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Degradation of Calcium Gels of Alginate and Periodate Oxidised Alginate

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Abstract

The extracellular matrix (ECM) functions as a structural scaffold for cells and controls cellular function through cell-matrix interactions. These interactions are required by most cells in order to maintain viable and promote proliferation. Therefore, an appropriate synthetic extracellular matrix is necessary for tissue engineering applications. Hydrogels, either derived from synthetic or natural polymers, are an alternative.

Alginate is a linear co-polymer formed by (1→4)-linked α-D-mannuronate (M) and its C-5 epimer β-L-guluronate (G). This natural polymer can be grafted with short bioactive peptide sequences by resorting to periodate oxidation and reductive amination, which can promote cell attachment.

This work studied the effects of saline treatment on the mechanical properties and stability of calcium gels of stipe alginate and oxidised alginate. The results showed that Ca-alginate gels of chemically modified alginate are more prone to degradation than stipe alginate when subjected to saline treatments. This was viewed as a decreasing Young's modulus and storage modulus of the gels as a function of saline treatments. Additionally, the leaked material from the gels presented an initially low G-content and average G-block length, which then increased, indicating that the mixed-in partially oxidised alginate leaked out first.

In sum, the use of periodate oxidation allowed to tailor the degradation rate of calcium alginate gels by varying the content of chemically modified alginate.

Preface

This master's thesis was conducted at the Norwegian University of Science and Technology (NTNU), Trondheim, Norway. Additional tests were conducted at the 3B's Research Group, Guimarães, Portugal.

First and foremost, I would like to thank my supervisors, Professor Berit L. Strand and Professor João Mano for their guidance and support. I am very grateful for the opportunity they presented me with. I would also like to thank PhD candidate Line A. Omtvedt for her help in the laboratory and with data analysis. I would like to extend my thanks to Staff Engineers Wenche Strand and Ann-Sissel Ulset, as well as Doctor Sofia Caridade, for their guidance with laboratory work and data acquisition.

Additionally, I would like to thank Carlos for his support and encouragement. Lastly, I would also like to extend my thanks to my friends and family, in particular my mother.

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Abbreviations

^{1}H-NMR	Proton Nuclear Magnetic Resonance
Ca-alginate gel	Calcium alginate gel
D_2O	Deuterium
D_{ox}	Degree of oxidation
E	Young's modulus
EDTA	Ethylenediaminetetraacetic acid
G	α-L-guluronic acid
G-blocks	Homopolymeric regions of α-L-guluronic acid
GDL	D-glucono-δ-lactone
^{1}H-NMR	Hydrogen nuclear magnetic resonance
M	β-D-mannuronic acid
M-blocks	Homopolymeric regions of β-D-mannuronic acid
MeOTyr	L-tyrosine methyl ester
MG-blocks	Alternating regions of α-L-guluronic acid and β-D-mannuronic acid
MQ-water	MilliQ-water (ultra-pure water)
M_w	Average molecular weight
M_w/M_n	Polydispersity
$N_{G>1}$	Average G-block length larger than 1
POA	Partially oxidised alginate
POA MeOTyr	Partially oxidised alginate coupled with L-tyrosine methyl ester
SEC-MALS	Size Exclusion Chromatography with Multi-Angle Light Scattering
TSP	3-(trimethylsiyl)-propionic-2,2,3,3,d4 acid sodium salt

1 Introduction

1.1 Background

Tissue engineering involves the stimulation of cells towards the formation of new tissue, with resort to molecular and mechanical signals [1]. The delivery of these signals may require a combination of stem cells and biomaterial-based scaffolds. Whilst stem cells present a high self-renewal and multilineage differentiation potential, scaffolds mimic the extracellular matrix by providing the 3-D environment and structural integrity necessary for cell culture. By allowing for cell-to-cell and cell-material communications, scaffolds facilitate differentiation into the desired lineage, while still allowing for nutrient and metabolite diffusion [2]–[5].

An option for extracellular mimicking is the use of hydrogels. They are hydrophilic polymer networks with high water absorption properties, which result in swelling of the network [6]. The chemical bonds and physical interactions between chains govern the degree of crosslinking and consequently the structural integrity. The degradation mechanism and rate should also be taken into account, as these may vary according to the application [4].

Alginate can be crosslinked by divalent cations such as calcium in order to form calcium alginate hydrogels [7]. This linear co-polymer is composed of guluronate (G) and mannuronate (M) residues, which are found in blocks of G-, M- and MG-. It is present in brown algae and secreted by some species of bacteria [8].

This natural polymer has been widely used for cell culture and in pharmaceutical applications, for the delivery of small chemical drugs and proteins. The combination of protein and cell delivery for tissue engineering has also been explored for the regeneration of blood vessels, bone, cartilage, among others [9].

However, alginate gel degradation is slow and poorly controlled. Cell attachment is also not promoted in alginate gels. Hence by chemically modifying alginate it is possible to tailor the degradation of calcium alginate gels and covalently link biologically active peptides, which mimic the ECM. Since this is a critical factor in new tissue formation, modified alginate is an attractive option for tissue engineering strategies [10], [11], [12].

1.2 Aim of Study

The aim of this study was to analyse the effects of saline treatment on the degradation of calcium alginate hydrogels contained mixed ratios of stipe alginate, partially oxidised alginate and partially oxidised alginate coupled with a model compound. This degradation will be followed as a variation in mechanical strength and swelling of the hydrogels. Analysis of the saline bath by NMR and SEC-MALS will be performed to indirectly view gel degradation through alterations in the guluronate content and molecular weight of the leached molecules, as a function of the saline treatments.

2 Theory

2.1 Extracellular Matrix

The extracellular matrix (ECM) functions as a structural scaffold for cells, being therefore responsible for tissue strength under physical load. It controls cellular function through cell-matrix interactions and has a crucial role in morphogenesis. The ECM is located between cells and is composed of organic matter, namely multidomain proteins, which can be classified as glycoproteins, proteoglycans and collagens. They have the ability to self-associate and form assemblies with other ECM proteins due to interaction potentials between domains [13].

In order to maintain viability and functionality, and ultimately lead to enhanced cellular migration, proliferation and growth, most cells require matrix interaction [12], [14]. Cell adhesion to the ECM is possible through specific membrane receptors, mainly integrins, but also through cell surface proteoglycans and glycoproteins. The arginine-glycine-aspartate (RGD) sequence is the main integrin-binding sequence, specifically through the aspartate residue [13]. This sequence can be found in a series of proteins, and short peptides containing it can be used to mimic cell adhesion proteins [15].

The production of a synthetic extracellular matrix can be challenging and involves the choice of the appropriate material for the envisioned application, as it should enhance cellular proliferation and distribution during its lifetime. An approach is the use of synthetic or naturally derived polymers for the production of hydrogels due to their mechanical and structural similarity with the natural ECM. Both types of hydrogels present tuneable chemistry and properties. Furthermore, hydrogels produced from naturally derived polymers, such as alginate, may possess macromolecular properties similar to the natural ECM, namely chemical and morphological properties [4], [14].

These hydrogels should organise the cells three-dimensionally, offering structural and mechanical integrity to direct tissue growth and formation, whilst still enabling the diffusion of nutrients and metabolites [4], [5]. However, it is important to consider the degradation products and material leaked from the hydrogels as they may affect the immune system differently than the initial material used to produce the gels [16].

Material selection for a certain application relies on physical, biological and mass transport properties. The physical properties of the material, such as its ability to form a gel, its mechanical characteristics and degradation rate, are dependent on the properties of the main chain polymer and its proclivity to crosslink. In regard to biological properties, the material should incite desirable cellular functions for the application it has been selected for, while not provoking a severe or chronic inflammatory response. Many hydrogels do not stimulate cellular adhesion and function. This is due to the fact that cells lack receptors for hydrogel forming polymers and also due to the hydrophilic nature of hydrogels. There is therefore a need to attach molecules to the polymers that stimulate the appropriate cellular response, such as RGD sequences. Regarding mass transport properties, diffusion rates are affected by the nanoporous structure of the gel and should also be considered [4].

