also catalyzes the hydrolysis of the glycosidic linkages in chitin molecules, causing depolymerization of the chitin chains. In addition, de-*N*-acetylation (i.e. the hydrolysis of the N-acetyl groups of chitin) also occurs. However, the rate of acid-catalyzed de-*N*-acetylation is much lower than the rate of depolymerization (Einbu & Vårum, 2008). Thus, in order to preserve the molecular weight of the chitin, DM should be performed using low acid concentrations and temperatures. Attempts to replace HCl with organic acids such as CH_3COOH, HCOOH, $C_6H_8O_7$ have been made; the highest yields obtained in this way were achieved using a mixture of 0.25 M HCOOH and 0.25 M of $C_6H_8O_7$ (Charoenvuttitham *et al.*, 2006). Sonication does not seem to enhance the acid-mediated removal of minerals from shrimp shells (Kjartansson *et al.*, 2006).

The purpose of deproteinization is to dissolve protein content of the crustacean by-products using alkaline solutions (usually of NaOH or KOH) at elevated temperatures. Other basic salts such as Na_2CO_3, $NaHCO_3$, K_2CO_3, $Ca(OH)_2$, Na_2S, $NaHSO_3$, and Na_3PO_4, have also been used for DPr (Charoenvuttitham *et al.*, 2006). DPr is often performed using modest base concentrations of 1-10% at temperatures ranging from 30 to 100 ^{o}C for 0.5-12h (Bruck *et al.*, 2009). The optimal DPr conditions depend on the raw material being processed, temperature, base concentration, time, and solid-to-solvent ratio (Synowiecki & Al-Khateeb, 2003). For example, when performing deproteinization with 1M NaOH, the reaction time and temperature do not significantly affect the molecular weight and DA of chitin provided that the temperature does not exceed 70 °C and the reaction time is less than 24h (Percot *et al.*, 2003a). In terms of the residual protein content, the optimal conditions for deproteinizing pink shrimp (*Solenocera melantho*) shell waste involve heating at 75 °C in 2.5 M NaOH with a solid-to-solvent ratio of 1:5 (Chang & Tsai, 1997). Sonication can be used to promote the removal of protein from North Atlantic shrimp shell waste (Kjartansson *et al.*, 2006).

Biological methods for chitin isolation

The reagents used in chemical deproteinizing protocols are hazardous to human health and the environment, and generally destroy the non-chitin components of the treated waste (e.g. proteins and pigments). Biological deproteinizing techniques use proteases or bacterial fermentation rather than harsh chemical agents to separate chitin from the other waste components, potentially enabling the recovery of all of the useful components of the waste material. However, the existing implementations of these methods produce chitin with a high residual protein content (from 1% to 7%) and require long reaction times. Together with the high cost of the relevant proteases and bacteria, these factors have limited the industrial uses of biological methods (Percot *et al.*, 2003b).

Some commercial proteases have been used for chitin isolation, including alcalase (De Holanda & Netto, 2006; Gildberg & Stenberg, 2001; Synowiecki & Al-Khateeb, 2000), pancreatin (De Holanda & Netto, 2006), papain (Gartner *et al.*, 2010), and bromelain (Wang *et al.*, 2008); alcalase proved to be more efficient than pancreatin for this purpose. Both bromelain and papain are claimed to possess both proteolytic and chitinolytic activity and have been used to produce chitosan oligosaccharides (Wang *et al.*, 2008).

Chitin isolation by lactic fermentation is an interesting new technology for chitin extraction (Kandra *et al.*, 2012) that involves treating the raw material with a bacterial inoculum. The fermentation process produces two fractions: solid chitin and a liquor containing proteins, minerals, pigments, and nutrients. The low pH generated during the process suppresses the growth of spoilage microorganisms and enhances DM by dissolving calcium carbonate (Beaney *et al.*, 2005; Bueno-Solano *et al.*, 2009; Ghorbel-Bellaaj *et al.*, 2011; Healy *et al.*, 2003; Oh *et al.*, 2007; Pacheco *et al.*, 2011). Fermentation can also be driven by autolytic processes catalyzed by endogenous enzymes in the crustacean by-products (Cao *et al.*, 2008; Kandra *et al.*, 2012).

2.4. Characterization

The chemical and physical characterization of chitin samples is very important because many of chitin's biological, physicochemical, and mechanical properties are determined by its chemical and physical characteristics. However, the insolubility of chitin in most common solvents limits the number of viable methods for its characterization and tends to reduce the accuracy of those methods that can be used. The parameters that have the greatest impact on the properties of a chitin sample are its degree of acetylation (DA), its molecular weight/molecular weight distribution, and its crystallinity. In addition, parameters such as the residual protein, ash, pigment, heavy metal and moisture contents can be important for certain applications.

2.4.1. Degree of acetylation

Various methods for determination of DA of chitin/chitosan have been developed. Broadly, they fall into three classes: (1) spectroscopic methods (NMR, UV, IR); (2) conventional methods (titrations, ninhydrin test); and (3) destructive methods (elemental analysis, acid/enzyme hydrolysis followed by colorimetry or chromatographic analysis, and thermal analysis using Differential Scanning Calorimetry (DSC)). Each method has its advantages in specific cases, so it is not straightforward to identify the optimal method for any given application (Kasaai, 2009). However, methods belonging to groups (1) and (3) are most widely used when working with chitin.

Nuclear Magnetic Resonance (NMR)

NMR is the most powerful method for determining the DA of chitin samples. However, the need for well-trained analysts and technicians as well as the availability of suitable instruments can limit the applicability of this method in some cases.

Due to chitin's poor solubility in aqueous solution, little effort has been invested into the development of liquid-state ^{1}H NMR spectroscopic methods for DA determination. However, Einbu & Vårum (2008) dissolved chitin in concentrated DCl (37% wt.) and recorded the resulting ^{1}H NMR spectra. Dissolution in such experiments can be promoted by heating the suspension of chitin in DCl at 40 °C for less than 30 minutes.

The DA is then calculated based on the resonances of the H-1 and H-2 protons of acetylated and de-*N*-acetylated residues. However, the intensities of the signals arising from H-1 and H-2 are weaker than those arising from H-2 to H-6, and so the reliability of the results obtained using this method is very sensitive to the accuracy of the integrals. It is possible that more accurate results could be obtained using this approach if it were combined with Hirai's method for chitosan (Hirai *et al.*, 1991), which estimates the DA based on the integral for the CH_3 signal and the sum of the integrals for H-2 to H-6. Moreover, it should be noted that dissolving chitin in concentrated DCl will cause some level of de-*N*-acetylation, which is reflected in the presence of an acetate peak in the spectrum. The extent of de-*N*-acetylation can thus be determined, and the acetate peak can be accounted for when determining the DA.

Solid-state ^{13}C NMR spectroscopy is a non-destructive method and has been used for DA determination by some researchers. Two key parameters that affect the quality of solid-state ^{13}C NMR spectra are the contact time and the relaxation time (Kasaai, 2009). The DA is calculated by dividing the intensity of the resonance of the methyl carbon by the average intensities of the resonance of the carbons in the pyranose ring. The method can be used regardless of the sample's DA, solubility, and crystallinity, and has minimal sensitivity to impurities. However, large amounts of sample (about 300 mg) are required and the analysis is time-consuming (Hein *et al.*, 2008).

Because chitin molecules contain nitrogen atoms in their amino and *N*-acetyl groups, ^{15}N NMR spectroscopy can be used to determine the DA of chitin samples. However, for samples with low DA values (<10%), the broad peaks of the resulting spectra reduce the method's sensitivity (Hein *et al.*, 2008).

Infra-red spectroscopy (IR)

IR spectroscopy is another non-destructive method that can be used to determine the DA of chitin. The DA is calculated based on the ratio of the absorbance of a probe band and a reference band. The probe band provides a measure of the sample's N-acetyl or amine content, while the reference band has an intensity that does not change with the DA. Different procedures for calculating DA values in this way have been proposed. However, impurities such as proteins in the sample affect the positions and intensities of

some peaks in the spectrum and therefore the calculated DA values. IR methods are therefore generally used for qualitative rather than quantitative DA analysis (Kasaai, 2008).

Ultra-violet spectroscopy (UV)

The wide availability of UV spectrometers means that they can be easily used for DA determination. The method was first developed for use with chitosan samples dissolved in dilute acids such as acetic or hydrochloric acid. However, chitin does not dissolve in these acids, so Hsiao (2004) used 85% phosphoric acid to solubilize chitin samples for UV analysis. Wu & Zivanovic (2008) and Hein et al. (2008) further modified this method by basing their analyses on the first- and zero-order derivatives of the UV spectra of chitin solutions, respectively. A number of factors can cause problems when preparing samples for UV analysis, including the formation and hydrolysis of the glucofuranosyl oxazolinium ion, the formation of 5-hydroxymethyl furfural (HMF), and the de-*N*-acetylation of the chitin. It is therefore essential to optimize the temperature at which the chitin is dissolved in the acid and the duration of the period allocated for dissolution (Wu & Zivanovic, 2008). The results obtained using this method have been shown to correlate extremely well with those achieved using solid-state ^{13}C NMR, suggesting that UV spectroscopy may be suitable for routine high-accuracy DA determinations (Hein *et al.*, 2008).

Acid hydrolysis/HPLC analysis

Under acidic conditions, the N-acetyl groups of chitin can be hydrolyzed, liberating acetic acid that can be determined by HPLC to evaluate the DA of the sample. This method was initially developed by Holan et al. (1980) and subsequently modified by Ng et al (2006). In the most recent version of the protocol, the chitin is hydrolyzed using a mixture of 12 M H_2SO_4 and 1.4 mM oxalic acid and then incubated at 110 °C in a sealed glass ampule for 40 min. The acetic acid released during this process is determined quantitatively by HPLC, eluting with 1 mM H_2SO_4. Sample preparation takes at least 3 hours and requires around 10 mg of material. The results obtained in this way correlate well with those achieved using solid-state ^{13}C NMR and first derivative UV methods (Ng *et al.*, 2006).

Pyrolysis/GC analysis

When chitin is pyrolyzed, acetic acid and various nitrogen-containing products such as acetonitrile and acetoamide are liberated. These compounds can be analyzed by gas chromatography (GC) and the results used to calculate the DA for the sample. Sato et al. (1998) used 3 µL of 1.0 M oxalic acid to hydrolyze chitin at 450^0C, with a 10 min incubation period at ambient temperature before the sample was placed into the furnace. This method is rapid and simple, and can be used for samples irrespective of their degree of acetylation.

Elemental analysis

The C/N ratio of chitin samples can be used to estimate their DA values. The ratio ranges from 5.145 for completely de-*N*-acetylated chitosan to 6.861 for fully acetylated chitin (Kasaai, 2009). However, impurities such as proteins and minerals in the sample will affect the accuracy of the results obtained (Hein *et al.*, 2008).

2.4.2. Molecular weight

Chitin is polydisperse with respect to molecular weight. Therefore, the molecular weight of a chitin sample is an average. The two most commonly used averages are the weight-average (M_w) and the number-average (M_n). The ratio of the weight-average and the number-average molecular weight (M_w/M_n) is defined as the polydispersity index (PI). M_w can be determined by measuring the sample's light scattering or sedimentation, while M_n is determined based on osmotic pressure measurements or by end group determination. The viscosity-average molecular weight, determined by the viscometric method, is generally closer to the weight-average molecular weight than the number-average.

$$M_n = \frac{\sum_i N_i M_i}{\sum_i N_i}, \quad M_w = \frac{\sum_i w_i M_i}{\sum_i w_i} = \frac{\sum_i w_i M_i^2}{\sum_i N_i M_i}$$

Here, N_i is the number of molecules and w_i is the weight of molecules having a specific molecular weight M_i.

Because of chitin's limited solubility, relatively few solvents can be used to solubilize it in order to determine its molecular weight. For a long time, the most widely used

solvent for chitin has been a mixture of N, N-dimethylacetamide (DMAc) and LiCl (Rinaudo, 2006). Chitin dissolves readily in DMAc/LiCl 5% without being degraded (Terbojevich *et al.*, 1988). Einbu et al. (2004) studied the solution properties of chitin dissolved in 2.77 M NaOH. The chitin was found to be stable for 8h at 20 °C with no significant change in molecular weight. Recently, Li et al. (2010) investigated the properties of dilute chitin solutions in aqueous NaOH/urea. Chitin was more stable in this system than in simple NaOH solutions: chitin samples stored in this mixture at 25 °C exhibited no evidence of degradation after 36h.

The molecular weight of a polymer is linked to its intrinsic viscosity by the Mark-Houwink-Sakurada (MHS) equation.

$$[\eta] = K.M^a$$

where [η] is the intrinsic viscosity, *K* is a constant and *a* is the exponent. The values of *K* and *a* vary according to both the polymer-solvent system and the temperature. The value of *a* also indicates the shapes of the polymer in solution. For compact spheres *a* = 0 while for rigid rods *a* = 1.8. Polysaccharides commonly have *a* values between 0.5 and 1.0.

The MHS equations for chitin in some solvent systems have been determined using light scattering and viscosity measurements and are shown in Table 2.2.

Table 2.2 MHS parameters of chitin

Solvent	***K* (mL/g)**	***a***	**t (^{0}C)**	**Reference**
DMAc/5%LiCl	$2.4.10^{-1}$	0.69	25	(Terbojevich *et al.*, 1988)
2.77 M NaOH	0.10	0.68	20	(Einbu *et al.*, 2004)
8%NaOH/4% urea	0.26	0.56	25	(Li *et al.*, 2010)

2.4.3. Crystallinity

The crystalline structure of chitin has been studied by several research groups using X-ray diffraction. The α and β allomorphs can be distinguished based on their XRD spectra, and the dimensions of the unit cells for each allomorph have been determined

(Rinaudo, 2006). The crystalline structure of chitin can be altered during isolation and evaluated using the crystallinity index proposed by Struszczyk (1987).

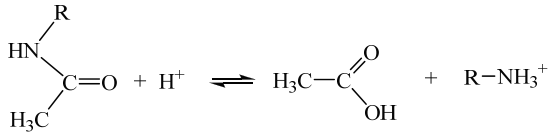
$$CrI = \frac{(I_{peak} - I_{am})x100}{I_{peak}} \quad (\%)$$

Here, I_{peak} is the intensity of the peak at the 020 or 110 reflection and I_{am} is the intensity of the amorphous diffraction.

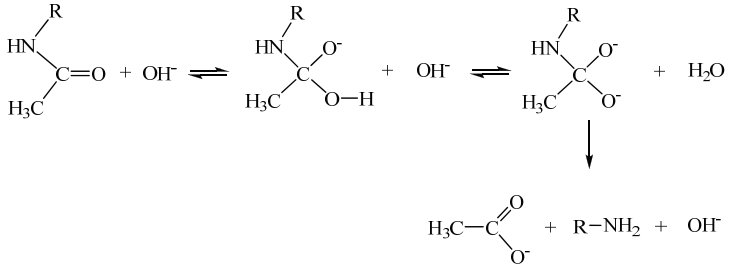
2.5. De-*N*-acetylation of chitin

The N-acetyl group in chitin can undergo hydrolysis (de-*N*-acetylation) in aqueous acid or base. The reactions in acid and base are fundamentally different and proceed via the following mechanisms (Solomons & Fryhle, 2009):

Acidic hydrolysis



Basic hydrolysis



Under basic conditions, the reaction is initiated by the attack of a hydroxide ion on the acyl carbon of the amide. A second hydroxide ion then deprotonates the resulting anionic tetrahedral intermediate to form a dianion, which collapses to give the products. Thus, two hydroxide ions are needed in the reaction.

The only way to obtain polymeric chitosan is to de-*N*-acetylate chitin in alkaline solution. The reaction can be performed under either homogeneous conditions with the

chitin in solution or under heterogeneous conditions where the chitin remains in the solid state (Kurita *et al.*, 1977). The alkaline hydrolysis of the glycosidic linkage of chitin is slow. However, it can undergo a rapid oxidative-reductive depolymerization reaction under basic conditions. This can be largely suppressed by performing the reaction in an oxygen-free atmosphere. In addition, de-*N*-acetylation can be performed enzymatically using chitin deacetylase, although this is not straightforward because of the crystallinity of the chitin substrate.

Heterogeneous de-*N*-acetylation is simple, cost effective, and widely used to produce commercial chitosan. It has been shown that the de-*N*-acetylation reaction exhibits pseudo-first-order kinetics with respect to acetyl concentration (Chang *et al.*, 1997; Galed *et al.*, 2005; Lamarque *et al.*, 2004a; Yaghobi & Hormozi, 2010). The DD (degree of de-*N*-acetylation) of chitin/chitosan increases linearly with time in the initial stage of the process then levels off to a maximal value that depends on the concentration of alkali and reaction temperature (Kurita *et al.*, 1977). At low alkali concentrations (< 35%), the rate of reaction is very slow and the DD remains low even with long reaction times at high temperatures (Chang *et al.*, 1997). Therefore, de-*N*-acetylation is often performed in 50% (w/w) NaOH solution at high temperatures. Chitosan with a high DD and high molecular weight can be obtained via a multi-step de-*N*-acetylation process (Lamarque *et al.*, 2004b). The reported activation energies for heterogeneous de-*N*-acetylation range from 16.2 kJ/mol to 56 kJ/mol (Chang *et al.*, 1997; Lamarque *et al.*, 2004b; Yaghobi & Hormozi, 2010). Chitin's insolubility and crystallinity make these results somewhat difficult to interpret for various reasons. For example, the concentration of hydroxide ions at the solvent-exposed surface may be quite different from the known concentration in solution. Kurita et al. (1977) suggested that heterogeneous de-*N*-acetylation produces block-type copolymers of GlcNAc and GlcN (judged by X-ray diffraction). Ottøy et al (1996) subsequently fractionated heterogeneously deacetylated chitosans into acid-soluble and acid-insoluble fractions. The DA of the acid-soluble fractions decreased as the de-*N*-acetylation time increased, whereas the DA of the acid-insoluble fractions remained almost unchanged. They concluded that virtually no de-*N*-acetylation took place in the chitin-like acid-insoluble fractions, suggesting that the product is actually a heterogeneous mixture of chitin and

chitosan rather than a material with the block-type distribution of units proposed by Kurita et al. (1977). Vårum et al. (1991a, b) used ^{1}H and ^{13}C NMR to study the degree of acetylation and the distribution of N-acetyl groups in heterogeneous de-*N*-acetylated chitins. The frequencies of diads and triads in chitosan prepared in both homogeneous and heterogeneous process were consistent with a random distribution of GlcNAc and GlcN residues. It is therefore likely that during heterogeneous de-*N*-acetylation, the chitin particles swell and undergo de-*N*-acetylation from the outside in, and that swelling is the rate limiting step of the process (Ottøy *et al.*, 1996).

Homogeneous de-*N*-acetylation has been reported to yield chitosan molecules with a random distribution of GlcNAc and GlcN (Kurita *et al.*, 1977; Vårum *et al.*, 1991a, b). Chitin is first dissolved in a relatively dilute alkaline solution (e.g. 8% or 10% NaOH), by prolonged stirring at low temperature. The homogeneous process is slow at temperatures below 40 °C, but the chitin precipitates at higher temperatures. The reaction has been reported to be pseudo-first-order with respect to the concentration of acetamide groups and its activation energy has been determined to be 22 kcal/mol (92 kJ/mol) (Sannan *et al.*, 1977). Sannan et al. also attempted to study the de-*N*-acetylation of the GlcNAc monomer but were unsuccessful because it decomposed under the tested conditions. The effects of varying the concentration of the alkaline solution on the kinetics of homogeneous de-*N*-acetylation have not yet been investigated.

3. Chitosan

3.1. Chemical structure

Chitosan, a deacetylated derivative of chitin, is a linear copolymer of N-acetyl-glucosamine (GlcNAc, the "**A**" unit) and glucosamine (GlcN, the "**D**" unit). Chitosan is differentiated from chitin based on its solubility in dilute aqueous solutions of acids such as acetic acid. The term refers to a family of polysaccharides whose degree of acetylation can range from 0 to 60 – 70%, with a wide variety of functional properties. When solubilized in acidic solutions, the amino groups of chitosan are protonated, making it a natural cationic polysaccharide. It has diverse applications in fields including agriculture, food production, the pharmaceutical industry, nanotechnology,

and biotechnology. In nature, chitosan occurs in the cell walls of fungi but is much less abundant than chitin.

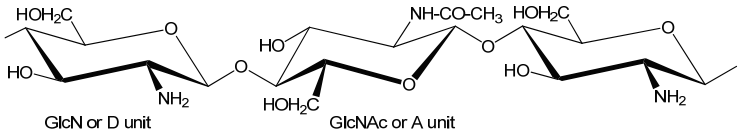


Figure 3.1 Chemical structure of chitosan

The most important functional parameters of chitosan are the degree of acetylation (DA), molecular weight, molecular weight distribution, and the distribution of acetyl groups along the chitosan chains. In addition, the crystallinity of chitosan samples is sometimes determined to evaluate their quality.

3.2. Chitosan in solution

The amino groups of chitosan can be protonated, making it unique among the polysaccharides.

$$R\text{-}NH_2 + H^+ \rightleftharpoons R\text{-}NH_3^+$$

The dissociation constant of chitosan (i.e. its pK_a value) is reported to be between 6.2 and 7, depending on type of chitosan in question and the conditions of measurement (Anthonsen *et al.*, 1993; Park *et al.*, 1983; Strand *et al.*, 2001). The literature data on the relationship between pK_a and DA are somewhat inconsistent. Anthonsen et al. (1993) and Strand et al. (2001) found that the pK_a values of chitosans (DA = 0 – 0.5) are 6.5 – 6.6, irrespective of DA value. On the other hand, Sorlier et al. (2001) reported that the pK_a of chitin/chitosan samples increased from 6.3 to 7.2 as the DA increased from 0.05 to 0.89.

Protonation of the amino groups also makes chitosan soluble in acidic solution, a property that is essential in many of its applications. The solubility and solution properties of chitosan are governed by the pH and ionic strength of the solution and the structure of the chitosan (i.e. its DA, the distribution of GlcNAc groups, and its molecular weight).

All chitosan samples are soluble in water with a pH of less than 6, and their solubility decreases as the pH is increased. The extent to which generic chitosan (i.e. heterogeneously de-*N*-acetylated material) precipitates as the pH is increased from 6 to 8 depends on its DA; higher DA values are associated with increased solubility at higher pH values. For instance, in chitosan samples with DA values of 0.37, 0.17, and 0.01, the percentages of insoluble material at a pH of 6.5 were 18%, 50%, and 90%, respectively (Vårum *et al.*, 1994). Thus, the solubility of chitosan depends strongly on the DA. This may explain why Sorlier et al. (2001) obtained different pK_a values when using potentiometric titration to study chitin/chitosan samples with widely varying DA values.

The solubility of chitosan at neutral pH is very important because its accessibility to enzymes is dependent on its solubility and because it can be used at biologically neutral pH values. Fully deacetylated chitosan is only soluble under neutral conditions if it has been degraded into oligomers. The neutral solubility of partially N-acetylated chitosan is governed by both its chemical structure and its molecular weight: neutral solubility increases as the DA increases and the degree of polymerization decreases (Vårum *et al.*, 1994). Chitosan samples with DA values between 0.4 and 0.6 exhibit complete neutral solubility even when their molecular weight is relatively high (Sannan *et al.*, 1976; Vårum *et al.*, 1994).

Chitosan is a polyelectrolyte when dissolved in acidic media, and there is electrostatic repulsion between neighbouring protonated amino groups. Increasing the ionic strength of the solution increases the abundance of dissolved anions that can screen the polymer's fixed charges from one-another, reducing the magnitude of these repulsive interactions. Consequently, chitosan expands in low ionic strength media and contracts at higher ionic strengths (Smidsrød & Moe, 2008). The conformation and degree of extension of chitosan under specific conditions can be estimated using the Mark-Houwink-Sakurada (MHS) equation. As the ionic strength of the medium increases the *K* (constant) in the MHS equation for chitosan decreases, while the *a* (exponent) increases as the ionic strength decreases (Anthonsen *et al.*, 1993). However, Berth and Dautzenberg (2002) conducted an independent review of the literature in this area and

concluded that chitosan samples with DA values ranging from 5% to 55% could be fitted to a common MHS equation with an exponent of 0.92.

3.3. Chitosan gels

Chitosan is biocompatible, biodegradable, non-toxic, anti-bacterial, anti-flammable, and bioadhesive (Kurita, 2006). It therefore has potential applications in biomedicine and the pharmaceutical industry. It can be used to create hydrogels, films, beads, microspheres, and solutions. Of these, hydrogels are particularly interesting because they can be designed to respond to the physiological environment (Kang De *et al.*, 2008).

Hydrogels can be defined as macromolecular networks that swell in water or biological fluid (Peppas, 1986). The network is held together by linkages whose lifetime is greater than the window of observation (Smidsrød & Moe, 2008). Chitosan-based hydrogels are considered to have great potential in biomedical and pharmaceutical applications such as drug delivery, protein delivery, gene (DNA) delivery, and as scaffolds in tissue engineering. Properties of chitosan gels that have been exploited in biomedical applications include their pH and temperature sensitivity as well as their swelling capacity and mechanical strength (Kang De *et al.*, 2008).

Chitosan can be used to prepare both chemical and physical hydrogels (Berger *et al.*, 2004). In chemical chitosan hydrogels, the individual polymer chains are linked by strong covalent bonds whose formation is not reversible, whereas the chains in physical hydrogels are linked by weaker ionic bonds or secondary interactions such as hydrogen bonding or hydrophobic interactions whose formation is reversible. Chitosan hydrogels can be formed with or without the addition of a crosslinker (Berger *et al.*, 2004).

Chitosan hydrogels formed using crosslinkers may be covalently or ionically cross-linked, depending on the nature of the interactions between the crosslinker and the polymer. Covalently cross-linked gels can be further divided into three types as shown in Figure 3.2 (a-c) (Berger *et al.*, 2004).

In hydrogels that are made of chitosan crosslinked with itself, the crosslinkers connect structural units from the same or different polymeric chains (Oyrton *et al.*, 1999).

