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Strategies for Stabilising Calcium Alginate Gel Beads: Studies of Chitosan Oligomers, Alginate Molecular Weight and Concentration

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Biotechnology (5 year)
Submission date: May 2017
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Preface

The work for this master's project was carried out at the Department of Biotechnology at the Norwegian University of Science and Technology (NTNU) in the time period August 2016 to May 2017.

Firstly, I would like to express my sincere gratitude to my supervisor Berit Løkensgard Strand for all the guidance throughout this work. I would also like to express my sincere gratitude to Abba Elisabeth Coron for her help throughout this work and for always being available for guidance in the laboratory and in the process of writing this thesis.

My sincerest thanks also goes to Wenche Iren Strand for invaluable help in the laboratory, and for performing the NMR analysis needed for my work. In addition, much appreciation is given to Ann-Sissel for performing the SEC-MALLS analysis and for helping me with the viscosity measurements. I would also like to kindly thank Olav Andreas Aarstad for assisting me with the compression analysis.

Lastly, I would like to thank my fellow students and my family for their endless encouragement and support.

Trondheim, May 15, 2017

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Abstract

Microencapsulation of cells or tissues in alginate-based capsules is the most commonly applied technique for immunoisolation. However, the biocompatibility of the capsules and their long-time stability under physiological conditions are two major problems related to cell encapsulation. Alginate is a linear binary copolymer consisting of β-**D**-mannuronic acid and α-**L**-guluronic acid linked by 1,4-glycosidic bonds, and is known to form gels with divalent ions like calcium. To increase the stability of alginate gel beads in physiological solutions, a polycation layer has traditionally been used to coat the alginate gel beads. However, most polycations are toxic to cells and the polycation layer have been associated with cellular overgrowth. Recently, alginates isolated from *Laminaria hyperborea* leaf with intermediate guluronic acid content was found to be highly biocompatible. However, gels prepared from alginates with intermediate guluronic acid content are known to exhibit lower mechanical strength and stability compared to alginate gels rich in guluronic acid.

In the present work, the stability and mechanical strength of *L. hyp.* leaf alginate gels were investigated to potentially provide a strategy to increase the stability of leaf alginate gel beads. The possibilities of generating leaf alginate gel beads crosslinked with calcium and chitosan oligomers were investigated, and the stability in terms of size and polymer leakage of the alginate-CO gel beads was studied. The mechanical properties in terms of Young's modulus and rupture strength were investigated for *L. hyp.* leaf alginate gel cylinders of varying molecular weights and concentrations, where gel cylinders prepared from low molecular weight *L. hyp.* stipe alginate were included as a reference. The effect of increasing the alginate concentration with very low molecular weight leaf or stipe alginate was also studied. In addition, syneresis was measured for all the alginate gel cylinders. Furthermore, the stability of low molecular weight *L. hyp*. leaf alginate gel beads in saline, in terms of swelling and polymer leakage, was investigated. High molecular weight *L. hyp.* leaf was included in the study for comparison. The chemical compositions of the leaked alginate from alginate-CO gel beads and low molecular weight leaf alginate gel beads were determined by ^{1}H-NMR. For the low molecular weight leaf alginate gel beads, average molecular weights of the leaked alginates were obtained with SEC-MALLS.

Introducing chitosan oligomers into the crosslinking of alginate gel beads with calcium had a minor stabilizing effect with respect to size at pH 5.0, whereas alginate-CO gel beads prepared at pH 6.5 showed no stabilizing effect, compared to alginate gel beads gelled only

with calcium. At pH 5.0, both alginate and chitosan oligomers are fully charged, hence expected to form strong interactions. The leaked alginate from the alginate-CO gel beads had a similar chemical composition to the starting material of alginate. Increasing the alginate concentration for the high and low molecular weight leaf alginate gel cylinders led to higher gel strength in terms of Young's modulus and rupture strength. The Young's modulus of the alginate gels were independent of the molecular weight in the given range (M_w = 115-200 kDa), while rupture strength increased with increasing molecular weight in the same range. Syneresis of the alginate gels decreased with increasing alginate concentration and decreasing molecular weight. The size stability of low molecular weight *L. hyp*. leaf alginate gel beads increased with increasing alginate concentration. No significant difference was seen between the 1.8 % (w/v) low and high molecular weight leaf alginate gel beads with regards to size stability. The degree of polymer leakage decreased with increasing alginate concentration and increasing molecular weight. The leaked materials from the low molecular weight leaf alginate gel beads were enriched in mannuronic acid compared to the starting material of alginate.

Increasing the alginate concentration of the *L. hyp.* leaf alginate gel beads showed promising results with regards to increasing stability of the gel beads. The molecular weight of the alginate had only minor effects on the stability. Introducing chitosan oligomers into the crosslinking of alginate with calcium had a slightly stabilizing effect at pH 5.0. However, pH 5.0 might compromise the viability of the cells being encapsulated.

Sammendrag

Mikroenkapsulering av celler og vev i alginatbaserte kuler er den vanligste metoden for immunoisolering. Hovedutfordringene med alginatkulene er biokompatibiliteten og stabiliteten under fysiologiske forhold på lang sikt. Alginat er et binært heteropolymer som består av β-**D**-mannuronsyre og α-**L**-guluronsyre koblet sammen av 1,4-glykosidbindinger, og er kjent for å danne geler med divalente ioner som kalsium. Tradisjonelt har alginatkulene blitt belagt med et polycation for øke stabiliteten i fysiologiske løsninger, men de fleste polykationer er giftige i tillegg til å ha blitt koblet til cellulær overvekst. En nylig publisert studie har vist at kuler av alginater med at lavt innhold av guluronsyre isolert fra *Laminaria hyperborea* blad er høyst biokompatible. Samtidig er geler av alginater med lavt innhold av guluronsyre kjent for å ha lavere mekanisk styrke og stabilitet sammenlignet med alginat geler med høyt innhold av guluronsyre.

Gjennom arbeidet med denne masteroppgaven ble stabiliteten og den mekaniske styrken til geler av *L. hyp.* bladalginat undersøkt for potensielt å finne en strategi for å øke stabiliteten til bladalginatkuler. Muligheten for å lage bladalginatkuler kryssbundet med både kalsium og kitosan oligomerer ble undersøkt, og stabiliteten til alginat-CO-kuler i form av størrelse og lekkasje av alginat og chitosan oligomerer ble studert. Den mekaniske styrken i form av Youngs modul og bruddstyrke ble undersøkt for gelsylindre av *L. hyp.* bladalginat av varierende molekylvekt og alginatkonsentrasjoner, hvor gelsylindre av lavmolekylært *L. hyp.* stilkalginat ble inkludert som en referanse. Effekten av å øke konsentrasjonen med lavmolekylært blad- eller stilkalginat ble også undersøkt. I tillegg ble syneresis målt for alle alginatgelesylindrene. Videre ble stabiliteten til alginatkuler av lavmolekylært *L. hyp.* bladalginat i saltløsning undersøkt i form av størrelse og lekkasje av alginat. Alginatkuler av høymolekylært *L. hyp.* bladalginat ble inkludert for sammenligning. Den kjemiske sammensetningen av det lekkede alginatet fra alginat-CO-kuler og fra alginatkuler av lavmolekylært bladalginat ble bestemt med ^{1}H-NMR. Den gjennomsnittlige molekylvekten ble funnet for alginatet som lekket ut av alginatkulene av lavmolekylært bladalginat med SEC-MALLS.

Å kryssbinde bladalginat med både kalsium og kitosanoligomerer hadde en mindre stabiliserende effekt med tanke på størrelse ved pH 5.0 sammenlignet med alginatkuler kryssbundet med bare kalsium. Alginat-CO-kuler fremstilt ved pH 6.5 viste derimot samme stabilitet som alginatekulene kryssbundet med bare kalsium. Ved pH 5.0 er både alginat og kitosanoligomerer fullstendig ladet, og det er dermed forventet at alginat og

kitosanoligomerer skal kunne danne sterke interaksjoner ved denne pH-verdien. Alginatet som lekket fra alginat-CO-kulene hadde samme kjemiske sammensetning som startmaterialet av alginat. En økning alginatkonsentrasjonen for gelesylindrene av høy- og lavmolekylært bladalginat ga økt gelstyrke i form av Youngs modul og bruddstyrke. Youngs modul for alginatgelene var uavhengig av molekylvekt i området undersøkt (M_w = 115-200 kDa), mens bruddstyrken derimot økte med økende molekylvekt i det samme området. Syneresis for alginatkulene ble redusert med økende alginatkonsentrasjon og synkende molekylvekt. Stabiliteten til alginatkuler av lavmolekylært *L. hyp*. bladalginat i form av størrelse økte med økende alginatkonsentrasjon. Ingen signifikant forskjell ble sett mellom alginatkuler av 1.8 % (w/v) lav- og høymolekylært bladalginat med hensyn til stabilitet i form av størrelse. Mengden lekket alginat fra kulene ble redusert med økende alginatkonsentrasjon og økende molekylvekt. Det lekkede alginatet fra kulene var anriket i mannuronsyre sammenlignet med startmaterial av alginat.

Ved å øke alginatkonsentrasjon kan alginatkuler av *L. hyp.* bladalginat bli stabilisert til en viss grad. Molekylvekten hadde mindre å si for stabiliteten til bladalginatkulene. Å kryssbinde alginat med både kalsium og kitosan oligomerer hadde en mindre stabiliserende effekt ved pH 5.0. Denne lave pH kan derimot påvirke levedyktigheten til cellene som blir innkapslet.

Symbols and Abbreviations

$\overline{M_n}$	Number average molecular weight
$\overline{M_w}$	Weight average molecular weight
$\overline{M_w}/\overline{M_n}$	Polydispersity index (PI)
% (w/v)	Weight/volume percentage (1 g/100 mL)
% (w/w)	Weight percentage
^{1}H NMR	Proton nuclear magnetic resonance
η	Viscosity
$\dot{\gamma}$	Shear rate
σ	Stress
ε	Strain
μ	Chemical potential
A	2-acetamide-2-deoxy-D-glucos (GlcNAc)
CLSM	Confocal laser scanning microscopy
D	2-amino-2-deoxy-β-D-glucose (GlcN)
DP_n	Number average degree of polymerization
DP_w	Weight average degree of polymerization
E	Young's modulus
EDTA	Ethylenediaminetetraacetic acid
F_A	Degree of N-acetylation
F_G	Guluronic acid frequency
F_M	Mannuronic acid frequency
G	α-L-Guluronic acid
GDL	D-glucono-δ-lactone
kDa	Kilo daltons
kV	Kilo volt
M	β-D-Mannuronic acid
MQ	Milli Q
MWCO	Molecular weight cut off
$N_{G>1}$	Average length of G-blocks
pK_a	Acid dissociate constant
rpm	Rotations per minute

Saline	0.9 % NaCl
SEC-MALLS	Size exclusion chromatography with inline multi-angle laser light scattering
TSP	Trimethylsilylpropinate
TTHA	Triethylenetetraaminehexaacetic acid

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1 Introduction

1.1 Background

1.1.1 Cell Therapy as a Treatment for Type 1 Diabetes

Diabetes is a group of metabolic diseases characterized by hyperglycemia (high levels of blood glucose) caused by defects in insulin secretion, insulin action, or both. Chronic hyperglycemia leads to long-term damage, dysfunction, and failure of different organs, particularly the eyes, kidneys, nerves, heart, and blood vessels. Several processes are involved in the development of diabetes, which range from autoimmune destruction of the pancreatic β-cells with following insulin deficiency to abnormalities that result in resistance to insulin action. Most cases of diabetes falls into two categories, type 1 diabetes, caused by an absolute deficiency of insulin secretion, and type 2 diabetes, caused by a combination of resistance to insulin action and inadequate insulin secretory response (American Diabetes Association, 2014). In 2013, the occurrence of people with diabetes world wide was estimated to be 382 million with an expected rise to 592 million by 2035, making diabetes a high disease burden for many countries and a large global health problem (Forouhi and Wareham, 2014).

Type 1 diabetes is not curable, but the disease can be treated through self-monitoring and insulin injections. The treatment, however, provide only limited control over blood sugar levels, and both short- and long-term negative effects are not unusual. Today, a tighter control of the blood sugar levels can be achieved by cell therapy, involving transplantation of a whole pancreas or just the insulin-producing cells (islets of Langerhans). But in order to prevent graft rejection, the recipients are in lifelong need for immunosuppressive drugs, which have significant side effects (Andersen et al., 2012). For cell therapy to be a relevant treatment option, an alternative to immunosuppressive drugs for overcoming the immune barrier is necessary. One such method is immunoisolation, referring to the practice where the tissues or the cells to be transplanted is enclosed in a device composed of a semipermeable membrane. The membrane should allow for diffusion of small molecules like oxygen, nutrients and waste products to maintain the viability of the cells, as well as the therapeutic products secreted by the encapsulated cells, but at the same time prevent contact between the graft and immune cells or other immunological factors such as antibodies with higher molecular weights (Stockley and Chang, 1997, Pareta et al., 2013), see Figure 1.1.

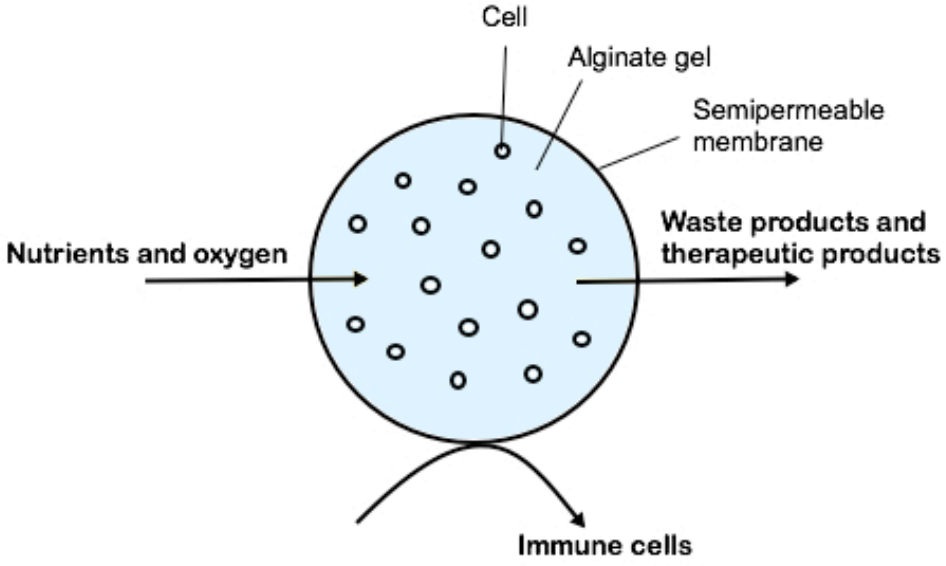

Figure 1.1: Principle of immunoisolation. The transplanted cells are enclosed in a semipermeable membrane that allows diffusion of small molecules like oxygen, nutrients and waste products, as well as therapeutic products produced by the cells, but prevent contact between the transplanted cells and the host immune cells. Alginate-based capsules is the most commonly applied technique for immunoisolation. Adapted from (Mørch, 2008).

1.1.2 Alginate as an Immunoisolating Material

Microencapsulation of cells or tissues in alginate-based capsules is the most commonly applied technique for immunoisolation. Alginate provide some advantages over other systems. The material do not interfere with the cellular function of the islets, the capsules can be formed rapid and in a single step under physiological conditions, and the microcapsules ensures a favorable micro-environment which gives growth support and prevents fusion of the islets (de Vos et al., 2006). In addition, alginate is a highly characterized and well-understood polymer where the relationship between structure and function is well-known (Skjåk-Bræk and Espevik, 1996). Cell encapsulation is a multidisciplinary and complex research field, which makes it a field with many ongoing challenges. Disciplines associated with the field include material science, polymer chemistry, immunology, cell biology, medicine and surgery. The complexity is connected to the many factors that influence the functional and safe performance of the microcapsules. These factors include physico-chemical properties like mechanical stability, permeability, morphology and surface properties, and biocompatibility (Rokstad et al., 2014).

The biocompatibility of the capsules and their long-time stability at physiological conditions are two major problems related to cell capsulation. The capsules are under a positive osmotic pressure due to the Donnan equilibrium of the non-cooperatively bound counter ions, which is counterbalanced at equilibrium by a negative pressure because of the elasticity of the gel network. Chelating agent (e.g. phosphate, citrate) and non-gelling ions (e.g. Na^+, Mg^{2+}), both present at physiological conditions, destabilize the gel network. This lead to swelling, which cause an increase in pore size and eventually possible dissolution or breakage of the capsules (Smidsrød and Skjåk-Bræk, 1990, Skjåk-Bræk and Espevik, 1996, Strand et al., 2003). To increase the mechanical stability of alginate microcapsules in physiological solutions, a polycation layer is often used to coat the capsules. However, most polycations are toxic to cells. Furthermore, poly-L-lysine (PLL), most often used to coat alginate beads, has been shown to be a main cause of cellular overgrowth (Strand et al., 2001).

Growth of host cells on the capsules reduce the diffusion of oxygen and nutrients to the transplanted cells resulting in necrosis of the encapsulated cells. The cells on the capsule surface are also found to be mainly inflammatory cells secreting cytokines and chemokines which can have a negative effect on the function of encapsulated cells (de Vos et al., 2006). The molecular weight of the alginate, the solution viscosity and the mannuronic (M) and guluronic (G) acid content have all been suggested in studies to have an effect on the biocompatibility of purified alginate in the absence of a polycation. However, the conclusions of such studies have not been consistent (Tam et al., 2011). Early studies have demonstrated that alginates with high M content induce an inflammatory response by stimulating monocytes to produce proinflammatory cytokines such as TNF, IL-1, and IL-6. In the same studies this was not seen for alginates with a high content of G (Otterlei et al., 1991). However, more recently conducted studies show that gel beads based on alginates with intermediate G content are more biocompatible in terms of host cells adhesion than gel beads based on alginates with high G content (Tam et al., 2011). In general, gels prepared from alginates with intermediate G content exhibit lower mechanical strength and lower stability under physiological conditions than gels prepared from alginates with high G content (Martinsen et al., 1989).

1.1.3 Aim of Study

The aim of this thesis has been to investigate the stability and mechanical properties of *Laminaria hyperborea* leaf alginate gels, which has an intermediate guluronic acid content, as a strategy to increase biocompatibility of alginate gels intended for use as immunoisolating material without compromising the stability of the gel under physiological conditions. The specific aims were:

1. Investigate the possibility of generating *L. hyp.* leaf alginate gel beads crosslinked with calcium and chitosan oligomers and the eventual effect on the stability with respect to size and polymer leakage.
2. Investigate the mechanical properties and syneresis of *L. hyp.* leaf alginate gel cylinders and the effect of alginate concentration and molecular weight.
3. Investigate the stability of low molecular weight *L. hyp.* leaf alginate gel beads with respect to size and polymer leakage and the effect of alginate concentration.

1.2 Alginate

1.2.1 Source and Application

Alginate is a family of anionic polysaccharides mainly occurring as structural components in marine brown algae (*Phaeophyceae*) (Painter, 1983). Alginate is the most abundant polysaccharide in brown algae, comprising up to 40 % of the dry matter, making alginate quite abundant in nature. It is located in the intercellular matrix as a gel containing sodium, calcium, magnesium, strontium and barium ions, giving both strength and flexibility to the algal tissue (Skjåk-Bræk et al., 2006, Donati and Paoletti, 2009). Soil bacteria such as *Azotobacter vinelandii* (Gorin and Spencer, 1966) and several species of *Pseudomonas* (Linker and Jones, 1966) produce an extracellular polymeric material that resembles alginate produced by brown algae

Commercial alginates are produced mainly form the brown algae *Laminaria hyperborea*, *Macrocystis pyrifera*, *Laminaria digitata* and *Ascophyllum nodosum* (Skjåk-Bræk et al., 2006, Donati and Paoletti, 2009). The highest content of guluronic acid is found in alginate prepared from stipes of *L. hyperborea*, while *A. nodosum* are characterized by a low content of G-blocks. The chemical composition and sequential structure of alginate depend not only on the species and the part of the plant but may also vary according to season and growth conditions. Alginates with more extreme compositions can be isolated from bacteria or from special algae tissues such as the other cortex (Haug et al., 1974, Indergaard and Skjåk-Bræk, 1987). Novel alginates with tailored properties can be prepared with C5-epimerases from *A. vinelandii* (Skják-Bræk et al., 1986).

Most industrial applications of alginates are related to their gelling, viscosifying and stabilizing properties, and their ability to retain water (Donati and Paoletti, 2009). In the food industry alginates are used as additives to improve, modify and stabilize the texture of certain foods such as jams and reconstructed foodstuffs (Skjåk-Bræk et al., 2006). In the pharmaceutical industry alginates have for many years been used in drug delivery systems, in wound dressings, as dental impression materials and in formulations preventing gastric reflux (Donati and Paoletti, 2009, Draget and Taylor, 2011). More advanced biotechnological and biomedical applications include entrapment of living cells within alginate calcium spheres (Smidsrød and Skjåk-Bræk, 1990). As stated earlier, immobilized cells have the potential to be used in cell transplantation, where the purpose of the gel is to act as a barrier between the transplant and the immune system of the host (de Vos et al., 2006).

1.2.2 Structure

Alginate is a linear binary copolymer consisting of β-**D**-mannuronic acid (M) and α-**L**-guluronic acid (G) (Figure 1.2 a) linked by 1,4-glycosidic bonds (Smidsrød and Draget, 1996). The polymer can be described as a block polymer containing homopolymeric regions termed M- and G-blocks and regions of alternating structure termed MG-blocks (Figure 1.2 c) (Haug et al., 1966). The guluronate residues and the mannuronate residues adopt different conformations, $^{1}C_{4}$ and $^{4}C_{1}$ respectively. As a consequence, alginate contains all four possible glycosidic linkages: diequatorial (MM), diaxial (GG), equatorial-axial (MG) and axial-equatorial (GM) (Figure 1.2 b) (Grasdalen et al., 1977). M-blocks will adopt a flat ribbonlike chain conformation, while G-blocks will have a buckled and more rigid structure. MG-blocks will have the greatest flexibility of the block structures owing to the alternating axial-equatorial and equatorial-axial glycosidic bonds connecting the residues (Donati and Paoletti, 2009). Thus, the rigidity of the blocks decrease in the order GG>MM>MG (Smidsrød et al., 1973).

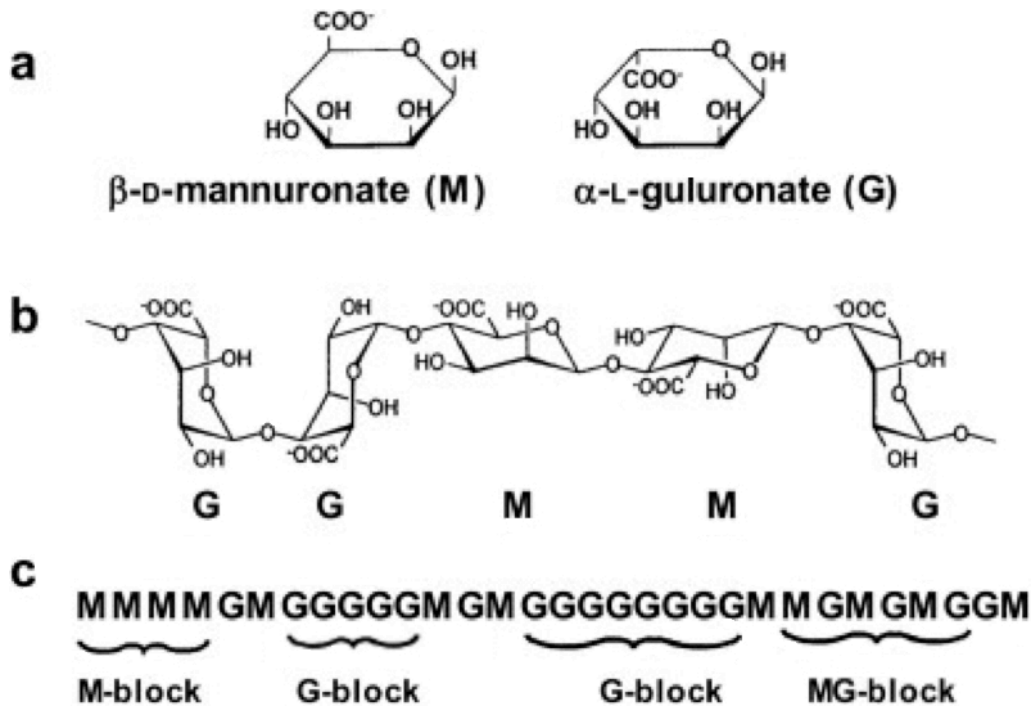

Figure 1.2: The chemical structure of alginate: (a) Haworth projection of the β-D-mannuronic acid and α-L-guluronic acid monomers, (b) the β-D-mannuronic acid in the $^{4}C_{1}$ chair conformation and α-L-guluronic acid in the $^{1}C_{4}$ chair conformation and the possible linkages between them, and (c) block structures in alginate (Draget and Taylor, 2011).

1.2.3 Characterization

Molecular weight and molecular weight distribution

The molecular weight of commercially available sodium alginates range from 32 000 to 400 000 g/mol (Lee and Mooney, 2012). Alginate, like other polysaccharides, are polydisperse when it comes to molecular weight. As a consequence, the molecular weight of an alginate is an average of the whole distribution The two most common methods for averaging are the number-average, $\overline{M_n}$, and the weight-average, $\overline{M_w}$. The number average weighs the polymer molecules according to the number of molecules having a specific weight and is defined as

$$\overline{M_n} = \frac{\sum_i N_i M_i}{\sum_i N_i}$$

where N_i is the number for molecules having a specific molecular weight M_i. The weight average weighs the polymer molecules according to the weight of molecules having a specific molecular weight and is defined as

$$\overline{M_w} = \frac{\sum_i w_i M_i}{\sum_i w_i} = \frac{\sum_i N_i M_i^2}{\sum_i N_i}$$

where w_i is the weight of molecules having a specific weight M_i. The weight average will always be greater than the number average ($\overline{M_w} > \overline{M_n}$) for polydisperse polymers, and for a random degraded polymer the weight average molecular weight is approximately two times the number average molecular weight ($\overline{M_w} \approx 2\overline{M_n}$). The ratio $\overline{M_w}/\overline{M_n}$ is called the polydispersity index (PI) and give information about the molecular weight distribution in an alginate sample. Typically, alginate samples have a PI ranging from 1.5 to 3, although values as high as 6 have been reported. A PI less than 2.0 may indicate that some fractionation has occurred during processing, while a PI higher than 2.0 indicate a wider distribution that might be a result of mixing of products with different molecular weights or a nonrandom degradation of the polymer. The PI is 1 in the case of monodisperse polymers (proteins) (Skjåk-Bræk et al., 2006).

Size-exclusion chromatography combined with on-line multi angle laser light scattering (SEC-MALLS) is the most common method for determine molecular distribution and averages. SEC-MALLS separate the polymers according to differences in the hydrodynamic size. The separation mechanism is the passive diffusion of macromolecules into porous particles, where the distribution of pore sizes matches the size of the molecules to be

separated. Smaller molecules will to a larger extent than larger molecules diffuse into the pores, and since the solvent inside the pores is stagnant, molecules in the pores will not be transported along the column until they diffuse out. Two detectors are included in the set-up, a concentration sensitive detector (refractive index or UV detector) and a light scattering detector, which measure scattering at several angles at very small volumes at the time, providing information on weight average for each small volume (Christensen, 2015, Smidsrød and Moe, 2008).