2.2 Alginate

Alginate is a linear co-polymer present in brown algae and secreted by some species of bacteria. This polysaccharide is formed by a (1→4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G). It is considered a block polymer, containing M-, G- and MG-blocks, as shown in Figure 2.1 [8] M-blocks are composed of consecutive M residues (MMMM), whilst G-blocks contain consecutive G residues (GGGG) and MG-blocks contain alternating G and M residues (GMGM) [9]. Homopolymeric mannuronan is initially synthesized and is then modified by mannuronan-C5-epimerases, which convert M into G [12]. The source of the alginate influences the block composition and distribution [7].

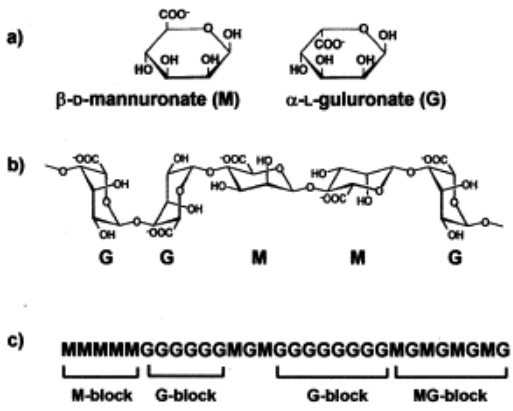

Figure 2.1 Alginate monomer (a) and chain (b) conformations and a schematic alginate chain sequence (c) [17]**.**

Alginate with a G-content superior to 50% does not initiate an immune response. Nonetheless, the resulting leakage of low molecular weight molecules and polymannuronic blocks from the stipe alginate gel can lead to immunostimulation [18]. Moreover, due to its high hydrophilicity and negative charge it does not promote cell attachment, which in return hinders the survival of cells requiring substrate attachment. Protein adsorption is also limited [12].

Grafting of alginate with short bioactive peptide sequences (ligands) that are recognized by cell receptors, such as RGD, can promote cell attachment. Due to pore sizes between 50 and 1500 Å, there is a fast release of encapsulated small molecules and proteins [9], [19]. This can be altered by chemical modification of the native structure and the use of different crosslinking strategies [20].

2.2.1 Chain Conformation

Four types of glycosidic linkages are present in alginate, namely the diequatorial (MM), diaxial (GG), equatorial-axial (MG) and axial-equatorial (GM) linkages [21]. These linkages can be cleaved by acid and alkaline degradation mechanisms, as well as by oxidation with free radicals [17]. However, it is the diaxial linkage that affects the stiffness and extended nature of the chain due to a large, hindered rotation around the glycosidic linkage. The electrostatic repulsion between charged groups in the polymer chain also contributes to the chain extension and consequent intrinsic viscosity. Parameter a in the Mark-Houwink-Sakurada equation (Equation 2-1) reflects the chain stiffness and extension, where $[\eta]$ is the intrinsic viscosity and M is the molecular weight [21].

$$[\eta] = K.M^{a} \qquad \textbf{Equation 2-1}$$

Seen as this parameter increases with an increasing chain extension, low a-values are associated with MG-blocks and high a-values for G-blocks [21]. Chain flexibility is therefore lowest for G-blocks, increases in M-blocks and flexibility is highest for MG-blocks [7].

2.2.2 Chemical Modification of Alginate

Periodate oxidation alters the hydrolytic stability of alginate by stoichiometrically cleaving the C2-C3 bond and introducing reactive dialdehydes, which are more prone to alkaline β-elimination [12], [22], [23]. The degree of oxidation can be determined by the concentration of the oxidant used [24]. The ring opening enhances chain flexibility and compaction. Periodate oxidation can cause a decrease in molecular weight and G-block length ($N_{G>1}$), which also decreases gel strength. Periodate oxidation of alginate usually leads to some depolymerisation [25].

Reductive amination can then be used for the attachment of substituents or crosslinking agents [12]. A model compound that mimics coupling of peptides, such as L-tyrosine methyl ester (MeOTyr), can be used for preliminary testing in order to reduce costs. The reaction involves the formation of a Schiff base due to the reaction of the open-ring form with the amine group, followed by reduction to a stable secondary amine using 2-picoline borane (pic-BH_3) as reducing agent (Figure 2.2) [12], [22], [26]. This agent is less toxic and more environmentally friendly in comparison to the generally used $NaBH_3CN$ [26]. Reduction reportedly increases the susceptibility of alginate towards acid hydrolysis [23].

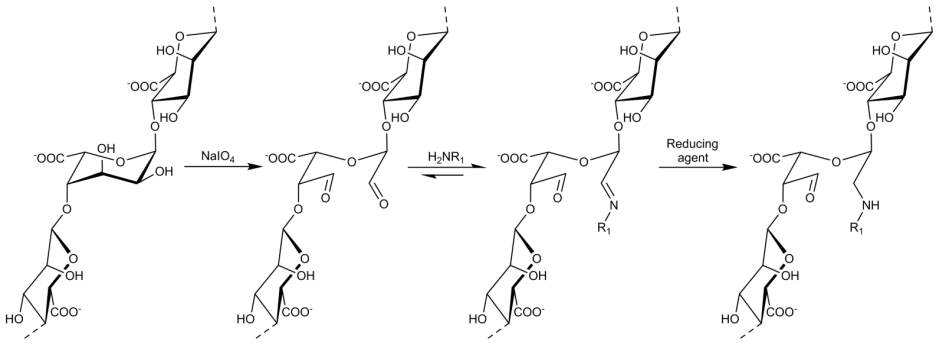

Figure 2.2 Partial oxidation of alginate followed by reductive amination. Representation of an alginate chain by an MGM fragment that is first partially oxidised and then reacted with a primary amine in order to form a Schiff base which is further reduced to a stable amine [22].

2.3 Calcium Alginate Gels

2.3.1 Gel Formation by Ionic Crosslinking

Divalent cations such as Ba^{2+}, Sr^{2+} and Ca^{2+} are bound to M-, G- and MG-blocks with different affinity, allowing for the crosslinking of chains and the consequent formation of hydrogels [7]. For G-blocks, barium ions bind with greater affinity, followed by strontium ions and lastly calcium ions. For M-blocks barium ions present a higher affinity, whilst the affinity for strontium and calcium ions is comparable [27].

In the "egg-box" model, each divalent ion interacts with two adjacent and two opposing G-residues from two chains. G-residues present a higher affinity for these ions and therefore the length of these blocks greatly influences the mechanical properties of the alginate gels formed [7]. MG-blocks have also been shown to take part in crosslinking by calcium ions with MG-blocks present in another alginate chain or with G-blocks (Figure 2.3), making it is therefore possible to form polyMG calcium alginate gels but not polyM [12], [28].

It is therefore important to consider block affinity for gelling ions, chain stiffness and intrinsic viscosity when discussing alginate gels [7], [21]. Due to these aspects, stipe alginate calcium gels containing many and long G-blocks are stiffer and more stable than alginates with a higher content of M, which form elastic and less stable gels [12].

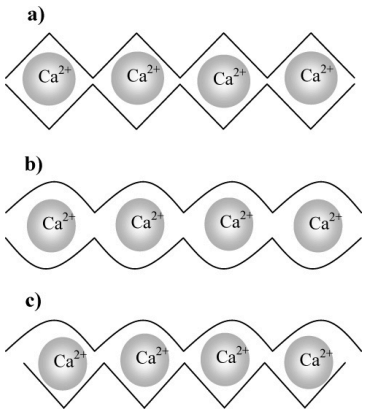

Figure 2.3 Graphical description of the three possible junctions in alginate gels. (a) GG/GG junctions, (b) MG/MG junctions, and (c) mixed GG/MG junctions [28]**.**

Calcium alginate gels can be formed by external and internal gelation. The first case can be accomplished by dripping an alginate solution into a calcium solution. It is characterised by rapid gelling kinetics, but the resulting gel exhibits an inhomogeneous alginate distribution. Internal gelation involves a slow leakage from an inert calcium source within the hydrogel, normally in the form of $CaCO_3$. The addition of slowly hydrolyzing lactones (ex: GDL) lowers the pH and consequently releases Ca^{2+} ions [12], [21]. By the use of equivalent molar amounts of GDL and carbonate, it is possible to form neutral gels [29].

2.3.2 Gel Stability

Solubility is an important factor during alginate gel formation. It is determined by the acidic properties of the alginate, the effects of ionic strength and the effect caused by gelling ions. For Ca-alginate gels, the most important factor is the presence of gelling ions, which limit solubility. In particular, a higher affinity of the polymer for the crosslinking ions increases gel strength [30]. Apart from the crosslinking strength it is important to consider the number of crosslinks, as a weakening or reduction in the number of crosslinking zones leads to a decrease in Young's modulus and storage modulus [28]. Chain length and stiffness should also be taken into considered. In turn, ionic strength affects chain extension and solution viscosity [30].