Conversely, in a hybrid polymer network (HPN), linkages are formed between a structural unit of a chitosan chain and a structural unit of a crosslinking polymer. In a semi-penetrating network (semi-IPN), a non-reacting polymer is added to the chitosan solution prior to crosslinking. If this additional polymer is further cross-linked, one obtains a full-IPN gel whose microstructure and properties can be quite different from those of a semi-IPN. In all three types of covalently cross-linked hydrogels, the main crosslinking interactions are covalent bonds but other interactions such as hydrogen bonds and hydrophobic interactions may also be important (Berger *et al.*, 2004; Draget, 1996). In contrast, ionic interactions between the crosslinkers and chitosan chains are the dominant crosslinking forces in ionically cross-linked hydrogels (Figure 3.2, d).

The formation of covalently cross-linked hydrogels requires a chitosan and a crosslinker with at least two reactive functional groups. The most common crosslinkers are dialdehydes such as glyoxal and glutaraldehydes, which condense with the free amino groups of the chitosan strands to form diimines (Kang De *et al.*, 2008). Covalently cross-linked hydrogels have good mechanical properties and are resistant to dissolution even at extreme pH values. However, most of the crosslinkers investigated to date are relatively toxic or have unknown effects on human health. For instance, glutaraldehyde is known to be neurotoxic (Beauchamp *et al.*, 1992) and glyoxal is mutagenic (MurataKamiya *et al.*, 1997). Genipin is a naturally occurring material that offers an interesting alternative to dialdehydes and is not cytotoxic *in vitro*, but its biocompatibility has not been assessed yet (Mi *et al.*, 2000). Because of these issues with crosslinker toxicity, hydrogels need to be purified and verified prior to use to avoid problems arising from the presence of free unreacted crosslinkers. At the moment, the main drawback of these systems is the lack of safe, biocompatible covalent crosslinkers. This can be avoided by using reversible ionically crosslinked hydrogels.

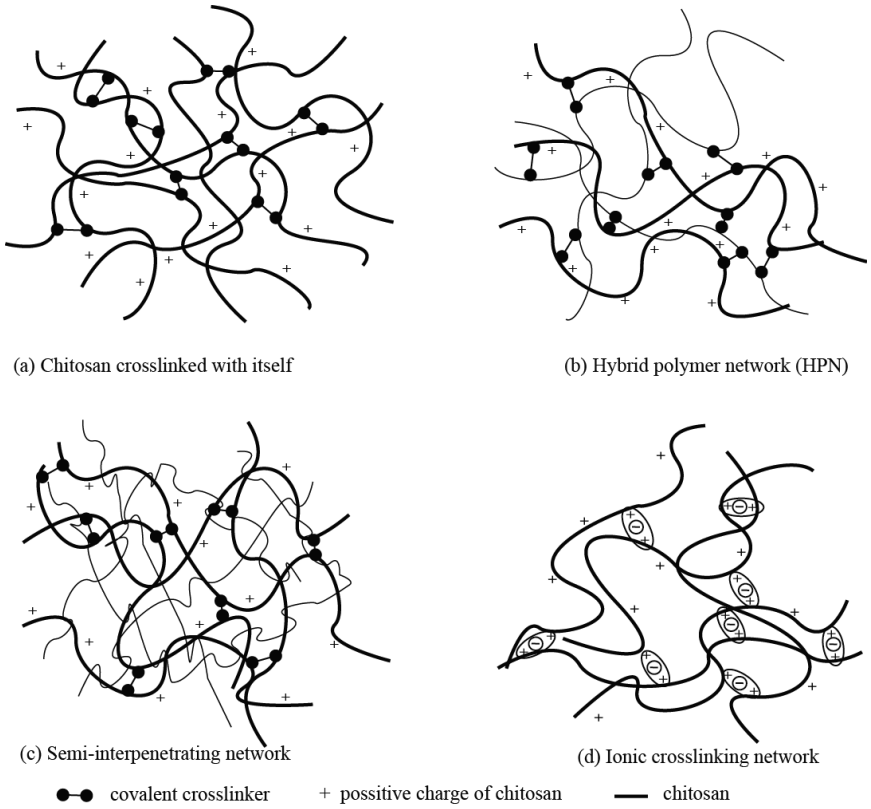


Figure 3.2 Different types of cross-linked chitosan hydrogels (Berger *et al.*, 2004)

In ionically cross-linked systems, the crosslinkers are either anions or anionic molecules that form a network by making ionic bridges between the cationic polymer chains. These interactions are similar to those formed in polyelectrolyte complexes (PEC), in which the crosslinkers have relatively high molecular weights. The main crosslinking interactions in the network are ionic bonds between the negative charges of the crosslinkers and the cationic ammonium groups of the chitosan chains. A range of crosslinkers have been examined for this purpose, including anionic complexes of metallic ions such as Mo(VI) (Draget *et al.*, 1992) and Pt (II) (Brack *et al.*, 1997). In addition, the suitability of sulfate, citrate and tripolyphosphate (TTP) anions as ionic

crosslinkers for chitosan/gelatin bead formation has been investigated (Shu & Zhu, 2002). Shchipunov et al. (2009) investigated the formation of chitosan hydrogels with some anionic polysaccharides such as alginate, hyaluronate, xanthan, lambda-carrageenan, and carboxymethylcellulose. The chitosan/glycerophosphate gelling system has been extensively studied, although glycerophosphate does not seem to induce ionic crosslinking (Chenite *et al.*, 2001; Kempe *et al.*, 2008; Ruel-Gariepy *et al.*, 2000; Zhou *et al.*, 2008).

The preparation of ionically cross-linked chitosan hydrogels is more straightforward than that of covalent systems. One method is to either dissolve (Ruel-Gariepy *et al.*, 2000) or disperse (Brack *et al.*, 1997; Draget *et al.*, 1992) the crosslinker in a chitosan solution. These methods produce relatively homogeneous hydrogels by random crosslinking. Alternatively, one can use a syringe to slowly add a chitosan solution to a crosslinker solution (Mi *et al.*, 1999a). This produces gel particles with an unreacted core and a highly cross-linked surface. Crosslinkers gradually diffuse into the core of these particles, making them more homogeneous over time. Shchipunov (2009) formed hydrogels by dispersing solid chitosan particles into anionic polymer solutions (e.g. alginate, xanthan, carrageenan) at pH values of 6-7, and then reduced the pH by adding solid glucono-δ-lactone (GDL) to dissolve and protonate the chitosan.

For gelation to occur, the crosslinkers must be negatively charged and the amino groups of the chitosan chains must be protonated. The charge density of anions in solution depends on their degree of oxidation and is independent of the pH, whereas the global charge of anionic molecules depends on their pK_a values and the pH of the solution. Therefore, the pH during gelation must be within the interval defined by the pK_a values of chitosan (pK_a = 6.5) and the crosslinkers; the pK_a value of the crosslinker should be lower than that of chitosan. It should also be noted that at pH values of more than 6.5, generic chitosan will precipitate. The need to maintain relatively acidic pH values reduces the biocompatibility of the hydrogels. However, chitosan can be stabilized in solution at neutral pH by adding glycerophosphate, which can increase the biocompatibility of the resulting gel (Chenite *et al.*, 2001).

Important properties of hydrogels such as their mechanical strength, swelling and rate of drug release are strongly influenced by their density of crosslinking. This in turn depends on the unit molar ratio and size of the crosslinkers, and on the global charges of chitosan and the crosslinkers (Remunán-López & Bodmeier, 1997). Smaller crosslinkers will diffuse more rapidly, increasing the rate of the crosslinking reaction (Mi *et al.*, 1999b). The global charge of chitosan and crosslinkers depends on the pH of the solution in which the gelation is performed. Therefore, the properties of the network can be modulated by adjusting the reaction conditions.

4. Rheological background

Rheology is the science of flow and deformation of matter, and rheological methods are useful in the characterization of gels, providing important information on the gelation process (kinetics) and the apparent gel equilibrium. Stress/strain controlled rheometers have recently become available commercially, making it possible to perform oscillatory measurements of hydrogels on a routine basis. In these measurements, the sample is subjected to a sinusoidal oscillating deformation (strain) with a known angular velocity (frequency * 2π) and the resulting force (stress) is monitored. Because a hydrogel is a viscoelastic material with both liquid-like and solid-like properties, the gel can be characterized in terms of its elastic (storage) modulus (G', which provides a measure of its solid-like properties), viscous (loss) modulus (G'', which provides a measure of its liquid-like properties), and phase angle (δ, the shift between applied deformation and the material's response).

For a perfect solid, the stress is in phase with the strain, with $\delta = 0^0$ and G'' = 0. The material then regains its original form when the force that caused the deformation is removed. For a perfect liquid, the stress is in quadrature with the strain, with $\delta = 90^0$ and G' = 0. This means that all of the energy used to deform the material is lost as heat. A real material will have a phase angle between 0^0 and 90^0 and will behave either as a solid or a liquid depending on the timescale of the rheological experiment. For instance, window glass is not a perfect solid, but rather exhibits fluid like behaviour over a long time scale. Similarly, over very short timescales (or equivalently in oscillatory

measurements, high frequencies) water can be regarded as a solid (Kavanagh & Ross-Murphy, 1998).

Gelation involves a gradual transition from a viscoelastic liquid to a viscoelastic solid and can be monitored using time sweep measurements or curing experiments. In curing experiments, a fluid system is loaded onto, for example, the lower plate of a cone-and-plate geometry. The upper cone is then lowered and oscillated at a fixed frequency and fixed maximum strain, and the response signal (whose phase and amplitude will differ from those for the cone) is monitored. Initially, the material will exhibit fluid properties and G'' will be greater than G'. Subsequently, both G' and G'' increase, but G' increases more rapidly than G''. Therefore, at some point there will be a crossover between G' and G''. This point is known as the gelling point, where G' = G'' and $\delta = 45^0$. G' and G'' continue to increase over time and then level off, at which point the system can be considered to be in equilibrium and stable (Figure 4.1, a). Once the gel has stabilized (i.e. when G' stops increasing significantly), the sample can be subjected to strain and frequency sweeps to investigate the gel's properties.

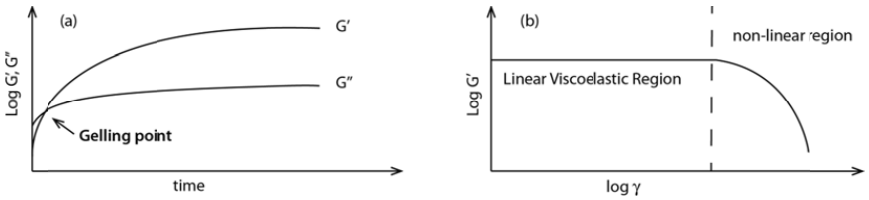


Figure 4.1 (a) Time sweep measurement (gelling kinetics), (b) Strain sweep

The data obtained from frequency sweeps can be displayed in mechanical spectra, i.e. plots of G' and G'' as functions of frequency. For a typical gel, the plot over a range of frequencies (for instance, from 0.01 to 10 Hz) consists of two nearly horizontal straight lines, showing that G' and G'' are independent of the frequency (Figure 4.2, a). G' is typically greater than G'' by a factor of 1-2 and both may increase slightly at high frequencies (Clark & Rossmurphy, 1987). Entanglement networks tend to behave as liquids at low frequencies but act like "false" gels (pseudogels) at higher frequencies (Figure 4.2, b).

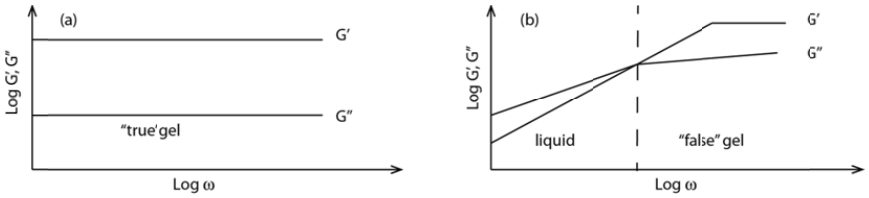


Figure 4.2 Mechanical spectra for (a) a typical gel and (b) an entanglement network.

A strain sweep (strain dependence) is used to determine the linear viscoelastic region (LVR), and also to qualitatively distinguish between strong gel and weak gels (Figure 4.1, b). In the LVR, G' is independent of the strain; for biopolymer gels, this region only extends to γ values of approximately 1 under exceptional conditions (Clark & Rossmurphy, 1987).

5. Alginate and Alginate gels

5.1. Alginate

Alginate is a linear copolymer of β-D-Mannuronic and α-L-Guluronic acid linked by 1-4 glycosidic bonds. It is a structural component in marine brown algae and a capsular polysaccharide in certain bacteria (Draget *et al.*, 2006). In brown algae, which are the current source of commercial alginates, it occurs as a mixed sodium-magnesium-calcium-strontium salt and the relative abundance of these ions in a given sample is probably determined by ion-exchange equilibria with the surrounding sea water (Haug & Smidsrød, 1967). It is likely that the structural role of alginates in algal cells is dependent on the accumulation of divalent ions from the environment and subsequent gel formation.

The biosynthesis of alginate begins with the enzymatically synthesis of mannuronan or polymannuronic acid from the precursor GDP-D-mannuronic acid (Lin & Hassid, 1966). However, mannuronan does not occur naturally except in certain mutated bacteria (Skjåk-bræk *et al.*, 1986). The mannuronan is then modified by a family of extracellular enzymes, the mannuronan-C5-epimerases, which flip the configuration of the C5 stereocenter and convert the monomer from β-D-Mannuronic acid to α-L-

Guluronic acid (Larsen & Haug, 1971). This also has the consequence that the $^4C_1$ conformation of the mannuronic acid residue is converted into the $^4C_1$ conformation of guluronic acid. Because several different epimerases act together in this process, there is a wide variety of alginates with different M and G contents and sequences. For example, the enzyme AlgE4 produces poly alternating (poly MG) alginate, whereas AlgE6 produces poly guluronan (poly G) (Ertesvåg *et al.*, 1995). Most studies on the mannuronan-C5-epimerases have been conducted using bacteria. However, some genes corresponding to bacterial epimerase genes have been found in brown algae (Nyvall *et al.*, 2003). It is therefore believed that the biosynthesis of alginates in seaweed is similar to that in bacteria (Draget *et al.*, 2006).

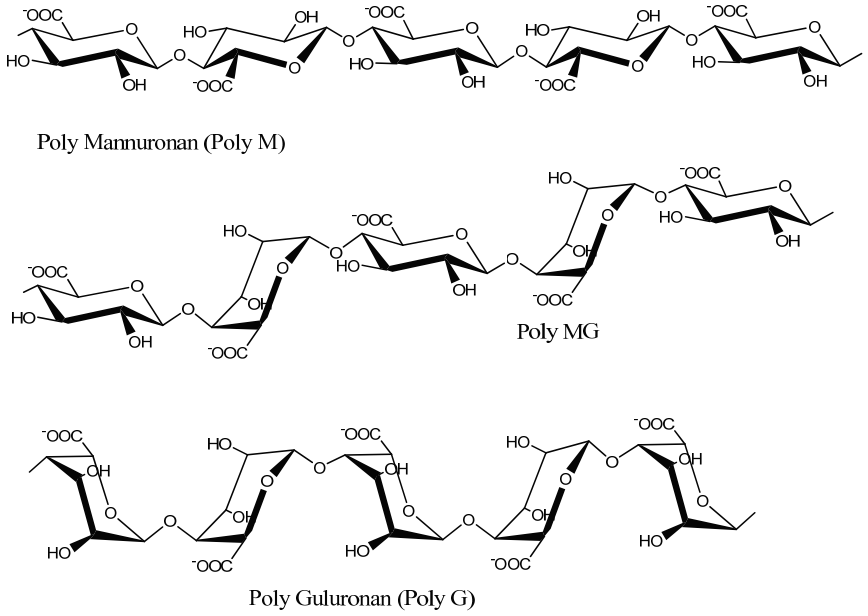


Figure 5.1 Chemical structures of some alginates: poly M, poly MG and poly G

Alginates have a block-type structure with two homopolymeric blocks (G- and M-block) and one alternating block (MG-block). The content and sequence of the two monomers strongly affects the properties of alginates, and so there is a need for

parameters that describe accurately their composition. The fractions or frequencies of M and G residues in a given alginate sample can be determined and are defined as follows:

$$F_G = \frac{n_G}{n_G + n_M} \quad F_M = \frac{n_M}{n_G + n_M}$$

More detailed information about the sequence can be obtained from the fractions of diads (e.g. MM, MG) or triads (e.g. MMM, GGG, MMG), which can be determined by NMR spectroscopy (Grasdalen, 1983; Grasdalen et al., 1979). Recently, Aarstad et al. (2012) proposed a new enzyme-based method of alginate sequencing. In this method, alginate is degraded into smaller fragments using specific lyases. These fragments are then analyzed by ^{1}H NMR spectroscopy, HPAEC-PAD and SEC-MALLS. This yields information on the distribution of block lengths for each of the three block types (M, G, and MG blocks) within the sample.

As discussed above, poly M, β-D-Mannuronic acid exists in the $^4C_1$ conformation. Consequently, all of the glycosidic linkages in poly M are diequatorial and the bond length is 0.515 nm (Atkins *et al.*, 1970). This is almost identical to the length of the glycosidic bond in chitosan, i.e. 0.516 nm (Minke & Blackwell, 1978). In poly MG and poly G, α-L-Guluronic acid exists in the $^1C_4$ conformation and the glycosidic linkages are diaxial (GG), equatorial-axial (MG) and axial-equatorial (GM). The glycosidic bond length in GG is 0.435 nm (Atkins *et al.*, 1970) which is shorter than that in poly M.

Alginic acid and its salts with divalent metals (aside from Mg) are insoluble in water, so studies on the properties of alginate solutions have been confined to alginates of monovalent metals, e.g. sodium (Smidsrød, 1975). The pK_a values of the two monomeric acids are 3.38 and 3.65, respectively (Haug, 1964). The pK_a values of polymeric alginates differ slightly from these values, depending on the ionic strength of the solution and the alginate concentration (Haug, 1964). Alginates in solutions with pH values that are below their pK_a tend to precipitate or to form gels.

5.2. Alginate gels

In biomedicine, alginate is often used in hydrogels to promote wound healing, for drug delivery, and in tissue engineering (Lee & Mooney, 2012). Like chitosan hydrogels,

alginate hydrogels can be formed by either ionic (physical) or covalent (chemical) cross-linking.

The most widely used crosslinkers in the preparation of ionically cross-linked alginate hydrogels are divalent ions (e.g. Ca^{2+}). The divalent ions are believed to bind strongly and in a cooperative manner to the G-blocks of alginate, so alginate samples with higher G contents will form stronger gels with Ca^{2+}. Directly mixing calcium-containing solutions (typically made using $CaCl_2$) with aqueous alginate solutions produces precipitates (usually in the form of lumps or fisheyes) rather than gels due to the rapid and irreversible formation of strong junctions between polymer chains. Therefore, there are two ways of making Ca-alginate gels: the dialysis method and the internal gelation method (Draget *et al.*, 2006).

In the dialysis method, an aqueous alginate solution is typically extruded into a $CaCl_2$ solution. The resulting gels are not homogeneous, having a denser outer envelope and a thinner center due to the inwards diffusion of Ca^{2+} and the outward movement of alginate chains from the center to the gelling zone. The homogeneity of the gel can be modulated by carefully selecting the molecular weight of the alginate used and the concentration of the alginate solution, as well as the concentrations of the gelling and non-gelling ions (Skjåk-Bræk *et al.*, 1989).

In the internal gelation method, inactive forms of the cross-linking ions (complexes of phosphate, citrate, EDTA or insoluble salts such as $CaSO_4$, $CaCO_3$) are mixed with alginate solution. A reagent that slowly releases protons, such as glucono-δ-lactone (GDL) is then added. The resulting gradual reduction in the solution's pH will cause the dissociation of the Ca^{2+} salt, initiating the gelation of the alginate solution (Draget *et al.*, 1989). The final pH values of alginate gels can be controlled by adjusting the relative proportions of calcium salt and GDL (Draget *et al.*, 1991). However, if the final pH is lower than the pK_a value of alginate (pK_a = 3.5), the alginate will be protonated to alginic acid, which may result in the formation of an alginic acid gel (Draget *et al.*, 1994). The advantage of this method is that it produces homogeneous alginate gels because of the relatively slow gelation process.

Covalent cross-linking has been investigated extensively as a potential way of improving the physical properties of alginate gels (Lee & Mooney, 2012). Covalently cross-linked alginate gels can be formed with adipic hydrazide, methyl ester L-lysine and polyethylene glycol (PEG) (Eiselt *et al.*, 1999; Lee *et al.*, 2000).

In aqueous solution, alginate is an anionic polymer at pH above its pK_a, while chitosan is a cationic one at pH below its pK_a. In the pH interval defined by the pK_a values of alginate (3.5) and chitosan (6.5), a direct mixing of alginate and chitosan solutions will result in precipitation. Therefore, alginate is often incorporated with chitosan in microcapsules or chitosan-coated alginate beads. Microcapsules can be prepared by dripping alginate solution into a chitosan solution that contains $CaCl_2$ (Gåserød *et al.*, 1998; Lucinda-Silva *et al.*, 2010). Ca-alginate beads can be formed in $CaCl_2$, after which the beads are incubated in chitosan solution to coat them with chitosan (Huguet & Dellacherie, 1996). Alginate-chitosan systems have some attractive physical and biological properties and have been used as carriers for the controlled release of proteins and drugs (Lucinda-Silva *et al.*, 2010).

Objectives of the thesis

This project has been a part of the project "*Improving training and research capacity of the Nha Trang University, Vietnam – Phase 2*". The main objectives of this thesis are to characterize Vietnamese chitin raw materials, especially in relation to the isolation of high quality chitin, to study the conversion of chitin to chitosan by performing a detailed study of the de-*N*-acetylation reaction with the chitin disaccharide as a model substance, and finally to investigate an advanced application of a new chitosan – alginate gelling system.

Results and Discussion

6. Characterization of Vietnamese shrimp shells and heads

Because of manual shrimp production in Vietnam, head and shell is removed in two different operation units meaning that head and shell can be separately collected in the processing areas. At present, most of shrimp by-product (head and shell together) has been used for chitin production using chemical procedures. In paper 1, we have characterized separately head and shell from the two major species of Vietnamese aquacultured shrimps (black tiger shrimp, *Penaeus monodon*, and white shrimp, *Penaeus vannamei*). Moreover, we determined the conditions for isolation of a pure chitin with high molecular weight.

6.1. Chemical composition of shrimp shells and heads

Table 6.1 Chemical composition of the shells and heads of WS and BT as percentages of their dry weight.

Composition	White shrimp (WS)		Black tiger shrimp (BT)	
	Shell (WS-S)	Head (WS-H)	Shell (BT-S)	Head (BT-H)
Ash	26.66 ± 1.81^{a}	25.61 ± 1.03^{a}	32.18 ± 1.13^{b}	27.09 ± 1.58^{a}
Protein	33.11 ± 1.56^{a}	44.39 ± 0.50^{b}	34.92 ± 0.59^{a}	48.56 ± 1.33^{c}
Chitin	27.37 ± 1.82^{a}	11.40 ± 1.83^{b}	29.29 ± 1.78^{a}	12.14 ± 0.94^{b}

* Values are given as means ± standard deviation (n = 3 – 6)

** Different superscript in the same row indicate significant differences ($p < 0.05$)

The three main chemical components of shrimp heads and shells are shown in Table 6.1. It was found that the two species yielded by-products with very similar compositions. However, the ash and protein content of BT waste was slightly higher than that of WS waste. These results are consistent with those of Ngoan et al. (2000), who investigated the chemical compositions of by-products from three Vietnamese shrimp species. There is a striking difference between the chemical compositions of the heads and the shells: the protein contents of the heads were 48.56 % (BT) and 44.39% (WS) whereas those of the shells were noticeably lower: 34.92 % for BT and 33.11%

for WS. The chitin contents of the shells were 29.29% (BT) and 27.37% (WS), while those of the heads were only 12.14% (BT) and 11.40% (WS). The ash contents of the heads and shells were quite similar in both shrimp species, but BT has a slightly higher overall ash content than WS due to its thicker and more rigid shell. Because the heads and shells have largely different compositions, it is possible that the optimal conditions for chitin isolation will differ in the two cases. However, no investigation along these lines was undertaken.

The data on the amino acid composition (Table 2, paper 1) and heavy metal content (Table 3, paper 1) of the heads and shells show that shrimp by-products could be a useful protein source with an acceptably low heavy metal content. Chitin is thus not the only potentially valuable substance that could be isolated from shrimp by-products.

6.2. Isolation and characterization of chitin from shrimp heads and shells

Separate studies were conducted on the heads and shells from the two shrimp species in order to identify conditions that would yield isolated chitin of a high quality. A goal when developing the isolation conditions was that the ash and protein content of the chitin should be minimized without disrupting the chitin's molecular weight and DA.

For shrimp shell, DM was carried out in 0.25 M HCl at room temperature and the ash content of the isolated chitin was monitored as a function of time. As can be seen in Figure 6.1 (a), the demineralization process was quite effective when applied to WS shells, reducing the ash content of the chitin to less than 3% within 3h and then slowly reducing it further to around 1.5% within 24h. However, the process was much slower with BT shells (Figure 6.1, b): in this case, the ash content of the chitin was 7% after 7 hours of acid treatment and 5% after 24 h. This is probably due to the difference in the thicknesses of the two species' shells - BT shrimp are larger than WS. However, differences in the properties of the biomaterials (such as the chitin-protein interactions involved in each case) may also contribute.