Chemical composition and sequential structure

The physical properties of alginates are highly dependent on the chemical composition and sequential structure. Information about monad frequencies (F_M and F_G), diad frequencies (F_{GG}, F_{GM}, F_{MG} and F_{MM}) and triad frequencies (F_{GGG}, F_{GGM}, F_{GMM}, F_{GMG}, F_{MMM}, F_{MMG}, F_{MGG} and F_{MGM}) can be obtained from ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) (Grasdalen et al., 1981, Grasdalen, 1983). NMR exploit the quantum mechanical properties of atomic nuclei. Spin is a type of angular momentum and for a atomic nucleus the spin has an associated quantum number, I, which depend on both the nuclear mass and the atomic number. Nuclei with I $\neq$ 0 exhibit a nuclear magnetic moment, which in a magnetic field orient itself in only certain allowed orientations. Isotopes with I = ½ (e.g. ^{1}H, ^{13}C) have two orientations, or levels. Electromagnetic radiation can transfer spins from a lower energy level to a higher energy level, and the frequency of the irradiation needed to perform this transfer is characteristic for the nucleus. Different nuclei have slightly different frequencies due to modification of the external magnetic field by nuclear shielding. This lead to a chemical shift and is related to the electron density around the nucleus. The instrument detect the energy released when the excited nuclei return back to equilibrium. In NMR spectra chemical shift is plotted against the intensity (Christensen, 2015). Figure 1.3 illustrate at typical ^{1}H NMR spectrum of alginate. The monad, diad, and G-centered triad frequencies can be calculated from the peak areas. For long chains in which the end effect can be neglected, the following relations holds

$$F_G + F_M = F_{GG} + F_{GM} + F_{MM} + F_{MG} = 1$$

$$F_{MG} = F_{GM} = F_{GGM} + F_{MGM}$$

$$F_G = F_{GGG} + F_{GGM} + F_{MGG} + F_{MGM}$$

$$F_{GGM} = F_{MGG}$$

When the triad frequencies are determined, the average length of G-blocks ($N_{G>1}$) can be calculated

$$N_{G>1} = \frac{n_G - n_{MGM}}{n_{GGM}} = \frac{F_G - F_{MGM}}{F_{GGM}}$$

(Donati and Paoletti, 2009)

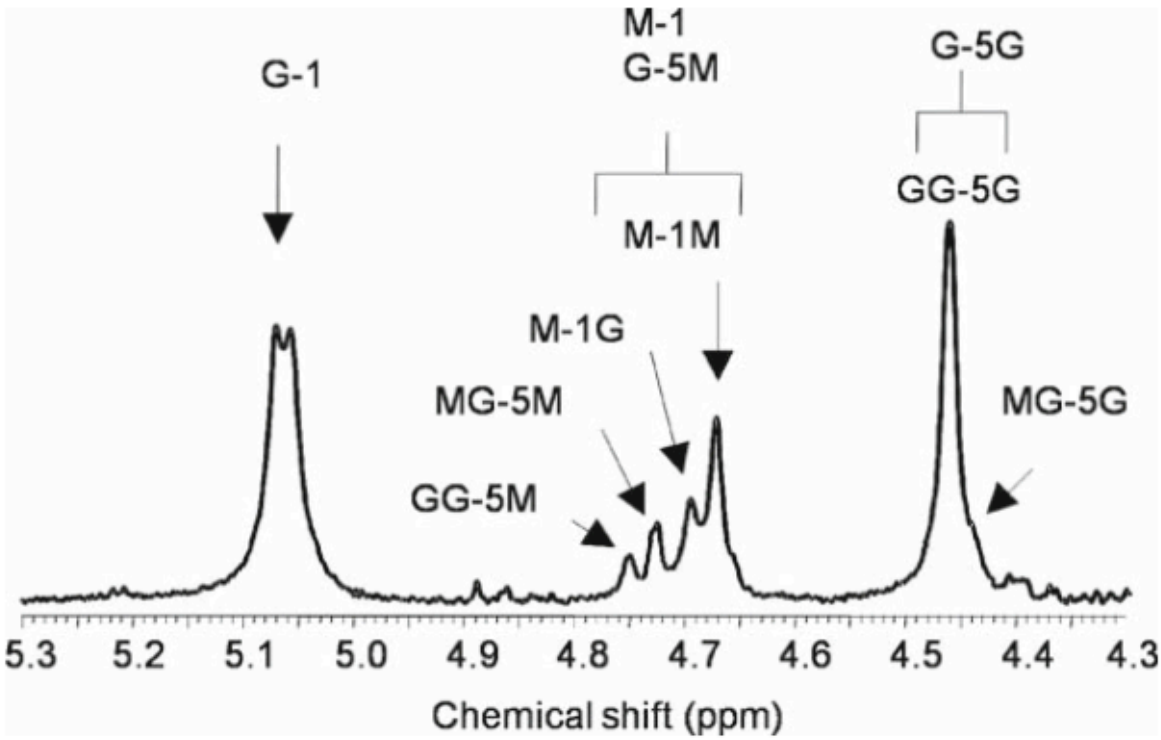

Figure 1.3: ^{1}H-NMR spectrum of *Laminaria hyperborea* stipe ($F_G \sim 0.63$). M-1M and M-1G denote the anomeric proton of an M residue neighboring another M residue or a G residue, respectively. MG-5M, GG-5M, and MG-5G refer to the H-5 proton of the central G residue in an MGM, GGM, or MGG triad, respectively. G-1 refers to the anomeric proton of G residues and GG-5G represent the anomeric proton of G residues in G-blocks (Donati and Paoletti, 2009).

1.2.4 Physical Properties

Solubility

Sodium alginate, like most other monovalent salts of alginate, is soluble in water. However, the solubility depends on parameters like pH and ionic strength. When alginate is dissolved in water, the associated counter ions dissociate and increase the entropy of mixing for the whole system. High concentration of any salt will reduce the solubility due to reduction in entropic gain from the release of the counter ions. In fully protonated form alginate is insoluble in water. The protonation and deprotonation of the carboxyl group depend on the acid dissociation constant (pK_a), which is 3.38 for mannuronic acid and 3.65 for guluronic acid.

The pK_a value of alginate chains does differ slightly from that of its monomeric units, depending somewhat on alginate composition, alginate concentration and ionic strength. If the pH is lowered below pK_a, precipitation or gel formation occurs. The molecular weight and composition of the alginate play a role in determining the pH of precipitation. Most divalent ions will also reduce the solubility as they act as cross linkers during hydrogel formation (Donati and Paoletti, 2009).

Viscosity

In general alginate solutions are highly viscous. This is caused by the stiff and extended conformation of the alginate chain, which gives alginate a large hydrodynamic volume. Polysaccharides in general are stiff molecules due to the restricted rotation around the glycosidic bonds. G-block have in particular a rigid structure because of diaxial linked guluronic acid residues. The electrostatic repulsion between the charged groups on the alginate molecule will additionally contribute to the extension of the polymer chain. As a consequence, the viscosity will depend on ionic strength of the solution. Naturally, the viscosity depends on molecular weight of the alginate and alginate concentration as well. Temperature will also have small effects on the viscosity of the solution (Skjåk-Bræk et al., 2006). High viscosity can be beneficial for some applications, such as in the food industry, however, high viscous solutions can be difficult to handle (e.g. sterile filtration) and may reduce cell viability due to the shear force required to mix it with cells (Kong et al., 2003).

Viscosity (η) is the internal friction of a liquid or its tendency to resist flow. It is defined as the ratio of shear stress (σ) and shear rate ($\dot{\gamma}$)

$$\eta = \sigma/\dot{\gamma}$$

The unit of measurements for viscosity is Pascal second ($Pa \cdot s$). For Newtonian fluids, the shear rate is directly proportional to the shear rate and viscosity is independent of the shear rate. However, most fluids are non-Newtonian: The viscosity can either decrease (pseudoplastic/shear thinning) or increase (dilatant/shear thickening) with increasing shear rate, or a minimum shear stress must be exceeded before flow begins (Bingham plastic). In general, alginate solutions are pseudoplastic. The viscosity can be measured by a rotational rheometer equipped with a cone and plate. The fluid is held by its own surface tension between a flat surface and a cone of small angels that barely touches the surface, see Figure 1.4. The flat surface is held stationary, while the cone is rotated, and the torque caused by the drag of the fluid is measured. Alternatively, the torque can be controlled and the displacement

of the cone can be measured. The viscosity can be calculated from the torque (M), the angel of the cone (δ), the radius of the cone (R), and angular velocity (ω) of the cone

$$\eta = 3\delta M/2\pi R^3\omega$$

A special feature with the cone and plate geometry is that the shear rate ($\dot{\gamma} = \omega/\delta$) is identical at all points in the fluid, provided that the cone angel is small (<5°) (Bourne, 2002).

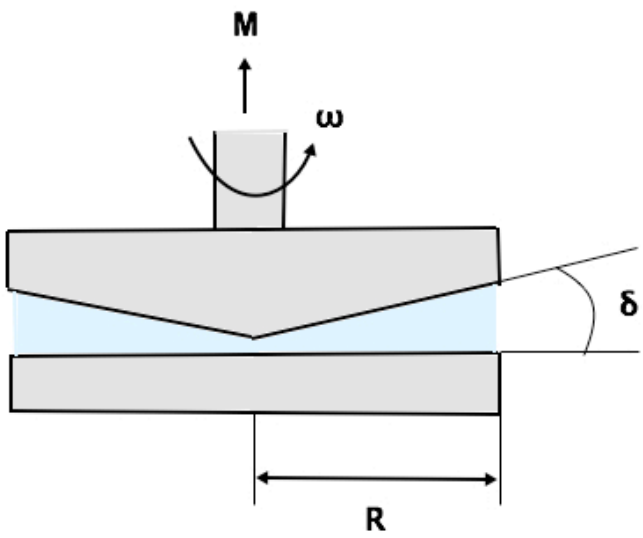

Figure 1.4: Cone and plate geometry. Adapted from (Bourne, 2002).

Ion binding

The ion-binding properties of alginates are the basis for their gelling properties. Most divalent metal ions, except Mg^{2+}, form gels or precipitates with alginate. The affinity of alginates for alkaline earth metals increases in the order $Mg^{2+} << Ca^{2+} < Sr^{2+} < Ba^{2+}$ (Haug and Smidsrod, 1970). The binding of ions is highly selective and the affinity depends strongly on the alginate composition. More specifically, the affinity towards divalent ions increase with increasing amount of G-blocks present in the alginate. The binding strength of divalent ions for the three block structures are as follows

GG-blocks: $Ba^{2+} > Sr^{2+} > Ca^{2+} >> Mg^{2+}$

MM-blocks: $Ba^{2+} > Sr^{2+} \sim Ca^{2+} \sim Mg^{2+}$

MG-blocks: $Ca^{2+} > Ba^{2+} \sim Sr^{2+} \sim Mg^{2+}$

(Smidsrød, 1974, Mørch et al., 2006). The ability to selectively bind similar ions can not be explained by non-specific electrostatic binding alone, but can be explained by chelation

caused by structural features in the G-blocks. This characteristic property is well described by the egg-box model (Grant et al., 1973, Braccini and Pérez, 2001, Sikorski et al., 2007), see Figure 1.5. When two or more G-blocks align, cavities between them form due to the perpendicular position of the G-monomers relative to the chain axis caused by diaxial glycosidic linkages. These cavities act as a binding site for divalent ions, where each cross linking ion interacts with two adjacent G residues in one chain and two adjacent G residues in an opposing chain, leading to the formation of junction zones. The binding process can be described as a cooperative process with an unfavorable binding of the first ion and a more favorable binding of the following ones. In the case of Ca^{2+}, a minimum of eight adjacent G residues are required for the formation of a stable junction (Stokke et al., 1993, Bowman et al., 2016). It has later been proposed that junction zones associate laterally beyond a pure dimerization, which increase with increasing calcium concentration and G content (Stokke et al., 2000). Studies have also suggested that MG-blocks are involved in junction formation with calcium as well, both as mixed MG/GG junctions and as pure MG/MG junctions (Donati et al., 2005).

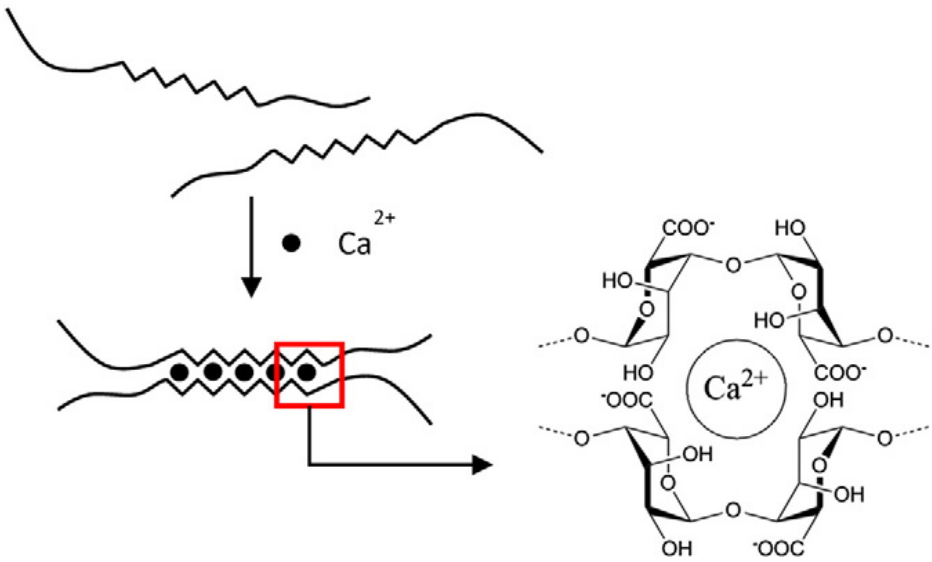

Figure 1.5: The egg-box model for binding for divalent cations to G-blocks in alginate (Paques et al., 2014).

1.3 Alginate Hydrogels

Hydrogels are three-dimensional networks composed of hydrophilic polymer chains that are capable of holding large amounts of water. Alginate hydrogels are physical gels, or more specific ionotropic gels, as the network of alginate chains are held together by ionic forces (Hoffman, 2012). The driving force for hydrogel formation is the significantly higher affinity of alginate for divalent ions, like Ca^{2+}, than for monovalent ions, typically Na^{+} (Donati and Paoletti, 2009). The properties of alginate gels are mainly determined by the chemical composition and sequential structure of the alginate, as well as by the molecular weight and molecular weight distribution. Important properties include gel strength, porosity, polymer distribution, syneresis, swelling, and polymer leakage (Draget et al., 1997).

1.3.1 Hydrogel Formation

There are two fundamental methods for preparing alginate hydrogels: the diffusion setting method and the internal gelation method. With the internal gelation method the calcium ions are released in a slow and controlled manner. This is achieved by using an inaccessible form of calcium (e.g. $CaCO_2$, Ca-EDTA, calcium citrate) in combination with a slowly hydrolyzing molecule such as D-glucono-δ-lactone (GDL). When the pH is slowly lowered, the calcium ions will be released gradually to form a homogeneous gel (Draget et al., 1990). In general, internally set gels are prepared in calcium-limited conditions to favor the formation of homogeneous gels, but compared to saturated calcium alginate gels their mechanical strength will be lower (Donati and Paoletti, 2009). In the diffusion setting method the cross-linking ions (e.g. Ca^{2+}) are allowed to diffuse from a large outer reservoir into an alginate solution. Alginate gel beads are prepared with this method (Skjåk-Bræk et al., 1989), see chapter 1.4. Alginate gels are special in the sense that the setting of the gel is independent of temperature, and that the gel is heat stable (Smidsrød and Draget, 1997).

1.3.2 Syneresis

Alginate gels are regarded as non-equilibrium gels. The binding of divalent ions to binding sites in alginate is very rapid, but the cross-linking is sub-optimal due to the limited diffusion of the polymer chains. To form a network with the maximum possible number of interguluronate sites, a series of dissociation/association steps after the initial crosslinking is necessary. However, dissociation of junction zones is kinetically unfavorably. Therefore, the building of a network structure with the maximum possible number of interguluronate sites is slow (Skjåk-Bræk et al., 2006, Andresen and Smidsørod, 1977).

Syneresis refers to the shrinkage of alginate gels after preparation, which leads to loss of water and an increased polymer concentration in the gel relative to that in the alginate solution. Syneresis depends on the alginate composition and sequence. Due to the formation of strong, irreversible junctions, gel prepared from alginates containing long G blocks will shrink less than gel prepared from alginates with shorter G blocks. The strong irreversible junctions will hinder the reorganization of the network structure (Martinsen et al., 1989). Alginates containing high amounts of alternating sequences (MG-blocks) will, however, undergo syneresis to a large extent. This have been related to the flexibility of MG-blocks, thus allowing denser packing of the gel network (Draget et al., 2001). In addition, it is believed that lateral association of junction zones beyond dimerization (Stokke et al., 2000) and secondary MG/MG junctions upon increasing calcium concentration (Donati et al., 2005) contribute to shrinkage of the alginate gel.

Besides G-block length and introduction of elastic segments, the molecular weight also has an effect on the syneresis of alginate gels. Alginate gels prepared from alginates with low molecular weight show a reduced syneresis. When a fraction of a high molecular weight alginate is replaced by short alginate molecules enriched in guluronate residues, the same effect is observed. This is suggested to stem from an increased loose-end fraction, which leads to a reduction in possible attachment sites for the slowly forming junctions, and thereby restrict further contraction of the primary network (Draget et al., 2001). In addition, syneresis increase with increasing calcium concentration and longer gelling time up to a certain point (Martinsen et al., 1989). Syneresis often represent a challenge in the manufacturing of food gels, but the partial collapse of the gel network increases the stability of the hydrogel and reduces its porosity, which is advantageous in the case of encapsulation for cells for transplantation (Martinsen et al., 1992).

1.3.3 Mechanical Properties

Alginate gels used in food and biomedical applications should have mechanical properties that enables them to withstand relevant stresses (e.g. compression, shear) (Donati and Paoletti, 2009). The elasticity of a network is a measure of its deformation under stress and is often described by the rubber elasticity theory (Smidsrød and Moe, 2008). In an ideal rubber elastic body, all the energy from deformation is stored as reduced entropy of the chain network. When the force of deformation is removed, the body will regain its original shape. The theory predicts that the elastic modulus will increase in proportion with the temperature, however most biopolymer gels do not show this behavior. In biopolymer gels, the cross-links are low

energy physical interaction that are not limited to single points but are extended junction zones, and the chains are stiff and extended. The energy of deformation must therefore be used for bending of bond angles and lengths, and distortion of the junction zones. The energy will be lost by heat dissipation, and biopolymer networks are thus called enthalpic rather than entropic. The elastic modulus will for these networks decrease with increasing temperature (Smidsrød and Moe, 2008).

Young's modulus – small deformation

Young's modulus describes the elastic properties of a solid undergoing tension or compression in only one direction. Young's modulus is a measure of the ability of a material to withstand changes in length when under lengthwise tension or compression (stiffness). Sometimes it is referred to as modulus of elasticity. Young's modulus (E) can be expressed mathematically as

$$E = \frac{\sigma}{\varepsilon} = \frac{F/A}{\Delta L/L}$$

where σ is the stress, ε is the strain, F is the tensile/compression force, A is the cross-sectional area, L is the original length, and ΔL is the change in length, see Figure 1.6. The unit of Young's modulus is newtons per square meters (N/m^2). This is a specific form of Hooke's law of elasticity, which states that for relative small deformations of an object, the deformation is directly proportional to the force. Under these conditions the object returns to its original shape and size upon removal of the force. Therefore, Young's modulus is only meaningful in the range in which the stress is proportional to the strain (Bourne, 2002). Young's modulus is frequently used to measure the gel strength of alginate gels (Donati and Paoletti, 2009). A mechanical deformation curve, or a stress-strain curve, can be obtained for an alginate gel by compressing the gel at a constant rate of deformation and measure the force. Young's modulus is calculated from the slope in the initial part of the curve.

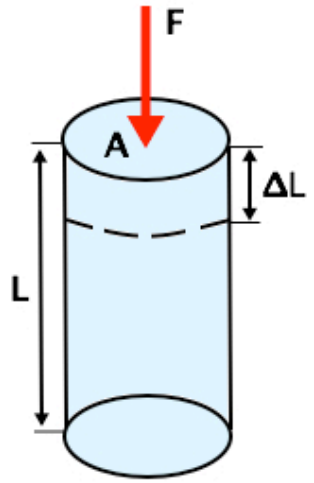

Figure 1.6: Young's modulus is defined as the ratio of stress to strain where stress is defined as the compression force (F) per unit area (A) and strain is defined as the relative deformation, i.e. change in length (ΔL). Adapted from (Smidsrød and Moe, 2008)

In general, the elastic modulus depends on the number and the strength of the cross-links, as well as the length and stiffness of the chains between the cross-links (Skjåk-Bræk et al., 2006). Consequently, the elastic modules depends on the concentration of cross-linking ions. As mentioned earlier, calcium saturated alginate gels have higher mechanical strength than calcium-limited gels. When a higher number of cross-linking ions are provided to the system, more junctions will be formed (Martinsen et al., 1989). In addition to ion concentration, the type of cross-linking ion has an effect on the gel strength. It has been reported that the minimum G-block length required for junction formation decreases upon the increase in affinity of the ion towards alginate, which gives a higher number of junctions per volume (Stokke et al., 1993).

Moreover, the mechanical properties are influence by the alginate composition and sequence. In general, alginates rich in guluronic acid residues form stronger gels, while M-rich alginates form softer gels (Smidsrød and Haug, 1972). Studies have shown that introducing alternating structures (MG-blocks) in alginates give gels with higher elastic modulus (Draget et al., 2000). This effect was first attributed to the fact that MG-blocks have higher intrinsic flexibility compared to other block structures in alginate, but the involvement of MG-blocks in calcium-induced junctions have later been proposed as an additional explanation (Donati et al., 2005). In addition to alginate composition and sequence, the concentration and molecular weight of the alginate have to be considered. The gel strength increases proportional to the square of the alginate concentration

$$E \propto c^2$$

(Smidsrød et al., 1972). The elastic modulus of a gel is independent of molecular weight above a certain limiting value, which depend on the method of preparation. For molecular weight below this value the gel strength decrease with decreasing molecular weight (Martinsen et al., 1989).

Rupture strength – large deformation

The rupture strength is the stress required to cause network breakage. The fracture of alginate gels result from disruption of junction zones. There is no direct correlation between the rupture strength and the elastic modulus (Mitchell, 1980). The rupture strength can though be correlated to the strength, length and number of cross-links. But whereas the elastic modulus becomes constant above a certain limiting molecular weight, the rupture strength continues to rise with increasing molecular weight (Mitchell, 1980). The deformation rate will also affect the fracture characteristics. Interesting, the gel fracture stress is correlated with Ca^{2+} and alginate concentration, whereas the fracture strain is independent of the same factors (Zhang et al., 2005). Gels prepared from M-rich alginates, despite having lower elastic modulus, typically show a higher rupture strength than those prepared from G-rich alginate. Therefore, M-rich alginates are characterized as more elastic, while G-rich alginates produce more brittle gels (Donati and Paoletti, 2009). The shorter, stiffer polymer chains of the latter transmit more energy to the network junction zones, thereby facilitating rupture (Skjåk-Bræk et al., 2006).

1.4 Alginate Gel Beads

1.4.1 Formation of Microbeads

Immobilization of cells in alginate gel beads is carried out in a single step by the mixing of a cell suspension with a sodium alginate solution and dripping the mixture into a solution containing divalent cations (usually Ca^{2+}), see Figure 1.7. The alginate droplet forms gel spheres instantaneously, entrapping the cells in a three dimensional lattice of cross-linked alginate (Smidsrød and Skjåk-Bræk, 1990). Several techniques have been established to control the size of the droplets, hence the gel beads, including systems based on electrostatic forces to pull the droplet off the needle (Strand et al., 2002). The size of the capsules depend on the voltage, flow and needle diameter. The viscosity of the alginate solution, which depend on the concentration and the molecular weight of the alginate, will as well influence the size and shape of the beads. Due to syneresis, the composition and sequential structure of the alginate will also have an impact on the size and permeability (Strand et al., 2002).

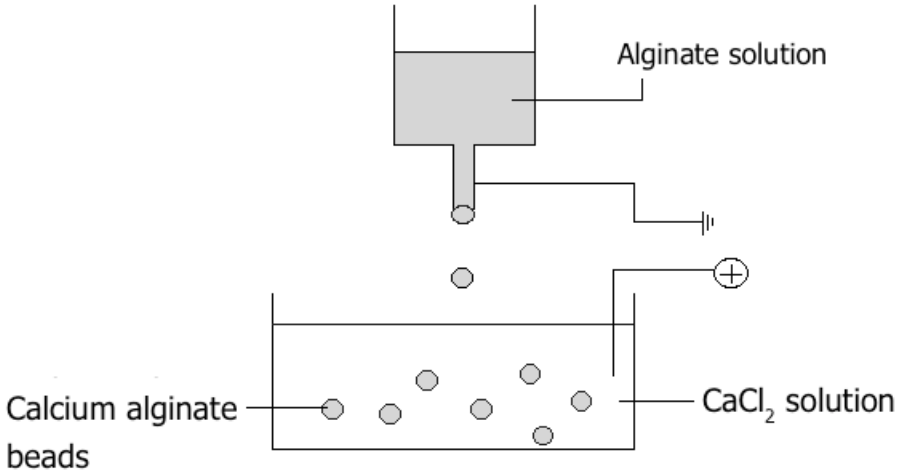

Figure 1.7: Formation of alginate gel beads by a electrostatic bead generator. Adapted from (Gåserød, 1998).

1.4.2 Stability and Biocompatibility

The main cause of breakage of capsules under physiological conditions is osmotic swelling (Thu et al., 1996). The gel can be seen as an osmotic pressure system where the gel surface acts as a semipermeable membrane through which the polymer cannot diffuse (Moe, 1993). The volume of the gel is determined by the equilibrium between the osmotic pressure and the elastic retractive force of the gel network,

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

where the osmotic pressure works to increase the volume of the gel and the elastic works to decrease the volume of the gel. The osmotic pressure results from the uneven distribution of the mobile counter ions of the fixed charges of alginate between the inside and the outside of the gel. Their concentration will always be greater inside the gel than outside since electroneutrality always must be fulfilled. Hence, a positive osmotic pressure is exerted on the semipermeable membrane of the gel, causing an inward flow of water and swelling of the gel. The elastic pressure of the gel network depends in the number and strength of the crosslinks (Moe, 1993). The physical interactions between alginate and divalent ions are far less resistant than chemical linkages. Alginate gels are especially sensitive towards chelating agents (e.g. phosphate and citrate) which have high affinity towards calcium and high concentration of non-gelling ions (e.g. Na^+ and Mg^{2+}) which can exchange calcium (Mørch et al., 2006).

To increase the stability and reduce the permeability of Ca-alginate gel beads, a polycation layer is often added to the alginate gel core. Most polycation are, however, toxic to cells, and the most commonly used polycation for encapsulation purposes, poly-L-lysine (PLL), has shown to be the main cause of cellular overgrowth (Strand et al., 2001). Both the composition and sequential structure of alginate and type of the cross-linking ion have a significant effect on the swelling properties of alginate microbeads. Guluronic acid-rich alginate gel beads swell less than corresponding mannuronic acid-rich gel beads (Darrabie et al., 2006). In addition, alginates containing long stretches of alternating structure, introduced enzymatically, have been shown to be extremely stable under physiological conditions (Mørch et al., 2007). Using barium and strontium as the crosslinking-ion in alginate gels have a strong beneficial effect with regard to size stability of high-G alginate capsules. Both barium and strontium as the crosslinking-ion in alginate gels have higher selectivity for G-blocks than calcium, but the toxicity of these ions limits their use in biomedical applications (Thu et al., 1996, Darrabie et al., 2006, Mørch et al., 2006).

Alginate gel beads will leak polymeric material to different degrees depending on the average G-block length, gelling ions, and molecular weight distribution. The leaked material has been shown to comprise the low molecular weight tail of the starting material's molecular weight distribution. In general, it has been found that the leaked material is enriched in both M content and M-doublets, and depleted in G content, G-doublets and G-triplets (Stokke et al., 1993). This can cause challenges when it comes to biocompatibility as alginates enriched in mannuronic acid has been related to stimulation of human monocytes to produce cytokines (e.g. TNF, IL-1, IL-6) (Otterlei et al., 1991, Kulseng et al., 1996). However, an *in vivo* study

performed in mice, *L. hyperborea* leaf alginate beads with intermediate G-content did not promote an immune response in form of host cell adhesion, unlike *L. hyperborea* stipe alginate beads with high G-content (Tam et al., 2011).

1.4.3 Polymer Distribution

Alginate gels prepared by diffusion of calcium ions into solutions of sodium alginate exhibit a concentration inhomogeneity. The polymer concentration is considerably higher at the surface than in the center of the gel (Skjåk-Bræk et al., 1989). The gelling mechanism of alginate is regarded as the main cause for this uneven distribution. The cross-linking ions rapidly diffuse towards the center of the droplet due to the difference in the concentration between the reservoir containing the gelling solution and the alginate bead. The rapid, and essentially irreversible, ion binding and formation of a network produce an inwardly moving gelling zone in which the activity of alginate will be zero. Alginate molecules will therefore diffuse from the center of the gel towards the gelling zone, leading to a lower concentration of alginate in the center. The diffusion of alginate is enhanced by the coupled diffusion of the counter-ion (Na). The polymer gradient is governed by the relative diffusion rate between the soluble alginate molecules and the cross-linking ion (Skjåk-Bræk et al., 1989). In general, low molecular weight alginate, low concentration of gelling ions relative to the polymer concentration and the absence of non-gelling ions give rise to the greatest inhomogeneity, while high molecular weight alginate and high concentration of both gelling and non-gelling ions give the lowest inhomogeneity (Skjåk-Bræk et al., 1989). Inhomogeneous alginate gels may be preferred in the preparation of microcapsules due to lower porosity (Martinsen et al., 1992) and higher resistance against swelling (Thu et al., 1996).

