



Norwegian University of
Science and Technology

Alginate gels cross-linked with mixtures of calcium and chitosan oligomers: Effect on swelling properties and leakage from the gel

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Biotechnology (5 year)

Submission date: May 2016

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Preface

This thesis was conducted and submitted at the Department of Biotechnology at the Norwegian University of Science and Technology (NTNU) in the time period August 2015 to May 2016.

First, I would like to express my deepest gratitude to my main supervisor, Professor Kjell Morten Vårum, for all the guidance, helpful discussions and time he has provided me with throughout this work. His support, encouragement and trust in my abilities have been essential for my motivation and dedication for the project. I would like to show my sincere appreciation to PhD Candidate Georg Kopplin for being my co-supervisor, for all the help and advices in the laboratory and during the writing process, and for all the laughs we shared. I would also like to kindly thank Olav A. Aarstad for constructive conversations and for assisting me with the SEC analyses, and Yiming Feng for teaching me the gel preparation protocol. I also cherish the conversations with Berit L. Strand, Kurt I. Draget and Gudmund Skjåk-Bræk, which improved my understanding of the swelling behaviour of alginate gels.

I am also grateful for the care and technical support I received from the senior engineers Ann-Sissel T. Ulset and Wenche I. Strand, and for the good counselling and administrative work performed by adviser Jo Esten Hafsmo. Lastly, I would like to thank my fellow students, family and friends for believing in me and for the continuous support I have received during these five years.

Trondheim, May 27th, 2016

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Abstract

A new alginate gelling system was recently reported where chitosan oligomers were used to cross-link alginate (poly-M) (Khong et al., 2013). These results have been followed up by determining the gel strength and syneresis of two different commercial alginates, said leaf alginate ($F_M = 0.54$) and stipe alginate ($F_M = 0.32$) from *Laminaria hyperborea*, that were cross-linked with combinations of calcium and a chitosan oligomer mixture (Article in preparation: “Alginate Gels with a Combination of Calcium and Chitosan Oligomer Mixtures as Crosslinkers”).

The purpose of the present master thesis has been to determine the swelling properties of two alginate gels, the first is a leaf alginate gel cross-linked with a combination of calcium and chitosan oligomer mixture (50/50 mixture), and the second a stipe alginate gel cross-linked with a combination of calcium and chitosan oligomer mixture (75/25 mixture). These two gels were selected because they exhibited a similar gel strength as pure calcium gels without significant syneresis (Article in preparation: “Alginate Gels with a Combination of Calcium and Chitosan Oligomer Mixtures as Crosslinkers”).

The chitosan oligomer mixture and the two alginates were first characterized with respect to their chemical composition and for the alginate, also their diad and triad sequences and average block lengths. The swelling of the gels was determined at four different pH-values (4.5, 5.5, 7.5 and 8.5) both in buffer solutions and where 100 mM NaCl was added. In order to compare the swelling of the combined gels, the swelling behaviour of pure calcium alginate gels at the four different pH-values were first determined. Both swelling kinetics and swelling after 24 and 48 hours were investigated.

At pH 4.5 and 5.5, where alginate and chitosan oligomers are both fully charged, the mixed alginate gels exhibited marginally lower swelling compared to the pure calcium gels in buffer solutions with and without salt. A significant increase in swelling was observed for the mixed alginate gels at higher pH of 7.5 and 8.5, whereas swelling behaviour of calcium alginate gels was similar to what was observed in the acidic conditions. These results suggested that chitosan oligomers contributed to cross-links, both above and below the pK_a of chitosan. This was supported by a swelling control study where the calcium content of the alginate gels was reduced to a similar amount of the mixed gels (but without the chitosan oligomers). The swelling kinetic results revealed that the mixed alginate gels swelled faster at pH values above the pK_a of chitosan's amino groups (pK_a -value of 6.5) compared to at the lower pH values and compared to calcium alginate gels. This more non-Fickian behaviour was most likely attributed to the reduced charge density of chitosan oligomers at the higher pH-values, decreasing the ionic association with alginate which consequently increase the stiffness of the gel as the polymer chain repulsion is increased. In addition, a size exclusion chromatography (SEC) study of the chain lengths of chitosan oligomers leaking out of the gel was performed on leaf alginate combined with a chitosan oligomer mixture. It was found that higher DP chitosan oligomers ($DP > 3$) diffused slower out of the gel, indicating an increased interaction of longer oligomers within the leaf alginate.

Sammendrag

Det ble nylig rapportert om ett nytt alginat gelsystem hvor kitosan-oligomerer ble brukt til å kryssbinde alginat (poly-M). Disse resultatene ble fulgt opp ved å bestemme gelstyrke og synerese av to forskjellige kommersielle alginater, henholdsvis blad-alginat ($F_M = 0.54$) og stilk-alginat ($F_M = 0.32$) fra *Laminara hyperborea*, som ble kryssbundet med en kombinasjon av kalsium og en blanding av kitosan-oligomerer (Artikkel i forberedelsesfase: «Alginate Gels with a Combination of Calcium and Chitosan Oligomer Mixtures as Crosslinkers”).

Formålet med denne masteroppgaven har vært å bestemme svellingsegenskapene til to alginatgeler, hvorav den første er en blad-alginatgel kryssbundet med en kombinasjon av kalsium og en blanding av kitosan-oligomerer (50/50 forhold), og den andre er en stilk-alginat kryssbundet med en kombinasjon av kalsium og en blanding av kitosan-oligomerer (75/25 forhold). Disse to gelene ble valgt på grunnlag av en gelstyrke tilsvarende rene kalsium alginatgeler uten signifikant forekomst av synerese (Artikkel i forberedelsesfase: «Alginate Gels with a Combination of Calcium and Chitosan Oligomer Mixtures as Crosslinkers”).

Kitosan oligomer blandingen og begge alginattypene ble karakterisert med hensyn til kjemisk komposisjon, og for alginat ble også monade, diade og triade sekvenser samt gjennomsnittlige blokk lengder funnet. Svellingsgraden for de ulike gelene ble bestemt for ulike pH-verdier (4.5, 5.5, 7.5 og 8.5) både i bufferløsninger og ved 100 mM tilsatt NaCl. Svellingsatferden til kalsium-alginatgeler ved de ulike pH-verdiene ble bestemt i forkant for å kunne sammenligne svellingen av gelene med kombinerte kryssbindere. Både svellingskinetikk og svelling etter 24 og 48 timer ble undersøkt.

Ved pH 4.5 og 5.5, hvor både alginat og kitosan-oligomerer er fulladet, uttrykte gelene med kombinerte kryssbindere en marginalt lavere svellingsgrad sammenlignet med rene kalsium-alginatgeler i bufferløsninger med og uten tilsatt salt. En signifikant økning i svelling for alginatgeler med kombinerte kryssbindere ble observert for ved pH 7.5 og 8.5, hvorav svellingsatferden for kalsium-alginatgeler var lignende til oppførselen som ble observert ved lavere pH. Disse resultatene indikerte at kitosan-oligomerene bidro i kryssbindinger, både over og under pK_a -verdien til kitosan. Dette inntrykket ble forsterket av ett kontrollstudie hvor kalsiuminnholdet i alginatgeler ble redusert til samme mengde som alginatgelene med kombinerte kryssbindere (men uten tilsetning av kitosan-oligomerer). Resultater fra svellingskinetikken viste at alginatgeler med kombinerte kryssbindere svellet raskere ved pH høyere enn pK_a -verdien til aminogruppene til kitosan (pK_a -verdi 6.5), sammenlignet med lavere pH verdier, samt sammenlignet med kalsium-alginatgeler. Denne atferden, som uttrykte mer non-Fickian oppførsel, var mest sannsynlig forårsaket av en redusert ladningstetthet av kitosan-oligomerer ved høye pH-verdier, som førte til redusert ionisk assosiasjon med alginat og en videre økning av stivhetsgrad for gelen som følger av økende elektrostatisk frastøtning av polymerkjeder.

I tillegg ble det utført ett size exclusion chromatography (SEC) studie av kjedelengden til kitosan-oligomerer som var lekket ut av blad-alginatgeler kryssbundet med en blanding av kitosan-oligomerer. Det ble oppdaget at kitosan-oligomere med høyere DP ($DP > 3$) duffunderte saktere ut av gelen, som var en indikasjon på at økt interaksjon med blad-alginat for lengre oligomerer.

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List of Abbreviations

A-residue	2-acetamide-2-deoxy-D-glucose (GlcNAc)
AmAc	Ammonium acetate
DP	Degree of polymerisation
D-residue	2-amino-2-deoxy- β -D-glucose (GlcN)
EMA	European Medicines Agency
FDA	Food and Drug Administration
G-residue	α -L-Guluronate
GDL	D-glucono- δ -lactone
GP3350	<i>Laminaria hyperborea</i> leaf alginate
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
<i>L. hyperborea</i>	<i>Laminaria hyperborea</i>
LF200S	<i>Laminaria hyperborea</i> stipe alginate
M-residue	β -D-Mannuronate
MALLS	Multi angle laser light scattering
MQ-water	MilliQ-water
NMR	Nuclear magnetic resonance
PEC	Polyelectrolyte complexes
Poly-M	Polymannuronic acid
pK _a	Acid dissociation constant
RI	Refractive index
SEC	Size exclusion chromatography

1 Introduction

1.1 Polymeric hydrogels

Hydrophilic gels or hydrogels are viscoelastic materials consisting of three-dimensionally cross-linked polymers, which exhibit the ability to absorb a large quantity of water in a hydrophilic network without dissolving. These complex networks of cross-linked polymer chains form a unique group of materials while being characterized with both solid and liquid like properties. The cross-links vary between covalent, ionic, hydrogen or strongly hydrophobic nature (Moe, 1993). Hydrogels possess a great variety of properties combined with the ability to store large amounts of water, allowing free diffusion in a mechanically stable network. Understanding hydrogel properties has been shown to share many similarities upon analysing biological tissues. Mechanical strength and swelling behaviour are common characteristics to investigate upon describing hydrogel systems. These features have made hydrogels a very interesting material for biomedical and industrial applications, such as tissue engineering and drug delivery systems (Ganji et al., 2010).

The high complexity of native tissue and physiological biochemistry request certain properties and performance criteria for a suitable material, such as biocompatibility, biodegradability, low toxicity, non-immunogenic, mechanical properties similar to native tissue, etc. Synthetic polymers may be rejected or causing undesirable inflammatory responses in biological systems, due to their synthetic non-biological origin. Hence, the search for applicable hydrogel materials has been extended towards naturally occurring materials or biomaterials produced by living organisms. Hydrogels of biopolymers have gained increased attention due to their application in e.g. tissue engineering, immobilization of cells and controlled drug delivery. Alginate gels is an example of a biomaterial abundant in nature that is extensively used in biomedical science and engineering while featuring many favourable properties such as biocompatibility, low toxicity and ease of gelation (Draget, 2006, Lee and Mooney, 2012, Sun and Tan, 2013).

1.2 Alginate

Alginates are a family of natural anionic polysaccharides, occurring in marine brown algae (*Phaeophyceae*) such as *Laminaria hyperborea*, *Saccharina japonica* and *Laminara digitate*. In brown algae, alginate primarily function as a structural component in the inter cellular matrix, providing skeletal strength and flexibility to the surrounding tissue. Commercial alginates are

mainly extracted from brown algae, wherein it occurs as mixed sodium, magnesium, calcium and strontium salts (Haug and Smidsrod, 1967). Alginates are also produced as a capsular polysaccharide in the extracellular matrix of a few soil bacteria, such as *Azotobacter vinelandii* and several *Pseudomonas* species (Haug and Larsen, 1971). The main industrial uses of alginates are in food and pharmaceutical applications, featuring its high performing gelling, viscofying and stabilising properties (Draget, 2006).

Alginate is characterized as a uronic acid where a primary hydroxyl group (-OH) on carbon six (C6) is oxidized, forming a carboxyl-group (-COO⁻). The protonation and deprotonation of the carboxyl group is directly dependent on the acid dissociation constant (pK_a), which is 3.38 and 3.65 for M- and G-monomers, respectively (Myklestad and Haug, 1966). The solubility is influenced by the pH, wherein alginate is insoluble in low pH solutions below pK_a due to protonation of the carboxyl group. The degree of charge density influences the hydrodynamic properties of alginate as neighbouring charges exhibit intramolecular repulsion which extends the random coil conformation. Extension of the polymer chain increases stiffness and induces a more rod like behaviour, which can lead to shear thinning and high viscosity even for low concentration alginate solutions (Smidsrød and Moe, 2008). Functional properties are also affected by the molecular weight of alginate, which is categorized as a polydisperse macromolecule where the molecular weight is given as an average of the total distribution. Commercial alginates are commonly produced in molecular ranges of 32-400 kDa (Draget, 2006).

1.2.1 Chemistry and Structure

In molecular terms, alginates comprise linear polymeric chains of β-D-Mannuronate (M) and α-L-Guluronate (G) residues linked by 1-4 glycosidic bonds (Figure 1). The composition and sequence of the two monomers constituting alginate chains may vary widely, dependent on what organism or tissue these biopolymers are isolated from (Draget and Taylor, 2011). The M and G residues are arranged in homopolymeric chain segments called M- and G-blocks, as well as alternating regions termed MG-blocks. The distribution of the M and G residues greatly influence the functional properties of the material, such as viscosity, gelation strength, stiffness, etc. (Draget, 2006).

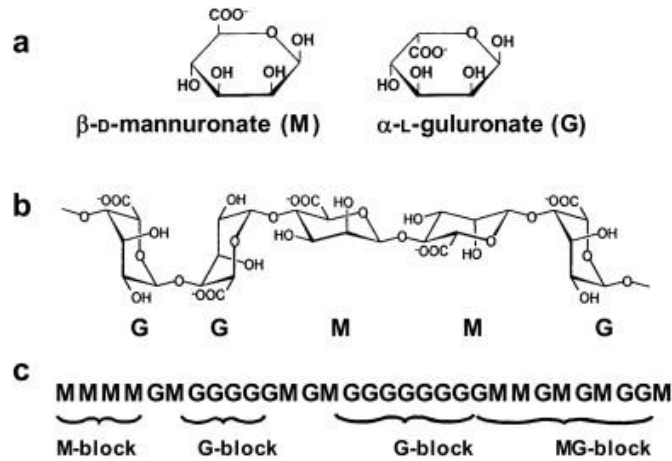


Figure 1. Structural characteristics of alginate: Haworth structure of monomers (a), ⁴C₁ M-residue and ¹C₄ G-residue chain conformations (b) and schematic chain sequence with block-segments (c) (Draget et al., 1997).

The biosynthesis of alginate is initiated through enzymatically synthesis of mannuronan or polymannuronic acid (poly-M) from the precursor GDP-D mannuronic acid (Lin and Hassid, 1966). The β -1,4 linkages are introduced by an enzymatically induced polymerization reaction. The Haworth formula of a M-monomer shows that the carboxyl points up from the ring structure (Figure 1. **Structural characteristics of alginate**). Henceforth, M-residues are most abundant in ⁴C₁ conformation, wherein the large functional group (-COOH) is stabilised in equatorial position (Haug and Larsen, 1969). Studies of bacterial synthesis of alginate have revealed that mannuronan-C5-epimerises perform an epimerisation of mannuronan chains through several reaction steps, which causes a configurational change at carbon 5 that eventually produces guluronate or G-residues (Figure 2) (Haug and Larsen, 1971). A C5 epimerisation exchanges the position of the carboxyl and the hydrogen group attached to carbon 5, such that the carboxyl group points inwards the ring structure. This configurational modification consequently converts the molecule from a D-mannuronate to an L-guluronate sugar as the epimerised molecule is most stable in ¹C₄ conformation. The configurational stereocenter at C5 is now flipped, placing the carboxyl group in equatorial rather than axial position (Figure 2) (Haug and Larsen, 1969).

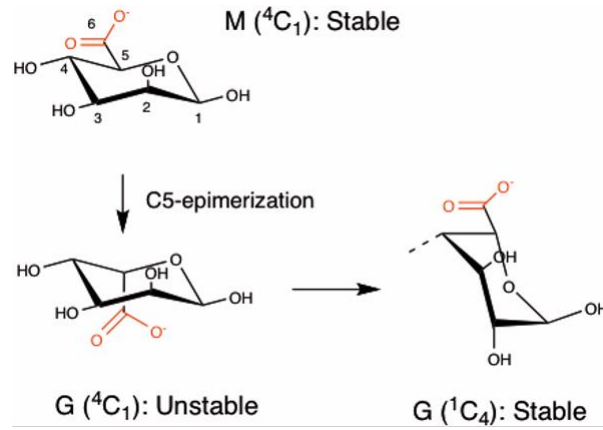


Figure 2. Conformational transition upon epimerisation of carbon number 5 (C5) for β -D-Mannuronate (M), to produce α -L-Guluronate (G) (Christensen, 2015).

The monomer composition is an important characteristic of alginates, which is described by the M and G fractions (F_M , F_G),

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

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

where n_M and n_G is the number of M and G residues. The sequence structure and monomer distribution of alginate is characterized by the fraction of diads (F_{MM} , F_{MG} , F_{GM} and F_{GG}) and triads (F_{MMM} , F_{MMG} , F_{MGG} , F_{MGM} , F_{GMG} , F_{GMM} , F_{GGM} , and F_{GGG}), rather than just the monomer composition. These parameters can be characterized by several nuclear magnetic resonance (NMR) spectroscopy techniques (Grasdalen et al., 1981, Grasdalen, 1983). Further, the average G and M block length can be calculated from these fractions.

$$\bar{N}_{G>1} = \frac{F_G - F_{MGM}}{F_{GGM}}$$

$$\bar{N}_{M>1} = \frac{F_M - F_{GMG}}{F_{MMG}}$$

Typically, the average G-content (F_G) and G-block length ($\bar{N}_{G>1}$) is significantly higher in alginate derived from brown algae tissue with high stiffness, such as *L. hyperborea* stipe tissue. Likewise, high average M-content (F_M) and M-block length ($\bar{N}_{M>1}$) is extracted from softer tissues such as *L. hyperborea* leaf tissue (Moe, 1993). Pure poly M can be produced in mutated or genetically modified bacterias, such as *P. aeruginosa* mutants and *P. fluorescens* strains (Gimmestad et al., 2003).

1.2.2 Gelling Properties

Naturally occurring alginates are often integrated in larger network structures and are mostly found as a gel substance in the extracellular matrix. The ability to form ionic gels may be claimed as the most important property of alginates (Draget, 2006). The gel-forming property is well explained by the “egg box model” as a result of selective affinity towards cations, which again varies upon the sequence and composition of the alginate chains. The diaxially linked G-residues in the G-block segments provides cavities which facilitates high affinity towards cations, especially divalent cations (Figure 3). The GG conformation function as a binding site for the ions and causes dimerization of alginate chains which enables the formation of a gel network with dimeric junction zones (Grant et al., 1973).

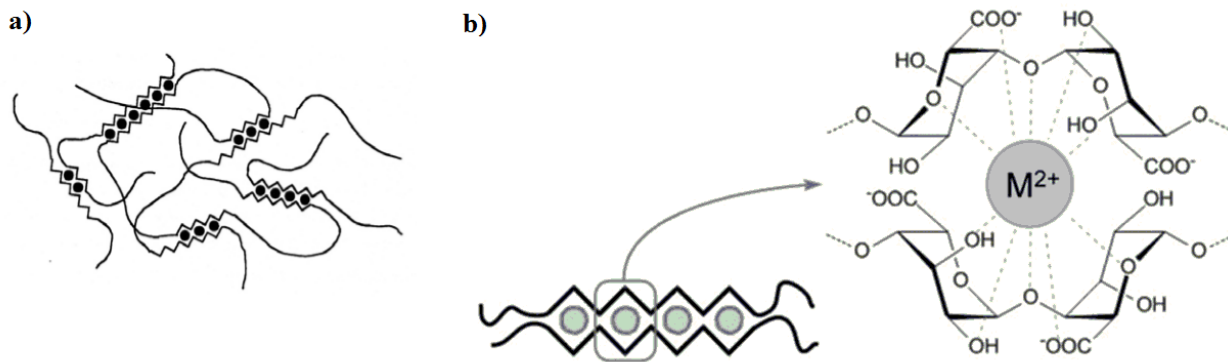


Figure 3. Egg box model: Schematic illustration of cross-linked alginate network (a) where chains are ionically cross-linked with divalent cations (M^{2+}) with high affinity to G-block junction zones (Moe, 1993, Pistone et al., 2015).

Ions are selectivity bound dependent on size, where the affinity towards divalent earth metal ions has been determined experimentally: $Pb > Cu > Cd > Ba > Sr > Ca > Co, Ni, Zn > Mn$ (Smidsrod,

1974). Calcium, strontium and barium are bound cooperatively between G-blocks when exceeding a certain average G-block length, $\bar{N}_{G>1}$, said between 8 and 20 monomers (Stokke et al., 1991). Alginate form strong gels with divalent cations due to high degree of cross-linking, however the gel properties are highly dependent on the choice of cross linking agent and the alginate composition. Alginate with high F_G produce hard and brittle gels with divalent ions, while alginate with high F_M produce weaker and more elastic gels. Calcium is frequently used as cross-linker in alginate gels due to its prevalence in biological systems, low costs and ability to form relatively strong and rigid gels (Draget, 2006).

1.2.3 Preparation of Homogeneous Gels by Internal Gelation

There are several methods for preparation of alginate gels. Homogeneous calcium alginate gels are commonly prepared by an *in situ* or internal gelation technique, where a calcium salt together with a sequestering agent is utilized. The preference calcium salt and sequestering agent is often finely ground calcium carbonate (CaCO_3) together with D-glucono- δ -lactone (GDL). In aqueous solution, a slowly hydrolysis of proton donor GDL induces liberation of calcium ions (Ca^{2+}) as CaCO_3 is protonated (Figure 4). In the same reaction step, the intermediate bicarbonate (HCO_3^-) is also produced which is further processed to carbon dioxide (CO_2) and water (H_2O). The total reaction can be induced by degassing an alginate CaCO_3 solution before adding GDL, where the CO_2 deficiency consequently shifts the reaction towards initiation of gelation by production of Ca^{2+} , following Le Châtelier's principle. Degassing also reduce gas capture within the gel and improves the gel turbidity. The released calcium ions associate with the alginate chains, forming ionic bonds with the G-blocks and weaker interactions with the M-blocks. The kinetics of the gelation process is observed independent of F_G , however the highest gel strength is obtained with equal Ca^{2+} and GDL concentrations within the mixture (Draget, 1990).

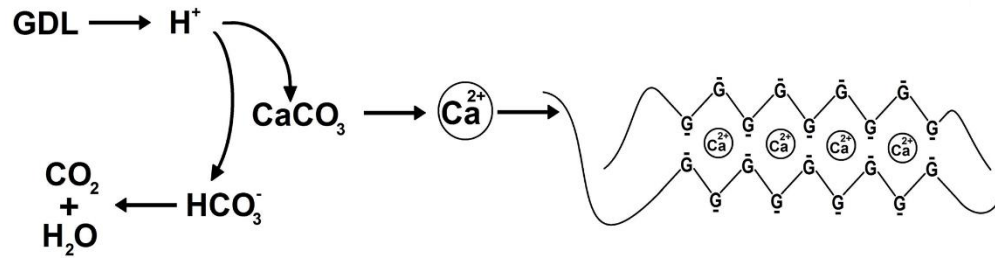


Figure 4. Internal gelation of guluronic acid (G) segments in alginate with calcium carbonate (CaCO_3) and D-glucono- δ -lactone (GDL). GDL is slowly hydrolysed and adequately CaCO_3 is protonated to release calcium ions (Ca^{2+}) (adapted from Georg Kopplin).

1.2.4 Applications

Today, alginate is one of the most employed biopolymers in agricultural, food and life science related industry, mostly due to its high performance gelling and viscosity properties. The initial industrial uses of alginates were as adhesive binders, however more recently they are extensively used as thickeners, emulsifiers, film and gels making substances. However, the price of alginate is relatively high compared to other biopolymers such as carboxymethyl cellulose (CMC) (Guiry and Blunden, 1991, Brownlee et al., 2009). As a U.S. Food and Drug Administration (FDA) approved material, alginates are one of the most significant biomaterials for various applications in regeneration medicine, nutrition supplements, semipermeable separation, etc. (Sun and Tan, 2013). Conventional role of alginates in pharmaceuticals are as thickening, gel forming and stabilizing agents, where oral dosage forms are currently the most frequent use. Nevertheless, there is an emerging interest for the use of alginate hydrogels as depots in tissue localized drug delivery and supporting scaffolds in regenerative medicine (Draget, 2006, Sun and Tan, 2013).

Alginate calcium spheres have been successfully used as immobilisation matrix for living plant and animal cells (Smidsrød and Skjåk-Bræk, 1990). Alginate gels have been shown to provide a moist wound dressing material which accelerates wound regeneration rates in pigs with acute surgical wounds (Rabbany et al., 2010). Alginate-based microcapsules have also been used for immunoisolation of pancreatic islets (de Vos et al., 2006), and targeted secretion of therapeutic molecules by microencapsulation of genetically engineered cells (Rokstad et al., 2002). Most recently, soft alginate hydrogels have been shown to generate a scaffold in which support growth of neuronal cells (Matyash et al., 2014). Another advantage of alginate is that it can be easily

modified and can be combined with other biomaterials, as a way of tuning its properties or discover new abilities that may be relevant for emerging applications (Sun and Tan, 2013).

1.3 Chitosan

Chitosan is another naturally occurring polyelectrolyte, and is the deacetylated derivative of chitin, one of the most abundant polysaccharides in nature. Chitin is a structural component found as crystalline microfibrils in the exoskeleton of arthropods, such as insects and crustaceans, and in the cell walls of algae, fungi and yeast. Industrial chitin and chitosan is mainly harvested from shrimps and crabs where it is retrieved by acid treatment followed by alkaline extraction. Direct processing and uses of chitin as raw material is limited by its insolubility, caused by its dense crystalline morphology and uncharged polymeric chain. Solid state chitin can however be processed into chitosan by partial hydrolysis (de-N-acetylation) in highly alkaline or acidic conditions, or by enzymatic hydrolysis induced by chitin deacetylases (Figure 5). Chitosan are semi-crystalline in solid state and water-soluble in acidic solutions, and are therefore much easier to process. Currently, there is a continuous search for a main application for chitosan and thus the annual production is still relatively moderate. However, the unique pseudo-natural cationic character, film forming properties, low toxicity and biocompatibility constitutes a biomaterial with high potential within many various fields, especially in biomedical industry (Vårum and Smidsrød, 2005, Rinaudo, 2006).

1.3.1 Chemistry and Structure

Chitin is a linear homopolymer of β -(1 \rightarrow 4)-linked 2-acetamide-2-deoxy-D-glucose (GlcNAc; A) where every sugar unit is in the 4C_1 conformation (Figure 5). The deacetylation reaction removes the acetyl-group (-COCH₃) and introduce a free amino-group (-NH₂) that carry charges in acidic solutions, hence making chitosan water-soluble. Thus, chitosan are polymeric chains composed of both A-residues and the same positively charged 2-amino-2-deoxy- β -D-glucose (GlcN; D) residue, linked together by 1-4 glycosidic bonds (Figure 5) (Vårum and Smidsrød, 2005).

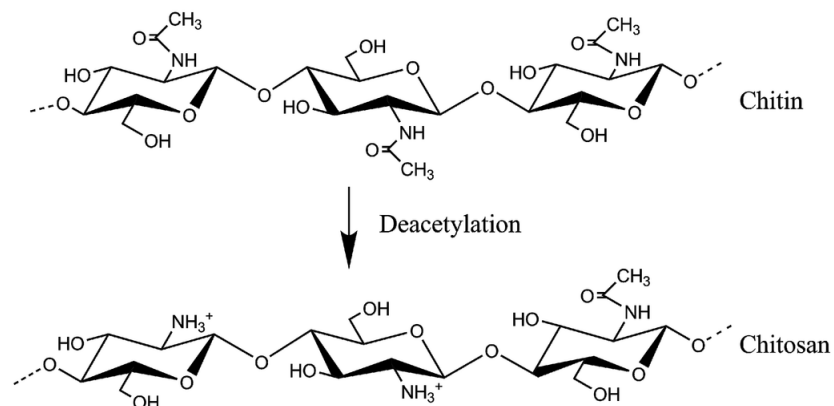


Figure 5. Structural conformation of chitin and chitosan where deacetylation of β -(1 \rightarrow 4) linked 2-acetamide-2-deoxy-D-glucose (GlcNAc; A) chitin produces β -(1 \rightarrow 4) linked 2-amino-2-deoxy- β -D-glucose (GlcN; D) chitosan (Nilsen-Nygaard et al., 2015).

Many chitins contain a certain amount of D-residues, and when the deacetylation degree of chitin reaches above 50% ($F_D = 0.5$) the polymer is characterised as chitosan. The solution properties of chitosan are governed by the relative content of acetylated amino-groups, which is reflected by the A- and D-residue fraction parameters (F_A , F_D). A- and D-residues are distributed randomly along the chain, and the composition directly influences functional properties of the polymer, such as solubility, flexibility and viscosity (Vårum et al., 1994).

The pK_a value of the amino-group is approximately 6.5-6.6, meaning chitosan is water-soluble when these functional groups are predominantly protonated ($-\text{NH}_3^+$). For a chitosan solution with pH equal to the pK_a , 50% of the amino-groups will be charged (Strand et al., 2001). The degree of ionization (α), and thus solubilisation of chitosan, is highly dependent on the pH and pK_a of the solvent. Chitosans are manufactured as “free amines”, that do not directly dissolve in pure water, or water soluble chitosan salts, typically with chloride or acetate counterions (Vårum et al., 1994). Similar to alginate, chitosan is a polydisperse material where the average molecular weight distribution of polymer molecules has great impact on the hydrodynamic and mechanical properties. As a polyelectrolyte, chitosan is also affected by the electrostatic repulsion between neighbouring charges, where high and low ionic strength solutions consequently contracts and extends the polymer chain, respectively (Smidsrød and Moe, 2008).

The characterization of chitosan requires determination of parameters such as intrinsic viscosity, deacetylation degree, average molecular weight distribution, etc. The average molecular weight distribution can be determined by several analytical methods, such as high performance liquid chromatography (HPLC), intrinsic viscosity measurements or NMR spectroscopy (Wu et al., 1976) (Smidsrød and Moe, 2008). Similar to alginate, F_D and F_A of chitosan is commonly characterized by NMR techniques, primarily liquid state ^1H NMR (Vårum et al., 1991).

1.3.2 Oligosaccharides

Recently an increased attention towards chitosan oligosaccharides have been developed, as this type of material has shown to generate antibacterial, antifungal and antitumor activities as well as immune-enhancing effects in some animals. Oligosaccharides typically consist of 2-20 sugar units defined by the degree polymerisation (DP). Chitosan can be used to chemically synthesize homogeneous series of chitosan oligosaccharides, or oligomers, by different depolymerisation processes. Conventional methods for preparing chitosan oligomers relies on either acidic, alkaline or enzymatically induced depolymerisation (Trombotto et al., 2008, Aam et al., 2010). Enzymatic production of chitosan oligomers was demonstrated by Heggset and co-workers, which performed degradation of chitosan with 46 different chitosanases isolated from *Streptomyces coelicolor* (Heggset et al., 2010). Tømmerraas and co-workers used nitrous acid (HNO_2) to hydrolyse chitosan and produce chitosan oligomers, which was further isolated and characterized by applying an analytical method based on chromatographic separation. DP, size distribution and degree of acetylation are common parameters to characterize a chitosan oligomer mixture (Tommeraas et al., 2001). Fully N-deacetylated or GlcN oligomers can be fabricated by hydrolysing fully N-deacetylated chitosan. The use of GlcN oligomers may provide an advantage in many applications as they are water soluble and produce low viscosity in solution (Trombotto et al., 2008, Aam et al., 2010).

1.3.3 Applications

Although chitosan might not have experienced its true potential in an industrial context, it is used in a broad range of applications within agricultural, cosmetic, food, water and waste treatment, and pharmaceutical applications. Chitosan is used coagulation and flocculation agent to purify water and waste water and treatment of oil waste from food and oil industry (Ahmad et al., 2006, Renault

et al., 2009). Chemical and physical chitosan gels with relevance for biological systems can be made by using various cross linkers (Nilsen-Nygaard et al., 2015). Alginate-chitosan complexes have been used as tissue engineered scaffolds in wound dressing and bone tissue regeneration applications (Wang et al., 2002, Li et al., 2005). Extensive research is done on alginate-chitosan microcapsules as a drug delivery system (Pasparakis and Bouropoulos, 2006). Nevertheless, there are currently no FDA or European Medicines Agency (EMA) approved chitosan based products on the global market (Dornish et al., 2012). Another major advantages of chitosan are that they are an easy target for chemical modifications, tailoring functional properties and design a polymer towards applications with highly specific requirements. However, challenges such as solubility in physiological conditions and regulatory issues is a concern that needs to be addressed to expand the uses of chitosan (Vårum and Smidsrød, 2005).

1.4 Cross-linking Alginate with Chitosan Oligomers

When mixing alginate and chitosan, a polyelectrolyte complexes (PEC) is formed through ionic interactions between the oppositely charged groups, typically constituted in an unordered polymeric network. The formation of an alginate-chitosan PEC is commonly done in pH range 3.5 to 6.5, where both polymers are soluble (Figure 6). However, when a fully charged polyanion and a polycation are mixed they generally precipitate and do not form a gel. Several PEC systems remain stable in physiological conditions, which indicates that amino-groups may retain their charge in an ionic interaction even though the pH is above the pK_a of chitosan (Lawrie et al., 2007).