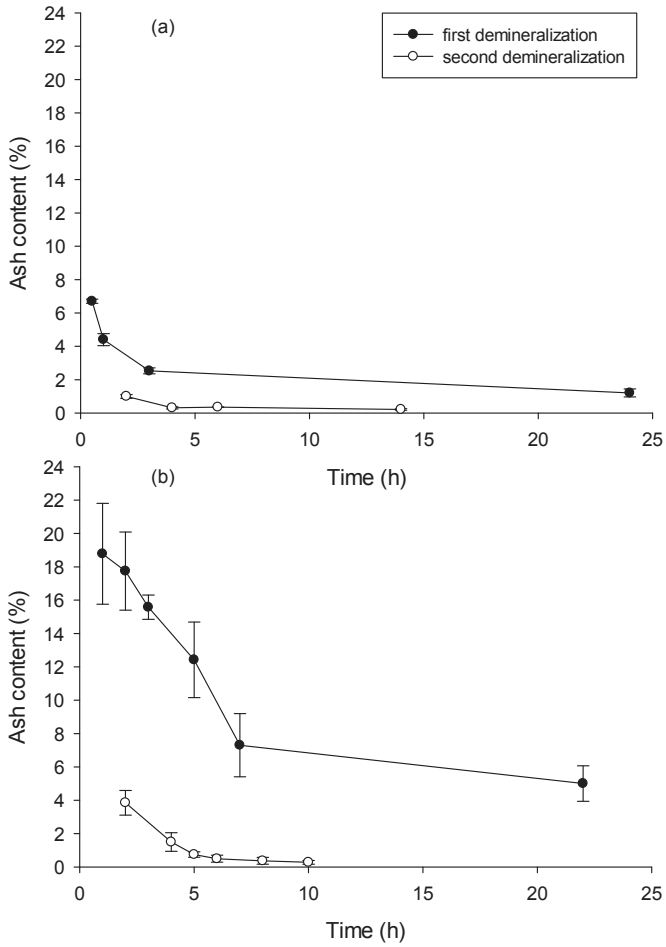


Figure 6.1 Effect of acid treatment on the ash contents of chitin from WS (a) and BT shells (b) as a function of time

Because of the relatively high ash contents of the shell chitin samples after the first acid treatment (1.5% and 5% after 24h for WS and BT shells, respectively), a second demineralization step was performed (after initial DM steps of 3h and 7h for WS and BT, respectively). This second acid treatment reduced the ash content of the chitin to less than 0.3% after 4h and 10h for WS and BT, respectively.

In conclusion, if chitin with an ash content of less than 1% is required, it is necessary to perform two successive DM steps when working with WS and BT shells. The total time required is 5h for WS and 12h for BT. Lower ash contents (0.3%) can be achieved with total acid treatment times of 7h and 17h for WS and BT shells, respectively. The acid treatment time (with 0.25M HCl) used in this work was longer than those used in previous studies (Percot *et al.*, 2003b; Rodde *et al.*, 2008), presumably because the previous works dealt with species that had thinner shells. If the shells were ground into a very fine powder (<80µm) prior to demineralization, complete DM was achieved with excess 0.25M HCl within 15 min. Rodde et al. examined shells obtained by processing small Northern shrimp species (230-260 shrimps/kg) with peeling machines and performed DM using a two step process with cold 0.25 M HCl with an initial treatment of 5 min and a second treatment of 30 min. However, no data on the ash content of the resulting chitin was reported. The shrimp species examined in our studies were substantially larger (50-60 and 30-40 shrimps/kg for WS and BT, respectively) and had thicker shells, which is presumably the reason for the longer DM times required.

We initially attempted demineralization of the shrimp heads using 0.5 M HCl at room temperature. However, under these conditions, the first demineralization step proceeded very slowly (data not shown). We therefore conducted DM at a higher HCl concentration (0.75M). Even after having taken this measure, it remained difficult to reduce the ash content of the isolated chitin to less than 1%. As shown in Figure 6.2 (a), the chitin from WS heads had an ash content of more than 3% after 24h treatment. In the second demineralization step (following an initial 8h DM step), the ash content of the chitin from WS heads decreased to around 1% after 20h. A similar trend was observed for BT heads, for which the chitin ash content was only reduced to around 1% after a second demineralization step of around 10h (with an initial DM period of 15h) (Figure 6.2, b)

In general, less severe DM conditions are required for shells than for heads in order to produce chitin with a low ash content. This may be due to the thicker and more complicated structure of the shell around the head, which restricts the accessibility of the chitin to the solvent, and hinders the diffusion of Ca^{++} and CO_2 to and from the bulk

solution. The relatively high lipid and protein contents of the heads may also contribute to these effects, further reducing the efficiency of the DM process.

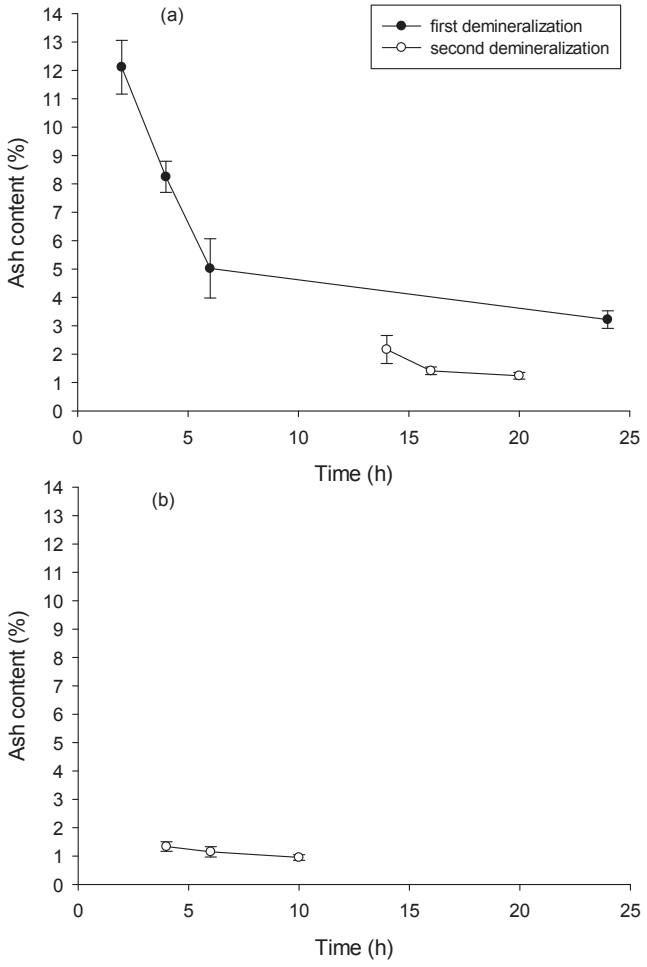


Figure 6.2 Effect of acid treatment on the ash contents of chitin from WS (a) and BT heads (b) as a function of time

When performing chemical isolation, the DM step can cause significant depolymerization of chitin chains. For instance, treatment of chitin with 3M HCl for

24h resulted in extensive depolymerization (Einbu *et al.*, 2004). The intrinsic viscosities and molecular weights of the isolated chitin samples obtained using our protocols are shown in Table 6.2. The WS shell chitin had the highest average molecular weights (1356, 1259, and 1110 kDa) after second demineralization steps of 4, 6, and 15h, respectively. The BT shell chitin samples had similar average molecular weights, ranging from 1112 to 1149 kDa. These values are somewhat higher than those reported for chitin isolated from northern shrimp, which range from 940 to 1060 kDa (Rodde *et al.*, 2008) but slightly lower than the highest reported value of 1508 kDa (Percot *et al.*, 2003b) (which was ourselves determined for chitin with a maximum intrinsic viscosity of 4400 mL/g after 60 minutes of DM from the data of Percot et al. and a molecular weight calculated using the MHS equation proposed by Terbojevich et al. (1988)). The chitin samples obtained from WS and BT heads had average molecular weights of 949 and 1021 kDa, respectively, which is slightly lower than the values obtained for shell chitin. This is probably due to the higher acid concentrations and longer DM times required when working with heads.

Table 6.2 Intrinsic viscosities and molecular weights of the isolated chitins

Chitin from	First DM (h)	Second DM (h)	Viscosity (mL/g)	Viscosity-average MW (kDa)*
WS-S	3	4	1479 ± 20	1356
	3	6	1406 ± 67	1259
	3	15	1291	1110
BT-S	7	4	1292 ± 12	1112
	7	6	1321 ± 31	1149
	7	8	1307 ± 41	1131
WS-H	8	16	1160 ± 11	949
BT-H	15	6	1219	1021

* calculated using the Mark-Houwink-Sakurada (MHS) equation with K=0.10, a=0.68 (Einbu *et al.*, 2004)

After DM under optimal conditions, the decalcified shrimp shells and heads were deproteinized in dilute NaOH solution. It was expected that DPr under alkaline conditions would have negligible effects on the molecular weight of the chitin

compared to those of acid-mediated DM, but that it would have a more severe impact on the degree of acetylation (Rodde *et al.*, 2008). We used a 2% NaOH solution for the shells and a 3% NaOH solution for the heads, with both processes being performed at 75 °C. Table 6.3 shows the protein contents and degrees of acetylation (DA) of the isolated chitins. For shells, alkali treatment for 10h was sufficient to reduce the protein level to less than 1%. For heads, longer treatment times with NaOH were required to reduce the protein content of the chitin to below 1%, as would be expected given the higher protein content of the raw material.

Table 6.3 Characterization of the isolated chitin samples

Chitin from	Deproteinization time (h)	Protein content (%)	Degree of acetylation
WS-S	8	1.11 ± 0.06	n.d.
	12	0.83 ± 0.02	0.982
	15	0.71 ± 0.03	0.988
BT-S	8	1.17 ± 0.05	n.d.
	10	0.52 ± 0.13	0.981
	12	0.38 ± 0.03	0.971
WS-H	9	1.23 ± 0.17	n.d.
	15	0.87 ± 0.17	0.962
BT-H	8	1.12 ± 0.09	0.973
	12	1.16 ± 0.18	0.968

n.d.: not determined

Alkaline deproteinization can cause de-*N*-acetylation, which will reduce the degree of acetylation (DA) of the isolated chitin. The DA values of the isolated chitin samples are shown in Table 6.3. All of the samples have similar and high DA values, ranging from 96% to 99%. This indicates that the rate of de-*N*-acetylation under the DPr conditions used is low. Notably, these DA values are somewhat higher than that of 96% reported by Einbu & Vårum (2008) for the DA of chitin from shrimp shells. The DA values presented in this work were determined using the method proposed by Einbu & Vårum (2008), so the difference in the DA values presented here and those determined in the

previous study (Einbu et al., (2008) is probably due to the different equations used when calculating the DA value. The ^{1}H NMR spectrum of a chitin sample isolated from WS shells in concentrated DCl at 25 °C is shown in Figure 6.3. This spectrum was used in conjunction with equation (1) to calculate the sample's DA value from the intensities of resonances arising from the protons of the acetyl, acetate groups and H-2/6. In contrast, Einbu & Vårum determined their DA values using the resonances arising from H-1 and H-2.

$$DA = \frac{2\ x\ (I_{Acetyl} + I_{Acetate})}{(2\ x\ I_{H-3*} + I_{H-2/6} + I_{H-2-D})} \qquad (1)$$

The data on DA values of the isolated chitins also show that the deproteinization conditions used in this study caused very little de-*N*-acetylation, which is consistent with the conclusions of Percot et al. (2003b). The highly acetylated chitins isolated from Vietnamese raw materials using the conditions described herein are very well suited as raw materials for the production of N-acetyl glucosamine in high yields (Einbu & Vårum, 2008).

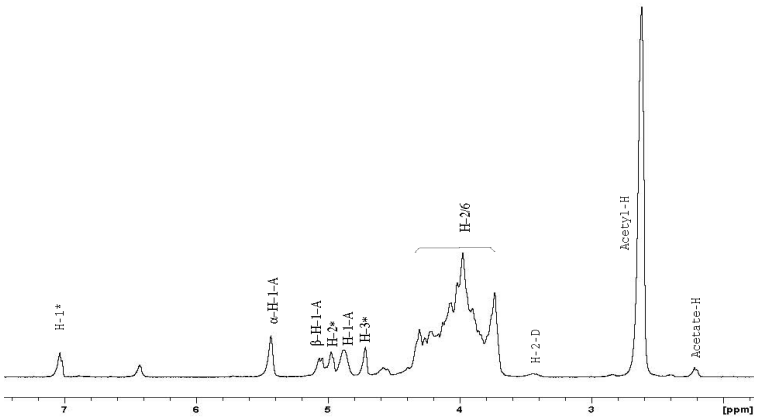


Figure 6.3 ^{1}H-NMR spectrum of chitin from WS shells in concentrated DCl (heated to 40 °C for 30 minutes before measuring) recorded on a 300 MHz spectrometer. Peaks arising from the glucofuranosyl oxazolinium ion are indicated with asterisks.

7. De-N-acetylation of chitin disaccharide

Chitosan is prepared from chitin by de-*N*-acetylation, which can be performed homogeneously or heterogeneously. Paper 2 describes a detailed investigation into homogeneous de-*N*-acetylation using chitin disaccharide as a model substance.

7.1. ^{1}H-NMR spectrum of chitin disaccharide in NaOD/D_2O

Prior to the publication of paper 2, the ^{1}H NMR spectrum of chitin disaccharide (GlcNAc-GlcNAc or **AA**) in NaOD had not been reported. Therefore, the resonances in the spectrum were assigned by comparison to the spectra of N-acetyl glucosamine (GlcNAc or **A**) and chitosan disaccharide (GlcN-GlcN or **DD**) at the same NaOD concentration. Figure 7.1 shows the ^{1}H NMR spectra of **AA** (top), **A** (middle) and **DD** (bottom) in 2.77 M NaOD at 25 °C. The spectra of **AA** and **A** in deuterated aqueous

Table 7.1 ^{1}H NMR chemical shifts of different resonances of chitin/chitosan disaccharides in 4.13 M NaOD at 45 ^{o}C

Chemical group	^{1}H chemical shift (ppm)
Acetate	1.93
Acetyl	2.03, 2.07
H-2 (**D**), non-reducing end	2.57
H-2 to H-6	3.21 – 3.92
H-1 (**D**) non-reducing end	4.37
H-1 (**A**) non-reducing end	4.48
β-H-1 (**AD**)	4.58
β-H-1 (**DD**)	4.60
β-H-1 (**AA**)	4.78
β-H-1 (**DA**)	4.80

sodium hydroxide solution are remarkably similar to those obtained in deuterated water (Sugiyama *et al.*, 2001; Vårum *et al.*, 1991a), considering that the hydroxyl groups are

(partially) ionized in NaOD solution. The resonance at ca. 4.5 ppm was assigned to the H-1 proton at the non-reducing end because it does not appear in the spectrum of **A**. Three resonances arising from the acetyl protons can be seen: one from the acetyl at the non-reducing end and two reducing end acetyl resonances (α and β anomers). These acetyl protons are well separated from the acetate protons (at 1.93 ppm), making it easy to monitor the de-*N*-acetylation reaction. The β anomeric proton is assigned to the resonance at ca. 4.8 ppm, while the α anomeric proton is superimposed on the strong solvent signal at ca. 5.2 ppm. The signals of H-2 to H-6 are not well separated and appear between 3.2 ppm to 3.9 ppm in the spectrum.

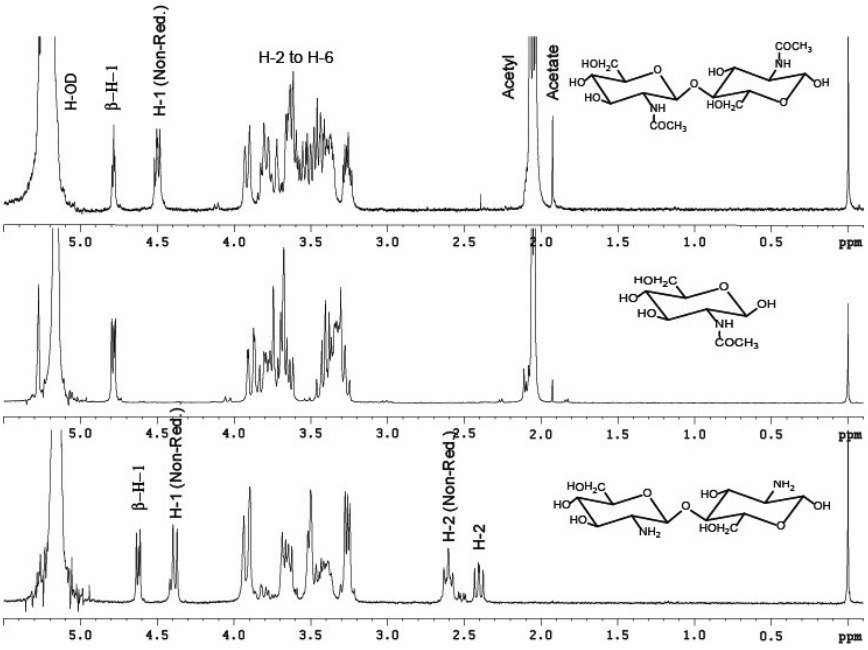


Figure 7.1 ^{1}H NMR spectra at 400 MHz of AA (top), A (middle) and DD (bottom) in 2.77 M NaOD recorded at 25 ^{o}C. Chemical shifts are given relative to TSP at 0.00 ppm.

The spectrum of **DD** in D_2O-NaOD is also very similar to the spectrum of the same compound in deuterated water (Sugiyama *et al.*, 2001; Vårum *et al.*, 1991a). The

resonances of the β anomeric proton and the non-reducing end H-1 are shifted slightly upfield relative to those in the spectrum of **AA**. The signals of the reducing and non-reducing end H-2 protons are well separated and shifted further upfield from those of H-3 to H-6, appearing at 2.4 and 2.6 ppm, respectively. The chemical shifts of the resonances of chitin/chitosan dimers in 4.13 M NaOD are shown in Table 7.1.

7.2. Kinetics of de-*N*-acetylation of the chitin disaccharide

The de-*N*-acetylation of the chitin disaccharides was followed by time-resolved NMR. The lower part of Figure 7.2 shows the spectra of **AA** at 4.13 M NaOD and 45 °C after 10 min and 1000 min, while the upper section of the figure shows time-resolved stacked plots for the anomeric region and the acetyl/H-2 (deacetylated) region.

As the de-*N*-acetylation of the dimer proceeds, new resonances appear in the spectrum. In the acetyl region, the intensity of the acetyl resonances (at ca. 2 – 2.1 ppm) decreases with a concomitant increase in the intensity of the resonances from the acetate protons (at ca. 1.9 ppm). In the anomeric region, the β anomeric H-1 protons of the newly-formed **DA**, **AD**, and **DD** dimers appear. The resonance of β-H-1 of **DA** is shifted downfield relative to that of **AA**, and is assigned as the signal at 4.8 ppm. The resonances of β-H-1 of **AD** and **DD** are shifted upfield relative to **AA**, appearing at 4.58 (**AD**) and 4.60 ppm (**DD**) (Table 7.1, Figure 7.2).

Interestingly, the resonances of the reducing end H-2 protons of the deacetylated dimers (**AD**, **DD**) did not appear in the time-resolved spectra, in which the resonances should be at ca. 2.4 ppm (Figure 7.1, bottom part). We also noticed that all of the β anomeric H-1 protons appeared as singlets rather than doublets. This is presumably due to the Lobry de Bruyn-Alberda van Ekenstein (LdB-AvE) epimerization and aldose-ketose interconversion reactions (Angyal, 2001). Since carbon C-2 of the chitin/chitosan oligosaccharides is connected to an amino or acetamido group rather than a hydroxyl group, only the epimerisation reaction is relevant in this case. Based on the ^{1}H-NMR spectra of the D-glucosamine monomer in 2.77 M NaOD-D_2O at different points in time it was determined that the H-2 resonance gradually decreased with time and the form of the H-1 resonance gradually changed from doublets to singlets. This is due to the ene-

diol equillibrium reaction, which causes a loss of stereochemical information at carbon C-2 and gradually replaces H-2 with deuterium from the solvent (deuterated water). Since deuterium will not be detected in the ^{1}H-NMR spectra and will not couple to the neighboring protons, this explains the change in the glucosamine spectrum over time as well as the absence of a signal arising from the reducing H-2 proton in the time-resolved spectra.

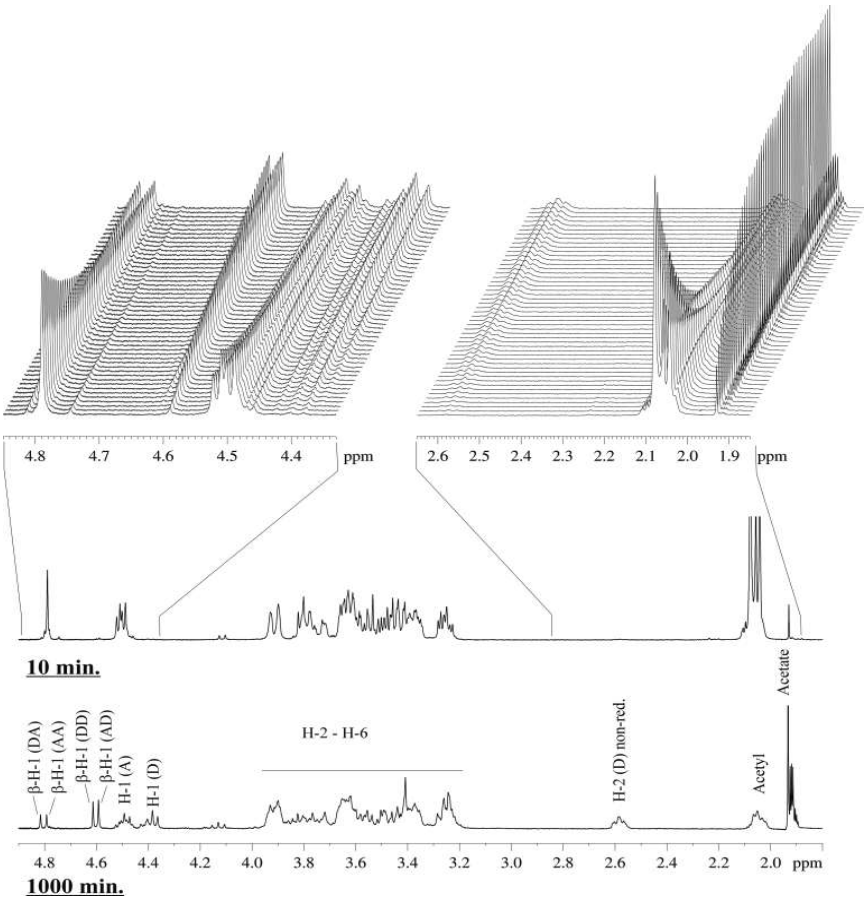


Figure 7.2 Time-resolved ^{1}H NMR spectra of AA in 4.13 M NaOD at 45 ^{o}C

In the anomeric region, we can identify separate resonances of β-H-1 from all four dimers (**AA**, **DA**, **AD** and **DD**), making it possible to determine the different rates of de-

N-acetylation of the chitin disaccharides (Table 7.1, Figure 7.2). In total, there are four different rate constants for de-*N*-acetylation. However, we only determined the initial rates of the first de-*N*-acetylation steps, i.e. the rate of hydrolysis of an acetyl group from the reducing or the non-reducing end:

$$\boldsymbol{AA} \xrightarrow{k_{DA}} \boldsymbol{DA} \qquad \boldsymbol{AA} \xrightarrow{k_{AD}} \boldsymbol{AD}$$

Here, k_{DA} and k_{AD} are the rate constants for the de-*N*-acetylation of the non-reducing and the reducing end sugar unit, respectively.

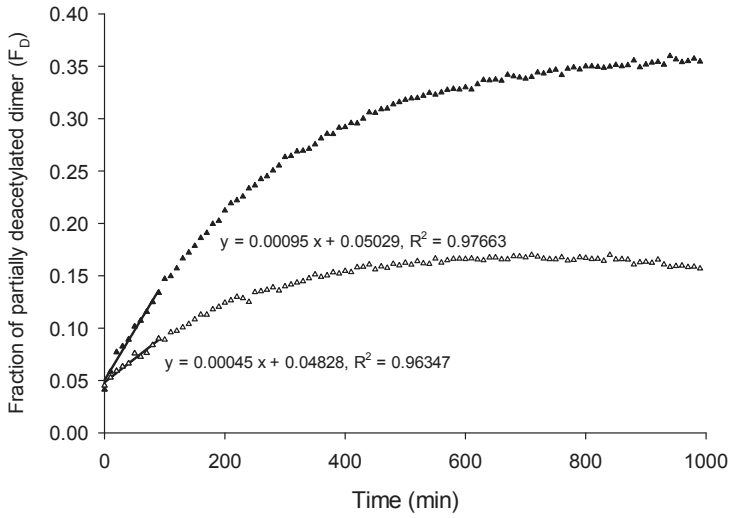


Figure 7.3 Molar fraction of the partially de-*N*-acetylated dimers AD (▲) and DA (Δ) as a function of time in 4.13 M NaOD at 45^{0}C.

Figure 7.3 shows the molar fraction of **AD** and **DA** as a function of time based on the relative intensities of the corresponding β-H-1 signals to the total intensities of all four β-H-1 signals. Note that in both cases, the extent of deacetylation will increase towards a maximum as the **AD** and **DA** dimers are gradually converted to the doubly-deacetylated dimer **DD**. Clearly, the de-*N*-acetylation of the non-reducing end is faster by a factor of around 2 (rates were determined from the initial slopes of the two curves shown in Figure 7.3). The activation energy of de-*N*-acetylation in 2.77 and 5.5 M

NaOD calculated from the Arrhenius plot were 114.4 and 98.6 kJ/mol, respectively. These results are consistent with a previous publication (Sannan *et al.*, 1977) in which the activation energy of chitin deacetylation was stated to be 22 kcal/mol (92 kJ/mol).

We also investigated the de-*N*-acetylation reaction of chitin in NaOD using time-resolved ^{1}H NMR. A chitin solution in NaOD-D_2O was prepared using the method described by Einbu (Einbu *et al.*, 2004). Figure 7.4 shows the spectra of chitin and chitin dimers in 2.77 M NaOD at 25 °C. The resolution of the resonances in the spectrum of chitin was not as good as for the chitin disaccharide. However, the acetyl resonance is well separated from the acetate peak, making it possible to calculate the degree of deacetylation as a function of time.