Calcium alginate gels are affected by syneresis, which can be defined as the shrinkage of the gels associated with an increase in calcium concentration due to water release [28], [31]. This leads to the compaction of the hydrogel network consequently increasing its strength. Seen as M-, G- and MG-blocks present different flexibilities, the monomer composition of the alginate chain will affect this shrinkage [31]. Long G-blocks contribute to a decrease in syneresis, while long flexible elastic segments, such as MG-blocks, lead to a high degree of syneresis [30], [32], [33]. This parameter can also be associated with the average molecular weight as a decrease in M_w leads to a reduced degree of syneresis [30].

The mixing of partially oxidised alginate with stipe alginate to form a gel leads to an overall loss of mechanical strength. This is due to the fact that partially oxidised alginate presents an enhanced chain flexibility and inferior average G-block length [25]. In addition, oxidised residues are not included in junction zones, which makes them more accessible towards degradation and also contributes to the lower strength of these gels [34].

When an external pressure is applied by the solvent, calcium alginate gels tend to swell [35]. By being placed in a saline solution, the crosslinking calcium ions will be replaced with non-crosslinking sodium ions. The exchange results in the destabilisation of the network junctions and increases the concentration of dissociable counterions, which in turn leads to water influx. This is translated into swelling and degradation of the gel by dissolution [12], [4], [31]. Hence the swelling ratio is lower for higher crosslinking densities [36].

2.4 Methods for Analysis

Mechanical testing of the calcium alginate gels in conjunction with NMR spectroscopy and SEC-MALS of the saline bath allowed to link alterations in gel strength and syneresis with the loss of material and corresponding composition and molecular weight.

2.4.1 Mechanical Testing

Mechanical testing was performed in order to obtain the Young's modulus (E), dynamic storage modulus (E') and loss factor (tan δ) of the Ca-alginate gels mixed with chemically modified alginate [37].

2.4.1.1 Compression testing

The determination of the Young's modulus for each Ca-alginate gel was performed through compression testing (Figure 2.4), where a load is applied to the sample. From the elastic region it is possible to obtain the Young's modulus (E), which describes the mechanical stress-strain behaviour of a material [38], [39]. The load applied divided by the application area is defined as stress, whilst the strain is the ratio of extension divided by the original length [40].

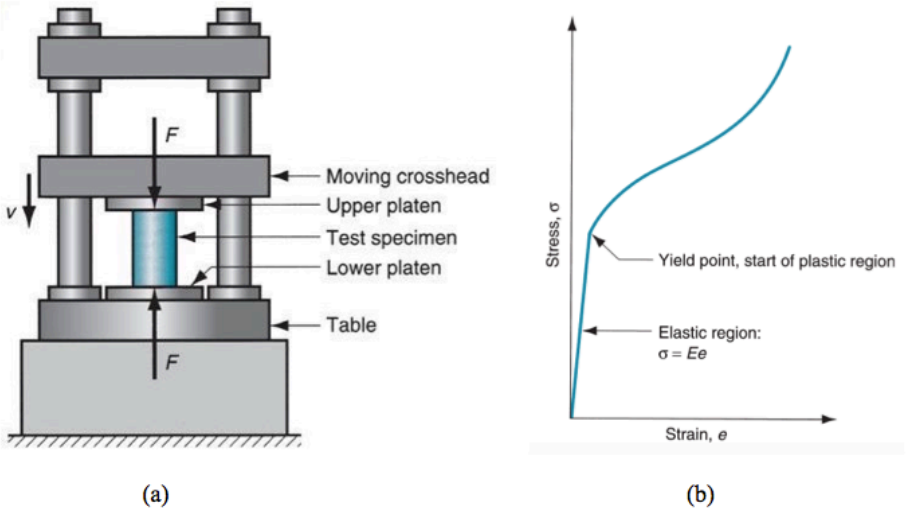

Figure 2.4 Compression test: (a) setup; (b) typical stress-strain curve [39]**.**

Under conditions of static loading, the stress to strain ratio is sufficient to determine Young's modulus. By contrast, under dynamic loading there is an internal friction exerted by the material, which resists the exciting force applied. This causes a phase shift between the stress and strain [41]. Ideally, the storage modulus (E') would be equivalent to Young's modulus (E), yet this is not verified in practice due to different testing conditions (oscillation vs. compression) and calculations (single point vs. slope) [42].

2.4.1.2 Dynamic Mechanical Analysis

Dynamic mechanical analysis (DMA) was used to study the mechanical properties of the calcium alginate gels further. This technique allows for the characterisation of the properties of a sample with known geometry while immersed in a fluid as a function of temperature, time, frequency, stress and atmosphere by application of a sinusoidal deformation in a cyclic manner [43].

An oscillatory force from the DMA leads to the application of a sinusoidal strain on the sample and allows to view its response to load (Figure 2.5). The phase lag (δ) gives information on sample viscosity, whilst its recovery can be used to calculate sample stiffness [42]. This stiffness is recorded as the amount of energy stored in the system per cycle and is defined as the dynamic storage modulus (E') [30]. From the tangent of the phase lag (tan δ) it

is possible to calculate damping, which describes the loss of energy due to molecular rearrangements and internal friction [42].

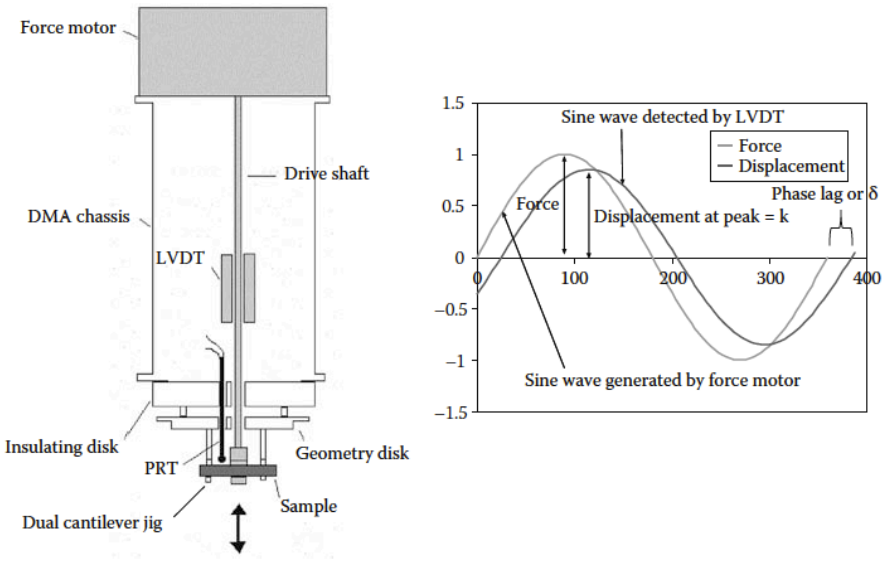

Figure 2.5 Dynamic mechanical analysis setup. An oscillatory force from the DMA leads to the application of a sinusoidal strain on the sample. The modulus, viscosity and damping of the sample can be calculated through the amplitude of the deformation at the peak of the sine wave, and through the lag between the stress and strain sine waves [42].

2.4.2 Stability Testing

^{1}H NMR and SEC-MALS were used for the analysis of the saline baths. These techniques allowed for the determination of the composition of these baths in regard to molecular weight, polydispersity, and uronate distribution.

2.4.2.1 ^{1}H Nuclear Magnetic Resonance Spectroscopy

The uronate composition and monomer sequence distribution determine the physical properties of alginates, and consequently their gelling capacity. As was mentioned previously, stipe alginates tend to form more stable gels than alginates with a higher M-content. Characterisation methods are therefore important in order to obtain detailed information regarding the uronate distribution [44].

Nuclear magnetic resonance provides information on molecular structure through the magnetic properties of nuclei [45]. In the case of alginate, ^{1}H NMR spectroscopy allows for the determination of block composition and M/G ratio. Mild acid hydrolysis can be used to reduce viscosity of the alginate solution, resulting in better spectral resolution. A chelator (such as EDTA) can be used to prevent interaction of divalent cations with the alginate. The acquisition is performed at a high temperature (~90°C) to further reduce the viscosity, increase the spectral resolution, and to shift the solvent peak (HDO) away from the spectral region of interest [44].