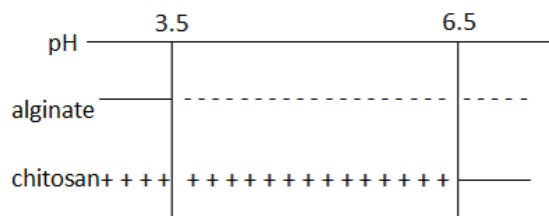


Figure 6. pH-solubility range of alginate and chitosan. pH range wherein alginate and chitosan are allegedly charged and soluble as a consequence of the pK_a of ~ 3.5 and ~ 6.5 for the carboxyl-groups of alginate and amino-group of chitosan (adapted from Georg Kopplin).

1.4.1 Alginate Chitosan Oligomer Gels

Evaluation of charge to charge distances for of alginate and chitosan chains led to the idea that more organised PEC network structure could be achieved using fully N-deacetylated chitosan oligosaccharides as cross linkers. This idea was based on basic chemical principles obtained from the chain conformation of alginate and chitosan chain segments. The charge to charge distances on each side of the conformational alternating M-, and G-block residues are 10.3-4 Å and 8.7 Å, respectively (Figure 7). In conformational terms, the poly-M of alginate and poly-D of chitosan are comparable, said both having the β -(1 \rightarrow 4) linked 4C_1 conformation that is rotated 180 degree angle relative to the neighbouring sugar unit. For poly-M alginate and poly-D chitosan, the sugars are linked together by a diequatorial linkage compared to the diaxial linkages between G-residues in G-block segments. Consequently, the charged groups on C5 is alternating for poly-M and poly-D, providing a charge distance of one disaccharide unit of 10.3-4 Å (Figure 7) (Khong et al., 2013).

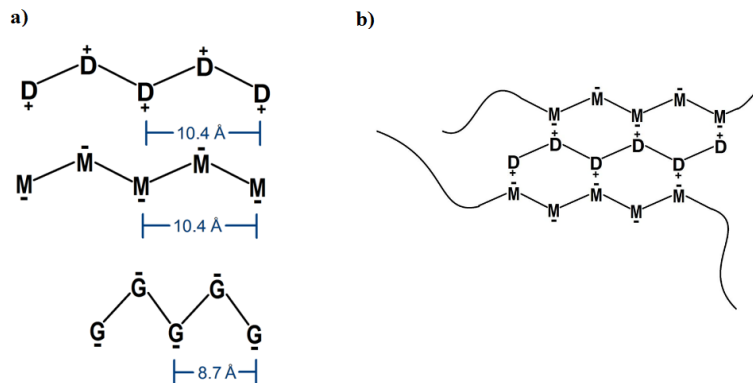


Figure 7. Charge distances between β -(1 \rightarrow 4)-linked glucosamine (D), β -(1 \rightarrow 4)-linked manuronate (M) and α -(1 \rightarrow 4)-linked guluronate (G) segments (a) and poly-M glucosamine gelling principle (adapted from Georg Kopplin).

Khong and co-workers managed to successfully prepare homogeneous alginate chitosan gels by cross-link poly-M alginate with a GlcN oligomers mixture. These ionic gels were prepared by internal gelation using GDL to slowly hydrolyse chitosan oligomers. To achieve homogeneous cross-linking, the pH of the chitosan oligomer solution was increased to ~ 8 prior to the mixing with the alginate solution. The gels showed relatively high mechanical strength, however not as high as conventional alginate calcium gelling systems. However, commercial alginates always contain

considerable M-fractions which provide minor contribution to the gel strength. As current alginate gelling systems mostly utilize G-residue cross-linking, the alginate chitosan oligomer gelation system provides an exciting alternative (Khong et al., 2013).

1.4.2 Alginate Gels Combined with Calcium and Chitosan Oligomers

A scientific group from Department of Biotechnology at the Norwegian University of Science (NTNU), led by Kjell Morten Vårum, have continued the work on the new gelling systems. This group managed to prepare a mixed gelling system where stipe and leaf alginates were cross-linked with both CaCO_3 and chitosan oligomers. This was also done using the same internal gelation protocol where GDL was used to protonate both CaCO_3 and chitosan oligomers simultaneously (Figure 8).

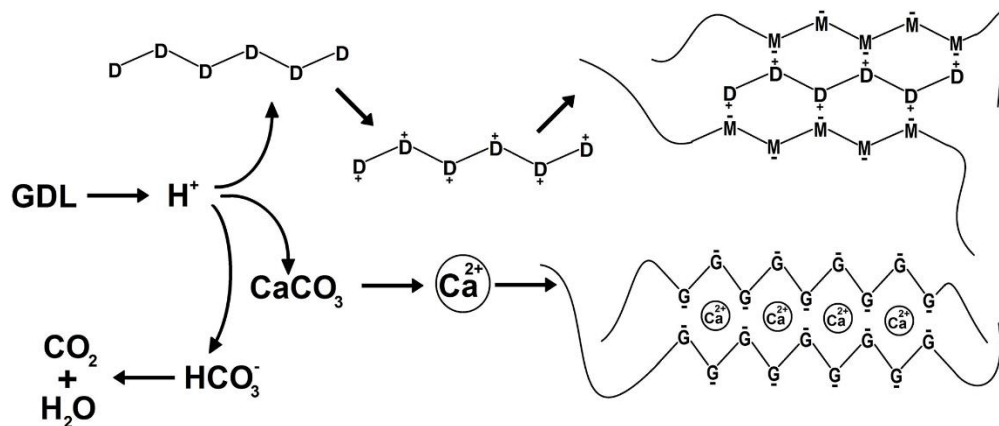


Figure 8. Dual internal gelation of mannuronate (M) and guluronate (G) segments in alginate with both calcium carbonate (CaCO_3) and fully deacetylated chitosan (poly-D), using D-glucono- δ -lactone (GDL). By adjusting the pH of the chitosan oligomer solution sufficiently above its pK_a value of 6.5, the oligomers are slowly charged and calcium ions (Ca^{2+}) are released when GDL is added and hydrolysed in the alginate mixture (adapted from Georg Kopplin).

The mechanical properties and syneresis of these mixed gels were investigated systematically by rheology and mechanical measurements. The group determined a maximum concentration of CaCO_3 (100%) before syneresis occurred in *L. hyperborea* leaf and stipe alginate calcium gels. Similarly, a maximum concentration of chitosan oligomer mixture (100%) before syneresis occurred was determined for *L. hyperborea* leaf alginate chitosan oligomer gels. Furthermore, an experiment was conducted where the gel strength was investigated as a function of different ratios

of CaCO_3 and chitosan oligomers, starting from the maximum concentration (100%) of each component. This was done for both M rich leaf and G rich stipe alginate. A remarkable effect in gel strength was observed for leaf alginate gels, whereas the gel strength was maintained upon substituting 50% of calcium with 50% of chitosan oligomers (Figure 9). For stipe alginate gels, the gels strength was maintained upon substituting a lower amount of calcium with chitosan oligomers (Figure 10). These results are yet to be published.

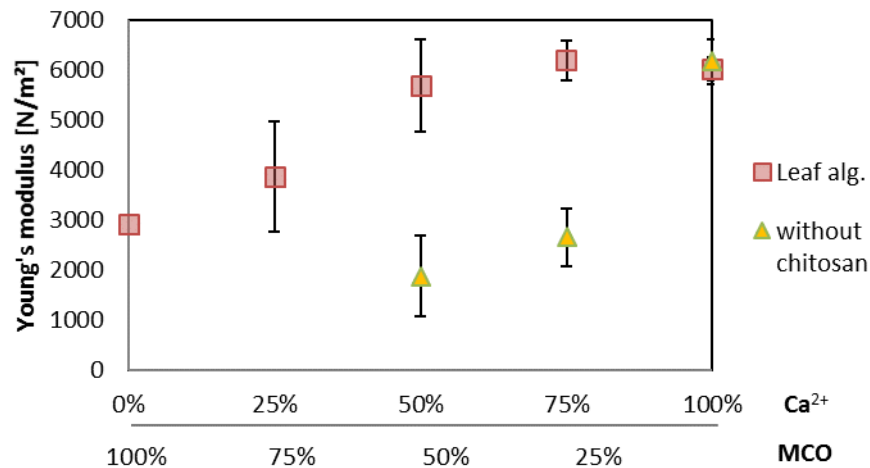


Figure 9. Gel strength *L. hyperborea* leaf alginate gels as a function of different mixing ratios of chitosan oligomers mixture (MCO) and calcium ions (Ca^{2+}). 100% calcium represents an internal calcium concentration of 15.1 mM, which was observed as the maximum concentration of calcium before syneresis occurred in leaf alginate calcium gels. Similarly, 100% MCO represents an internal MCO concentration of 37.6 mM, which was observed as the maximum concentration of MCO before syneresis occurred in alginate MCO gels. The gel strength was maintained upon substituting 50% of the calcium with 50% MCO. This figure was obtained from and belongs to Yiming Feng and Georg Kopplin.

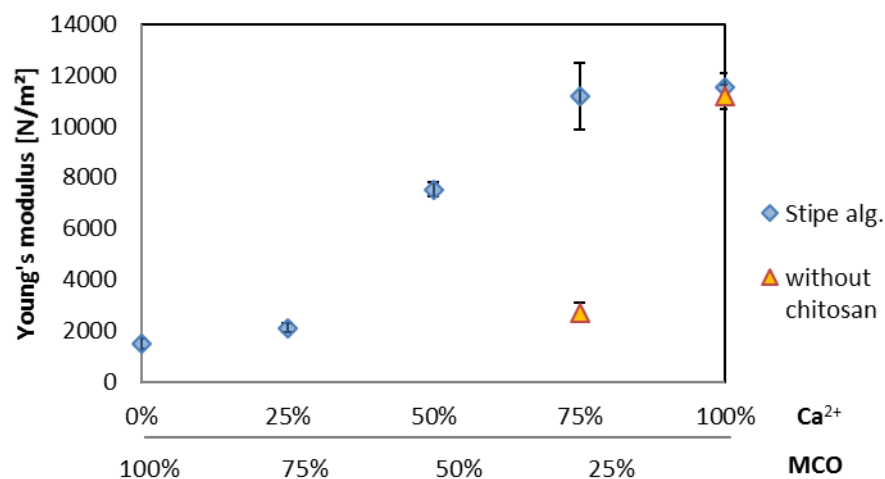


Figure 10. Gel strength *L. hyperborea* stipe alginate gels as a function of different mixing ratios of chitosan oligomers mixture (MCO) and calcium ions (Ca^{2+}). 100% calcium represents an internal calcium concentration of 16.6 mM, which was observed as the maximum concentration of calcium before syneresis occurred in stipe alginate calcium gels. Similarly, 100% MCO represents an internal MCO concentration of 37.6 mM, which was observed as the maximum concentration of MCO before syneresis occurred in alginate MCO gels. The gel strength was maintained upon substituting 75% of the calcium with 25% MCO. This figure was obtained from and belongs to Yiming Feng and Georg Kopplin.

An alginate gelling system with cross linking agents with high affinity towards both M- and G-blocks may provide exciting possibilities. M rich alginate exhibits lower calcium affinity, which produces hydrogels with significantly lower mechanical strength. The M and G content of commercial alginates often vary considerably, hence reducing quality and reproducibility of alginate gel products (Draget, 2006). In biomedical terms, certain cells have been observed sensitive to extracellular calcium concentration exceeding physiological conditions ranging from 1 to 3 mM. Maeno and co-workers reported that calcium concentrations above 10 mM induced cytotoxic effects for osteoblasts cultivated in type II collagen gels comprising hydroxyapatite (Maeno et al., 2005). Hence, it might be beneficial to reduce the amount of calcium in hydrogels applicable in some biological systems. However, further characterization is necessary before speculating in potential applications for these gels. For instance, the swelling behaviour of these gels have been undisclosed.

1.5 Swelling Behaviour of Gels

The swelling behaviour is an important parameter in characterization of polymeric hydrogels and have gained increased attention over the recent years as this type of fluid rich materials is suitable for many industrial, pharmaceutical and medical applications. The rate and degree of swelling are for instance the most significant parameters upon evaluating materials applicable in drug delivery systems. The swelling phenomenon is a rather delicate matter, and many studies and interpretations have been done in order to understand describe the thermodynamics and kinetics behind swelling of polymeric hydrogels (Ganji et al., 2010).

Flory's swelling theory is often applied as the basis to describe the swelling properties at equilibrium for polymer network structures. However, this theory has been modified by researches such as Tanaka et al., Ilavsky et al. and Vashegani-Farahani et al. in an attempt to establish a more quantitative prediction of the swelling phenomena at given conditions (Ganji et al., 2010). The swelling kinetics are mostly related to Fick's first law of diffusion, where empirical equations are frequently utilized to determine the major mechanism of diffusion, and advanced mathematical models provide more precise estimations (Masaro and Zhu, 1999). In this section the basic swelling theory of Flory, Fick's diffusion process is elaborated, as well as a few modified models to identify main factors that contribute to swelling equilibrium and kinetics in relation to alginate gels.

1.5.1 Basic Thermodynamical Treatment of Polymer Network Structures

Swelling of hydrogels in aqueous solution is often described as a two-component system involving a gel phase (g), containing a polymeric network, and a solvent (s). The cross links between polymer chains prevents polymer segments from dissolving into the solvent phase. Contrariwise, mobile moieties such as free ions and water molecules, can freely diffuse through the network interface and junction zones, and redistribute between the two phases (Höpfner et al., 2013). A gel can be better understood as a osmotic pressure system where the gel surface function as a semipermeable membrane, entrapping the polymer and allowing only mobile species to diffuse through (Moe et al., 1993). To establish thermodynamic equilibrium, the chemical potential of each mobile species must identical in both phases:

$$\mu_i^g = \mu_i^s$$

where μ_i is the chemical potential for species i (Höpfner et al., 2013).

Swelling at equilibrium can be described as a free energy change corresponding to the volume change of the gel. To describe the volume changes of a gel as a two-phase osmotic pressure system, the following relationship between chemical potential and pressure from the laws of thermodynamics have been applied:

$$\left(\frac{\partial G}{\partial p}\right)_T = V$$

$$\left(\frac{\partial \mu}{\partial p}\right)_T = \bar{V}$$

Further, the chemical potential is defined as the partial molar free energy:

$$\mu_i = \left(\frac{\partial G}{\partial n_i}\right)_{P,T,n_{j \neq i}}$$

where G represents (Gibb's) free energy and the number n_i of moles of component i (Smidsrød and Moe, 2008). At thermodynamic equilibrium, the chemical potential μ_i of component i is associated with its activity a_i in the system:

$$\mu_i - \mu_i^0 = RT \ln a_i$$

where R is the gas constant, T the absolute temperature, μ_i^0 is the chemical potential at standard state and $\mu_i - \mu_i^0$ the partial molar Gibbs free energy change. The change in chemical potential caused by different osmotic pressures, from p_0 to $p_0 + \Pi_i$, in a two-phase polymer-solvent system is related to the formula:

$$\Delta \mu_i^p = \int_{p_0}^{p_0 + \Pi_i} \left(\frac{\partial \mu_i}{\partial P}\right)_T dP = \int_{p_0}^{p_0 + \Pi_i} \bar{V}_1 dp \approx \int_{p_0}^{p_0 + \Pi_i} V_1^0 dp \approx V_1^0 \Pi_i$$

Here, \bar{V}_1 represents the partial volume and V_1^0 the molar volume of the solvent (Moe, 1993).

Flory uses the analogy of an elastic polymer solution to describe a swollen gel. Swelling is an opportunity for a polymer network to increase its entropy, afforded by the volume increase created upon absorbing the solvent. Hence, the “swelling pressure” in a gel is comparable to the osmotic pressure of a polymer solution. The absorption of solvent generates elongation of polymer chains between network junctions, which produces an elastic retractive force opposing the force of the swelling process (Flory, 1953). Swelling equilibrium is achieved when the osmotic pressure and the elastic reaction are in balance:

$$\Pi_{osm} + \Pi_{el} = 0$$

The difference in chemical potential inside and outside the gel is often preferred in the theoretical treatment of swelling rather than the osmotic pressure. Equilibrium is established when the chemical potential of the solvent inside and outside the gel is equal (Moe et al., 1993).

Hence, the swelling equilibrium is rather expressed:

$$\Delta\mu_{osm} + \Delta\mu_{el} = 0$$

According to Flory, the osmotic contribution related to swelling is distributed between two factors: the chemical potential difference caused by the spontaneous polymer-solvent mixing (μ_{mix}), and the chemical potential difference caused by the difference in ion concentration within and outside the gel (μ_{ion}):

$$\Delta\mu_{osm} = \Delta\mu_{mix} + \Delta\mu_{ion}$$

In the means of non-ionic networks, there is no alteration in the presence of ions inside the gel and in the exterior solution, thus the difference in chemical potential of ions equals zero. The swelling of such gels can be described exclusively by the mixing and elastic term:

$$\Delta\mu_{mix} = RT[\ln(1 - \phi_2) + \phi_2 + \chi\phi_2^2]$$

$$\Delta\mu_{el} = RTV_1^0 \frac{v}{V_0} \left(\phi_2^{\frac{1}{3}} + \frac{1}{2}\phi_2 \right)$$

where V_0 represents the polymer network volume at cross-linking, ν the molar number of cross-linked chains, ϕ_2 polymer volume fraction and χ is the Flory-Huggins interaction parameter. χ characterizes polymer and the solvent interactions, and is $\chi < 0.5$ for a good solvent, $\chi > 0.5$ for a poor solvent and $\chi = 0.5$ for θ conditions (Moe et al., 1993). The mixing term is based on the osmotic pressure of a polymer solution under ideal conditions, provided by the Flory – Huggin’s equation for the Gibbs free energy of mixing (Flory, 1953). The elastic term is based on the theory of rubber elasticity under the assumption of a Gaussian polymer network cross-linked in dry state (Flory and Rehner, 1943). Using the elastic and mixing term to define the dynamics of cross-linked polymer networks is often referred to as the Flory-Rehner approach (Ganji et al., 2010).

Tanaka et al. suggested that the stress and drag forces between the gel and solvent phase counterbalance the motion of the polymer network. By assuming that the polymer network end-to-end distances are provided by the random walk configuration of the polymer chains, said upon a gel volume identical to the volume as cross-links are formed (V_0), the elastic expression is modified (Tanaka, 1978):

$$\Delta\mu_{el} = RTV_1^0 \frac{\nu}{V_0} \left(\left(\frac{\phi}{\phi_0} \right)^{\frac{1}{3}} - \frac{1}{2} \frac{\phi}{\phi_0} \right)$$

Here, ϕ represents the volume fraction of polymer in the gel and ϕ_0 is the gel volume fraction during cross-link formation.

1.5.2 Equilibrium Swelling of Polyelectrolyte Gels

If the polymer chains in the gel network contains ionisable groups, the swelling forces may be greatly influenced by unequal distribution of mobile ions between inside and outside the gel. The presence of fixed charges in the polymer network attracts charged substituents and prevents diffusion of these mobile ions into the external solution (Moe, 1993). Consequently, the ionic term now contributes to the volume changes of the polyelectrolyte gel, and swelling at equilibrium is expressed (Flory, 1953):

$$\Delta\mu_{mix} + \Delta\mu_{ion} + \Delta\mu_{el} = 0$$

Ilavský et al introduced Gaussian and non-Gaussian contributions into the elastic term to correct for the effect of electrostatic interactions of charges on the polymer chain during phase transition in polyelectrolyte gels:

$$\Delta\mu_{el} = \Delta\mu_{el}^G + \Delta\mu_{el}^{NG}$$

Here, $\Delta\mu_{el}^{NG}$ is calculated under the assumptions of a Langevin distribution of the finitely extensible chains (Ilavský, 1981)

Tanaka and co-workers suggested an ionic term based on osmotic pressure under ideal conditions:

$$\Delta\mu_{ion} = fRTV_1^0 \frac{v}{V_0} \left(\frac{\phi}{\phi_0} \right)$$

where the number of counterions per chain f is introduced. This model assumes an ideal polymer solution at infinite dilution. Under these conditions, where an equal number of mobile ions and fixed charges inside the gel is assumed, the ionic term is proportional to the number of counterions in the ionic network. Hence, the osmotic effect is calculated from the number of particles in the system, independent of the ionic strength of the solution (Tanaka et al., 1980). The Tanaka group also showed that thermo-responsive ionic gels have a discontinuous volume transition, while a continuous transition was observed for non-ionic gels (Sato Matsuo and Tanaka, 1988). They stated a parameter S determines whether the transition of a gel is continuous or discontinuous:

$$S = \frac{vV_1^0}{V_0\phi_0^3} (2f + 1)^4 = S_0(2f + 1)^4 = \left(\frac{b}{a} \right)^3 S_0(2f + 1)^4$$

Here, a and b represents the effective radius and persistence length of the polymer chain, respectively (Tanaka et al., 1980).

Tanaka's and Ilavsky's modification of Flory's swelling theory provides an expansion of the basic swelling theory by including parameters in relation to the elastic forces to adjust for the influence of electrostatic interactions and number of counterions in the polymer network. Nevertheless, these

model neglects the strong non-ideal behaviour observed for polyelectrolytes, caused by contributions such as ionic strength of the solvent, internal polymer concentration and counterion condensation. These factors can somewhat be included by pursuing Flory's analogy of the gel as a semipermeable membrane. To further adapt the swelling theory towards the properties of ionic gels, it is feasible to utilize the theoretical terms of Donnan membrane equilibrium and counterion condensation theory (Manning condensation) for a polyelectrolyte solution (Moe et al., 1993).

A Donnan equilibrium appears in a two-phase system comprising a polyelectrolyte in solution and a solvent, said where the polyelectrolyte is retained by a semipermeable membrane that exclusively allows free diffusion of low molecular weight salts. In a polyelectrolyte solution, counterions are attracted electrostatically to the fixed charges on the polymer chain in order to maintain electroneutrality. When salt is added to the exterior solvent, some co-ions, ions with similar charge as the fixed residues on the polymer, will diffuse into the polyelectrolyte side of the membrane. These co-ions must be accompanied by an equivalent number of charges from counterions. If the accompanied ions and the counterions are identical salts, there will be an uneven distribution of counter and co-ions between polyelectrolyte solution and the exterior solvent. The uneven distribution generates an osmotic driving force that restricts further sorption of co-ions into the polyelectrolyte solution and desorption of counterions into the exterior solvent. The exclusion of co-ion migration into the gel, referred to as Donnan exclusion, results in a higher concentration of counterions in the polyelectrolyte phase and a higher concentration of co-ions in the solvent (Smidsrød and Moe, 2008).

In a typical Donnan equilibrium, the two criteria's of electroneutrality between ions and an equal chemical potential between diffusible species across the membrane, is fulfilled. The Donnan equilibrium contributes to the osmotic pressure with the sum of the concentrations of anions and cations, providing the osmolarity of the solution. At high salt concentrations or at infinite dilution, the contribution from the Donnan equilibrium becomes insignificant. In ideal Donnan equilibrium the activity coefficients for the salt on each side of the membrane is assumed to be identical (Smidsrød and Moe, 2008).

The Donnan term can be expressed mathematically and included into the ionic term by considering the molar concentration of mobile ions inside at outside the gel:

$$\Delta\mu_{ion} = RT[\ln(1 - X_{mobile\ ions}^{inside}) - \ln(1 - X_{mobile\ ions}^{outside})] \approx -RTV_1^0 \Delta C_{mobile\ ions}$$

where $X_{mobile\ ions}$ and $\Delta C_{mobile\ ions}$ represents the mole fraction and difference in molar concentration of mobile ions, respectively. Further, $\Delta C_{mobile\ ions}$ is defined:

$$\Delta C_{mobile\ ions} = (C_+ + C_-) - (C'_+ + C'_-)$$

wherein C_+ and C_- abbreviates the concentration of positive and negative mobile ions inside the gel, and C'_+ and C'_- are the negative and positive ions outside the gel. The Donnan equilibrium term allows calculations of the concentration of mobile charged species inside e.g. an anionic polymer gel:

$$\begin{aligned} z_+ C'_+ &= z_+ C'_- \\ z_+ C'_+ &= z_- C_- + z_P C_P \\ \gamma_{\mp}^2 C_+ C_- &= \gamma'_{\mp}{}^2 C'_+ C'_- \end{aligned}$$

where C_+ and C_- represents the counter- and co-ions inside the gel, respectively. z_+ and z_- is the absolute values of the valence of the positively charged counterion and the negatively charges co-ion, z_P is the number of uncondensed counterion charges per monomer unit, C_P is the polymer concentration expressed as the molar concentration of monomer units, and γ_{\pm} and γ'_{\pm} are the internal and exterior activity coefficients of salt in the gel and the solvent, respectively. This equation applies for anionic polymers in 1:1, 1:2 and 2:1 electrolytes (Tanford, 1961).

Uncondensed mobile ions are treated by the Debye-Hückel Limiting Law, under the assumptions of; complete dissociation of the electrolyte, each ion is strictly spherical and surrounded by ions of opposite charge, and the electrolyte solution is a highly dilute solution. The mean activity coefficient γ_{\pm} is then calculated by the Debye-Hückel equation:

$$\log \gamma_{\pm} = -\frac{z_i^2 e^2 N_A^{1/2}}{4\pi(\epsilon_r \epsilon_0 k_B T)^{3/2}} \sqrt{\frac{I}{2}} = -\frac{1.824 \times 10^6}{(\epsilon T)^{3/2}} |z_+ z_-| \sqrt{I}$$

Here, N_A is Avogadro's number, the ϵ_r is the relative permittivity, ϵ_0 is the permittivity of free space, ϵ is the relative dielectric constant and T is the temperature of the solution. The concentration of mobile ions is expressed in terms of the ionic strength:

$$I = \frac{1}{2} \sum_i C_i z_i^2$$

where C_i represents any mobile ion and z_i is its charge (Tanford, 1961).

The number of uncondensed ions z_p can be calculated from Manning's theory of counter ion condensation. When operating with long densely charged polyelectrolytes at low salt concentrations, the behaviour appears to be rather dominated by the electrostatic repulsion of the charged groups causing a chain extension and localization of mobile ions in the vicinity of chain segments. Hence, the charge density of the polymer and the number of uncondensed ions becomes an important parameter to describe polyelectrolyte behaviour in dilute solutions. Manning's theory states that the electrostatic attraction between the fixed charges on a polyelectrolyte and counterions in solution results in condensation of the counterions on the polymer chain. This is a consequence of the interchange between the electrostatic forces and the loss of translational entropy due to the counterion localization in the vicinity of the polyelectrolyte chain (Manning, 1969).

Manning defined a parameter ξ expressing an average charge distance between free or uncondensed charges on monovalent ions:

$$\begin{aligned} z_p &= \theta z_p^0 \\ \theta &= (z_+ \xi)^{-1}, \xi > 1 \\ \theta &= 1, \xi < 1 \\ \xi &= \frac{e_2}{4\pi\epsilon k T b} \end{aligned}$$

Here, z_p^0 represents number of uncondensed ions per monomer when counterion condensation is not considered, k the Boltzmann constant, b average distance between fixed charges and e the electron charge. For water at room temperature (25°C), the critical value $\xi = 1$ corresponds to a minimum distance of 7.14 Å (0.714 nm) between charges before condensation will occur, hence reducing the polymer charge density to this limited distance during such conditions (Manning, 1969).

Vasheghani-Farahani and co-workers suggested a model where swelling of ionic gels was described by assuming non-ideal behaviour of the polyelectrolyte and include the Donnan equilibrium term (Vasheghani-Farahani et al., 1990). The ideal osmotic pressure Π_{ideal} is calculated upon full dissociation of counter ions, expressed by the Van't Hoff equation:

$$\Pi_{ideal} = RT(n_m\alpha + n_p)$$

where n_m represents the monomer molarity, n_p is polymer molarity. Further, the ideal condition is corrected by introducing the osmotic activity coefficient Φ , or dissociation constant, accounting for the fraction of dissociated counterions in the gel phase:

$$\Phi = \frac{\Pi_p}{\Pi_{ideal}}$$

$$\Pi_p = RT(n_m\alpha\phi_p + n_p)$$

where α is the degree of ionization. A combination of these equations leads to an expression for the osmotic activity coefficient:

$$\Phi = \frac{\left(\phi_p + \frac{n_p}{n_m\alpha}\right)}{\left(1 + \frac{n_p}{n_m}\right)}$$

By assuming ideal Donnan condition, the concentration of counterions in the gel phase in an external solution containing both monovalent and divalent ions is included in Flory's ionic term:

$$\Delta\mu_{ion} = RT \left\{ \Phi \left[n_m \alpha (1 + f) \frac{1}{2} \right] + \phi \sum_{i=1}^n C_i \right\}$$

These interpretations were mainly based on the work of Alexandrowicz et al. and Katchalsky et al. on polyelectrolyte solutions (Alexandrowicz, 1960, Katchalsky et al., 1961). Katchalsky and co-workers showed that the osmotic activity coefficient decrease as the degree of ionization increase, and is almost completely independent of polymer concentration and the presence of salt. Dilution of the polyelectrolyte solution did not lead to higher dissociation of counterions (Katchalsky et al., 1961).

1.5.3 Kinetics of Hydrogel Swelling

Understanding the kinetic mechanism governing the swelling of hydrogels towards equilibrium is a major challenged that has concerned many researches. Diffusion in polymer structures is a complex process dependent on many elements, such as the properties of the diffusing particles, the polymer network and the solvent. Numerous theories and mathematical models have been developed to explaining the swelling kinetics or dynamics of gels, however no specific concept have been established as a superior explanation to the phenomenon. Swelling kinetic models are mostly derived from the first mathematical treatment of fluid diffusion, Fick's law for diffusion in one dimension:

$$J = -Aj = -AD \frac{\partial c}{\partial z}$$

Here, J represents the flux of the fluid, j the flux per unit area, A the cross-section of the area of diffusion, D the diffusion coefficient, c the concentration, z the distance and $\frac{\partial c}{\partial z}$ the concentration gradient along the z-axis of the network. A general approach is to divide the swelling models into two main categories, said Fickian or non-Fickian diffusion (Masaro and Zhu, 1999).

Fickian diffusion, also called Case I diffusion, is often exhibited by rubbery polymer structures above the glass transition temperature (T_g). A rubbery polymer structure enables greater chain mobility compared to a more brittle glassy polymer structure, hence allowing higher diffusion of solvent into the network. In characteristic Fickian behaviour, the solvent diffusion rate, R_{diff} , is

significantly slower than the polymer relaxation rate, R_{relax} , ($R_{\text{diff}} \ll R_{\text{relax}}$). When the fluid penetrates the network, the solvent concentration profile decreases exponentially as the fluid front progresses towards the core or centre of the polymer structure (Figure 11). The fluid uptake per time, M_t , corresponds to the diffusion distance, which is proportional to the square root of time t :

$$M_t = kt^{\frac{1}{2}}$$

Here, k represents a constant characteristic for the polymer-solvent system. It should be emphasized that this behaviour is strictly temperature dependent, following the extensive polymer network transition between glassy and rubbery state at the critical temperature T_g . Other diffusion influencing factors are the geometric shape of the structure, network interactions, configuration of network interfaces and junctions, pH, solvent composition, etc. (Masaro and Zhu, 1999).

Non-Fickian diffusion is often observed for glassy and stiff polymer structures. A rigid and highly ordered structure reduce chain mobility and inhibits immediate penetration of the solvent towards the core structure (Figure 11). Non-Fickian diffusion may be further divided into Case II and anomalous diffusion. In Case II the solvent diffusion rate is much faster than of the polymer relaxation ($R_{\text{diff}} \gg R_{\text{relax}}$). This type of diffusion is due to high solvent activities, often associated with a sharp solvent penetration front that advances in different rates towards the core of the structure. If the solvent is penetrating the network at a constant rate, the fluid uptake is given:

$$M_t = kt$$

Anomalous diffusion is observed when the solvent diffusion rate and polymer relaxation is approximately in the same order of magnitude ($R_{\text{diff}} \sim R_{\text{relax}}$). Moreover, anomalous diffusion is an intermediate swelling process between Case I and Case II diffusion (Masaro and Zhu, 1999), expressed

$$M_t = kt^n$$

Here, n represents the diffusional exponent or swelling power number describing the transport process of the penetrant. For anomalous diffusion, n is said to be in between the range of 0.5 to 1, meaning $n = 0.5$ and $n = 1$ indicates Fickian and Case II behaviour (Ganji et al., 2010).

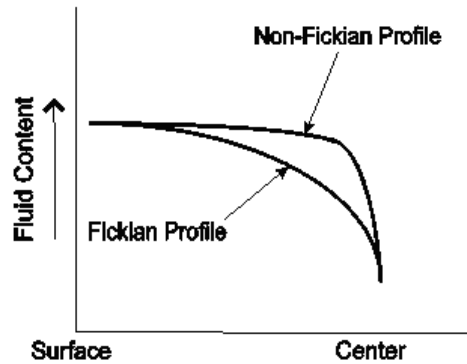


Figure 11. Fluid content as a function of swelling distance, progressing from the gel surface towards the core of a gel to demonstrate Fickian or non-Fickian diffusion profile. Fickian behaviour is exhibited by flexible and rubbery polymer structures, and non-Fickian behaviour occur in stiff and glassy polymer structures (Peppas and Sahlin, 1989).

An empirical equation that is commonly utilized to determine the major mechanism of diffusion in polymer gels is given:

$$\frac{M_t}{M_\infty} = kt^n$$

where M_t/M_∞ is the fractional water uptake by the polymer. This expression is retrieved from the Higuchi model, explaining fluid flux in planar systems derived under pseudo-steady state assumptions. By plotting swelling kinetics data in a log-log plot, n can be estimated through linear regression (Siepmann and Peppas, 2001).

$$\log\left(\frac{M_t}{M_\infty}\right) = \log k + n \log t$$

As previously mentioned, the n value can be used as an empirical parameter to determine if the fluid transport process in the network is governed by Fickian, anomalous or Case II diffusion. However, the n value obtained from the above expression only describes the major portion of the

swelling behaviour and does not provide a comprehensive and precise analysis, especially when the M_t/M_∞ ratio is above 0.60 (Bartil et al., 2007).