1.5 Chitosan

1.5.1 Source and Application

The raw material for all commercial production of chitosans is chitin. Chitin is a structural polysaccharide in the outer skeleton of crustaceans (e.g. shrimps, crabs and crayfish) and insects. It is also a component of the cell walls of certain fungi and algae, making it the second most abundant polymer in nature. Chitin is isolated from crustacean shells, mostly crabs and shrimps, in two steps, first by acid treatment to extract the calcium carbonate ($CaCO_3$) and then by alkaline treatment at elevated temperatures to remove proteins. Chitosan is produced from chitin by deacetylation, which is performed by hydrolysis under alkaline conditions (Younes and Rinaudo, 2015, Vårum and Smidsrød, 2006).

Chitin is highly hydrophobic and is insoluble in water and in most organic solvents, which limits its utilization. Chitosan, however, is soluble in dilute acidic aqueous solutions due to protonation of the amino groups, and is used in a wide range of applications in areas such as agriculture, cosmetics, food, water treatment and biomedicine (Ravi Kumar, 2000).Chitosan is known to be non-toxic, biocompatible and biodegradable, and exhibits diverse bioactivities such as antimicrobial activity, antifungal activity, antitumor activity and antioxidant activity (Cheung et al., 2015). In addition, chitosan can easily be molded into different shapes and forms including gels, films, sponges and fibers (Croisier and Jérôme, 2013). Lysozyme, present in all body fluids, are able to degrade chitosan, though to non-toxic oligosaccharides, which has to be considered for all application in the human body (Croisier and Jérôme, 2013). Because of the amino groups, chitosan efficiently complexes various species, such as metal ions, and is therefore often used in waste water treatment (Bassi et al., 2000, Ishii et al., 1995) The cationic character of chitosan also enables the polymer to form ionic complexes with a variety of anionic species, such as lipids, proteins, alginate, DNA and some negatively charged synthetic polymers (Rinaudo, 2006). Biomedical application for chitosan is especially related to tissue engineering, drug delivery systems and wound healing (Cheung et al., 2015). Today chitosan is also used in health food due to its cholesterol-lowering and supposed weight-reducing effects (Vårum and Smidsrød, 2006).

1.5.2 Structure and Chemistry

Chitin is a linear polymer composed of β-(1-4) linked 2-acetamide-2-deoxy-β-D-glucose (GlcNAc; A) and share many structurally similarities with cellulose. The deacetylation reaction removes the acetyl group ($-COCH_3$) and introduces a free amino group ($-NH_2$) that carries a charge at acidic conditions, see Figure 1.8. Chitosan consists therefore of two

monomers, A-residues and 2-amino-2-deoxy-D-glucose (GlcN; D), and can be prepared with different chemical compositions (denoted as the content of A in the polymer chains; F_A), where the A- and D-residues are believed to be randomly distributed along the chains, with varying chain lengths. Chitosans are amphiphilic polymers as the amino groups can become positively charged and as the acetylated amino groups are hydrophobic. As a result, the chemical, physical and biological properties of chitosans vary considerably depending on the chemical composition (Vårum and Smidsrød, 2006).

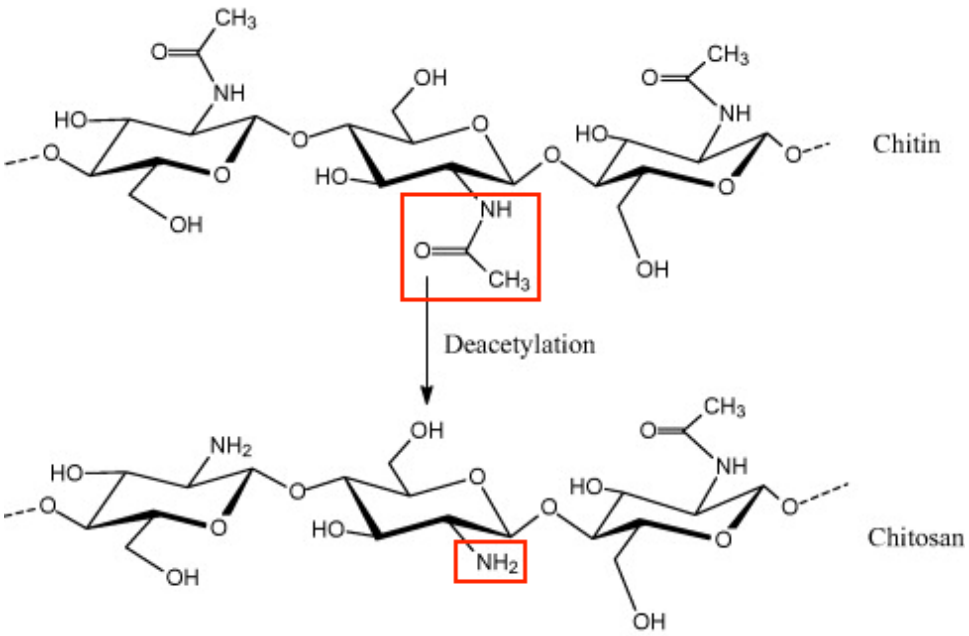

Figure 1.8: The chemical structure of chitin and chitosan (Tran et al., 2011)

The charge density of chitosans depends on several factors such as chemical composition (F_A), pH and ionic strength. The dissociation constant (pK_a) for chitosans ranges from 6.2 to 7.0. At pH values below 6, all chitosans are soluble, but with increasing pH the solubility decreases. However, the solubility increases with increasing F_A and chitosans with F_A between 0.4 and 0.6 can be considered to be soluble at neutral pH. Low-molecular-weight chitosans are also soluble at neutral pH where the solubility increases with decreasing molecular weight (Vårum and Smidsrød, 2006, Khong et al., 2013).

1.5.3 Gel Formation

No simple ionic and nontoxic crosslinking agent have been found that gives reproducible chitosan gels at low concentrations, such as calcium ions for gelling of alginates (Vårum and Smidsrød, 2006). However, many chitosan gelling systems have been reported, including both chemical and physical gels (Nilsen-Nygaard et al., 2015). The primary amino group of the D-residue is a good target for covalent crosslinking and many different covalent crosslinking

agents have been suggested, such as glutaraldehyde (Argüelles-Monal et al., 1998) and diethyl squarate (Neimert-Andersson et al., 2011), however not all of them are biocompatible (Nilsen-Nygaard et al., 2015). One way to prepare physical chitosan gels is by the use of glycerol phosphate in combination with temperature, which leads to protons being transferred from chitosan to glycerol phosphate, resulting in more uncharged chitosan chains that aggregate (Chenite et al., 2001). Alternatively, physical chitosan gels can be formed by controlled addition of alkali (Montembault et al., 2005), or addition of ionic crosslinkers such as phosphates and citrates (Shu and Zhu, 2002) or transition metal ions such as molybdate (Draget et al., 1992).

1.6 Alginate Chitosan Oligomer Gels

Polyelectrolyte complexation is usually associated with associative phase separation and precipitation, but can be a mechanism for physical gelling under the right conditions. By adjusting parameters like relative polymer concentration and molecular weight (e.g. using oligomers), precipitation can be avoided (Nilsen-Nygaard et al., 2015). Chitosan can form complexes with a large number of different poly-anions like DNA, xanthan, pectin and alginate (Luo and Wang, 2014), and gelling systems composed of alginate and chitosan where one of them are present as oligomers have been reported (Khong et al., 2013).

A pK_a-value of ca. 6.5 for the amino groups of chitosan and a pK_a-value of ca. 3.5 for the carboxyl groups of alginate indicate that there should be a strong ionic interaction between the positively charged chitosans and the negatively charged alginates in the pH interval from 4 to 6. The reason chitosan oligomers are especially suitable for cross-linking alginate are the structural similarities between chitosan and M-blocks in alginate: the monomers exist in the $^4C_1$ conformation, the monomers are linked through β-(1,4) diequatorial glycosidic linkage, and the monomers are rotated 180° relative to the neighbouring monomer. Consequently, every second sugar unit in both chitosan and poly-M will have a charged group pointing in the same direction with a distance between the charges of 10.3-10.4 Å (Khong et al., 2013).

Khong and co-workers managed to successfully prepare alginate chitosan oligomer gels by crosslinking alginate containing only mannuronic acid (poly-M) with fully de-N-acetylated (F_A = 0.002) chitosan oligomers (CO). The gels were prepared by mixing the alginate solution with the CO-mixture at a pH well above 7 and then lowering the pH in a controlled manner by adding the slowly hydrolysing D-glucono-δ-lactone (GDL) (Khong et al., 2013). Later, Feng and co-workers have managed to successfully prepare alginate gels with a combination of calcium and chitosan oligomers as crosslinkers. The alginates had a mannuronic acid content

of 54 and 32 %. The gels were prepared by the same internal gelation protocol, where GDL was used to protonate both $CaCO_3$ and chitosan oligomers simultaneously (Feng et al., 2017), see Figure 1.9.

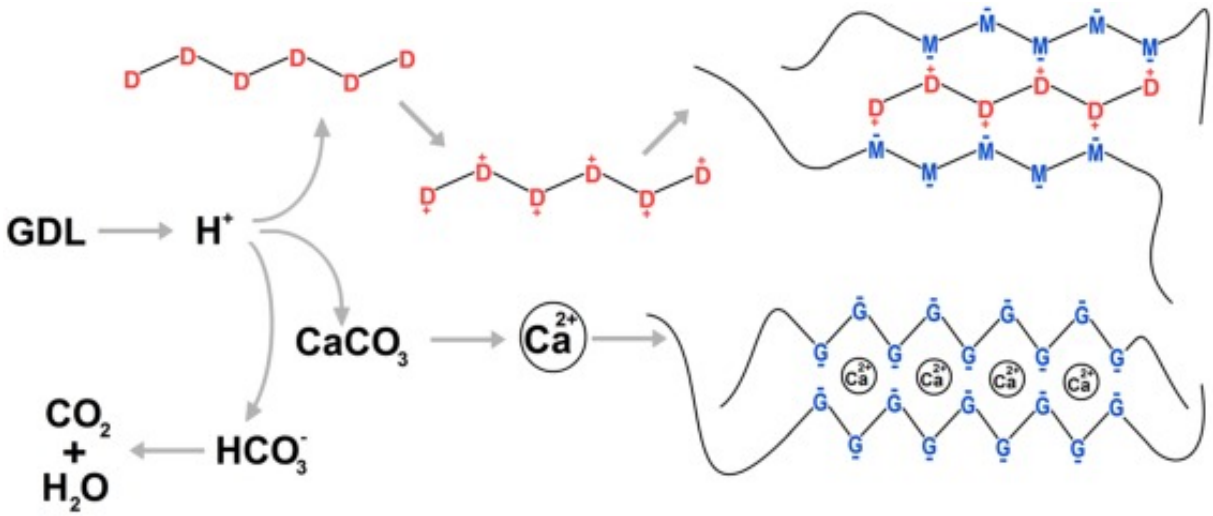

Figure 1.9: Schematic illustration of internal gelation of alginate crosslinked with calcium and chitosan oligomers. D: glucosamine, M: mannuronic acid, G: guluronic acid, GDL: D-glucono-δ-lactone (Feng et al., 2017).

An alginate gelling system containing crosslinkers with high affinity towards both M- and G-blocks may have some advantages. One advantage is higher mechanical strength for gels prepared from alginates rich in mannuronic acid. A second advantage is the possibility to drastically reduce the calcium concentration without a reduction in gel strength (Feng et al., 2017). It has been shown that Ca^{2+} released from calcium alginate gels can promote inflammatory responses for certain cell lines (Chan and Mooney, 2013).

2 Materials and Methods

2.1 Materials

High molecular weight sodium alginate isolated from *Laminaria hyperborea* leaf (GP3350) and low molecular weight sodium alginate isolated from *L. hyp.* stipe (LF10/60, lot number S22039) were purchased from FMC Biopolymer (Norway). Low molecular weight (LF10/40) and very low molecular weight sodium alginate isolated from *L. hyp.* leaf (LFR5/60 RB) and stipe (LFR5/60, lot number S21799) were purchased from the same company. Detailed chemical information about the alginates is displayed in Table 2.1. Koyo Chemical (Japan) provided the fully de-N-acetylated chitosan oligomer mixture (lot number 121017WG). The mixture contained oligomers ranging in length from dimers to twentymers with an average degree of polymerization (DP_n) of 4. Oligomers with $DP_n < 6$ was fully de-N-acetylated, while the average acetylation degree for the complete mixture was 0.05.

Table 2.1: Chemical composition, molecular weights and intrinsic viscosity of alginate isolated from *L. hyperborea* leaf and stipe.

	F_G	F_{GG}	F_{MG}/ F_{GM}	F_{MM}	F_{MGG}/ F_{GGM}	F_{MGM}	F_{GGG}	$N_{G>1}$	[η] (ml/g)	M_w^* (kDa)	M_n^* (kDa)
High MW leaf	0.46	0.29	0.17	0.37	0.05	0.15	0.24	7	958	200	105
Low MW leaf	0.50								560	115	43
Very low MW leaf	0.51	0.34						7		34	17
Low MW stipe	0.68	0.57	0.11	0.21	0.04	0.08	0.53	14	592	103	53
Very low MW stipe	0.68	0.58	0.10	0.21	0.04	0.07	0.54	16	147	33	22

* The standards were 10-20 % underestimated, molecular weight averages of alginate samples may therefore be correspondingly underestimated.

Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), sodium chloride (NaCl), sodium nitrate ($NaNO_3$,) and sodium acetate (CH_3COONa) were purchased from Merck KGaA (Germany). Calcium carbonate ($CaCO_3$) was provided by KSL staubtechnik gmbh (Germany). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, $C_8H_{18}N_2O_4S$), deuterium oxide (D_2O) and GDL (D-(+)-gluconic acid δ-lactone, $C_6H_{10}O_6$) were all manufactured by Sigma Aldrich (USA). TTHA (triethylenetetraaminehexaacetic acid, $C_{18}H_{30}N_4O_{12}$, 0.3 M, pH ~ 7), TSP

(trimethylsilylpropinate, $C_6H_{14}O_2Si$, 1 %) and NMR sampling tubes were also purchased from Sigma Aldrich (USA). EDTA (ethylenediaminetetraacetic acid, 100 mM, pH 7.0) was provided by VWR BDH Prolabo Chemicals (USA).

2.2 Stability of Alginate Chitosan-Oligomer Gel Beads in Saline

2.2.1 Preparation

Alginate was dissolved in Milli-Q water at a concentration of 3.6 % (w/v). The chitosan oligomer (CO) mixture was dissolved in HEPES buffer (20 mM) with at a concentration of either 0.76 % (w/v) or 1.36 % (w/v) and the pH was adjusted to 7.5. Equal volumes of the alginate solution and the chitosan oligomer mixture solution were mixed vigorously to make an alginate – CO solution. For comparison, a solution containing only alginate was prepared by mixing equal volumes of alginate solution (3.6 % (w/v)) and HEPES buffer (20 mM) and adjust the pH to 7.5. The final alginate concentration was 1.8 % for all the samples and the final CO-mixture concentration was either 0.38 or 0.68 %. The gelling solutions were prepared by dissolving $CaCl_2$ (50 mM) in acetate buffer (10 mM) with a pH adjusted to 5.0 or in HEPES buffer (10 mM) with a pH adjusted to 6.5.

Gel beads were prepared using an electrostatic bead generator constructed at the Department of Physics at NTNU, see Figure 2.1. The voltage was set to 7 kV and the alginate-CO solutions were injected into the bead generator with a 10 mL syringe at a rate of 10 mL/h. The needle had a diameter of 0.35 mm and was placed 2-3 cm above the gelling solution. The gelling solution had a volume of 150-300 mL. The gel beads were prepared from a certain volume of the alginate-CO solutions (0.5-5 mL) depending on the amount needed for further use by controlling the time of operation. The gel beads were left in the gelling solution for 10 min after the last droplet.

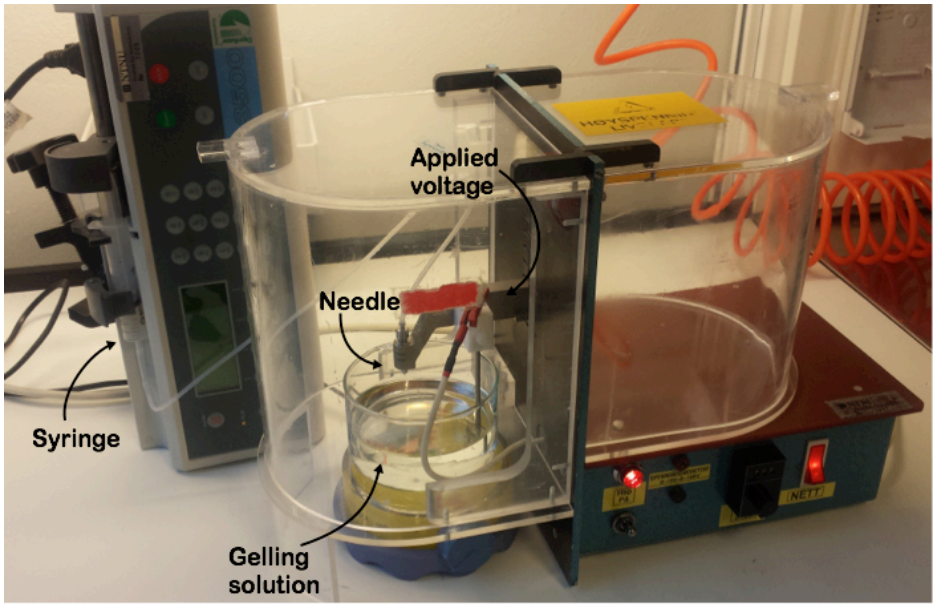

Figure 2.1: Electrostatic bead generator with a syringe pump. Manufactured at the Department of Physics, NTNU.

2.2.2 Swelling

Three samples were prepared: Leaf alginate-CO gel beads (high molecular weight alginate) with a CO-mixture concentration of 0.68 % gelled in $CaCl_2$ solution with a pH of either 5.0 or 6.5, and leaf alginate gel beads gelled in $CaCl_2$ solution with a pH of 6.5. Most of the gelling solution was poured off and the gel beads were transferred to 5 mL tubes. Any remaining gelling solution was removed with a pipette and 0.9 % NaCl (3 mL) was subsequently added. The diameter of the gel beads (n=20) was measured in a microscope (Eclipse TS100, Nikon) (Figure 2.2) before the beads were incubated on a gyrator shaker (10 rpm) at room temperature for one hour. After one hour the swelling of the gel beads was measured before the saline was exchanged. This was repeated five times (six treatments in saline in total). To compare the samples, the relative increase in diameter was calculated from the initial size and the size after six saline treatments.

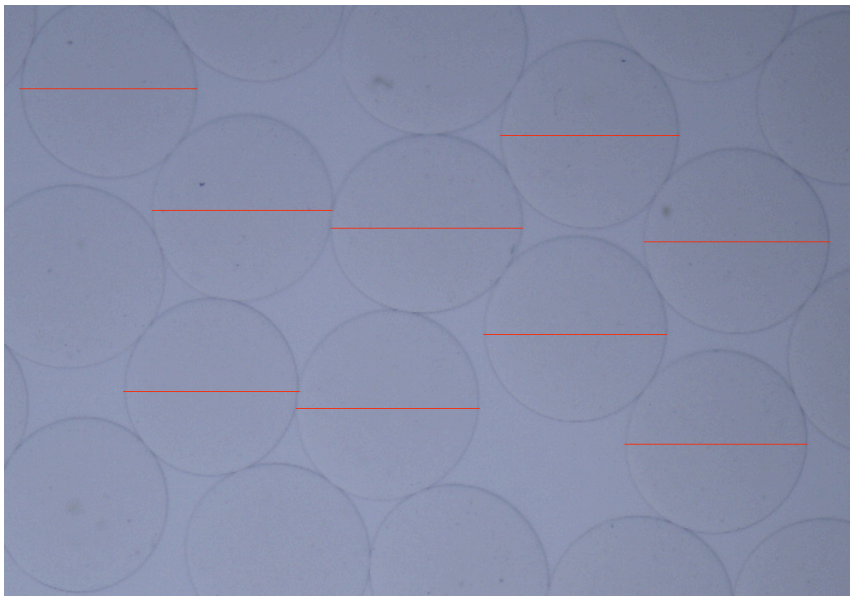

Figure 2.2: Alginate-CO gel beads at 4X magnification (Eclipse TS100, Nikon). The diameter of 20 beads was measured for each sample.

2.2.3 Polymer Leakage

Three samples were prepared: Leaf alginate-CO gel beads with a CO-mixture concentration of 0.68 % gelled in a $CaCl_2$ solution with a pH of either 5.0 or 6.5, and leaf alginate gel beads gelled in a $CaCl_2$ solution with a pH of 6.5. The gelling solution was removed by using a filter, and washed three times with 15 mL 0.9 % NaCl. The saline was collected (45 mL in total for each sample), and the gel beads were transferred from the filter to a blue-cap bottle together with 40 ml 0.9 % NaCl solution. The bottles were kept on a gyrator shaker (10 rpm) at room temperature overnight. The saline was collected and pooled with corresponding

samples of previous collected saline (resulting in 85 mL in total for each sample). The samples were dialyzed (MWCO 150-500 Da. Spectrum Laboratories, Inc., USA) against MQ water, until the conductivity had sunk below 1 S/m. The sample pH was adjusted to 7.0 and the samples lyophilized overnight (Alpha 1-4 LD freeze dryer).

To remove excess calcium, the leaked material was dissolved in MQ water by adding EDTA (final concentration of 10 mM). The samples were dialyzed (MWCO 100-500 Da) first against 50 mM NaCl (4 shifts) and then against MQ-water (6 shifts). The sample pH was adjusted to 7.0 and the samples lyophilized overnight. The leaked material was dissolved in 15 mL MQ water and transferred to a smaller container. The samples were lyophilized overnight before the leaked material finally was weighted.

NMR

^{1}H-NMR analysis was performed by Wenche Irene Strand (NTNU). About 5 mg sample was transferred to Eppendorf tubes and dissolved in 600 µL D_2O. The pH was adjusted to 8. The samples were transferred to NMR sample tubes, where 5 µL TSP was added as a chemical shift reference and 20 µL TTHA was added as a chelator for divalent ions. The NMR analysis was performed on a Bruker 400 MHz Advance III HD equipped with a 5-mm SmartProbe z-gradient probe and SampleCase at 90 ^{o}C. The spectra was recorded and further processed by the software TopSpin^{TM}. The monad, the diad and G-centered triad frequencies were determined from the ^{1}H spectra as described by Grasdalen and coworkers (Grasdalen, 1983).

2.3 Physical Properties of Leaf Alginate Gel Cylinders

2.3.1 Preparation

The alginate content of the gel cylinders is displayed in Table 2.2. Gel cylinders with an alginate concentration of 1.0 %, 1.8 % and 2.8 % were prepared by dissolving 375 mg, 675 mg and 1050 mg alginate, respectively, in 25 mL MQ water using a 250 mL flask with suction over night. 56 mg $CaCO_3$ was dissolved in 5 mL MQ water (corresponding to a final concentration of 15 mM) and added to the alginate solution. The mixture was degassed using a vacuum suction for 15-30 minutes. 200 mg GDL was dissolved in 7.5 ml MQ water and added to the alginate/$CaCO_3$ solution. The mixture was stirred gently for 10 seconds before poured into the eight middle positions of a 24 well plate, see Figure 2.3. The lid was closed after every position had a positive meniscus. After 20 hours the gel cylinders were removed from the wells to a 1 L beaker and dialyzed against 50 mM $CaCl_2$ in 0.2 NaCl for 24 hours at 4 ^{o}C.

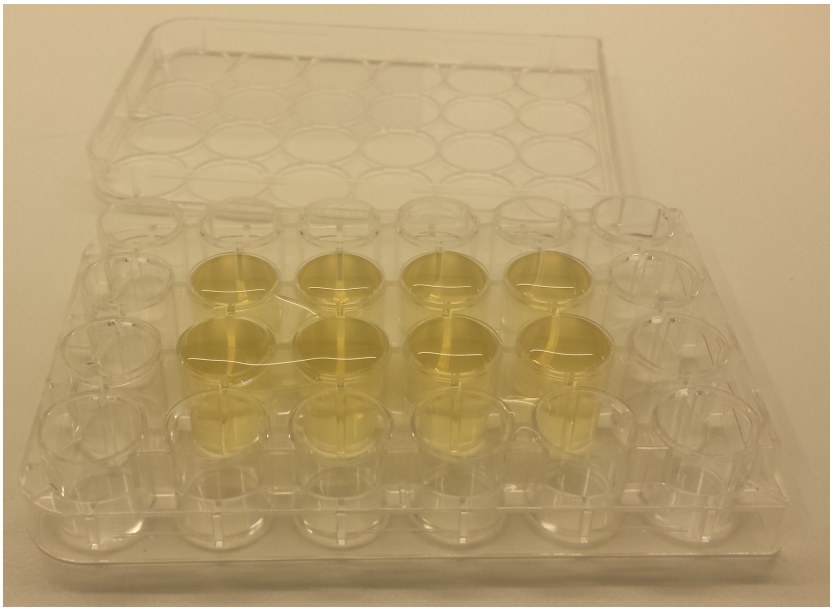

Figure 2.3: Gel cylinders casted in the eight middle positions of 24-well plates. The alginate solutions were poured into the wells until a positive meniscus was made before the lid was put on.

Table 2.2: Alginate content in gel cylinders prepared for measurement of gel strength and syneresis.

No.	Samples
1.	High MW leaf (1.0 %)
2.	High MW leaf (1.8 %)
3.	High MW leaf (2.8 %)
4.	Low MW leaf (1.0 %)
5.	Low MW leaf (1.8 %)
6.	Low MW leaf (2.8 %)
7.	Low MW stipe (1.0 %)
8.	Low MW stipe (1.8 %)
9.	Low MW stipe (2.8 %)
10.	High MW leaf (1.8 %) + very low M_w leaf (1.0 %)
11.	Low MW leaf (1.8 %) + very low M_w leaf (1.0 %)
12.	Low MW stipe (1.8 %) + very low M_w leaf (1.0 %)
13.	High M_w leaf (1.8 %) + very low M_w stipe (1.0 %)
14.	Low MW leaf (1.8 %) + very low M_w stipe (1.0 %)
15.	Low MW stipe (1.8 %) + very low M_w stipe (1.0 %)

2.3.2 Young's Modulus, Rupture Strength and Syneresis

After calcium saturation of the gel cylinders the diameter and the radius of the gel cylinders were measured using a digital caliper from PreciTech AS (Norway). The gel cylinders were also weighted. Syneresis was calculated as the weight loss of the gels in percentage, see appendix C. The initial weight is a theoretical weight that is based on the volume of the wells and the assumption that the density of the alginate solution are the same as water. The mechanical properties were analyzed by uniaxial compression (0.10 mm/s test speed) using a texture analyzer (TA.XT *plus* texture analyser, Stable Micro Systems, UK) equipped with a 5 kg or a 30 kg load cell and a cylindrical 35 mm diameter aluminum probe. Young's modulus, rupture strength and deformation at rupture were calculated from the stress/strain curves by Texture Exponent software v.5.1.2.0, see appendix D for more details. Young's modulus was corrected with regard to new alginate concentration, as a result of syneresis, calculated from the weight of the gel cylinders.

2.3.3 Viscosity

The samples listed in Table 2.3 were prepared using a solution volume of 5 mL and the shear viscosity was measured using a rotational rheometer (Kinexus Ultra +, Malvern, UK) equipped with a cone/plate (4°/40 mm). The measurements was conducted at 20 ^{o}C for shear rates between 0.5 and 30.0 s^{-1} or between 0.1 and 100.0 s^{-1} with six measurements per decade with a logarithmic progression. rSpace Software was used for test settings and data processing.

Table 2.3: Alginate solutions in which viscosity measurements were conducted on.

	Samples
1.	High MW leaf (1.8 %)
2.	High MW leaf (2.8 %)
3.	Low MW leaf (2.8 %)
4.	Low MW stipe (2.8 %)
5.	High MW leaf (1.8 %) + Very low MW leaf (1.0 %)
6.	Low MW leaf (1.8 %) + Very low MW leaf (1.0 %)
7.	Low MW stipe (1.8 %) + Very low MW leaf (1.0 %)
8.	High MW leaf (1.8 %) + Very low MW stipe (1.0 %)
9.	Low MW leaf (1.8 %) + Very low MW stipe (1.0 %)
10.	Low MW stipe (1.8 %) + Very low MW stipe (1.0 %)

2.4 Stability of Low Molecular Weight Leaf Alginate Gel Beads in Saline

2.4.1 Preparation

The studied gel beads were prepared as described in section 2.2.1 and are listed in Table 2.4. The gel beads were prepared from 5.0 mL alginate solution.

Table 2.4: Alginate type and concentrations (w/v) in alginate gel beads prepared for investigation of stability in terms of swelling and polymer leakage.