Block structure can be determined by integrating the H1 and H5 protons of the uronic acids using the maximum averaging approach [46]. Through this approach the fraction of monad (F_M and F_G), diad (F_{MM}, F_{MG}, F_{GM} and F_{GG}) and triad (F_{MMM}, F_{MMG}, F_{MGM}, F_{MGG}, F_{GMM}, F_{GGM}, F_{GMG} and F_{GGG}) frequencies are obtained. From these frequencies it is possible to calculate the G-block length larger than one ($N_{G>1}$), which correlates with the gelling properties of alginate [21].

Figure 2.6 illustrates the NMR spectra for a high-G alginate and high-M alginate in solid and broken line, respectively [46]. Exemplified in Figure 2.6 and Figure 2.7 is the chemical shift of the anomeric protons in alginates, typically in the 4.3 – 5.3 ppm region. When alginate is reduced and coupled with MeOTyr additional peaks due to the four aromatic protons of the substituent are found at chemical shifts 6.8 – 7.3 ppm [22].

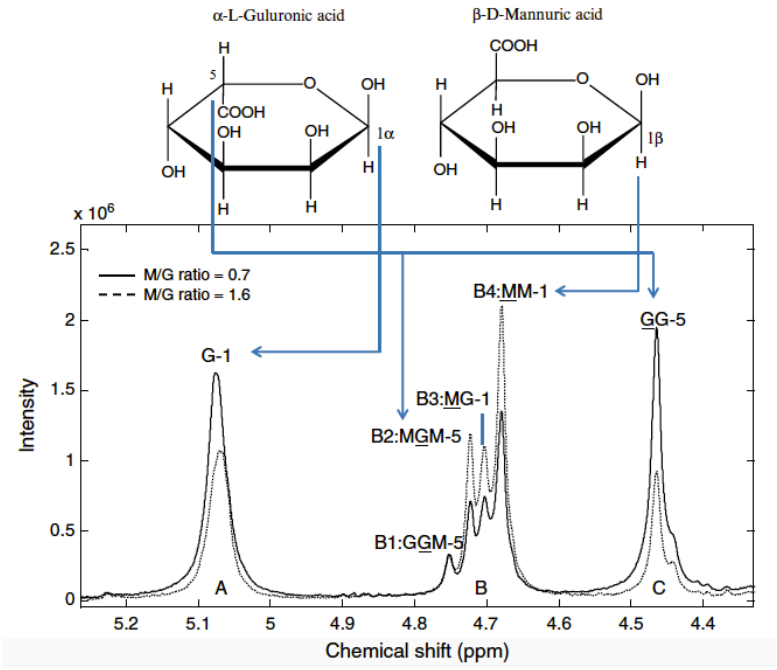

Figure 2.6 ^{1}H NMR spectra (4.35 – 5.25 ppm) of stipe alginate and alginate with a higher M-content in solid and broken line, respectively. Arrows indicate the assignment of peaks [46].

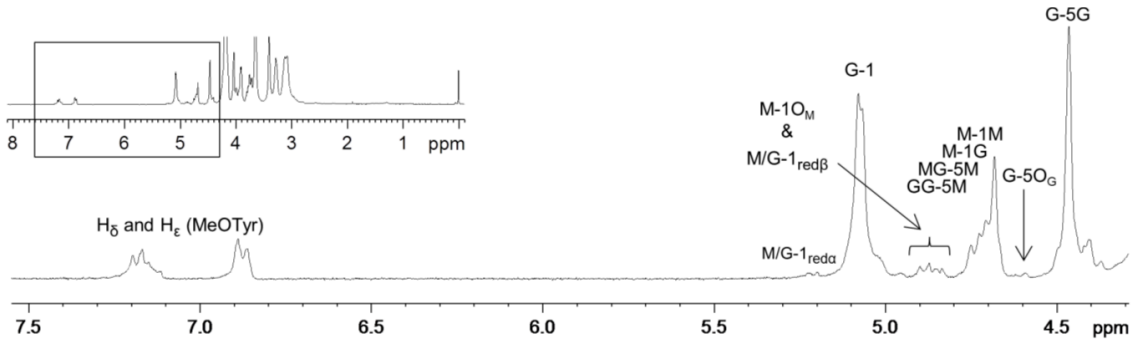

Figure 2.7 ^{1}H-NMR spectra (300 MHz, 90°C), of *L. hyperborea* stipe POA-MeOTyr [22]**.**

2.4.2.2 Size Exclusion Chromatography with Multi-Angle Light Scattering

Size exclusion chromatography (SEC) allows for the determination of the average molecular weight and molecular weight distribution. The separation relies on the molecular hydrodynamic volume or size. The polymer is dissolved and injected into a column with porous particles, where it will elute. Large molecules are not able to penetrate the pores, resulting in a short retention time, whilst smaller molecules penetrate the pores and have a longer retention time. This results in the primary elution of high molecular weight material, followed by low molecular weight materials [47].

SEC-MALS combines size exclusion chromatography with multi-angle light scattering (MALS), which detects the scattered radiation from the molecule after it exits the SEC column due to the incidence of a beam. The intensity of the scattered radiation is dependent on the molecular weight, molecular size, number, shape of the molecule and the scattering angle [47].

Due to the fact that alginate presents a polydispersity index (PI) close to 2, its molecular weight should be considered as an average over the distribution of all molecular weights [12], [30]. Alginate typically presents a high molecular weight ranging from 10^5 to 10^6 Da, and a decrease is an indication of degradation [12], [34].

3 Materials and Methods

3.1 Alginate

Starting materials referenced in Table 3.1 were used to study the stability and degradation of calcium gels of 1% stipe alginate mixed with 1% stipe alginate with an 8% oxidation degree (POA; D_{ox}=8%) and 1% stipe alginate with an 8% oxidation degree coupled with tyrosine methyl ester (POA MeOTyr; D_{ox}=8%). 1% *Laminaria hyperborea* stipe (stipe alginate) (FMC Biopolymer, Drammen, Norway) was used as control.

Table 3.1 Initial material used for the preparation of calcium alginate hydrogels and corresponding molecular weight, polydispersity, guluronate and mannuronate content, average G-block length larger than one and MeOTyr content. MeoTyr content is not applicable for stipe alginate and POA (-). POA refers to partially oxidised alginate and POA MeOTyr refers to partially oxidised alginate coupled with methyl tyrosine ester.

Sample	M_w (kDa)	M_w/M_n	F_G	F_M	$N_{G>1}$	F_{MeOTyr}
Stipe alginate	143	2.0	0.67	0.33	14	-
POA	102	1.9	0.64	0.36	8	-
POA MeOTyr	114	1.9	0.67	0.33	13	0.07

3.2 Preparation of Calcium Alginate Hydrogels

Alginate hydrogels were prepared according to a standard protocol for preparation of alginate gel cylinders using D-(+)-Gluconic acid δ-lactone (GDL) (SIGMA) and natural calcium carbonate ($CaCO_3$), 4µm (ESKAL 500, KSL Staubtechnik GMBH) for gel crosslinking [33], [48].

For the preparation of the first batch of Ca-alginate gels (8 gels), a total of 375 mg of alginate was dissolved in 25 mL of MQ-water. Dissolution occurred overnight, after which 15 mM $CaCO_3$ in 5 mL MQ-water was added to the alginate solution and left to degass using vacuum suction. Following degassing, 30 mM GDL in 7.5 mL MQ-water was added to the solution and stirred carefully for a few seconds before pouring into the 8 centre wells of a 24-well

plate, making a positive meniscus. The gels were removed from the wells after 20 hours and saturated with Ca^{2+} by dialysing against 800 mL of 50 mM $CaCl_2$ in 0.2 M NaCl for 24 hours at 4ºC.

For the first and second batch of gels with different mixed ratios of stipe alginate, POA and POA MeOTyr were prepared following the percentages described in Table 3.2.

Table 3.2 Mixing ratio of stipe alginate, POA and POA MeOTyr for each gel tested. Calcium alginate hydrogels were prepared by mixing stipe alginate with a varying amount of POA and POA MeOTyr. Stipe alginate refers to 1% stipe alginate gels, POA is 1% stipe alginate partially oxidised (D_{ox}=8%), whilst POA MeOTyr is 1% stipe alginate partially oxidised (D_{ox}=8%) coupled with methyl tyrosine ester.