Berens and Hopfenberg suggested a modified model to enable an improved analysis upon a M_t/M_∞ ratio beyond 0.60. By assuming first order relaxation process for the concentration difference regarding fluid absorption for polymeric microspheres, the following two expressions applies:

$$\frac{dM_t}{dt} = k_2(M_\infty - M_t)$$

$$\frac{M_t}{M_\infty} = (1 - Ae^{-k_2t})$$

Here, A and k_2 are constants retrieved from linear regression of $\ln(1-M_t/M_\infty)$ as a function of time (Berens and Hopfenberg, 1978). Further, Peppas and co-workers developed model for anomalous diffusion, where the contributions of relaxation-controlled transport and diffusion-controlled transport were described by the two parameters k_1t and $k_2t^{1/2}$, respectively (Peppas and Sahlin, 1989).

$$\frac{M_t}{M_\infty} = \left(k_1t + k_2t^{1/2} \right)$$

Karadag and co-workers determined diffusional exponent n for cylindrical shaped acrylamide/crotonic acid (AAM/CA) hydrogels by utilizing a simplified empirical equation:

$$Q = \frac{M_t - M_0}{M_0} = Kt^n$$

Here, Q represents the swelling rate, M_0 is the dry mass of the gel before swelling is initiated. Fickian or non-Fickian behaviour was distinguished by plotting $\log((M_t - M_0)/M_0)$ as a function of $\log(t)$ and solving n through linear regression of the swelling data:

$$\log\left(\frac{M_t - M_0}{M_0}\right) = \log(k) + n \log(t)$$

For cylindrical gels, $n = 0.45 - 0.50$ resembles Fickian behaviour while $0.50 < n < 1.0$ is non-Fickian behaviour (Karadag and Saraydin, 2002).

More advanced swelling kinetics models have been developed, including Tanaka and co-workers model based on Fickian diffusion and the rubber elastic theory (Tanaka et al., 1973, Tanaka and Fillmore, 1979). Wang and co-workers showed that the kinetics are highly influenced by the nature of solvent motion in the network, dependent on the geometry and shape of the gel. For a cylinder with radius a , the velocity of the solvent is proportional to $r/2a$ along the radial direction and z/a along the axial direction:

$$\frac{\partial w_r(r, t)}{\partial t} = -\frac{r}{2a} \frac{\partial u_r(a, t)}{\partial t}$$

$$\frac{\partial w_z(z, t)}{\partial t} = \frac{z}{a} \frac{\partial u_r(a, t)}{\partial t}$$

Where $\partial w_r/\partial t$ and $\partial w_z/\partial t$ represents the solvent velocity, and $\partial u_r(a, t)/\partial t$ is the change of radius of the gel. From these expressions it is possible to sketch a diffusion velocity profile or solvent motion pattern in a long cylindrical gel (Figure 12.).

Similarly, for a large disk gel, the diffusion velocity profile is expressed:

$$\frac{\partial w_r(r, t)}{\partial t} = \frac{r}{a} \frac{\partial u_r(a, t)}{\partial t}$$

$$\frac{\partial w_z(z, t)}{\partial t} = -2 \frac{z}{a} \frac{\partial u_r(a, t)}{\partial t}$$

Here, a represents the half thickness of the disk and the velocity of the solvent is proportional to r/a along the radial direction and $2z/a$ along the axial direction. Different geometrical shapes exhibit two somewhat different diffusion profiles (Wang et al., 1997).

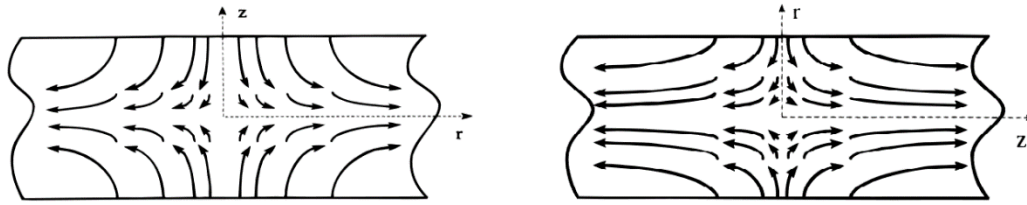


Figure 12. Estimated solvent diffusion patterns in long disk gel (left) and long cylindrical gels (right) (Wang, 1997).

1.5.4 Main Contributing Factors with Relation to Swelling Behaviour

After reviewing the basic swelling theory and some modified interpretations, it is possible to get an overview of contributing forces that are governing gel swelling behaviour at equilibrium. The main contributing factors are the osmotic pressure forces, electrostatic forces and viscoelastic restoring forces. Hence, it must be appreciated that the swelling properties of hydrogels is highly dependent on the environmental conditions in which they are tested (Ganji et al., 2010). Ionic gels contain polyelectrolytes and do often exhibit non-ideal behaviour due to the presence of fixed charged groups on the polymer chain, which attracts mobile ions.

In ionic gels where salt is absent, the swelling pressure is mainly caused by the degree of counterion condensation inside the gel. Some counterions will be dissociated and be in contact with a water reservoir, while others associated in the vicinity of the polymer chain. In highly dilute solutions, the counterions tend to diffuse further away from the polymer backbone, thus increasing the net charge that induces charge-charge repulsion. This induces expansion of the polymer chain, resulting in a more rod like behaviour (Young and Lovell, 2011).

When salt is present in the gel, the swelling pressure is associated with the osmotic forces due to an establishment of a Donnan equilibrium between the inside and outside of the gel (Skouri et al., 1995). According to the terms of the Donnan equilibrium, there are three ways of affecting the ionic swelling term $\Delta\mu_{ion}$ in an ionic gel: Changing the salt concentration of the solution surrounding the gel, the valence of the counterions, and the effective charge density of the polymer (Moe, 1993).

The swelling kinetics are relatively easy to measure and observe, but difficult to estimate and evaluate. Great care should be taken upon applying models, interpreting and comparing the dynamics of different polymeric system under specific condition. Empirical models, such as Higuchi equation, may be utilized to estimate the diffusion exponent n , and provide a quantitative differentiation between Fickian and non-Fickian behaviour. However, while performing such interpretation, the dissolution of the polymer network, compatibility of the polymer, solvent and solute should be considered (Masaro and Zhu, 1999).

The presented swelling theory is adapted to provide a respectable qualitative description of the swelling phenomenon, and will not be utilized to give quantitative predictions of the swelling behaviour as this was not the purpose of this thesis.

1.5.5 Swelling of Alginate Gels

The functional properties of alginate gels have been extensively investigated due to its prevalence in many different applications. Characterization of the swelling properties have been of particularly interest in relation to pharmaceutical uses, especially in drug delivery systems. In general, alginate gels are known to provide stiff, highly ionized polymer networks that exhibit a strong non-Gaussian behaviour due to long junction zones and discontinuous volume changes should be expected. Henceforth, alginate gels differs from the main assumptions in the theory of rubber elasticity of a random-coil behaviour of the elastic chains, and that the network chains are linked together by point-like cross-links (Stokke et al., 1991). The alginate calcium gels form porous networks where the pore sizes are dependent on its structural composition, said the GG junction zones provides more free volume space for fluid diffusion (Draget et al., 2006).

Martinsen and co-workers have previously investigated swelling calcium alginate gel beads. They reported that where the elasticity of calcium alginate gels is dependent of the number and strength of the cross-links, and swelling was observed to decreased when the concentration of calcium cross-linker in the external solution was increased. The observations suggested that the swelling was reduced by an increase of the elastic retractive force which opposes the swelling pressure, which corresponds well to the swelling theory (Martinsen et al., 1989). Gel shrinking of alginate calcium gel cylinders was also reported by Golmohamadi and co-workers shrinking when sodium

and calcium salts were added to the solution surrounding the gel, whereas the largest impact was observed when calcium was added to the solution (Golmohamadi and Wilkinson, 2013).

Moe and co-workers investigated the equilibrium swelling volume of covalently cross-linked sodium alginate gel beads. They also claimed that the swelling degree for alginate gels is fundamentally different from predictions based on the rubber elastic theory. A numerical analysis of Flory's theory regarding swelling of ionic gels showed that the main determining factor for volume changes in covalently cross-linked sodium alginate gels was mainly attributed to the ionic contribution μ_{ion} . The same analysis suggested that the influence of the ionic term was mainly caused by the Donnan equilibrium effect, which was found to contribute more than 10 times more to the swelling pressure compared to the polymer-solvent mixing μ_{mix} . (Moe et al., 1993). From swelling experiments, a market hysteresis was observed by Moe and co-workers upon adding divalent ions Ca^{2+} , Sr^{2+} and Ba^{2+} to the solution surrounding the gel. The hysteresis was explained as a consequence of changing the valence of the counterions. The divalent ions introduce additional cross-links formed between the ions and G-block segments, which increase the elastic network restoring force and adds to the elastic term μ_{el} .

Furthermore, Moe and co-workers demonstrated that by reducing pH of the surrounding solution from 5 to 1 at constant ionic strength in different buffer systems, the swelling degree of the gels was significantly decreased. This was elucidated by the protonation of carboxyl-groups following the pH reduction towards the pK_a of alginate. The protonation of alginate reduces the effective charge (z_p) of the polymer and equally the charge difference in ion concentration between the inside and outside the gel. This is in accordance to the predictions from the Donnan equilibrium, and leads to reduced swelling degree for an ionic gel. Reduced swelling was also seen when adding monovalent salts, such as sodium chloride (NaCl) to the solution. Again, this was attributed to the Donnan equilibrium effects, said the addition of salt reduce the difference in mobile ion concentration between the two phases and hence reduce the fluid penetration of the gel driven by the osmotic force (Moe et al., 1993). Similar observations were also reported by both Kuo and colleagues and Golmohamadi and co-workers upon swelling alginate calcium disks and cylinders, respectively. Kuo showed that the pH had little effect when the pH was sufficiently above the pK_a of alginate (Golmohamadi and Wilkinson, 2013, Kuo and Ma, 2008).

1.6 Size Exclusion Chromatography

Chromatography is a generic term for a series of separation techniques where compounds are separated based on physical properties such as charge, volatility, size, etc. The basic concept of chromatography relies on distributing molecules between a mobile phase and a stationary phase. The molecules are separated differently between the two phases due to retention towards the stationary phase. Chromatography is a powerful tool utilized for many purposes, especially in downstream processing, purification and analytical chemistry (Lundanes et al., 2014).

HPLC is a widespread chromatographic method used for separation, identification and quantification of molecules in a mixture. A typical HPLC instrumentation setup is composed of a pump (s), injector, column (s), detector and a datalogger connected to a data processor (Figure 13). The pump generates pressure to transfer a mobile phase through the injector, where the sample is introduced into the system, and further into a column packed with a stationary phase material. Post to the separation, the different sample fractions enter the detector cell along with the mobile phase before it is either discarded or collected with a fraction collector (Lundanes et al., 2014).

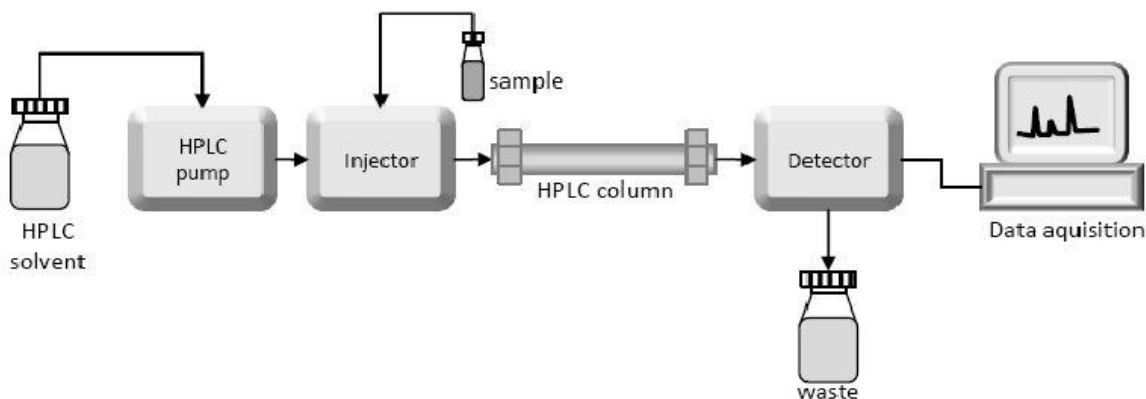


Figure 13. Schematic illustration of a High Performance Liquid Chromatography (HPLC) system. A mobile phase is pumped through the column at a constant flow rate, and the sample is introduced and solvated in the mobile phase through the injector. A detector obtains a detection signal and a logger registers the signal and transfers it to a data acquisition device. The sample is pumped through the detector where it can be either collected or discarded (Czaplicki, 2013).

Size exclusion chromatography (SEC) is a high performance liquid chromatography technique where a mixture is separated on the basis of effective size or hydrodynamic volume of the molecule. The SEC system was developed during the late 50's and early 60's, and is also referred to as gel filtration or gel permeation chromatography (GPC). SEC can be differentiated from GPC whereas using an aqueous mobile phase to transport the macromolecules through the column, instead of polymeric or nonaqueous mobile phases. SEC is frequently applied on macromolecules in biological and polymer chemistry industry for both preparative and analytical purposes, e.g. for detecting impurities or obtaining an estimation of the molecular mass (Lundanes et al., 2014).

The separation mechanism of a SEC system is based on passive diffusion of macromolecules on porous spherical particles in the stationary phase with a defined pore size (Figure 14). The separation is conducted according to the establishment of a local equilibrium between the mobile and stationary phase. Molecules that are significantly larger than the pore size are excluded from the particles and transported along the column with the mobile phase. These molecules will elute out first at a retention volume V_0 , also called the void volume. Small molecules will distribute freely in the column and is retained in the stationary phase as a consequence of diffusion into all particle pores. Henceforth, small molecules such as salts and ions have a high retention time and elute last at a retention volume V_i . Intermediate sized molecules have access to a selection of the pores, and elute between V_0 and V_i dependent on their physical size. The elution volume of a compound V_e is expressed:

$$V_e = V_0 + k'V_i$$

where k' represents the distribution or partition coefficient. The partition coefficient is designated the molecular concentrations between the mobile and stationary phases at equilibrium. It is dependent on the ratio of gyration, R_G , of the molecules as well as the average pore diameter in the column packing diameter. The degree of retention for one particularly sized molecule is dependent on its hydrodynamic volume, relative to the particle pore size and the flow rate of the mobile phase. Chain extension of polymers will therefore effects the hydrodynamic volume and thus the retention time (Smidsrød and Moe, 2008, Lundanes et al., 2014).

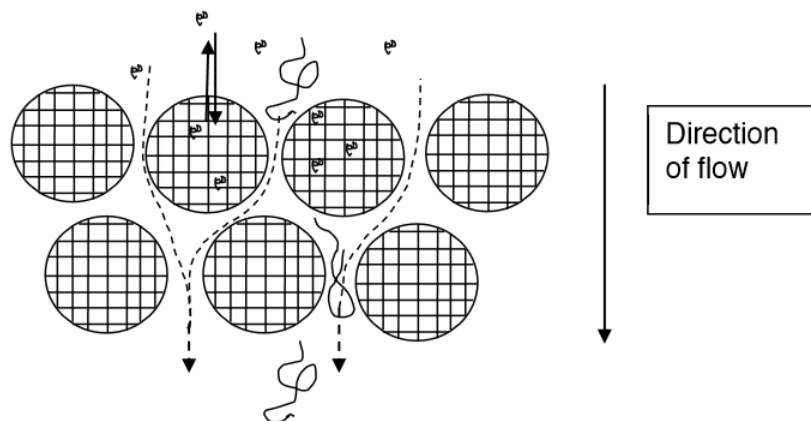


Figure 14. Size Exclusion Chromatography (SEC) separation principal of a disperse mixture of macromolecules. Small molecules will be retained in the pores of the stationary phase, while larger molecules are excluded from the pores and is further transferred with the mobile phase (Christensen, 2015).

The choice of column material is essential to obtain the high performance separation. An optimal separation is obtained when the distribution of pore sizes for the packing material is equal to the size distribution of the analyte. Macroporous, incompressible, monodisperse polymer resins are often successfully used in SEC columns as this type of material is inert and generates minimal chemical absorption (Smidsrød and Moe, 2008).

Choice of detection advice is an important factor to consider in relation to the sample analysed and the purpose of the analysis. UV-absorption and refractive index (RI) detectors are commonly used in ordinary SEC systems. Conventional UV detectors measure the light absorption at a specific wavelength, which is useful upon detection UV absorbing compounds, such as aromatic or conjugated molecules. RI detectors measure the refraction of light in a flow cell in comparison to a reference cell, which enables detection of analytes without UV absorbance. Multi angle laser light scattering (MALS) and mass spectrometry (MS) detection can also be combined with SEC to provide analytical methods for quantitative and qualitative predictions of characterization parameters, such as number (M_n) and weight (M_w) average molecular weight distributions, radius of gyration (R_G) and intrinsic viscosity ($[\eta]$) (Smidsrød and Moe, 2008, Lundanes et al., 2014).

SEC also provides an excellent tool to separate and analyse oligomers and is often utilized as characterization method in oligosaccharide chemistry (Smidsrød and Moe, 2008). Tømmeraas and co-workers managed to separate fully N-acetylated and fully N-deacetylated chitosan oligomers by SEC (Tommeraas et al., 2001). This was also demonstrated by Sorbotten and co-workers separated chitosan oligomers with varying degree of acetylation with a SEC RI system. In this work, SEC was also utilized to monitor the enzymatic degradation rate of chitosans by following the size-distribution of oligomers. The SEC system was calibrated with a partially deacetylated chitosan oligomer mixture according to degree of polymerisation and not chemical composition. The amount of each oligosaccharide was quantified by using the exact amounts of a fully acetylated and a de-N-acetylated as the basis to determine the area under each detection peak (Sorbotten et al., 2005). This method was also used by Khong and co-workers to characterize a fully de-N-acetylated chitosan oligomer mixture and obtain the oligomer size distribution (Khong et al., 2013).

1.7 Aim of Study

This master thesis has been connected to an ongoing project at the Department of Biotechnology at NTNU in relation to the interesting findings of Khong and co-workers upon cross-linking alginate with chitosan oligomers. The overall object of this project is to study a novel alginate chitosan oligomer gelling system to understand the macroscopic and chemical structure and interactions of these gels, in order to apply this technology for e.g. new capsules for immobilization of living cells.

In this master thesis the focus has been to investigate and compare the swelling properties of *L. hyperborea* leaf and stipe alginate gels cross-linked with calcium, chitosan oligomers or combinations of calcium and chitosan oligomers. To achieve this, an empirical comparative study has been conducted, where the swelling kinetics and equilibrium of these gels were measured after submersion in solutions of different pH and ionic strengths. In addition, a SEC RI system have been utilized in order to detect the relative cross-linking activity of oligomers of different sizes towards leaf alginate. Interpretations of the results obtained from performed experiments have been done qualitatively.

2 Materials and Method

2.1 Materials

Alginate gel cylinders prepared in this thesis comprised either *L. hyperborea* stipe (LF200S, $F_G = 0.68$) or leaf (GP3350, $F_G = 0.46$) alginate purchased from FMC Biopolymer. Fully de-N-acetylated chitosan oligomer mixture (CO, $F_A =$) kindly provided by Koyo Chemical (Japan). The alginate gels also contained calcium carbonate (CaCO_3 , $M_w = 100.09$ g/mol) and D-glucono- δ -lactone (GDL, $\text{C}_6\text{H}_{13}\text{NO}_5$, $M_w = 178.14$ g/mol) bought from Merck KGaA and Sigma Aldrich, respectively.

Sodium chloride (NaCl , $M_w = 58.44$ g/mol) and calcium chloride dihydrate (CaCl_2 , $M_w = 147.02$ g/mol) used in some swelling solutions was produced by Merck KGaA and EMD Millipore. The glacial acetic acid (HAc , CH_3COOH , $M_w = 60.05$ g/mole, $\rho = 1.05$ g/cm³) and hydrochloric acid (HCl , $M_w = 36.46$ g/mole $\rho = 1.19$ g/cm³) utilized in preparation of acetate and tris buffer was provided by Merck KGaA and EMD Millipore, respectively. These buffer solutions also contained sodium acetate (NaAc , NaCH_3COO , $M_w = 82.04$ g/mole) or Trizma[®] base (2-Amino-2-(hydroxymethyl)-1,3-propanediol, $\text{C}_4\text{H}_{11}\text{NO}_3$, $M_w = 121.14$ g/mole) bought from Sigma Aldrich.

Sodium hydroxide (NaOH , $M_w = 40.00$ g/mole $\rho = 2.13$ g/cm³) from Merck KGaA was used in pH adjustments of hydrolysate, used in the NMR characterization of alginate, and chitosan oligomer stock solutions. Deuterium oxide (D_2O , $M_w = 20.03$ g/mole) from CDN Isotopes, triethylenetetraminehexaacetic acid (TTHA, $M_w = 494.45$ g/mole) and trimethylsilyl propanoic acid (TMSP or TSP, $\text{C}_6\text{H}_{14}\text{O}_2\text{Si}$, $M_w = 172.27$ g/mole) from Sigma Aldrich was utilized as solvents in NMR experiments. The mobile phase used in SEC characterisations consisted of ammonium acetate (AmAc , $\text{CH}_3\text{COONH}_4$, $M_w = 77.08$ g/mole) produced by Merck KGaA and EMD Millipore.

The water used in preparation of buffer solutions and gel cylinders was Milli-Q (MQ) filtered and deionized water with a resistivity of 18.2 M Ω cm.

2.2 Stipe and Leaf Alginate Characterization

The chemical composition of leaf and stipe alginate was determined utilizing ^1H and ^{13}C NMR spectroscopy. Prior to the NMR analysis, the alginate was subjected to partial acid hydrolysis, reducing DP to approximately 50, to decrease the viscosity. Low viscosity was necessary while a NMR analysis is performed at relatively high sample concentrations. A DP of 50 also ensures minimal influence of reducing and non-reducing ends on the spectra, which is often associated with low molecular weight compounds, and consequently disrupts sequence parameter calculations. D_2O was used as aqueous solvent to exchange OH protons with deuterium and avoid an interfering peak at 4.8 ppm in the ^1H NMR spectra. Chelator TTHA was added to bind traces of divalent cations that could cause gelation and TSP was utilized as internal reference. The number and weight average molecular weight distributions had been determined in previously performed experiments by SEC MALLS analysis.

2.2.1 Partial Acid Hydrolysis

The degradation of alginate was carried out as a stepwise acid hydrolysis. 20 mg alginate was dissolved in a 100 mL vial containing 60 ml MQ water. The hydrolysis was initiated by adjusting the pH to 5.6 with 0.1 M HCl followed by 60 min incubation in a 95°C water bath. The sample was cooled to room temperature (25°C) and titrated with 0.1 M HCl, reducing the pH to 3.8. The hydrolysis was proceeded as the sample was incubated a second time at 95°C for 45 min. Further, the alginate hydrolysate was titrated with 0.1 and 0.01 M NaOH until pH 6.8 was measured. Eventually, the alginate hydrolysate solution was transferred into a round bottom flask, frozen with liquid nitrogen and freeze-dried overnight utilizing an Alpha 1-4 LD freeze dryer.

2.2.2 NMR Characterization

32 mg of freeze dried stipe alginate hydrolysate was added to a small vial and mixed with 550 μl D_2O , 25 μl 3 M TTHA and 80 μl TSP. The mixture was then transferred into a NMR tube and analysed overnight by high resolution 400 MHz ^{13}C NMR spectroscopy at 85°C . 7 mg of freeze dried leaf alginate hydrolysate was mixed with 575 μl D_2O , 20 μl TTHA and 5 μl TSP and analysed overnight by high resolution 400 MHz ^1H NMR spectroscopy at 85°C . Average G-block length, M/G ratio and triad frequencies was calculated by analysing the ^1H and ^{13}C NMR spectra presented in Appendix A, respectively. The NMR spectra was further analysed by the software TopspinTM.

2.3 Chitosan Oligomer Characterization by Size Exclusive Chromatography

The size distribution of the chitosan oligomer batch with lot number 121017WG was determined by separating the chitosan oligomers in an oligomer SEC system optimized for oligosaccharide separation based on DP. The system was composed of three serially connected HiLoad™ 26mm/60mm columns packed with Superdex™ 30 preparative grade delivered by GE Healthcare Life Sciences. Degassed 0.15 M AmAc buffer pH 4.5 was used as mobile phase with a flow rate of 0.8 ml/min during the entire analysis. The relative amount of oligomers was monitored with a Shodex RI-101 refractive index (RI) over 20 hours (1200 min). 21.4 mg of CO mixture was dissolved in 3 mL 0.15 AmAc and mixed for 15 min with a PTR-60 Multi-Rotator. When completely dissolved, the sample was filtered with through a 0.2 µm polypropylene membrane and injected into the columns. The chromatogram was retrieved by plotting the relative amount of oligomers as a function of retention time. DP and weight fraction of each oligomer was determined from the area in each peak in the chromatogram by integration in excel. In addition, the degree of acetylation has been determined in preciously performed experiments by NMR spectroscopy characterization for every oligomer fraction separated by SEC.

2.4 Preparation of Alginate Gel Cylinders

Leaf and stipe alginate gels crosslinked with calcium, chitosan oligomers or a combination of calcium and chitosan oligomers were prepared using the same *in situ* gelation protocol, however with different mixing ratios of CaCO₃, chitosan oligomers and GDL. A detailed description of the mixing ratios for the different gelling systems is given Appendix B.

As previously mentioned, different defined mixing ratios was suggested through previous experiments performed by Vårum K.M.'s group at the Department of Biotechnology at NTNU. Identical cross-linking mixing ratios and gel forming protocols was used in gel preparation for the swelling experiments performed in this thesis. Leaf alginate calcium gels (GP3350 100/0) and stipe alginate calcium gels (LF200S 100/0) contained 15.1 mM and 16.6 mM CaCO₃, respectively. Mixed leaf alginate gels (GP3350 50/50) contained 7.5 mM CaCO₃ and 18.8 mM chitosan oligomer mixture, and mixed stipe alginate gels (LF200S 75/25) contained 12.4 mM CaCO₃ and 9.4 mM chitosan oligomer mixture. Leaf alginate chitosan oligomer gels (LF200S 0/100) contained 37.6

mM chitosan oligomer mixture. Leaf alginate calcium gels with 50% reduced calcium content (GP3350 50/0) and stipe alginate calcium gels with 25% reduced calcium content (LF200S 75/0) contained 7.5 mM and 8.3 mM CaCO_3 , respectively (Figure 9, Figure 10).

The next subsections demonstrate the preparation of 6 leaf alginate gel cylinders cross-linked a mixture of calcium and chitosan oligomers.

2.4.1 Preparation of Alginate and Chitosan Oligomer Stock Solutions

A 1.5 % stock solution was prepared by adding 1308.5 mg leaf alginate to 80 ml MQ water in a 250 mL blue cap bottle. The solution was sealed and dissolved overnight utilizing a IKA VXR Vibrax[®] shaker. A 10% stock solution was prepared the next day by dissolving 150 mg CO in 0.800 ml MQ water in a 2.5 mL Eppendorf tube. The pH was adjusted from 4 to 7.9 to deprotonate most of the amino groups of chitosan and avoid inhomogeneous gel formation when adding CO solution to the solution comprising negatively charged alginate. This was done by titrating the CO solution with 0.1M and 1 M NaOH. Eventually the volume was brought up to 1.5 mL with MQ water, after obtaining a pH measurement of 7.9 ± 0.1 utilizing an Orion Ross combination pH Micro electrode.

2.4.2 Preparation of Homogeneous Alginate Gel Cylinders by *In Situ* Gelation

18.00 g of 1.5 % GP3350 alginate solution was added to a 100 mL flask with suction. 20.4 mg of CaCO_3 was mixed into 2.00 mL MQ water in a small glass vial. The CaCO_3 solution and 1.026 mL CO stock solution was carefully added to the alginate solution and mixed for 10 min using a magnetic stirrer. Both solutions were added dropwise using a 100-1000 μl pipette and the mixture and stirred at relatively low frequency to avoid any gelation. Further, the mixture was degassed for 10 minutes by vacuum suction to remove CO_2 from the solution. Meanwhile, 163.1 mg GDL was dissolved in 5.974 ml MQ water in a small glass vial. After degassing, the GDL solution was added dropwise to the alginate gelling mixture with a 100-1000 μl pipette and stirred at low frequency for one minute. Adding GDL starts the gelation process and gradually protonates the calcium and chitosan oligomers. The gelling mixture was therefore poured into six cylindrical wells in a levelled 24-well plate, immediately after the mixing step. The eight middle positions in the well plate was used to minimize contamination. Each well was cylindrical with a height of 16.2 mm and a diameter of 17.1 mm. Each well was completely filled with solution, generating a positive meniscus as

shown in (Figure 15). The lid was put on gently and the plate was left in room temperature for 20 hours to ensure complete gelation.

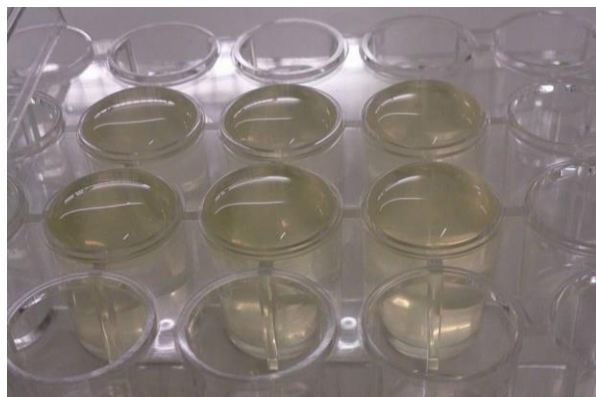


Figure 15. Illustration of gel preparation of six leaf alginate gels with mixed cross-linkers. Leaf alginate mixture containing D-glucono- δ -lactone (GDL), calcium carbonate (CaCO_3) and chitosan oligomer mixture was poured into six wells with a positive meniscus before the lid was put on.

2.5 Preparation of Swelling Solutions

Eight different buffer solutions (Table 1) were prepared in order to study how changes in salt content, ionic strength and pH effected the swelling behaviour for different alginate gelling systems. Some additional solutions were also prepared to determine the how CaCl_2 and the buffer component might influence the swelling behaviour of leaf and stipe alginate calcium gels. A detailed overview of all buffers prepared for this thesis is presented in Appendix C.

Table 1. Acetate and tris buffer solutions for systematic swelling experiment.

Buffer system	pH	C_{buffer}	C_{NaCl}
	[-]	[mM]	[mM]
Acetate	4.5	50	0
Acetate	4.5	50	100
Acetate	5.5	50	0
Acetate	5.5	50	100
Tris	7.5	50	0
Tris	7.5	50	100
Tris	8.5	50	0
Tris	8.5	50	100

2.5.1 Acetate Buffer with pH 4.5 and 5.5

10.25 g sodium acetate was dissolved in a 400 mL beaker containing 250 mL of MQ water to produce a 0.5 M sodium acetate solution. A 0.5 M acetic acid solution was prepared by adding 7.15 mL of 100% glacial acetic acid into 242.85 mL MQ water in a 400 mL beaker. A 0.5 M buffer solution with pH 4.5 was prepared by titrating 228 mL of the 0.5 M sodium acetate with solution with 182 mL of the 0.5 M acetic acid solution. For the pH 5.5 buffer, 175 mL of the 0.5 M acetate solution was titrated with 31 mL of the 0.5 M acetic acid. When pH was measured to 4.50 ± 0.01 or 5.50 ± 0.01 with a pH meter electrode, the solution was transferred to a sealed 500 mL flask and stored in a refrigerator. The solution was diluted to 50 mM with MQ water and pH was controlled before of swelling experiments were initiated.

2.5.2 Tris Buffer with pH 7.5 and 8.5

30.285 g of Trizma-Base was dissolved in 400 mL MQ water in a 600 mL beaker. The beaker was titrated with 20.529 mL 12.18 M HCl to make a 0.5 M Tris HCl solution. The solution volume was brought up to 500 mL by adding MQ water and pH was measured to 7.23 ± 0.01 . Further, 6.057 g Trizma Base was dissolved in 100 mL MQ water in a 250 mL beaker to prepare a 0.5 M Tris Base solution. In order to prepare a pH 7.5 Tris buffer, 201.5 mL of the 0.5 M Tris HCl solution was transferred to a 400 mL beaker and titrated with 13.0 mL 0.5 M Tris Base solution. The 8.5 buffer

was prepared by titrating 105 mL of the tris HCl was titrated with 112.5 mL tris base, preparing a tris buffer. After the pH was measured to 7.50 ± 0.01 or 8.50 ± 0.01 the solution was transferred to a 500 mL sealed flask and stored at room temperature. The solution was diluted to 50 mM with MQ water and pH was controlled before of swelling experiments were initiated.

2.5.3 Swelling Solutions for Control Experiments

A control experiment was conducted where 10 mM CaCl_2 was added to 50 mM tris and acetate buffer solutions in order to investigate the effect of adding a cross-linking agent into the external swelling solution. The tris and acetate buffers were prepared as previously described, before 73.5 mg was added to 50 mL of the buffer solutions.

A control experiment investigating a potential effect of the buffer components on the swelling behaviour of alginate gels was conducted. A 50 mM pH 5.5 acetate buffer solution was prepared as previously described, however the pH was adjusted to 7.5 with 100 mM NaOH. Similarly, a 50 mM pH 7.5 tris buffer solution was prepared and pH was adjusted to 5.5.