No.	Samples
1.	High MW leaf (1.8 %)
2.	Low MW leaf (1.8 %)
3.	Low MW leaf (2.8 %)
4.	Low MW leaf (1.8 %) + Very low MW leaf (1.0 %)
5.	Low MW leaf (1.8 %) + Very low MW stipe (1.0 %)

2.4.2 Swelling and Polymer Leakage

After the preparation of the gel beads, the diameter of the gel beads (n=20) were measured in a microscope (Eclipse TS100, Nikon). The gelling solution was removed by using a filter, and the gel beads were washed three times with 15 mL 0.9 % NaCl. The saline was collected (45 mL for each sample), and the size of the gel beads were measured before the gel beads were transferred to a blue cap bottle together with 40 mL 0.9 % NaCl. The gel beads were kept in saline on a gyrator shaker (10 rpm) at room temperature overnight. The saline was collected and pooled with the corresponding samples of previous collected saline (resulting in 85 mL in total for each sample). The diameter of the gel beads were measured, and the relative increase in bead diameter was calculated from the initial size and the size of the gel beads after six consecutive saline treatments.

The collected saline was dialyzed (MWCO 14-16 kDa, Spectrum Laboratories, Inc., USA) against MQ water (6 shift) until the conductivity had sunk below 1 S/m. The sample pH was adjusted to 7.0 before the samples were lyophilized overnight (Alpha 1-4 LD freeze dryer). The leaked material was dissolved in MQ water by adding EDTA (final concentration of 10 mM). The samples were dialyzed (MWCO 12-14 kDa, Spectrum Laboratories, Inc., USA) against 50 mM NaCl (4 shifts) and MQ-water (6 shifts). The sample pH was adjusted to 7.0 before the samples were lyophilized overnight. Finally, the leaked material was weighted.

NMR

Before NMR analysis the leaked alginate was degraded by a stepwise partial acid hydrolysis. The leaked material was dissolved in 60 ml MQ water in a blue cap bottle. The pH was adjusted to 5.6 with 0.1 M HCl and the samples were incubated at 95 ^{o}C in a water bath for one hour. The samples were immediately cooled down to room temperature and the pH was adjusted to 3.8. The samples were further incubated at 95 ^{o}C in a water bath for 50 minutes. The samples were immediately cooled down, and the pH was adjusted to 6.8 with NaOH. The samples were lyophilized overnight. ^{1}H-NMR analysis was performed by Wenche Irene Strand (NTNU) as described in 2.2.3, except the pH of the samples was not adjusted to 8.

SEC-MALLS

Size exclusion chromatography with multi-angular laser light scattering (SEC-MALLS) was performed by Ann-Sissel Teialeret Ulset (NTNU). The mobile phase was made up of 0.15 M $NaNO_3$ with 0.01 M EDTA, pH 6. The samples (1-3 mg) were dissolved in the mobile phase and filtered (0.8 µm) before injection. The system was composed of a mobile phase reservoir, an on-line degasser (Biotech, Degassi Classic), a HCLP isocratic pump (LC-10AD, Shimadzu) an autoinjector (SCL-10A, VP, Shimadzu), a precolumn, two serially connected columns: TSK G-6000 PWXL and TSK G-5000 PWXL (Toso Haas), and two serially connected detectors: a Dawn Heleos II laser light scattering photometer and an Optilab T-rEX differential refractormeter (Wyatt Technology corporation, USA). The analysis was carried out at ambient temperature. Astra software v. 6.1 was used to collect and process the data. The refractive index increment value (dn/dc_{μ}) for alginate of 0.150 mL/g was obtained from literature (Vold et al., 2006).

3 Results

3.1 Stability of Alginate Chitosan Oligomer Gel Beads in Saline

The possibilities for generating leaf alginate gel beads cross-linked with both calcium and chitosan oligomers (CO) were investigated. The gel beads were prepared with different chitosan concentrations (0.38 % and 0.68 % w/v) and gelled in solutions with different pH (5.0 and 6.5). The sample combinations and the shape and size of the resulting gel beads are displayed in Table 3.1. Gel beads were successfully prepared for all combinations of CO concentrations and pH values. All the gel beads had a regular and spherical shape, and the size of the gel beads varied between 358 µm and 410 µm.

Table 3.1: Shape and size of alginate gel beads prepared with different chitosan oligomer concentrations and gelling solution pH-values.

Sample	Gelation	Shape	Size
0.38 % CO / pH 6.5	Yes	Regular and spherical	401 ± 23
0.68 % CO / pH 6.5	Yes	Regular and spherical	358 ± 16
0.38 % CO / pH 5.0	Yes	Regular and spherical	371 ± 32
0.68 % CO / pH 5.0	Yes	Regular and spherical	410 ± 8

3.1.1 Swelling

The swelling properties of leaf alginate gel beads crosslinked with both calcium and chitosan oligomers prepared in gelling solutions with different pH values were investigated and compared to the swelling properties of leaf alginate gel beads crosslinked with only calcium (pH 6.5). The relative increase in size was calculated after six following saline treatments, see Figure 3.1 A. All the gel bead samples showed a significant degree of swelling but remained intact during the six saline treatments. The initial size of the gel beads was measured to be between 396 and 416 µm. There was not a significant difference between the gel bead samples with regards to initial size. The alginate-CO gel beads gelled at pH 5.0 had a degree of swelling of 63 %, while the calcium alginate gel beads and alginate-CO gel beads gelled at pH 6.5 had a degree of swelling of 84 and 88 %, respectively (Figure 3.1 B). A significant difference in size was not seen between the alginate-CO gel beads gelled at pH 6.5 and the alginate gel beads gelled only with calcium. However, the alginate-CO gel beads gelled at pH 5.0 showed a significant (p-value < 0.001) lower degree of swelling compared to the calcium alginate gel beads.

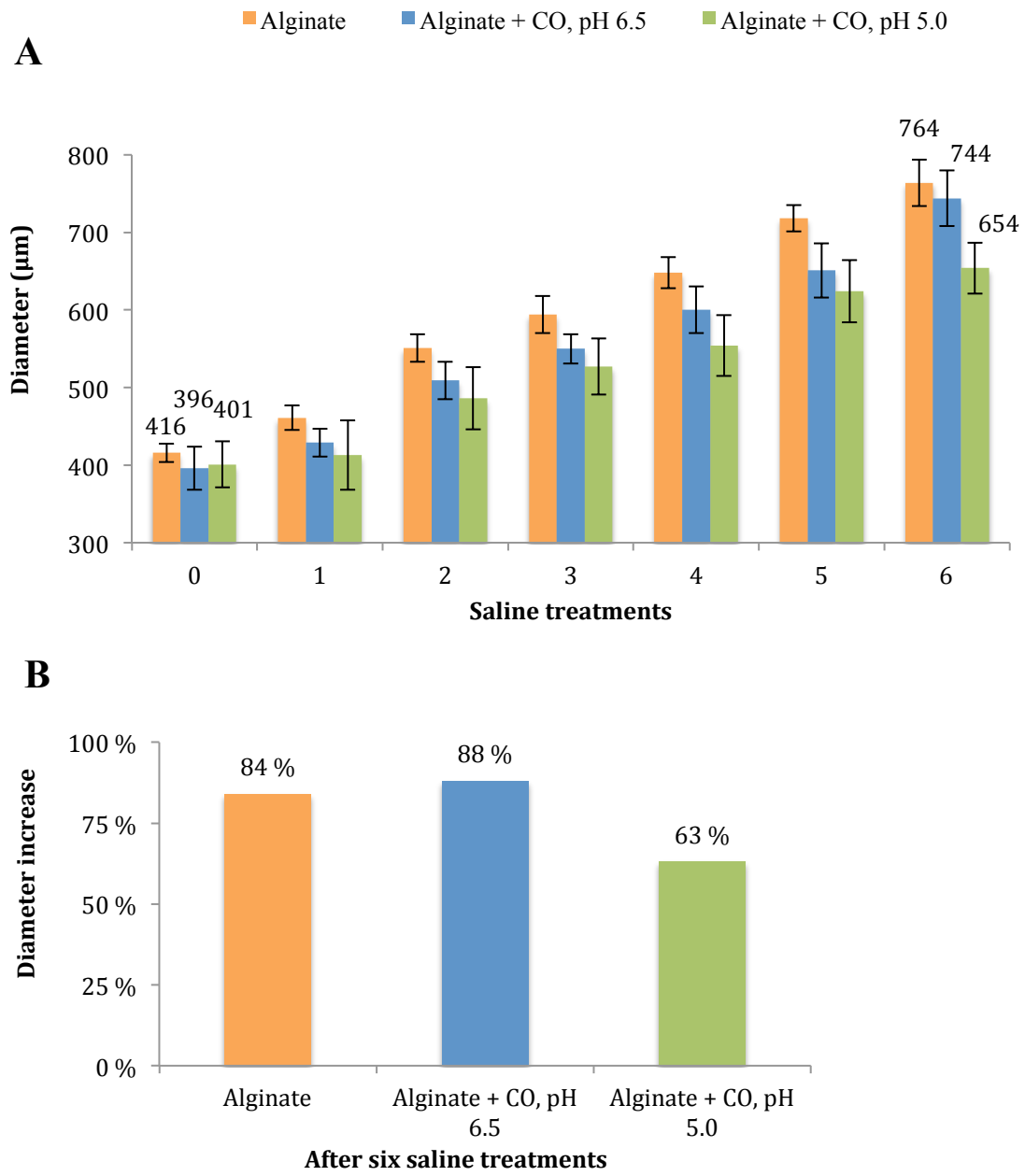

Figure 3.1: Swelling of leaf alginate gel beads crosslinked with calcium and chitosan oligomers (CO) compared to swelling of leaf alginate gel beads crosslinked with only calcium in 0.9 % NaCl. The gel beads were prepared in a gelling solution with a pH of either 5.0 or 6.5. A: The saline solution was exchanged every hour for six hours and the bead diameter was measured before each exchange. B: The relative increase in bead diameter (%) was calculated after six consecutive saline treatments. The bead diameters are the mean ± SD of 20 beads.

3.1.2 Polymer Leakage

The stability of alginate chitosan oligomer gel beads during saline treatment was also investigated with respect to polymer leakage. In general, a considerable proportion of the starting material leaked out during saline treatment for all the gel bead samples, see Table 3.2. The alginate-CO gel beads gelled at pH 6.5 had especially a high degree of leakage (46 %).

Table 3.2: The weight of the starting material and the material leaked from alginate-CO gel beads and calcium alginate gel beads, where the degree of leakage was calculated. The gel beads were washed with saline and kept in saline overnight. Dialysis tubes with a MWCO of 100-500 Da were used to remove the sodium chloride.

Sample	Starting material	Leaked material	Degree of leakage
Leaf alginate	90 mg	29 mg	32 %
Leaf alginate + CO (pH 6.5)	90 mg + 34 mg	57 mg	46 %
Leaf alginate + CO (pH 5.0)	90 mg + 34 mg	15 mg	12 %

The chemical composition of the leaked material was determined by NMR analysis, see Table 3.3. In general, the composition of the leaked material was similar to the starting material. However, the average G-block length of the leaked material was lower compared to the starting material. From Figure 3.2, the NMR spectra of the leaked material from the alginate-CO gel beads gelled at pH 5.0 (Figure 3.2 B) indicate that chitosan oligomers (Figure 3.2 C) are present in the leaked material, by the presence of peaks around 4.6 and between 5.2-5.3 that are not present in the pure alginate sample (Figure 3.2 A). The signal intensity for the alginate-CO gel beads gelled at pH 6.5 (Figure 3.2 D) was insufficient in giving information about the composition of the leaked alginate as well as the presence of chitosan oligomers.

Table 3.3: The chemical composition of alginate leaked from alginate-CO gel beads and calcium alginate gel beads during saline treatment, compared to the chemical composition of the starting material. The chemical composition of alginate leaked from alginate-CO gel beads gelled at pH 6.5 could not be obtained to due low signal intensity.

Sample		F_G	F_M	F_{GG}	$F_{GM/MG}$	F_{MM}	$F_{GGM/MGG}$	F_{MGM}	F_{GGG}	$N_{G>1}$
Leaf alginate	Start	0.46	0.54	0.29	0.17	0.37	0.05	0.15	0.24	7.0
	Leaked	0.43	0.57	0.28	0.15	0.42	0.08	0.09	0.20	4.1
Leaf alginate + CO (pH 6.5)	Start	0.46	0.54	0.29	0.17	0.37	0.05	0.15	0.24	7.0
	Leaked	-	-	-	-	-	-	-	-	-
Leaf alginate + CO (pH 5.0)	Start	0.46	0.54	0.29	0.17	0.37	0.05	0.15	0.24	7.0
	Leaked	0.45	0.55	0.29	0.16	0.39	0.09	0.08	0.20	4.2

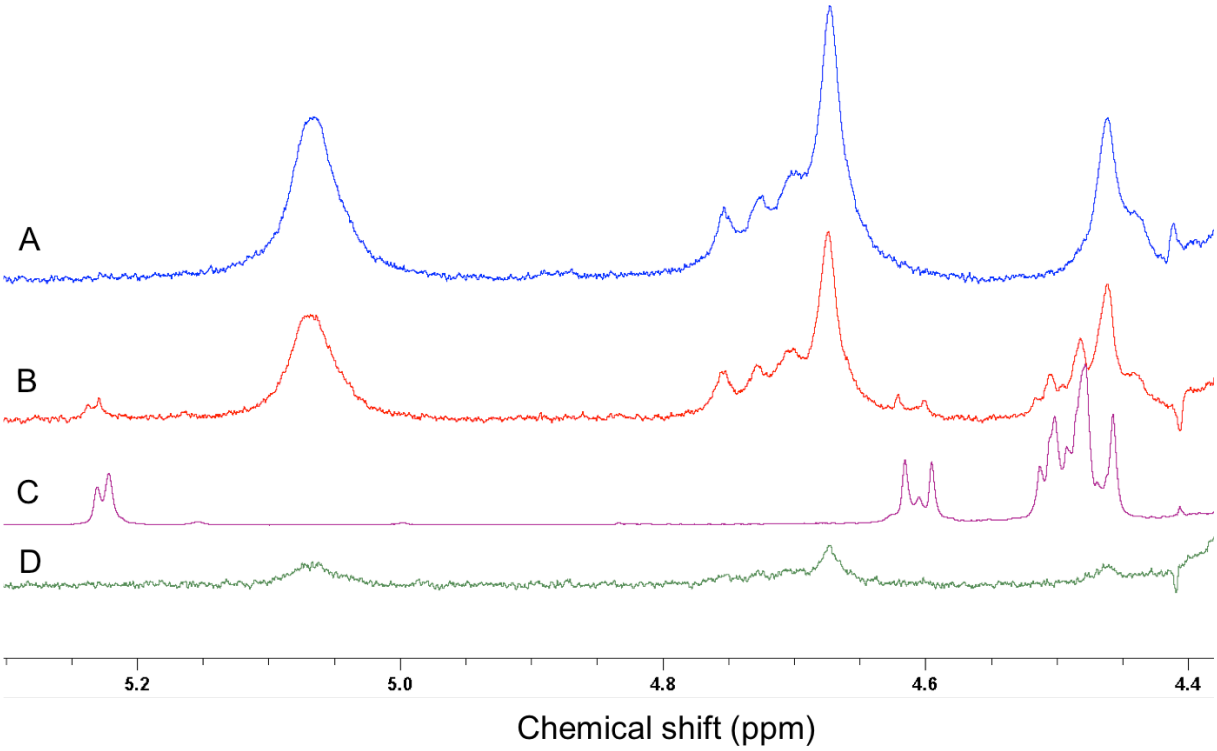

Figure 3.2: NMR spectra of the material leaked from calcium alginate gel beads (A) and alginate CO gel beads gelled at pH 5.0 (B) and pH 6.5 (D). In addition, the NMR spectrum of the chitosan oligomer mixture (C) is included.

3.2 Physical Properties of Leaf Alginate Gel Cylinders

The mechanical properties of leaf alginate gel cylinders were investigated in terms of Young's modulus and rupture strength, and were compared to the properties of stipe alginate gel cylinders. The effect of molecular weight and concentration on Young's modulus and rupture strength was examined. Before compression analysis, the degree of syneresis was determined for all the gel cylinders. In addition, the viscosity was measured for the samples with the highest alginate concentration (2.8 % w/v).

3.2.1 Syneresis

As shown in Figure 3.3, syneresis in the alginate gels decreased considerably with increasing alginate concentration. Syneresis also decreased with decreasing molecular weight of the alginate. A small difference in syneresis was observed between the gels of leaf and stipe alginate, comprising intermediate and high guluronic acid content, respectively. Although, the low molecular weight stipe alginate had a slightly lower syneresis compared to the low molecular weight leaf alginate for all the concentrations. For the high molecular weight leaf alginate, syneresis decreased from 40 ± 1 to 16 ± 1 % (w/w) when the concentration was increased from 1.0 to 2.8 % (w/v). For the low molecular weight stipe and leaf alginate gels, syneresis decreased from 24 ± 1 and 28 ± 1 %, respectively, to -1 and 4 % syneresis when the concentration was increased from 1.0 to 2.8 % (w/v).

When 1.0 % (w/v) of the alginate in a given alginate sample of 2.8 % (w/v) was substituted with very low molecular weight alginate, syneresis decreased more than that of the nonsubstituted gels with the same alginate concentration. There was a slight difference between adding very low molecular weight leaf or stipe alginate to high molecular weight leaf alginate. Syneresis decreased from 15 ± 1 to 7 ± 1 and 4 ± 3 % (w/w), respectively, when 1.0 % of the high molecular weight alginate was substituted with very low molecular weight leaf or stipe alginate.

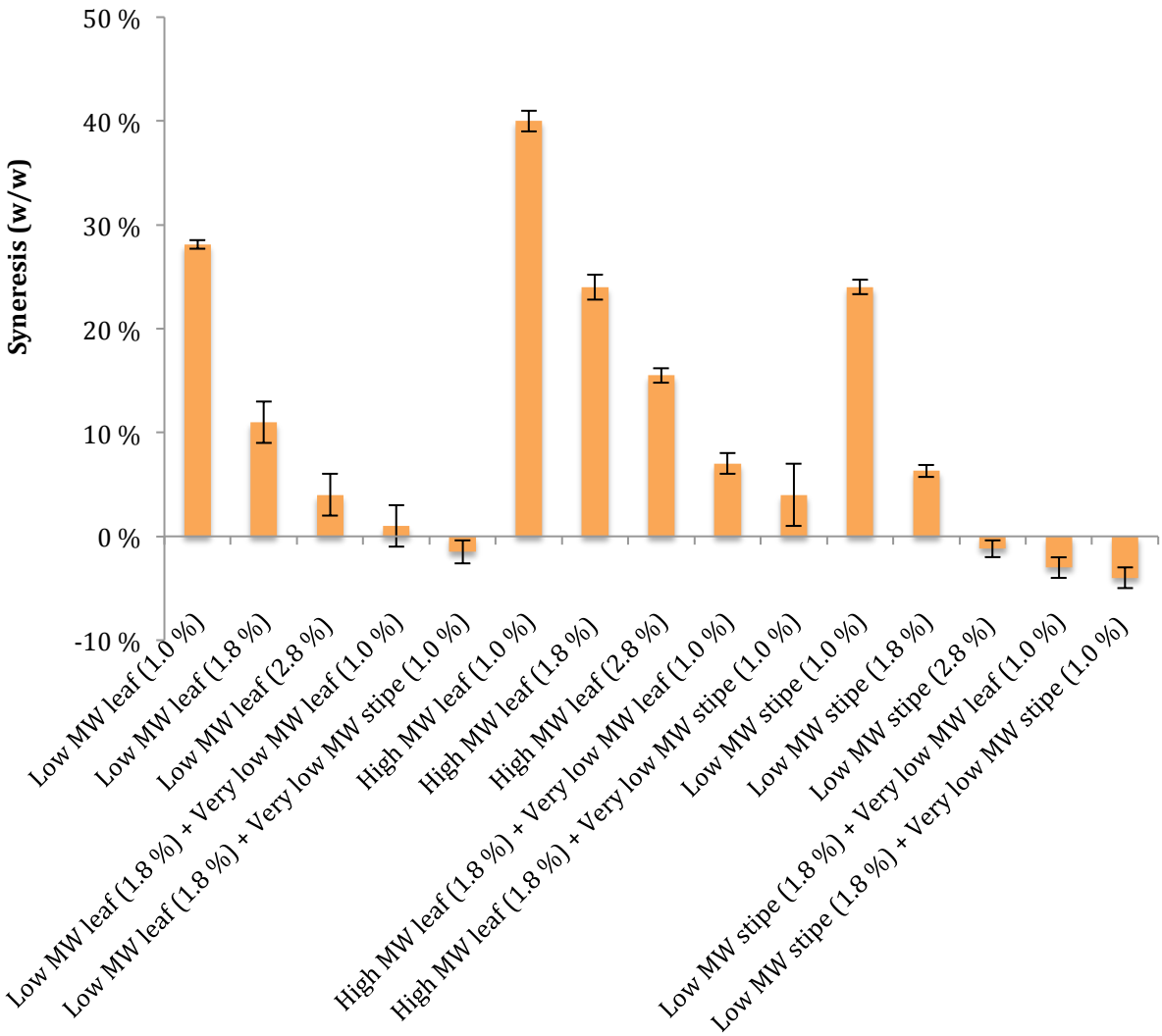

Figure 3.3: Syneresis for gels prepared from low molecular weight leaf or stipe alginate and high molecular weight leaf alginate. For every sample three concentrations were prepared: 1.0 %, 1.8 % and 2.8 % (w/v). Syneresis was also measured for gels where 1.0 % of the alginate was substituted with very low molecular weight leaf or stipe alginate. Syneresis was calculated from the difference in weight before gelling (based on the volume of well and the assumption that the density of alginate solution is the same as water) and the weight after gelling and Ca^{2+} saturation. The values are given as the average ± SD of 5-9 gel cylinders.

3.2.2 Young's modulus

As illustrated in Figure 3.4, Young's modulus of the alginate gels increased with increasing alginate concentration. Young's modulus also increased with increasing guluronic acid content in the gels. No difference was observed between the low and high molecular weight leaf alginate gels with respect to Young's modulus. For the low molecular weight stipe alginate gels, Young's modulus increased from 31 to 92 kPa when the concentration was increased from 1.0 to 2.8 % (w/v). For the low and high molecular weight leaf alginate gels, Young's modulus increased from 15 and 12 kPa, respectively, to 50 kPa for both gels when the concentration was increased from 1.0 to 2.8 % (w/v).

In regards to the Young's modulus, increasing the alginate concentration with very low molecular weight leaf or stipe alginate had a similar effect as increasing the concentration with alginate of higher molecular weight. In comparison to the 2.8 % (w/v) gel cylinders, the gels with added very low molecular weight leaf alginate (total concentration of 2.8 % w/v) showed a slightly lower Young's modulus. For the low molecular weight leaf alginate, Young's modulus decreased from 50 to 44 kPa when 1.0 % (w/v) of the low molecular weight leaf alginate was substituted with very low molecular weight leaf alginate. For the high molecular weight leaf alginate, Young's modulus decreased from 50 to 45 kPa when 1.0 % (w/v) of the high molecular weight leaf alginate was substituted with very low molecular weight leaf alginate. For the low molecular weight stipe alginate, Young's modulus decreased from 92 to 76 kPa. Substituting 1.0 % (w/v) of the alginate of one of the given alginate samples of 2.8 % (w/v) with of very low molecular weight stipe alginate, gave on the other hand a slightly higher Young's modulus, except for the low molecular weight stipe alginate. For the low and the high molecular weight leaf alginate, Young's modulus increased from 50 to 61 and 57 kPa, respectively, when 1.0 % of the alginate was substituted with very low molecular weight stipe alginate. For the low molecular weight stipe alginate, Young's modulus decreased from 92 to 90 kPa when 1.0 % of the alginate was substituted with very low molecular weight alginate.

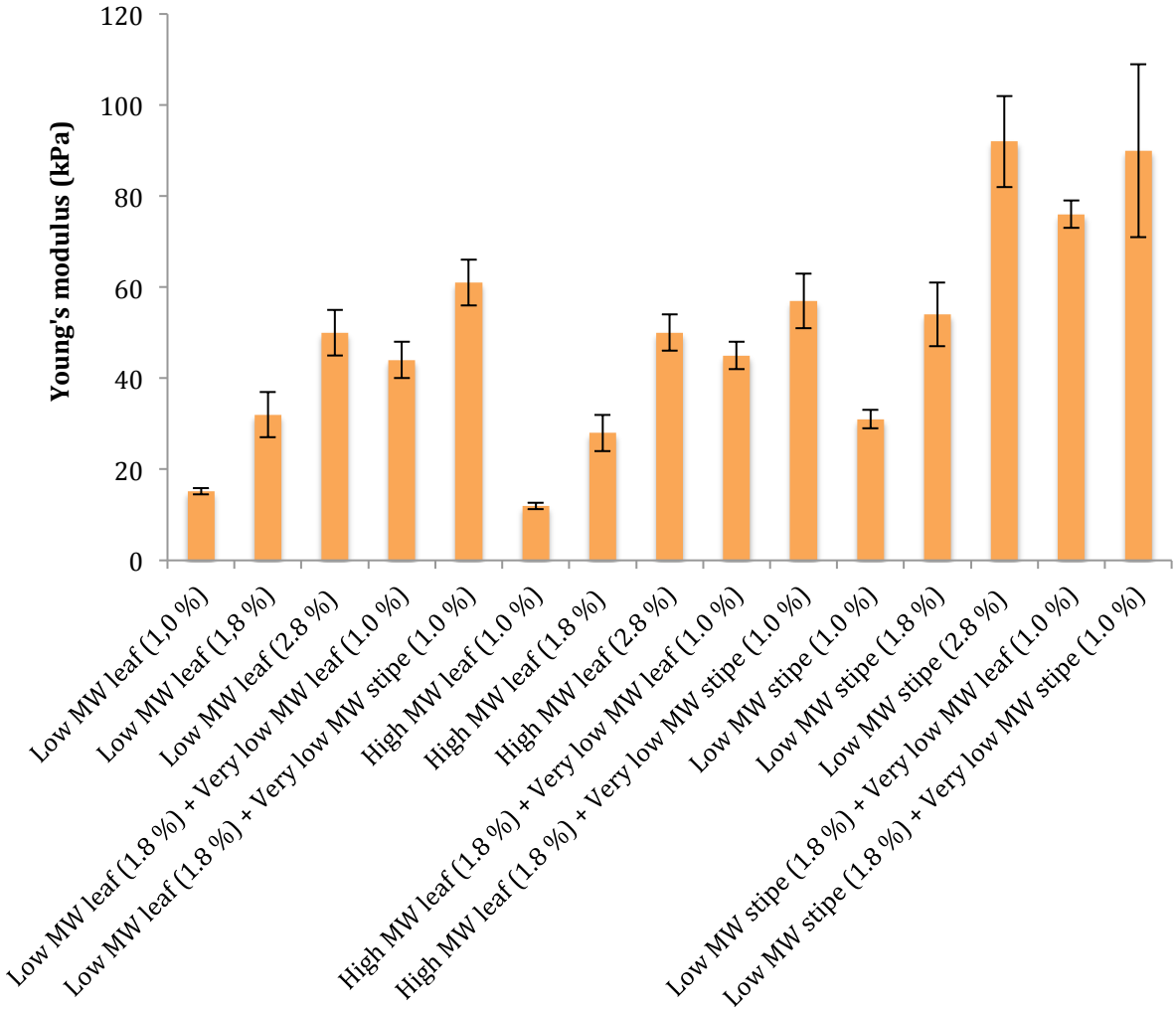

Figure 3.4: Young's modulus (kPa) for gel cylinders prepared from low molecular weight leaf or stipe alginate and high molecular weight leaf alginate. For every sample three concentrations were prepared: 1.0 %, 1.8 % and 2.8 %. Young's modulus was also measured for gels with added very low molecular weight leaf or stipe alginate (total concentration of 2.8 % (w/v)). Young's modulus was calculated from the initial part (0.1-0.3 mm) of the stress/strain curve obtained during compression (0.1 mm/sec) of the gel cylinders. The values are given as the average ± SD of 5-9 gel cylinders.

3.2.3 Rupture strength

As for Young's modulus, the rupture strength of the alginate gels increased with increasing alginate concentration, see Figure 3.5. For example, the rupture strength for the low molecular weight leaf alginate increased from 1.8 ± 0.2 to 5.7 ± 1.2 kg when the concentration was increased from 1.0 to 2.8 % (w/v). However, for the low molecular weight stipe alginate the rupture strength did not increase further when the concentration was increased from 1.8 to 2.8 % (w/v). Rupture strength also increased with increasing molecular weight. The high molecular weight leaf alginate (M_w = 200 kDa) had about twice as high rupture strength as the low molecular weight leaf alginate (M_w = 115 kDa) for all the concentrations. The stipe alginate showed a higher rupture strength than the leaf alginate of the same molecular weight. At 2.8 % (w/v), the rupture strength for the low molecular weight stipe alginate was 8.4 ± 1.7 kg compared to 5.7 ± 1.2 kg for the low molecular weight leaf alginate. It should be noticed that for most of the measurements the uncertainty was quite high.