Mixing ratio / Sample name	Stipe alginate	POA	POA MeOTyr
Stipe alginate	100%	-	-
POA (25%)	75%	25%	-
POA (50%)	50%	50%	-
POA (75%)	25%	75%	-
POA MeOTyr (50%)	50%	-	50%
POA MeOTyr (75%)	25%	-	75%

3.3 Mechanical Properties

3.3.1 Change in Mechanical Properties upon Saline Treatments

Posterior to dialysis against $CaCl_2$ and NaCl solution, five of the eight hydrogels were weighed, measured and subjected to compression testing (1 mm) to determine Young's modulus. The gels were then placed in a 0.15 M NaCl solution at 4 ºC with gentle stirring (100 rpm) for 24 hours, after which the gels were again measured, compressed and placed in a new NaCl solution. This cycle was repeated for each type of hydrogel until the gel had lost its cylindrical form and it was no longer possible to perform mechanical measurements (3 – 4 days).

3.3.1.1 Determination of Young's Modulus

Mechanical strength of the gels was measured using Texture Analyser with a P/35 probe and a 5 kg loading cell. Exponent 32 was the chosen analysis software.

Posterior to weight (g), height (mm) and width (mm) measurements, gels from the first batch were placed under the loading cell and compressed until 1mm. These parameters and the correction factor for weight were taken into account when calculating the corrected Young's modulus through Equation 3-1.

$$E_{corr}(Pa) = \frac{Gradient\ (N/m) \times Height\ (mm) \times 10^3}{Area\ (mm^2) \times \left(Correction\ factor_{weight}\right)^2} \qquad \textbf{Equation 3-1}$$

3.3.1.2 Dynamic Mechanical Analysis

Mechanical/viscoelastic measurements were performed at the 3B's Research Group, Portugal, by Sofia Caridade using a TRITEC 2000 DMA from Triton Technology (UK), equipped with the compressive mode.

A second batch of calcium alginate gels was prepared as indicated in the section on hydrogel preparation, using the same mixing ratios present in Table 3.2. Only stipe alginate with mixed partially oxidised alginate gels were produced.

After production, the properties of the samples were evaluated and placed in a 0.15 M NaCl solution. Mechanical/viscoelastic properties were reassessed every day until their complete degradation. The measurements were carried out at room temperature. All samples were measured accurately for each sample. The samples were always analysed immersed in a liquid bath containing the 0.15 M NaCl solution placed in a Teflon® reservoir. The samples were then clamped in the DMA apparatus and immersed in the NaCl solution. DMA spectra were obtained during a frequency scan between 0.2 and 15 Hz. The experiments were performed under constant strain amplitude (50 μm). A small preload was applied to each sample to ensure that the entire scaffold surface was in contact with the compression plates before testing and the distance between plates was equal for all hydrogels being tested. At least three

samples were used for each condition with the same experimental settings; average values are presented.

In order to correct the storage modulus values of the second batch of gels for weight, the correction factor for weight obtained for each type of gel of the first batch, as a function of the saline baths, was applied accordingly.

3.4 Treatment of Saline Bath

2 mL EDTA was added to each NaCl solution in which the gels had been kept. This solution was dialysed twice in a 3.5 kDa dialysis membrane against 50 mM NaCl and the remaining times against MQ-water until the conductivity was below 4 µS/cm. This was done to all solutions with the exception of the stipe alginate and POA (50%), making it necessary to add EDTA after dialysis in order to enable dissolution in MQ-water for NMR and SEC-MALS sample preparation.

3.5 ^{1}H Nuclear Magnetic Resonance and Sample Preparation

For NMR testing it was necessary for samples to undergo degradation by mild acid hydrolysis. Approximately 15 mg of each sample was dissolved in 60 mL of MQ-water. The pH of the samples was lowered to 5.6 and the samples were placed in a 95 ºC water-bath for one hour. Samples were cooled to room temperature, and the pH was lowered further to 3.8. Thereafter, the samples were placed in the water-bath for 40 minutes. Samples were again cooled to room temperature, and the pH was adjusted to neutral.

After degradation, 8 mg of each sample (20 mg for POA (50%) and stipe alginate) were freeze-dried and transferred to Eppendorf tubes were 700 µL of deuterium (D_2O) and 5 µL of the chemical shift reference sample 1% TSP (3-(trimethylsiyl)-propionic-2,2,3,3,d4 acid sodium salt) was added. 600 µL of each sample was placed in an NMR pipette and analysed.

^{1}H NMR was performed at NTNU, Trondheim, with BRUKER Avance DPX 300 MHz with 5 mm z-grad dual probe at 95 ºC. Measurements and data processing were done by Wenche Strand and Line A. Omtvedt at the Department of Biotechnology, NTNU, using TopSpin 3.0.

3.6 Size Exclusion Chromatography with Multi-Angle Light Scattering and Sample Preparation

A minimum of 5 mg was weighed for each sample to which a certain amount of MQ-water was added in order to reach a concentration of 5 mg/mL. The same volume of 2x buffer $NaNO_3$/EDTA (0.15 M $NaNO_3$; 0.01 M EDTA) was added and the solution was filtered (pore size 0.80 μm) prior to injection. A 200 μL volume of injection was set to obtain an optimal light scattering signal.

The first attempt at obtaining data was not successful due to the presence of aggregates. In order to dissolve these clusters a drop of EDTA was added to each sample and left on stirring overnight.

Ann Sissel Ulset at NTNU performed measurements and Astra software v. 6.1 (Wyatt, USA) was used to collect and process the obtained data. A refractive index increment (dn/dc_μ) of 0.15 mL/g was used.

4 Results

4.1 Mechanical Properties

In order to establish how the inclusion of chemically modified alginate in calcium alginate hydrogels affected gel behaviour, compression testing was conducted. This testing allowed to obtain the initial gradient values in N/m (Figure 4.1). The corresponding initial Young's modulus values are given in Figure 4.2.

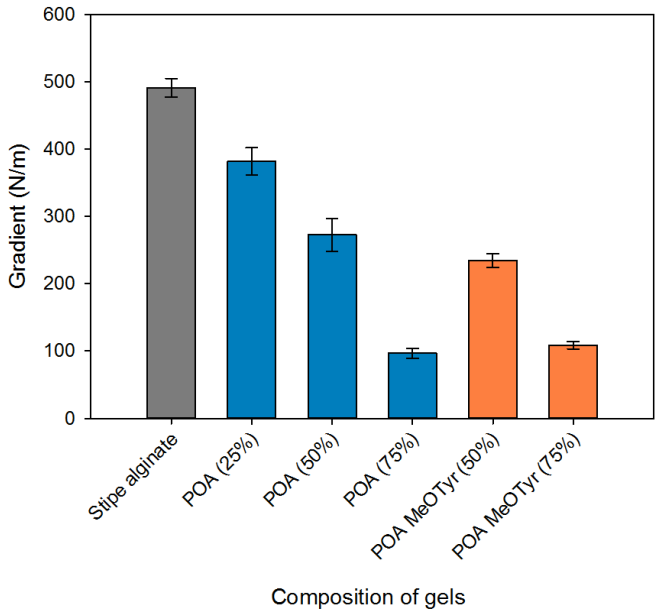

Figure 4.1 Initial gradient values in N/m for each type of calcium alginate hydrogel before being subjected to saline treatments. Stipe alginate was used as control to establish a comparison between the incorporation of an increasing ratio in partially oxidised alginate (POA; D_{ox}=8%) and partially oxidised alginate coupled with tyrosine methyl ester (POA MeOTyr; D_{ox}=8%). The graph depicts the average gradient values and standard deviation measured from 5 gel cylinders for each sample.

Stipe alginate was used as a reference and presented the highest initial Young's modulus value of 29.5 ± 0.8 kPa. A decrease in Young's modulus was shown for an increasing inclusion of oxidised and coupled material, reaching a minimum at 7.9 ± 0.7 kPa for POA (75%). POA (50%) and POA MeOTyr (50%) hydrogels demonstrated similar initial values, as did POA (75%) and POA MeOTyr (75%).