2.6 Swelling Kinetics and Equilibrium Measurements

The gel cylinder was gently cut out of the well with a spatula, weighted and submersed into a sealed plastic container holding 50 ml swelling solution. The plastic container was then stirred at 40 rpm on a bench-top shaker to circulate the liquid surrounding the gel cylinder during the swelling experiment. Seven swelling measurements were taken at different times after submersion where gel weight changes were recorded. While performing such measurements, the gel was transferred with a spoon to a plastic weighing scale, excessive fluid was removed by a thin paper tissue and the gel was put back in solution after the weight was determined utilizing a Mettler Toledo AG204 DeltaRange[®] scale (Figure 16). Swelling kinetics was investigated by measuring the weight at 0.5, 1.5, 2.5 and 5 hours post submersion. The weight was also measured at 24 hours to investigate the swelling behaviour at equilibrium. A final swelling equilibrium measurement was obtained by transferring the swollen gel into a new plastic container comprising freshly prepared swelling solution, and measure weight changes after another 24 hours.

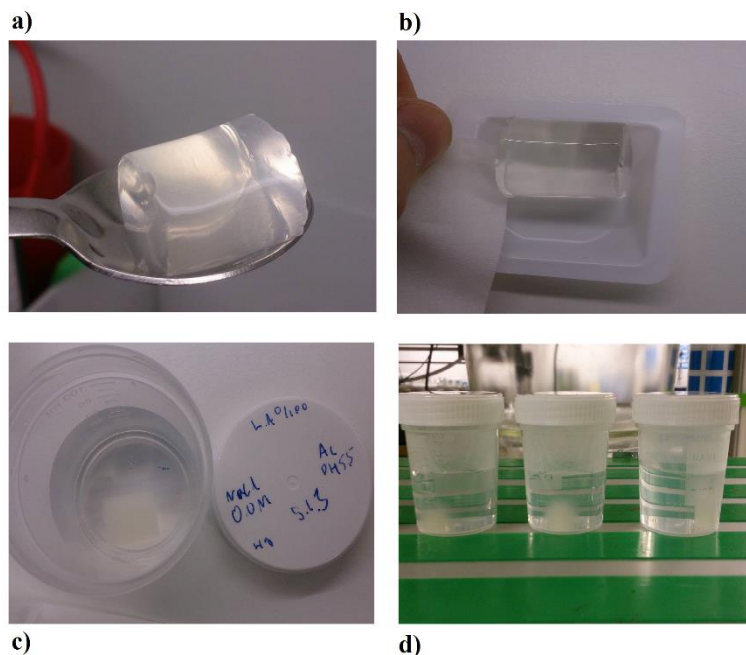


Figure 16. Illustration of swelling measurement procedure. The gel was carefully taken out of the solution with a spoon (a) and placed in a plastic container where excessive fluid was removed with a thin paper tissue (b). After obtaining the weight at a given time, the gel was immediately put back into the swelling solution (c) and placed on a bench top shaker (d) where it was shaken at 40 rpm.

2.7 Sample Purification of Leaf Alginate Chitosan Oligomer Swelling Fractions

Six swelling fractions obtained from swelling solutions of leaf alginate chitosan oligomer gels was prepared for SEC analysis to analyse the sol fraction content (Figure 2). These samples were freeze-dried to increase the concentration, an essential step to in order enhance the detection signal in a RI detector. Samples with pH 5.5 were pH adjusted to 3.5 with concentrated HCl to avoid Schiff base formation of the chitosan. 50 mL swelling solution was transferred to a round bottom flask and cryopreserved while rotating in liquid nitrogen. The majority of water was effectively removed from the sample through sublimation under low pressure using an Alpha 1-4 LD freeze dryer. The solid samples were stored in in room temperature in round bottom flasks sealed with parafilm until further analysis.

Table 2. Swelling fraction samples for size exclusion chromatography (SEC) analysis of oligomer content in different swelling solutions. The fractions were collected from swelling solutions where leaf alginate chitosan oligomer gels (GP3350 0/100) were swelled at different times in 50 mM pH 5.5 acetate buffer with and without salt. Two samples were obtained after swelling the gels for 24 hours in two swelling solutions.

Sample	t	C _{NaCl}
GP3350 0/100	5 h	0 mM
GP3350 0/100	24 h	0 mM
GP3350 0/100	(24 h) ^{2nd}	0 mM
GP3350 0/100	5 h	100 mM
GP3350 0/100	24 h	100 mM
GP3350 0/100	(24 h) ^{2nd}	100 mM

2.8 Size Exclusive Chromatography Analysis of Chitosan Oligomer Content in Swelling Fractions

SEC analysis was applied to six freeze dried samples in order to disclose presence of oligomers in the sol fraction solutions. Similar to the system applied for chitosan oligomers mixture characterization described in section 3.3, this SEC system consisted of three connected HiLoadTM 26/60 SuperdexTM 30 columns. However, 0.15 M AmAc with pH 6.9 was used as mobile phase and relative amount of oligomers was monitored by Shimadzu RID -6A detector. pH 6.9 was used to due to avoid potential aggregation of alginate present in the swelling solution. The plate number (N) of the SEC system was calculated to be 26 000 from the chromatogram given in Appendix D, which was obtained by injecting 2 mg of de-N-acetylated dimer (A2).

5 ml MQ was separated into three fractions and added one by one into the round bottom flask containing the sample. As the dried sample was gradually dissolved, the three fractions were collected and transferred to a 5 mL vial and mixed for 15 min. The sample solution was then filtered through a 0.2 µm polypropylene membrane filter and injected to the SEC system with a 01 mm syringe.

3 Results and Discussion

In this section, results from the material characterization, swelling experiments and SEC analysis of swelling solutions are presented and further discussed. Initially, the material characterization of leaf and stipe alginate, and chitosan oligomer mixture is assessed, followed by a review of the swelling behaviour of alginate calcium gels and alginate chitosan oligomer gels. Furthermore, the swelling of leaf and stipe alginate gels combined with calcium and chitosan oligomers, is interpreted. Evaluation of results obtained from the SEC analysis of leaf alginate gels comprising chitosan oligomers is also included.

3.1 Material Characterization

3.1.1 Chemical Composition of *L. hyperborea* Leaf and Stipe Alginate Samples

The chemical composition in the means of M and G monad, diad and triad frequencies of *L. hyperborea* leaf and stipe alginate samples was determined by ^1H and ^{13}C NMR spectroscopy (Table 3). As expected, the *L. hyperborea* stipe alginate had a higher G-residue content ($F_G = 0.68$) compared to *L. hyperborea* leaf alginate ($F_G = 0.46$). The M and G frequencies were obtained from the NMR spectra by integrating peaks characteristic for the different sequences, applying Grasdalen's method (Grasdalen et al., 1981, Grasdalen, 1983). The peak integration was obtained using TopspinTM. ^1H and ^{13}C NMR spectrums are presented in Appendix A.

Table 3. Chemical composition determined by ^1H and $(*)^{13}\text{C}$ NMR spectroscopy of *L. hyperborea* leaf and stipe alginate samples. The result is given as $\beta(1\rightarrow4)$ -D-Mannuronate (M) and $\alpha(1\rightarrow4)$ -L-Guluronate (G) monad, diad and triad frequencies.

Alginate Source	F_G	F_M	F_{GG}	F_{MG}	F_{GGG}	F_{MGM}	F_{GGM}
<i>L. hyperborea</i> leaf (GP3350)	0.46*	0.54*	0.36*	0.43*	0.18*	0.20*	0.19*
<i>L. hyperborea</i> stipe (LF200S)	0.68	0.32	0.57	0.28*	0.10*	0.53	0.17*

Average block lengths (Table 4) was calculated from the different M and G segment frequencies, according to Grasdalen's method (Grasdalen et al., 1981, Grasdalen, 1983). The average G-block length was calculated to 4.8 for leaf alginate and 14.0 for stipe alginate, from the frequencies obtained from ^{13}C NMR and ^1H NMR spectra, respectively. Similarly, the average M-block length

was calculated to 3.6 for leaf alginate and 3.2 for stipe alginate, from frequencies obtained from the ^{13}C NMR spectra. The chemical composition obtained from the NMR characterisation of *L. hyperborea* leaf and stipe alginate was highly similar to results from previously performed characterization of the same alginate source (Draget, 2006).

Table 4. Average G- and M-block lengths ($N_{G>1}$, $N_{M>1}$), weight and number average molecular weight distributions (M_w , M_n) and polydispersity index (PI) of *L. hyperborea* leaf and stipe alginate determined by ^1H and (*) ^{13}C NMR spectroscopy and SEC MALLS.

Alginate Source	$N_{G>1}$	$N_{M>1}$	M_w (g mol^{-1})	M_n (g mol^{-1})	PI
<i>L. hyperborea</i> leaf (GP3350)	4.8*	3.6*	220 000	105 400	2.09
<i>L. hyperborea</i> stipe (LF200S)	14	3.2*	270 000	97 400	2.78

Molecular weight distributions of the two alginate samples were characterized SEC MALLS (Table 4). The weight average molecular weight distribution (M_w) was significantly higher for stipe alginate compared to leaf alginate, said 270 000 and 220 000 respectively. The number average molecular weight distribution (M_n) was 105 400 for leaf and 97 400 for stipe alginate. The polydispersity index (PI) parameter is simply the M_w/M_n ratio, which expresses the molar mass dispersity of the sample. In a polydisperse sample containing molecules of different weight, M_w is naturally higher than M_n . Both alginate samples had $\text{PI} \sim 2$, exhibiting a polydisperse distribution similar to a Kuhn distribution (Smidsrød and Moe, 2008).

3.1.2 Chemical Composition and Size Distribution of Chitosan Oligomer Mixture

A high amount of oligomers was required to prepare sufficient gel cylinders in experiments performed in this thesis. Thus, a commercial batch of chitosan oligomer (lot number 121017WG) produced by Koyo Chemical was utilized in the gel preparation. This batch was produced by enzymatic degradation of chitosan. A structural characterization of the chitosan oligomer mixture was performed in order to determine certain physical and structural parameters, e.g. average degree of polymerisation (DP_n) and degree of acetylation F_A , that are important in the means of hydrogel properties. Oligomer mixtures with dissimilar DP_n have previously been shown to alter the hydrogel behaviour, such gel strength (Khong et al., 2013).

A SEC-RI system was used to separate oligomers and obtain a size distribution of the chitosan oligomer mixture. The oligomers were separated in a AmAc mobile phase at pH 4.5, where chitosan is fully soluble. By plotting the detector signal from the RI detector as a function of elution time, the relative amount of each oligomer in the mixture was determined (Figure 17). The chromatogram clearly demonstrates the polydispersity of the oligomer mixture, where fragments ranging from the dimer (DP = 2) to the twentymer (DP = 20) is detectable. The area under each peak was determined by integration, as described by Sorbotten and co-workers (Sorbotten et al., 2005). The relative content of the different oligomers was used to calculate the, which was determined to 3.96 for the mixture. The DP_n was also confirmed by 1H NMR where the integral of reducing ends was related to the integral of internal anomeric proton-signals. Thus, the tetramer (DP = 4) was shown as the most abundant oligomer in the mixture, which also is easily detected by observing the highest peak in the presented chromatogram (Figure 17).

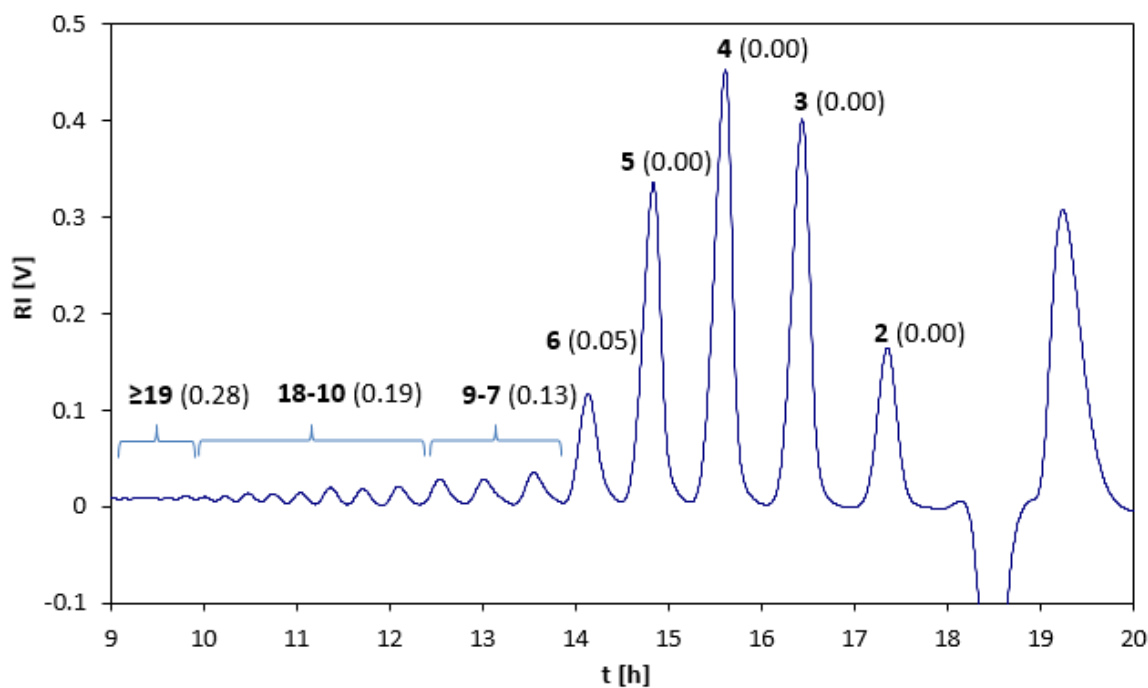


Figure 17. SEC chromatogram of a chitosan oligomer mixture with degree of polymerisation and N-acetylation. The relative amount of oligomers is plotted as a function of elution time (t). Each peak represents a chitosan oligomer where the degree of polymerisation, equal to the number of chitosan monomer units, and N-acetylation degree is written above, e.g. **6** (0.05) represents the sixmere with an N-acetylation degree of 0.05. The drop in the baseline observed after 18 hours is the water front and the peak with elution time of 19.5 hours is the salt peak. The chromatogram was obtained from a SEC RI using a flow rate of 0.8 mL/min and pH 4.5 150 mM AmAc mobile phase

The degree of acetylation (F_A) of each oligomer (Table 5) was obtained from previously performed experiments at the Department of Biotechnology, where the chemical structure of the oligomer mixture was characterised by ^1H NMR spectroscopy according to Tømmeraas and co-worker's method (Tommeraas et al., 2001). Chitosan oligomers with $\text{DP} < 6$ was shown to be fully de-N-acetylated, while the average acetylation degree for the complete mixture was 0.045.

Table 5. Size distribution and N-acetylation degree (F_A) of a chitosan oligomer mixture. The size distribution is given as relative amount of each oligomer, which is characterized by the degree of polymerisation (DP). The degree of N-acetylation (F_A) for each oligomer is also provided.

DP [-]	2	3	4	5	6	7-9	10-18	≥ 19
Content [%]	9.8	22.7	25.5	19.1	7.0	5.7	6.6	3.6
F_A [-]	0.00	0.00	0.00	0.00	0.05	0.13	0.19	0.28

3.2 Swelling Behaviour of Alginate Gels Crosslinked with Calcium

The swelling behaviour of leaf and stipe alginate calcium gels was investigated in various buffer solutions in order to connect the trends observed in this thesis to established swelling principles and other studies performed on alginate calcium gels. In addition, the calcium gels provided a control of which the swelling behaviour of the mixed alginate gelling system could be compared to. The next to subsection will focus on the effect of pH and salt on the swelling behaviour of alginate calcium gels.

The swelling ratio (Q_m) for all gels was calculated as the ratio between original mass (M_0) and the mass of the swollen gel at a given time (M_t). Henceforth, $Q_m = 1$ indicates neither gel swelling or shrinking, $Q_m > 1$ designates gel swelling and $Q_m < 1$ implies gel shrinking. All swelling experiments were performed with minimum 3 gels, where the error margin was calculated by an excel standard deviation formula. The general trend observed in early test experiments suggested that the different gelling systems were more or less equilibrated with the surrounding environment after 24 hours in the same solution, said few physical changes was observed beyond this point. Upon transferring the swollen gel into fresh solution, further swelling was observed for the next 24 hours. To be able to compare results obtained from the swelling experiments, the same experimental setup was used for both alginate calcium gels and the mixed alginate gels.

3.2.1 Swelling Across a pH Range from 4.5 to 8.5

According to the literature, the pH should not have a significant effect on the swelling behaviour on alginate calcium gels. Nevertheless, the effects of pH on swelling at equilibrium for leaf and stipe calcium alginate gels were investigated to provide a comparison control for the mixed alginate gels. Swelling was measured after 24 hours in buffer solutions across the pH range of 4.5-8.5, said both in 100 mM NaCl and no-salt solutions (Figure 18, Figure 19). The swelling ratio was also measured after transferring the gels into fresh solution for another 24 hours (48 hours in total). As expected, the pH changes did not seem to constitute any significant effect on the swelling ratio for either leaf or stipe alginate calcium gels, said both in solutions with and without salt (Figure 18, Figure 19). The same trend was observed at both 24 and 48 hours in solution. Minimal differences in the swelling behaviour for alginate calcium gel cylinders as a function of pH was also reported by Golmohamadi and co-workers across the pH range 4-9 upon swelling alginate calcium gel cylinders (Golmohamadi and Wilkinson, 2013).

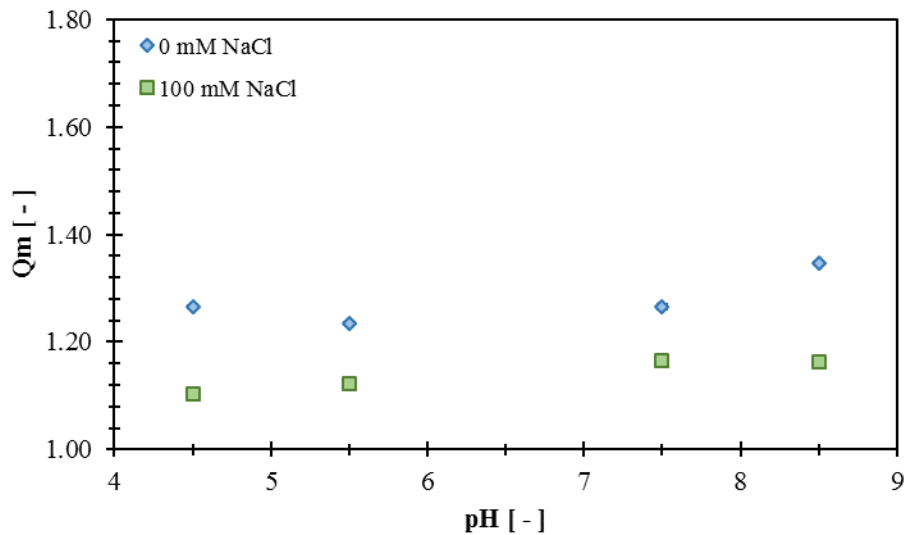


Figure 18. Swelling ratio (Q_m) as a function of pH of leaf alginate calcium gels swollen in solutions with and without 100 mM NaCl for 24 hours. Three gels ($n = 3$) were swelled in each solution where the standard deviation was calculated in excel.

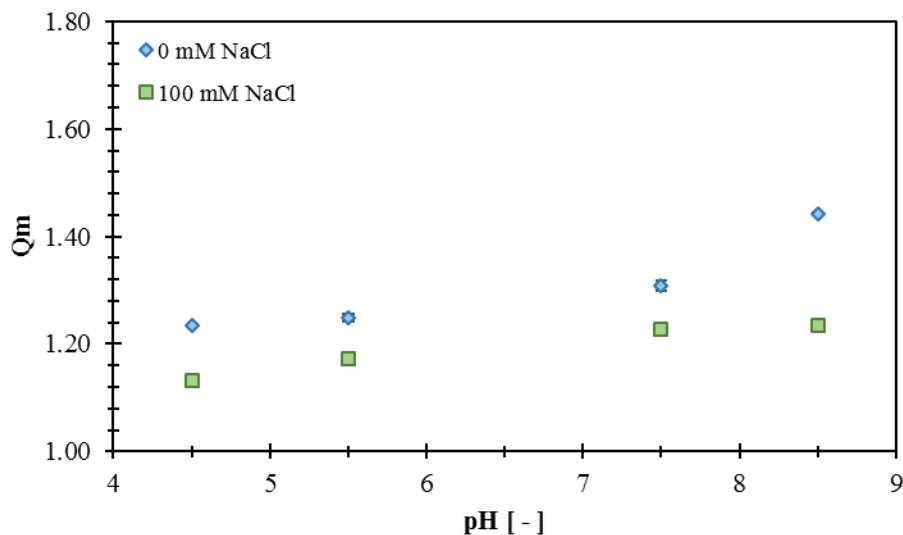


Figure 19. Swelling ratio (Q_m) as a function of pH of stipe alginate calcium gels swollen in solutions with and without 100 mM NaCl for 24 hours. Three gels ($n = 3$) were swelled in each solution where the standard deviation was calculated in excel.

From the swelling theory it is clear that Donnan potential and hence swelling is effected by the protonation degree of functional carboxyl-groups on the alginate chain (Moe et al., 1993). For alginate gels, the charge density of the carboxyl-groups is more or less determined by the pK_a of M and G monomers of 3.38 and 3.65, respectively. Thus, for pH values above \sim pH 5.5, more than approximately 99% of the carboxyl-groups will be deprotonated and carry charges (Draget, 2006). Hence, pH is most likely not governing swelling of alginate calcium gels above pH 5.5, which explains the low variation observed in the means of swelling behaviour of alginate calcium gels.

When interpreting the swelling results for alginate calcium gels in pH 4.5 solutions, a significant increase in protonation of the carboxyl-group is expected, said approximately 90% of the carboxyl-groups are charged at pH 4.5. A small decrease of the swelling ratio could be expected when the pH approaches the pK_a region of alginate, due to the decrease in ionic strength inside the gel which reduces $\Delta\mu_{ion}$ (Moe et al., 1993). However, a decreased swelling effect might also be compensated by the reduction of cross-links which would have decreased the elastic retractive force and reduce $\Delta\mu_{el}$, consequently causing relaxation of the network and reswelling of the gel. Hence, the relatively small increase in protonation of carboxyl-groups at pH 4.5 would most likely not have generated

any large response related to the swelling behaviour. A small decrease in swelling ratio was observed for all alginate calcium gels submerged in salt containing solutions between pH 4.5 and 5.5, said from 1.173 ± 0.001 to 1.13 ± 0.005 for stipe alginate calcium gels (Figure 19). It is however difficult to attribute this effect directly to the protonation of carboxyl-groups.

A marginally increase was observed for stipe alginate gels at pH 7.5 and 8.5, which was unexpected. A control study presented in subsection 3.5.1 revealed that this observation was most likely explained by the use of two different buffer systems. Larger variation was observed in the swelling data of solutions without NaCl compared to solutions with 100 mM NaCl (Figure 20, Figure 21). These variations were most likely related to different ionic strengths in the acetate and tris buffer solutions, which will be further elaborated in subsection 3.2.3. A significantly higher swelling ratios was observed in non-salt containing solutions compared to 100 mM NaCl solutions across all pH values. The salt effect on equilibrium swelling of alginate calcium gels will be elaborated in the next subsection. All gels where observed to experience increased swelling after being submerged in the second solution, which indicates the gels did not reach the maximum swelling capacity after 24 hours. Nevertheless, the main observation was that pH did not cause large effects on the swelling behaviour of leaf and stipe alginate calcium gels after 24 hours and 48 hours of swelling (Figure 20, Figure 21).

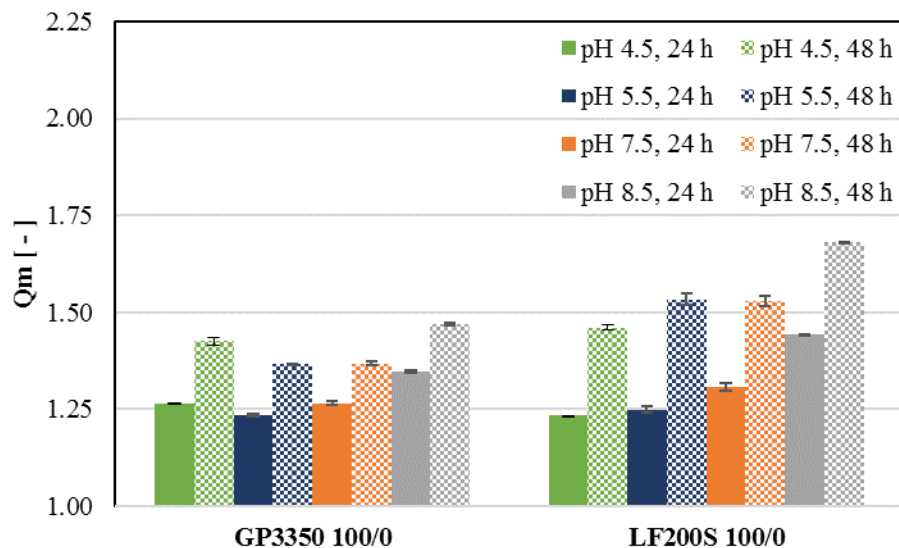


Figure 20. Swelling ratio (Q_m) of leaf (GP3350 100/0) and stipe (LF200S 100/0) alginate calcium gels after 24 and 48 hours in 50 mM buffer solutions with pH ranging from 4.5 to 8.5. Three gels ($n = 3$) were swelled in each solution where the standard deviation was calculated in excel.

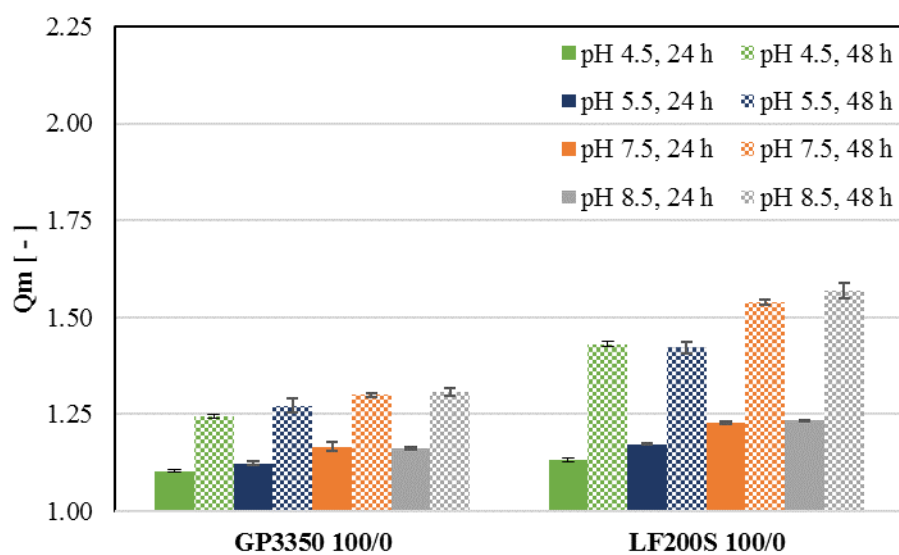


Figure 21. Swelling ratio (Q_m) of leaf (GP3350 100/0) and stipe (LF200S 100/0) alginate calcium gels after 24 and 48 hours in 50 mM buffer solutions with 100 mM NaCl and pH ranging from 4.5 to 8.5. Three gels ($n = 3$) were swelled in each solution where the standard deviation was calculated in excel.

3.2.2 Effect of Adding Salts to the Swelling Solutions

The swelling of leaf and stipe alginate calcium gels was measured after 24 hours in buffer solutions with and without salt. 100 mM NaCl was added as a part of the systematic setup to study how increasing ionic strength effected the swelling behaviour of alginate gels. In addition, an experiment where 10 mM CaCl₂ was added was conducted. The CaCl₂ experiment was included in order to demonstrate the swelling effect upon introducing additional cross-links by adding Ca²⁺ to the swelling solution, which would also connect our work to previously performed studies concerning swelling of alginate calcium gels.

As a demonstration of how adding sodium and calcium salts to the external buffer effect the swelling behaviour, results obtain from swelling leaf and stipe alginate calcium gels in different pH 7.5 tris buffer solutions are presented (Figure 22). Adding salts to the solutions significantly reduced the swelling for both leaf and stipe alginate gels. For instance, leaf alginate calcium gels was observed with a swelling ratio of 1.309 ± 0.010 , 1.265 ± 0.005 and 0.784 ± 0.005 in solutions where no salt, 100 mM NaCl and 10 mM CaCl₂ plus 100 mM NaCl was added, respectively (Figure 22). In solutions with 100 mM NaCl, swelling was decreased by approximately 10% for both leaf and stipe alginate calcium gels. An even larger effect was occurred in solutions containing both 100 mM NaCl and 10 mM CaCl₂, whereas gel shrinking was observed for both alginate calcium gels. The introduction of CaCl₂ in the external solution was shown to reduce swelling for leaf and stipe alginate calcium gels with approximately 50% and 40% compared to gels in non-salt and NaCl solutions, respectively. Very similar trends were also observed when performing the same experiment in pH 5.5 acetate solutions, whereas these results are given in Appendix E.

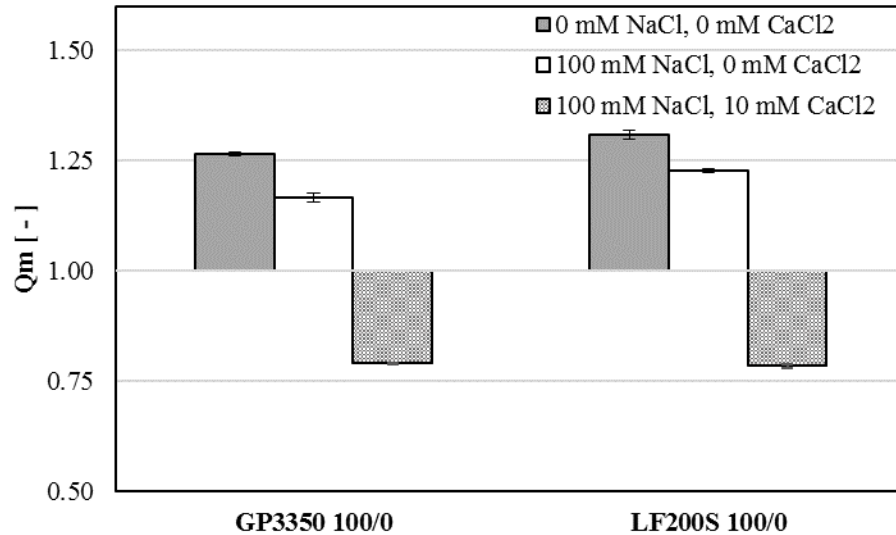


Figure 22. Swelling ratio (Q_m) of leaf (GP3350 100/0) and stipe (LF200S 100/0) alginate calcium gels after 24 hours in 50 mM tris buffer solutions with 0 mM salt, 100 mM NaCl and 10 mM plus 100 mM NaCl at pH 7.5. Three gels ($n = 3$) were swelled in each solution where the standard deviation was calculated in excel.

A weight increase of roughly 30% may be significantly lower than what many other researches have observed upon studying alginate calcium hydrogels. Martinesen with co-workers reported on a threefold volume increase upon 24 hour swelling in 0.1 M pH 7.0 tris buffer with 0.9% (w/v) NaCl concentration (Martinsen et al., 1989). However, as emphasized in the swelling theory, there are many influencing parameters to account for upon interpreting the swelling behaviour of such systems. Gel beads are much smaller, have a different geometrical shape and possess a considerably higher surface to volume ratio, which will greatly influence the swelling capacity. Other variables are the alginate source, consecutive G-residue content, calcium concentration and particle size, gel to solvent volume ratio, etc. Most swelling experiment performed with gel beads, microspheres or capsules are also fundamentally different following the different gel preparation methods, and hence cross-linking process which is known to have direct influence on swelling elastic term. Hence, one should be cautious upon performing direct comparisons of the swelling ratio between the results obtained in this work and other studies. Kuo and co-workers reported on an approximately 40% weight increase for small cylindrical *L. hyperborea* stipe alginate calcium gel disks swelled until equilibrium in physiological saline. These gels were prepared by *in situ* gelation using CaCO_3 and GDL, and were submerged in various solutions for 24 hours. Then again, direct

comparison would not be appropriate while these gels were 1.5% alginate with significantly lower calcium ion to alginate carboxyl group ratio of 0.72 (Kuo and Ma, 2008).

In terms of the salt effects on swelling behaviour, a reduced swelling ratio upon adding relatively high concentrations of NaCl to the swelling solution have been observed in many studies investigating the swelling behaviour of alginate gels (Kuo and Ma, 2008, Moe et al., 1993, Martinsen et al., 1989, Golmohamadi and Wilkinson, 2013, Segeren et al., 1974, Matyash et al., 2014). The phenomenon is most likely caused by the presence of counterion Na^+ , and the increase in mobile ions and hence ionic strength in the solution surrounding the gel. The Donnan equilibrium predicts that the difference in ionic concentration between the inside and the outside of the gel decreases when the ionic strength of the surrounding solution is increased (Moe et al., 1993). Hence, the reduced swelling ratio observed when NaCl is added to the solution may be attributed to the decreasing Donnan potential due to charge screening and increased concentration of mobile ions outside the gel.

Sodium ions are also counterions for the negatively charged alginate chain, that will diffuse into the gel network and most likely interact electrostatically with the carboxyl-groups. Even though it will not form cross-links with alginate, it may decrease charge to charge repulsion on the alginate chains and compete with cross-linked calcium ions. The calcium-sodium selectivity coefficient $k_{\text{Ca/Na}}$ is 21.1 in *L. hyperborea* (Haug and Smidsrod, 1967), suggesting that high presence of Na^+ in the solution may exchange some of the calcium ions inside the gel, said two Na^+ for one Ca^{2+} . Consequently, this may weaken the cross-links over time and hence increase the osmotic pressure and decrease elasticity. This would lead to an increased swelling ratio and dissolving of the gel (Strand, 2002). This effect was present in our alginate systems, as gels submerged in 100 mM NaCl solution for 2x24 hours typically showed a tendency to be less stable with loss of the cylindrical shape (Figure 26). However, the significantly lower swelling ratio observed for alginate calcium gels in salt containing solution compared to non-salt solution gels suggests that the Donnan effect was predominant during the specific circumstances in our swelling experiments.