In regards to rupture strength, increasing the alginate concentration with very low molecular weight leaf or stipe alginate did not have the same effect as increasing the concentration with alginate of higher molecular weight. For example, the rupture strength decrease from 5.7 ± 1.2 for the 2.8 % (w/v) low molecular weight leaf alginate to 3.5 ± 0.7 and 3.0 ± 1.0 kg, when 1.0 % (w/v) of the alginate was substituted with very low molecular weight leaf or stipe alginate (total concentration of 2.8 % (w/v)), respectively. In comparison, the rupture strength for the 1.8 % (w/v) low molecular weight leaf alginate was 3.0 ± 0.8 kg, showing that increasing the concentration with very low molecular weight alginate did not have an effect. For the low molecular weight stipe alginate, the rupture strength of the gels with added very low molecular weight leaf or stipe alginate (total concentration of 2.8 % (w/v)) decreased even below the 1.8 % (w/v) gel consisting purely of low molecular weight stipe alginate.

The deformation at rupture seemed to be independent of alginate concentration, molecular weight and guluronic acid content, see appendix D – 3. The gel cylinders had an initial height of 18 mm, and the deformation at rupture for all the samples were between 9.2-11.0 mm.

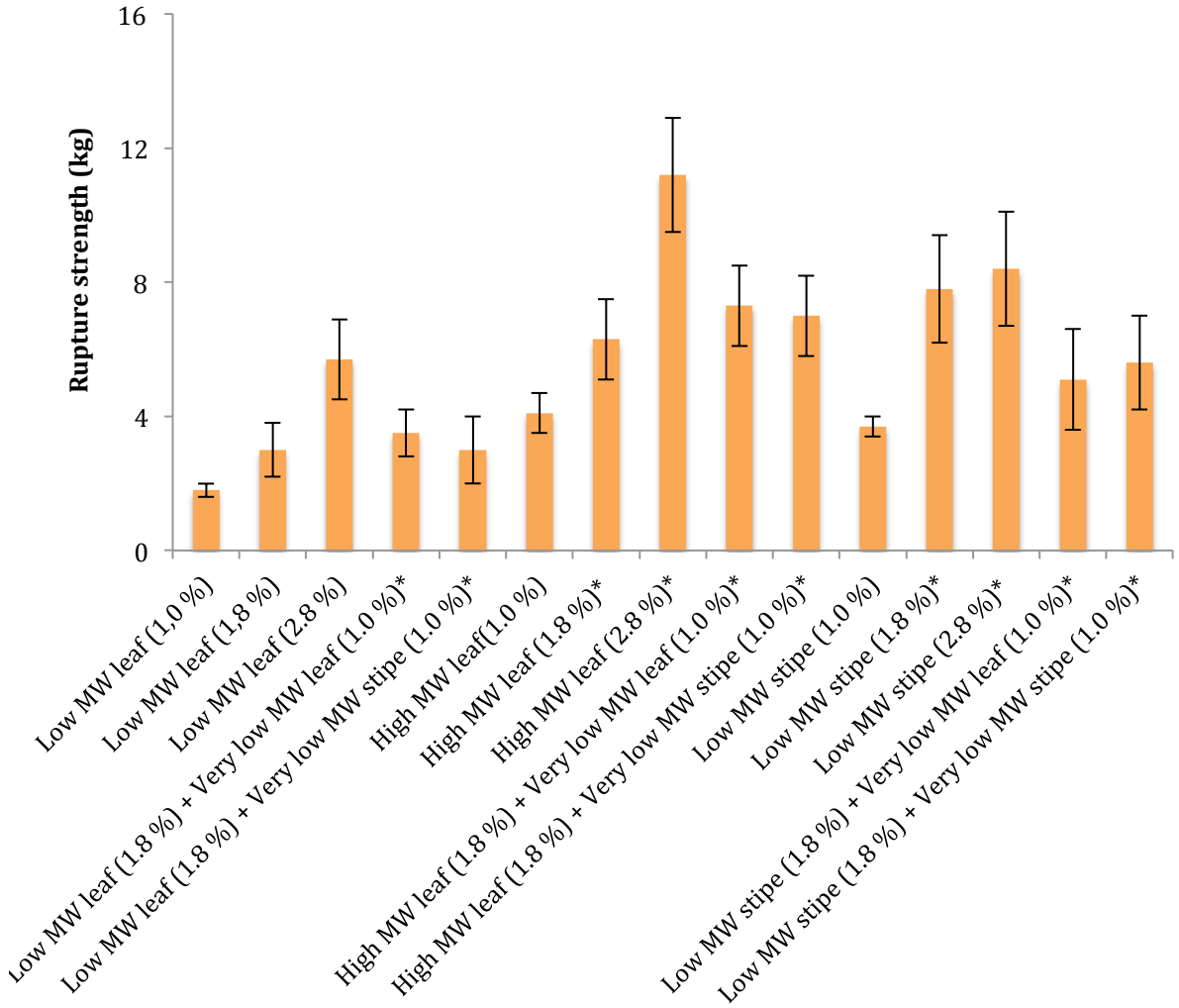

Figure 3.5: Rupture strength (kg) measured for gel cylinders prepared from low molecular weight leaf or stipe alginate and high molecular weight leaf alginate. For every sample three concentrations were prepared: 1.0 %, 1.8 % and 2.8 %. Rupture strength was also measured for gels with added very low molecular weight leaf or stipe alginate with a total concentration of 2.8 % (w/v). The rupture strength was calculated from the stress/strain curve obtained during compression (0.1 mm/sec test speed) of the gel cylinders. For the weakest gels a 5 kg load cell was used, while for the stronger gels a 30 kg load cell (*) was used. The values are given as the average ± SD of 5-9 gel cylinders.

3.2.4 Viscosity

As displayed in Table 3.4, the high molecular weight alginate gave the most viscous solutions. All the solutions containing the high molecular weight alginate, independent of concentration and added very low molecular weight alginate, were more viscous than all the low molecular weight alginate samples, including those with the highest alginate concentration. The high molecular weight leaf alginate with a concentration of 2.8 % (w/v) stood out as especially viscous. The shear viscosity at 23 s^{-1} was measured to be 1.65 Pa s for this sample. The stipe alginate was shown to be more viscous than the leaf alginate of the same molecular weight. The shear viscosity at 22 s^{-1} for the 2.8 % (w/v) low molecular weight stipe alginate solution was shown to be 0.41 Pa s, while the shear viscosity at the same shear rate was 0.16 for the 2.8 % (w/v) low molecular weight leaf alginate solution. In addition, upon substitution of 1.0 % (w/v) of the low molecular weight stipe alginate with very low molecular weight leaf or stipe alginate, the viscosity was considerably lower than for the nonsubstituted stipe alginate sample (from 0.41 to 0.16 and 0.18 Pa s for very low molecular weight leaf and stipe, respectively).

Table 3.4: The shear viscosity was measured using a rotational rheometer (Kinexus Ultra +, Malvern, UK) equipped with a cone/plate (4°/40 mm) for shear rates between 0.5 and 30 s^{-1} or between 0.1 and 100 s^{-1}, see appendix E. The value listed in this table are for 22-23 s^{-1}.

Sample	Shear Viscosity (Pa s)
High MW leaf (2.8 %)	1.65
High MW leaf (1.8 %) + very low MW stipe (1.0 %)	0.66
High MW leaf (1.8 %) + very low MW leaf (1.0 %)	0.60
High MW leaf (1.8 %)	0.49
Low MW stipe (2.8 %)	0.41
Low MW stipe (1.8 %) + very low MW stipe (1.0 %)	0.18
Low MW stipe (1.8 %) + very low MW leaf (1.0 %)	0.16
Low MW leaf (2.8 %)	0.15
Low MW leaf (1.8 %) + very low MW stipe (1.0 %)	0.084
Low MW leaf (1.8 %) + very low MW leaf (1.0 %)	0.075

3.3 Stability of Low Molecular Weight Leaf Alginate Gel Beads in Saline

The stability of low molecular weight leaf alginate gel beads in saline was investigated in terms of swelling and polymer leakage. The gel beads were washed three times in saline and kept in saline overnight. The effect of increasing the alginate concentration was examined, by adding either more low molecular weight leaf alginate or by adding very low molecular weight leaf or stipe alginate. The total alginate concentration was either 1.8 or 2.8 % (w/v). For comparison, stability of high molecular weight leaf alginate gel beads was measured.

3.3.1 Swelling

The size of the gel beads was measured three times: after gelation, after the washing procedure, and after being suspended in saline overnight. To compare the swelling properties of the gel bead samples, the relative increase in diameter from initial size (in gelling solution) was calculated after the gel beads were kept in saline overnight. The initial size was similar for all the gel bead samples, except for the 1.8 % (w/v) low molecular weight alginate gel beads, see Figure 3.6 A. The latter had an initial diameter of 366 ± 45 µm, while the other gel beads had an initial diameter between 453 ± 17 and 475 ± 25 µm. The 1.8 % (w/v) low molecular weight leaf alginate showed the highest degree of swelling (36 %), see Figure 3.6 B. The lowest degree of swelling was seen for the gel beads with added very low molecular weight stipe alginate (total concentration 2.8 % (w/v)). It proved to be challenging to prepare evenly sized gel beads from the 1.8 % (w/v) low molecular weight alginate, see Appendix F, resulting in high uncertainty in the size of these gel beads.

In general, increasing the alginate concentration in the gel bead formulations led to increased dimensional stability. Increasing the alginate concentration with very low molecular weight alginate had a larger effect on the size stability than increasing the concentration with more low molecular weight leaf alginate. Adding very low molecular weight stipe alginate to the alginate bead formulation of very low molecular weight leaf alginate gave the largest increase in stability. All the different low molecular weight leaf alginate gel bead formulations with a total alginate concentration of 2.8 % (w/v) showed a higher degree of stability than the 1.8 % (w/v) high molecular weight leaf alginate. The high molecular weight leaf alginate showed a slightly lower degree of swelling than the low molecular weight leaf alginate. However, as mentioned, due to the high uncertainty in the size of the 1.8 % (w/v) low molecular weight leaf alginate gel beads this observation is inconclusive.

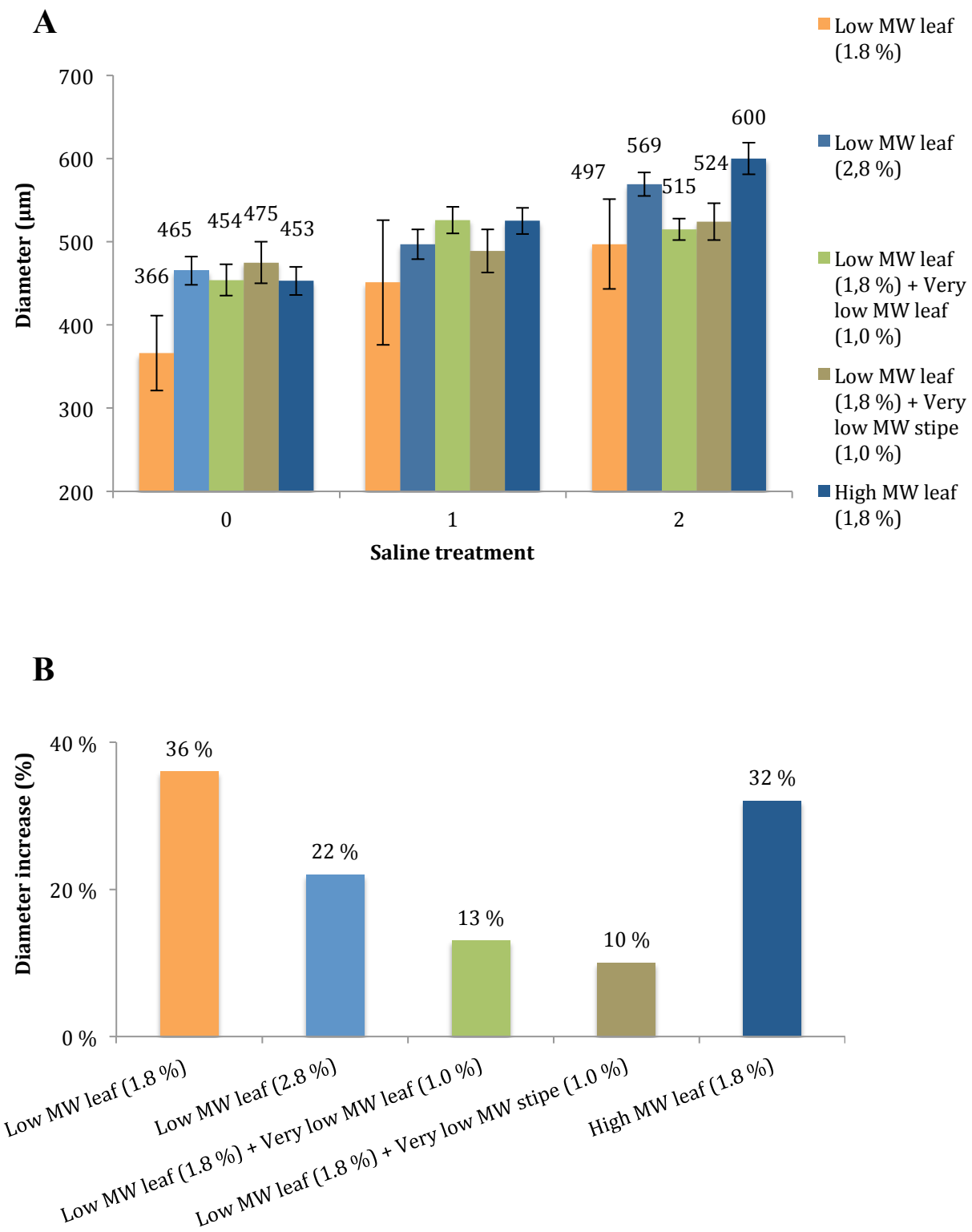

Figure 3.6: Swelling of different formulations of low molecular weight leaf alginate gel beads in 0.9 % NaCl, with or without added very low molecular weight leaf or stipe alginate. The total alginate concentration was either 1.8 or 2.8 % (w/v). The high molecular weight alginate gel bead sample was included for comparison. A: The bead diameter was measured after gelation, after washing with 15 ml 0.9 % NaCl x 3, and after the beads had been submerged in 40 ml 0.9 % NaCl over night. B: The relative increase in bead diameter (%) after the gel beads had been kept in saline overnight compared to the initial size. The bead diameters are the mean ± SD of 20 beads.

3.3.2 Polymer Leakage

The material leaked from the alginate gel beads was weighted for leakage quantification (% w/w), and the chemical composition and molecular weight averages was determined by ^{1}H NMR and SEC-MALLS, respectively. The highest degree of leakage (17 %) was seen for the 1.8 % (w/v) low molecular weight leaf alginate gel beads, whereas the lowest degree of leakage (4 %) was observed for the 1.8 % (w/v) high molecular weight leaf alginate (see Table 3.5); showing a clear effect of molecular weight, where the leakage from the gel beads was reduced by using high molecular weight alginate. The high molecular weight leaf alginate in this experiment was a new sample showing considerably lower leakage than in the first experiment (section 3.1.2). In addition, increased alginate concentration led to a decrease in the amount of leaked material. By increasing the alginate concentration of the gel beads from 1.8 to 2.8 % (w/v), the degree of leakage decreased from 17 to 6 % for the low molecular weight leaf alginate gel beads. Increasing the total alginate concentration by adding very low molecular weight leaf or stipe alginate also led to a decrease in the relative amount of leaked material, from 17 to 12 and 7 %, respectively.

Table 3.5: Weight of different alginate formulations used to prepare gel beads and the alginate leaked from the gel beads during treatment with saline. The degree of leakage is calculated as the ratio between leaked material and starting material (% w/w). Dialysis tubes with a MWCO of 12-14 kDa were used to remove sodium chloride.

Sample	Starting material	Leaked material	Degree of leakage
High MW leaf (1.8 %)	90 mg	4 mg	4 %
Low MW leaf (1.8 %)	90 mg	15 mg	17 %
Low MW leaf (2.8 %)	180 mg	11 mg	6 %
Low MW leaf (1.8 %) + very low MW leaf (1.0 %)	180 mg	13 mg	7 %
Low MW leaf (1.8 %) + very low MW stipe (1.0 %)	180 mg	21 mg	12 %

The polymeric material leaked from the alginate gel beads during treatment with saline was enriched in mannuronic acid and had a shorter average G-block length compared to the starting material, see Table 3.6.

Table 3.6: Chemical composition of the starting material of alginate and the alginate leaked from the gel beads during treatment with saline. The composition is obtained with ^{1}H-NMR.

Sample		F_G	F_M	F_{GG}	$F_{GM/MG}$	F_{MM}	$F_{GGM/MGG}$	F_{MGM}	F_{GGG}	$N_{G>1}$
High MW leaf (1.8 %)	Start	0.46	0.54	0.29	0.17	0.37	0.05	0.15	0.24	7.0
	Leaked	0.37	0.63	0.25	0.13	0.50	0.06	0.06	0.19	5.3
Low MW leaf (1.8 %)	Start	0.50	0.50	-	-	-	-	-	-	-
	Leaked	0.40	0.60	0.26	0.14	0.46	0.06	0.08	0.20	5.4
Low MW leaf (2.8 %)	Start	0.50	0.50	-	-	-	-	-	-	-
	Leaked	0.32	0.68	0.17	0.14	0.54	0.06	0.08	0.11	3.9
Low MW leaf (1.8 %) + very low MW leaf (1.0 %)	Start	0.50/ 0.51	0.50/ 0.49	-/0.34	-/-	-/-	-/-	-/-	-/-	-/-
	Leaked	0.30	0.70	0.16	0.14	0.57	0.06	0.07	0.11	4.2
Low MW leaf (1.8 %) + very low MW stipe (1.0 %)	Start	0.50/ 0.68	0.50/ 0.32	-/0.58	-/0.104	-/0.21	-/0.04	-/0.07	-/0.54	-/16
	Leaked	0.45	0.55	0.31	0.14	0.42	0.07	0.07	0.25	5.8

The molecular weights of the leaked alginate, obtained by SEC-MALLS, were considerably lower compared to the starting material, see Table 3.7. The molecular weights of the leaked material was in the same range for all the samples, regardless of the average molecular weight of the starting material. In addition, the polydispersity index is lower for the leaked alginate.

Table 3.7: The number average molecular weight (M_n), the weight average molecular weight (M_w) and the polydispersity index of the starting material of alginate and alginate leaked from the gel beads during treatment with saline. The molecular weight averages were obtained from SEC-MALLS.

Sample		M_n (kDa)	M_w (kDa)	Polydispersity (M_w/M_n)
High MW leaf (1.8 %)	Start	105	200	1.90
	Leaked	27.3 ± 0.4	36.5 ± 0.3	1.37 ± 0.03
Low MW leaf (1.8 %)	Start	43	115.0	2.67
	Leaked	26.6 ± 0.4	45.0 ± 0.3	1.69 ± 0.03
Low MW leaf (2.8 %)	Start	43	115	2.67
	Leaked	21.9 ± 0.7	32.4 ± 0.4	1.49 ± 0.05
Low MW leaf (1.8 %) + very low MW leaf (1.0 %)	Start	43/17	115/34	2.67/ 2.00
	Leaked	20.68 ± 0.14	28.4 ± 0.1	1.37 ± 0.01
Low Mw leaf (1.8 %) + very low MW stipe (1.0 %)	Start	43/21	115/33	2.67/1.55
	Leaked	22.6 ± 0.2	34.2 ± 0.2	1.56 ± 0.02

4 Discussion

4.1 Stability of Alginate Chitosan Oligomer Gel Beads in Saline

Alginate chitosan oligomer gel beads were successfully prepared with different chitosan concentrations (0.38 % and 0.68 % w/v) and gelled in calcium chloride solutions (50 mM) with different pH (5.0 and 6.5). However, the addition of chitosan oligomers as crosslinkers had a minor effect on the dimensional stability in saline for the gel beads gelled at pH 5.0, and no effect on the dimensional stability for the gel beads gelled at pH 6.5. In general, a large proportion of the starting material leaked out during saline treatment for all the gel bead samples.

4.1.1 Preparation of Alginate Chitosan Oligomer Gel Beads

In the present work, gel beads were prepared from *L. hyperborea* leaf alginate crosslinked with calcium and chitosan oligomers. *L. hyperborea* leaf alginate has an intermediate guluronic acid content (Table 2.1). Studies have shown that gels prepared from alginates rich in mannuronic acid generally exhibit lower mechanical strength and lower stability than gels prepared from alginates rich in guluronic acid, owing to the strong cooperative binding of calcium ions to long stretches of guluronic acid residues forming stable junctions in the gel network (Smidsrød and Haug, 1972, Martinsen et al., 1989). The idea behind crosslinking *L. hyp.* leaf alginate with both calcium and chitosan oligomers was to potentially increase the mechanical strength and the stability of gel beads despite the intermediate guluronic acid content. Khong et al. (2013) showed that poly M forms relative strong gels with chitosan oligomers compared to alginates with a high content of guluronic acid, which form gels with chitosan oligomers of very limited mechanical strength. In a later study, Feng et al. (2017) successfully prepared *L. hyp.* leaf and stipe alginate gels crosslinked with calcium and chitosan oligomers by internal gelation. The study showed that for alginate gels with intermediate guluronic acid content, 50 % of the calcium could be substituted with chitosan oligomers without reduction in gel strength. Hence, it seems that calcium and chitosan oligomers can provide crosslinkers for alginates where calcium ions are crosslinking through the G-blocks and the chitosan oligomers through the M-blocks.

The pK_a values of the carboxyl groups on alginate and the amino groups on chitosan are approximately 3.5 and 6.5, respectively. Therefore, it is expected that a strong ionic interaction will form between negatively charged alginates and positively charged chitosan

oligomers in the pH range 4 to 6. The chitosan oligomers and the alginates was mixed at pH 7.5, like in previous work (Khong et al., 2013, Feng et al., 2017), without any precipitation. The chitosan oligomers will essentially be uncharged at this pH, hence unable to form interactions with alginates. In general, chitosans are considered to be insoluble at neutral pH, but the solubility at neutral pH increase with decreasing molecular weight and increasing degree of acetylation (Vårum et al., 1994). The chitosan oligomer mixture had an average degree of polymerization as low as 4 (section 2.1). The mixture contained though chitosan oligomers with a degree of polymerization up to 20. However, the longer chains had a higher degree of acetylation.

The gel beads were prepared by dripping the alginate chitosan oligomer mixture into a calcium chloride solution (50 mM) with a pH of either 5.0 or 6.5. As mentioned above, at pH 5.0 chitosan oligomers are expected to form strong ionic interactions with alginates. However, pH 6.5 is closer to the pH requirement of cells, which is relevant for biomedical applications. In the present work, the alginate-CO gel beads prepared at pH 5.0 showed a slightly lower degree of swelling compared to the alginate-CO gel beads prepared at pH 6.5 (Figure 3.1). Previous studies have shown that the gel strength increase with increasing amounts of slowly hydrolyzing **D**-glucono- δ-lactone (GDL) during gelation down to a pH of just below 4 (Khong et al., 2013, Feng et al., 2017). Chitosan oligomers are only able to crosslink alginate when the amino groups on the oligomers are protonated and charged. At pH 5.0 97 % of the amino groups on the chitosan oligomers will be protonated and charged, while at pH 6.5 only 50 % of the amino groups on the chitosan oligomers will be protonated and charged. Hence, at pH 6.5 the chitosan oligomers are less likely to interact with the negatively charged carboxyl groups on alginate. The alginate-CO gel beads gelled pH 6.5 showed a similar degree of swelling as the alginate gel beads crosslinked with only calcium. All the gel bead samples were prepared with the same calcium concentration (50 mM), hence if the chitosan oligomers do not crosslink alginate, the alginate-CO gel beads are expected to exhibit the same swelling behavior as the alginate gel beads crosslinked with only calcium. The alginate-CO gel beads gelled at pH 5.0 showed a slightly lower degree of swelling compared to the alginate gel beads crosslinked with only calcium. When most of the amino groups on the chitosan oligomers are protonated and charged, as they are at pH 5.0, the chitosan oligomers are expected to contribute to increase the crosslinking density of the *L. hyp.* leaf alginate gel beads. As mentioned, studies have shown that poly M and chitosan oligomers form relative strong gels compared to alginates enriched in guluronic acid (Khong et al., 2013). Hence, it is reasonable to assume that chitosan oligomers have higher affinity than calcium towards M-

blocks. This is supported by the fact that the gel strength of alginate gels with intermediate guluronic acid content is maintained upon replacing a certain amount of calcium with chitosan oligomers (Feng et al., 2017). The elasticity of the gel network depend on the strength and the number of crosslinks (Moe, 1993). By increasing the number of crosslinks, the alginate-CO gel beads gelled at pH 5.0 was able to withstand a greater osmotic pressure. However, pH 5 is less compatible with cell encapsulation than pH 6.5.

It should be mentioned that in all the studies mentioned above the internal gelation method was used for preparation of alginate-CO gel cylinders. However, in this thesis the diffusional setting method was used for preparation of alginate-CO gel beads. If the gelation method has an effect on the crosslinking of alginate with chitosan oligomers is unclear.

4.1.2 Swelling

All the gel beads showed significant degree of swelling but remained intact during saline treatment (Figure 3.1). The alginate-CO gel beads and the alginate gel beads crosslinked with only calcium were prepared from *L. hyp.* leaf alginate with intermediate guluronic acid content. Gel beads prepared from alginates with intermediate guluronic acid content have been shown to exhibit lower size stability than gel beads prepared from alginate rich in guluronic acid (Martinsen et al., 1989). The alginate-CO gel beads prepared at pH 5.0 showed a slightly lower degree of swelling than the alginate gel beads crosslinked with only calcium (50 mM). As discussed above, chitosan oligomers crosslink alginate through M-blocks and contribute to increase the crosslinking density of the leaf alginate gel beads (Khong et al., 2013, Feng et al., 2017). The alginate-CO gel beads prepared at pH 6.5 showed a similar degree of swelling as the alginate gel beads crosslinked with only calcium. Chitosan oligomers form strong interaction with alginate only in the pH range 4 to 6, as the pK_a value of the amino groups is 6.5. Above pH 6.5, most of the amino groups are deprotonated and uncharged.

The pH of the saline solution (0.9 % NaCl) was not controlled, but was assumed to be close neutral pH. The swelling properties of alginate gels are not affected by the pH of the saline solution in the pH range 4-9 (Golmohamadi and Wilkinson, 2013), as the pK_a values of the guluronic acid and mannuronic acid residues are 3.65 and 3.28, respectively. However, at neutral pH only 24 % of the amino groups on chitosan oligomers will be protonated and charged. The neutral pH of the saline solution used in the size stability experiment in the present work likely caused chitosan oligomers to dissociate from the alginate. Chitosan oligomers with shorter chain lengths are expected to form weaker interactions with alginates

as they interact through fewer amino groups (Khong, 2013), hence shorter oligomers are more likely to dissociate when the pH of the surrounding solution is above the pK_a value. A study conducted on the swelling properties of alginate gels crosslinked with calcium and chitosan oligomers showed that the size stability of the gels was influenced by the pH of the saline solution (Myrnes, 2016). In the study, internally gelled alginate gel cylinders crosslinked with calcium and chitosan oligomers swelled slightly less than the alginate gels crosslinked with only calcium in saline solutions with pH 4.5 and 5.5, while in saline solutions with pH 7.5 and 8.5 the alginate gels crosslinked with calcium and chitosan oligomers swelled significantly more than the calcium alginate gels. It should be noticed that in the study, the calcium concentration was reduced with 50 % in the alginate-CO gels compared to the concentration in the calcium alginate gels. Calcium ions form crosslinks with alginate through G-blocks, hence reducing the calcium concentration will lead to lower gel strength (Martinsen et al., 1989). Therefore, a larger effect on the size stability might have been seen for the CO-alginate gel beads gelled at pH 5.0 in the present work if the pH of the saline solution had been lower. However, the size stability at lower pH is not relevant as the physiological pH is close to neutral pH.

Alginate gel beads swell under physiological conditions partly because the gelling ions (e.g. calcium ions) are being replaced by non-gelling ions such as sodium ions (Thu et al., 1996). The swelling behavior of the alginate-CO gel beads were investigated in 0.9 % NaCl. When sodium ions are present at such high amounts, the crosslinking junctions will be destabilized due to the exchange with calcium. The number of crosslinks will decrease, and hence will the elasticity of the gel network and the elastic pressure which work to reduce the volume of gel (Moe, 1993). In addition, the osmotic pressure will increase as one calcium ion is exchanged with two sodium ions (Strand, 2002). More water will flow into the gel beads and the volume of the gel beads will increase. The calcium-sodium selectivity coefficient depends on the composition of the alginate (Smidsrød and Haug, 1968). Alginates rich in mannuronic acid display a much lower resistance against ion exchange in the present of non-gelling sodium ions than high G alginates (Martinsen et al., 1989, Mørch et al., 2006). In the present study, the alginate-CO gel beads were prepared from *L. hyperborea* leaf alginate which has an intermediate guluronic acid content. At the right pH during and after gelling, chitosan oligomers can contribute to increasing the crosslinking density (Khong et al., 2013), hence the elasticity of the gel network. However, the selectivity between sodium ions and chitosan oligomers has not been characterized.