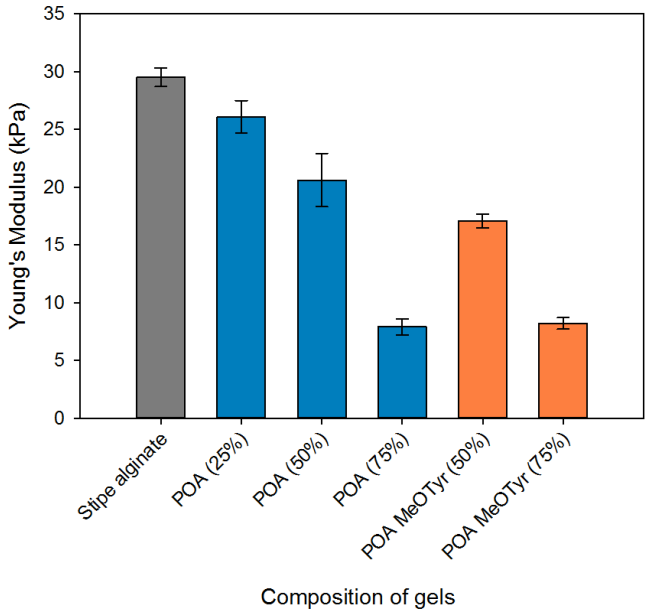

Figure 4.2 Initial Young's modulus values in kPa for each type of calcium alginate hydrogel before being subjected to saline treatments (0.15 M NaCl). Stipe alginate was used as control to establish a comparison between the incorporation of an increasing ratio in partially oxidised alginate (POA; D_{ox}=8%) and partially oxidised alginate coupled with tyrosine methyl ester (POA MeOTyr; D_{ox}=8%). The graph depicts the average Young's modulus values and standard deviation measured from 5 gel cylinders for each sample.

Figure 4.3 shows the initial syneresis values for calcium alginate gels with different ratios of partially oxidised alginate and partially oxidised alginate coupled with tyrosine methyl ester. Syneresis also decreased with an increase in the incorporated amount of oxidised and coupled alginate. Stipe alginate had a syneresis of 24 ± 0 %, whilst the lowest value, 10 ± 1 %, was reported for POA (75%).

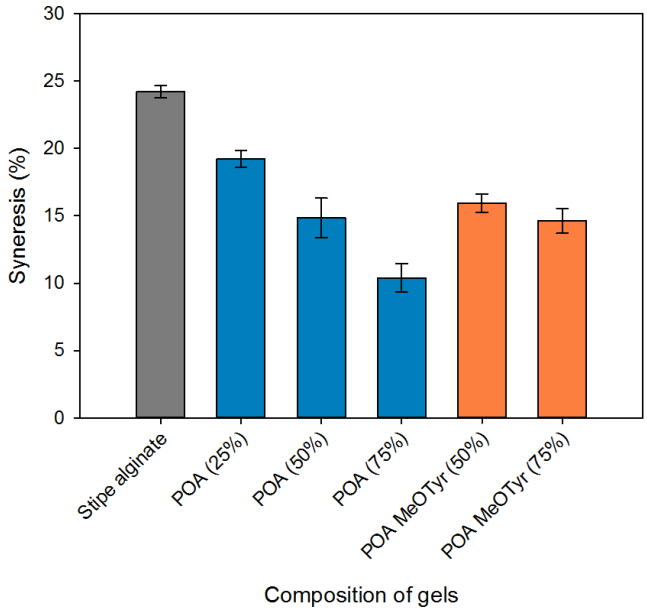

Figure 4.3 Syneresis of calcium alginate gels with different compositions. Stipe alginate was used as control to establish a comparison between the incorporation of an increasing ratio in partially oxidised alginate (POA; D_{ox}=8%) and partially oxidised alginate coupled with tyrosine methyl ester (POA MeOTyr; D_{ox}=8%). The graph depicts the average syneresis values and standard deviation measured from 5 gel cylinders for each sample.

The curves in Figure 4.4 show that each gel batch had a reduction in Young's modulus when subjected to saline treatment. Stipe alginate maintained its structure for the highest number of saline treatments. Measurement of Young's modulus on POA (25%), POA (50%) and POA MeOTyr (50%) was performed until the third treatment. It was not possible to handle POA (75%) and POA MeOTyr (75%) gels after the second treatment and thus no more values were obtained.

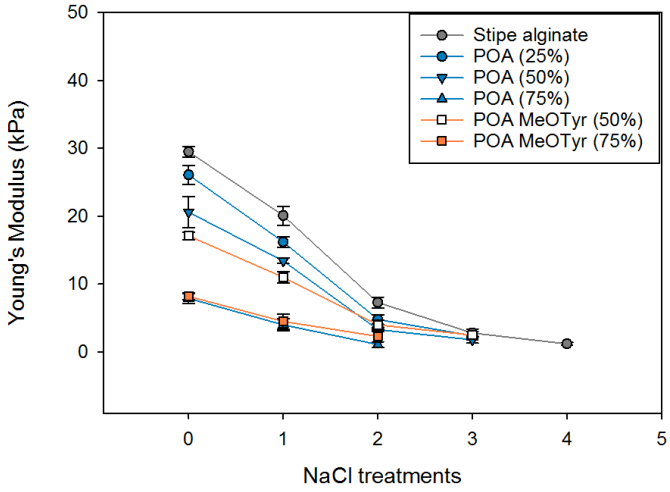

Figure 4.4 Young's modulus variation with successive NaCl treatments (0.15 M NaCl). Each treatment can be understood as a 24-hour period during which the gels were kept at gentle stirring in a saline bath. Loss of mechanical strength prevented from performing measurements on POA (75%) and POA MeOTyr (75%) after the second treatment, and after the third treatment for POA (25%), POA (50%) and POA MeOTyr (50%). Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. The graph depicts the average Young's modulus values and standard deviation measured from 5 gel cylinders for each sample. Guidelines are drawn between the measured points.

Variation in swelling due to saline treatments was also studied for each hydrogel batch (Figure 4.5). The weight of the hydrogels was considerably stable for the first 24 hours, after which there was an increase for all except POA (75%).

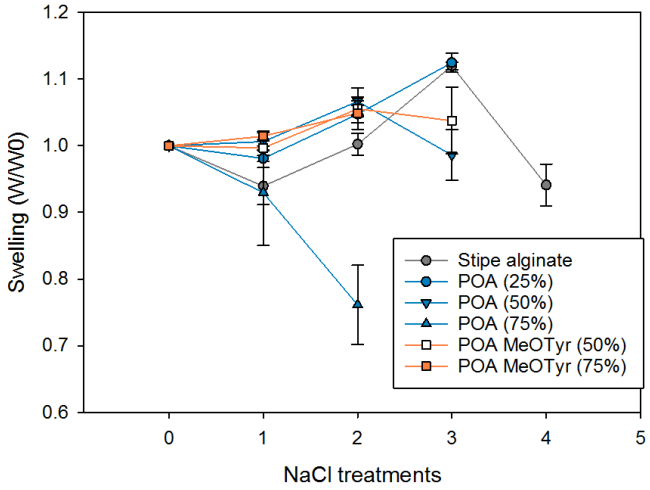

Figure 4.5 Swelling variation with saline treatments (0.15 M NaCl). Swelling was calculated as the ratio between the initial weight of the gel prior to saline treatments (W_0) and the weight at each time point (W). Each saline treatment can be understood as a 24-hour period during which the gels were kept at gentle stirring. Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were

analysed. The graph depicts the average swelling values and standard deviation measured from 5 gel cylinders for each sample. Guidelines are drawn between the measured points.

4.1.1 Dynamic Mechanical Analysis

DMA experiments were used to assess the mechanical/viscoelastic properties of the produced hydrogels and such properties were reassessed daily until their complete degradation. The variation of the storage (elastic) modulus (E') along time is presented in Figure 4.6. Stipe alginate hydrogels were stiffest, presenting an E' of 44.2 ± 5.4 kPa at 1 Hz before saline treatments. As the content of partially oxidised alginate increased, the stiffness of the hydrogels decreased (e.g. POA (75%) presented an E' of 11.6 ± 2.2 kPa at 1 Hz before saline treatment, being the less stiff condition). For all conditions the stiffness of the hydrogels decreased along time. Moreover, only stipe alginate and POA (25%) hydrogels were the ones in which it was possible to evaluate the mechanical/viscoelastic properties after the third treatment. Such properties were possible to be evaluated until the second saline treatment for POA (50%) and (POA 75%).