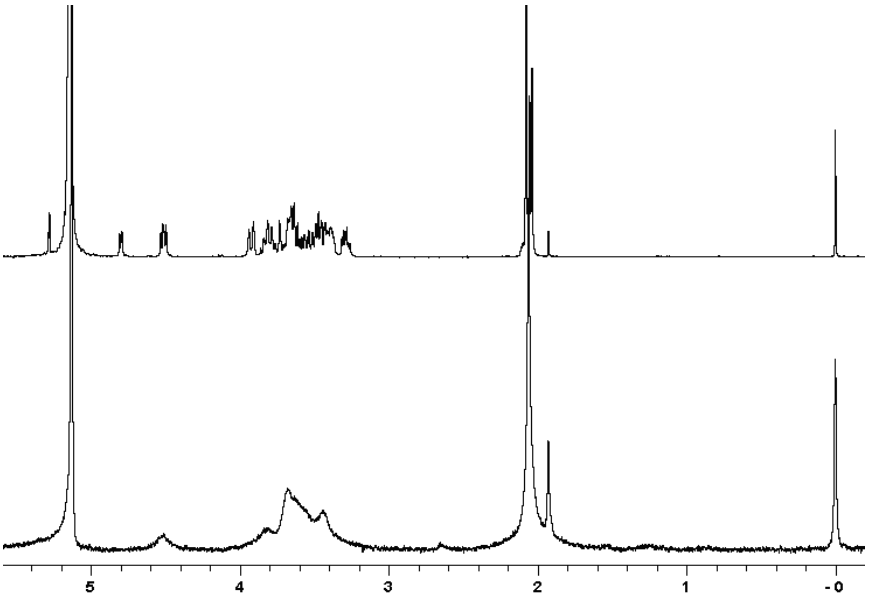


Figure 7.4 ^{1}H NMR spectra of chitin disaccharide AA (top) and chitin (bottom) and in 2.77 M NaOD at 25 ^{o}C.

The initial rate of de-N-acetylation of chitin is very similar to that of chitin disaccharide (0.116 and 0.108, respectively) as shown in Figure 7.5. This suggests that the chitin

disaccharide is indeed a good model substance for studying the de-*N*-acetylation of chitin.

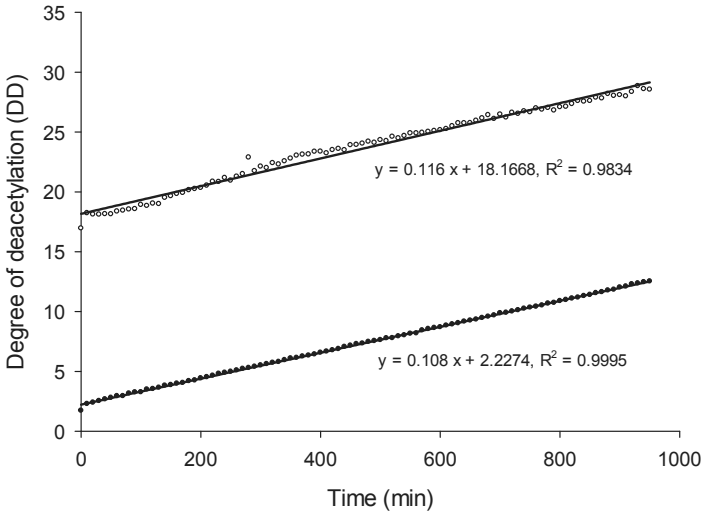


Figure 7.5 Degree of deacetylation of chitin (top) and chitin disaccharide AA (bottom) as a function of time in 2.77 M NaOD at 25 ^{o}C

7.3. Mechanism of de-*N*-acetylation of chitin disaccharide

A previous study on the kinetics of chitin deacetylation by Sannan et al. (1977) found that the reaction exhibits pseudo-first-order kinetics with respect to the concentration of acetamide groups. However, these authors did not report data on the order of the reaction with respect to the hydroxide concentration. We therefore conducted experiments to obtain this information.

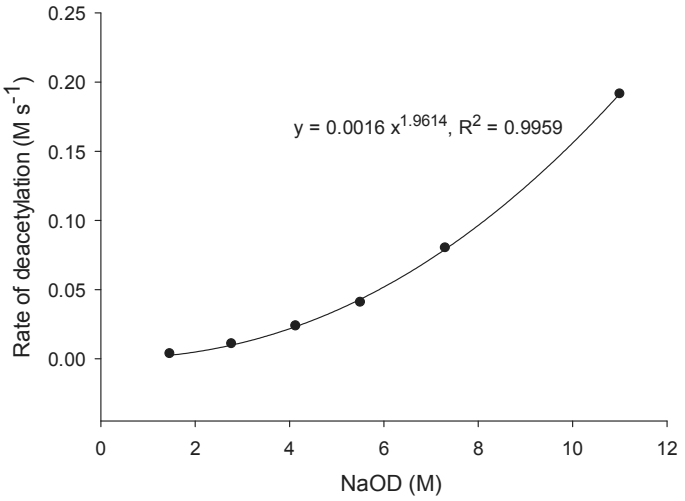
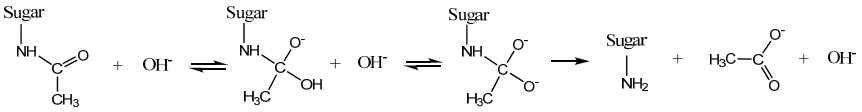


Figure 7.6 Rate of de-*N*-acetylation (at 25 °C) as a function of NaOD concentration

Figure 7.6 shows the reaction rate as a function of NaOD concentration. Clearly, the rate increases non-linearly with increasing base concentration, and the best power fit of the curve gave an exponent of 1.96. Thus, the de-*N*-acetylation reaction of chitin dimer is second order with respect to the concentration of hydroxide ion, involving two hydroxide ions and a doubly-charged intermediate (Scheme 7.1). This may explain why the heterogeneous reaction is slow, and why the rate-determining step of the heterogeneous reaction is the swelling step, i.e. the step that permits the diffusion of the hydroxide ion into the insoluble crystalline chitin molecules.



Scheme 7.1. Proposed reaction mechanism for alkaline de-*N*-acetylation

8. Chitosan and Alginate gelling system

Chitosan and alginate are both biopolymers which have attracted much attention in relation to their ability to form hydrogels. Both in chitosan and poly-mannuronate (poly-M) the sugar units occur in the $^4C_1$ conformation, and are linked through β-(1-4) glycosidic linkage where the sugars are rotated by 180^0 relative to its neighbor. Thus, every second sugar unit in the chain of chitosan or poly-M will have a charged group pointing to the same side, with a distance between the charges of 10.3 – 10.4 Å. This distance is the same as the length of a disaccharide unit (Atkins *et al.*, 1970; Minke & Blackwell, 1978).

In paper 3, we report on a new gelling system that is composed of chitosan and alginate, either poly-M and chitosan oligomers (CO) or chitosan and mannuronan oligomers (MO). In both systems, the components are mixed at a pH where the chitosan/chitosan oligomers are almost uncharged, while poly-M/mannuronan oligomers are fully negatively charged. The neutral-soluble medium-viscosity chitosan has a degree of acetylation of 40% so that it is soluble at a pH well above the pK_a-value of the amino group. The pH is subsequently lowered by adding a proton donating substance, D-glucono-δ-lactone (GDL), which releases H^+ in a controlled manner so that the amino groups of chitosan/CO become positively charged and a chitosan-alginate gel is formed. The kinetics of gelling, the effect of GDL concentration and ionic strength on the gel strength have been investigated. The importance of the matching distance between positive and negative charges was also tested.

8.1. Gelation kinetics and characterization of the gels

The gelation kinetics of alginate – CO and chitosan – MO gels made with different concentrations of oligomers are shown in Figure 8.1. In the early stages, there is a clear and substantial difference in the initial gelling rates (the slope of the curve) of the two gels. In both systems, the rates increase with increasing oligomer concentration. This can be attributed to the higher number of junctions formed in the network as the crosslinker concentration is increased, which would increase the gel's strength (G').

Because a fixed chitosan concentration (0.9 mg/mL final gel) was used when preparing the chitosan–MO gels, the same amount of GDL was used in all cases (4.8 mg/mL final gel). Conversely, the CO concentration was varied when preparing the alginate – CO gels, so in this case we maintained a fixed GDL-CO ratio in order to achieve a similar final pH in all experiments. This means that a higher GDL concentration was used with a higher chitosan oligomer concentrations. The higher GDL concentrations may have increased the rate of gelling in poly-M – CO gels prepared with a higher concentration of chitosan oligomers. In homogeneous internally-gelled Ca-alginate gels, the dissolution and protonation of $CaCO_3$ rather than the hydrolysis of GDL is likely to be the rate limiting step (Draget *et al.*, 1991).

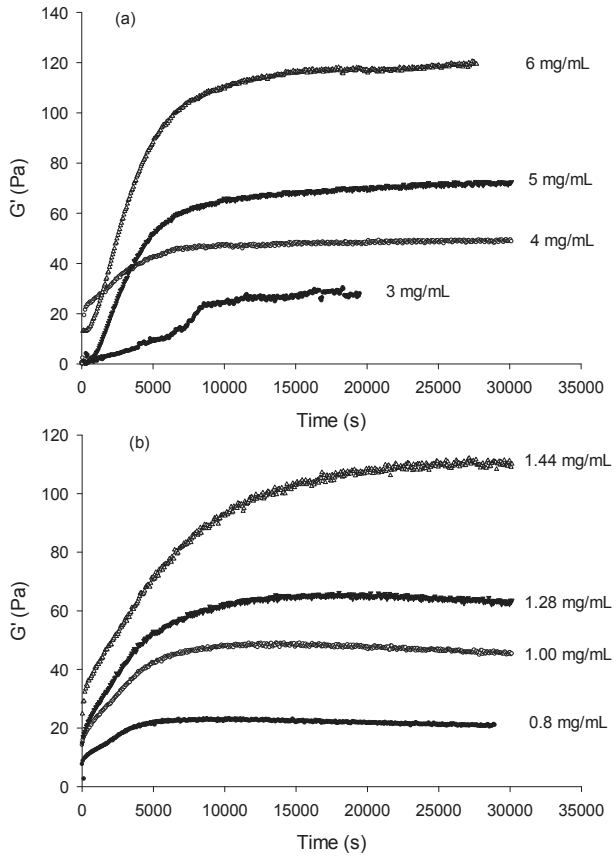


Figure 8.1 Kinetics of gelling (time sweep) at 20^0C. (a) poly-M – CO gel with different concentration of CO (mg/mL final gel), (b) chitosan – MO gel with different concentration of MO mixture (mg/mL final gel)

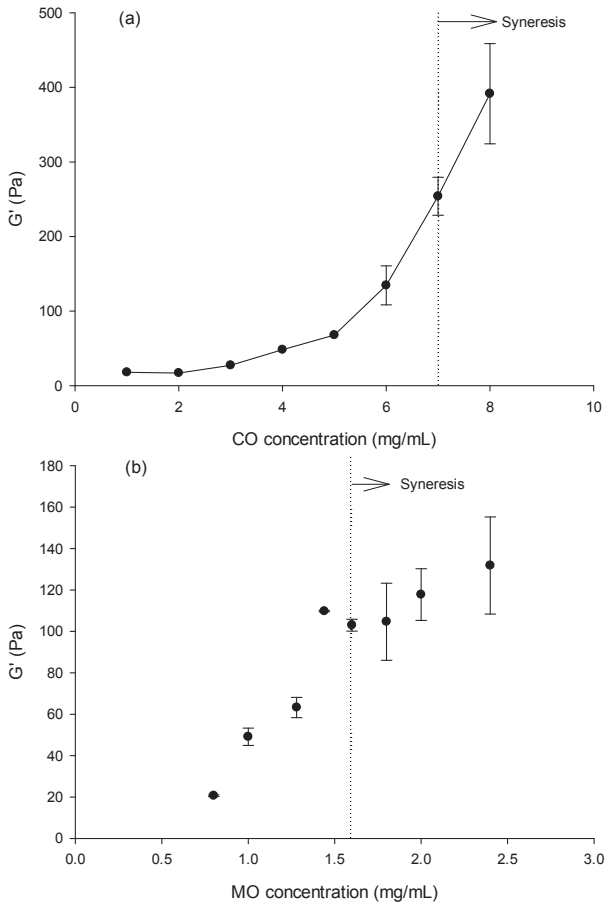


Figure 8.2 Gel strength (G') as a function of oligomer mixture concentration, (a) poly-M - CO mixture, (b) chitosan – MO mixture

Figure 8.2 shows that the gel strength (G') of the cured gels increased with the oligomer concentration in both gelling systems. When the concentration of oligomers reached a certain point (7 mg/mL for alginate – CO gel and 1.6 mg/mL for chitosan – MO gel), syneresis occurred. Syneresis results in a higher polymer concentration in the gel compared to the starting solution due to water release (Draget *et al.*, 2006). Thus, in the following experiments we chose concentrations of 6 mg/mL for CO and 1.44 mg/mL

for MO in order to maximize the strength of the resulting gels without inducing syneresis.

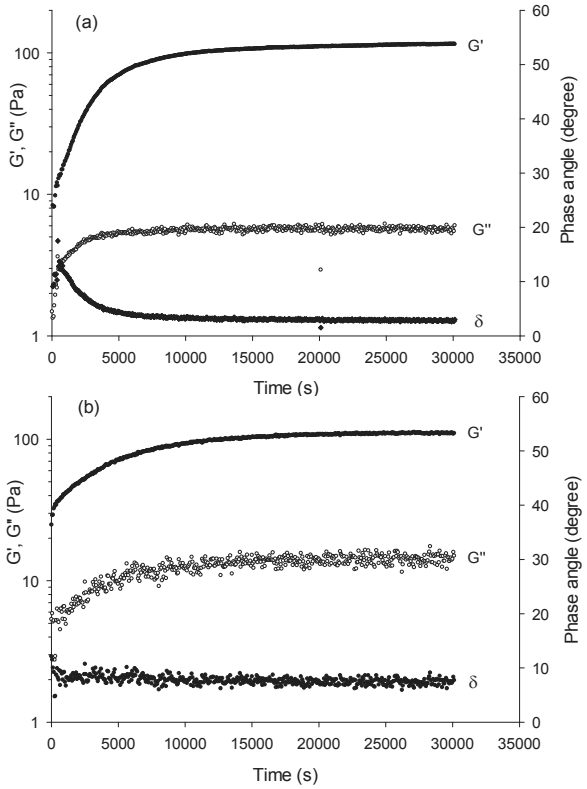


Figure 8.3 Kinetics of gelling (time sweep) at 20 ^{o}C. (a) poly-M - CO gel (poly-M, 10 mg/mL; CO mixture, 0.6 mg/mL; GDL, 4 mg/mL). (b) chitosan – MO gel (chitosan, 9 mg/mL; MO mixture, 1.44 mg/mL; GDL, 4.8 mg/mL).

Figure 8.3 shows the change of G', G'' and δ as a function of time in both gelling systems. Both systems exhibit a rapid and sharp sol-gel transition so that the gelling points can only be probed in poly-M – CO gel with low oligomer concentration, but not in the chitosan – MO gel. After a rapid sol-gel transition the crosslinking process continues with a fast increase of the gel strength in the first hour, then a slower increase

in the next few hours. The time to reach an apparent equilibrium state depends on the gelling system, the concentration of oligomers, and the amount of GDL added. However, this time does not often extend to beyond 7 h. The MO used in the chitosan – MO gel have an average chain length ($\overline{DP_n}$~ 11) larger than that of CO ($\overline{DP_n}$ ~ 5) (Figure 2, paper 3). The smaller DP crosslinkers lead to a faster initial gelling rate in the poly-M - CO gel as compared to the chitosan – MO system as judged from the slopes of the curves in Figure 8.3. Both gelling systems result in comparable gel strength even though the concentration of the crosslinkers in the chitosan – MO gel is lower than that in the poly-M – CO gel (MO, 1.44 mg/mL; CO, 6 mg/mL). This could be due to the higher numbers of charges of the crosslinker (MO in this case) resulting in longer gelation time and stronger mechanical strength as reported in TTP/chitosan beads (Shu & Zhu, 2002).

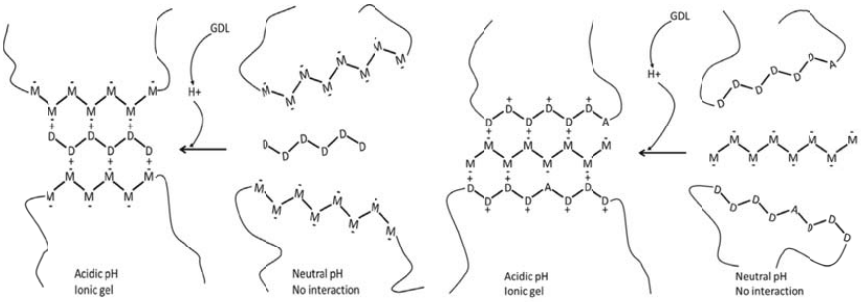


Figure 8.4 Schematic illustration of the gelling systems

The gelling principle is schematically illustrated in Figure 8.4. When GDL is added to the gelling solution, it is hydrolyzed to liberate H^+ which then protonate the amino group of chitosan/chitosan oligomers. Because of ionic interaction between the opposite charge of chitosan/chitosan oligomers and poly-M/mannuronan oligomers, gels are formed.

Poly-M would not form a gel with Ca^{++} because of complete lacking of guluronic acid blocks (Smidsrød & Skjåk-bræk, 1990). However, poly-M – CO gives a gel with G' of ca. 100 Pa (Figure 8.3). This gel is weaker than calcium-alginate gels with high G-

content alginate (G' is often greater than 1000 Pa). The gel strength of the chitosan – MO gel is comparable to that of chitosan – Mo(VI) gel (G' ~ 100 Pa, (Draget *et al.*, 1992)), but lower than the chitosan – glycerophosphate gel (G' > 1000 Pa, (Chenite *et al.*, 2001)). Figure 8.3 also shows that the chitosan – MO gel is more viscous than poly-M – CO gel. At equilibrium, the phase angle of chitosan – MO gel is around 10^0, while for the poly-M – CO gel it is only around 2^0. A typical mechanical spectrum of a true gel is shown in Figure 8.5, with G' larger than G'' over wide range of frequencies.

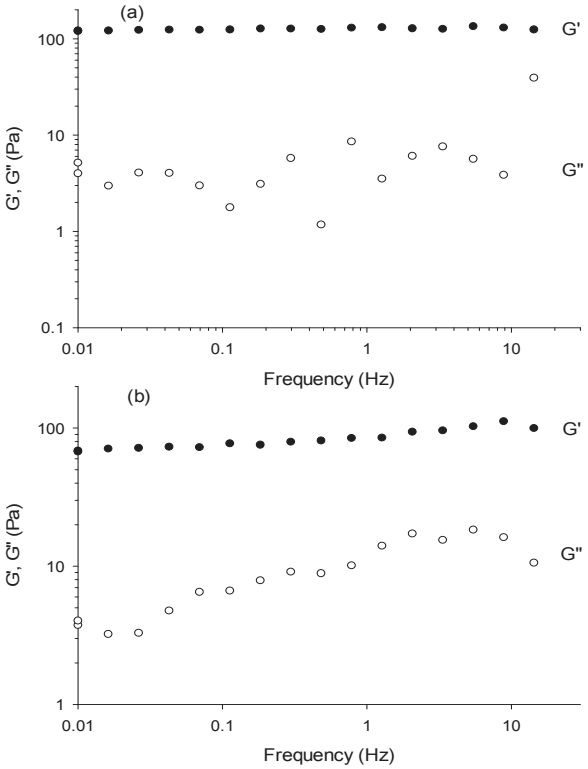


Figure 8.5 Frequency dependence (after 15h curing at 20^0C) of storage (G') and loss (G'') moduli of (a) poly-M – CO gel (poly-M, 10 mg/mL, CO mixture, 0.6 mg/mL; GDL, 4.8 mg/mL); (b) chitosan – MO gel (chitosan, 9 mg/mL; MO mixture, 1.44 mg/mL; GDL, 4.8 mg/mL)

8.2. Stress – strain dependence

Figure 8.6 shows the storage modulus (G') – strain dependence of both gels after 15h of curing at 20^0C. G' remains constant with increasing amplitude of oscillation up to a strain of 0.1. It means that the chosen strain (0.001) for time-resolved measurements and stress sweep is well within the Linear Viscoelastic Region (LVR). Figure 8.6 also shows that the chitosan – MO gel behaves as expected (strain weakening), while the poly-M – CO gel seems to become more rigid with increased deformation (strain hardening). For chitosan – MO gel, at strains greater than ca. 0.8, G' drops sharply, presumably due to the breakdown of the polymer network (Goycoolea *et al.*, 2007). The rather unexpected result for the poly-M – CO gel needs further investigation in order to give a more detailed explanation.

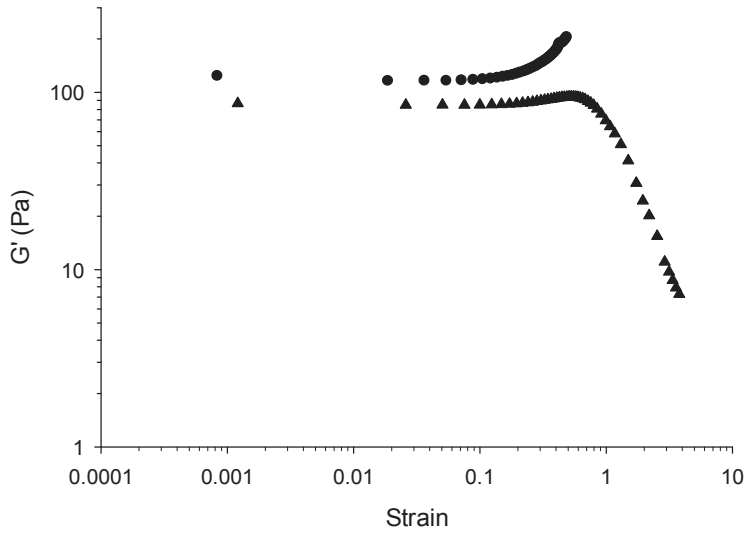


Figure 8.6 Storage modulus - strain dependence of (●) poly-M – CO gel (poly-M, 10 mg/mL, CO mixture, 0.6 mg/mL; GDL, 4.8 mg/mL); (▲) chitosan – MO gel (chitosan, 9 mg/mL; MO mixture, 1.44 mg/mL; GDL, 4.8 mg/mL)

8.3. Gel strength as a function of added GDL

The properties of ionically cross-linked hydrogels, such as mechanical, swelling or drug release, depend on the global charge of polymer and crosslinkers (Berger *et al.*, 2004). At the neutral pH of the chitosan – alginate mixture before adding GDL, alginate/alginate oligomers are fully negatively charged. The global charge of chitosan/chitosan oligomers will increase when decreasing the pH from neutral. Therefore, with lower final pH of a gel, a higher gel strength is to be expected as long as the final pH is well above the pK_a-value of the carboxyl groups (ca. 3.5).

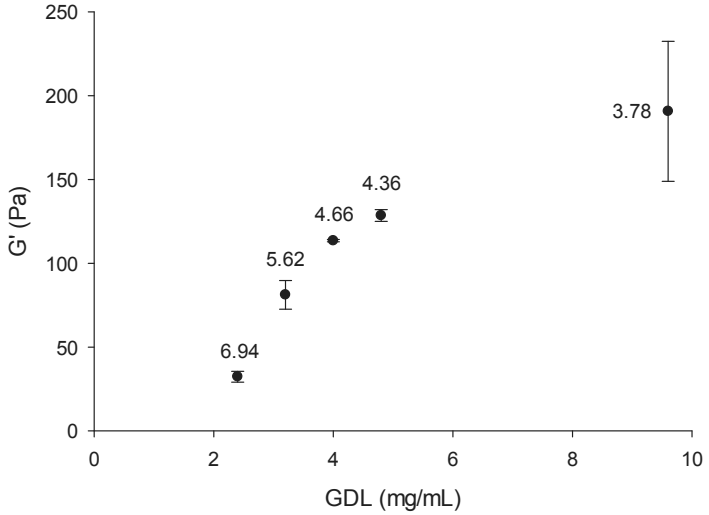


Figure 8.7 Gel strength (G') of poly-M – CO gel as a function of GDL concentration (poly-M, 10 mg/mL; CO mixture, 0.6 mg/mL). Numbers above data points indicating corresponding final pH values of the gel

The effect of increasing amount of GDL (and thereby decreasing pH) on the gel strength of poly-M – CO gel is shown in Figure 8.7. The results show an increased gel strength with increasing amount of GDL down to a pH of 3.78. However, with increasing amount of GDL, there is a higher variation in the gel strength, probably due to the faster initial gelation kinetics that follows from the higher GDL concentration. Moreover, at

low pH close to pK_a of alginate, because of the protonation of carboxyl groups, there may be a possibility of forming alginic acid gels which could partly contribute to the higher gel strength of poly-M –CO gel at the lowest pH.

For the chitosan – MO gel, similar results were obtained (data not shown). However, this gelling system is somewhat less reproducible compared to the poly-M – CO system. This lower reproducibility could be due to different kinetics of protonization of the amine groups in a polymeric chitosan chain compared to the protonization of oligomeric chitosan molecules. Furthermore, the degree of acetylation of the chitosan used in this study is 40%, and the hydrophobic interaction between *N*-acetyl groups may occur (Draget, 1996) and contribute to the lower reproducibility.

8.4. Gel strength as a function of ionic strength

In the preparation of chitosan – alginate gels, chitosan/chitosan oligomers are used in the form of chloride salts, while the alginate/alginate oligomers as their sodium salts. It means that when one ionic bridge between amino- and carboxyl-group is formed, one molecule of NaCl is released. The concentration of polymeric alginate and chitosan (with DA of 0.4) in the gels is 10 and 9 mg/mL, respectively. Thus, assuming that all charged groups are involved in amino-carboxyl ionic bonds, the maximum internal concentration of NaCl is 50 mM for the chitosan – alginate oligomer gel while it is 27 mM for the alginate – CO gel. Because of the screening off effect of counter-ions on the fixed charge, the ionic interaction (and hence the degree of cross-linking) is expected to decrease when the ionic strength increases. Figure 8.8 shows the effect of ionic strength on the gel strength of both gelling systems. A clear decrease in the dynamic storage modulus with increasing added salt concentration can be observed, as expected for ionic gels. For chitosan – MO gels (Figure 8.8, b) the decrease is somewhat more pronounced compared to that of the poly-M – CO gels (Figure 8.8, a). However, since the ionic strength without any added salt is not equal and the effect of the ionic strength on poly-electrolytes are most pronounced at the lowest ionic strengths (Smidsrød, 1971; Smidsrød & Haug, 1971), care should be taken in interpretation of the ionic strength effects in the two gelling systems.