The gel contraction observed upon swelling in 10 mM CaCl_2 and 100 mM NaCl swelling solutions is most likely due to the presence of divalent ions with strong affinity towards sequential G

segments in the alginate chains. When sufficient CaCl_2 is present in the swelling solution, Ca^{2+} will diffuse into the alginate network and saturate the GG binding sites. According to the counterion condensation theory, not all Ca^{2+} present inside the gel network are associating with the alginate chains (Manning, 1969). Since alginate calcium gels used in our experiments are unsaturated with calcium, additional Ca^{2+} may promote further cross-linking. An increase in cross-link density contributes to the elastic term, $\Delta\mu_{el}$, in the means of reinforcing the elastic retractive force, which leads contraction of the polymer network. The additional mobile ions also increase the ionic strength of the external solution and which reduces the ionic term $\Delta\mu_{el}$. Henceforth, a secondary effect is generated that may amplify the gel shrinking by enhancing the Donnan effect (Moe et al., 1993). It is difficult to separate these two effects since the increase of external ionic strength and overall charge-density both reduce swelling. Previously performed studies have indicated that Ca^{2+} binding effect to carboxyl group is the main contributing factor rather than the effect of the electrostatic interactions (Golmohamadi and Wilkinson, 2013).

By reviewing previously performed experiments it was expected that stipe-alginate calcium gels would have a lower swelling degree compared to leaf alginate gels due to a higher G-residue content (Martinsen et al., 1989, Kuo and Ma, 2008, Strand, 2002). G rich alginate gels have been shown to be more permeable than M-rich alginate due to the densely cross-linked network which provide a highly ordered structure with more free volume space. The M-rich alginate calcium gels are more flexible due to the less effective cross-linking of the M-blocks. However, alginates with long average G-block length have been shown to possess slightly lower swelling capacity compared to M-rich alginates due to the higher elastic restoring force which opposes the osmotic swelling pressure (Martinsen et al., 1989, Kuo and Ma, 2008). Rather interestingly, in these experiments, the general trend was that the G-rich stipe alginate calcium gels always swelled marginally more than M-rich leaf alginate calcium gels. It may be speculated if this phenomenon may be related to the small difference in M_w , different degree of calcium saturation or the potential maximum swelling capacity of the network when it is pushed to its viscoelastic limits, etc. However, the observed effect in the means of leaf versus stipe alginate calcium gels was marginal and will not be further pursued in this thesis.

3.2.3 Effect of salt, ionic strength and pH on swelling kinetics

The swelling kinetics of alginate calcium gels were also monitored across the pH range of 4.5-8.5 in solutions with and without 100 mM NaCl (Figure 23, Figure 24,). The swelling kinetic curve provide an indication of how fast and with what quantity the fluid diffuses into the polymer network. A slack swelling slope would according to the swelling theory be equivalent to a Fickian behaviour, indicating a more flexible and rubbery polymer network. Intuitively, a rather steep swelling slope would indicate more non-Fickian behaviour caused by a more rigid and brittle polymer network that is easily penetrated (Masaro and Zhu, 1999). As we will observe from the swelling kinetic results, the kinetic behaviour is highly influenced by the environment surrounding the gel. The aim of this study was to observe difference in terms of swelling behaviour for different gelling systems and perform a qualitative analysis, thus no model has been fitted to the swelling kinetic data. The swelling kinetics for stipe alginate calcium gels are shown in Appendix F.

For the leaf and stipe alginate gels swollen in buffer solutions without salt, there were significantly different swelling rates across the pH range of 4.5 to 8.5. For instance, a slower swelling rate was observed for both leaf and stipe alginate calcium gels in the low pH Ac solutions, compared to swelling at pH 7.5 and 8.5. An interesting observation was that alginate calcium gels seemed to swell slower when pH was increased from 4.5 to 5.5. In terms of protonation of functional carboxyl group on the alginate chain, the opposite effect would be expected due to the increased osmotic driving force in the establishment of a Donnan equilibrium when the charge density of alginate is increased. This phenomenon was however most likely compensated by another osmotic driving force attributed to variations in the ionic strength between the different solutions. The pK_a of Ac is 4.76, meaning ~ 36% of the Ac carboxyl-group are charged at pH 4.5 compared to ~ 85% at pH 5.5, according to the Henderson-Hasselbalch equation.

The difference in degree of protonation results in significantly different ionic strengths for all the solutions, e.g. 18 mM and 43 mM for a 50 mM Ac solution with pH 4.5 and 5.5, respectively. This would also the case for the tris buffer that has a pK_a value of 8.06. Henceforth, the ionic strength of all buffer solutions used in the swelling experiments in this thesis were calculated, and are presented in Appendix G. Two numerical examples for ionic strength calculations are given in

Appendix G. The difference in ionic strength of the buffer solutions may explain some of the smaller variations observed in the equilibrium swelling of alginate calcium gels (Figure 20).

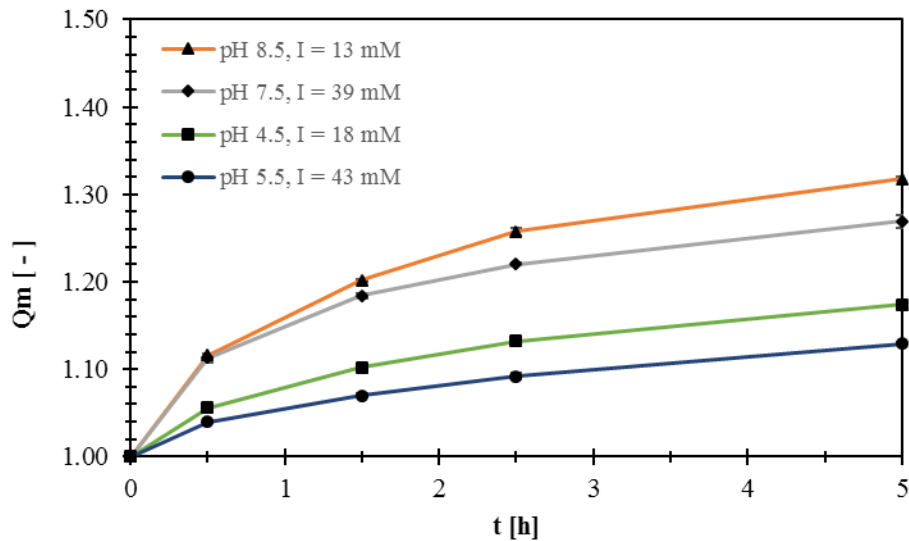


Figure 23. Swelling kinetics of leaf alginate calcium gels in 50 mM buffer solutions of different pH. Swelling ratio (Q_m) is plotted as a function of time after the gels were submerged in the solution. An Ac buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non/Fickian diffusion. Three gels ($n = 3$) were measured at each given time where the standard deviation was calculated in excel.

Another trend was a faster swelling ratio at pH 7.5 and 8.5, compared to pH 4.5 and 5.5. For instance, after 5 hours in solution, leaf alginate calcium gels had a swelling ratio of 1.129 ± 0.004 at pH 5.5 compared to 1.318 ± 0.001 at pH 8.5 (Figure 24). This observation proposed that it might be an additional effect caused by the different buffer system, possibly connected to the opposite charges on the functional groups of tris and acetate molecules. A buffer control experiment was performed in order to enlighten this effect, which is presented in subsection 3.5.1.

For leaf alginate calcium gels submerged in NaCl containing solutions, little impact was observed in the means differences in swelling kinetic behaviour across a pH range of 4.5 to 8.5 (Figure 24). The relatively slack curve indicates a more Fickian diffusion profile of leaf and stipe alginate calcium gels when NaCl is present in the solution. A significant difference could however be observed between the acetate and tris buffer systems, said between pH 5.5 and 7.5, respectively,

again suggesting that the buffer component or high pH may influence the swelling of these gels. Highly similar trends were observed for the stipe alginate calcium gels, showed in Appendix F.

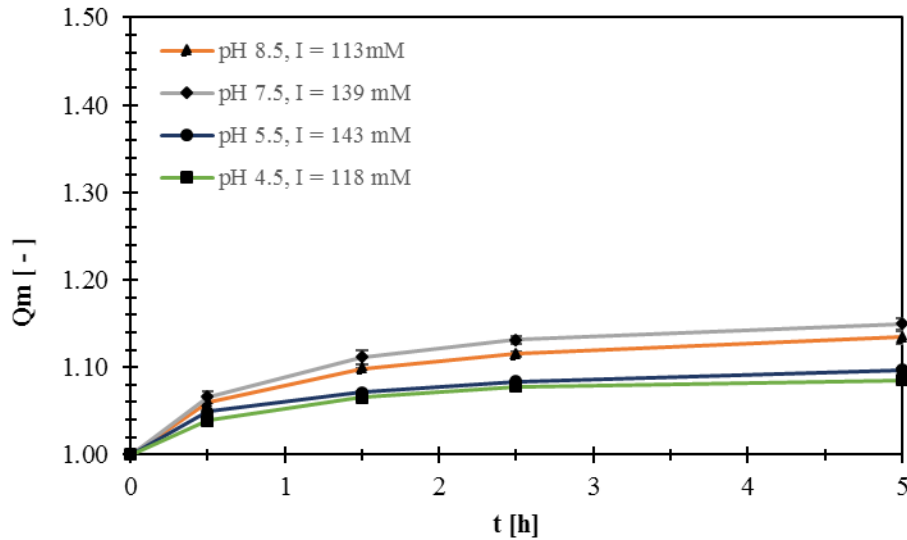


Figure 24. Swelling kinetics of leaf alginate calcium gels in 50 mM buffer solutions of different pH and with 100 mM NaCl. Swelling ratio (Q_m) is plotted as a function of time after the gels were submerged in the solution. An Ac buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non/Fickian diffusion. Three gels ($n = 3$) were measured at each given time where the standard deviation was calculated in excel.

3.3 Swelling Behaviour of Alginate Gels Cross-linked with Chitosan Oligomers

An experiment investigating swelling behaviour of alginate gels cross-linked with chitosan oligomers was conducted in order to support the interpretation of swelling of alginate gels with mixed cross-linkers. A certain stability and mechanical strength was required to be able to prepare stable cylindrical gels and perform the swelling measurements. Stipe alginate gels crosslinked with chitosan oligomers possess relatively low gel strength, and did not provide cylindrical gels stable enough for the swelling measurements. The low mechanical strength could be due to the low F_M and $N_{M > 1}$ of 0.32 and 3.2, respectively (Table 3, Table 4). Leaf alginate gels cross-linked with chitosan oligomers provided sufficient stability to perform swelling measurements in pH 5.5 Ac solutions after pH 24-hour submersion (Figure 25).

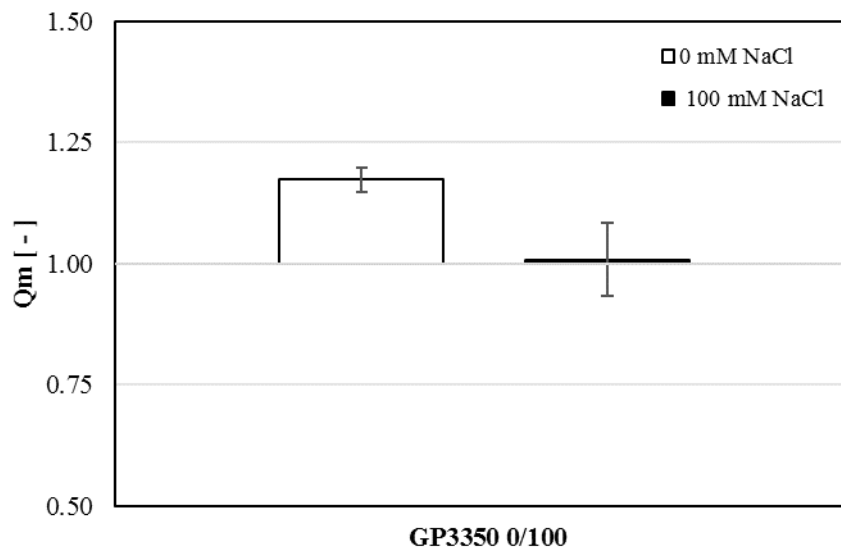


Figure 25. Swelling ratio (Q_m) of leaf alginate gels cross-linked with chitosan oligomers (GP3350 0/100) in pH 5.5 acetate solutions with and without 100 mM NaCl. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

A decrease of the swelling ratio from 1.173 ± 0.025 and 1.009 ± 0.076 was obtained upon introducing NaCl into the swelling solution (Figure 25), as well as reduced stability of the leaf alginate chitosan oligomer gel shape (Figure 26). The relatively high deviation was most likely due to the instability of the system, which amplified when NaCl was added. One reasonable explanation could be that Na^+ is competing with the cross-linker, as for the alginate calcium gels, which would slowly dissolve the gel.

Swelling beyond 24 hours or performing more than one measurement was complicated for leaf alginate chitosan oligomer gels, because these gels were easily deformed when taken out of the swelling solution. When submerged at higher pH in the tris buffer system, the leaf alginate chitosan gels were observed to dissolve within few hours (Figure 26). This phenomenon may be related to solubility and the pK_a of the chitosan oligomers, or the matter of cation association with the negatively charged alginate chain. In theoretical terms, the deprotonation of chitosan oligomers at high pH might cause dissociation of chitosan oligomers. However, several studies have reported that chitosan-polycation PEC formed below the pK_a of chitosan have been observed to maintain its stability in physiological conditions (Vårum and Smidsrød, 2005).

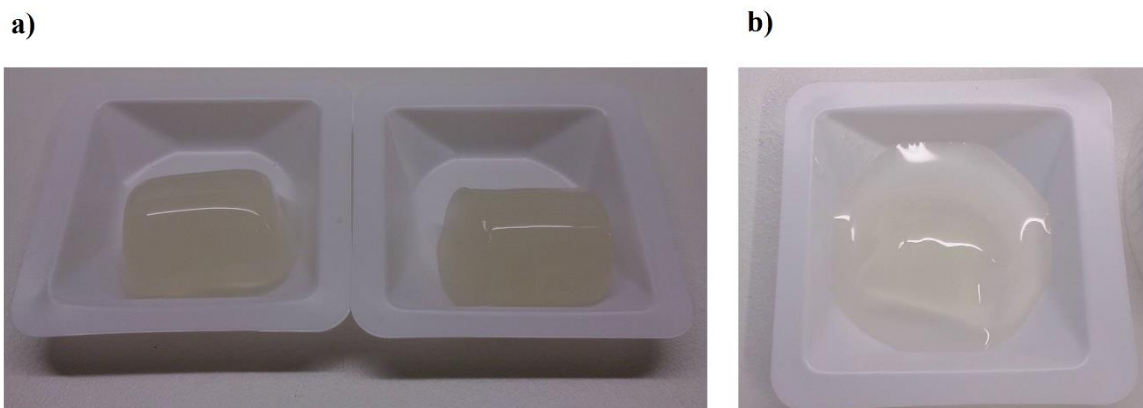


Figure 26. Physical shape of swollen leaf alginate gels cross-linked with chitosan oligomers. The gels were swelled in 50 mM pH 5.5 Ac solution (a) with (left) and without (right) 100 mM NaCl, and in 50 mM pH 7.5 tris solution without salt (b). After 25 hours in pH 5.5 Ac solution, the gel was observed with softer edges and less defined cylindrical shape when 100 mM NaCl was added to the solution. In pH 7.5 tris solutions, the gels dissolved few hours after being submerged into the solution.

3.4 Swelling Behaviour of Alginate Gels with Mixed Cross-linkers

Upon investigating the swelling properties of leaf and stipe alginate gels cross-linked with a combination of calcium and chitosan oligomers, the focus was to study the behaviour in different environments. Initially, swelling was studied at pH 4.5 and 5.5 where both alginate and chitosan was allegedly charged and soluble. In these conditions, pH was sufficiently above the pKa of alginate of 3.5, ensuring that the majority of the alginate carboxyl groups remain negatively charged. In addition, the pH was below the pKa value of chitosan of 6.5, meaning most of the amino groups would be positively charged. Buffering solutions were necessary to control the pH and avoid swelling conditions close to 6.5, where small variations in pH might cause larger and irregular impacts on the charge of the chitosan oligomers.

It was desirable to investigate how the swelling behaviour of the mixed gels was in an alkaline environment close to physiological pH, which is relevant in relation to biomedical applications. It was also interesting to observe swelling above the pKa of chitosan where the chitosan oligomers are theoretically insoluble. Swelling experiments was conducted at pH 7.5 and pH 8.5 for this purpose. Furthermore, in order to investigate the behaviour in an environment close to the ionic strength in physiological conditions, the mixed gels were also swelled across the pH range of 4.5 to 8.5 with 100 mM NaCl added to the solution.

The overall aim was to describe and compared the swelling behaviour of the two mixed gelling systems with respect to each other, and also the alginate calcium and alginate chitosan oligomer gels. While this thesis was the first swelling study performed on the mixed alginate gelling system, one should be careful upon concluding what forces and structure related properties that are governing the swelling mechanism of these gels.

3.4.1 Swelling in Ac Solution at pH 4.5 and 5.5

After 24 hours in pure 50mM Ac solution at pH 4.5, a swelling ratio of 1.151 ± 0.001 and 1.173 ± 0.008 was measured for mixed leaf and stipe alginate gels, respectively (Figure 27). A significant increase of the swelling ratio was observed for both systems when the gels were submerged in fresh solution for additional 24 hours, said 1.214 ± 0.002 for leaf and 1.276 ± 0.002 for stipe alginate gels (Figure 27). The swelling ratio was generally lower for the mixed gelling system compared to the alginate calcium system (Figure 20), said approximately 20% lower after 48 hours (2x24 h). In addition, the swelling for the mixed leaf alginate gels was observed as marginally higher compared to mixed stipe alginate gels in at pH 4.5.

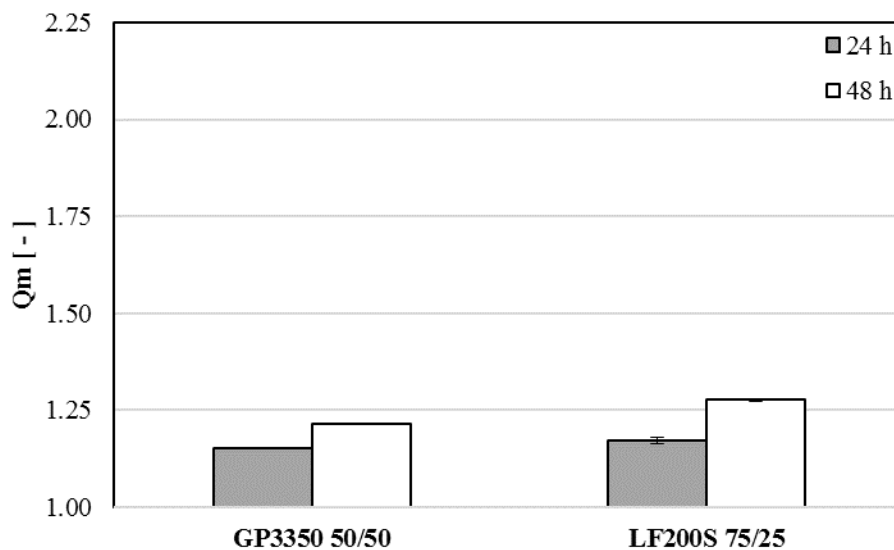


Figure 27. Swelling ratio (Q_m) for mixed leaf (GP3350 50/50) and stipe (LF200S 75/25) alginate gels cross-linked with calcium and chitosan oligomers after 24 and 48 (2x24) hours swelling in 50 mM pH 4.5 Ac buffer solution. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

The fact that both mixed gelling systems was observed to swell even more in the second solution demonstrates that similar to what was observed for the alginate calcium gels, the maximum swelling potential was not reached after 24 hours. The lower swelling ratio observed for the mixed alginate gels compared to the corresponding alginate calcium gels may in theoretical terms be attributed to either a reduced internal ionic strength, increased valence of counterions, or higher cross-linking degree (Moe et al., 1993). Thus, one might speculate if any of these arguments could be used to explain the observed behaviour of the mixed gels in these conditions.

In pure pH 5.5 Ac solutions, a swelling ratio of 1.161 ± 0.009 and 1.170 ± 0.006 was observed after 24 hours for mixed alginate and stipe alginate gels, respectively (Figure 28). A significant increase of the swelling ratio was obtained after 48 hours, said 1.272 ± 0.009 for leaf and 1.343 ± 0.004 for stipe alginate gels (Figure 28) Once again, the swelling ratio of the mixed gels was significantly lower (~10%) compared to the corresponding alginate calcium gels (Figure 18, Figure 19), whereas the mixed leaf alginate gel exhibited the lowest swelling ratio. A marginal increase (~5%) of the swelling ratio was observed for the mixed gels at 48 hours in pH 5.5 solution, compared to what was observed in the 4.5 solutions.

Since the mixed alginate gels exhibited a lower swelling ratio compared to the alginate calcium gels at both pH 4.5 and 5.5, one might discuss plausible reasons for the observed trend. It is however difficult to predict the interaction mechanisms that most likely occurs when chitosan oligomers are introduced into the alginate calcium gels. Hence, it is also complicated to describe what governs swelling in these gels. One might speculate that in theoretical terms, a decreased swelling ratio could hypothetically be an effect of a lower ionic strength inside the gel. A lower internal ionic strength would reduce the osmotic pressure due to a reduced difference between mobile ions inside and outside the gel, $\Delta C_{\text{mobile ions}}$. Nevertheless, as for the mixed leaf alginate gels, the substitution of calcium with chitosan oligomers should not affect the overall concentration of mobile ions inside the gel in terms of positive charges per negatively charged carboxyl group. A numerical example demonstrating the theoretical internal ionic strength of the different gels is shown in Appendix X. Thus, it is more likely that the explanation was related to the matter of cross-linking.

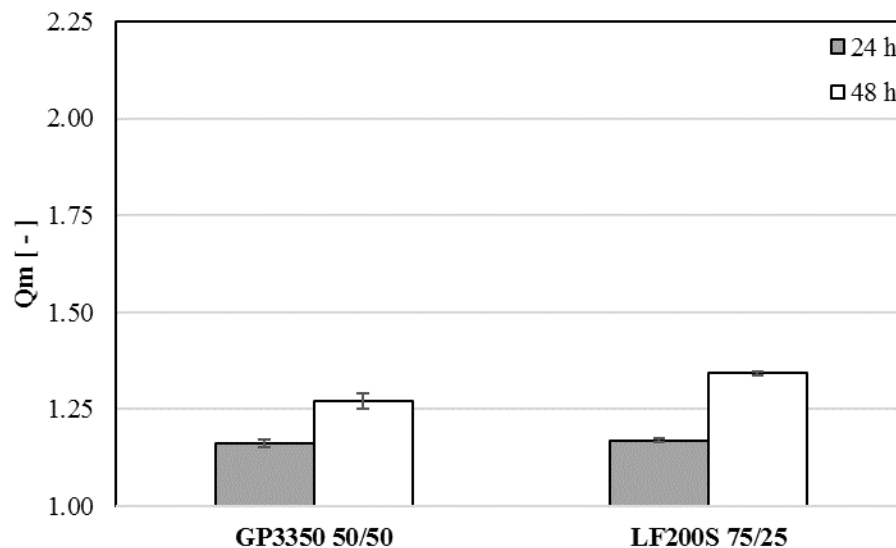


Figure 28. Swelling ratio (Q_m) for mixed leaf (GP3350 50/50) and stipe (LF200S 75/25) alginate gels cross-linked with calcium and chitosan oligomers after 24 and 48 (2x24) hours swelling in 50 mM pH 5.5 Ac buffer solution. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

Hypothetically speaking, the substitution of calcium with chitosan oligomers may have increased the total number of cross-links compared to the alginate calcium gels. Since it has previously been shown that poly-M and chitosan oligomers form gels, it is likely that additional cross-links are formed between chitosan and M-blocks segments on the alginate chain (Khong et al., 2013). It has previously been reported that the calcium has low affinity towards M-blocks (Draget, 2006). The *L. hyperborea* leaf and stipe alginate have both a respectable amount of M-block segments, where $N_{M>1}$ is 3.6 for leaf and 3.2 for stipe alginate (Table 4). The selectivity between calcium and chitosan oligomers in relation to alginate is undescribed. However, one might speculate if the chitosan oligomers would have a higher affinity towards the M-block than calcium due to the conformational similarities between chemical structure of poly-M and deacetylated chitosan (Figure 7). Hence, the M-block segments in leaf and stipe alginate may be accessible for chitosan oligomer cross-link formation, a theory which is supported by the maintenance of the gel strength upon substituting a certain amount of calcium with chitosan oligomers (Figure 9, Figure 10) If this was the case, there might have been a small increase in the total number of cross-links in the mixed gels compared to the alginate calcium gels, which could explain the small reduction of swelling for exhibited by the mixed gels.

According to Manning theory, a significant fraction of the calcium ions will be uncondensed and dissociated from alginate chain (Manning, 1969). However, since the cross-linking of the mixed gels may be based on cross-links with both G- and M-blocks with different cross-linkers, it would be very complicated to apply counter-condensation theory based predictions to elaborate the swelling behaviour of the mixed gels. One might speculate that when reducing the calcium content in the gel, the majority of the calcium ions removed would be the uncondensed ions or the calcium associating in weaker interactions with the M-blocks. Hence, when introducing chitosan oligomers, the M-blocks may be available for cross-linking and the total number of cross-links would thus increase. Since leaf alginate is rich in M-residues and have a higher $N_{M>1}$ compared, more cross-links will be introduced here compared to stipe alginate. A potential increase of cross-link density related to the chemical composition of alginate might explain why the swelling is lower for the mixed alginate gel compared to the alginate calcium gels, and additionally why the mixed leaf alginate had a lower swelling ratio compared to the mixed stipe alginate gels.

The mechanism of how chitosan oligomer actually interact with alginate in the mixed gelling system still unknown. Therefore, the above arguments represent unconfirmed speculations, and a larger quantity of scientific data is required to substantiate such assumptions. It should also be kept in mind that the leaf alginate singularly cross-linked with chitosan oligomers had a swelling ratio comparable to the mixed leaf alginate gels in pH 5.5 solutions (). Mechanical and rheological studies of these gels have reported a significantly lower gel strength for the alginate chitosan oligomer gels compared to the alginate calcium gels (Figure 9, Figure 10), demonstrating that it is not necessarily the strength of the cross-links that is reducing the swelling in these conditions. Measuring the mechanical strength of the swollen gel would provide a good indication of the cross-linking degree in the different gelling systems at swollen state. Measurements of gel strength was performed on some of the swollen gels, however this was challenging due to irregular shapes and low reproducibility and was not included or further pursued in this thesis.

When operating within the pH range of 4.5-5.5, one should also consider that approximately 10% of carboxyl-groups for the alginate and 90% of the amino-groups for chitosan oligomers will be protonated at pH 4.5 and 5.5, respectively, as a result of the different pK_a . In addition, as previously

discussed, the ionic strength of the buffer solution was significantly higher at pH 5.5 compared to 4.5. In theory, these influencing factors may cause effects on the swelling behaviour of the mixed gels, such as dissociation of cross-linked chitosan oligomers or alginate chains, changes of ionic strength and the osmotic swelling pressure, etc. However, marginal differences in the swelling ratio was observed for the mixed gels upon varying the pH between 4.5 and 5.5, suggesting that the pH does not have a predominant influence on the swelling behaviour in these conditions. Nonetheless, one cannot exclude that opposing osmotic and elastic forces might have been present, but compensated for each other.

3.4.2 Swelling in Tris Buffer Solutions at pH 7.5 and 8.5

The swelling ratio was increased by more than 50% for both mixed leaf and stipe alginate gels upon increasing the pH to 7.5. After 24 hours in pure 50mM tris solution at pH 7.5, a swelling ratio of 1.404 ± 0.009 and 1.405 ± 0.002 was measured for mixed alginate and stipe alginate gels, respectively (Figure 29). Further, a swelling ratio of 1.610 ± 0.015 and 1.777 ± 0.027 was measured after 48 hours, wherein the gels were still stable (Figure 29). The swelling ratio observed at pH 7.5 represents a remarkable response in great contrast to what was observed for the mixed gels in the acidic conditions. The swelling ratio was significantly higher compared to the alginate calcium gels (Figure 20), said approximately 10% after 24 hours and 25-30% after 48 hours (Figure 29).

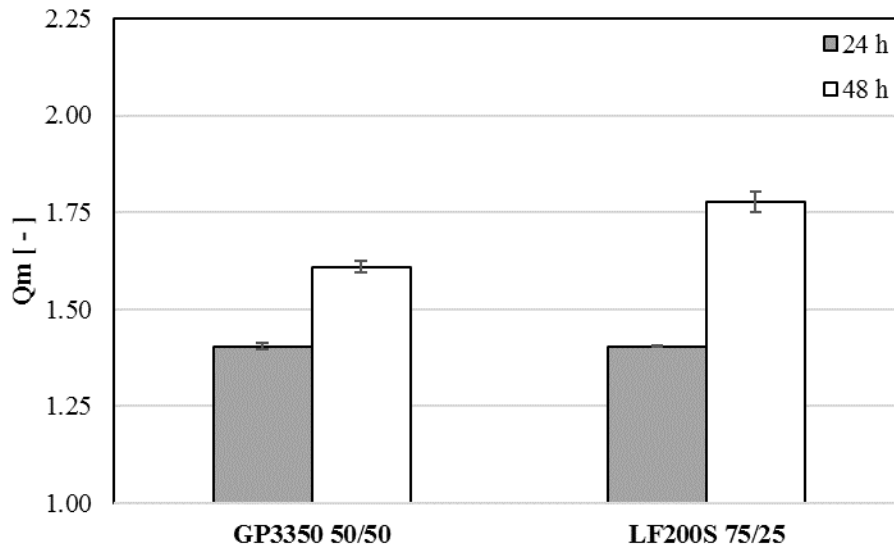


Figure 29. Swelling ratio (Q_m) for mixed leaf (GP3350 50/50) and stipe (LF200S 75/25) alginate gels cross-linked with calcium and chitosan oligomers after 24 and 48 (2x24) hours swelling in 50 mM pH 7.5 tris buffer solution. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

Before discussing possible reasons explaining the observed behaviour, the results from swelling mixed gels in pH 8.5 solution is also presented (Figure 30). Similar results were obtained at pH 8.5 in the means of observations of a significantly higher swelling ratio of the mixed alginate gels compared to the alginate calcium gels. After 24 hours at pH 8.5, a swelling ratio of 1.762 ± 0.011 and 1.654 ± 0.010 was observed for mixed alginate and stipe alginate gels, respectively (Figure 30). Further, a swelling ratio of 1.998 ± 0.064 and 1.982 ± 0.017 was obtained after 48 hours (Figure 30). Moreover, the swelling ratio was observed to be significantly higher at pH 8.5 compared to 7.5 (Figure 29, Figure 30). Marginal differences in the swelling behaviour was observed between the mixed leaf and stipe alginate gels in pH 7.5 and pH 8.5 solutions.

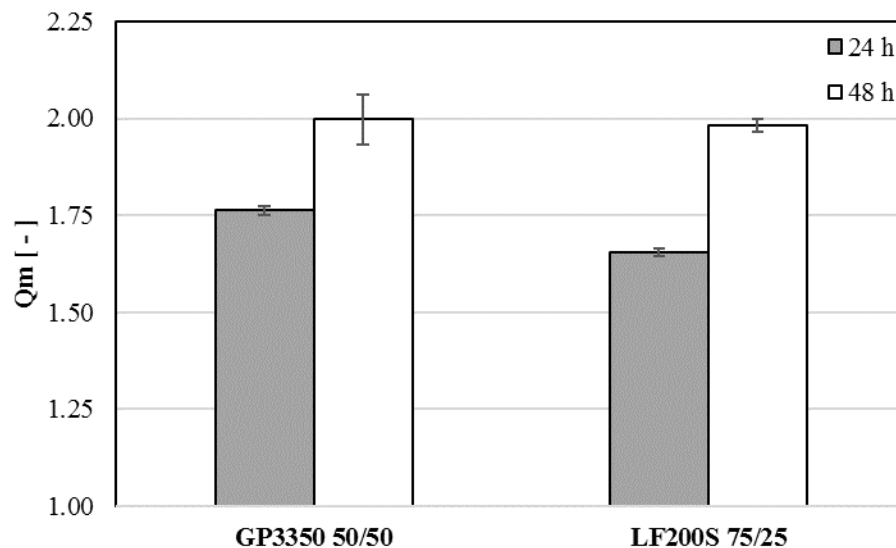


Figure 30. Swelling ratio (Q_m) for mixed leaf (GP3350 50/50) and stipe (LF200S 75/25) alginate gels cross-linked with calcium and chitosan oligomers after 24 and 48 (2x24) hours swelling in 50 mM pH 8.5 tris buffer solution. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

Observations of the swelling behaviour in pH 7.5 and 8.5 solutions without salt suggest a change of character for the mixed gels compared to the alginate calcium gels. It is intuitive to suspect that by increasing the pH of the solution surrounding the mixed gels above the pK_a value of chitosan, the swelling behaviour of the mixed alginate gels might have been affected as a result of the charge density of chitosan oligomers. For a pure chitosan solution, the majority of the amino-groups would be deprotonated and uncharged at physiological conditions, said approximately 10% at pH 7.5 and 1% at pH 8.5. The increase of pH or change of buffer component in the swelling solution may have caused circumstantial changes of chitosan oligomer related interactions, which consequently increase the swelling capacity of the gels. In theoretical terms, increased swelling effects of polyelectrolyte gels in dilute solutions is most likely related to a reduction of counter-ion condensation, decreased cross-linking density, and increased internal ionic strength (Moe et al., 1993).