In comparison with Young's modulus obtained for Ca-alginate gels of stipe alginate and POA, the storage modulus was generally higher prior to saline treatments and after the first saline treatment. For example, the initial E for POA (25%) was 26.1 ± 1.4 kPa, whilst E' was 36.8 ± 2.1 kPa. After the second and third saline treatments, Young's modulus was either higher than the storage modulus, or both values were similar (e.g. POA (25%) presented an E of 4.8 ± 0.7 kPa after the second saline treatment, versus a E' of 2.2 ± 0.3 kPa).

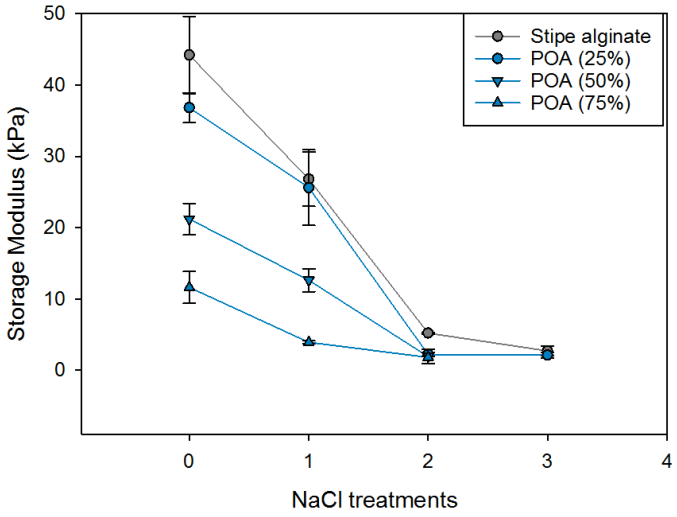

Figure 4.6 Storage (elastic) modulus (E') variation with NaCl treatments (0.15 M NaCl) corrected for weight. Each treatment can be understood as a 24-hour period during which the gels were kept at gentle stirring in a saline bath. Loss of mechanical strength prevented from performing measurements on POA (25%) and POA (50%) after the second treatment, and after the third treatment for the remaining two samples. Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) samples were analysed. The graph depicts the average storage modulus values and standard deviation measured from three gel cylinders for each sample. Guidelines are drawn between the measured points.

The variation of the loss factor (tan δ) along time is presented in Figure 4.7. The tan δ is the ratio of the amount of energy dissipated by viscous mechanisms relative to energy stored in the elastic component providing information about the damping properties of the membranes. For all hydrogels, it was observed that tan δ increased with the content of partially oxidised alginate thus presenting higher dissipative properties.

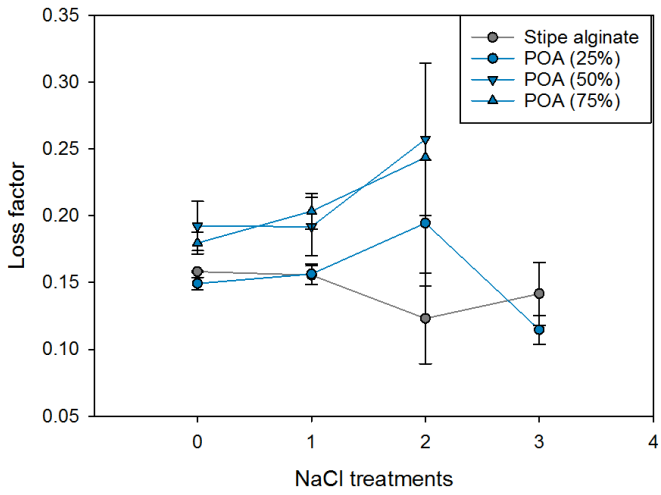

Figure 4.7 Loss factor variation with NaCl treatments (0.15 M NaCl). Each treatment can be understood as a 24-hour period during which the gels were kept at gentle stirring in a saline bath. Loss of mechanical strength prevented from performing measurements on POA (25%) and POA (50%) after the second treatment, and after the third treatment for the remaining two samples. Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) samples were analysed. The graph depicts the average loss factor values and standard deviation measured from three gel cylinders for each sample. Guidelines are drawn between the measured points.

4.2 Stability Characterisation

In order to study the stability of the calcium alginate hydrogels, ^{1}H NMR and SEC-MALS were used to analyse the leaked material from stipe alginate and chemically modified alginate gels.

Table 4.1 contains the mass fraction (wt%) for each hydrogel sample associated to each saline treatment. For stipe alginate an error occurred during compression testing resulting in the need to repeat the measurement corresponding to the fourth saline treatment. The saline bath values obtained for this repetition were accounted for in the fifth saline treatment.

For all gel cylinders there was an increase in percentage of leaked material as the gels were transferred through the saline baths. Stipe alginate presented the overall lowest percentage of leaked material, with a final leaked percentage of 32%. The highest percentage was for POA (75%) and POA MeOTyr (75%), with 43.7% and 52.4% for the final treatment, respectively. Leaked material was relatively low for POA (50%), with 31.9% leaked material by the final treatment.

Table 4.1 Percentage of leaked material from calcium alginate hydrogels when placed in NaCl baths with gentle stirring. The mass fraction (wt%) was calculated by accounting for the leaked material present in the saline bath in regard to the theoretical initial mass of alginate contained in each hydrogel. Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. Posterior to a certain amount of treatments, gels lost mechanical strength and were too degraded to allow for further testing (-). Leaked material from the repetition of the fourth saline treatment for stipe alginate was added to the fifth saline treatment (*).

No. of saline treatments / Sample	1	2	3	4	5*
Stipe alginate	0.8%	3.3%	5.1%	7.2%	32.0%
POA (25%)	15.5%	18.5%	17.3%	68.4%	-
POA (50%)	4.1%	7.9%	14.1%	31.9%	-
POA (75%)	17.2%	32.0%	43.7%	-	-
POA MeOTyr (50%)	14.0%	16.9%	23.5%	58.3%	-
POA MeOTyr (75%)	17.7%	22.3%	52.4%	-	-

From Figure 4.8 and Figure 4.9 it is shown that low molecular weight molecules were released first, and that polydispersity decreased as a function of saline treatments. For partially oxidised alginate, the average molecular weight was lowest for POA (50%), followed by POA (25%) and finally POA (75%). These values increased as a function of the saline treatments and the final M_w value obtained for POA (25%) was 132 kDa, which was closer to the initial material used as control (143 kDa). On the other hand, the average molecular weights for POA (50%) and POA (75%) were 101 kDa and 110 kDa, respectively, which were closer to the initial partially oxidised material (102 kDa). Polydispersity was initially highest for POA (50%) with a value of 3.6, followed by 2.7 for POA (75%) and 2.4 for POA (25%). This parameter decreased for all samples as a function of the saline treatments until it reached 1.8 for POA (25%), 2.0 for POA (50%) and 1.9 for POA (75%). Considering that the polydispersity for stipe alginate start material was 2 and that for POA and POA MeOTyr it was 1.9, the values obtained from the leaked alginates was comparable.

Gels mixed with partially oxidised alginate coupled with MeOTyr also displayed similar behaviour with an initially low M_w and high polydispersity. POA MeOTyr (50%) had an initial M_w of 46 kDa that increased to 123 kDa after four treatments, an intermediate value with regards to the initial material used as control (143 kDa) and of POA MeOTyr (114 kDa). For POA MeOTyr (75%) M_w increased from 79 kDa to 103 kDa. The final average molecular

weight value was closer to that of POA initial material (102 kDa). Polydispersity for POA MeOTyr (50%) and POA MeOTyr (75%) decreased from 2.1 to 1.9, and from 2.4 to 1.9, respectively.

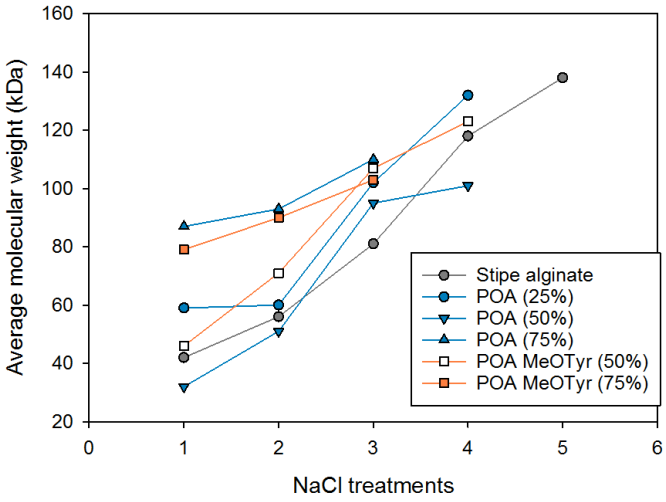

Figure 4.8 Average molecular weight in kDa obtained due to leakage from calcium alginate gels when these were placed in sucessive saline treatments (0.15 M NaCl). Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. Guidelines are drawn between the measured points.