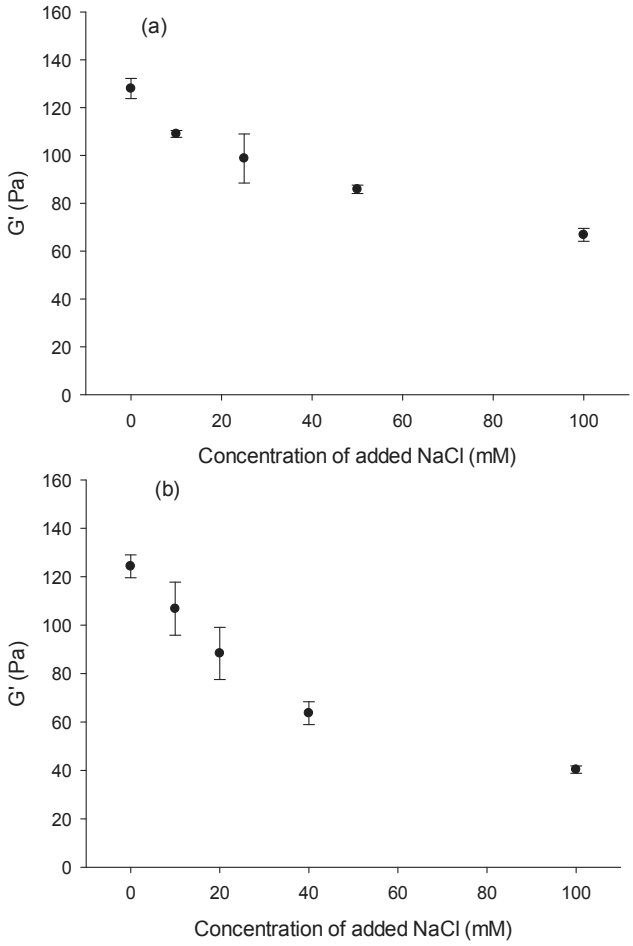


Figure 8.8 Gel strength (G') as a function of concentration of added NaCl. (a) poly-M – CO gel (poly-M, 10 mg/mL; CO mixture, 0.6 mg/mL; GDL, 4.8 mg/mL). (b) chitosan – MO gel (chitosan, 9 mg/mL; MO mixture, 1.44 mg/mL; GDL, 4.8 mg/mL).

8.5. Gelling of poly-guluronate/guluronate oligomers with chitosan/chitosan oligomers

A poly-guluronate (poly-G) and a guluronate oligomer mixture were selected to test their ability to gel with chitosan oligomers and chitosan. The results are given in Figure 8.9, showing a large difference in the gel strengths. The G' of chitosan – guluronate oligomer gel is ca. 30 Pa, as compared to ca. 110 Pa of chitosan – MO gel, whereas G' of poly-G – CO gel is of ca. 10 Pa compared to ca. 130 Pa of poly-M - CO gel.

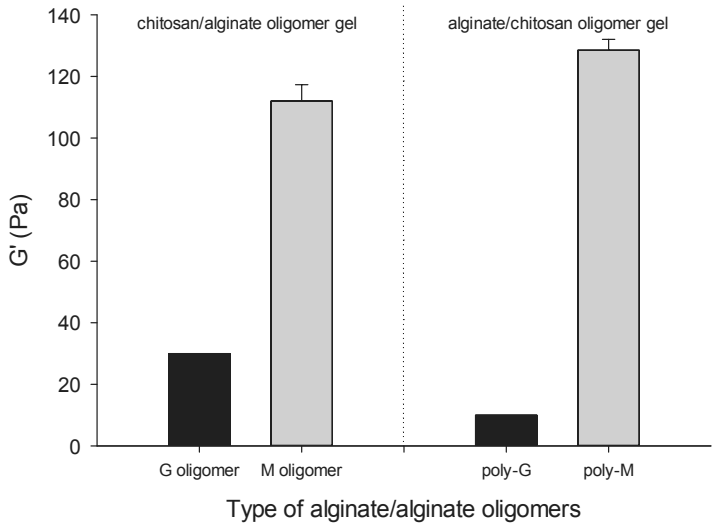


Figure 8.9 Gel strength (G') of both gelling system with different types of alginate/alginate oligomers (poly-M, poly-G, 10 mg/mL; M oligomer, G oligomer, 1.44 mg/mL)

As previously mentioned, there is a nearly perfect match in the distance between the negative charges on the same side of poly-M or MO and the positive charges on the same side of chitosan or CO. In contrast, the poly-G exists in the $^{1}C_{4}$ chair conformation with diaxial glycosidic linkage, giving a less extended structure and where the disaccharide length would be reduced to 8.7 Å (Atkins *et al.*, 1973). Therefore, it seems

that the matching conformation and thereby distance between the charges in poly-M and chitosan is crucial for the investigated gelling systems.

8.6. Gelling of poly-M with chitosan oligomer having defined chain length.

In order to further investigate the poly-M – CO gel, chitosan oligomers with defined chain length (degree of polymerization, DP) were used, from dimer (DP2) to nonamer (DP9) in gelation with poly-M.

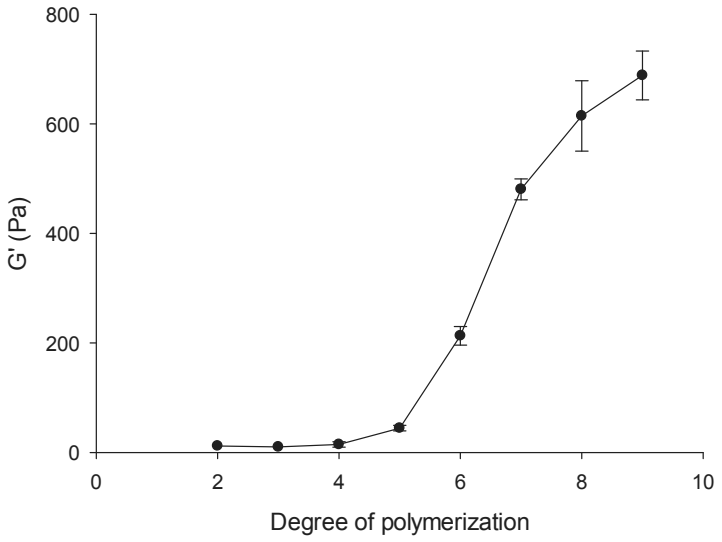


Figure 8.10 Gel strength of poly-M - MO gel with different degree of polymerization of CO (same weight concentration). (poly-M, 10 mg/mL; chitosan oligomer, 4 mg/mL)

Figure 8.10 shows the effect of chain length on the gel strength (G') of the poly-M – CO gel having the same weight concentration of chitosan oligomers, This means that the higher DP have lower number of molecules. For lower DP, i.e. DP2, DP3, DP4, the gels are very weak with a G' of ca. 10 Pa. For DP greater than 4, the gel strength increases sharply, even when the number of molecules (molar concentration) of higher

DP oligomers decreases. However, the mixture of DP10-DP12 exhibits a precipitation when it is adjusted to pH 8 before mixing with poly-M solution, resulting in less reproducibility of the gel strength. The increase in gel strength with increasing DP is probably due to the stronger interaction between poly-M and the longer chain length of chitosan oligomers. Shu & Zhu (2002) investigated the strength of chitosan/gelatin beads ionically cross-linked with different anions, sulfate, citrate and tripolyphosphate (TTP). Their results show that although the cross-linking time of TTP/chitosan beads are longer, their mechanical strength is much higher than those of sulfate or citrate/chitosan beads, probably due to higher charge numbers of TTP.

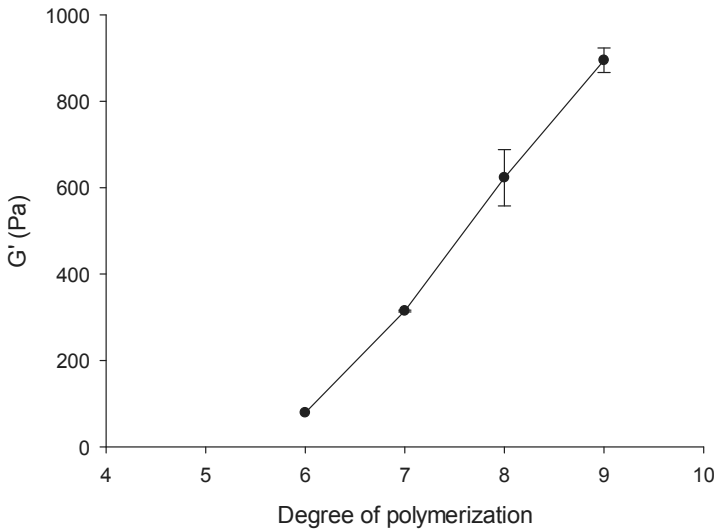


Figure 8.11 Gel strength (G') of poly-M - CO gel with different degree of polymerization of CO (same molar concentration, ca. 2.5 mM).

Figure 8.11 shows the gel strength (G') as a function of the degree of polymerization with the same molar concentration of chitosan oligomers. It seems that the gel strength increases linearly with the number of charges on a chitosan oligomer chain.

Conclusions

The chemical composition of head and shell of black tiger and white shrimp was studied, showing a much higher protein content in the head as compared to the shell, while the chitin content of the shell is much higher than in the head. As the head and shell are separated in the manual processing of shrimps in Vietnam, this points to a separate utilization of head and shell. The high protein content of the head can be utilized for application in e. g. fish feed, while the high chitin and low protein content of the shell for high-quality chitin production. The alkaline de-*N*-acetylation reaction in the process of preparing chitosan from chitin was studied in detail with the chitin disaccharide as a model substance. The reaction was found to be second order with respect to sodium hydroxide concentration, which contributes to explain the differences between the homogeneous and heterogeneous de-*N*-acetylation process.

A new chitosan – alginate gelling system that is suitable for advanced biomedical applications was studied, and its proof of principle demonstrated. The two components are mixed at a pH where chitosan is essentially neutral, the pH is lowered in a controlled way by adding a controlled amounts of the slowly hydrolysing D-glucono-δ-lactone, and a gel is formed upon leaving the mixture. The match in length between the opposite charges on the same side of chitosan and poly-mannuronate was important, as poly-mannuronate formed relatively strong gels with chitosan oligomers while poly-guluronate formed gels of very limited mechanical strength.

References

1. Aarstad, O. A., Tøndervik, A., Sletta, H., & Skjåk-Bræk, G. (2012). Alginate Sequencing: An Analysis of Block Distribution in Alginates Using Specific Alginate Degrading Enzymes. *Biomacromolecules, 13*(1), 106-116.

2. Abdou, E. S., Nagy, K. S. A., & Elsabee, M. Z. (2008). Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technology, 99*(5), 1359-1367.

3. Angyal, S. J. (2001). The Lobry de Bruyn-Alberda van Ekenstein Transformation and Related Reactions. In A. Stütz. *Glycoscience* pp. 1-14): Springer Berlin Heidelberg.

4. Anthonsen, M. W., Vårum, K. M., & Smidsrød, O. (1993). Solution Properties of Chitosans: Conformation and Chain Stiffness of Chitosans with Different Degrees of N-Acetylation. *Carbohydrate Polymers, 22*(3), 193-201.

5. Atkins, E. D. T., Mackie, W., & Smolko, E. E. (1970). Crystalline Structures of Alginic Acids. *Nature, 225*(5233), 626-628.

6. Atkins, E. D. T., Nieduszynski, I. A., Mackie, W., Parker, K. D., & Smolko, E. E. (1973). Structural Components of Alginic Acid .1. The Crystalline-Structure of Poly-Beta-D-Mannuronic Acid. Results of X-Ray-Diffraction and Polarized Infrared Studies. *Biopolymers, 12*(8), 1865-1878.

7. Bautista, J., Jover, M., Gutierrez, J. F., Corpas, R., Cremades, O., Fontiveros, E., Iglesias, F., & Vega, J. (2001). Preparation of crayfish chitin by in situ lactic acid production. *Process Biochemistry, 37*(3), 229-234.

8. Beaney, P., Lizardi-Mendoza, J., & Healy, M. (2005). Comparison of chitins produced by chemical and bioprocessing methods. *Journal of Chemical Technology and Biotechnology, 80*(2), 145-150.

9. Beauchamp, R. O., Clair, M. B. G. S., Fennell, T. R., Clarke, D. O., Morgan, K. T., & Kari, F. W. (1992). A Critical Review of the Toxicology of Glutaraldehyde. *Critical Reviews in Toxicology, 22*(3-4), 143-174.

10. Berger, J., Reist, M., Mayer, J. M., Felt, O., Peppas, N. A., & Gurny, R. (2004). Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics, 57*(1), 19-34.

11. Berth, G., & Dautzenberg, H. (2002). The degree of acetylation of chitosans and its effect on the chain conformation in aqueous solution. *Carbohydrate Polymers, 47*(1), 39-51.

12. Brack, H. P., Tirmizi, S. A., & Risen Jr, W. M. (1997). A spectroscopic and viscometric study of the metal ion-induced gelation of the biopolymer chitosan. *Polymer, 38*(10), 2351-2362.

13. Brine, C. J., & Austin, P. R. (1981). Chitin Isolates: Species Variation in Residual Amino Acids. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 70*(2), 173-178.

14. Bruck, W. M., Slater, J. W., & Carney, B. F. (2009). Chitin and Chitosan from Marine Organisms. In S.-K. Kim. *Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological Activities and Applications* (p. 643). New York: Taylor & Francis.

15. Bueno-Solano, C., Lopez-Cervantes, J., Campas-Baypoli, O. N., Lauterio-Garcia, R., Adan-Bante, N. P., & Sanchez-Machado, D. I. (2009). Chemical and biological characteristics of protein hydrolysates from fermented shrimp by-products. *Food Chemistry, 112*(3), 671-675.

16. Cao, W. H., Zhang, C. H., Hong, P. Z., & Ji, H. W. (2008). Response surface methodology for autolysis parameters optimization of shrimp head and amino acids released during autolysis. *Food Chemistry, 109*(1), 176-183.

17. Chang, K. L. B., & Tsai, G. (1997). Response surface optimization and kinetics of isolating chitin from pink shrimp (*Solenocera melantho*) shell waste. *Journal of Agricultural and Food Chemistry, 45*(5), 1900-1904.

18. Chang, K. L. B., Tsai, G., Lee, J., & Fu, W. R. (1997). Heterogeneous N-deacetylation of chitin in alkaline solution. *Carbohydrate Research, 303*(3), 327-332.

19. Charoenvuttitham, P., Shi, J., & Mittal, G. S. (2006). Chitin extraction from black tiger shrimp (*Penaeus monodon*) waste using organic acids. *Separation Science and Technology, 41*(6), 1135-1153.

20. Chen, P. Y., Lin, A. Y. M., McKittrick, J., & Meyers, M. A. (2008). Structure and mechanical properties of crab exoskeletons. *Acta Biomaterialia, 4*(3), 587-596.

21. Chenite, A., Buschmann, M., Wang, D., Chaput, C., & Kandani, N. (2001). Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions. *Carbohydrate Polymers, 46*(1), 39-47.

22. Cianci, M., Rizkallah, P. J., Olczak, A., Raftery, J., Chayen, N. E., Zagalsky, P. F., & Helliwell, J. R. (2001). Structure of lobster apocrustacyanin A(1) using softer X-rays. *Acta Crystallographica Section D-Biological Crystallography, 57*, 1219-1229.

23. Clark, A. H., & Rossmurphy, S. B. (1987). Structural and Mechanical-Properties of Bio-Polymer Gels. *Advances in Polymer Science, 83*, 57-192.

24. Cohen, E. (1987). Chitin Biochemistry: Synthesis and Inhibition. *Annual Review of Entomology, 32*, 71-93.

25. De Holanda, H. D., & Netto, F. M. (2006). Recovery of components from shrimp (*Xiphopenaeus kroyeri*) processing waste by enzymatic hydrolysis. *Journal of Food Science, 71*(5), C298-C303.

26. Diaz-Rojas, E. I., Arguelles-Monal, W. M., Higuera-Ciapara, I., Hernandez, J., Lizardi-Mendoza, J., & Goycoolea, F. M. (2006). Determination of chitin and protein contents during the isolation of chitin from shrimp waste. *Macromolecular Bioscience, 6*(5), 340-347.

27. Draget, K. I. (1996). Associating phenomena in highly acetylated chitosan gels. *Polymer Gels and Networks, 4*(2), 143-151.

28. Draget, K. I., Bræk, G. S., & Smidsrød, O. (1994). Alginic Acid Gels: the Effect of Alginate Chemical Composition and Molecular Weight. *Carbohydrate Polymers, 25*(1), 31-38.

29. Draget, K. I., Moe, S. T., Skjåk-Bræk, G., & Smidsrød, O. (2006). Alginates. *Food Polysaccharides and Their Applications* pp. 289-334): CRC Press.

30. Draget, K. I., østgaard, K., & Smidsrød, O. (1989). Alginate-Based Solid Media for Plant Tissue Culture. *Applied Microbiology and Biotechnology, 31*(1), 79-83.

31. Draget, K. I., østgaard, K., & Smidsrød, O. (1991). Homogeneous Alginate Gels: A Technical Approach. *Carbohydrate Polymers, 14*(2), 159-178.

32. Draget, K. I., Vårum, K. M., Moen, E., Gynnild, H., & Smidsrød, O. (1992). Chitosan cross-linked with Mo(VI) polyoxyanions: a new gelling system. *Biomaterials, 13*(9), 635-638.

33. Einbu, A., Naess, S. N., Elgsaeter, A., & Vårum, K. M. (2004). Solution properties of chitin in alkali. *Biomacromolecules, 5*(5), 2048-2054.

34. Einbu, A., & Vårum, K. M. (2008). Characterization of chitin and its hydrolysis to GlcNAc and GlcN. *Biomacromolecules, 9*(7), 1870-1875.

35. Eiselt, P., Lee, K. Y., & Mooney, D. J. (1999). Rigidity of two-component hydrogels prepared from alginate and poly(ethylene glycol)-diamines. *Macromolecules, 32*(17), 5561-5566.

36. Ertesvåg, H., Hoidal, H. K., Hals, I. K., Rian, A., Doseth, B., & Valla, S. (1995). A Family of Modular Type Mannuronan C-5-Epimerase Genes Controls

Alginate Structure in Azotobacter-Vinelandii. *Molecular Microbiology, 16*(4), 719-731.

37. Fox, D. L. (1973). Chitin-Bound Keto-Carotenoids in a Crustacean Carapace. *Comparative Biochemistry and Physiology, 44*(4B), 953-962.

38. Galed, G., Miralles, B., Panos, I., Santiago, A., & Heras, A. (2005). N-Deacetylation and depolymerization reactions of chitin/chitosan: Influence of the source of chitin. *Carbohydrate Polymers, 62*(4), 316-320.

39. Gartner, C., Pelaez, C. A., & Lopez, B. L. (2010). Characterization of chitin and chitosan extracted from shrimp shells by two methods. *E-Polymers*.

40. Gåserød, O., Smidsrød, O., & Skjåk-Bræk, G. (1998). Microcapsules of alginate-chitosan - I - A quantitative study of the interaction between alginate and chitosan. *Biomaterials, 19*(20), 1815-1825.

41. Ghorbel-Bellaaj, O., Hmidet, N., Jellouli, K., Younes, I., Maalej, L., Hachicha, R., & Nasri, M. (2011). Shrimp waste fermentation with *Pseudomonas aeruginosa* A2: Optimization of chitin extraction conditions through Plackett-Burman and response surface methodology approaches. *International Journal of Biological Macromolecules, 48*(4), 596-602.

42. Gildberg, A., & Stenberg, E. (2001). A new process for advanced utilisation of shrimp waste. *Process Biochemistry, 36*(8-9), 809-812.

43. Goycoolea, F. M., Arguelles-Monal, W. M., Lizardi, J., Peniche, C., Heras, A., Galed, G., & Diaz, E. I. (2007). Temperature and pH-sensitive chitosan hydrogels: DSC, rheological and swelling evidence of a volume phase transition. *Polymer Bulletin, 58*(1), 225-234.

44. Grasdalen, H. (1983). High-Field ^{1}H-NMR Spectroscopy of Alginate: Sequential Structure and Linkage Conformations. *Carbohydrate Research, 118*(Jul), 255-260.

45. Grasdalen, H., Larsen, B., & Smidsrød, O. (1979). A n.m.r Study of the Composition and Sequence of Uronate Residues in Alginates. *Carbohydrate Research, 68*(1), 23-31.

46. Hackman, R. H. (1960). Studies on Chitin IV. The Occurrence of Complexes in Which Chitin and Protein are Covalently Linked. *Australian Journal of Biological Sciences, 13*(4), 568-577.

47. Hamodrakas, S. J., Willis, J. H., & Iconomidou, V. A. (2002). A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochemistry and Molecular Biology, 32*(11), 1577-1583.

48. Haug, A. (1964). Composition and Properties of Alginates. Trondheim: Norwegian Institute of Technology.

49. Haug, A., & Smidsrød, O. (1967). Strontium Calcium and Magnesium in Brown Algae. *Nature, 215*(5106), 1167-1168.

50. Healy, M., Green, A., & Healy, A. (2003). Bioprocessing of marine crustacean shell waste. *Acta Biotechnologica, 23*(2-3), 151-160.

51. Hein, S., Ng, C. H., Stevens, W. F., & Wang, K. (2008). Selection of a practical assay for the determination of the entire range of acetyl content in chitin and chitosan: UV spectrophotometry with phosphoric acid as solvent. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials, 86B*(2), 558-568.

52. Hirai, A., Odani, H., & Nakajima, A. (1991). Determination of Degree of Deacetylation of Chitosan by ^{1}H-NMR Spectroscopy. *Polymer Bulletin, 26*(1), 87-94.

53. Holan, Z., Votruba, J., & Vlasakova, V. (1980). New Method of Chitin Determination Based on Deacetylation and Gas-Liquid-Chromatographic Assay of Liberated Acetic-Acid. *Journal of Chromatography, 190*(1), 67-76.

54. Hsiao, H. Y., Tsai, C. C., Chen, S. M., Hsieh, B. C., & Chen, R. L. C. (2004). Spectrophotometric determination of deacetylation degree of chitinous materials dissolved in phosphoric acid. *Macromolecular Bioscience, 4*(10), 919-921.

55. Huguet, M. L., & Dellacherie, E. (1996). Calcium alginate beads coated with chitosan: Effect of the structure of encapsulated materials on their release. *Process Biochemistry, 31*(8), 745-751.

56. Iconomidou, V. A., Willis, J. H., & Hamodrakas, S. J. (2005). Unique features of the structural model of 'hard' cuticle proteins: implications for chitin-protein interactions and cross-linking in cuticle. *Insect Biochemistry and Molecular Biology, 35*(6), 553-560.

57. Kameda, T., Miyazawa, M., Ono, H., & Yoshida, M. (2005). Hydrogen bonding structure and stability of alpha-chitin studied by ^{13}C solid-state NMR. *Macromolecular Bioscience, 5*(2), 103-106.

58. Kandra, P., Challa, M. M., & Jyothi, H. K. P. (2012). Efficient use of shrimp waste: present and future trends. *Applied Microbiology and Biotechnology, 93*(1), 17-29.

59. Kang De, Y., Fang Lian, Y., Jun Jie, L., Yu Ji, Y., Claire, J., & Matthew, S. (2008). Chitosan-Based Gels and Hydrogels. *Smart Materials*: CRC Press.

60. Kasaai, M. R. (2008). A review of several reported procedures to determine the degree of N-acetylation for chitin and chitosan using infrared spectroscopy. *Carbohydrate Polymers, 71*(4), 497-508.

61. Kasaai, M. R. (2009). Various Methods for Determination of the Degree of N-Acetylation of Chitin and Chitosan: A Review. *Journal of Agricultural and Food Chemistry, 57*(5), 1667-1676.

62. Kavanagh, G. M., & Ross-Murphy, S. B. (1998). Rheological characterisation of polymer gels. *Progress in Polymer Science, 23*(3), 533-562.

63. Kempe, S., Metz, H., Bastrop, M., Hvilsorn, A., Contri, R. V., & Maeder, K. (2008). Characterization of thermosensitive chitosan-based hydrogels by rheology and electron paramagnetic resonance spectroscopy. *European Journal of Pharmaceutics and Biopharmaceutics, 68*(1), 26-33.

64. Kjartansson, G. T., Zivanovic, S., Kristbergsson, K., & Weiss, J. (2006). Sonication-assisted extraction of chitin from North Atlantic shrimps (*Pandalus borealis*). *Journal of Agricultural and Food Chemistry, 54*(16), 5894-5902.

65. Kumar, M. N. V. R., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews, 104*(12), 6017-6084.

66. Kurita, K. (2006). Chitin and chitosan: Functional biopolymers from marine crustaceans. *Marine Biotechnology, 8*(3), 203-226.

67. Kurita, K., Sannan, T., & Iwakura, Y. (1977). Studies on Chitin, .4. Evidence for Formation of Block and Random Copolymers of N-Acetyl-D-Glucosamine and D-Glucosamine by Heterogeneous and Homogeneous Hydrolyses. *Makromolekulare Chemie-Macromolecular Chemistry and Physics, 178*(12), 3197-3202.

68. Lamarque, G., Viton, C., & Domard, A. (2004a). Comparative study of the first heterogeneous deacetylation of alpha- and beta-chitins in a multistep process. *Biomacromolecules, 5*(3), 992-1001.

69. Lamarque, G., Viton, C., & Domard, A. (2004b). Comparative study of the second and third heterogeneous deacetylations of alpha- and beta-chitins in a multistep process. *Biomacromolecules, 5*(5), 1899-1907.

70. Larsen, B., & Haug, A. (1971). Biosynthesis of Alginate .1. Composition and Structure of Alginate Produced by *Azotobacter-Vinelandii* (Lipman). *Carbohydrate Research, 17*(2), 287-296.

71. Lee, K. Y., & Mooney, D. J. (2012). Alginate: Properties and biomedical applications. *Progress in Polymer Science, 37*(1), 106-126.

72. Lee, K. Y., Rowley, J. A., Eiselt, P., Moy, E. M., Bouhadir, K. H., & Mooney, D. J. (2000). Controlling mechanical and swelling properties of alginate hydrogels independently by cross-linker type and cross-linking density. *Macromolecules, 33*(11), 4291-4294.

73. Lertsutthiwong, P., How, N. C., Chandrkrachang, S., & Stevens, W. F. (2002). Effect of chemical treatment on the characteristics of shrimp chitosan. *Journal of Metals, Materials and Minerals, 12*(1), 11-18.