According to Manning's theory, the polymer behaviour is dominated by electrostatic repulsion and thus the charge density and the number of uncondensed counterions (z_p) in dilute solutions (Manning, 1969). An assumed fraction of 60-70% condensation of counterions have previously

been used to predicted the average charge to charge distance parameter b for sodium alginate to explain the swelling behaviour (Katchalsky et al., 1961, Moe et al., 1993). Since the mixed alginate gelling system and the actual gel forming mechanism is unknown, we can only assume that the degree of counter-ion condensation is similar to other alginate gelling systems. We assume that not all calcium and chitosan oligomer molecules are forming cross-links with alginate and rather focus on the observed differences and similarities between the two systems. The fact that both the mixed alginate gel types swelled significantly more than the conventional alginate calcium gels at low external ionic strength at high pH may indicate that the cross-link density for these gels is now lower than for the alginate calcium gels. A reduced cross-linking degree would reduce the elastic retractive force and thus $\Delta\mu_{el}$, allowing more fluid to enter the polymeric network (Moe et al., 1993).

Hypothetically, the aggregation of chitosan oligomer cross-linkers may have reduced the amount of mobile ions and hence the ionic strength in the mixed gels. However, if the internal ionic strength and hence $\Delta\mu_{ion}$ is reduced, a gel shrinking response is expected. Thus, it is more likely to be a cross-linking related effect was causing the increased swelling response. A reduction of the elastic term, $\Delta\mu_{el}$, caused by a decreased number of cross-links would allow the network to expand, resulting in a higher swelling ratio. It is likely that the high pH caused dissociation of chitosan oligomers, which lead to reduced association with alginate and therefore a decreased number of cross-links in the mixed gels. Alginate remains fully charged and the calcium induced cross-links would probably be intact, thus avoiding gel dissolving as was observed for the leaf alginate singularly cross-linked with chitosan oligomers. Since there were more oligomers in the M-rich leaf alginate gels, it is in these gels one would expect a larger effect on swelling. This may explain why the mixed leaf alginate gels exhibit the largest transformation in terms of weight increase as a function of pH ranging from 4.5 to 8.5.

One must also not forget that similar to the Ac, the pK_a of the tris buffer results in a higher ionic strength at pH 7.5 compared to 8.5, which would have a larger influence on the swelling behaviour in dilute conditions. The lower ionic strength at pH 8.5 might be the reason for the significantly increased swelling observed at this pH due to an increase in mobile ions inside and outside the gel. Anyhow, it was evident that either the increased pH or the change of buffer component significantly

changed the swelling behaviour for both mixed alginate gel types. Another interesting feature is that the calcium ions clearly prevent the mixed leaf alginate gel from dissolving in the tris buffer, in great contrast to what was observed for the leaf alginate calcium gels in pH 7.5 tris solutions (Figure 26).

3.4.3 Swelling in Salt Containing Solutions Across pH Range 4.5 to 8.5

As previously mentioned, one of the objects of this thesis was to investigate the influence of salt on swelling behaviour of the mixed alginate gelling system in order to relate the results to physiological conditions. Therefore, swelling was monitored in 50 mM solutions containing 100 mM NaCl across the pH range 4.5 to 8.5. As expected for an ionic gel, the swelling was significantly reduced for all mixed alginate gels when NaCl was added to the Ac solution. Once again, swelling was lower in the mixed gelling system compared to the alginate calcium gels (Figure 21), and the mixed leaf alginate gels was observed with the lowest swelling ratio (Figure 31). As previously mentioned, this might have been related to additional cross-links introduced by the chitosan oligomers.

After 24 hours in pH 4.5 Ac solution, the swelling ratio was 1.015 ± 0.002 for mixed leaf alginate gels and 1.094 ± 0.004 for mixed stipe alginate gels (Figure 31), which is almost equal to the original gel size before submersion. Swelling was significantly reduced compared to what was observed for the same gels in the Ac solution without salt (Figure 27), said an approximate reduction of 15% for mixed leaf alginate gels and 10% for mixed stipe alginate gels (Figure 31). A small increase of the swelling ratio was observed the gels were put in fresh solution after 24 hours (Figure 31). The NaCl caused a marginally larger impact on the swelling behaviour for the mixed gels than observed for the alginate calcium gels in the same swelling conditions (Figure 22). Similar trends were also detected at pH 5.5, said a swelling ratio of 1.026 ± 0.012 and 1.151 ± 0.003 was obtained for mixed alginate and stipe alginate gels, respectively (Figure 31). The mixed leaf alginate gels exhibited almost identical response to the additional 100 mM NaCl compared to the leaf alginate gels singularly cross-linked with chitosan oligomers (Figure 25). A relatively large difference (~30%) was detected between the two mixed gel types after 48 hours of in pH 5.5 solution, where stipe alginate expressed the highest swelling ratio (Figure 31). In the means of physical stability,

small deformations in terms of less defined cylindrical shape was observed for the mixed alginate gels, typically after 48 hours in the salt containing solution at both pH 4.5 and 5.5.

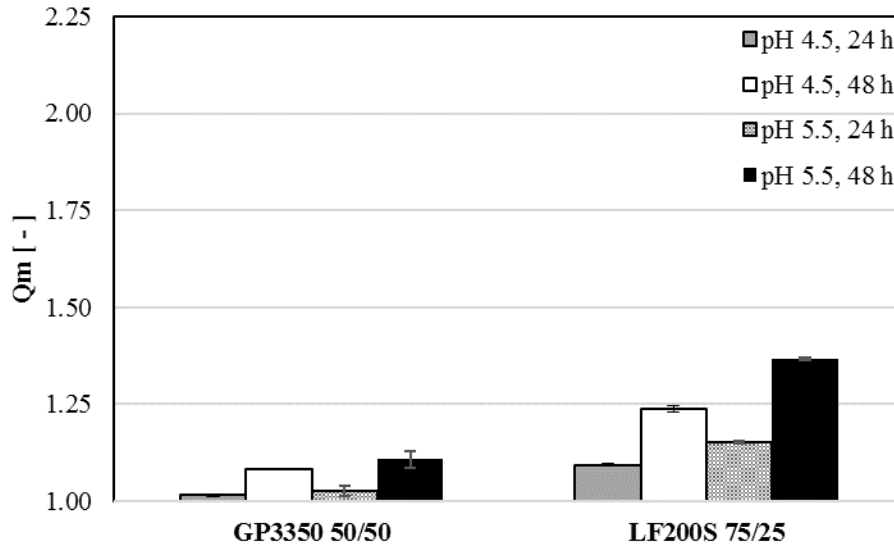


Figure 31. Swelling ratio (Q_m) for mixed leaf (GP3350 50/50) and stipe (LF200S 75/25) alginate gels cross-linked with calcium and chitosan oligomers after 24 and 48 (2x24) hours swelling in 50 mM Ac buffer solutions with pH 4.5 and 5.5. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

The decrease in swelling observed when NaCl was added to the solution at pH 4.5 and 5.5 indicates that the mixed alginate gels are most likely subjected to a similar NaCl response as the alginate calcium gels. As previously discussed for the alginate calcium gels in this thesis and in many other studies, the swelling of a polyelectrolyte in salt containing solutions is probably highly influenced by the Donnan equilibrium. Thus, the swelling behaviour of the mixed alginate gelling system in salt containing solution may also be interpreted as a result of electrostatic interactions between Na^+ counterions or an adjustment of the Donnan equilibrium potential, caused by a decrease of $\Delta\mu_{\text{ion}}$. This will be further elaborated later in this subsection.

After 24 hours in pH 7.5 tris solution with NaCl, a swelling ratio of 1.218 ± 0.007 and 1.314 ± 0.014 was observed for mixed leaf and stipe alginate gels, respectively (Figure 32). In comparison to the results obtained from swelling in pure buffer solutions, swelling was again reduced when NaCl was added, said 22.2% for mixed leaf alginate gels compared to 9.0% for the stipe alginate

gels. Similar trends were also observed for after 48 hours in pH 7.5 solution (Figure 32). As a confirmation of the previous observations from swelling of mixed gels in pH 7.5 and 8.5 solutions without salt, the swelling ratio of the mixed gels was significantly higher (~10%) compared to alginate calcium gels in solutions comparable to physiological conditions (Figure 21). Furthermore, a significant weight increase was also observed at pH 8.5, whereas a swelling ratio of 1.344 ± 0.007 was measured for mixed leaf alginate gels and 1.265 ± 0.016 for mixed stipe alginate gels (Figure 32). Thus, the effect of NaCl was also observed to induce a gel shrinking effect at pH values above the pK_a of chitosan, when comparing to swelling in pure buffer solutions.

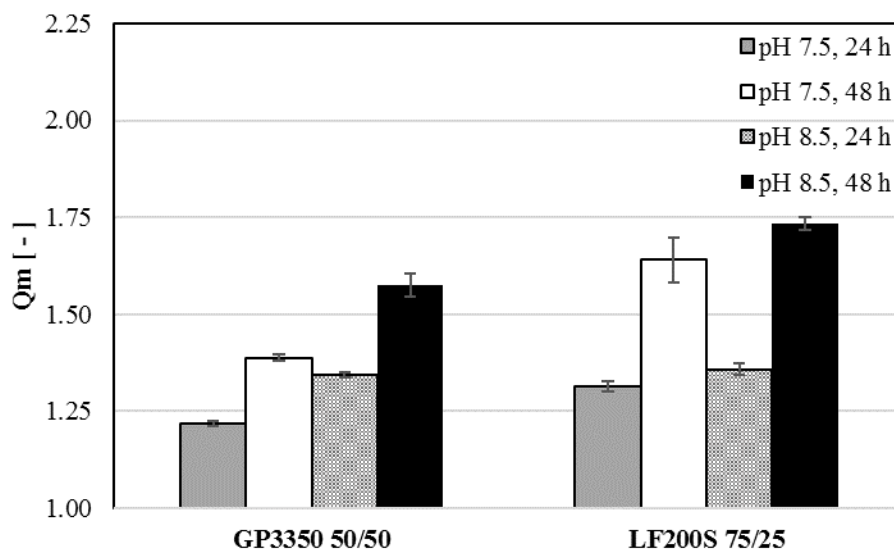


Figure 32. Swelling ratio (Q_m) for mixed leaf (GP3350 50/50) and stipe (LF200S 75/25) alginate gels cross-linked with calcium and chitosan oligomers after 24 and 48 (2x24) hours swelling in 50 mM tris buffer solutions with pH 7.5 and 8.5. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

In the means of swelling behaviour, the general trends for mixed alginate gels in salt containing solutions above the pK_a value for chitosan was highly similar to what was observed in the buffer solutions without salt at pH 7.5 and 8.5. Once again, the high swelling ratio for the mixed alginate gels indicates a reduced charge density of chitosan oligomers reduces the total number of cross-links in the mixed gels. This was also indicated by a control experiment where the calcium content in alginate calcium gels was reduced to be equal to the mixed alginate calcium gels, which will be evaluated later in this thesis.

In pH 7.5 and 8.5 solutions with added salt, a significantly decreased swelling was observed compared to salt-free solutions. The reduced swelling ratio in salt containing solutions was most likely related to the high presence of Na⁺. Na⁺ counterions tend to diffuse into the gel network where it will most likely interact electrostatically with the alginate carboxyl-groups and decrease the charge to charge repulsion and compete with cross-linking moieties. Cross-linking alginate with chitosan oligomers is a relatively new concept, and the selectivity between Na⁺ and chitosan oligomers in *L. hyperborea* is uncharacterised. Henceforth, it is difficult to predict if and how Na⁺ might affect or compete with cross-linked chitosan oligomers, and how this can be related to the present calcium ions inside the gel. Keeping in mind that the alginate chitosan oligomer gels dissolved at pH 7.5, one might speculate if Na⁺ and positively tris molecules have a more competitive effect towards chitosan oligomer formed cross-links than for calcium cross-links. The significantly lower swelling ratio observed for the mixed gels in salt containing solution compared to non-salt solution does however provide a strong indication that the Donnan equilibrium effect and the decreased $\Delta\mu_{\text{ion}}$ could have been main contributing factors in the acidic conditions.

3.4.4 Swelling of Mixed Gels as a Function of pH with and without Salt

The swelling ratios obtained from measuring the weight of mixed leaf and stipe alginate gels, after 24 hour submerged in different buffer solutions, were plotted as a function of pH to visualize the environmental influence on the swelling behaviour (Figure 33, Figure 34).

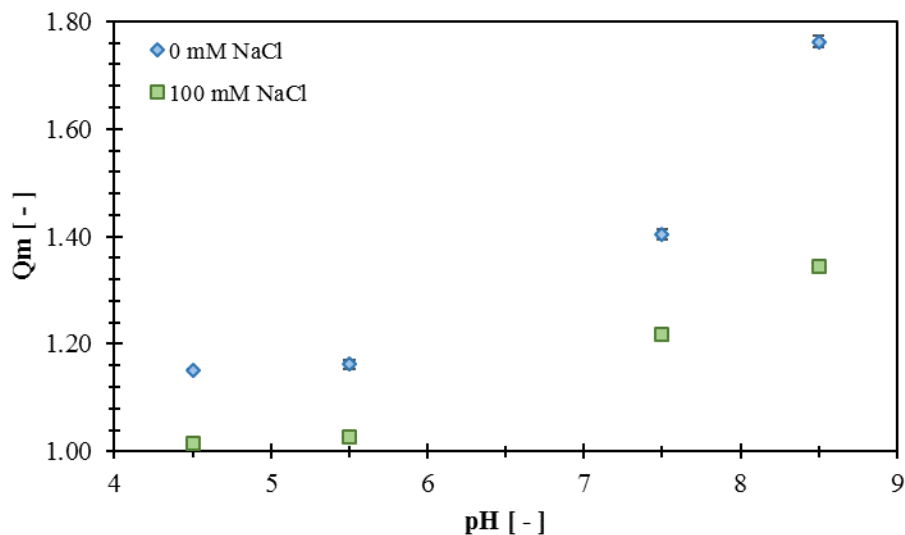


Figure 33. Swelling ratio (Q_m) of mixed leaf alginate gels in solutions with and without 100 mM NaCl as a function of pH. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

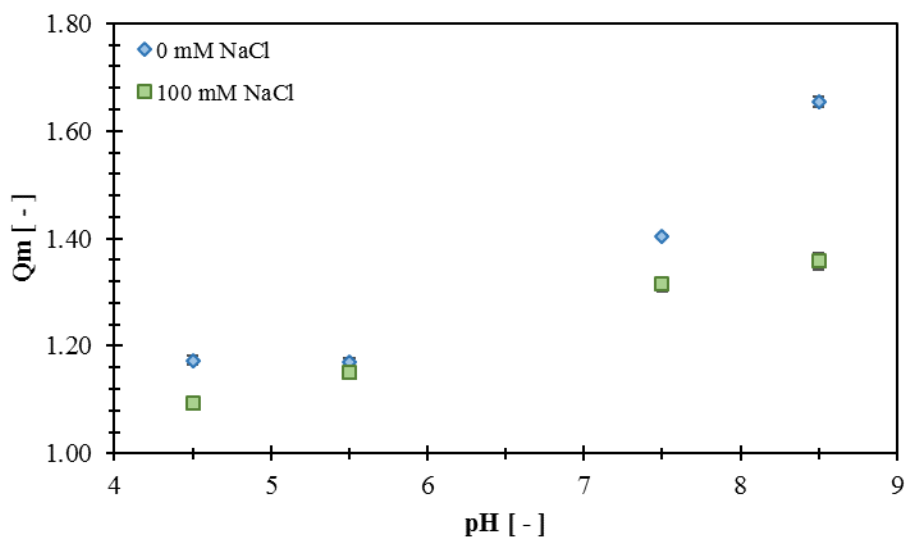


Figure 34. Swelling ratio (Q_m) of mixed stipe alginate gels in solutions with and without 100 mM NaCl as a function of pH. Three gels ($n = 3$) were swelled in each solution and the standard deviation was calculated in excel.

In contrast to the alginate calcium gels (Figure 18, Figure 19), there was a significant increase in the swelling ratio above the pK_a of chitosan that seemed to correlate with the increasing pH. This trend was observed for both mixed gelling systems after 24 and 48 hours in solutions with and

without salt, thus suggesting that the deprotonation the amino-groups may induce increased swelling due to a decreased number of cross-links within the gel. The presence of Ca^{2+} may have prevented the mixed gels from dissolving in pH above pK_a of chitosan, in contrast to leaf alginate gels singularly cross-linked with chitosan oligomers (Figure 26). These results would be very relevant in relation to biomedical applications, where a high water content is often required to simulate tissue similar properties. One might speculate if the changes in swelling behaviour exhibited by the mixed alginate gels may be applicable for drug delivery systems tailored to be stable and bypass the low pH in gastro fluids, and swell and deliver pharmaceuticals when exposed to physiological conditions (Pasparakis and Bouropoulos, 2006).

In addition, a significantly reduced swelling ratio was observed for both mixed gelling systems across the pH range of 4.5 to 8.5 when 100 mM NaCl was present in the solution (Figure 33, Figure 34). The general trend was that the salt seemed to have caused a significantly larger effect on the mixed leaf alginate gels compared to the mixed stipe alginate gels. A marginally larger impact of NaCl was also observed for the leaf alginate calcium gels compared to the stipe alginate calcium gels (Figure 18, Figure 19). Thus suggesting that the stability provided by the Ca^{2+} cross-linking with the G-blocks might produce increased resistance towards competing Na^+ or other cationic counterions and Donnan equilibrium induced effects. The large reduction of swelling ratio observed for alginate chitosan oligomer gels upon adding NaCl to the swelling solution (Figure 18, Figure 19) supports this argument, and may together with the observed behaviour for mixed alginate calcium gels indicate that the chitosan oligomer induced cross-links are more susceptible towards cationic counterions and the Donnan equilibrium effect.

3.4.5 Swelling Kinetics in Pure and Salt Containing Buffer Solutions

The weight of mixed leaf and stipe alginate gels was measured at several specific times (0.5, 1.5, 2.5 and 5 hours) after submersion in buffer solutions with and without salt across the pH range from 4.5 to 8.5 in order to study the fluid penetration kinetics, or swelling kinetics. In pure buffer solutions, there were significantly different kinetic profiles in the various solutions for the mixed alginate gels (Figure 35, Figure 36). Similar to the alginate calcium gels, both mixed gelling systems exhibited rather slow swelling profile, indicating a Fickian behaviour, in acidic conditions. In both mixed systems, there was marginally faster swelling in when the pH was decreased from

5.5 to 4.5, most likely do to the lower ionic strength at pH 4.5 which consequently increases the osmotic driving force. Only minor differences were observed between the mixed leaf and stipe alginate gels, said stipe alginate gels expressed a marginally faster swelling rate in pH 4.5 solution.

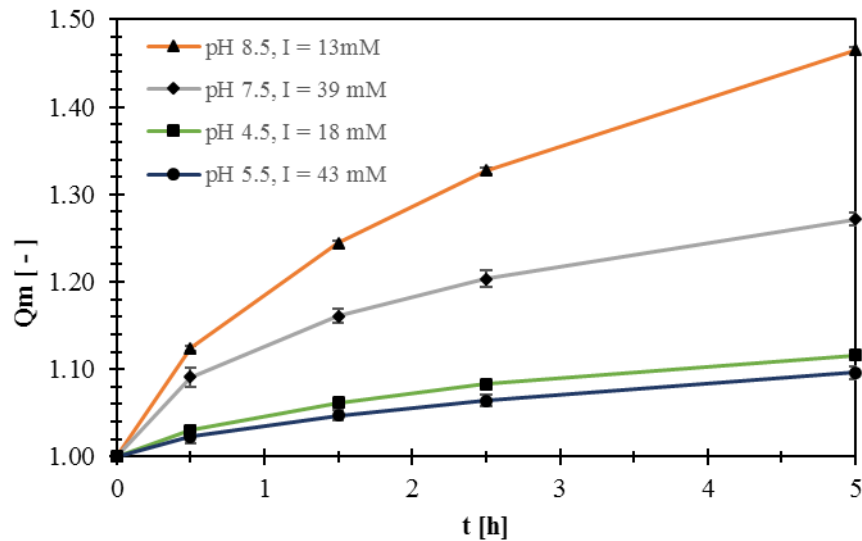


Figure 35. Swelling kinetics of leaf alginate gels cross-linked with calcium and chitosan oligomers in 50 mM buffer solutions of different pH. Swelling ratio (Q_m) is plotted as a function of time (t) after the gels were submerged in the solution. An acetate buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non/Fickian diffusion.

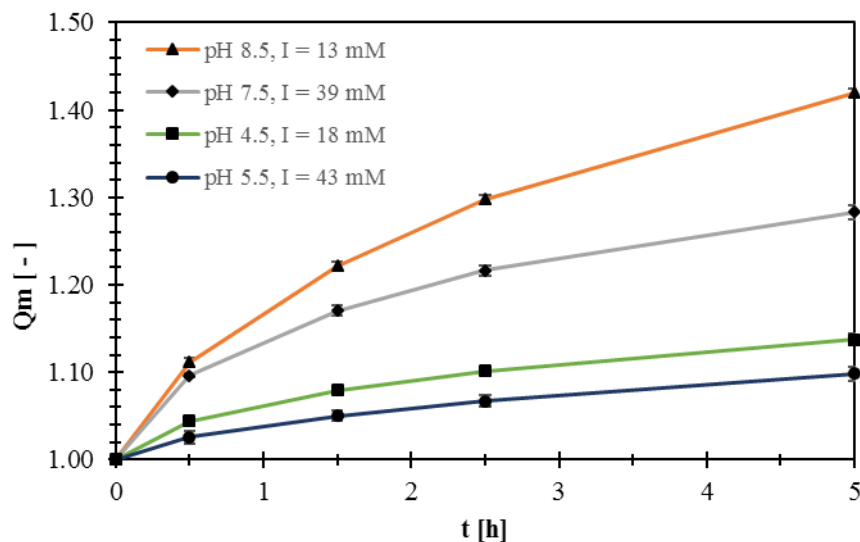


Figure 36. Swelling kinetics of stipe alginate gels cross-linked with calcium and chitosan oligomers in 50 mM buffer solutions of different pH. Swelling ratio (Q_m) is plotted as a function of time after the gels were submerged in the solution. An acetate buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non/Fickian diffusion.

When the pH was increased above the pK_a of chitosan, a significantly steeper swelling profile was observed for both mixed gel types, indicating a more non-Fickian or anomalous behaviour of the gels. The same trend was observed for the alginate calcium gels (Figure 23), however a significantly larger response and faster swelling rate was exhibited by the mixed alginate gels at pH 7.5 and 8.5. The mixed stipe alginate gels were observed with a marginally faster swelling rate at pH 7.5 compared to the mixed leaf alginate gels, however the opposite result was seen at pH 8.5. The significantly different swelling rate observed at pH 7.5 and 8.5 is most likely due to the difference in ionic strength of the buffer solutions. The difference in swelling kinetics rate observed at pH above the pK_a of chitosan was expected for the mixed alginate gels. When pH of the solution surrounding the gel is above the pK_a of chitosan, a deprotonation of the amino-groups would cause dissociation of chitosan oligomers. Dissociation of chitosan oligomers would significantly reduce the total amount of counterions associating with the fully charged alginate, which would consequently increase the charge to charge repulsion between the carboxyl-group and eventually cause extension of the alginate chains. Extended chains generate a more rod like behaviour of the polymer, which results in a stiffer and more rigid gel structure that is more easily penetrated by

fluids. Hence, the faster swelling kinetics observed for the mixed alginate gels compared to the calcium alginate gels represents an indication that the chitosan oligomers are dissociating at pH 7.5 and pH 8.5. The higher swelling rate at pH 8.5 compared to 7.5 may be attributed to a decrease of the ionic strength of the buffer solution or the lower charge density of the chitosan oligomers.

When NaCl was added to the buffer solutions, the swelling rate of the mixed gels was significantly decreased (Figure 37, Figure 38), which is similar to the observations made for the alginate calcium gels (Figure 24). This was also as expected due to the increased concentration of Na⁺ counterions, which most likely would have associated with the carboxyl-groups on alginate and thus decreased the electrostatic repulsion. Decreased electrostatic repulsion induces a more random coil distribution of alginate, consequently forming a denser and rubberier network that takes more time for the fluid to penetrate. Thus, the NaCl enhances the opposite response of what is the case when pH is increased for the mixed gels. Only minor differences were observed in acidic conditions and at pH 7.5 and 8.5. A higher swelling rate and more non-Fickian behaviour was monitored for the mixed gels at high pH, which again may be the effect of the cationic tris buffer or the reduced cross-link density due to the decreased charge density of the oligomers (Figure 37, Figure 38).

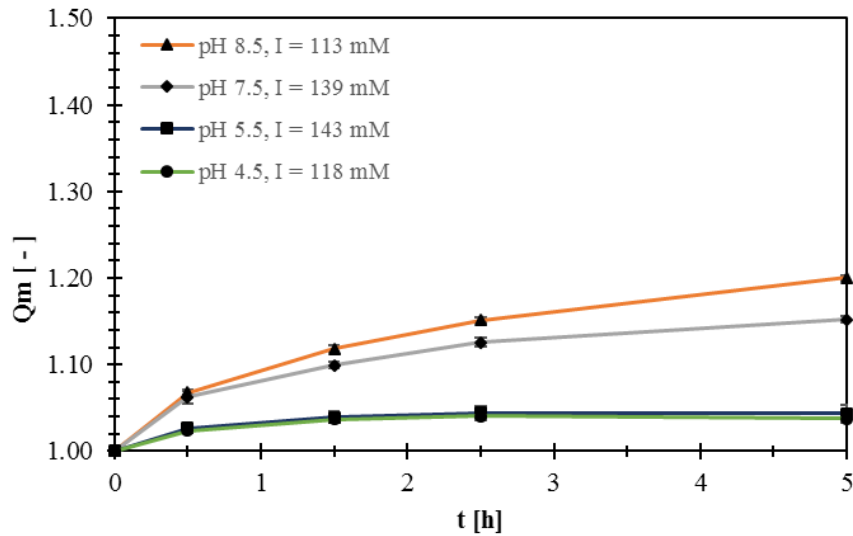


Figure 37. Swelling kinetics of leaf alginate gels cross-linked with calcium and chitosan oligomers in 50 mM buffer solutions of different pH and 100 mM NaCl. Swelling ratio (Q_m) is plotted as a function of time after the gels were submerged in the solution. An acetate buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non/Fickian diffusion.

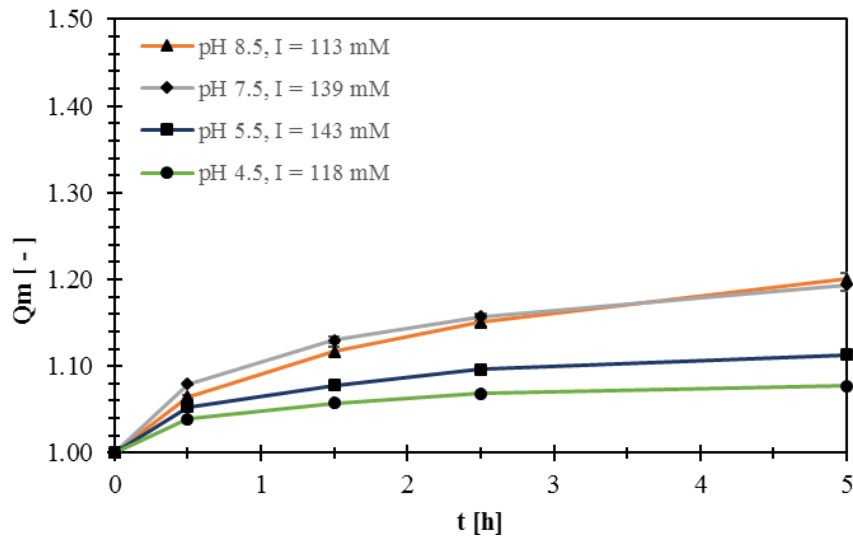


Figure 38. Swelling kinetics of stipe alginate gels cross-linked with calcium and chitosan oligomers in 50 mM buffer solutions of different pH and 100 mM NaCl. Swelling ratio (Q_m) is plotted as a function of time after the gels were submerged in the solution. An acetate buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non-Fickian diffusion

There were only small differences between the different gelling systems when NaCl was present in the solution. The mixed leaf alginate gels exhibited marginally slower swelling compared to the alginate calcium gels in acidic conditions, indicating a slightly rubberier network. One might speculate if this can be attributed to an increased number of cations associating with the alginate chain, however it is difficult to relate this to the mixed gels without knowing more details regarding the association mechanism between chitosan oligomers and alginate e.g. the alginate selectivity of chitosan versus sodium. However, it is evident that the mixed alginate gels exhibit a more Fickian like behaviour in NaCl containing solutions or in acidic conditions, and a more non-Fickian or anomalous behaviour when pH was increased above the pK_a of chitosan.

3.5 Control Experiments

3.5.1 Acetate and Tris Influence on Swelling of Alginate Gels

To investigate if either of the different buffer systems had a swelling inducing effect, a control study was performed with leaf and stipe alginate calcium gels (Figure 39). The alginate calcium gels were submerged in four different solutions, said two Ac solutions with pH 5.5 and 7.5, and two tris solutions with pH 5.5 and 7.5. The experiment was performed with 100 mM NaCl in each solution to minimize the effect of the ionic strength of the solution. Thus, the only two variables between the different solutions: first, the buffer component as in tris or Ac. Secondly, the presence of a buffering system as the tris pH 5.5 and Ac pH 7.5 was outside the buffer range. The results obtained from swelling of leaf alginate calcium gel is given in Appendix E.

There was no significant difference in the swelling ratio between a buffering system or a non-buffering system, both after 24 and 48 hours (Figure 39). However, a relatively small but however significant difference of the swelling ratio was observed between the Ac solutions and the tris solution, said approximately 5% after 24 hours and 15% after 48 hours. Conclusively, the tris buffer was observed to induce a marginally higher swelling ratio for calcium alginate gels. Thus, one cannot exclude that the increased swelling ratio observed for both mixed alginate gels and calcium alginate gels might to some extent have been enhanced by the tris buffer system at pH 7.5 and 8.5. Nevertheless, this effect does not likely explain the entire increase observed for the mixed alginate gels. The reasons for the enhanced swelling response may be attributed to the additional cations that might associate with the alginate chain and compete with cationic cross-linkers. This

experiment was not performed with the mixed gels due to the requirement of a buffering system to prevent the pH from approaching the pK_a of chitosan, which may conceal other influencing effects.

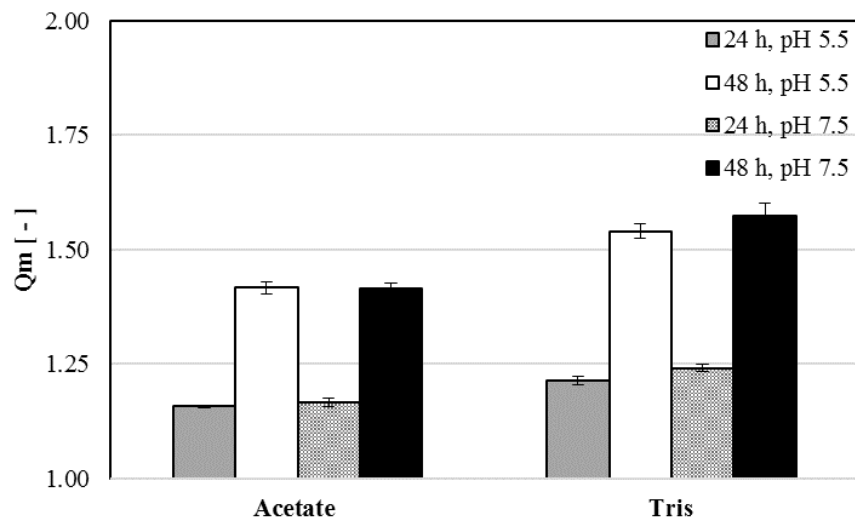


Figure 39. Swelling ratio (Q_m) of stipe alginate calcium gels after 24 and 48 (2x24) in 50 mM Ac and tris solutions of pH 5.5 and 7.5.

3.5.2 Swelling of Alginate Calcium Gels with Reduced Calcium Content

A control experiment was performed with alginate calcium gels where the calcium content was equal to what was in the mixed gels. This was done in order to demonstrate the cross-linking effect of chitosan oligomers. Leaf and stipe alginate calcium gels with a calcium amount equal to the what was in mixed leaf and stipe alginate gels where swollen for 24 in pH 7.5 tris solutions with and without 100 mM NaCl. Leaf alginate calcium gels with 50% reduced calcium concentration are called GP3350 50/0 and similarly, stipe alginate calcium gels with 25% reduced calcium concentration were called LF200S 75/0.

As expected, a significantly higher swelling ratio was obtained for the reduced calcium alginate gels compared to the other gelling systems, both in solutions with and without added NaCl (Figure 40, Figure 41). A swelling ratio of 1.821 ± 0.019 and 1.473 ± 0.034 was measured for GP3350 50/0 gels after 24 hours in salt and non-salt solutions, respectively (Figure 40). The results in solution without salt represents an increase of 41.7% and 55.6% of the swelling ratio relative to the mixed leaf alginate and leaf alginate calcium gelling system. Similarly, the swelling ratio was increased

by 25.5% and 30.7% when 100 mM NaCl was present to the solution. The remarkable increase in swelling suggest that the total number of cross-links is significantly reduced, which implies that the presence of chitosan oligomers in the mixed gels provides a significant contribution to the number of cross-links in the gel.