4.1.3 Polymer Leakage

In general, the degree of polymer leakage from the alginate-CO gel beads and the calcium alginate gel was high, especially for the alginate CO gel beads gelled at pH 6.5. There are indications that the leaked materials from the alginate-CO gel beads and the calcium alginate gel beads contained sodium chloride. Most of the gel beads seemed to remain intact during the saline treatment, which makes 46 % leakage from the alginate-CO gel beads (pH 6.5) seem excessive. If all the chitosan oligomers had leaked out of the gel beads that would have corresponded to 27 % leakage, still having a very high amount of alginate leakage. The NMR spectrum of the material leaked from the alginate CO gel beads gelled at pH 6.5, showed that only a small amount of alginate was present in the sample, resulting in a NMR spectrum of poor resolution where information on the chemical composition of the leaked alginate could not be obtained. All the NMR samples were prepared with the same weight of leaked material (after dialysis and lyophilization), however, the NMR spectra of the other samples with leaked alginate or alginate-CO showed a larger amount of alginate present in the samples (Figure 3.2). When the leaked material from the respective gel beads was dialyzed the second time with EDTA, the weight of the leaked material from the calcium alginate gel beads significantly increased, instead of decreasing as expected. At a later point in the study, the leakage from calcium alginate gel beads was measured again in a second experiment where the dialyzation procedure was altered, and the result showed 4 % leaked material (Table 3.5) compared to 32 % in the first experiment (Table 3.2). The degree of leakage obtained in the second experiment was in agreement with earlier work done on polymer leakage from alginate gel beads, showing degrees of leakage in the region of 0.8-7 % (Stokke et al., 1993, Coron, 2015). Coron (2015) followed the same dialyzation procedure as for the second experiment in the present work.

The alteration in the dialyzation procedure between the first (section 3.1.2) and the second (section 3.3.2) leakage experiment consisted of changing the molecular weight cut off (MWCO) of the dialysis tubes. In the first leakage experiment, dialysis tubes with a MWCO of 100-500 Da were used, while in the second leakage experiment dialysis tubes with a MWCO of 12-14 kDa were used. The reasoning for using a low MWCO (100-500 Da) in the first leakage experiment was to hinder the diffusion of chitosan oligomers across the membrane. Unpublished work performed by Ragnhild Bardal Roness at Department of Biotechnology (NTNU) showed that it takes over 16 days before 0.1 M NaCl completely exits a dialysis tube with a MWCO of 100-500 Da. In addition, the study showed that the conductivity in the water surrounding the dialysis tubes was drastically lower than the

conductivity measured inside the tubes even after 15 + hours. In the present work, the dialysis was only conducted over 5 days and conductivity was measured in the water surrounding the dialysis tubes, not inside the dialysis tubes; meaning that actual presence of ions within the sample was probably higher than measured in the surrounding solution of the dialysis tube. Also considering that the sodium chloride concentration was higher in this experiment (0.9 % w/v NaCl) than 0.1 M NaCl (0.6 % w/v), the possibility that most of the salt was not removed during dialysis is high, which makes the final measured weight of the leaked materials inconclusive.

Although the amounts of leaked material from the gel beads are uncertain, it is clear that both alginate and chitosan oligomers leaked out from the gel beads. The NMR analysis showed that there was nearly no differences between the leaked alginate and the starting material of alginate in regards to chemical composition, both for the calcium alginate gel beads and the alginate-CO gel beads gelled at pH 5.0 (Figure 3.2). The more or less unchanged chemical composition was not expected for the leaked material from calcium alginate gel beads, as studies have shown that alginate leaked from gel beads is enriched in mannuronic acid (Stokke et al., 1993). Stokke and coworker attributed the change in composition to chains not able to bind to the gel network, i.e. do not contain G-blocks longer than the minimum length required for formation of stable crosslinking junctions. Even though the leaked alginate was not particular enriched in mannuronic acid compared to the starting material, the average G-block length was lower in the leaked alginate. A minimum of eight adjacent G residues are required for the formation of a stable junction (Stokke et al., 1993, Bowman et al., 2016). The average G-block in the leaked alginate was 4. In that respect, the present result are in agreement with earlier work. Dissolution of parts of the gel beads might have caused the composition of the leaked alginate to be more similar to the starting material.

As it has been shown that chitosan oligomers form crosslinking junctions with M-blocks (Khong et al., 2013), one might speculate if crosslinking alginate gels with calcium and chitosan oligomer could reduce the amount of leaked material or change the composition of the alginate leaking out. However, as mentioned above, the interactions between alginate and chitosan oligomers have been shown to be sensitive to the pH both under and after gelling (Khong et al., 2013, Myrnes, 2016), and the selectivity between sodium and chitosan in *L. hyp.* leaf alginate is not known. In addition, shorter chitosan oligomers are likely to form weaker interaction with alginate (Khong, 2013). Chitosan oligomers that do not bind or form weak interactions with alginate will eventually diffuse out of gel. Myrnes (2016) conducted an experiment to investigate the length of chitosan oligomers leaking out of alginate CO gels.

The result showed that at pH 4.5 only shorter oligomers ($DP_n \leq 6$) leaked out of the gel, while at pH 8.5 a high proportion of longer chains leaked out. These result suggest that interactions between chitosan oligomers and alginate are only maintained at lower pH, which makes it probable that a great portion of chitosan oligomers leaked out from the gel beads in the present work. However, due to the probable presence of sodium chloride in the samples, it is not possible to quantify the amount of oligomers leaking out. The leaked alginate from the alginate CO gel beads gelled at pH 5.0 had nearly the same chemical composition as the starting material. This could be attributed to the crosslinking of M-blocks by chitosan oligomers, hindering the M-blocks in leaking out of the gel beads, except the same trend was seen for alginate gel beads crosslinked with only calcium.

4.2 Physical Properties of Leaf Alginate Gel Cylinders

An *in vivo* study performed in mice has shown that *L. hyp.* leaf alginate beads with intermediate guluronic acid content do not promote an immune response in terms of cellular overgrowth on transplanted gel beads, unlike *L. hyp.* stipe alginate beads rich in guluronic acid (Tam et al., 2011). However, Tam et al. (2011) also tested the stability of the alginate gel beads in terms of swelling and degradation *in vitro* and *in vivo*, and found the leaf alginate gel beads with intermediate guluronic acid content to be the least stable. Previous studies have also shown that gels prepared from alginate rich in mannuronic acid exhibit lower gel strength and lower size stability in saline compared to gels prepared from high G alginates (Smidsrød and Haug, 1972, Martinsen et al., 1989). Therefore, in addition to stability regarding size and polymer leakage (section 4.1), the mechanical properties of *L. hyp.* leaf alginate gels were also investigated, as physical properties of the alginate gel beads beyond the stability in terms of size and polymer leakage are important for how the alginate gel beads will behave in a given system. The effect of increasing the alginate concentration on the strength of leaf alginate gels was investigated. Both high and low molecular weight leaf alginates were studied, and compared to low molecular weight stipe alginate. Since high concentrations of alginate are known to give highly viscous solutions, which can have several disadvantages (Kong et al., 2003), viscosity measurements were conducted as well. In addition, the syneresis of all the alginate gels was also measured.

4.2.1 Syneresis

Syneresis of the prepared alginate gels decreased considerably with increasing alginate concentration. Since the calcium concentration was kept constant for all the gel samples, increasing the alginate concentration meant decreasing the calcium concentration relative to the alginate concentration. Studies have shown that syneresis become prominent only when the calcium content exceeds the optimal alginate concentration correlated to the content of guluronic acid residues (Draget et al., 1990, Martinsen et al., 1989). Syneresis have been suggested to be a result of lateral association of junction zones beyond dimerization (Stokke et al., 2000) and secondary MG/MG junctions (Donati et al., 2005) upon increasing the calcium concentration. However, without excess calcium these junction will not form, hence the gel network will not partially collapse, and water will not be released.

In the present study, syneresis decreased with decreasing molecular weight. Also, increasing the alginate concentration with very low molecular weight alginate led to a larger decrease in syneresis compared to increasing the concentration with alginates of higher molecular

weights. Studies have shown that decreasing the molecular weight of the alginate gives reduced syneresis (Draget et al., 2001). Draget and coworkers suggested that the decrease in syneresis with reduced molecular weight is a consequence of the increasing loose-end fraction, which reduces the possible attachment sites for the slowly forming junctions. They reported that the degree of syneresis was reduced with 50 % when the molecular weight was reduced from 320 to 50 kDa. In the present work, the degree of syneresis decreased with 30 % when the molecular weight was reduced from 200 to 115 kDa. Thus, the degrees of syneresis measured in the present work are in good agreement with previous work (Draget et al., 2001). The decrease in syneresis observed after replacing some of the higher molecular weight alginates with very low molecular weight alginate was likely caused by a reduction in the overall average molecular weight as these gels can be seen as bimodal blends with respect to molecular weight (Draget et al., 2001).

The stipe alginate gels showed slightly lower syneresis compared to the leaf alginate gels of the same molecular weight for all the concentrations. Martinsen and coworkers (1989) reported that high content of guluronic acid residues and high average G-block length led to low syneresis. The strong junctions G-blocks forms with calcium will hinder reorganization of the gel network. Mørch et al. (2008) reported 44 ± 1 % syneresis for 1.0 % (w/v) *L. hyp.* leaf alginate gels (η = 1200 mL/g) and 27 ± 0.6 % syneresis for 1.0 % (w/v) *L. hyp.* stipe alginate gels (η = 590 mL/g). The present work is thus consistent with previous work as the degree of syneresis was calculated to be 40 ± 1 % for the 1.0 % (w/v) high molecular weight *L. hyp.* leaf alginate gel ([η] = 958 mL/g) and 24 ± 1 % for the low molecular weight stipe alginate ([η] = 592 mL/g).

4.2.2 Young's Modulus

Young's modulus increased with increasing alginate concentration. Studies have shown that there is a linear relationship between Young's modulus and the square of the alginate concentration, which have been correlated to the biomolecular mechanism for junction formation (Smidsrød et al., 1972, Martinsen et al., 1989).

The Young's modulus was measured to be higher for the stipe alginate gels compared the leaf alginate gels. It has been reported that Young's modulus increases with increasing guluronic acid content and increasing average G block length (Smidsrød and Haug, 1972, Martinsen et al., 1989). In general, Young's modulus depends on the number and the strength of the crosslinks and on the length and stiffness of the chains between the crosslinks. As junctions are formed by chelation of calcium ions between guluronic acid blocks (Smidsrød, 1974,

Grant et al., 1973), a higher content of guluronic acid will give a higher number of crosslinks. *L. hyp.* stipe alginate gels have been found to have a considerably higher amount of long G-blocks ($N_{G>1} > 20$) compared to alginates rich in mannuronic acid (*M. Pyrifera* and *Durvillea potatorum*), and these long G-blocks have been correlated with high gel strength (Aarstad et al., 2012, Aarstad et al., 2013). Mørch et al (2008) conducted compression analysis on *L. hyp.* leaf and stipe alginate gels and the present results were consistent with those results. In the study by Mørch and coworkers, Young's modulus was measured to be 11 ± 1 and 27 ± 2 kPa for leaf and stipe alginate gels, respectively, while Young's modulus in this work was measured to be 12 ± 1 and 31 ± 3 kPa for leaf and stipe alginate gels, respectively.

No difference with regards to Young's modulus was seen between the high and low molecular weight leaf alginate gels, showing a molecular weight independence in terms of gel strength for the given molecular weights of alginate (M_w = 115-200 kDa). Studies have shown that Young's modulus is independent of molecular weight above a certain value. Smidsrød and Haug (1972) reported that Young's modulus was independent of the degree of polymerization down to $\overline{DP}_w \approx 400$. Martinsen et al. (1989) reported that Young's modulus was independent of molecular weight above η = 480 ml/g. The low molecular weight alginates was above this threshold, however, the very low molecular weight alginates were not. This explains why no difference in stiffness was seen between the high (M_w = 200 kDa) and low (M_w = 115) molecular weight leaf alginate gels, and why increasing the concentration with very low molecular weight leaf alginate had an effect on the gel strength, in giving a slightly lower Young's modulus compared to gels with the same alginate concentration. The reduction in Young's modulus is probably minor because the total average molecular weight was kept relatively close to the threshold. Increasing the concentration with very low molecular weight stipe alginate gave on the other hand a slightly higher Young's modulus compared to the alginate gels of the leaf alginate with the same concentration. The reduction in molecular weight was most likely counterbalanced by the high guluronic acid content in the stipe alginate.

4.2.3 Rupture Strength

The rupture strength of the alginate gels in the present work increased with increasing alginate concentration, which is in agreement with previous studies (Zhang et al., 2005, Mancini et al., 1999). The same studies reported that deformation at rupture was independent of alginate concentration, which also was seen in the present result. By increasing the alginate concentration, the crosslinking density is increased as long as calcium is present in sufficient

amounts (Stokke et al., 2000). The fracture of alginate gels results from the disruption of junctions zones as less energy is required to break the physical interactions which constitute the junctions zones in the gel network compared to the covalent bonds connecting the monomers in the network chains (Zhang et al., 2005). Hence, increasing the crosslinking density gives rise to increased rupture strength, as seen in the present study.

Furthermore, the rupture strength of the studied alginate gels increased with increasing molecular weight. In contrast to Young's modulus, it has been reported that rupture strength continues to rise with increasing molecular weight of alginate, i.e. does not become constant above a certain molecular weight (Mitchell, 1979). As a consequence, increasing the alginate concentration with very low molecular weight alginate had no effect on the rupture strength as the molecular weight of the added alginates was too low, in the region 33-34 kDa.

The rupture strength of the stipe alginate gels was significantly higher than the rupture strength of the leaf alginate gels of the same molecular weight for all the measured concentrations. Studies have shown that the rupture strength was greater for the gels prepared from alginates rich in guluronic acid (Mancini et al., 1999). However, other studies have shown that gels from alginates rich in mannuronic acid typically exhibit a higher rupture strength than gels prepared from alginates rich in guluronic acid (Mitchell and Blanshard, 1976, Mørch et al., 2008). This has been attributed to the shorter, stiffer chains of guluronic acid, which facilitate rupture by transmitting more energy to the junctions in the network. The rupture strength also depends on the strength and the number of crosslinks (Zhang et al., 2007), and a higher content of guluronic acid and longer average G-blocks will give more and longer junction zones. The longer the junction is, the higher stress is required to break it. However, after the breakage of the first junctions, the energy released will be transferred to the neighboring junction, hence accelerating the disruption of the other junctions (Zhang et al., 2007). The content of alternating sequences (MG-blocks) in the alginates may also affect the rupture strength (Mørch et al., 2008). MG-blocks are characterized by greater flexibility due to the alternating axial-equatorial and equatorial-axial linkages (Donati and Paoletti, 2009), which transfers less of the stress to the neighboring junctions. On the other hand, MG-blocks form weaker and fewer crosslinking junctions with calcium (Mørch et al., 2006).

4.2.4 Viscosity

Highly viscous solutions can be difficult to handle and can reduce cell viability due to the high forces required to mix the solution with cells (Kong et al., 2003). In addition, it can be difficult to prepare spherical and evenly sized gel beads from highly viscous alginate

solutions with an electrostatic bead generator. Therefore, it is desirable to keep the viscosity low. However, too low viscosity can make it difficult to prepare evenly sized gel beads, which was seen for the 1.8 % (w/v) low molecular weight leaf alginate gel beads. As expected, the 2.8 % (w/v) high molecular weight leaf alginate solution was much more viscous than the other alginate solutions. The stipe alginate solutions showed a higher viscosity than the leaf alginate solutions with the same molecular weight, which can be explained by the rigid structure of the G-block compared to the M- and MG-blocks due to the diaxial linkages (Skjåk-Bræk et al., 2006). In the present work, increasing the alginate concentration was shown to give greater gel strength in terms of Young's modulus and rupture strength. The present viscosity measurement showed that the concentration of the low molecular weight leaf alginate could be increased without drastically increase the viscosity of the solution. For the high molecular weight leaf alginate, the concentration could be increased with very low molecular weight alginate, however, this only gave an increase in Young's modulus, not rupture strength.

4.3 Stability of Low Molecular Weight Leaf Alginate Gel Beads

4.3.1 Swelling

The degree of swelling of the studied alginate gel beads decreased with increasing alginate concentration (Figure 3.6). To my knowledge, no studies have been conducted on the effect of alginate concentration on the swelling properties of alginate gels. However, unpublished work performed by Aurora Resell at Department of Biotechnology (NTNU) showed a similar correlation between alginate concentration and the degree of swelling. The swelling of alginate gel beads is determined by the equilibrium between the osmotic pressure and the elastic retraction force of the gel network, where the latter works to decrease the volume of the gel beads (Moe, 1993). For calcium alginate gels, the elasticity of the gel depends on the strength and the number of crosslinks. By increasing the alginate concentration, the number of crosslinks per unit volume, hence the number of elastically active chains per unit volume, will increase (Stokke et al., 2000). However, increasing the alginate concentration will also lead to a greater osmotic pressure due to a higher concentration of mobile counter ions. The results indicate that the latter has less impact on the volume of the gel beads, as the degree of swelling decrease with increasing alginate concentration. This might be due to the presence of high concentrations of sodium chloride in the solution, which reduces the osmotic pressure exerted on the gel beads. Nevertheless, calcium ions will be exchanged with the non-gelling sodium ions (Thu et al., 1996). However, the results indicate that a higher crosslinking density might counterbalance the destabilization of junction zones due to the exchange of calcium with sodium to some extent.

The high molecular weight leaf alginate gel beads showed a slightly lower degree of swelling compared to the low molecular weight leaf alginate gel beads. However, the uncertainty in the size of the 1.8 % (w/v) low molecular weight leaf alginate gel beads makes this difference insignificant. The results indicate that the degree of swelling is independent of the molecular weight, at least above a certain limit. In the current study, and in previous studies (Smidsrød and Haug, 1972, Martinsen et al., 1989), Young's modulus has been shown to be independent of the molecular weight above $M_w \approx 80$ kDa. Young's modulus reflects the number of crosslinks in the gel (Smidsrød and Haug, 1972). Hence, the elastic retraction force of the gel network will not be affected by the molecular weight of the alginate if Young's modulus is not affected by the molecular weight of the alginate. As a consequence, the degree of swelling will not change with molecular weight of the alginate.

Increasing the alginate concentration with very low molecular weight alginate had a greater effect on the size stability than increasing the concentration with higher molecular weight alginate. This might be attributed to greater inhomogeneity with respect to alginate distribution in the gel bead. Studies have shown that low molecular weight alginate give more inhomogeneous gels than higher molecular weight alginates, among other factors (Skjåk-Bræk et al., 1989). Increasing the alginate concentration at the surface has been correlated to greater stability (Thu et al., 1996). As expected, addition of stipe alginate reduced the degree of swelling to a large extent than addition of leaf alginate. Alginates rich in mannuronic acid have been shown to have a much lower resistance against ion exchange in the presence of non-gelling sodium ions than high G alginates (Martinsen et al., 1989, Mørch et al., 2006).

4.3.2 Polymer Leakage

The polymer leakage from the alginate gel beads of this study decreased with increasing alginate concentration and increasing molecular weight (Table 3.5). Previous studies have shown that the degree of polymer leakage from alginate gel beads depends on the molecular weight distribution, the average G-block length and the type of crosslinking ion (Stokke et al., 1991, Stokke et al., 1993). To my knowledge, no previous studies have investigated the effect of alginate concentration on the degree of polymer leakage. An increase in the polymer concentration induces a reduction in the pore size of the alginate gel (Martinsen et al., 1992). One might therefore speculate if higher alginate concentrations limit the diffusion of the short chains out of the gel beads to a greater extent. In addition, the degree of swelling has been shown in the present work to decrease with increasing alginate concentration. This might have been beneficial with respect to polymer leakage as swelling leads to larger pore size. Since the alginate samples varied widely in average molecular weight, it is natural to assume that the molecular weight distribution of the alginate samples varied equally. Stokke and coworkers (1993) found that the leaked material comprised the low molecular weight tail of the initial molecular weight distribution. This is consistent with the present results (Table 3.7), as the molecular weight of the leaked material was considerably lower than for the starting material of alginate. Hence, the total amount leaking out from the alginate gel beads depends on the fraction of the material that can leak out in the initial distribution. The results therefore indicate that the low molecular weight alginate sample contained a higher fraction of short chains not able to bind to the gel network. However, the average molecular weights of the leaked material were quite similar for all the samples regardless of the molecular weight of the starting material of alginate and there was no clear correlation between the two weights. This indicates that all the studied alginate samples contained short, non-gelling chains even

though the average molecular weight is high. Stokke and coworkers (1993) reported leakage to be in the order of 1-4 % of the starting material, which was consistent with the present work shown in Table 3.5.

The leaked materials were enriched in mannuronic acid (Table 3.6), which is consistent with previous work (Stokke et al., 1993). Stokke and coworkers assumed that the change in chemical composition arises from the chains that are not able to bind to gel network, i.e. do not contain G-blocks longer than the minimum length required for formation of stable junctions. They estimated that the minimum G-block length required for formation of stable crosslinking junctions was 8 ± 2, which later has been confirmed by other studies (Bowman et al., 2016). In the present work, the average G-block length of the leaked material was below 6 for all the alginate samples, which is in agreement with the assumption made by Stokke and coworkers (1993) . However, the leaked material was expected to be more enriched in mannuronic acid than that observed. Leakage of alginate from gel beads prepared from alginates with intermediate guluronic acid content, comprising lower amounts of long G-blocks, is expected to be less enriched in mannuronic acid (Stokke et al., 1993). Nevertheless, Stokke and coworkers reported a more profound difference in mannuronic acid content between the leaked alginate and the starting material of alginate, including alginate gel beads rich in mannuronic acid. It is possible that a fraction of the gel beads dissolved during saline treatment, which can explained the small difference in chemical composition is seen between the leaked alginate and the starting material of alginate. However, alginate gel beads were visible in the light microscope for all the samples.

The gel bead samples of alginate with added very low molecular weight leaf alginate showed a lower degree of leakage than the gel bead samples with added very low molecular weight stipe alginate, which was not expected. As mentioned above, it has been reported that decreasing the average G-block length increases the degree of leakage (Stokke et al., 1991), and the average G-block length was 16 for the very low molecular weight stipe alginate compared to 7 for the very low molecular weight leaf alginate. However, the uncertainty in the degree of leakage is not known, neither is the effect of the very low molecular weight alginate.

The amount and composition of leaked alginate are important from an immunological point of view. Studies have shown that alginates enriched in mannuronic acid stimulate the immune system (Otterlei et al., 1991, Kulseng et al., 1996). Alginate gel beads prepared from alginates with intermediate guluronic acid content are known to leak more compared to gel beads

prepared from alginates rich in guluronic acid (Stokke et al., 1993). However, an *in vivo* study performed in mice has shown that *L. hyp.* leaf alginate gel beads with intermediate G content did not promote an immune response as opposed to alginate gel beads rich in guluronic acid (Tam et al., 2011). The chemical composition of the leaked material may be more important than the amount leaking out. The current study has shown that increasing the alginate concentration and the molecular weight of the alginate will reduce the degree of leakage from *L. hyp.* leaf alginate gel beads. However, one should notice that increasing the concentration of the low molecular weight leaf alginate from 1.8 % to 2.8 % (w/v) did not reduce the degree of leakage below that of the 1.8 % (w/v) high molecular weight leaf alginate. As mentioned previous, increasing the concentration of the high molecular weight leaf alginate could be problematic due to high viscosity. On the other hand, increasing the concentration of the low molecular weight leaf alginate further could be an option, especially since the alginate gel beads with added very low molecular weight leaf alginate displayed about the same degree of leakage as the gel bead samples with corresponding concentrations.

4.4 Future work

The aim of this thesis has been to investigate the stability and mechanical properties of *Laminaria hyperborea* leaf alginate gels as a strategy to increase biocompatibility of alginate gels intended for use as a immunoisolating material without compromising the stability of the gel under physiological conditions.

The polymer leakage experiment conducted on the alginate-CO gel beads showed that both alginate and chitosan leaked out of the beads. However, the leakage from alginate-CO gel beads should be further investigated to obtain more quantitative data. By increasing the time of dialysis considerably, sodium chloride can be completely removed from the leaked material which would give more accurate estimates of the degree of polymer leakage from the gel beads. Alternatively, dialysis tubes with a higher molecular weight cut off (12-14 kDa) could be used for quantification of merely alginate leakage, as the chitosan oligomers will leak out from the dialysis tubes due to its small size. Furthermore, the average molecular weight of the alginate leaking out of the alginate-CO gel beads could be determined by SEC-MALLS. It has been suggested that the interaction between alginate and chitosan oligomers increases with the length of the chains of the oligomer (Khong, 2013). Therefore, investigations on the length of the chitosan oligomers leaking out of the gel beads could be studied.

Increasing the alginate concentration showed promising results in increasing the stability of low MW leaf alginate gel beads both with respect to size and polymer leakage. To achieve a greater understanding of the mechanism behind these results, it could be investigated if the same stabilizing effect is observed for gel beads treated with ion free water. In ion free water the osmotic pressure exerted on the gel beads would be greater, however, the junctions would not destabilized by the exchange of calcium with sodium. As the present study showed a concentration dependence regarding stability in size and polymer leakage, further investigations could be performed on the effect of increasing the alginate concentration beyond the concentrations studied in this work. Moreover, the effect of molecular weight of the alginate on the stability should be further studied. The present results indicated that the degree of swelling was independent of the molecular weight of the alginate. However, this should be studied more systematically with several different molecular weights, and at higher concentrations to avoid high uncertainty in the size of gel beads prepared from low molecular weight alginate. The gel bead samples with added very low MW stipe alginate showed greater

stability with respect to size but lower stability with respect to polymer leakage, compared to the gel bead samples with added very low MW leaf alginate. This was not expected and should be further investigated. One strategy could be to investigate the effect of adding very low MW stipe alginate to gel beads prepared from low MW stipe alginate on the stability, especially with regards to polymer leakage, and compare this to the present result.

Alginate gel beads with highly inhomogeneous polymer distributions, where most of the alginate is located at the surface of the gel beads, might be preferred for immunoisolation due to lower porosity at the gel surface (Martinsen et al., 1992) and higher dimensional stability (Thu et al., 1996). Therefore, it could be relevant to investigate the polymer distribution within both the alginate-CO gel beads and the low MW leaf alginate gel beads with confocal laser scanning microscopy (CLSM). Substituting a fraction (1.0 % w/v) of the low MW leaf alginate with very low MW leaf or stipe alginate had a stabilizing effect with respect to size. This might have been due to a more inhomogeneous polymer distribution in the gel beads with added very low MW alginate, which could be visualized by CLSM. Furthermore, it could be relevant to investigate the permeability of the gel beads, as permeability is an important factor determining the functionality of beads intended for immunoisolation, and the alginate concentration and chemical composition will affect the permeability of the gel (Martinsen et al., 1992).

5 Conclusion

In this thesis, the stability and mechanical properties of *L. hyperborea* leaf alginate gels were investigated. *L. hyp.* leaf alginate gel beads crosslinked with calcium and chitosan oligomers were prepared in calcium chloride solutions (50 mM) with pH 5.0 and 6.5. The stability of alginate-CO gel beads in saline was studied in terms of swelling and polymer leakage, and compared to alginate gel beads crosslinked with only calcium. The mechanical properties in terms of Young's modulus and rupture strength was investigated for *L. hyp.* leaf alginate gel cylinders of varying molecular weights and concentrations. The effect of increasing the alginate concentration with very low molecular weight leaf or stipe alginate was also studied, where gel cylinders prepared from low molecular weight *L. hyp.* stipe alginate were included as a reference. In addition, syneresis was measured for all the alginate gel cylinders. Furthermore, the stability of low molecular weight *L. hyp*. leaf alginate gel beads in saline, in terms of swelling and polymer leakage, was investigated. High molecular weight *L. hyp.* leaf was included for comparison.