74. Li, G. X., Du, Y. M., Tao, Y. Z., Liu, Y. T., Li, S., Hu, X. W., & Yang, J. H. (2010). Dilute solution properties of four natural chitin in NaOH/urea aqueous system. *Carbohydrate Polymers, 80*(3), 970-976.

75. Lin, T.-Y., & Hassid, W. Z. (1966). Pathway of Alginic Acid Synthesis in the Marine Brown Alga, *Fucus gardneri* Silva. *The Journal of Biological Chemistry, 241*, 5284-5297.

76. Lucinda-Silva, R. M., Salgado, H. R. N., & Evangelista, R. C. (2010). Alginate-chitosan systems: *In vitro* controlled release of triamcinolone and *in vivo* gastrointestinal transit. *Carbohydrate Polymers, 81*(2), 260-268.

77. Merzendorfer, H. (2006). Insect chitin synthases: a review. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology, 176*(1), 1-15.

78. Mi, F.-L., Shyu, S.-S., Wong, T.-B., Jang, S.-F., Lee, S.-T., & Lu, K.-T. (1999a). Chitosan–polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using tripolyphosphate or polyphosphate as reagent. *Journal of Applied Polymer Science, 74*(5), 1093-1107.

79. Mi, F. L., Shyu, S. S., Lee, S. T., & Wong, T. B. (1999b). Kinetic study of chitosan-tripolyphosphate complex reaction and acid-resistive properties of the chitosan-tripolyphosphate gel beads prepared by in-liquid curing method. *Journal of Polymer Science Part B-Polymer Physics, 37*(14), 1551-1564.

80. Mi, F. L., Sung, H. W., & Shyu, S. S. (2000). Synthesis and characterization of a novel chitosan-based network prepared using naturally occurring crosslinker. *Journal of Polymer Science Part A-Polymer Chemistry, 38*(15), 2804-2814.

81. Minke, R., & Blackwell, J. (1978). The structure of alpha-chitin. *Journal of Molecular Biology, 120*(2), 167-181.

82. MOIT (2012). Statistics on cultured shrimp production in Vietnam by provinces. Vietnamese Ministry of Industry and Trade (MOIT). http://tttm.vecita.gov.vn/dstk.aspx?NewID=181E&CateID=95

83. MurataKamiya, N., Kamiya, H., Kaji, H., & Kasai, H. (1997). Mutational specificity of glyoxal, a product of DNA oxidation, in the *lacI* gene of wild-type *Escherichia coli* W3110. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 377*(2), 255-262.

84. Ng, C. H., Hein, S., Chandrkrachang, S., & Stevens, W. F. (2006). Evaluation of an improved acid hydrolysis-HPLC assay for the acetyl content in chitin and chitosan. *Journal of Biomedical Materials Research Part B-Applied Biomaterials, 76B*(1), 155-160.

85. Ngoan, L. D., Lindberg, J. E., Ogle, B., & Thomke, S. (2000). Anatomical proportions and chemical and amino acid composition of common shrimp species in Central Vietnam. *Asian-Australasian Journal of Animal Sciences, 13*(10), 1422-1428.

86. Nikolov, S., Fabritius, H., Petrov, M., Friak, M., Lymperakis, L., Sachs, C., Raabe, D., & Neugebauer, J. (2011). Robustness and optimal use of design principles of arthropod exoskeletons studied by ab initio-based multiscale simulations. *Journal of the Mechanical Behavior of Biomedical Materials, 4*(2), 129-145.

87. Nyvall, P., Corre, E., Boisset, C., Barbeyron, T., Rousvoal, S., Scornet, D., Kloareg, B., & Boyen, C. (2003). Characterization of mannuronan C-5-epimerase genes from the brown alga *Laminaria digitata*. *Plant Physiology, 133*(2), 726-735.

88. Oh, K. T., Kim, Y. J., Nguyen, V. N., Jung, W. J., & Park, R. D. (2007). Demineralization of crab shell waste by *Pseudomonas aeruginosa* F722. *Process Biochemistry, 42*(7), 1069-1074.

89. Ottøy, M. H., Vårum, K. M., & Smidsrød, O. (1996). Compositional heterogeneity of heterogeneously deacetylated chitosans. *Carbohydrate Polymers, 29*(1), 17-24.

90. Oyrton, A. C., Monteiro, J., & Airoldi, C. (1999). Some studies of crosslinking chitosan–glutaraldehyde interaction in a homogeneous system. *International Journal of Biological Macromolecules, 26*(2–3), 119-128.

91. Pacheco, N., Garnica-Gonzalez, M., Gimeno, M., Barzana, E., Trombotto, S., David, L., & Shirai, K. (2011). Structural Characterization of Chitin and Chitosan Obtained by Biological and Chemical Methods. *Biomacromolecules, 12*(9), 3285-3290.

92. Park, J. W., Choi, K. H., & Park, K. K. (1983). Acid-Base Equilibria and Related Properties of Chitosan. *Bulletin of the Korean Chemical Society, 4*(2), 68-72.

93. Peppas, N. A. (1986). *Hydrogels in medicine and pharmacy: vol.1 fundamentals*. Boca Raton, Fla.: CRC Press.

94. Percot, A., Viton, C., & Domard, A. (2003a). Characterization of shrimp shell deproteinization. *Biomacromolecules, 4*(5), 1380-1385.

95. Percot, A., Viton, C., & Domard, A. (2003b). Optimization of chitin extraction from shrimp shells. *Biomacromolecules, 4*(1), 12-18.

96. Raabe, D., Sachs, C., & Romano, P. (2005). The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material. *Acta Materialia, 53*(15), 4281-4292.

97. Rao, M. S., Aye Nyein, K., Si Trung, T., & Stevens, W. F. (2007). Optimum parameters for production of chitin and chitosan from squilla (*S. empusa*). *Journal of Applied Polymer Science, 103*(6), 3694-3700.

98. Rebers, J. E., & Willis, J. H. (2001). A conserved domain in arthropod cuticular proteins binds chitin. *Insect Biochemistry and Molecular Biology, 31*(11), 1083-1093.

99. Remunán-López, C., & Bodmeier, R. (1997). Mechanical, water uptake and permeability properties of crosslinked chitosan glutamate and alginate films. *Journal of Controlled Release, 44*(2-3), 215-225.

100. Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science, 31*(7), 603-632.

101. Rodde, R. H., Einbu, A., & Vårum, K. M. (2008). A seasonal study of the chemical composition and chitin quality of shrimp shells obtained from northern shrimp (*Pandalus borealis*). *Carbohydrate Polymers, 71*(3), 388-393.

102. Ruel-Gariepy, E., Chenite, A., Chaput, C., Guirguis, S., & Leroux, J. C. (2000). Characterization of thermosensitive chitosan gels for the sustained delivery of drugs. *International Journal of Pharmaceutics, 203*(1-2), 89-98.

103. Sannan, T., Kurita, K., & Iwakura, Y. (1976). Studies on Chitin .2. Effect of Deacetylation on Solubility. *Makromolekulare Chemie-Macromolecular Chemistry and Physics, 177*(12), 3589-3600.

104. Sannan, T., Kurita, K., & Iwakura, Y. (1977). Studies on Chitin .5. Kinetics of Deacetylation Reaction. *Polymer Journal, 9*(6), 649-651.

105. Sato, H., Mizutani, S., Tsuge, S., Ohtani, H., Aoi, K., Takasu, A., Okada, M., Kobayashi, S., Kiyosada, T., & Shoda, S. (1998). Determination of the degree of acetylation of chitin/chitosan by pyrolysis gas chromatography in the presence of oxalic acid. *Analytical Chemistry, 70*(1), 7-12.

106. Shchipunov, Y. A., Ivanova, N. A., & Sarin, S. A. (2009). *In situ* formation of chitosan hydrogels with anionic polysaccharides. *Mendeleev Communications, 19*(3), 149-151.

107. Shu, X. Z., & Zhu, K. J. (2002). Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure. *International Journal of Pharmaceutics, 233*(1-2), 217-225.

108. Sikorski, P., Hori, R., & Wada, M. (2009). Revisit of alpha-chitin crystal structure using high resolution X-ray diffraction data. *Biomacromolecules, 10*(5), 1100-1105.

109. Skjåk-bræk, G., Grasdalen, H., & Larsen, B. (1986). Monomer Sequence and Acetylation Pattern in Some Bacterial Alginates. *Carbohydrate Research, 154*, 239-250.

110. Skjåk-Bræk, G., Grasdalen, H., & Smidsrød, O. (1989). Inhomogeneous Polysaccharide Ionic Gels. *Carbohydrate Polymers, 10*(1), 31-54.

111. Smidsrød, O. (1971). Estimation of Relative Stiffness of the Molecular Chain in Polyelectrolytes From Viscosity Measurements at Different Ionic Strengths - Comparison of Polycations and Polyanions. *Acta Chemica Scandinavica, 25*(7), 2770-2771.

112. Smidsrød, O. (1975). Molecular-Basis for Some Physical-Properties of Alginates in Gel State. *Faraday Discussions, 57*, 263-274.

113. Smidsrød, O., & Haug, A. (1971). Estimation of the relative stiffness of the molecular chain in polyelectrolytes from measurements of viscosity at different ionic strengths. *Biopolymers, 10*(7), 1213-1227.

114. Smidsrød, O., & Moe, S. T. (2008). *Biopolymer Chemistry*. Trondheim: Tapir academic press.

115. Smidsrød, O., & Skjåk-bræk, G. (1990). Alginate as Immobilization Matrix for Cells. *Trends in Biotechnology, 8*(3), 71-78.

116. Solomons, T. W. G., & Fryhle, C. (2009). *Organic Chemistry*. John Wiley & Sons.

117. Sorlier, P., Denuziere, A., Viton, C., & Domard, A. (2001). Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan. *Biomacromolecules, 2*(3), 765-772.

118. Strand, S. P., Tømmeraas, K., Vårum, K. M., & østgaard, K. (2001). Electrophoretic light scattering studies of chitosans with different degrees of N-acetylation. *Biomacromolecules, 2*(4), 1310-1314.

119. Struszczyk, H. (1987). Microcrystalline Chitosan .1. Preparation and Properties of Microcrystalline Chitosan. *Journal of Applied Polymer Science, 33*(1), 177-189.

120. Sugiyama, H., Hisamichi, K., Sakai, K., Usui, T., Ishiyama, J. I., Kudo, H., Ito, H., & Senda, Y. (2001). The conformational study of chitin and chitosan oligomers in solution. *Bioorganic & Medicinal Chemistry, 9*(2), 211-216.

121. Synowiecki, J., & Al-Khateeb, N. A. (2003). Production, properties, and some new applications of chitin and its derivatives. *Critical Reviews in Food Science and Nutrition, 43*(2), 145-171.

122. Synowiecki, J., & Al-Khateeb, N. A. A. Q. (2000). The recovery of protein hydrolysate during enzymatic isolation of chitin from shrimp *Crangon crangon* processing discards. *Food Chemistry, 68*(2), 147-152.

123. Terbojevich, M., Carraro, C., Cosani, A., & Marsano, E. (1988). Solution Studies of the Chitin Lithium Chloride-N,N-Dimethylacetamide System. *Carbohydrate Research, 180*(1), 73-86.

124. Tharanathan, R. N., & Kittur, F. S. (2003). Chitin — The Undisputed Biomolecule of Great Potential. *Critical Reviews in Food Science and Nutrition, 43*(1), 61 - 87.

125. Vårum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991a). ^{13}C-n.m.r. studies of the acetylation sequences in partially N-deacetylated chitins (chitosans). *Carbohydrate Research, 217*, 19-27.

126. Vårum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991b). Determination of the Degree of N-Acetylation and the Distribution of N-Acetyl Groups in Partially N-Deacetylated Chitins (Chitosans) by High-Field n.m.r. spectroscopy. *Carbohydrate Research, 211*(1), 17-23.

127. Vårum, K. M., Ottøy, M. H., & Smidsrød, O. (1994). Water-Solubility of Partially N-Acetylated Chitosans as a Function of pH: Effect of Chemical Composition and Depolymerization. *Carbohydrate Polymers, 25*(2), 65-70.

128. Wang, S. L., Lin, H. T., Liang, T. W., Chen, Y. J., Yen, Y. H., & Guo, S. P. (2008). Reclamation of chitinous materials by bromelain for the preparation of antitumor and antifungal materials. *Bioresource Technology, 99*(10), 4386-4393.

129. Wu, T., & Zivanovic, S. (2008). Determination of the degree of acetylation (DA) of chitin and chitosan by an improved first derivative UV method. *Carbohydrate Polymers, 73*(2), 248-253.

130. Yaghobi, N., & Hormozi, F. (2010). Multistage deacetylation of chitin: Kinetics study. *Carbohydrate Polymers, 81*(4), 892-896.

131. Zhou, H. Y., Chen, X. G., Kong, M., Liu, C. S., Cha, D. S., & Kennedy, J. F. (2008). Effect of molecular weight and degree of chitosan deacetylation on the preparation and characteristics of chitosan thermosensitive hydrogel as a delivery system. *Carbohydrate Polymers, 73*(2), 265-273.

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Kinetics of de-*N*-acetylation of the chitin disaccharide in aqueous sodium hydroxide solution

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ABSTRACT

Chitosan is prepared from chitin, a process which is carried out at highly alkaline conditions, and that can be performed either on chitin in solution (homogeneous deacetylation) or heterogeneously with the chitin as a solid throughout the reaction. We report here a study of the de-*N*-acetylation reaction of the chitin dimer (GlcNAc–GlcNAc) in solution. The reaction was followed by ^{1}H NMR spectroscopy in deuterated aqueous sodium hydroxide solution as a function of time, sodium-hydroxide concentration and temperature. The ^{1}H NMR spectrum of GlcNAc–GlcNAc in 2.77 M deuterated aqueous sodium hydroxide solution was assigned. The interpretation of the ^{1}H NMR spectra allowed us to determine the rates of de-*N*-acetylation of the reducing and non-reducing ends, showing that the reaction rate at the reducing end is twice the rate at the non-reducing end. The total deacetylation reaction rate was determined as a function of the hydroxide ion concentration, showing for the first time that this de-*N*-acetylation reaction is second order with respect to hydroxide ion concentration. No significant difference in the deacetylation rates in deuterated water compared to water was observed. The activation energy for the reaction (26–54 °C) was determined to 114.4 and 98.6 kJ/mol at 2.77 and 5.5 M in deuterated aqueous sodium hydroxide solution, respectively.

1. Introduction

Generic chitosans are prepared from chitin, and this process is currently performed by chemical de-*N*-acetylation in concentrated sodium-hydroxide at high temperatures in a heterogeneous process where the chitin/chitosan remains in the solid state during the process. However, the same process may also be performed in a homogeneous process with the chitin solubilized in the alkaline solution.[1] The homogeneous de-*N*-acetylation of chitin has been studied as a function of temperature, and the activation energy was determined to 22 kcal/mol (92 kJ/mol). The same authors assumed that the reaction was first order with respect to hydroxide ion (at 10% aqueous NaOH-concentration), and found that the reaction was pseudo-first order with respect to acetamido groups. However, their attempt to study the de-*N*-acetylation of the monomer GlcNAc was unsuccessful, which they attributed to decomposition.[2]

There is considerable interest in the heterogeneous deacetylation process, as this is a cost-effective process for producing chitosans from chitin and the functionality of chitosans has been found to depend on their degree of acetylation.[3] Studies of this process are more complicated due to the insolubility and crystalline nature of chitin. However, activation energies in the range from 40 to 45 kJ/mol have been reported,[4] which is less half the reported value in the homogeneous process mentioned above. It is, however, more difficult to interpret results from the heterogeneous processes, as the local concentrations of for example, hydroxyl ions at the solved exposed surface where the reaction occurs may be quite different from known concentration in solution.

In order to study the de-*N*-acetylation reaction in more detail, we decided to investigate the kinetics of the deacetylation reaction by directly following changes in time-resolved ^{1}H NMR spectra (in alkaline solutions) with the chitin dimer as a model substance. The results reveal that one of the Lobry de Bruyn-Alberda van Ekenstein (LdB-AvE) reactions occurs, that is, the epimerisation reaction as demonstrated by the disappearance of the H-2 proton as it was replaced by deuterium in the NMR spectra. Moreover, we demonstrate that the de-*N*-acetylation reaction is in second order with respect to the (deuterated) hydroxide ion, and that the reaction rate at the reducing end is twice the rate at the non-reducing end.

2. Experimental

2.1. NMR of the chitin dimer in deuterated aqueous sodium hydroxide solution

Chitin dimer (AA) was purchased from Seikagaku, Japan. (99.5%) D_2O and (99.5% D) NaOD 40% w/w were purchased from CDN isotopes, Canada. Stock solution was prepared by dissolving 30 mg/mL chitin dimer in D_2O and aliquant 100 μL into eppendorf tube and stored at −35 °C. Prior to dissolving the oligomers, the NaOD was saturated with nitrogen gas to remove oxygen in order to prevent side-reactions.

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All NMR experiments were recorded on BRUKER Avance DPX 400 or 300 spectrometer equipped with 5 mm QNP probe and 5 mm z-gradient DUL (C/H) probe, respectively. The NMR data were processed and analysed with Bruker XwinNMR ver 3.5 and TopSpin 3.0 software.

Different chitin dimer samples were prepared where ~3 mg (AA and DD) were dissolved in 2.77 M NaOD (in D_2O) with sodium 3-(trimethylsilyl) propionate-d_4 (TSP) as internal standard. Homonuclear 1D, 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and ^{13}C heteronuclear single quantum coherence (HSQC) experiment were recorded at 25 °C and used to assign the chemical shifts.

For time-resolved NMR, pseudo 2D spectrum (first dimension is ^1H spectrum and second dimension is time spaced), the samples were prepared by mixing the chitin dimer (3 mg) with D_2O and NaOD 40% w/w to a total volume of 500 μL with the desired NaOD concentration. Then the sample was immediately inserted into the preheated NMR instrument. The recorded spectrum is a pseudo-two-dimensional type experiment recording a 1D ^1H NMR spectrum every 10 min. The recorded 1D carbon ^1H spectrum contains 16 K data points, has a spectral width of 12 ppm, 16 scans with a 30 degree flip angle, relaxation delay of 1 s (total recording time of 49 s). In order to check the isotope effect a sample was prepared and a time-resolved spectrum was recorded (32 scans, with presaturation of the water resonance, total recording time of 89 s) as described above with 10% D_2O/90% H_2O instead of 99.9% D_2O.

2.2. Determination of de-*N*-acetylation rate constants

The de-*N*-acetylation of the chitin dimer was determined by monitoring the intensity of the acetate signal in relation to the total intensity for both acetyl and acetate signals in the NMR spectra. The fraction of deacetylated dimer AD or DA (F_D) was determined and plotted as a function of time, and the initial rate of de-*N*-acetylation was determined as the slope for the initial linear part of the curve.

The natural logarithm of the fraction of deacetylated units (F_D) plotted as a function of time resulted in an initial linear relation. The rate constants of the de-*N*-acetylation reaction were determined as the slope of the linear region in these plots.

Molar fractions of each dimer (AD and DA) in the reaction mixture were calculated from the ratio of the β-anomer proton of each dimer to the sum of β-anomer protons of the dimers. Rate constant of de-*N*-acetylation at reducing end (k_{DA}) and at non-reducing end (k_{AD}) was determined as the slopes of the plots of the natural logarithm of the DA and AD molar fractions against time at the initial stage of the reaction, respectively.

2.3. Determination of activation energies

Arrhenius plots were created from rate constants obtained for de-*N*-acetylation at two different concentrations of NaOD (2.77, 5.5 M) at three different temperatures (26, 45, 54 °C). Activation energies were calculated as the slopes of these plots.

3. Results and discussion

3.1. ^1H NMR spectra of chitin dimer in NaOD/D_2O

Figure 1 shows the ^1H spectra of the chitin dimer GlcNAc–GlcNAc (panel A) together with the monomer GlcNAc (panel B) and the fully de-*N*-acetylated dimer GlcN–GlcN (panel C) in deuterated aqueous sodium hydroxide solution at 2.77 M and 25 °C. The large resonance of the solvent signal appears at 5.2 ppm (at 25 °C).

The chitin dimer and monomer spectra in deuterated aqueous sodium hydroxide solution are remarkably similar to the same spectra in deuterated water,[5,6] considering that the hydroxyl groups at this high pH-value are (partially) ionized. The assignments of the non-reducing end anomeric proton at 4.5 ppm are evident from the comparison of the two spectra. Coupling constants are also similar to those in deuterated water,[5,6] indicating that the conformation of the chitin dimer is the same as in water, that is, with a diequatorial glycosidic linkage with the sugar rings in the $^4C_1$ conformation. The acetyl-protons appear as three resonances, that is, the non-reducing end acetyl and two reducing end acetyl resonances (α- and β-anomer). Note also that the acetate protons appear well separated from the acetyl-protons, making it easy to monitor the de-*N*-acetylation reaction.

The chitosan dimer (GlcN–GlcN) spectrum in deuterated aqueous sodium hydroxide solution is also similar to the spectrum of chitosan dimer in deuterated water.[5] However, the chemical shift of H-2 proton is shifted upfield about 0.5 ppm as compared to the shift for H-2 proton when the amino-group is protonated.[5] Also, the anomeric protons are shifted upfield at the high pD-values of this solvent. The assignments of the H-1/H-2 resonances are straightforward from the spectra (Fig. 1), and coupling constants are again similar to those in deuterated water. Resonances were also assigned using the 2D COSY, TOCSY, ^{13}C HSQC spectra of GlcNAc–GlcNAc (data not shown), and the assignments are summarized in Table 1.

Having assigned the resonances in the spectra in Figure 1, we went on to examine the de-*N*-acetylation of the chitin dimer. Figure 2 shows the spectra of the chitin dimer (GlcNAc–GlcNAc) at 4.13 M and 45 °C after 10 and 1000 min (bottom part). In the upper part of Figure 2 is shown a stacked plot of the anomer region and the acetyl/H-2 (deacetylated) region. As the de-*N*-acetylation of the chitin dimer proceeds, new resonances appear in the spectrum. In the acetyl region, the acetyl-resonances (at ~2–2.1 ppm) decrease with concomitant increase in the resonances from the acetate protons (at ~1.9 ppm). By integrating the acetyl and acetate resonances as a function of time, the total rate of de-*N*-acetylation could be determined with a high accuracy. From the previous assignments of the resonances of the chitin dimer (AA), the chitin monomer (A), and the chitosan dimer (DD) (see Fig. 1 and Table 1), we can also identify the resonances of the two partially deacetylated dimers GlcNAc-GlcN (AD) and GlcN-GlcNAc (DA) in the anomer region of the spectrum. However, we noticed that the anomeric resonance of the reducing ends of the various dimers did not appear as doublets but as singlets, and also that the resonance of the H-2 (deacetylated) gradually decreased with time.

In alkaline solution of carbohydrates, the Lobry de Bruyn-Alberda van Ekenstein (LdB-AvE) reactions may occur.[7] LdB-AvE involves two reactions, epimerization and aldose-ketose interconversion. Since carbon 2 in chitin/chitosan oligosaccharides is not connected to a hydroxyl group but an amino or acetamido group, we need only to consider the epimerisation reaction (Scheme 1). From the ^1H NMR spectra of the monomer D-glucosamine obtained at different time in 2.77 M NaOD (in D_2O), it was determined that the H-2 resonance gradually decreased with time and that the anomeric resonance gradually changed from a doublet to a singlet. The change of the β-anomeric resonance to a singlet was also observed with the *N*-acetylated monomer. This is due to the ene-diol equilibrium reaction with the loss of stereochemistry at carbon 2, which will gradually substitute H-2 with deuterium from the solvent (deuterated water), see Scheme 1. As deuterium will not be detected in the ^1H NMR spectra and since the quadrupole moment of deuterium will result in a collapse of the coupling fine structure for the neighbouring protons,[8] this explains the change in the glucosamine spectrum with time. It should also be noted that this epimerization reaction is fast in comparison to the de-*N*-acetylation reaction at the present conditions. Thus, the

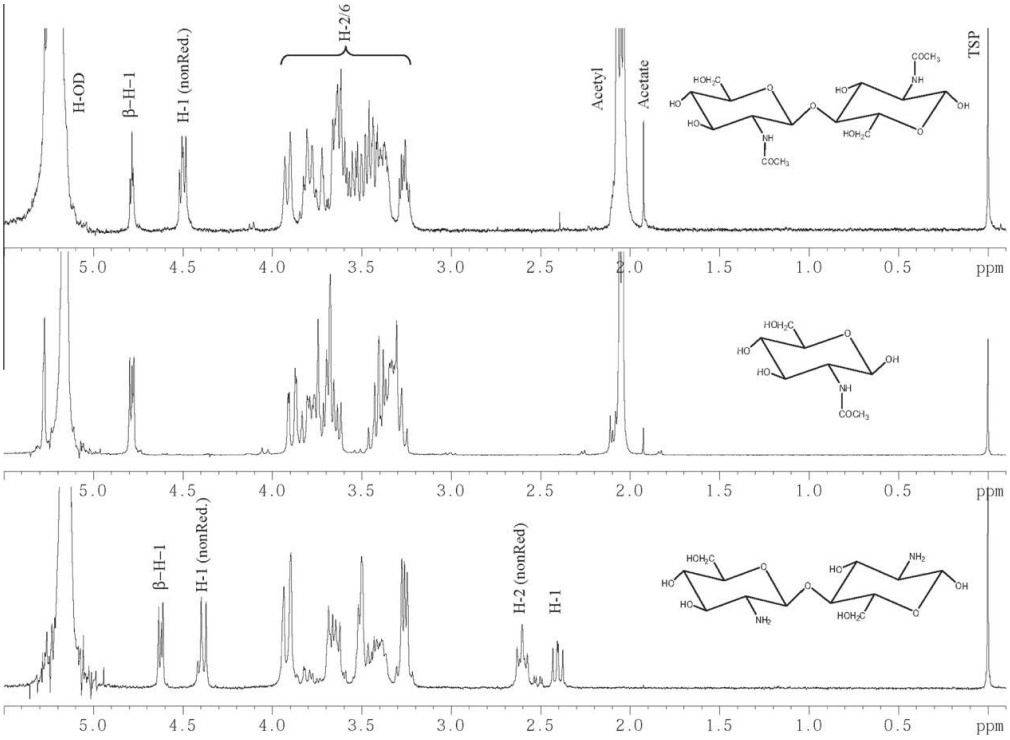


Figure 1. ^{1}H NMR spectra at 400 MHz of GlcNAc–GlcNAc (top), GlcNAc and GlcN–GlcN in 2.77 M NaOD recorded at 25 °C. Chemical shifts are given relative to TSP at 0.00 ppm.