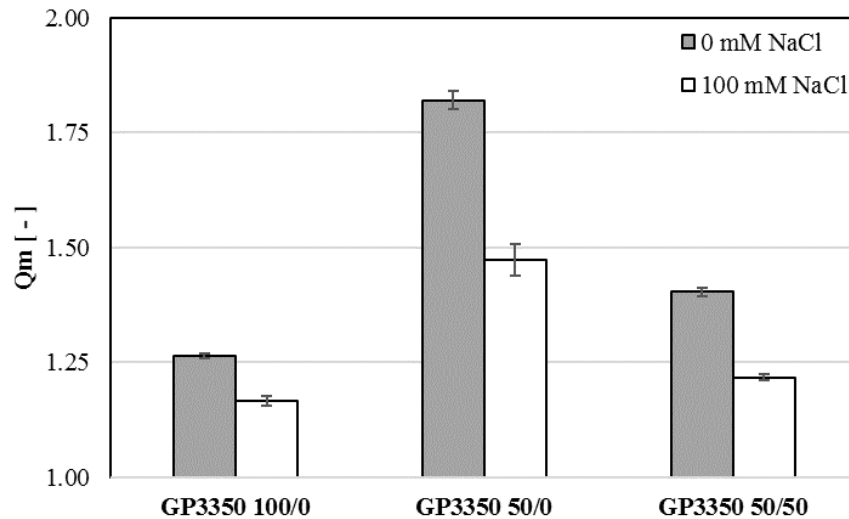


Figure 40. Swelling ratio (Q_m) of leaf alginate calcium gels, with a calcium concentration of 15.1 mM (GP3350 100/0) and 7.5 mM (GP3350 50/0), and mixed leaf alginate gels (GP3350 50/50). The gels were swollen for 24 hours in pH 7.5 tris buffer with and without 100 mM NaCl.

For the LF200S 75/0 gels, a swelling ratio of 1.653 ± 0.008 and 1.405 ± 0.027 was observed after 24 hours in salt and non-salt solutions, respectively (Figure 41). In pure buffer solution, swelling increased by 24.8% and 34.4% compared to the mixed leaf alginate and leaf alginate calcium gelling system. In 100 mM NaCl solutions, swelling was increased by 12.9% and 21.5% compared to the mixed leaf alginate and leaf alginate calcium gels.

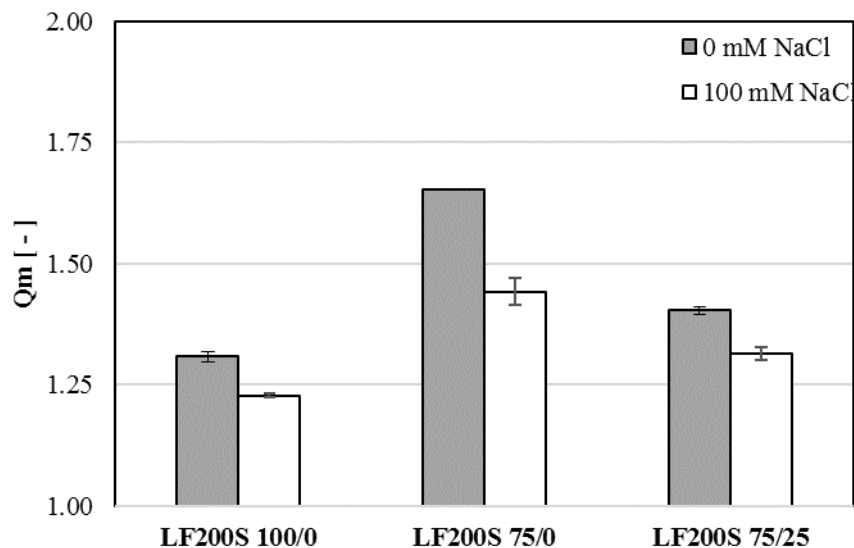


Figure 41. Swelling ratio (Q_m) of stipe alginate calcium gels, with a calcium concentration of 16.6 mM (LF200S 100/0) and 12.4 mM (LF200S 75/0), and mixed stipe alginate gels (LF200S 75/25). The gels were swollen for 24 hours in pH 7.5 tris buffer with and without 100 mM NaCl.

The increased swelling observed upon decreasing the calcium concentration in the gel was as expected, and was most likely related to the degree of cross-linking. A similar phenomenon was also observed by Kuo et al. upon decreasing the internal concentration of calcium in small alginate cylinders (Kuo and Ma, 2008). When the number of cross-linking agents is significantly reduced, the elastic retractive force opposing the swelling pressure is decreased and consequently, the gel network allows a larger quantity of fluid to enter the gel. The most interesting aspect was that these results makes it easier to observe the direct effect of adding chitosan oligomers. In the means of material composites, the only difference between the mixed gels and the gels with reduced calcium content is the presence of chitosan oligomers. Henceforth, introducing chitosan oligomers in alginate calcium gels appears to significantly reduce swelling even in an environment where a majority of the oligomers in theory are uncharged. These results thus represent another indication that the chitosan oligomers are interacting with the alginate chains and forming additional cross-links.

3.6 Size Exclusion Chromatography Analysis of Alginate Chitosan Oligomer Gel Swelling Fractions

It has previously been suggested that the strength of an interaction between poly-M and chitosan oligomers increase with the length of the chain of the oligomer (Khong, 2013). In order to possibly experimentally support this hypothesis, an experiment was conducted investigating the length of chitosan oligomers leaking out of the gel, in swelling sol fractions. Swelling fractions were obtained after swelling leaf alginate chitosan oligomer gels at different times in a pH 5.5 Ac buffer with and without 100 mM NaCl. Six different swelling fractions were analysed by a SEC RI system, said after 5, 24 and 2x24 hours in solutions with and without salt (Table 6). The fractions were obtained by removing the swollen gel, concentrating the remaining swelling solution by freeze drying and resolving in a smaller volume of water before analysis by SEC. Leaf alginate gels cross-linked with chitosan oligomers were chosen in this experiment. pH 5.5 was chosen to make sure both chitosan and alginate were charged and soluble. A different SEC RI system was utilized than in the material characterization of the chitosan oligomer mixture where a pH 4.5 150 mM AmAc mobile phase, which is usually used for separation of chitosan/chitin oligomers, was used (Figure 17). Instead, a SEC RI system with a pH 6.9 150 mM AmAc was used to avoid alginate induced interactions within the column. At pH 6.9, the chitosan oligomers are less charged and should not interact with alginate which is fully charged and soluble.

The chitosan oligomer mixture that was used as cross-linking substance, was characterized on the SEC RI system using the same pH 6.9 mobile phase to be able to identify different chitosan oligomers in the swelling fractions by the elution time (Figure 42). As can be observed by comparing two chromatograms of the chitosan oligomer mixture (Figure 17, Figure 42), the sensitivity and separation was different for the two SEC RI systems, due to the different pH of the mobile phases and the use of different RI detectors. However, the ratio of the relative amount between the oligomer was similar (Table 5).

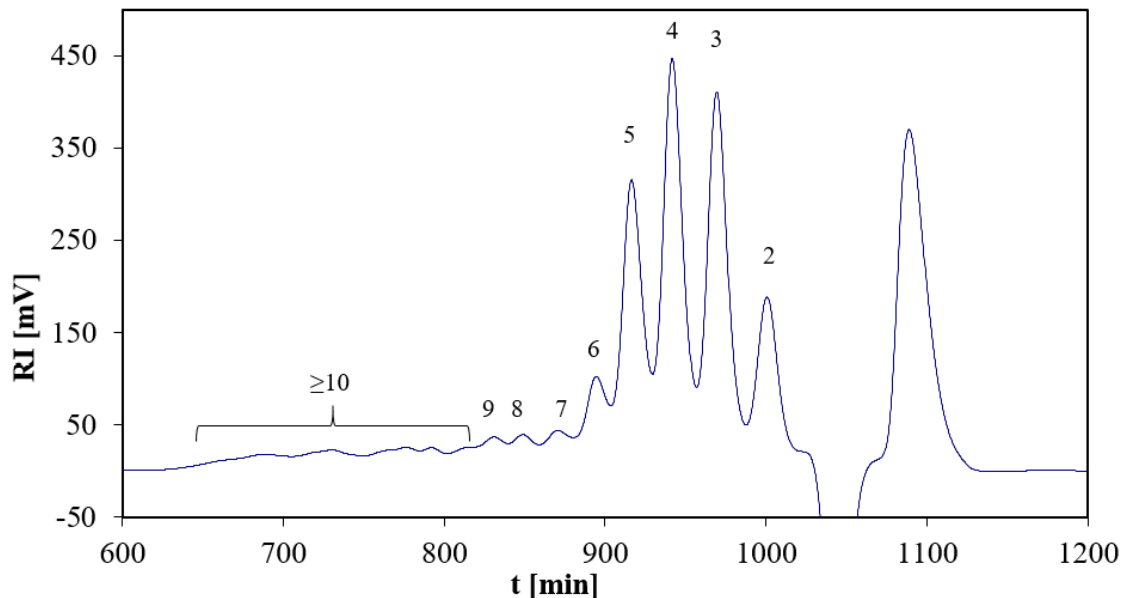


Figure 42. SEC RI analysis of chitosan oligomer mixture, used in gel preparations, dissolved in MQ upon using a pH 6.9 AmAc mobile phase. The refractive index (RI) equivalent to the relative amount of each oligomer was plotted as a function of elution time (t). Nine different oligomers were detected, ranging from the dimer (DP = 2) to the ninemere (DP = 9). The drop in the baseline represents the water front and the peak with the longest elution time represents the salt peak.

The results from the SEC analysis of the six different leaf alginate chitosan oligomer gel swelling fractions revealed the presence of low DP oligomers in all the samples (Table 3). This observation was as expected as oligomers that do not bind or form rather weak interactions with alginate will eventually diffuse out of the gel and into the surrounding solution. Previously studies have observed decreasing gel strength in poly-M chitosan oligomer gels with increasing chain length of the cross-linking oligomer, suggesting stronger interaction of the high DP oligomers with alginate (Khong, 2013). Studying the chemical conformation of poly-M and poly-D chains (Figure 8), it is more likely that low DP oligomers that interacts through fewer charged amino groups, will be released into the surrounding solution before the longer DP oligomers.

Only shorter oligomers ($DP \leq 6$) were detectable in the swelling fractions (Figure 43, Figure 44), which indicates that the higher DP oligomers were retained in the gel. The fully de-N-acetylated dimer (DP = 2) was partially overlapping with the large salt peak (Figure 43, Figure 44). The void peak observed in all chromatograms was most likely unbound alginate which had a significantly higher molecular weight (Figure 43, Figure 44). As for the chitosan oligomers, Alginate fractions

that do not crosslink will tend to leak out of the gel. One might speculate if the structural composition of the alginate would favour G-rich alginate chains that may have weaker interactions with the oligomers, however this was not further pursued in this thesis. The other chromatograms are shown in Appendix D.

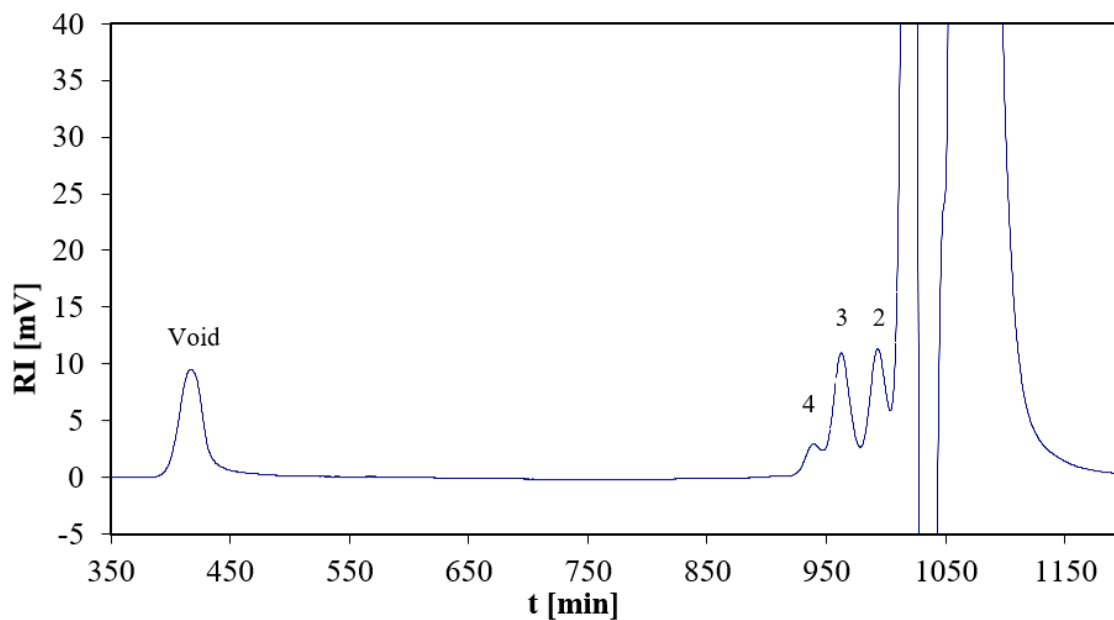


Figure 43. Separation of 24 hour swelling fractions obtained from swelling solution of leaf alginate gels cross-linked with chitosan oligomers. The gels were swelled in pH 5.5 50 mM acetate buffer solution before the gel was removed. The void peak represents alginate polymers in solution. Just before the salt peak, which is overlapped by the water front, the chitosan oligomer dimer (DP = 2), trimer (DP = 3) and tetramer (DP = 4) were detected, whereas the dimer and the tetramer are most abundant.

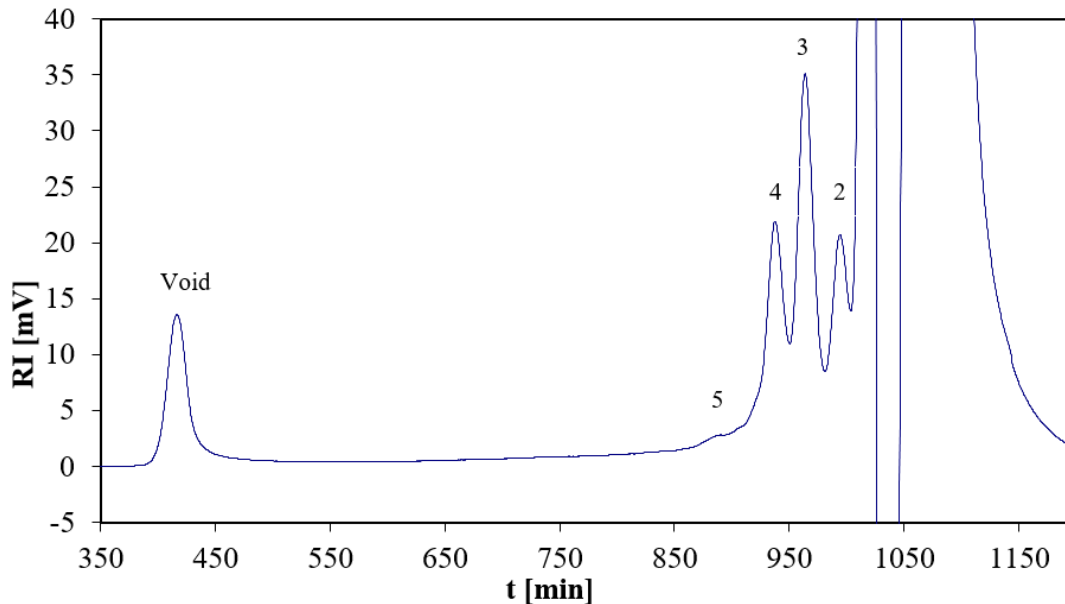


Figure 44. Separation of 24 hour swelling fractions with 100 mM NaCl obtained from swelling solutions of leaf alginate gels cross-linked with chitosan oligomers. The gels were swelled in pH 5.5 50 mM acetate buffer solution before the gel was removed. Just before the salt peak, the chitosan oligomer dimer (DP = 2), trimer (DP = 3) and tetramer (DP = 4) and the pentamer (DP = 5) were detected whereas the tetramer is the most abundant.

To further investigate the effect of salt and kinetics of the leaking oligomers from the leaf alginate gel, the ratio of the area under each peak for the different was compared (Table 6). The area under each peak was calculated by integration in excel. Since the salt peak was overlapping with the dimer and the shortest, henceforth the area under the peak for the trimer was used as an internal reference. Thus the assumption is made that the trimer will not have a specific interaction with the leaf alginate, and the amount of the oligomers leaking out of the gel with a DP higher than three will be compared with the trimer. Thus, in table 4 the ratio of the area of the given oligomers divided by the area of the trimer is given. The shortest oligomers were also most likely to leak out of the gel and exhibit the lowest variation in terms of area, and were therefore well suited as a reference peak.

The intention was also to investigate the leaf alginate gels cross-linked with chitosan oligomers at a pH above the pK_a value of chitosan. However as previously mentioned, these gels dissolved in at pH 7.5, meaning all the chitosan oligomers and alginate would be present in the swelling fractions. With a high concentration of fully charged alginate and partially charge chitosan oligomer

represented in the fraction samples, one would risk gelation in the column, which would damage the columns. Hence, these swelling fractions was not further investigated.

Table 6. Qualitative oligomer ratios of chitosan oligomers with low degree of polymerisation (DP) obtained from SEC analysis of various swelling fractions. The ratio of each oligomer is relative to chitosan trimer (DP = 3). The samples consist of a chitosan oligomer mixture and leaf alginate chitosan oligomer gel swelling fractions. The relative ratio between trimer, tetramer (DP = 4), pentamer (DP = 5) and higher oligomers (DP \geq 6) was obtained from SEC of chitosan oligomer mixture and gel swelling fraction with and without NaCl at different times in solution.

Sample	t	C _{NaCl}	DP			
			3	4	5	6 \leq
CO mixture	0 h	0 mM	1.0	1.05	0.75	1.08
GP3350 0/100	5 h	0 mM	1.0	0.32	0.11	0.21
GP3350 0/100	24 h	0 mM	1.0	0.25	0.00	0.00
GP3350 0/100	(24 h) ^{2nd}	0 mM	1.0	0.44	0.23	0.27
GP3350 0/100	5 h	100 mM	1.0	0.58	0.08	0.69
GP3350 0/100	24 h	100 mM	1.0	0.73	0.33	0.70
GP3350 0/100	(24 h) ^{2nd}	100 mM	1.0	1.21	0.33	0.61

For the swelling fractions without salt, the ratios for the chitosan tetramer, pentamer and oligomers equal or longer than the hexamer, was significantly lower compared to what was estimated for the oligomer mixture (Table 6), indicating that more of the oligomers with DP > 3 is diffusing out with time. Furthermore, the ratio was increasing from the first swelling solution (5 h, 24 h) to the second solution. This was expected as most of the shorter oligomers will diffuse out and exhibit a high presence in the first solution. When the gel was transferred to a new fresh solution, equilibrium is shifted again over the next 24 hours, inducing further swelling of the gels which forces some of the longer oligomers to leak out. These trends were also observed in the 100 mM NaCl containing swelling fractions, however the differences were not very clear. This may be attributed to the presence of Na⁺, which will compete against the cross-linked chitosan oligomers and hence increasing the amount of longer chitosan oligomers in the swelling solution. Conclusively, the results obtained from SEC analysis of oligomer fractions indicates that chitosan oligomers form cross-links in leaf alginate gels, and that the longer oligomers have stronger interaction with the

alginate chains compared to the shorter oligomers, said in pH 5.5 acetate solutions with and without NaCl. These results are in line with the results obtained by Khong and co-workers, where an increasing gel strength of poly-M observed with increasing length of the oligomers (Khong, 2013).

At the very end of this project, a SEC analysis of swelling fractions obtained from swelling mixed leaf alginate gels at a pH above and below the pK_a of chitosan was performed. The results, which are not presented in this thesis. However, this results indicated that there was a difference in the ratio between oligomers in relations to chain length between the pH 4.5 and 8.5. At low pH, the similar trends were observed as for the leaf alginate chitosan oligomer gels, said that longer oligomers were retained in the gel. At higher pH, there seemed to be a larger presence of longer oligomers, indicating that the longer oligomers are retained because of their interaction with alginate and not due to slower diffusion caused by their larger size. It would be interesting to pursue these findings in future experiments.

4 Future Prospects

In this project we have qualitatively demonstrated that there are differences in the means of swelling behaviour between leaf and stipe alginate gels with mixed cross-linkers and leaf and stipe alginate calcium gels. This was done by conducting a series of different empirical swelling experiments.

It would be interesting to further investigate the swelling behaviour of the mixed gelling system and provide a more quantitative characterization of the swelling properties for these gels. One option would be to perform more swelling experiments in similar conditions, however with more frequent measurements in relation to the kinetics, a longer time scale for the equilibrium swelling measurements and a larger variation of swelling solutions in terms of increased pH and ionic strength range. Moreover, well established theoretical models could be applied to the swelling data to provide more quantitative descriptions of the swelling behaviour, which could be related to structural properties of the gel with less divergence. One example of a theoretical model that could easily be fitted towards the kinetic data obtained in this work is Higuchi's based models, which was used by Karadag and co-workers. There, the diffusional exponent, n , can be estimated by plotting swelling kinetics data in a log-log plot, and determine the slope of the curve (Karadag and Saraydin, 2002). However, several more measurements are required to perform quantitative predictions of the swelling kinetics which will only describe the swelling behaviour in a particular environment.

If the equilibrium swelling data is combined with mechanical measurements of the swollen gels, it would provide more detailed description regarding the cross-link density of the mixed gels in various chemical environments. A method where that accounts for the irregular shape of the swollen cylinder is required in order to perform such measurements. The gel strength and the swelling ratio of a swollen gel have previously been used to apply established swelling equilibrium models to estimate the cross-link density (Moe et al., 1993). However, this is often done when the average charge distance of the polymer is provided, which might be challenging for the mixed gels that possess two different cross-linkers.

Investigating the size distribution of chitosan oligomers that are leaking out of the gel by SEC could be done quantitatively by adding a defined amount of internal oligomer standards to determine the accurate mass of each oligomer. Quantitative analysis of the oligomer fractions was performed experimentally for some samples in the very end of this project, but there was not sufficient time to pursue these findings. Improved separation of the oligomers could most likely be achieved by collecting SEC fractions across the elution range of the chitosan oligomers. In collected fractions, impurities such as the void and most of the salt would be removed, and the samples could be concentrated and applied on the columns a second time. A second SEC run with a mobile phase within the solubility range of chitosan most likely improve the quality of the chromatograms sufficient enough to conduct a quantitative analysis of the size-distribution. Furthermore, collecting the void enables the possibility to investigate whether alginate is present by performing a NMR characterization of a concentrated and hydrolysed sample. An NMR characterization would also provide the chemical composition relative to M and G frequencies and average block lengths of the alginate. Such results would be interesting in terms of investigate the characteristics for alginates that leak out of the gel and thus have weaker interactions with the chitosan oligomers.

In the means of applying the mixed alginate gelling system in future applications, it would be interesting to prepare mixed alginate calcium and chitosan oligomer beads and compare swelling results with previously performed swelling experiments of alginate beads. The mixed gel system was observed to be relatively stable up to at least 48 hours in 50 mM buffer solutions across the pH range of 4.5-8.5. This demonstrates a potential use in applications in relation to aqueous environments of similar ionic strengths and pH. Chitosan oligomers have previously been shown to induce antibacterial effects (Vårum and Smidsrød, 2005), and combined with the high water content and biocompatibility of alginate gels, the mixed gelling system represents an interesting material for biomedical applications e.g. as immobilisation matrices. The low swelling ratio observed in acidic condition and high swelling capacity in around physiological conditions may be applicable for drug delivery systems tailored to be stable and bypass the low pH in gastro fluids, and swell and deliver pharmaceuticals when exposed to physiological conditions. The mixed gelling system also provide an alternative to reduce the calcium content of alginate gels without interfering with the gel strength, said in dry state. Nevertheless, before applying the system, further characterization of the properties of these gels is recommended.

5 Conclusion

Throughout this thesis, the swelling behaviour of *L. hyperborea* leaf and stipe alginate cross-linked with mixtures of calcium and chitosan oligomers was studied and compared towards *L. hyperborea* leaf and stipe alginate calcium gels. The swelling experiments demonstrated that the swelling behaviour of mixed alginate gels was influenced by the pH and ionic strength of the swelling solution and varied from the swelling behaviour of the alginate gels only cross-linked calcium, despite the similar mechanical properties that both of these gels have in dry state.

In pH 4.5 and 5.5 solutions with and without salt the alginate gels cross-linked with chitosan and calcium were swelling marginally less than the once cross-linked with only calcium, whereas at a higher pH of 7.5 and 8.5 (well above the pK_a -value of chitosan) the gels with mixed crosslinkers are swelling significantly more than the calcium alginate gels. The increased swelling observed in solutions above the pK_a of chitosan was most likely attributed to a decreased cross-link density between alginate and chitosan oligomers. A control study investigating swelling of leaf and alginate calcium gels with an equal amount of calcium as corresponding leaf and stipe mixed gels, also indicated that the introduction of chitosan oligomers to alginate calcium gels most likely increased the total number of cross-links, even at pH well above the pK_a of chitosan.

The swelling kinetics showed a slower swelling for the mixed gels in the lower pH than in the higher pH. At lower pH alginate and chitosan are (almost) fully charged, producing cross-links that increase the elastic retractive force and oppose the swelling pressure. The swelling kinetics were similar to the alginate calcium gels in these conditions, however a marginally lower final swelling ratio was detected, which may indicate a higher cross-link density in the mixed gels.

At higher pH the mixed alginate gelling system was swelling significantly faster and had a higher swelling capacity compared to the conventional alginate calcium gels in pH 7.5 and 8.5 buffer solutions, both with and without added salt. This non-Fickian swelling behaviour can be explained by the lower number of cross-links and probably by the higher repulsive forces within the alginate chain when the chitosan oligomers are losing their charge.

As expected for an ionic gel, swelling of the mixed gels was significantly reduced and decelerated when the ionic strength of the solution was increased by either the buffer protonation degree or the addition of salt. This phenomenon was most likely attributed to a reduced osmotic swelling pressure due to a shift of the Donnan equilibrium when the difference of mobile ions inside and outside the gel was reduced. An increased deformation of the physical appearance of both mixed and alginate calcium gels was observed when NaCl was present in the solution. This might be related to Na⁺ counterions that are competing with the cationic cross-binders and associating with the alginate chain causing a more random coil behaviour, henceforth slowly dissolving the gel.

Lastly, a qualitative SEC analysis of swelling fractions, retrieved from the pH 5.5 acetate swelling solutions of *L. hyperborea* leaf alginate gels cross-linked with chitosan oligomers, showed that chitosan oligomers leak out of the gel in higher quantity and the faster the smaller the DP is. This effect is especially pronounced (relatively) at lower pH when both chitosan and alginate is charged, indicated that shorter chitosan oligomers have reduced affinity towards M-rich alginate than longer chitosan oligomer chains.

In addition, a higher presence of longer oligomers in the swelling fractions was observed when NaCl was present in the solution, suggesting that Na⁺ counterions are competing with chitosan oligomers that are cross-linked in the gel, similar to the competing behaviour observed in calcium alginate gels.

Appendix A – NMR Spectra of *L. hyperborea* Leaf and Stipe Alginate

^1H and ^{13}C NMR spectroscopy was used to characterize *L. hyperborea* leaf (GP3350) and stipe (LF200S) alginate samples and determine the chemical composition in the means of β -D-Mannuronate (M) and α -L-Guluronate (G) residue sequences. Grasdalen's ^1H and ^{13}C NMR methods were used to interpret the different spectra through integration of peaks characteristic for different M and G monad, diad and triad sequences (Grasdalen, 1983, Grasdalen et al., 1981). ^{13}C NMR spectroscopy was used to characterize the leaf alginate sample (Figure 1) and ^1H and ^{13}C NMR spectroscopy was used to characterize stipe alginate sample (Figure 2, Figure 3).

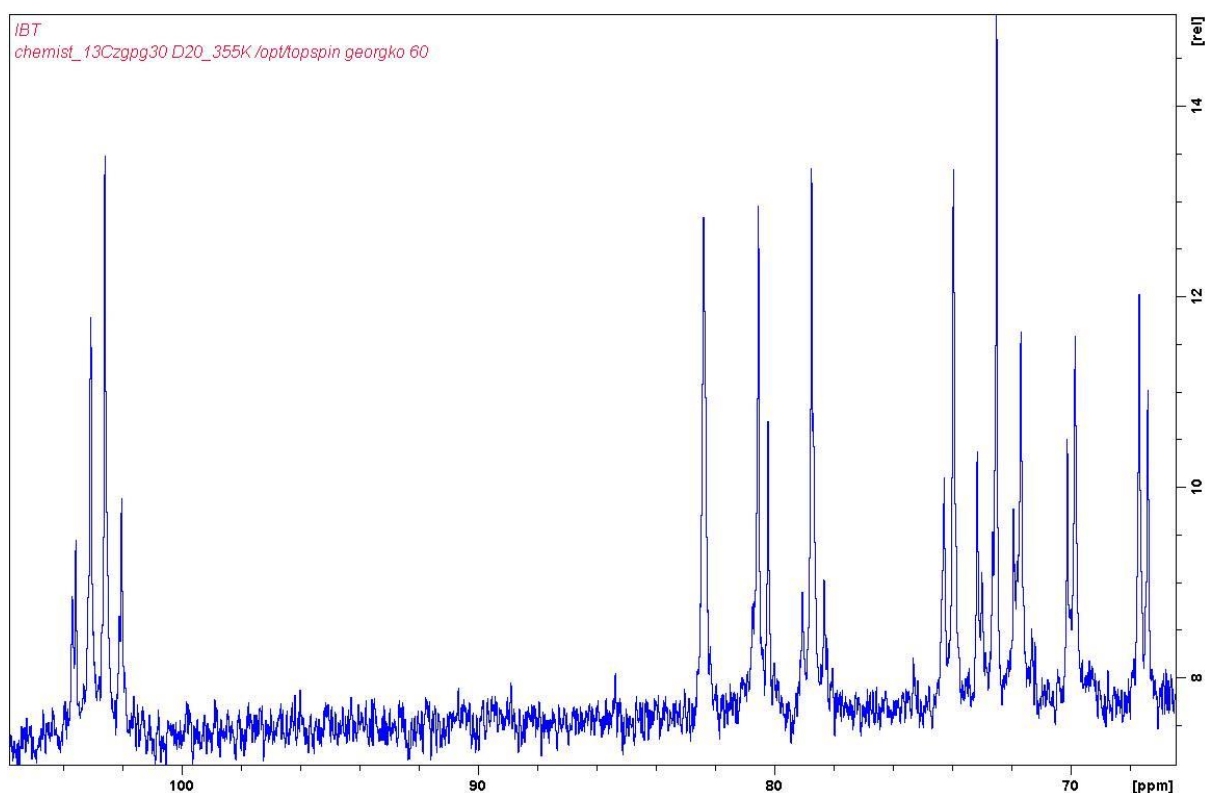


Figure 1. ^{13}C NMR spectra of hydrolysed *L. hyperborea* leaf alginate, showing chemical shift (ppm) of M and G sequences.

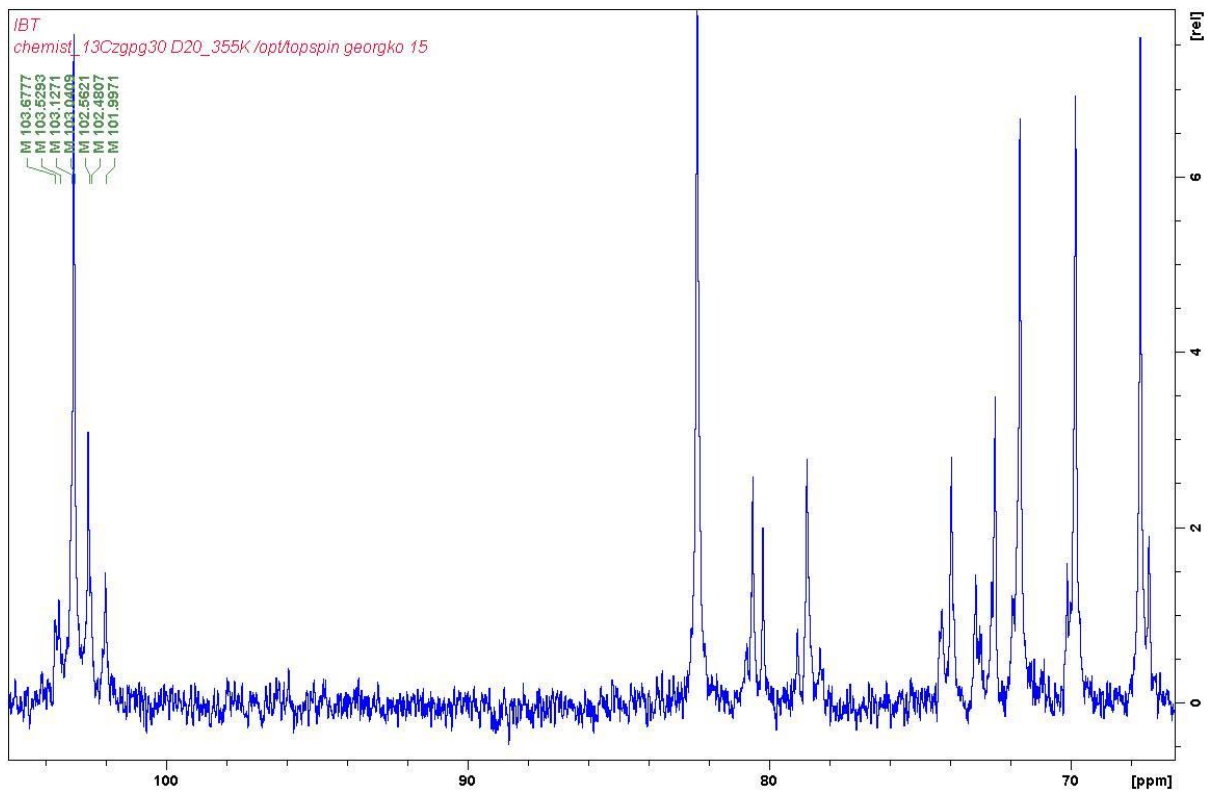


Figure 2. ^{13}C NMR spectra of hydrolysed *L.hyperborea* stipe alginate, showing chemical shift (ppm) of M and G sequences.