Introducing chitosan oligomers into the crosslinking of alginate gel beads with calcium had a minor stabilizing effect with respect to size at pH 5.0, whereas alginate-CO gel beads prepared at pH 6.5 showed no stabilizing effect, compared to alginate gel beads gelled only with calcium. The chitosan oligomers at pH 5.0 had a larger capacity to form crosslinks with alginate as a higher fraction of the amino groups were protonated, and therefore a stabilizing effect was seen for the alginate-CO gel beads gelled at pH 5.0 opposed to the alginate-CO gel beads gelled at pH 6.5. In regards to polymer leakage, both alginate and chitosan oligomers leaked out of the alginate-CO gel beads, however, the amounts were uncertain due to a high salt content in the samples. The leaked alginate from the alginate-CO gel beads had a similar chemical composition to the starting material of alginate, which is contradictory to previous studies that have shown a lower guluronic acid content in the leaked material from alginate gel beads (Stokke et al., 1993). The present finding may have been caused by the dissolution of parts of the gel beads, resulting in the composition of leaked alginate to be similar to the starting material of alginate.

Increasing the alginate concentration for low and high molecular weight *L. hyp.* leaf alginate gels led to higher Young's modulus and higher rupture strength. The Young's modulus of the alginate gels were independent of the molecular weight in the given range (M_w = 115-200 kDa), where increasing the alginate concentration of the gels with very low molecular weight leaf or stipe alginate had the same effect as increasing the alginate concentration with higher

molecular weight leaf alginate. On the other hand, the rupture strength increased with increasing molecular weight of the alginate in the given range (M_w = 115-200 kDa), where no effect was seen for the rupture strength when the alginate concentration was increased with very low molecular weight leaf or stipe alginate. The low molecular weight *L. hyp.* stipe alginate showed higher Young's modulus and slightly higher rupture strength compared to the leaf alginate of the same molecular weight. Syneresis of the alginate gels decreased considerably with increasing alginate concentration. The low molecular weight leaf alginate showed lower syneresis than the high molecular weight alginate. The low molecular weight stipe alginate showed slightly lower syneresis than the leaf alginate of the same molecular weight.

The size stability of low molecular weight *L. hyp*. leaf alginate gel beads in saline increased with increasing alginate concentration. Increasing the alginate concentration with very low molecular weight alginate had a larger effect on the size stability than increasing the alginate concentration correspondingly with low molecular weight leaf alginate. No significant difference was seen between the 1.8 % (w/v) low and high molecular weight leaf alginate gel beads with regards to size stability. The degree of polymer leakage decreased with increasing alginate concentration and increasing molecular weight. Increasing the alginate concentration with very low molecular weight leaf or stipe alginate also led to a decrease in the relative amount of leaked material, but the effect was smaller than increasing the concentration with more low molecular weight leaf alginate. The leaked material from the low molecular weight leaf alginate gel beads was enriched in mannuronic acid compared to the starting material.

Conclusively, the present work have shown that the studied leaf alginate gel beads can be stabilized in terms of swelling and polymer leakage by increasing the alginate concentration. However, the leaf alginate gel beads still exert lower stability than stipe alginate gel beads. Introducing chitosan oligomers into the crosslinking of alginate with calcium had only a slight stabilizing effect at pH 5.0, which is not compatible with immunoisolation purposes. The molecular weight of the alginate had also only minor effects on the stability of the leaf alginate gel beads.

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Appendices

A Swelling of Alginate Chitosan Oligomer Gel Beads in Saline

Table A – 1: Size measurements leaf alginate gel beads (1,8 % w/v) with and without added chitosan oligomers (CO) prepared in gelling solution with a pH of either 5.0 or 6.5 before six hourly treatments with 0.9 % NaCl. The bead diameters are the average ± the standard deviation of 20 beads.

Saline treatment	**Leaf alginate**		**Leaf alginate + CO, pH 6.5**		**Leaf alginate + CO, pH 5.0**	
	Diameter (µm)	SDOM (n=20)	Diameter (µm)	SDOM (n=20)	Diameter (µm)	SDOM (n=20)
0	416	12	396	28	401	30
1	461	16	429	18	413	45
2	551	18	509	24	486	40
3	594	24	550	19	527	36
4	648	20	600	30	554	39
5	718	17	651	35	624	40
6	764	30	744	36	654	33
Diameter increase	84 %		88 %		63 %	

B Polymer Leakage from Alginate Chitosan Oligomer Gel Beads

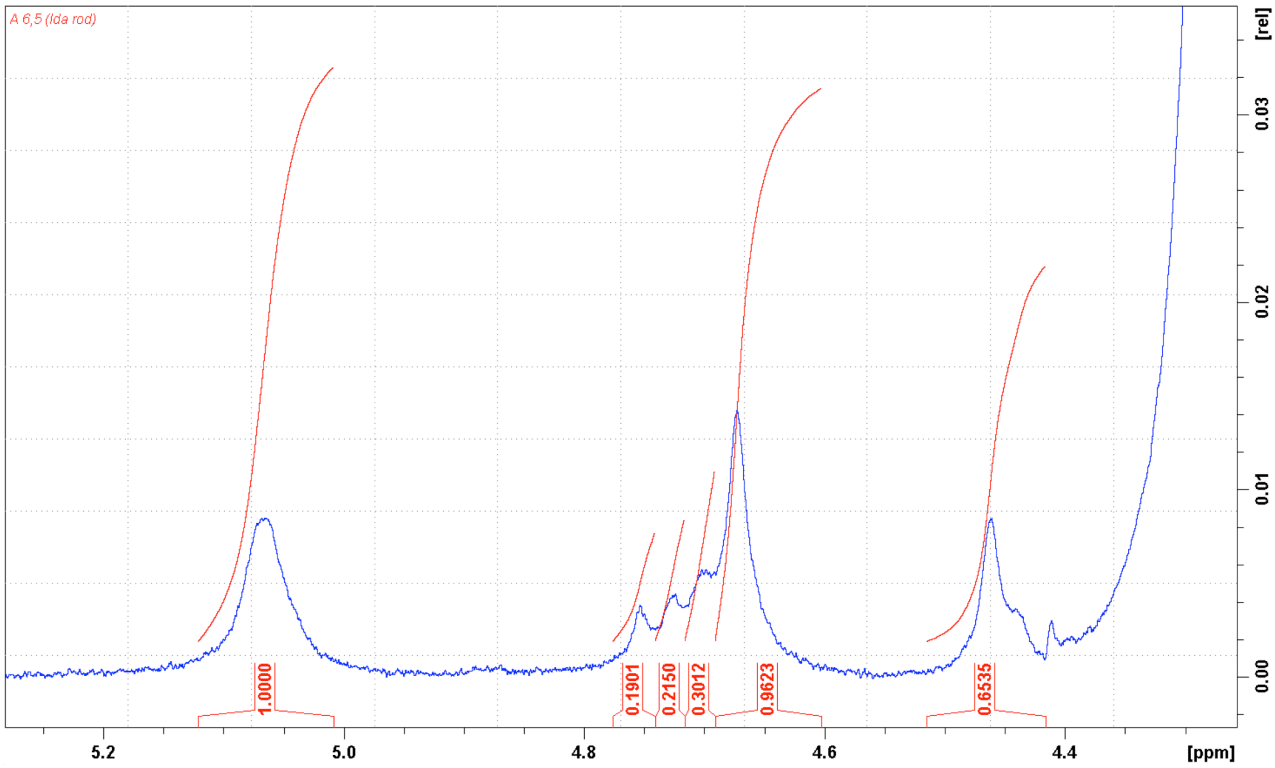

Figure B – 1: NMR spectrum of material leaked from leaf alginate gel beads.

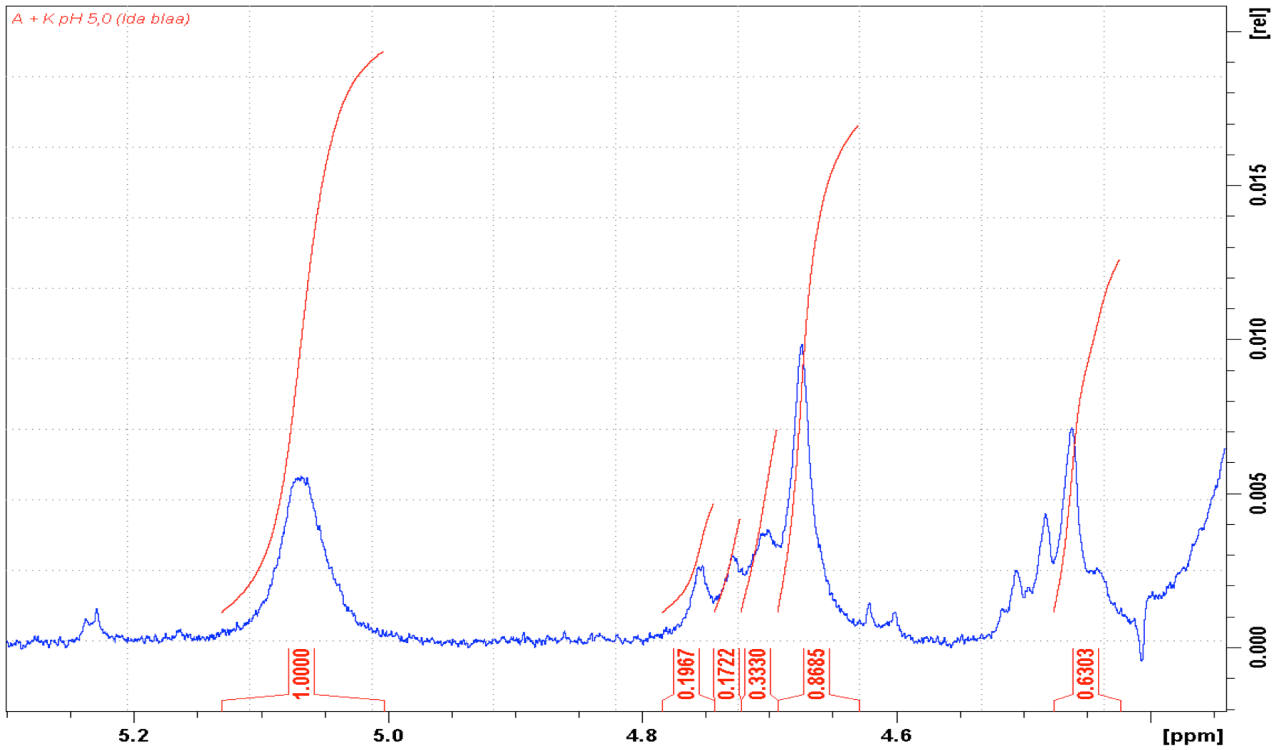

Figure B – 2: NMR spectrum of material leaked from alginate chitosan oligomer gel beads gelled at pH 5.0.

C Syneresis

The original weight of the gel cylinders are calculated from the height and the diameter of the well by assuming that the density of the alginate solution is the same as water

$$w_0 = (3.14 \cdot (16\,mm/2)^2 \cdot 18\,mm) \cdot 0.001\,g/mm^3 = 3.62\,g$$

After gelling and Ca^{2+} saturation the gel cylinders were weighted. Syneresis was calculated from the ration between weight loss and original weight

$$Syneresis\ (\%) = \frac{w_0 - w}{w_0} \cdot 100$$

The weight and syneresis data for all the alginate samples are displayed in table C – 1.

Table C – 1: Syneresis and weight data for gel cylinders prepared from high and low molecular weight leaf alginates and low molecular weight stipe alginate, including these alginates mixed with very low molecular weight leaf and stipe alginate. The mixed alginate samples had a total concentration of 2.8 % (w/v). The gel cylinders comprising only one alginate were prepared with three different concentrations: 1.0 %, 1.8 % and 2.8 %. The weight of the alginate solutions were calculated from the volume of the well and the density of the solution (assumed the density is the same as water). The values are given as the average ± SD of 5-9 gel cylinders.

Sample	**Weight of alginate solution, w_0 (g)**	**Weight after gelling and Ca^{2+} saturation, w (g)**	**Syneresis (% w/w)**
High MW leaf (1.0 %)	3.62	2.19 ± 0.04	40 ± 1
High MW leaf (1.8 %)	3.62	2.76 ± 0.04	24 ± 1
High MW leaf (2.8 %)	3.62	3.06 ± 0.03	15 ± 1
Low MW leaf (1.0 %)	3.62	2.60 ± 0.02	28 ± 1
Low MW leaf (1.8 %)	3.62	3.23 ± 0.07	11 ± 2
Low MW leaf (2.8 %)	3.62	3.48 ± 0.08	4 ± 2
Low MW stipe (1.0 %)	3.62	2.75 ± 0.02	24 ± 1
Low MW stipe (1.8 %)	3.62	3.39 ± 0.02	6 ± 1
Low MW stipe (2.8 %)	3.62	3.65 ± 0.03	-1 ± 1
High MW leaf (1.8 %) + very low MW leaf (1.0 %)	3.62	3.38 ± 0.05	7 ± 1
Low MW leaf (1.8 %) + very low MW leaf (1.0 %)	3.62	3.58 ± 0.07	1 ± 2
Low MW stipe (1.8 %) + very low MW leaf (1.0 %)	3.62	3.72 ± 0.02	-3 ± 1
High MW leaf (1.8 %) + very low MW stipe (1.0 %)	3.62	3.48 ± 0.10	4 ± 3
Low MW leaf (1.8 %) + very low MW stipe (1.0 %)	3.62	3.67 ± 0.04	-1 ± 1
Low MW stipe (1.8 %) + very low MW stipe (1.0 %)	3.62	3.76 ± 0.04	-4 ± 1

D Compression

D – 1 Example Stress/Strain Curve

The mechanical properties were analyzed by uniaxial compression (0.10 mm/s test speed) using a texture analyzer (TA.XT *plus* texture analyser, Stable Micro Systems, UK) equipped with a 5 kg or a 30 kg load cell and a cylindrical 35 mm diameter aluminum probe. Texture Exponent software v.5.1.2.0 was used to analyze the data. Figure D – 1 show an example of a stress/strain curve.

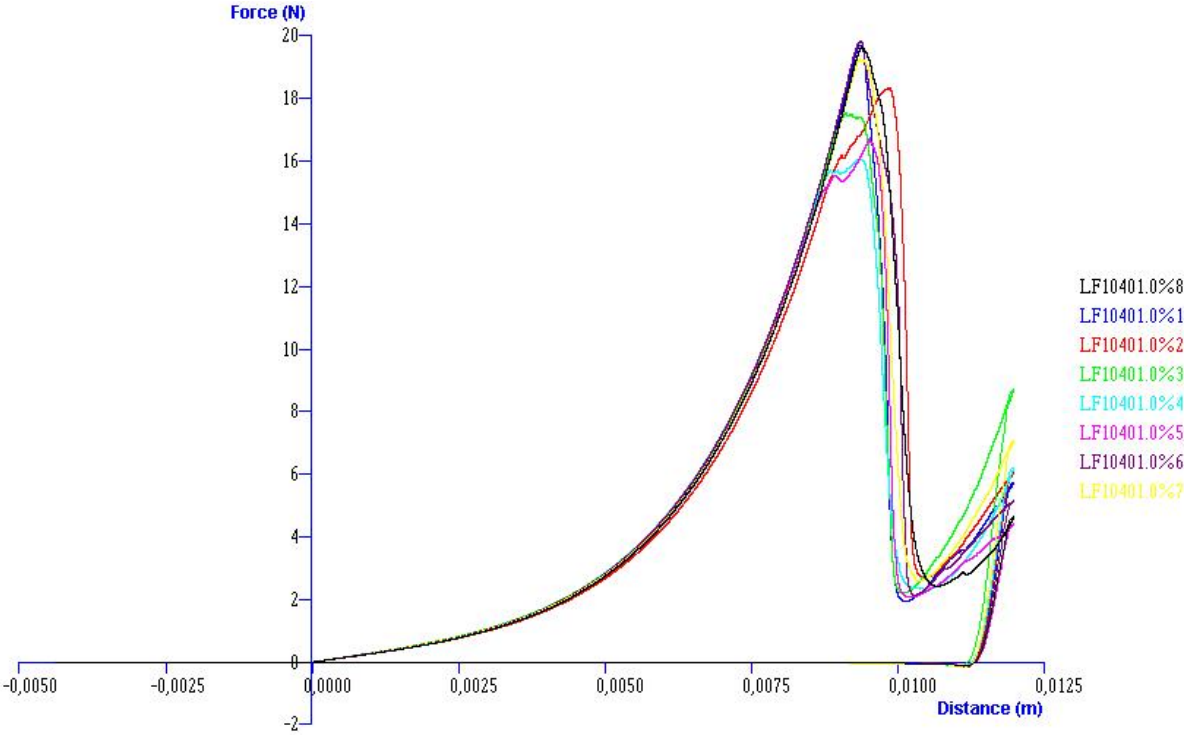

Figure D – 1: The stress/strain curves for gel cylinders made of 1.0 % LF10/40. Young's moduli is calculated from the initial part of the curves (0,1 - 0,3 mm). The first peak represent point of rupture.

D – 2 Young's Modulus

Young's modulus is defined as the ratio of stress to strain, where stress is force per unit area and strain as the relative amount of deformation

$$E = (F/A)/\,(\Delta L/L)$$

The gradient is given by the slope of the initial part of the stress/strain curve (0,1-0,3 mm) and has the unit N/m

$$Gradient = F/\Delta L$$

The area which the force is applied on is calculated from the diameter of the gel cylinder

$$A\ (m^2) = 3.14\ \cdot (diameter\ (m)/2)^2$$

Young's modulus was therefore calculated by multiplying the gradient with the length of the gel cylinder and dividing the product by the area of force impact

$$E = (Gradient\ \cdot L\ (m)/A$$

Young's modulus is then corrected with regard to weight due to change in alginate concentration following syneresis

$$E_{corr} = E\ \cdot (w_0/w)^2$$

The gradient, Young's modulus and Young's modulus corrected for change in alginate concentration for all the alginate samples are displayed in in table D – 1.

Table D – 1: Gradient, Young's modulus and Young's modulus corrected for change in alginate concentration for gel cylinders prepared from high and low molecular weight leaf alginates and low molecular weight stipe alginate, including these alginates mixed with very low molecular weight leaf and stipe alginate. The mixed alginate samples had a total concentration of 2.8 % (w/v). The gel cylinders comprising only one alginate were prepared with three different concentrations: 1.0 %, 1.8 % and 2.8 %. The gradients are obtained from the initial stress/strain curves (0,1 – 0,3 mm). The values are given as the average ± SD of 5-9 gel cylinders.

Sample	**Gradient (N/m)**	**Young's modulus (kPa)**	**Young's modulus corrected with regard to weight (Pa)**
High MW leaf (1.0 %)	305 ± 16	33 ± 2	12 ± 1
High MW leaf (1.8 %)	478 ± 54	47 ± 5	28 ± 5
High MW leaf (2.8 %)	719 ± 43	70 ± 6	50 ± 4
Low MW leaf (1.0 %)	285 ± 9	30 ± 1	15 ± 1
Low MW leaf (1.8 %)	440 ± 37	40 ± 5	32 ± 5
Low MW leaf (2.8 %)	559 ± 54	54 ± 4	50 ± 5
Low MW stipe (1.0 %)	525 ± 39	54 ± 4	31 ± 3
Low MW stipe (1.8 %)	646 ± 59	61 ± 7	54 ± 7
Low MW stipe (2.8 %)	896 ± 77	90 ± 9	92 ± 10
High MW leaf (1.8 %) + very low MW leaf (1.0 %)	544 ± 24	52 ± 3	45 ± 3
Low MW leaf (1.8 %) + very low MW leaf (1.0 %)	459 ± 37	45 ± 3	44 ± 4
Low MW stipe (1.8 %) + very low MW leaf (1.0 %)	674 ± 37	72 ± 4	76 ± 3
High MW leaf (1.8 %) + very low MW stipe (1.0 %)	645 ± 58	62 ± 5	57 ± 6
Low MW leaf (1.8 %) + very low MW stipe (1.0 %)	589 ± 46	59 ± 5	61 ± 5
Low MW stipe (1.8 %) + very low MW stipe (1.0 %)	787 ± 132	83 ± 15	90 ± 19

D – 3 Rupture Strength

The rupture strength (kg) and deformation at rupture was calculated by the Texture Exponent software from the stress/strain curve and is displayed in in table D – 2.

Table D – 2: Rupture strength and deformation at rupture for gel cylinders prepared from high and low molecular weight leaf alginate and low molecular weight stipe alginate, including these alginates mixed with very low molecular weight leaf and stipe alginate. The mixed alginate samples had a total concentration of 2.8 % (w/v). The gel cylinders comprising only one alginate were prepared with three different concentrations: 1.0 %, 1.8 % and 2.8 %. The values are given as the average ± SD of 5-9 gel cylinders.

Sample	**Rupture strength (kg)**	**Deformation at rupture (mm)**
High MW leaf (1.0 %)	4.1 ± 0.6	10.6 ± 0.3
High MW leaf (1.8 %)	6.3 ± 1.2	10.3 ± 0.3
High MW leaf (2.8 %)	11.2 ± 0.9	10.9 ± 0.2
Low MW leaf (1.0 %)	1.8 ± 0.2	9.2 ± 0.3
Low MW leaf (1.8 %)	3.0 ± 0.8	9.3 ± 0.5
Low MW leaf (2.8 %)	5.7 ± 1.2	10.2 ± 0.7
Low MW stipe (1.0 %)	3.7 ± 0.4	9.8 ± 0.3
Low MW stipe (1.8 %)	7.8 ± 1.6	10.5 ± 0.6
Low MW stipe (2.8 %)	8.4 ± 1.7	11.0 ± 0.7
High MW leaf (1.8 %) + very low MW leaf (1.0 %)	7.3 ± 1.2	10.9 ± 0.3
Low MW leaf (1.8 %) + very low MW leaf (1.0 %)	3.5 ± 0.7	10.2 ± 0.8
Low MW stipe (1.8 %) + very low MW leaf (1.0 %)	5.1 ± 1.5	10.8 ± 1.0
High MW leaf (1.8 %) + very low MW stipe (1.0 %)	7.0 ± 1.2	10.7 ± 0.7
Low MW leaf (1.8 %) + very low MW stipe (1.0 %)	3.0 ± 1.0	9.7 ± 0.9
Low MW stipe (1.8 %) + very low MW stipe (1.0 %)	5.6 ± 1.4	10.8 ± 0.2

E Viscosity

The viscosity of selected alginate solutions was measured with a rotational rheometer. The viscosity was measured at 20 °C for shear rates either between 0.5 and 30 s^{-1} or between 0.1 and 100 s^{-1}. The result is displayed in figure E – 1.

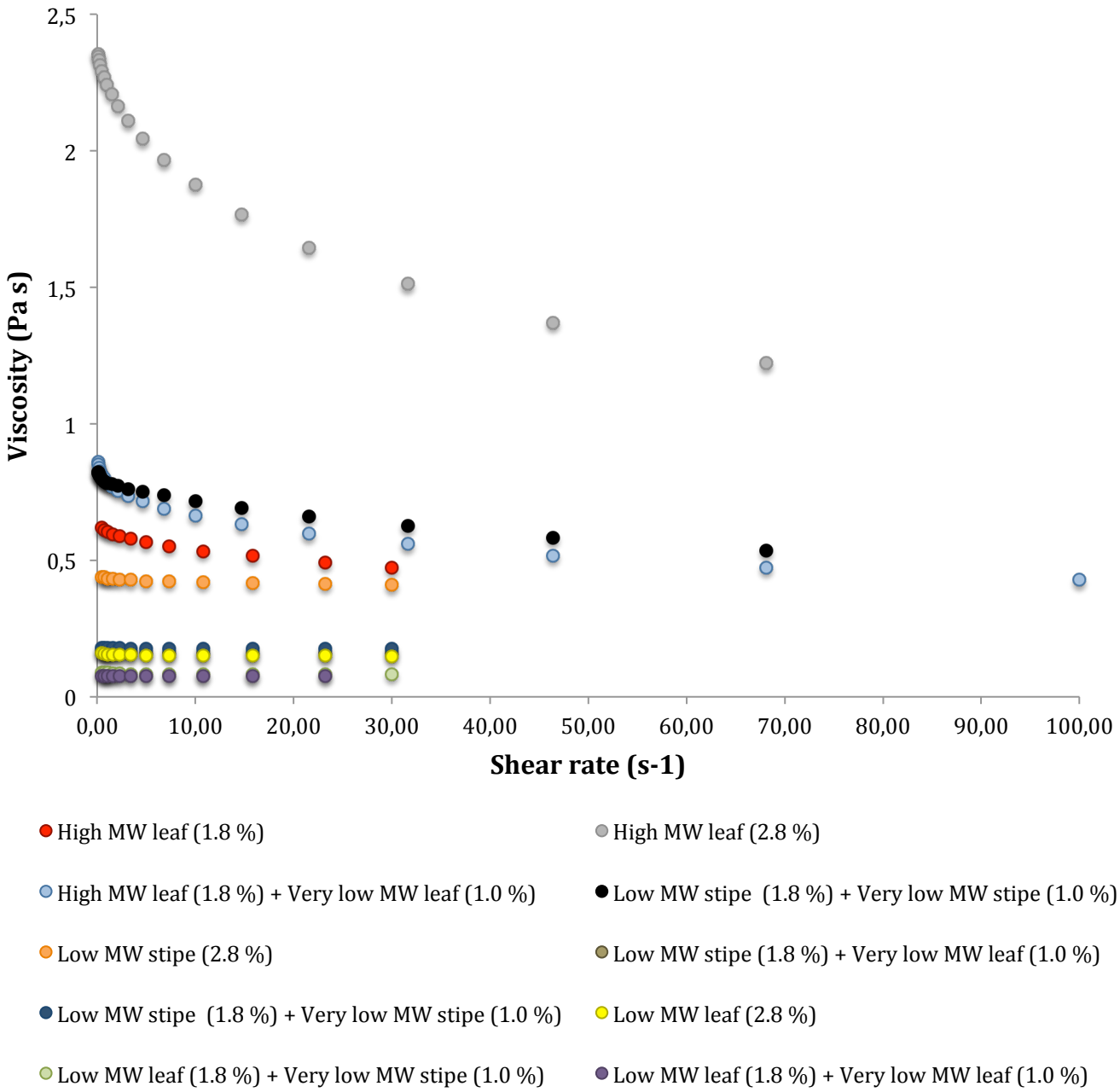

Figure E -1: The shear viscosity was measured using a rotational rheometer (Kinexus Ultra +, Malvern, UK) equipped with a cone/plate (4°/40 mm). The measurements was conducted at 20 °C for shear rates between 0.5 and 30 s^{-1} (except for the samples with high molecular weight leaf alginate with a total concentration of 2.8 %, which was measured for shear rates between 0.1 and 100 s^{-1}) with six measurements per decade with a logarithmic progression.

F Swelling of Low Molecular Weight Leaf Alginate Gel Beads in Saline

Table F – 1: Size measurements of low and high molecular weight leaf alginate gel beads with and without added very low molecular weight alginate after gelation, after being washed with 0.9 % NaCl, and after being submerged in 0.9 % NaCl over night. The degree of swelling (%) was calculated by comparing the final bead diameter with the initial diameter. The bead diameters are the average ± the standard deviation of 20 beads.

Saline treatment	**Low MW leaf (1.8 %)**		**High MW (1.8 %)**		**Low MW leaf (2.8 %)**		**Low MW leaf (1.8 %) + very low MW leaf (1.0 %)**		**Low MW leaf (1.8 %) + very low MW stipe (1.0 %)**	
	Diameter (µm)	SD (n=20)	Diameter (µm)	SD (n=20)	Diameter (µm)	SD (n=20)	Diameter (µm)	SD (n=20)	Diameter (µm)	SD (n=20)
0	366	45	453	17	465	17	454	19	475	25
1	451	75	525	16	497	18	526	16	489	26
2	497	54	600	19	569	14	515	13	524	22
Diameter increase (%)	36		32		22		13		10	

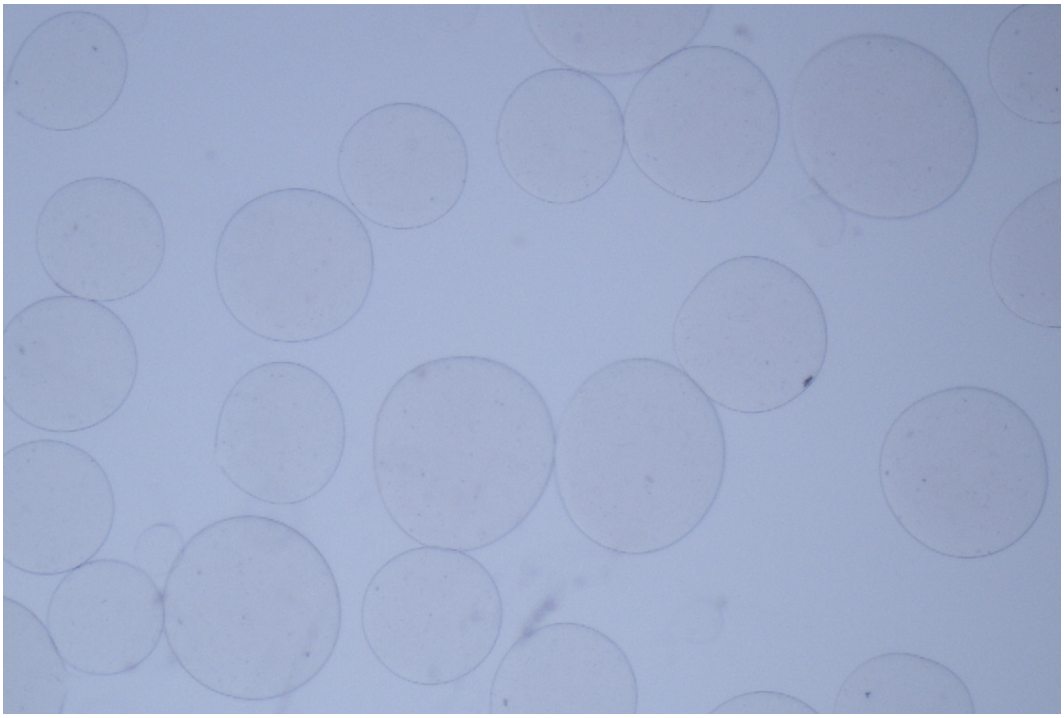

Figure F – 1: Alginate gel beads comprised of low molecular weight leaf alginate (1.8 % w/v) at 4X magnification. Due to low viscosity it was difficult to get evenly sized beads.