Table 1
^{1}H Chemical shift obtain for different chitin/chitosan dimers in 99.9% D_2O with 4.13 M NaOH recorded at 43 °C

Chemical group	^{1}H Chemical shift (ppm)
Acetate	1.93
Methyl	2.03, 2.07
H-2 (D), non-reducing end	2.57
H-2 to H-6	3.21, 3.92
H-1 (D) non-reducing end	4.37
H-1 (A) non-reducing end	4.48
β-H-1 (AD)	4.58
β-H-1 (DD)	4.60
β-H-1 (AA)	4.78
β-H-1 (DA)	4.80

anomer protons of all dimers formed initially (AA, AD and DA) appear as singlets in the spectra. The β-anomer of the reducing end proton of the dimer DA is assigned to the resonance just downfield (at 4.80 ppm) from the same proton in AA. Similarly, the β-anomer of the reducing end proton of the dimer AD is assigned to the resonance just upfield (at 4.58 ppm) from the same proton in DD. We can then identify separate resonances in the anomer region from all four dimers (AA, DA, AD and DD), allowing the determination of the different rates of de-*N*-acetylation of the chitin dimer from the relative intensities of the DA/AD anomer protons in Figure 2. There are in total four different rate constants for de-*N*-acetylation. However, here we will only consider the (initial) rates of the first de-*N*-acetylation steps, that is, the rate of hydrolysis of an acetyl group from the reducing and the non-reducing ends:

$AA \xrightarrow{k_{DA}} DA$ where

$AA \xrightarrow{k_{AD}} AD$ k_{DA} : Rate constants for the de-*N*-acetylation of the non-reducing

k_{AD} : Rate constants for the de-*N*-acetylation of the reducing end

The LdB-AvE epimerisation reaction will result in the exchange of the proton at carbon 2 with deuterium, and we need also to consider the possibility of the formation of a 2-amino-2-deoxy D-mannose unit as the water (or actually D_2O) when the reaction (Scheme 1) is reversed. However, the integral of the reducing end singlets was found to be constant with time, and we could not detect any new resonances in the anomer region of the spectra, suggesting that the formation of 2-amino-2-deoxy D-mannose (or the *N*-acetylated analogue) is low.

Figure 3 shows the de-*N*-acetylation of the reducing and non-reducing ends of the chitin dimer as determined from the formation of the partially *N*-acetylated dimers AD and DA, respectively. Note that the formation of both AD and DA will increase to a maximum, as both dimers will be further deacetylated to the dimer DD. Clearly, the de-*N*-acetylation of the reducing end is faster by a factor of about 2 (rates were determined from the initial slope of the two curves in Fig. 3). Further, the activation energies of the (total) de-*N*-acetylation reactions at 2.77 and 5.5 M [NaOD] were determined at 26, 45 and 54 °C, and the Arrhenius plot is shown in Figure 4. The resulting activation energies were calculated from the slopes of the curves, giving 114.4 and 98.6 kJ/mol at 2.77 and 5.5 M NaOD, respectively. The results are in reasonable agreement with previous results[2] who determined the activation energy of chitin to 22 kcal/mol, that is 92 kJ/mol.

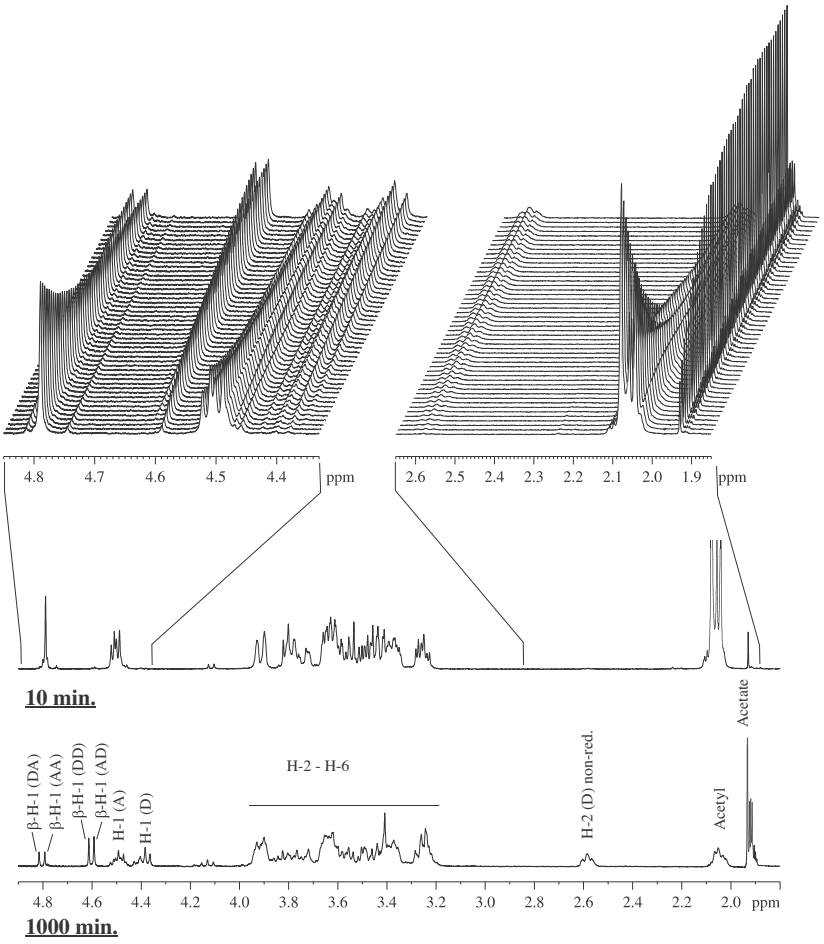
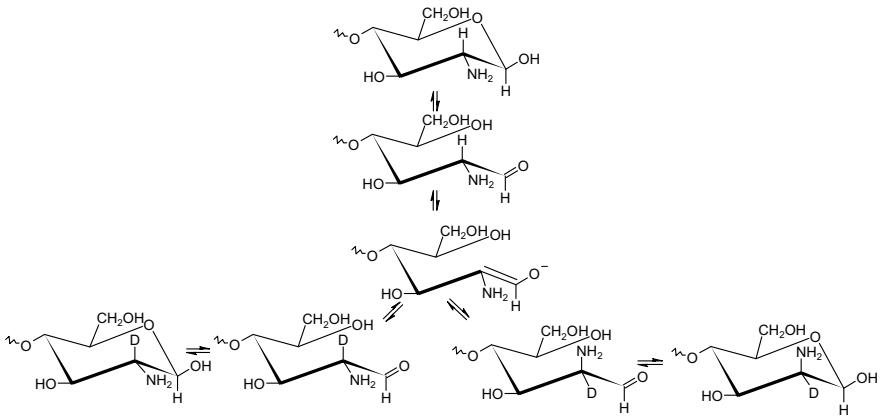


Figure 2. Anomer region of the ^{1}H NMR spectra of the chitin dimer (GlcNAc–GlcNAc) at 4.13 M and 45 °C after 10 and 1000 min. In the upper part of the Figure is shown a stacked plot of the anomer region and the acetyl/H-2 (deacetylated) region.



Scheme 1. Proposed reactions at the reducing end leading to exchange of the proton at C2 with deuterium.

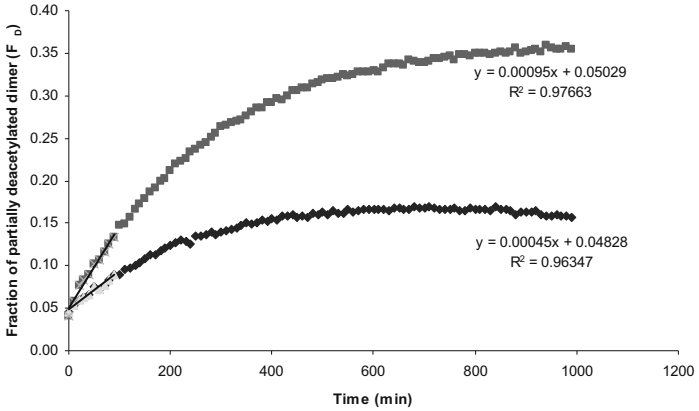


Figure 3. Molar fractions of the partially *N*-acetylated dimers AD (upper) and DA (lower) as a function of time in 4.13 M NaOD at 45 °C.

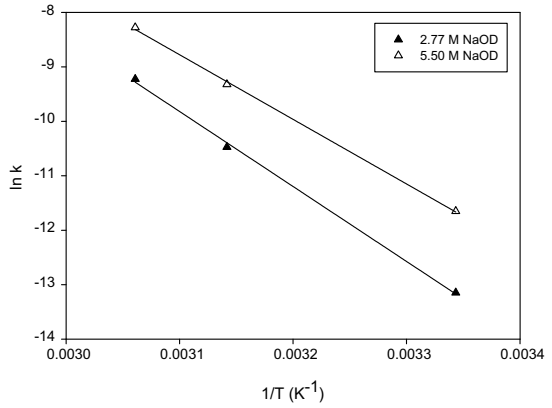


Figure 4. Rate constants of de-*N*-acetylation at 2.77 and 5.5 M sodium-hydroxide (NaOD) as a function of the inverse of the absolute temperature (Arrhenius plot).

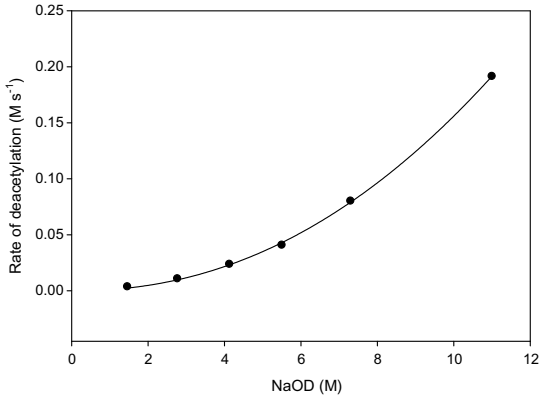
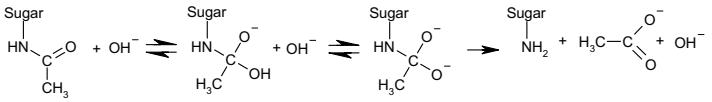


Figure 5. Rate constants of de-*N*-acetylation (at 25 °C) as a function of the concentration of NaOD.

Reaction rates in deuterated water may vary considerably from the rates in water,[9] as has been determined for the acid-catalysed hydrolysis of the chitin dimer.[10] Thus, we determined the rate of de-*N*-acetylation in 10% D_2O/90% H_2O and compared it to the rate in 99.9% D_2O. No significant difference in the rates could be determined which is conceivable considering that in the base-catalysed deacetylation reaction it is the hydroxide ion (OH^-/OD^-) that is active as compared to the proton (H^+/D^+) in the acid-catalysed hydrolysis.

We then moved on to determine the reaction order with respect to hydroxide ion concentration and the reaction rate as a function of the concentration of NaOD is shown in Figure 5. Clearly the rate increases non-linearly with increasing lye concentration, and the best power fit of the curve gave an exponent of 1.96. Thus, the de-*N*-acetylation reaction is second order with respect to the hydroxide ion, contrary to what was assumed previously.[2] These authors did not report results on the reaction order with respect to the hydroxide ion. However, the aqueous hydrolysis of acetamido groups at high pH-values has generally been assumed to be a second order reaction,[11] although this has to our best knowledge not previously been demonstrated for this particular reaction. The de-*N*-acetylation of chitin is performed to prepare water-soluble chitosan. Our results strongly suggest that the homogeneous reaction is second order with respect to the hydroxide ion, involving two hydroxide ions and a double-charged intermediate (Scheme 2). This explains why the heterogeneous reaction is slow, and may contribute to explain why the heterogeneous reaction creates a heterogeneous mixture of chitin and chitosan,[12] and why the rate-determining step of the heterogeneous deacetylation reaction is the swelling step, that is the diffusion of the hydroxide ion into the insoluble and crystalline chitin molecules.



Scheme 2. Proposed reaction mechanism for the alkaline de-*N*-acetylation reaction.

Acknowledgements

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References

1. Sannan, T.; Kurita, K.; Iwakura, Y. *Die Makromol. Chem.* **1975**, *176*, 1191–1195.
2. Sannan, T.; Kurita, K.; Iwakura, Y. *Polym. J.* **1977**, *9*, 649–651.
3. Vårum, K. M.; Smidsrød, O. In *Polysaccharides: Structural Diversity and Functional Versatility*; Dumitriu, S., Ed.; Marcel Dekker, 2004; pp 625–642.
4. Lamarque, G.; Cretenet, M.; Viton, C.; Domard, A. *Biomacromolecules* **2005**, *6*, 1380–1388.
5. Sugiyama, H.; Hisamichi, K.; Sakai, K.; Usui, T.; Ishiyama, J. I.; Kudo, H.; Ito, H.; Senda, Y. *Bioorg. Med. Chem.* **2001**, *9*, 211–216.
6. Vårum, K. M.; Anthonsen, M. W.; Grasdalen, H.; Smidsrød, O. *Carbohydr. Res.* **1991**, *211*, 17–23.
7. Angyal, S. In *Glycoscience: Epimerisation, Isomerisation and Rearrangement Reactions of Carbohydrates*; Stutz, A. E., Ed.; Springer-Verlag: Berlin, 2001; Vol. 215, pp 1–14.
8. Claridge, T. D. W. *High-resolution NMR Techniques in Organic Chemistry*; Pergamon: Oxford, 1999.
9. Wiberg, K. B. *Chem. Rev.* **1955**, *55*, 713–743.
10. Einbu, A.; Varum, K. M. *Biomacromolecules* **2007**, *8*, 309–314.
11. Atkins, P. W. *Physical Chemistry*, 5th ed.; Oxford University Press: Oxford, 1994.
12. Ottøy, M. H.; Vårum, K. M.; Smidsrød, O. *Carbohydr. Polym.* **1996**, *29*, 17–24.

Paper III

A new gelling concept combining chitosan and alginate – Proof of principle

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Keywords: chitosan, alginate, oligomer, GDL, ionic gel

Abbreviations:

CO: mixture of fully de-*N*-acetylated chitosan oligomers; **A**-units: GlcNAc units; **D**-units: GlcN units; M: Mannuronic acid unit; G: Guluronic acid unit; poly-M: high molecular weight bacterial alginate containing 100% β-D-Mannuronic Acid; MO: mixture of mannuronate oligomers (prepared from poly-M); poly-G: high molecular weight bacterial alginate containing 88% α-D-Guluronic Acid

ABSTRACT

A direct mixing of the polycation chitosan and the polyanion alginate will not result in formation of a homogeneous gel but rather a precipitation or formation of particles due to the fast and irreversible binding between the charged molecules. We here report on a new gelling system of chitosan and alginate which are prepared using the following steps: i) the chitosan and alginate are mixed at a pH well above 7 where the chitosan is mainly uncharged ii) the pH is lowered in a controlled way by adding the slowly hydrolyzing D-glucono-δ-lactone (GDL) iii) a homogeneous chitosan-alginate gel is formed upon leaving the mixture at room temperature. Some properties of the new gelling system is demonstrated herein by adding controlled amounts of GDL to i) a mixture of a polymeric and neutral-soluble chitosan with alginate oligomers and ii) a mixture of a polymeric alginate and neutral-soluble chitosan oligomers. The neutral-solubility of the polymeric chitosan is achieved by selecting a polymeric chitosan with a degree of acetylation of 40% (F_A of 0.4) while the neutral-solubility of the (fully de-*N*-acetylated) chitosan oligomers is obtained by selecting oligomers with a chain length below 10. The new gelling system is particularly interesting for alginates with a low content of guluronate residues, both because these alginates form weak gels with multivalent cations (e.g. calcium) and because the nearly perfect match in conformation and thereby distance between the charged groups in chitosans and alginates with a high content of mannuronate residues. This match in conformation and charge distance is due to the di-equatorial linkages between the sugar units and that the sugar residues occur in the $^4C_1$ chair conformation. A proof of concept of the new gelling system is demonstrated by measuring the gel strengths of a polymeric chitosan (F_A=0.4) – poly-M-oligomer mixture, and polymeric mannuronan – chitosan oligomer mixture. The results show that the gel strength increases with decreasing the pH from neutral to 5, and that the gel strength decrease with increasing ionic strength, indicating that in both cases an ionic gel is formed. Moreover, the importance of the match in conformation and thereby charge distance is demonstrated by the very different gel strengths of two alginates with extreme composition, i.e. a poly-Mannuronate and a poly-Guluronate, where the former formed relatively strong gels with chitosan oligomers while the latter formed gels of very limited mechanical strength.

1. INTRODUCTION

Chitosans are derived from chitin, one of Nature's most abundant biopolymers. These water-soluble linear polysaccharides are composed of β-(1-4)-linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc; **A**-unit) and the same de-*N*-acetylated sugar unit (GlcN; **D**-unit), they can be prepared with widely varying chemical composition and chain lengths, and the two building units are believed to be randomly distributed along the chains. [1]

Alginates are a family of polysaccharides produced by brown algae and bacteria. [2] They are linear copolymers of 1→4 linked *β*-D-mannuronate (M) in the $^4C_1$ conformation and its C5-epimer, *α*-L-guluronate (G) in the $^1C_4$ conformation. The two units are arranged in a blockwise pattern along the chain with homopolymeric regions of M (M-blocks) and G (G-blocks) interspaced with regions of alternating structure (MG-blocks). [2]

Hydrogels are three-dimentional networks composed of hydrophilic polymer chains that are crosslinked. Such networks have attracted much attention in recent years, especially in relation to e.g. their applications in tissue engineering, immobilization of cells and controlled drug release. [3, 4] Engineering of hydrogels is important for biomedical applications, especially related to their transport properties (e.g. controlled release), degradation, bioadhesion, bioactivity (e.g. immunological responses), and mechanical properties.[5] Biopolymers are especially interesting as they, in comparison to synthetic polymers, are well-tolerated by cells and organisms, show lower toxicity and higher biodegradability.

Different chitosan gelling systems have been reported. The chitosan / glycerol phosphate gelling system[6-9] has been extensively studied, taking advantage of the decreased pK_a-value of the amino-groups of chitosans upon heating to body temperature which releases protons that are taken up by glycerol-phosphate and thereby mediates more neutral chitosan chains which form a gel. A covalently crosslinked chitosan gelling system, where a chitosan with about equal amounts of **A**- and **D**-units (F_A=0.5) that are soluble at neutral pH, is crosslinked with diethyl squarate have recently been reported. [10]

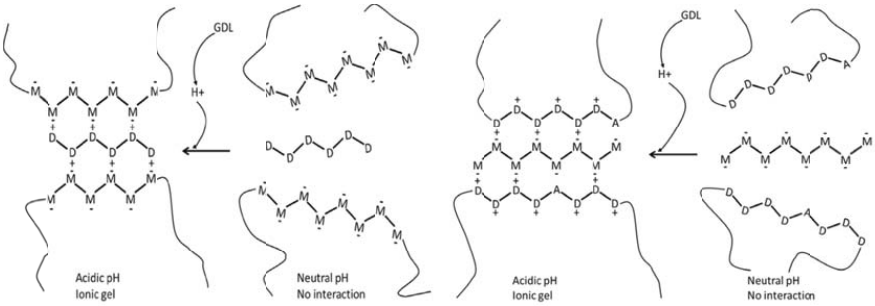
Alginates can form ionic gels in the presence of certain multivalent cations, such as Ca^{2+}. Calcium-alginate gels have been intensively studied, and such gels can be

prepared by internal gelation or the diffusion method. [2] The calcium ions show a specific interaction with the G-units in alginates, and brown algae have utilized this to synthesize alginates with different gelling properties, such as the high-G alginates of the stipes of *Laminiaria hyperborea* giving a rigid gel, and the lower-G alginates of the fronds of the same species, giving more flexibility. Homogeneous gels of calcium-alginate can be prepared by internal gelation, where (insoluble) calcium-carbonate is mixed with a solution of alginate, and the pH is subsequently lowered by adding a proton-donating substance (such as a slowly hydrolyzing lactone, D-Glucono-δ-lactone (GDL), which will release calcium ions in a controlled matter to form a homogeneous gel. [11]

When a polycation and a polyanion are mixed when fully charged, they will usually precipitate and not form a gel. The pK_a-values of the chitosan's amino-groups of ca. 6.5 and of the alginate's carboxyl-groups of ca. 3.5 indicates that in the pH-interval from 4 to 6 there should be a strong ionic interaction between the positively charged chitosans and the negatively charged alginates. Both chitosan and poly-M are polysaccharides where the sugar building units occur in the $^4C_1$ conformation, [12] the units are linked through β-(1-4) diequatorial glycosidic linkages, and the more stable conformation is where each sugar unit is rotated 180^0 relative to its neighbors. Consequently, both in chitosan and poly-M, every second sugar unit in the same chain will have a charged group pointing in the same direction with a distance between the charges which is of the same length as a disaccharide unit, i.e. 10.3-10.4 Å. [12, 13]. In poly-G, the G-units occur in the $^1C_4$ conformation so that the glycosidic linkages are diaxial and consequently the length of a disaccharide unit are 8.7 Å, [12] considerably shorter as compared to poly-M.

Here we report on a new gelling system composed of chitosan and alginate, or their corresponding oligomers, where the two components are mixed at a (high) pH where the chitosan/chitosan oligomers are uncharged, the pH is subsequently lowered by adding a proton-donating substance, such as GDL, which releases protons in a controlled way so that the chitosan amino-groups become positively charged and a chitosan-alginate gel is formed. The gelling principle as well as the matching distances between charges are illustrated schematically in Figure 1. Herein we report on a proof-of-principle of the

new gelling system, by using i) a neutral-soluble chitosan that are mixed with MO and GDL, and ii) a poly-M is mixed with CO and GDL. Both systems reveal that a homogeneous gel of reasonable strength are formed, as judged from the gelling kinetics of the systems. Interestingly, the importance of the matching distance between the positive and negative charges was tested by comparing the gel strength of poly-M and poly-G crosslinked with CO, revealing a much higher gel strength with poly-M.



(a) Alginate - Chitosan oligomers gel (b) Chitosan - Alginate oligomers gel

M - Mannuronate unit D - Glucosamine unit GDL - D-glucono-δ-lactone

Figure 1. Schematic illustration of the gelling systems

2 EXPERIMENTAL

2.1. Materials.

Fully de-*N*-acetylated chitosan (F_A=0.002) was prepared by further heterogeneous de-*N*-acetylation of a commercial chitosan. The CO were prepared by enzymatic hydrolysis, using a commercial chitosanase from *Streptomyces griseus* (Sigma C9830), of the fully de-*N*-acetylated chitosan. The CO content was analysed by Size Exclusion Chromatography. [14] A chitosan with a degree of acetylation (F_A) of 0.40 was provided by Advanced Biopolymers. This chitosan was converted to hydrochloric acid form by dialysis. [15] A high molecular weight mannuronan (poly-M) was isolated from a C-5 epimerase negative mutant of *Pseudomonas fluorescens* as described previously. [16] An alginate enriched in guluronic acid with 88% G (poly-G) was prepared by *in vitro* epimerization with recombinantly produced C-5 epimerase AlgE6. [17] D-Glucono δ-lactone (GDL) was purchased from Sigma-Aldrich. Other chemicals were of analytical grade and used without any further purification. The MO was prepared by

acid hydrolysis as previously described. [16] The MO content was characterized by HPAEC-PAD. [18] Details of the characterization of the polysaccharides and oligosaccharides are given in Table 1 and 2, and the chromatograms of the CO and MO are given in Figure 2.

Table 1. Characterization of polymeric chitosan and alginates

Polysaccharide	Degree of acetylation (F_A)	Mannuronan fraction (F_M)	Intrinsic viscosity (mL/g)
Chitosan	0.4		840
Poly Mannuronan		1.0	740
Poly Guluronan		0.12	610

Table 2. Characterization of chitosan oligomers and alginate oligomers

Oligosaccharide	Degree of acetylation (F_A)	Mannuronan fraction (F_M)	Number-average degree of polymerization
Chitosan	0.002		5
Mannuronan		1.0	11

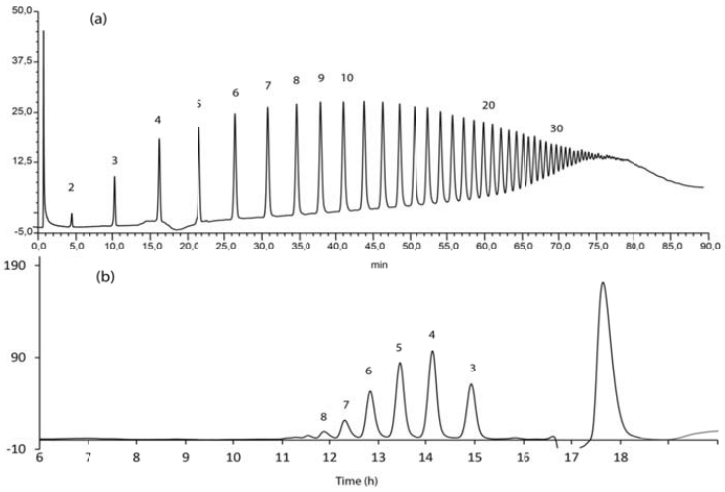


Figure 2. HPAEC-PAD chromatogram of the MO (a) and SEC-Chromatogram of the CO (b). The numbers above the peaks are the DP of the oligomer.

Chitosan – MO gels.

Chitosan hydrochloride (F_A = 0.4) was dissolved in distilled water at a concentration of 1.5%. 1.5 g of the chitosan solution was weighted out in glass vials and the pH was adjusted to 7.5 by adding 80 µL of 1M $NaHCO_3$ and mixed extensively for 15 min on a magnetic stirrer, then 120 µL of 0.1 M $NaHCO_3$ was added and mixed for 3 min. An aqueous solution (0.8 mL) of MO (3.6 mg or 1.44 mg/mL final gel) was mixed with the chitosan solution for 2 min (total volume of 2.5 mL). Solid GDL in varying amounts was finally added to the chitosan–MO mixture and mixed for 1 min, the mixture was then applied to the rheometer.