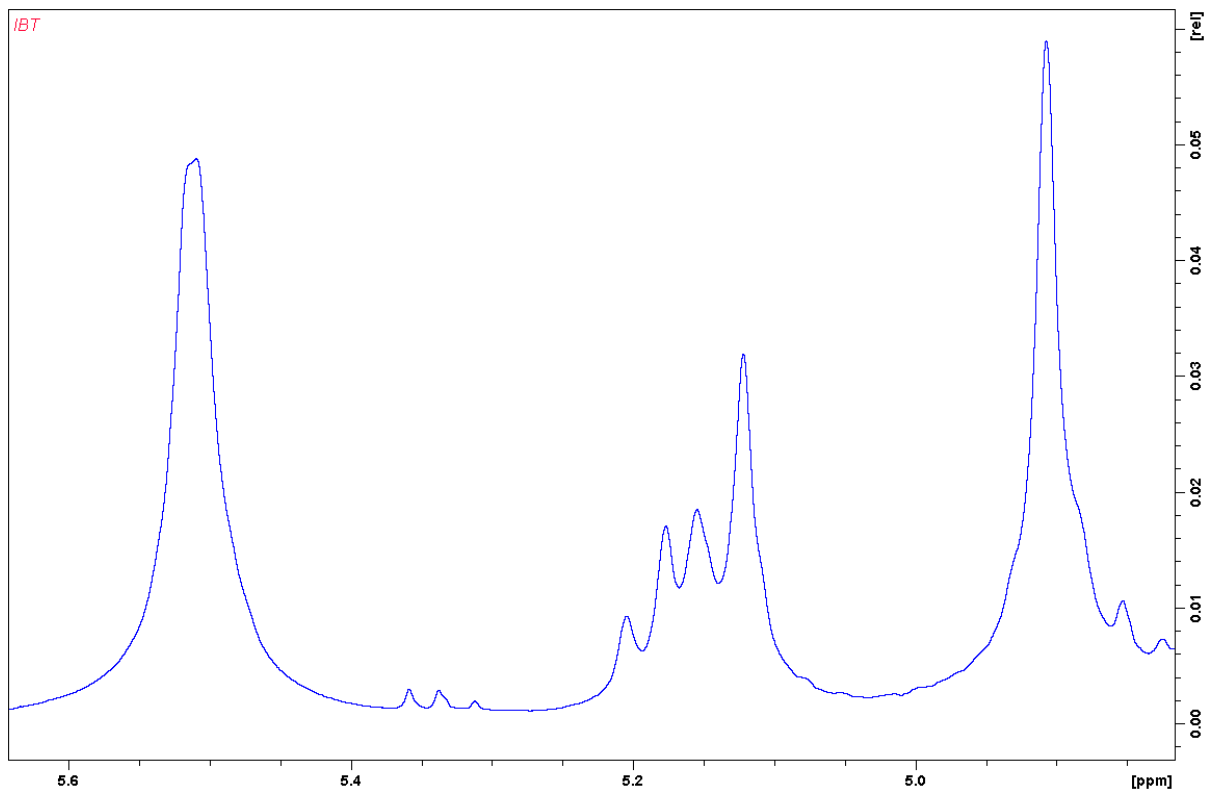


Figure 3. ^1H NMR spectra of hydrolysed *L. hyperborea* stipe alginate, showing chemical shift (ppm) of M and G sequences.

Appendix B – Gel preparation

Table 1. Mixing ratios for leaf (GP3350) and stipe (LF200S) alginate calcium and mixed leaf and stipe alginate calcium (Ca^{2+}) and chitosan oligomer (CO) gel preparation. The ratios was obtained from previously performed experiments at NTNU on mixed alginate gels

Alginate Sample	Unit	LF200S			GP3350			
		100/0	75/25	75/0	100 /0	50/50	50/0	0/100
$\text{Ca}^{2+}/\text{CO}^{2+}$	-	100/0	75/25	75/0	100 /0	50/50	50/0	0/100
$\underline{m}_{\text{alg}}$ stock solution	g	18.00	18.00	18.00	18.00	18.00	18.00	18.00
$\underline{m}_{\text{alg}}$	mg	270.00	270.00	270.00	270.00	270.00	270.00	270.00
C_{CO}	mM	0.0	9.4	0.0	0.0	18.8	0.0	37.7
$\underline{m}_{\text{CO}}$	mg	0.00	51.29	0.00	0.00	102.59	0.0	205.18
V_{CO} stock solution	ml	0.00	0.513	0.000	0.000	1.026	0.000	2.052
$\underline{m}_{\text{CO}} / \underline{m}_{\text{alg}}$	-	0.00	0.19	0.00	0.00	0.38	0.00	0.75
C_{CaCO_3}	mM	16.6	12.4	12.4	15.1	7.5	7.5	0.0
m_{CaCO_3}	mg	44.8	33.6	33.6	40.7	20.4	20.4	0.0
V_{MQ} for CaCO_3	ml	2.00	2.00	2.00	2.00	2.00	2.00	0.00
Ca/Alg.	-	1.10	0.83	0.83	1.00	0.50	0.50	0.00
$\underline{m}_{\text{GDL}}$ for CO	mg	0.05	45.34	0.00	0.05	90.63	0.00	181.21
$\underline{m}_{\text{GDL}}$ for CaCO_3	mg	159.42	119.57	119.57	144.93	72.47	72.47	0.00
$\underline{m}_{\text{total}}$ amount GDL	mg	159.47	164.91	119.57	144.98	163.10	72.47	181.21
V_{MQ} for GDL	ml	7.000	6.487	6.487	7.000	5.974	5.974	6.948

Appendix – C Swelling Solutions

All prepared buffer solutions used in systematic swelling experiment and control studies as presented in Table 1.

Table 1. All acetate and tris buffer solutions prepared for swelling experiments.

Buffer system	pH	C_{buffer}	C_{NaCl}	C_{CaCl₂}
	[-]	[mM]	[mM]	[mM]
Acetate	4.5	50	0	0
Acetate	4.5	50	100	0
Acetate	5.5	50	0	0
Acetate	5.5	50	100	0
Acetate	5.5	50	0	10
Acetate	7.5	50	0	0
Acetate	7.5	50	100	0
Tris	5.5	50	0	0
Tris	5.5	50	100	0
Tris	7.5	50	0	0
Tris	7.5	50	100	0
Tris	7.5	50	0	10
Tris	8.5	50	0	0
Tris	8.5	50	100	0

Appendix D – Size Exclusion Chromatography Analysis

In order to further characterize the swelling properties of alginate gels comprising chitosan oligomers, an experiment was conducted investigating traces of chitosan oligomers, which had leaked out of the gel, in swelling sol fractions. The swelling fractions were obtained after swelling leaf alginate chitosan oligomer gels at different times in a pH 5.5 Ac buffer without and with 100 mM NaCl. Six different swelling fractions were analysed by a SEC RI system, said after 5, 24 and 2x24 hours in solutions with and without salt. The chromatograms are shown in Figure 1, Figure 2, Figure 3 and Figure 4. A more sensitive detector was used for the 5 hour sample without salt as the previous detector was broken.

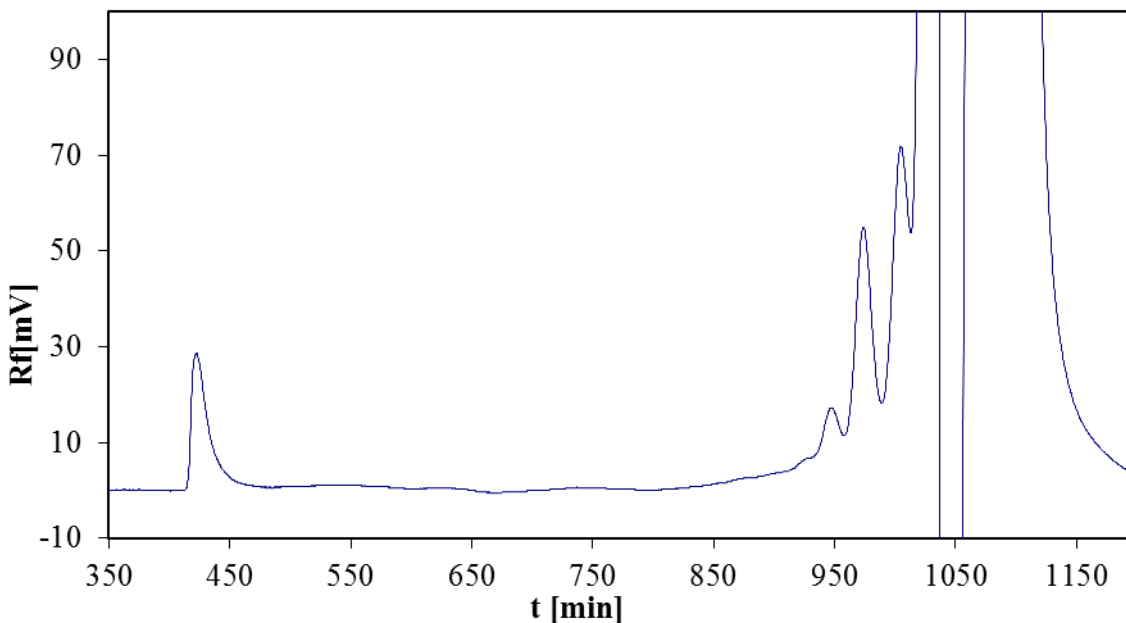


Figure 1. Separation of 5 hour swelling fractions obtained from swelling solution of leaf alginate gels cross-linked with chitosan oligomers. The gels were swelled in pH 5.5 50 mM acetate buffer solution before the gel was removed. The void peak had the shortest elution time, which indicates it consist alginate that possess higher molecular weight than the chitosan oligomers. Just before the salt peak, which is overlapped by the water front, the chitosan oligomer dimer (DP = 2), trimer (DP = 3), tetramer (DP = 4) and pentamer (DP = 5) were detected, whereas the dimer was the most abundant. This chromatogram was obtain with a more sensible detector compared to the other samples.

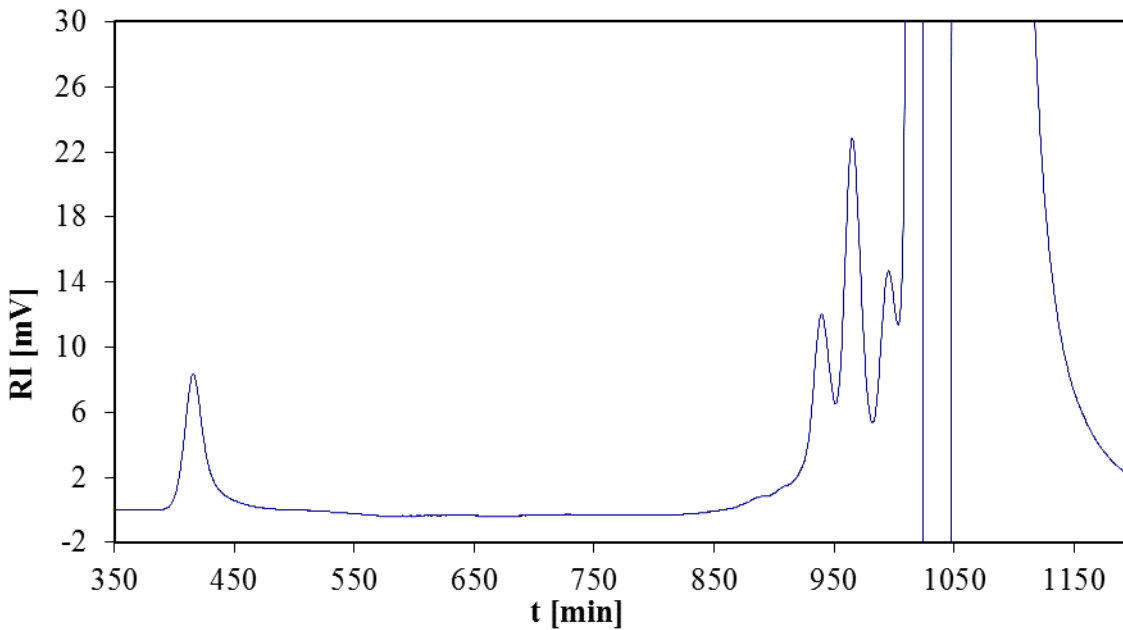


Figure 2. Separation of 5 h hour swelling fractions with 100 mM NaCl obtained from swelling solutions of leaf alginate gels cross-linked with chitosan oligomers. The gels were swelled in pH 5.5 50 mM acetate buffer solution before the gel was removed. Just before the salt peak, the chitosan oligomer dimer (DP = 2), trimer (DP = 3), tetramer (DP = 4), the pentamer (DP = 5) and the sixmere (DP = 6) were detected whereas the trimer was the most abundant.

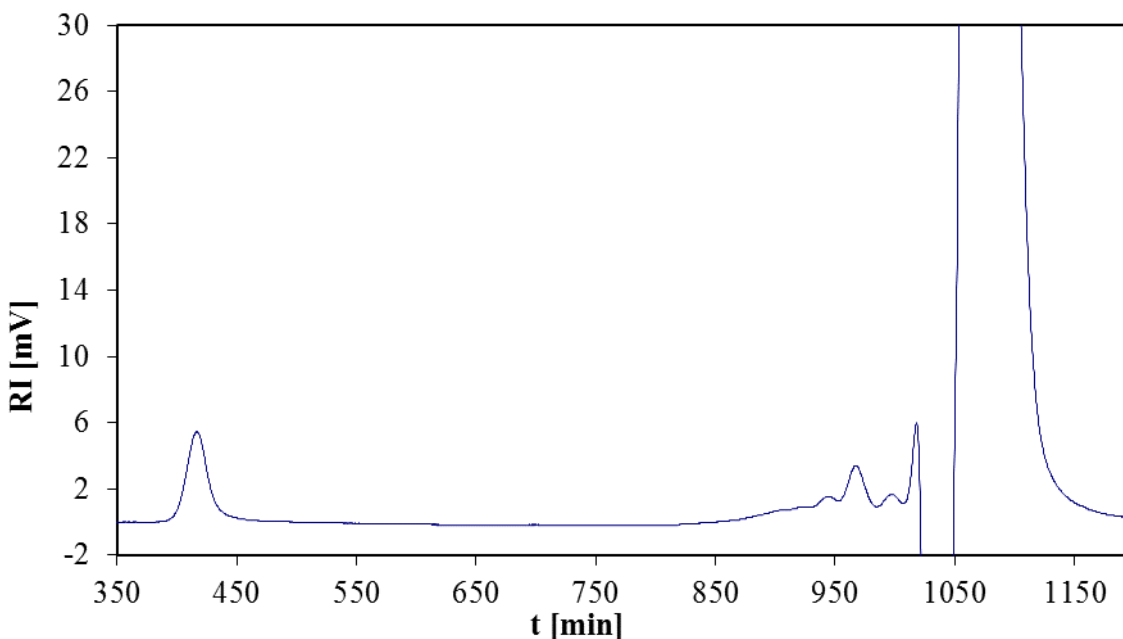


Figure 3. Separation of 48 hour swelling fractions obtained from swelling solution of leaf alginate gels cross-linked with chitosan oligomers. The gels were swelled in pH 5.5 50 mM acetate buffer solution before the gel was removed. The void peak had the shortest elution time, which indicates it consist alginate that possess higher molecular weight than the chitosan oligomers. Just before the salt peak, which is overlapped by the water front, the chitosan oligomer dimer (DP = 2), trimer (DP = 3) and tetramer (DP = 4) were detected, whereas the trimer was most abundant.

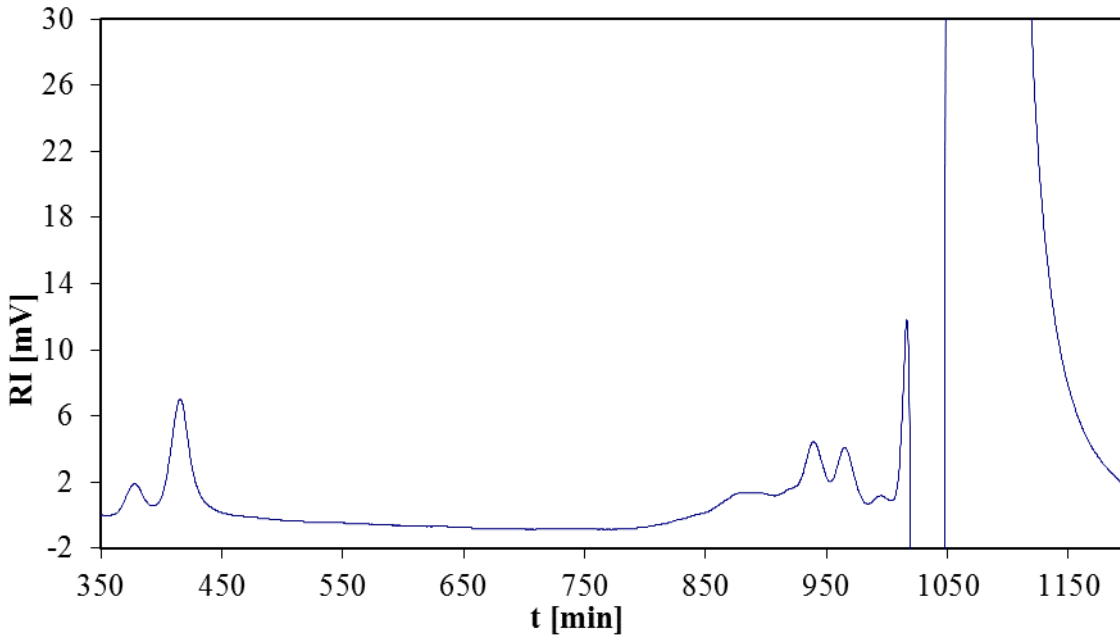


Figure 4. Separation of 48 h hour swelling fractions with 100 mM NaCl obtained from swelling solutions of leaf alginate gels cross-linked with chitosan oligomers. The gels were swelled in pH 5.5 50 mM acetate buffer solution before the gel was removed. Just before the salt peak, the chitosan oligomer dimer (DP = 2), trimer (DP = 3), tetramer (DP = 4), the pentamer (DP = 5), the sixmere (DP = 6) and longer oliomgers were detected whereas the tetramer was the most abundant.

Appendix E – Control Experiments

Several control experiments were performed in order to demonstrate different effects on the swelling behaviour of alginate gels of e.g. increasing cross-linking degree by adding CaCl_2 to the buffer solution.

Salt Effect on Swelling of Alginate Calcium Gels at pH 5.5

CaCl_2 and NaCl was added to the swelling solution in order to demonstrate the effect of increasing the ionic strength and the total number of cross-links on swelling behaviour of alginate calcium gels (Figure 1).

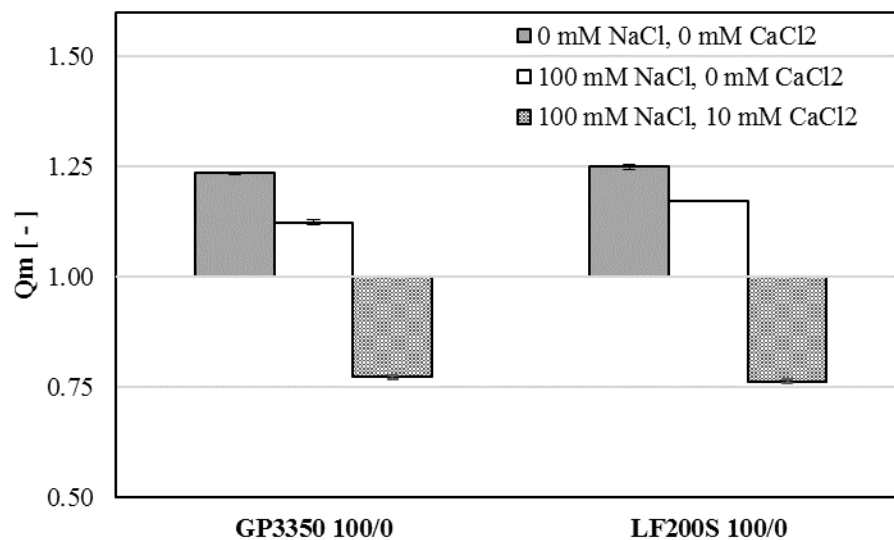


Figure 1. Swelling ratio (Q_m) of leaf (GP3350 100/0) and stipe (LF200S 100/0) alginate calcium gels after 24 hours in 50 mM acetate buffer solution with 0 mM salt, 100 mM NaCl and 10 mM CaCl_2 plus 100 mM NaCl at pH 5.5.

Acetate and Tris influence on Swelling of Leaf Alginate Calcium Gels

To investigate a potential effect of the tris and acetate buffer systems on the swelling behaviour of leaf alginate gels, leaf alginate calcium gels were swelled for 24 hours in acetate pH 7.5 and tris pH 5.5 solutions in addition to the regular acetate pH 5.5 and tris 7.5 solutions (Figure 2).

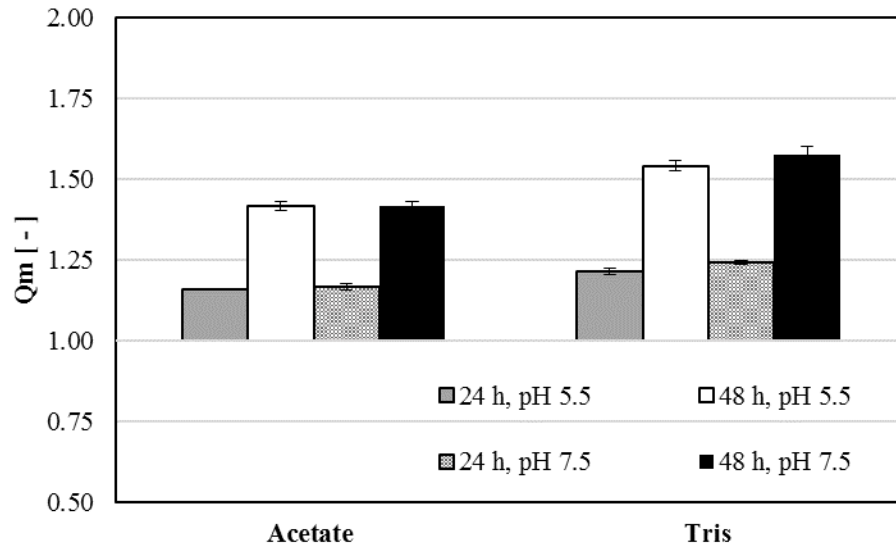


Figure 2. Swelling ratio (Q_m) of leaf alginate calcium gels after 24 and 48 (2x24) hours in 50 mM acetate and tris solutions of pH 5.5 and 7.5.

Appendix F – Swelling Kinetics of Stipe Alginate Calcium Gels

Swelling was measured at several times for stipe alginate calcium gels in order to investigate the swelling kinetics of these gels in different solution. The gels were swelled in solutions of pH 4.5, 5.5, 7.5 and 8.5 without (Figure 1) and with added salt (Figure 2).

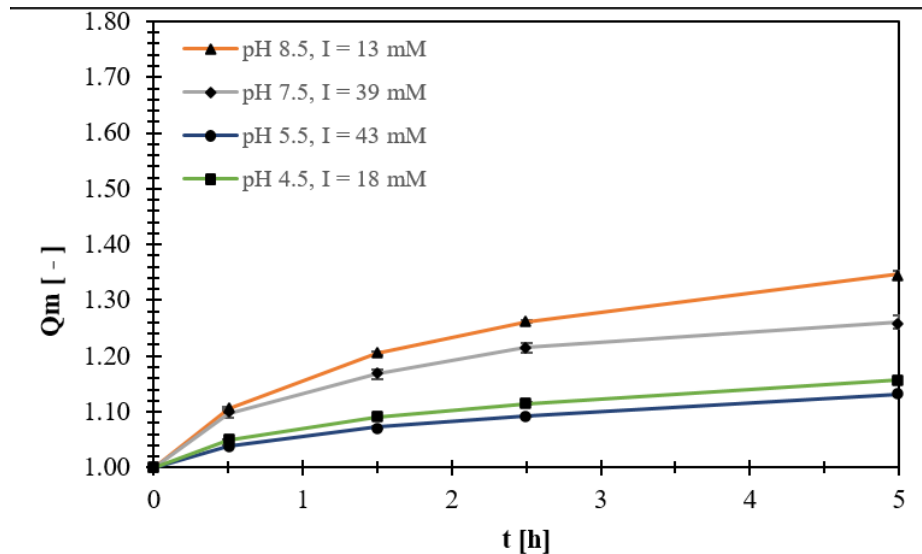


Figure 1. Swelling kinetics of leaf alginate calcium gels in 50 mM buffer solutions of different pH. Swelling ratio (Q_m) is plotted as a function of time after the gels were submerged in the solution. An Ac buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non/Fickian diffusion. Three gels ($n = 3$) were measured at each given time where the standard deviation was calculated in excel.

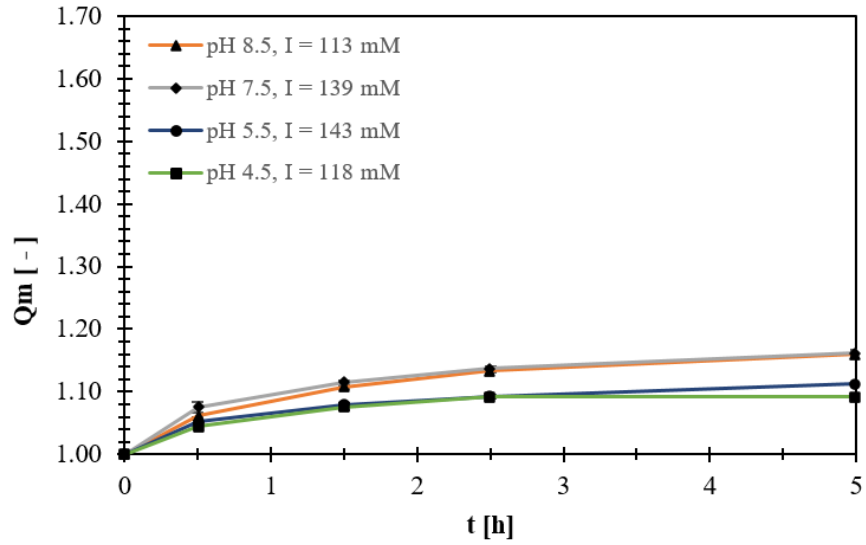


Figure 2. Swelling kinetics of stipe alginate calcium gels in 50 mM buffer solutions of different pH and with 100 mM NaCl. Swelling ratio (Q_m) is plotted as a function of time after the gels were submerged in the solution. An Ac buffer was used at pH 4.5 and 5.5, and a tris buffer was used for pH 7.5 and 8.5. The calculated ionic strength (I) of the four different buffer solutions is presented. A slack swelling slope indicates Fickian diffusion profile and a steep swelling slope implies non/Fickian diffusion.

Appendix G – Ionic strength calculations

Numerical Example of Ionic

Knowing the ionic strength (I) of the buffer is important to be able to investigate its influence on the swelling capacity for alginate hydrogels. When calculating the ionic strength, it is essential to determine the molar concentration (C_i) and the number of charges (z_i) of all ionic compounds in the solution following:

$$I = \frac{1}{2} \sum_i C_i z_i^2$$

The molar concentration of charged acetate molecules in the solution can be calculated utilizing the Henderson – Hasselbalch equation.

$$pH = pK_a + \log_{10} \frac{[A^-]}{[HA]}$$

The following two examples demonstrates how the ionic strength of four different swelling solutions used in this thesis was calculated.

Acetate Buffer with pH 4.5

Provided with the pK_a value for acetate of 4.76, the molar concentration ratio of protonated acetate molecules at pH 4.5 can be expressed.

$$\frac{[A^-]}{[HA]} = 10^{pH-pK_a} = 10^{4.50-4.76} = 0.55$$

A second expression is created by calculating the total molar concentration of acetate in the solution from the number of moles of the two components. The number of moles can be found from the concentration of and added volume of acetic acid and sodium acetate to the final solution.

$$[A^-] + [HA] = \frac{n_{CH_3COO^-} + n_{CH_3COOH}}{V} = \frac{C_{CH_3COO^-}V_{CH_3COO^-} + C_{CH_3COOH}V_{CH_3COOH}}{V_{final}}$$

$$C_{CH_3COO^-} = \frac{10.25g}{82.03 \frac{g}{mole} \times 250mL} = 0.50M$$

$$C_{CH_3COOH} = \frac{7.15 mL \times 1.05 \frac{g}{mL}}{60.05 \frac{g}{mole} \times 250mL} = 0.50M$$

$$[A^-] + [HA] = \frac{0.50M \times 228mL + 0.50M \times 182mL}{410mL} = 0.50M$$

The molar concentration of charged acetate molecules is decided by combining the two expressions for concentration of protonated and deprotonated acetate.

$$C_{CH_3COO^-} = [A^-] = \frac{1}{10} \times \frac{0.50M}{(1 + \frac{1}{0.55})} = 0.018M$$

As previously discussed, only opposing charges contributes to the ionic strength. For a pure acetate buffer, the contributing compounds will be the negatively charged acetate and the opposing positively charged sodium ions. Hence, the ionic strength of the buffer system for a 0.05 M acetate buffer at pH 4.5 is given

$$I = \frac{1}{2} (C_{Na^+} z_{Na^+}^2 + C_{CH_3COO^-} z_{CH_3COO^-}^2)$$

$$I = \frac{1}{2} (0.018M \times (-1)^2 + 0.018M \times (1)^2) = \mathbf{0.018 M}$$

The ionic strength is increased upon adding sodium chloride to the solution as the sodium and chloride ions are oppositely charged particles contributing to system. Typically, 2.922 g NaCl was added to 500 mL of 0.05 M buffer solution.

$$C_{NaCl} = 0.5 L^{-1} \times \frac{2.922g}{58.44 \frac{g}{mole}} = 0.10M$$

Hence, the new ionic strength when NaCl is added is given:

$$I = \frac{1}{2} (C_{Na^+} z_{Na^+}^2 + C_{CH_3COO^-} z_{CH_3COO^-}^2 + C_{Na^+} z_{Na^+}^2 + C_{Cl^-} z_{Cl^-}^2)$$

$$I = \frac{1}{2} (0.018M \times (1)^2 + 0.018M \times (-1)^2 + 0.1M \times (1)^2 + 0.1M \times (-1)^2) = \mathbf{0.118 M}$$

Tris Buffer with pH 7.5

The Tris molecules are positively charged when the amino group is protonated. The pK_a of the amino group is 8.06, meaning more than 50% of the Tris molecules will be positively charged below pH 8.06. Again, the Henderson – Hasselbalch equation was applied to calculate the molar concentration of charged Tris molecules.

$$pH = pK_a + \log_{10} \frac{[A]}{[HA^+]}$$

$$\frac{[A]}{[HA^+]} = 10^{pH-pK_a} = 10^{7.50-8.06} = 0.28$$

The total molar concentration can also be expressed by calculating the concentration of

$$[A] + [HA^+] = \left(\frac{C_{Tris\ Base} V_{Tris\ Base}}{V_{Total}} + \frac{C_{Tris\ Hcl} V_{Tris\ Hcl}}{V_{Total}} \right)$$

$$C_{Tris\ Base} = \frac{6.057\ g}{121.14 \frac{g}{mole} \times 100mL} = 0.5\ M$$

$$C_{Tris\ Hcl} = \frac{30.285\ g}{121.14 \frac{g}{mole} \times 500mL} = 0.5\ M$$

$$[A] + [HA^+] = \left(\frac{0.5M \times 13\ mL + 0.5M \times 201.46mL}{214.76mL} \right) = 0.50M$$

$$C_{(CH_2OH)_3CNH_3^+} = [HA^+] = \frac{1}{10} \times \frac{0.50M}{(1 + 0.28)} = 0.039M$$

Hence, the ionic strength is given:

$$I = \frac{1}{2}(C_{NH_3^+}z_{NH_3^+}^2 + C_{Cl^-}z_{Cl^-}^2)$$

$$I = \frac{1}{2}(0.039M \times (1)^2 + 0.039M \times (-1)^2) = \mathbf{0.039 M}$$

$$C_{NaCl} = 0.5 L^{-1} \times \frac{2.922 g}{58.44 \frac{g}{mole}} = 0.10M$$

Hence, the new ionic strength when NaCl is added is given:

$$I = \frac{1}{2}(C_{NH_3^+}z_{NH_3^+}^2 + C_{Cl^-}z_{Cl^-}^2 + C_{Na^+}z_{Na^+}^2 + C_{Cl^-}z_{Cl^-}^2)$$

$$I = \frac{1}{2}(0.039M \times (1)^2 + 0.039M \times (-1)^2 + 0.1M \times (1)^2 + 0.1M \times (-1)^2) = \mathbf{0.139 M}$$

Calculated Ionic Strength of Swelling Solutions

The ionic strength of the swelling solutions used in the systematic study of mixed leaf and stipe alginate gels and leaf and stipe alginate gels was calculated according to the above example.

Table 1. Ionic strength of different buffer solutions calculated by from the Henderson-Hasselbalch equation.

Buffer system	pH	C _{buffer}	C _A ⁻ /C _{HA} ⁺	C _{NaCl}	Ionic strength (I)
	[-]	[mM]	[mM]	[mM]	[mM]
Acetate	4.5	50	18	0	18
Acetate	4.5	50	18	100	118
Acetate	5.5	50	43	0	43
Acetate	5.5	50	43	100	143
Tris	7.5	50	39	0	39
Tris	7.5	50	39	100	139
Tris	8.5	50	13	0	13
Tris	8.5	50	13	100	113

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