G Polymer Leakage from Low Molecular Weight Leaf Alginate Gel Beads

G – 1 NMR Spectra

The chemical composition of the polymeric material leaked from alginate gel beads during treatment with saline was obtained with 1H-NMR spectroscopy. Figure G – 1 to G – 5 show the NMR spectra for the five samples the leakage experiment was conducted on. The method used for determining the chemical composition is one developed by Grasdalen and coworkers (Grasdalen, 1983).

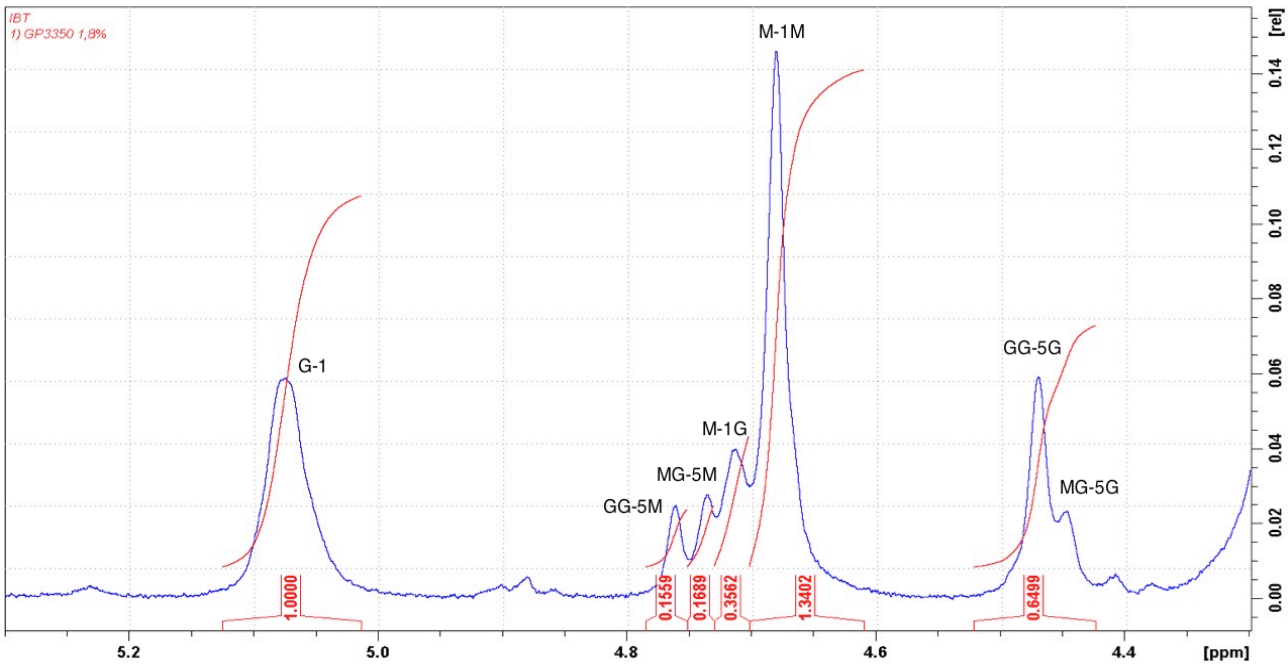

Figure G – 1: NMR spectrum of the leaked material from alginate gel beads prepared from high MW leaf (1.8 %).

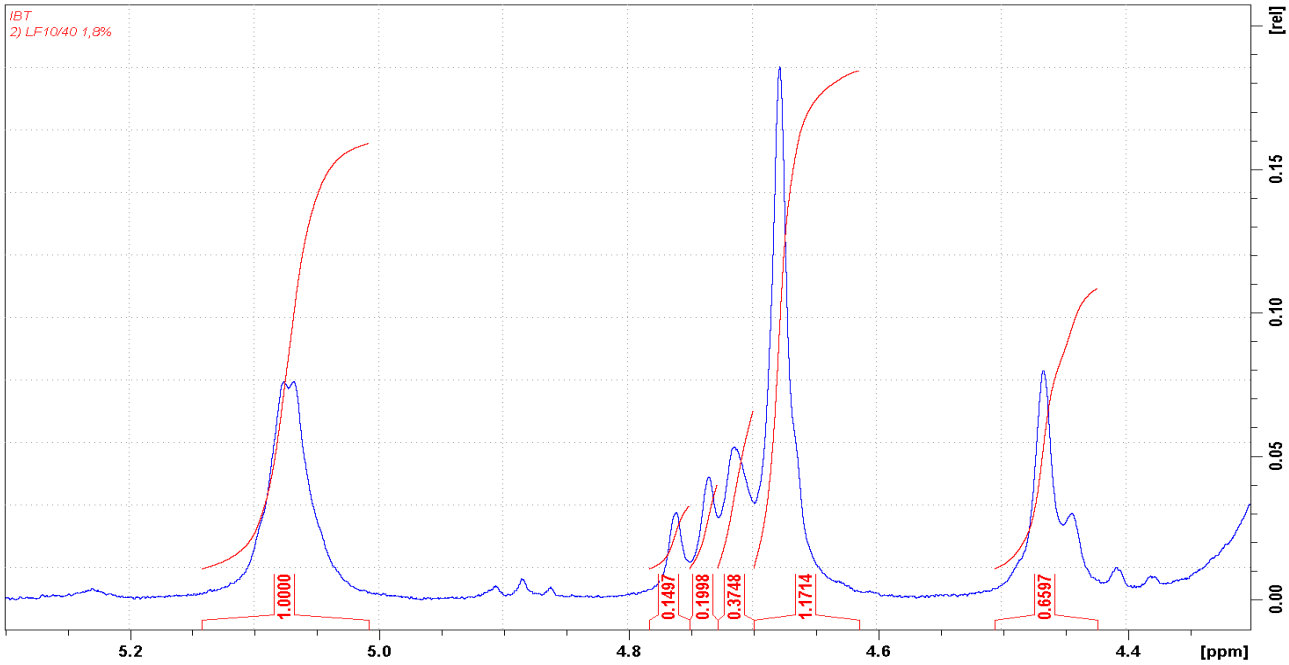

Figure G – 2: NMR spectrum of the leaked material from alginate gel beads prepared from low MW leaf (1.8 %)

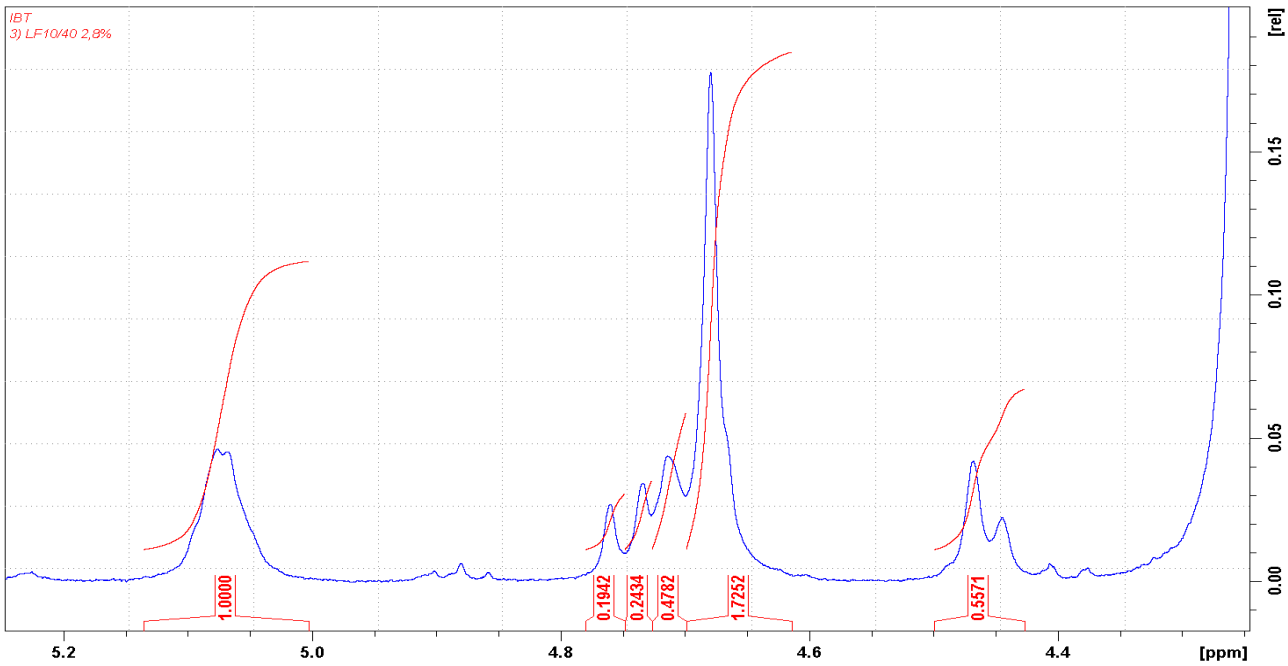

Figure G – 3: NMR spectrum of the leaked material from alginate gel beads prepared from low MW leaf (2.8 %).

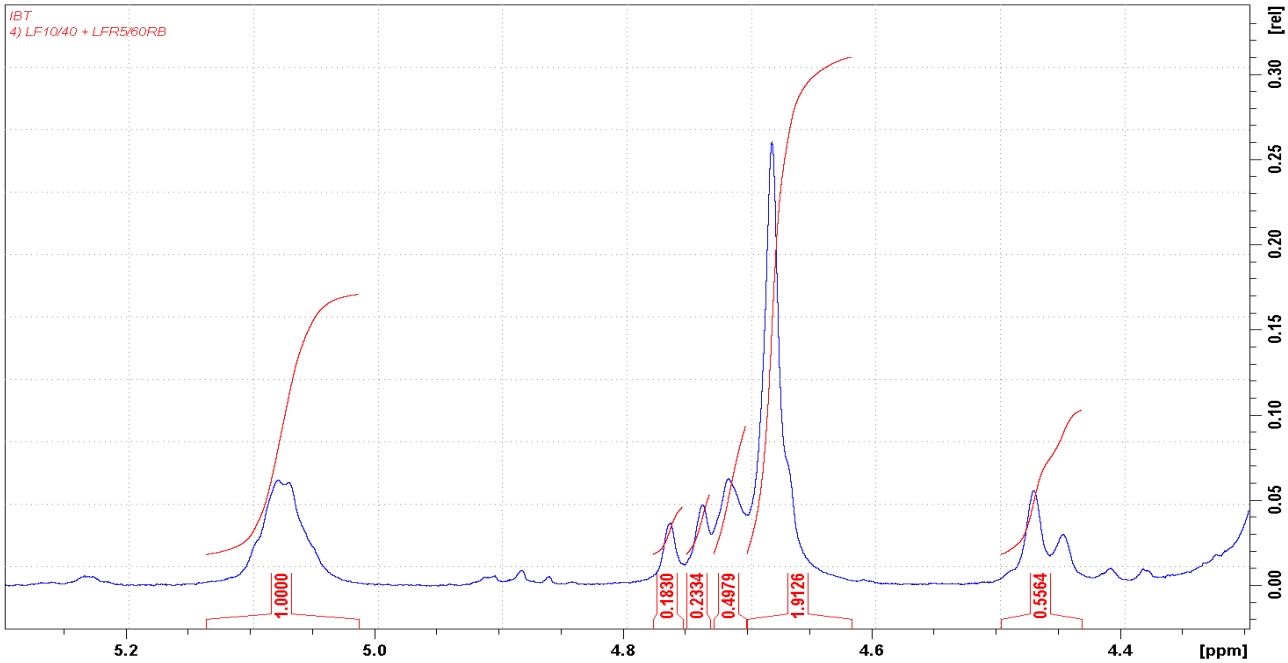

Figure G – 4: NMR spectrum of the leaked material from alginate gel beads prepared from low MW leaf (1.8 %) + very low MW leaf.

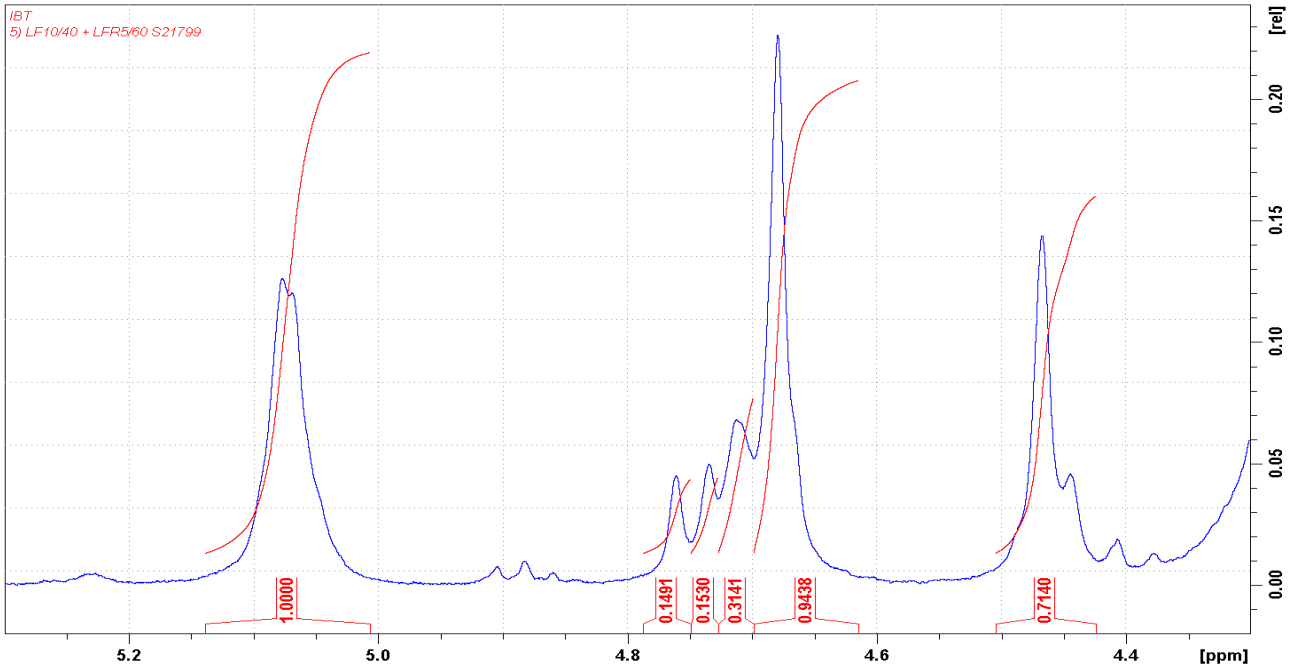

Figure G – 5: NMR spectrum of the leaked material from alginate gel beads prepared from low MW leaf (1.8 %) + very low MW stipe.

G – 2 SEC-MALLS Chromatograms

The average molecular weight and molecular weight distribution of alginate leaked from gel beads prepared from leaf alginate were determined by SEC-MALLS. The gel beads were washed in saline and kept in saline overnight. Figure G – 5 to G – 10 present the chromatograms of the leaked material for all the gel bead samples. The molecular weight and the concentration of alginate were varied.

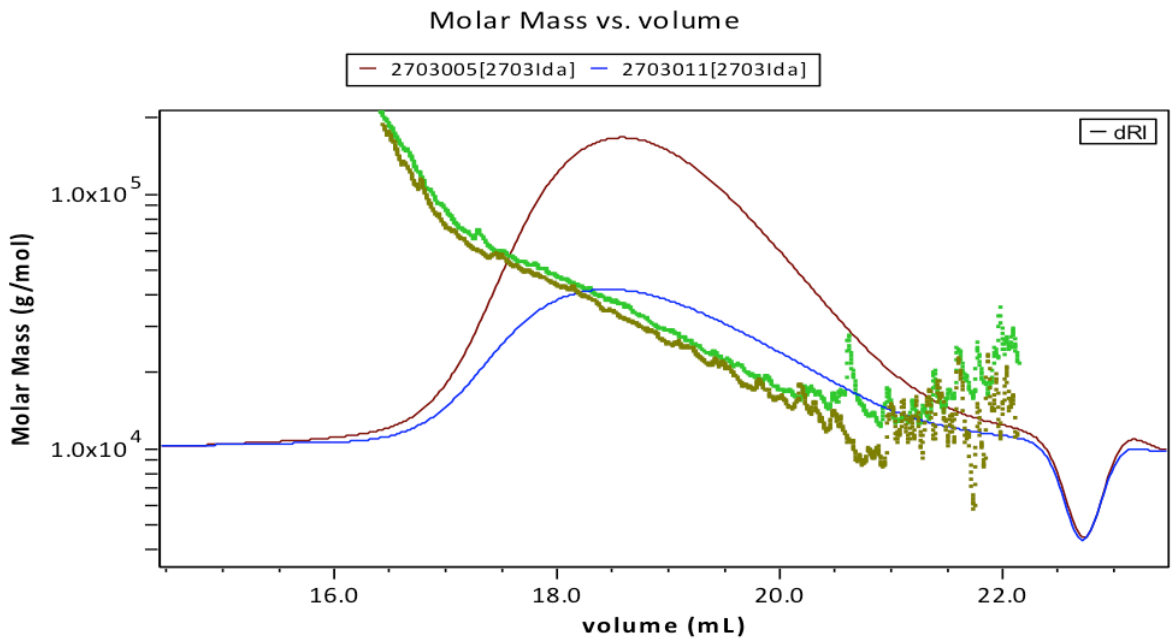

Figure G – 6: SEC MALLS chromatogram of alginate leaked from gel beads prepared from high molecular weight leaf alginate (1.8 %).

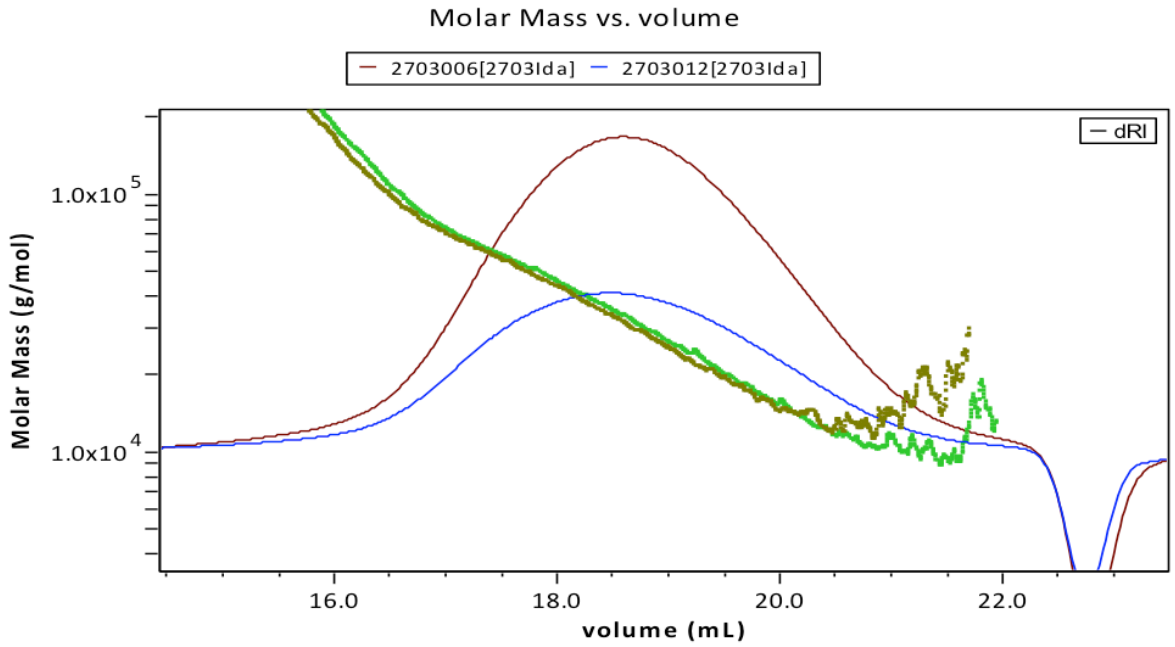

Figure G – 7: SEC MALLS chromatogram of alginate leaked from gel beads prepared from low molecular weight leaf alginate (1.8 %).

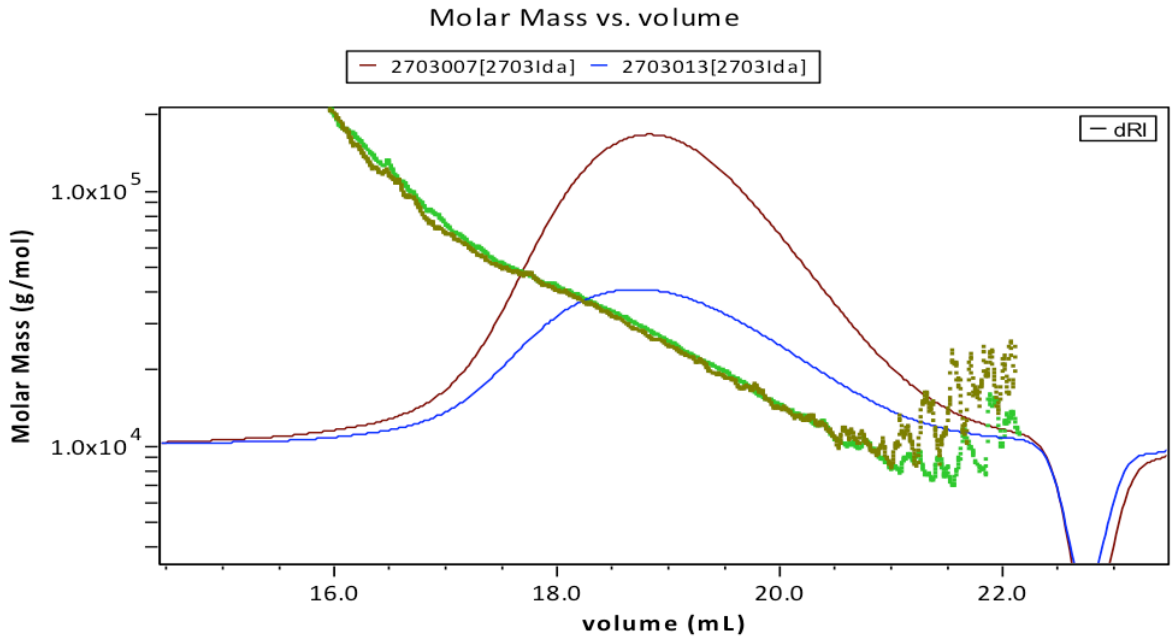

Figure G – 8: SEC MALLS chromatogram of alginate leaked from gel beads prepared from low molecular weight leaf alginate (2.8 %).

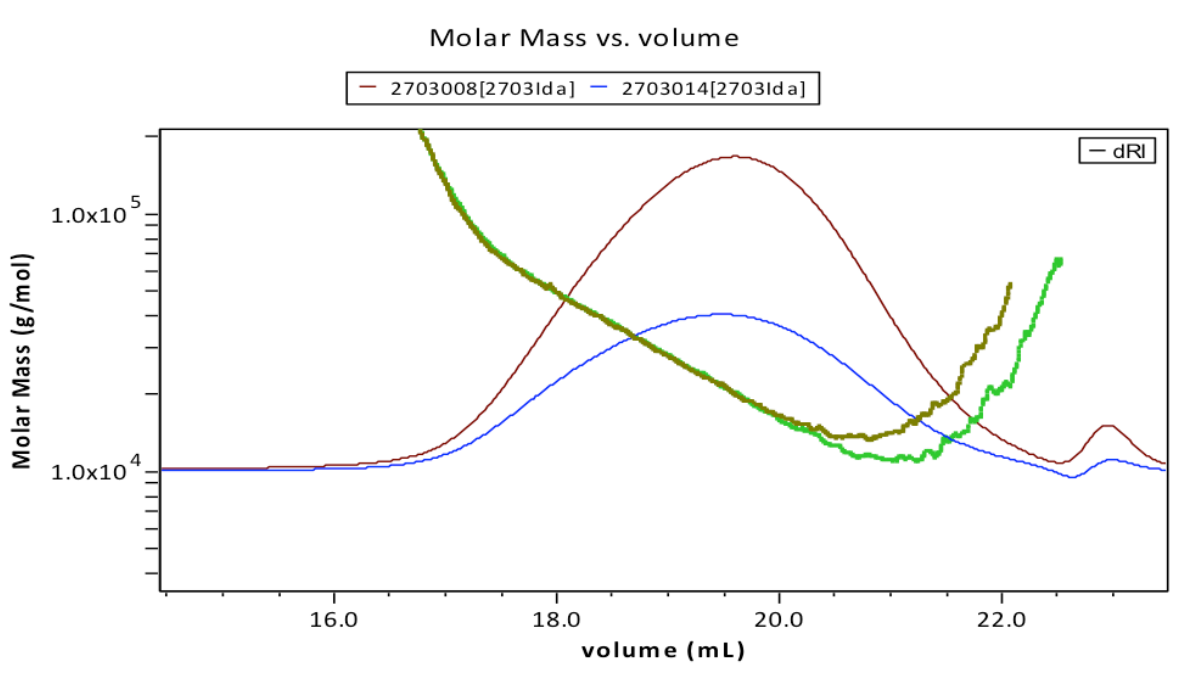

Figure G – 9: SEC MALLS chromatogram of alginate leaked from gel beads prepared from low molecular weight alginate (1.8 %) with very low molecular weight leaf alginate (1.0 %).

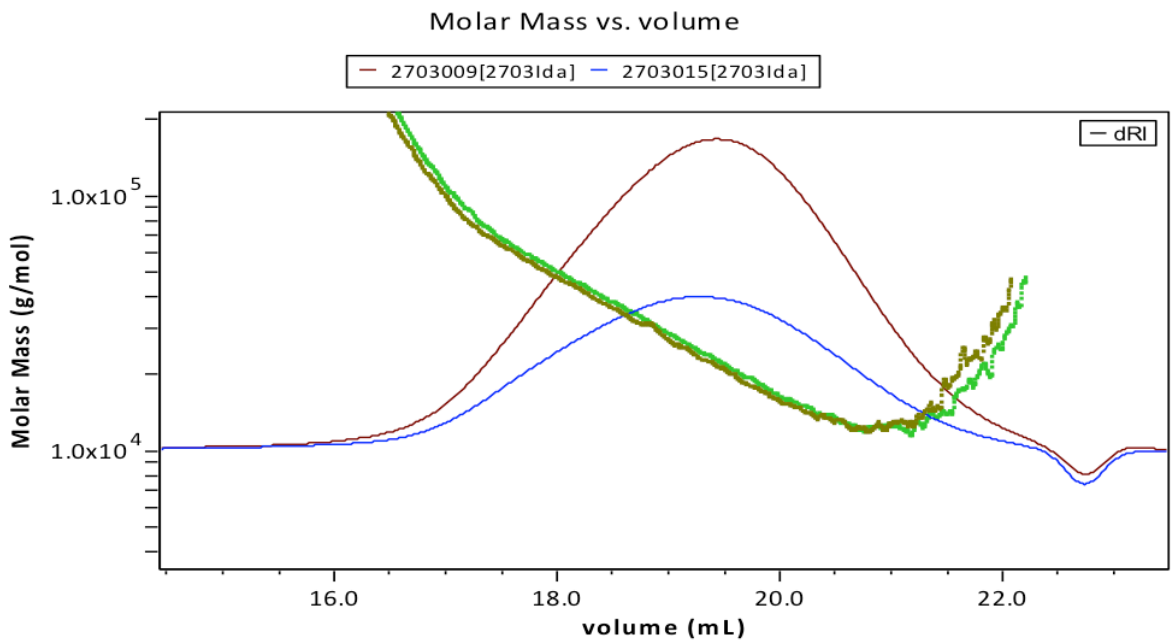

Figure G – 10: SEC MALLS chromatogram of alginate leaked from gel beads prepared from low molecular weight leaf alginate (1.8 %) with added very low molecular weight stipe alginate (1.0 %).