Alginate – CO gel

Alginate (poly-M or poly-G) was dissolved in distilled water at a concentration of 2% (20 mg/mL). 1.5 g of the alginate solution was weighted out into a glass vial and mixed with 1.3 mL of the CO previously pH-adjusted to 8 (using 0.1 M NaOH). A freshly-made GDL solution (200 µL of varying concentrations) was finally added to obtain a mixture with a total volume of 3.0 mL, and was applied to the rheometer.

2.2. Rheological measurement

The rheological characterization of chitosan or alginate gels was performed using a Stresstech Rheometer (RheoLogica Instruments, Sweden) fitted with a cone-and-plate geometry (cone angle of 4^0 and diameter of 35 or 40 mm). To prevent drying of the samples during measurement, the sample was covered with a layer of low-viscosity silicon oil. The test methods employed were oscillatory time, stress and frequency sweep at a constant temperature of 20^0C. For time sweep, the experiments were performed at a low oscillation frequency (1 Hz) and a small strain (0.001) to ensure that the measuring conditions did not disrupt the gelation process. The stress sweep, at a constant frequency of 1 Hz, was used to determine the linear viscoelastic region (LVR) of the hydrogels. Finally, the hydrogels were subjected to a frequency sweep in the linear viscoelastic region with a constant strain of 0.001 to characterize the viscoelastic properties as a function of frequency (0.01 to 10 Hz).

3. RESULTS AND DISCUSSION

3.1 Alginate gels crosslinked with chitosan oligomers and Chitosan gels crosslinked with alginate oligomers – Kinetics of gelling

A medium-viscosity bacterial alginate composed of only mannuronate units (poly-M) was selected. The carboxyl groups of alginates have a pK_a-value ca 3.5, [19] and the carboxyl groups will thus be fully negatively charged at neutral pH-values. The intrinsic pK_a-values of the amino-groups of fully de-*N*-acetylated CO of increasing chain lengths (monomer to heptamer) have been determined to ca 6.7 for the higher DP oligomers while the dimer had a much higher pK_a value of 7.6. [20] The solubilities at neutral pH-values of fully de-*N*-acetylated CO have to our knowledge not been systematically investigated. However, it is known that the neutral-solubility (i.e. at pH 7.5 and an ionic strength of 0.1) of low-molecular weight chitosans will increase with decreasing molecular weight and increasing F_A. [15] A mixture of fully de-*N*-acetylated CO, where the pH was adjusted to 7.5 so that the amino-groups of the oligosaccharides are essentially neutral, was added to the poly-M solution (pH 7.5), the solution mixed vigorously, and then the (slowly) proton-donating substance D-glucono-δ-lactone, GDL, was added to decrease the pH of the chitosan-alginate mixture in a controlled manner. The conditions were optimized with respect to the ratio of polymer to oligomer as well as the amount of GDL in relation to protonable amino-groups. Also, a too high concentration of oligomer was found to result in (unwanted) syneresis. The gelling kinetics was followed by measuring the dynamic storage modulus (G') and the loss modulus (G'') as a function of time. In Figure 3a is shown a frequency sweep of the poly-M together with the CO without any proton-donating substance, showing a liquid-like behaviour over a wide frequency range, Figure 3b shows the time dependent behaviour of the same mixture when GDL was added. It can be seen that upon adding GDL to the poly-M - CO mixture a dramatic change in G' and G'' with time can be observed, with G' exceeding G'' even in the first measurements, indicating an initial fast gelling kinetics of the system. The dynamic storage modulus continues to increase up to ca. 4 ks (1 h 7 minutes), although at a slower rate than initially, and then reaches an apparent equilibrium of about 100 Pa. Figure 3c shows the frequency dependence of

the resulting gel (after 30 ks of exposure to GDL). A typical mechanical spectrum of a true gel is observed, with G' larger than G'' over a wide frequency range.

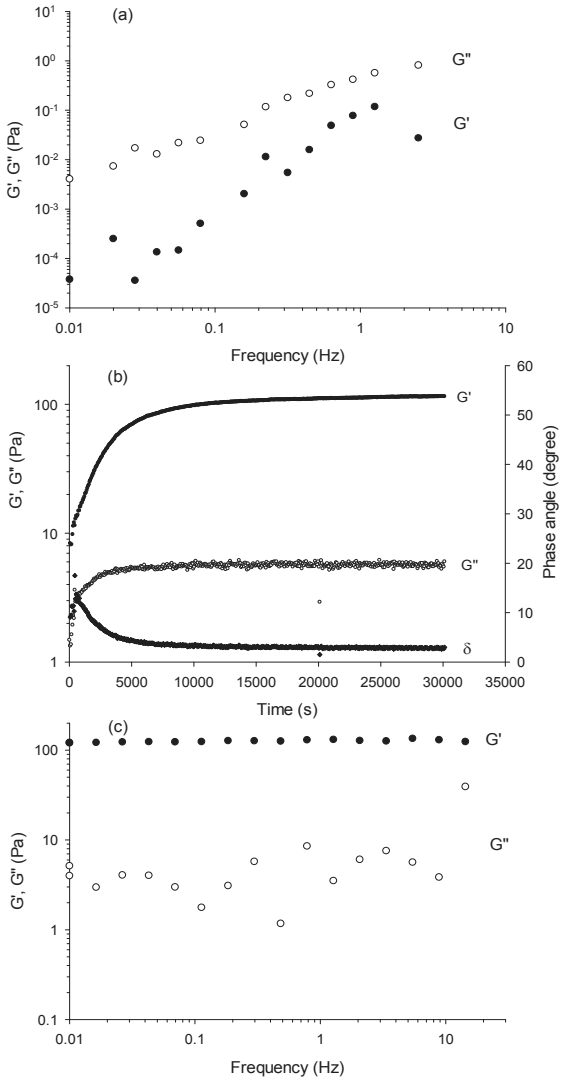


Figure 3. Frequency sweep of poly-M – CO gel without GDL (a) the kinetics of gelling with GDL (poly-M, 10 mg/mL; CO mixture, 0.6 mg/mL; GDL, 4 mg/mL), and frequency sweep after 30 ks (c). All measurements at 20^0C.

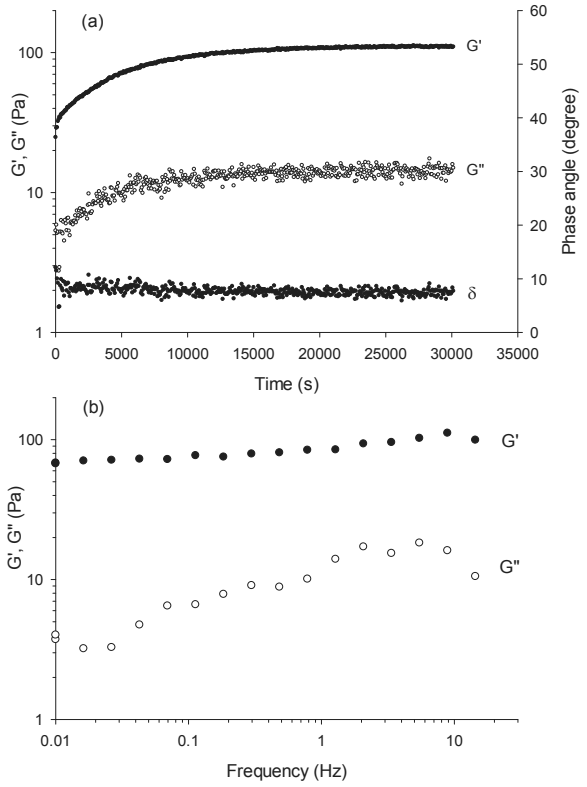


Figure 4. Kinetics of gelling of chitosan – MO gel (a) and frequency sweep after 30 ks (b). Concentrations of chitosan; 9 mg/mL; MO mixture, 1.44 mg/mL; GDL, 4.8 mg/mL. All measurements at 20^0C.

A medium viscosity (and thereby molecular weight) chitosan with a degree of acetylation (F_A) of 0.40 that was soluble at physiological (neutral) pH-values) was selected. The intrinsic pK_a-value of the amino-group of chitosan is ca. 6.5, [21, 22] , although others have reported an increase in the apparent pK_a with increasing F_A. [23, 24] Chitosans of medium-viscosity with a F_A of less than 0.4 will precipitate upon increasing the pH to neutrality, [15] while medium-viscosity chitosans with F_A between 0.4 and 0.6 are neutral-soluble meaning that they will not precipitate upon increasing the pH from below 6 to above 7. [15, 25] After solubilizing the chitosan at acidic pH and then increasing the pH to 7.5 so that the amino-groups of the chitosan are essentially

neutral, a MO was added, the solution mixed vigorously, and then the (slowly) proton-donating substance D-glucono-δ-lactone (GDL) was added to decrease the pH of the chitosan - MO mixture in a controlled manner. The gelling kinetics was followed by measuring the dynamic storage modulus (G') and the loss modulus (G'') as a function of time, as shown in Figure 4a. As with the poly-M – CO system (Figure 3b), it can be seen that G' exceeded G'' even in the first measurements, indicating an initial fast gelling kinetics of the system. In this system the dynamic storage modulus continues to increase up to ca 7 ks (ca 2 hours), at a slower rate than initially, and then reaches an apparent equilibrium of about 100 Pa. The gelling kinetics as well as the final gel strength seem qualitatively similar to the poly-M - CO. The two systems differ in the rate of the second phase of the kinetics (below 5 ks) as well as in the higher magnitude of the loss modulus (G'') of the chitosan – MO system. This could be due to quite different chain length distributions of the oligomers (Figure 2), where the mannuronan oligomers were of a much higher (average) chain length than the chitosan oligomers ($\overline{DP_n}$ of 11 vs. 5).

3.2. Stress – strain dependence

G' as a function of imposed stress (Figure 5) shows that the chosen strain for the time

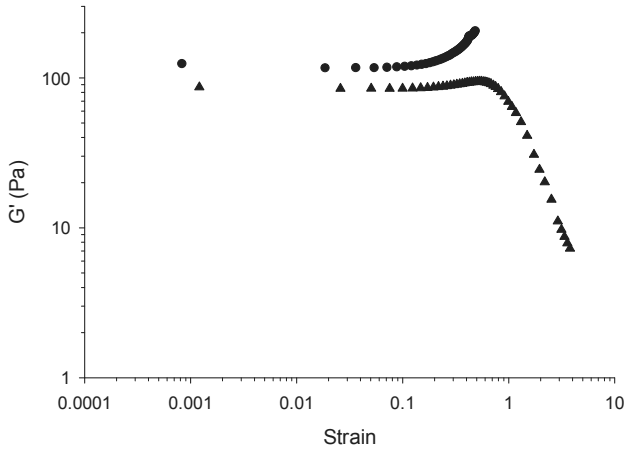


Figure 5. Storage modulus - strain dependence of (●) poly M – CO gel (poly M, 10 mg/mL, CO mixture, 0.6 mg/mL; GDL, 4.8 mg/mL); (▲) chitosan – MO gel (chitosan, 9 mg/mL; MO mixture, 1.44 mg/mL; GDL, 4.8 mg/mL).

resolved measurements and the frequency sweeps (0.001) is well within the linear viscoelastic regime for both systems. Furthermore, the results presented in Figure 5 suggest that the chitosan – MO gel system behaves as expected (strain weakening), whereas the poly-M – CO system seems to become more rigid with increased deformation (strain hardening). The latter result is quite unexpected, however it is far too early to speculate on the molecular explanation for such a behavior.

3.3. Gel strength as a function of added GDL

The addition of GDL to a mixture of Poly-M and (neutral) CO reproducibly resulted in homogeneous gels. The GDL added will protonize the amino-groups of the CO (pK_a-values of higher oligomers is ca. 6.7), [20] and we tested the effect of adding increasing amount of GDL on the gel strength. The results are given in Figure 6, showing an

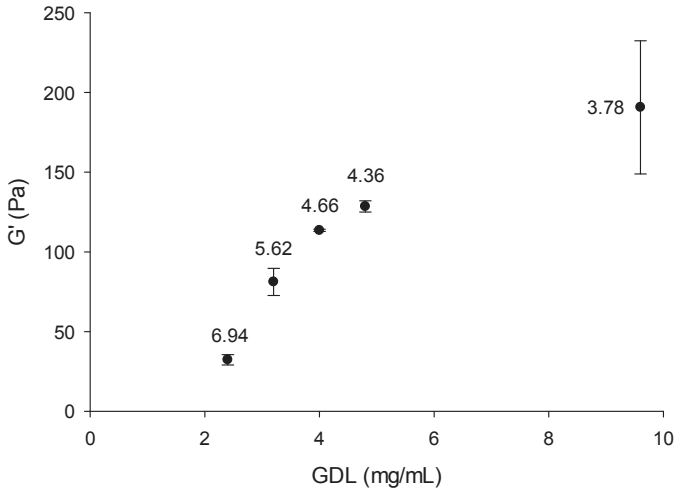


Figure 6. Gel strength (G') of poly-M – CO gel as a function of GDL concentration (poly-M, 10 mg/mL; CO mixture, 0.6 mg/mL). The numbers above the data points is the measured final pH-values of the gel.

increased gel strength (G') with increasing amount of GDL down to a pH of just below 4. With higher amounts of GDL the gel strengths were not reproducible, probably due to the faster initial gelation kinetics that follows from the higher GDL concentrations. At the lowest pH-values which is close to the pK_a-value of the carboxyl-groups, the

formation of alginic acid gels. Similar results were obtained with the chitosan – MO system, with an increasing gel strength (measured as G') with increasing GDL concentration (data not shown). Generally, this gelling system was somewhat less reproducible compared to the poly-M - CO system. This lower reproducibility could be due to different kinetics of the protonization of an amine group in a polymeric chitosan molecule as compared to the protonization of oligomeric chitosan molecules, or it could be due to hydrophobic interaction between the acetyl groups. [26]

3.4. Gel strength as a function of ionic strength

The previous results were obtained when mixing chitosan/chitosan oligomers as their

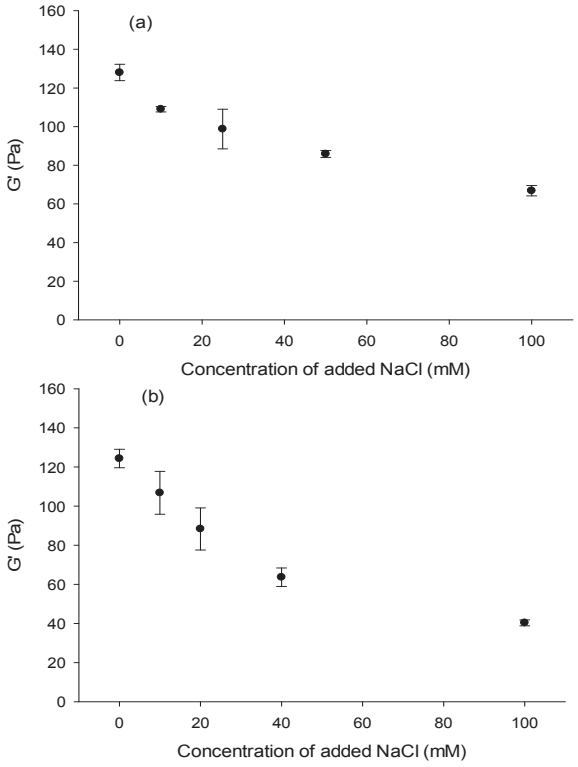


Figure 7. Gel strength (G') as a function of concentration of added NaCl. (a) poly-M – CO gel (poly-M, 10 mg/mL; CO mixture, 0.6 mg/mL; GDL, 4.8 mg/mL). (b) Chitosan – MO gel (Chitosan, 9 mg/mL; MO mixture, 1.44 mg/mL; GDL, 4.8 mg/mL).

corresponding chloride-salts and alginate/alginate oligomers as their corresponding sodium-salts, which means that one molecule of NaCl is released upon each formation of an ionic bond between the amino- and carboxyl-groups. At a concentration of 10 mg/mL of alginate, this means a (maximum) NaCl-concentration of ca. 50 mM, while for the polymeric chitosan with a degree of acetylation of 0.4 it means a maximum NaCl-concentration of ca. 30 mM, assuming that all charged groups are involved in amino-carboxyl ionic bonds. However, it should be noted that the Manning condensation of counterions is effective at the relatively short distances between electric charges, as in the alginate molecule. We measured the effect on the gel strength (measured as G') upon adding increasing concentrations of sodium-chloride. The results are shown in Figure 7 (a and b) for poly-M - CO and the chitosan - MO gels, respectively. For both systems a clear decrease in the dynamic storage modulus with increasing added salt concentration can be seen, which is in accordance of what is expected for ionic gels. The decrease is somewhat more pronounced for the chitosan – MO gels (Figure 7 b) than for the poly-M -CO gels (Figure 7 b). However, since the ionic strength without any added salt is not equal (see above) and the effect of the ionic strength on poly-electrolytes are most pronounced at the lowest ionic strengths, [27, 28] care should be taken with a detailed interpretation of the ionic strength effects in the two gelling systems.

3.5. Gelling of poly-mannuronate and poly-guluronate with chitosan oligomers

Two medium-viscosity alginates of extreme chemical compositions, a poly-M (F_M=1.0) and a poly-G (F_G=0.88), were selected and their ability to form alginate - CO gels were compared. The characterization of the alginates are given in Table 1. The gelling kinetics was followed as shown in Figure 8 by measuring the dynamic storage modulus (G') and the loss modulus (G'') as a function of time as described in the gelling kinetics previously. The results show that while the poly-M form relatively strong gels (G' of 100 Pa) with the CO upon addition of GDL, poly-G form very weak gels (G' of 10 Pa). As previously mentioned poly-M exist in the $^4C_1$ chair conformation resulting in diequatorial glycosidic linkages, [12, 29] the distance between the negative charges on the same side of the polymer chain is ca. 10.4 Å, which matches the distance between two positive charges on the same side of the similar chitosan chain, which is also in the $^4C_1$ chair conformation with diequatorial glycosydic linkages (Figure 1). In contrast, poly-G

exists in the $^1C_4$ chair conformation with diaxial glycosidic linkages, giving a less extended structure and where the dimer length is reduced to 8.7Å. [29, 30] It seems therefore that the matching conformation and thereby distance between the charges in poly-M and chitosan is important for the gelling systems presented herein.

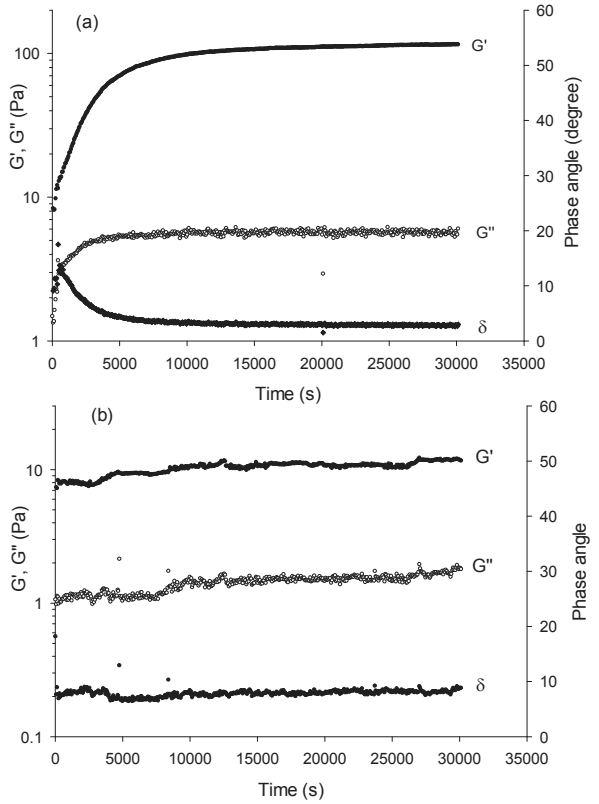


Figure 8. Kinetics of gelling (time sweep) at 20^0C of (a) poly M – CO mixture and (b) poly G – CO mixture. (poly-M, 10 mg/mL; poly G, 10 mg/mL; CO mixture, 0.6 mg/mL; GDL, 4 mg/mL).

4. CONCLUSIONS

We have demonstrated a new gelling system of the polycation chitosan (polymeric or oligomeric) and poly-M (polymeric or oligomeric), where the two oppositely charged polyelectrolytes are mixed at a pH where the amino-groups of chitosan is uncharged, then decreasing the pH in a controlled way by adding a proton donating substance which protonates the amino-groups of chitosan to form an ionic gel. The new gelling system is particularly interesting as there is a nearly perfect match in distance between the charged groups in chitosans and alginates with a high content of mannuronate residues, which importance is demonstrated by the large difference in gel strength between poly-M and poly-G crosslinked with chitosan oligomer. This new gelling system is also interesting as it represents an alternative and biocompatible gelling system for alginates with a low content of Guluronate which do not gel with calcium. Moreover, the crosslinking with oligomers, as demonstrated with mixtures of chitosan or alginate oligomers herein, represents a possibility to study the effect of the chain-length of the crosslinkers using oligomers of defined chain lengths.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

(1) Vårum, K. M.; Smidsrød, O., Structure - Property Relationship in Chitosans. In *Polysaccharides: Structural Diversity and Functional Versatility*, CRC Press: 2004.
(2) Draget, K. I.; Moe, S. T.; Skjåk-Bræk, G.; Smidsrød, O., Alginates. In *Food Polysaccharides and Their Applications*, CRC Press: 2006; pp 289-334.
(3) Berger, J.; Reist, M.; Mayer, J. M.; Felt, O.; Peppas, N. A.; Gurny, R., *Eur J Pharm Biopharm* **2004,** *57*, (1), 19-34.
(4) Kang De, Y.; Fang Lian, Y.; Jun Jie, L.; Yu Ji, Y.; Claire, J.; Matthew, S., Chitosan-Based Gels and Hydrogels. In *Smart Materials*, CRC Press: 2008.
(5) Seliktar, D., *Science* **2012,** *336*, (6085), 1124-1128.
(6) Chenite, A.; Buschmann, M.; Wang, D.; Chaput, C.; Kandani, N., *Carbohydr. Polym.* **2001,** *46*, (1), 39-47.
(7) Ruel-Gariepy, E.; Chenite, A.; Chaput, C.; Guirguis, S.; Leroux, J. C., *Int. J. Pharm.* **2000,** *203*, (1-2), 89-98.
(8) Zhou, H. Y.; Chen, X. G.; Kong, M.; Liu, C. S.; Cha, D. S.; Kennedy, J. F., *Carbohydr. Polym.* **2008,** *73*, (2), 265-273.

(9) Kempe, S.; Metz, H.; Bastrop, M.; Hvilsorn, A.; Contri, R. V.; Maeder, K., *Eur J Pharm Biopharm* **2008,** *68*, (1), 26-33.
(10) Neimert-Andersson, T.; Hallgren, A. C.; Andersson, M.; Langeback, J.; Zettergren, L.; Nilsen-Nygaard, J.; Draget, K. I.; van Hage, M.; Lindberg, A.; Gafvelin, G.; Gronlund, H., *Vaccine* **2011,** *29*, (48), 8965-8973.
(11) Draget, K. I.; østgaard, K.; Smidsrød, O., *Carbohydr. Polym.* **1991,** *14*, (2), 159-178.
(12) Atkins, E. D. T.; Mackie, W.; Smolko, E. E., *Nature* **1970,** *225*, (5233), 626-628.
(13) Minke, R.; Blackwell, J., *J Mol Biol* **1978,** *120*, (2), 167-81.
(14) Sorbotten, A.; Horn, S. J.; Eijsink, V. G. H.; Varum, K. M., *FEBS J.* **2005,** *272*, (2), 538-549.
(15) Vårum, K. M.; Ottøy, M. H.; Smidsrød, O., *Carbohydr. Polym.* **1994,** *25*, (2), 65-70.
(16) Holtan, S.; Zhang, Q. J.; Strand, W. I.; Skjak-Braek, G., *Biomacromolecules* **2006,** *7*, (7), 2108-2121.
(17) Aarstad, O. A. Alginate sequencing - Block distribution in alginates and its impact on macroscopic properties. The Norwegian University of Science and Technology (NTNU), 2013.
(18) Ballance, S.; Holtan, S.; Aarstad, O. A.; Sikorski, P.; Skjak-Braek, G.; Christensen, B. E., *J Chromatogr A* **2005,** *1093*, (1-2), 59-68.
(19) Haug, A. Composition and Properties of Alginates. Norwegian Institute of Technology, Trondheim, 1964.
(20) Tsukada, S.; Inoue, Y., *Carbohydr. Res.* **1981,** *88*, (1), 19-38.
(21) Anthonsen, M. W.; Smidsrød, O., *Carbohydr. Polym.* **1995,** *26*, (4), 303-305.
(22) Strand, S. P.; Tømmeraas, K.; Vårum, K. M.; østgaard, K., *Biomacromolecules* **2001,** *2*, (4), 1310-1314.
(23) Domard, A., *Int. J. Biol. Macromol.* **1987,** *9*, (2), 98-104.
(24) Sorlier, P.; Denuziere, A.; Viton, C.; Domard, A., *Biomacromolecules* **2001,** *2*, (3), 765-772.
(25) Sannan, T.; Kurita, K.; Iwakura, Y., *Makromolekulare Chemie-Macromolecular Chemistry and Physics* **1976,** *177*, (12), 3589-3600.
(26) Draget, K. I., *Polym Gels Netw* **1996,** *4*, (2), 143-151.
(27) Smidsrød, O.; Haug, A., *Biopolymers* **1971,** *10*, (7), 1213-27.
(28) Smidsrød, O., *Acta Chem. Scand.* **1971,** *25*, (7), 2770-2771.
(29) Atkins, E. D. T.; Nieduszynski, I. A.; Mackie, W.; Parker, K. D.; Smolko, E. E., *Biopolymers* **1973,** *12*, (8), 1865-1878.
(30) Sikorski, P.; Mo, F.; Skjåk-Bræk, G.; Stokke, B. T., *Biomacromolecules* **2007,** *8*, (7), 2098-2103.