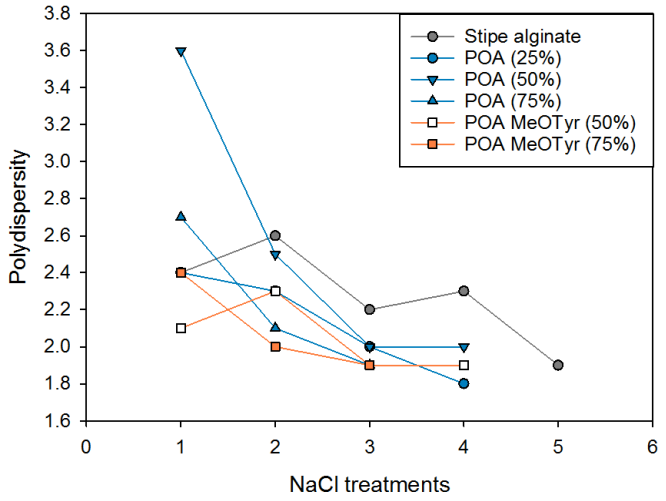

Figure 4.9 Polydispersity of molecules leached from calcium alginate gels when these were placed in sucessive saline treatments (0.15 M NaCl). Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. Guidelines are drawn between the measured points.

In regard to NMR data (Figure 4.10) there was an initially low guluronate content, which then increased as a function of saline treatments. The rate at which F_G increased between the first and second treatment was similar for all POA and POA MeOTyr batches. Average G-block length also tended to increase as the gels were placed in consecutive saline baths (Figure 4.11).

Due to the small amount of leaked material from the stipe alginate gels after the first saline treatment it was not possible perform NMR analysis. The remaining time points showed some stability for F_G and $N_{G>1}$ values, comparable with the ones presented in table 3.1 as the starting materials.

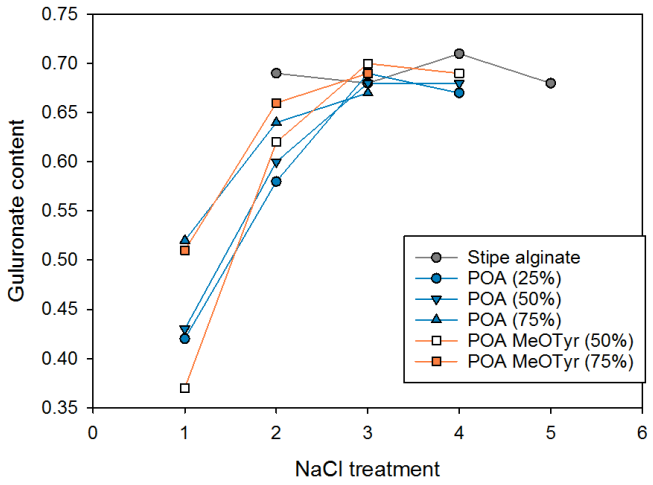

Figure 4.10 Guluronate content of molecules leached from calcium alginate gels when these were placed in successive saline treatments (0.15 M NaCl). Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. Guidelines are drawn between the measured points.

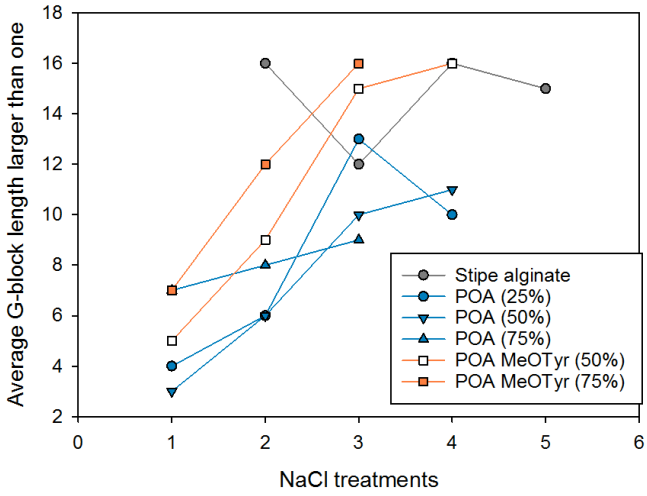

Figure 4.11 Average G-block length larger than one for the leaked molecules from calcium alginate gels when these were placed in successive saline treatments (0.15 M NaCl). Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. Guidelines are drawn between the measured points.

Guluronate content was initially low for mixed partially oxidised alginate gels (25% < 50% < 75%) and increased to a value comparable with that of stipe alginate. Average G-block length was initially lower for POA (25%) and POA (50%) with a respective 4 and 3, than for POA (75%), which was 7. After the final treatments the values obtained were 10, 11 and 9 for POA (25%), POA (50%) and POA (75%), respectively.

For POA MeOTyr (50%) and POA MeOTyr (75%), both G-content and $N_{G>1}$ were initially low and underwent an increase as a function of the saline treatments. The final treatment values were similar for both conditions, though POA MeOTyr (50%) showed a larger variation due to an originally lower F_G and $N_{G>1}$. In comparison to the initial stipe alginate and POA MeOTyr values evident in table 3.1, POA MeOTyr (50%) and POA MeOTyr (75%) presented higher guluronate and average G-block length after treatment 4 and 3, respectively. In regard to MeOTyr content, the initial values for 50% and 75% were similar and decreased as a function of saline treatments (Figure 4.12).

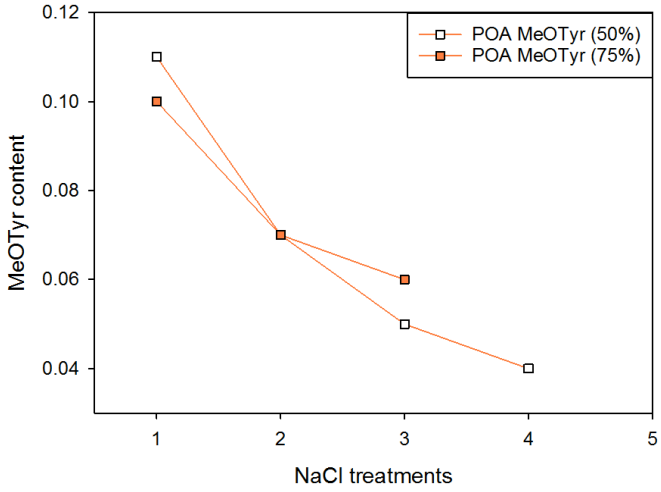

Figure 4.12 Methyl tyrosine ester content of the leaked molecules from calcium alginate gels when these were placed in successive saline treatments (0.15 M NaCl). Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. Guidelines are drawn between the measured points.

5 Discussion

5.1 Mechanical Properties

5.1.1 Young's Modulus and Storage Modulus

Young's modulus and syneresis values obtained for stipe alginate (F_G=0.66; $N_{G>1}$=13) were consistent with previously documented work by Mørch *et al.* [7] using an identical calcium gelation method. They obtained a Young's modulus of 27 ± 1.9 kPa and 27 ± 0.6 % syneresis, whilst the results for stipe alginate (F_G=0.67; $N_{G>1}$=14) for this work were 29.5 ± 0.8 kPa and 24 ± 0 %.

Stipe alginate gels presented a higher Young's modulus value than the gels with modified alginate (figure 4.2), as was expected. Painter *et al.* [49] demonstrated that periodate oxidation occurs at a 50% higher rate on G-residues than on M-residues. These higher oxidation rates on G-residues will therefore lead to a consequent reduction in strength, as those residues will no longer partake in the ionic linkage. Regarding reductive amination, Dalheim *et al.* [22] concluded that coupling of MeOTyr will also occur mainly on G-residues.

Gels containing POA showed a decrease in Young's modulus as the percentage of included POA increased (figure 4.2). Bouhadir *et al.* [50] described a lower mechanical strength for partially oxidised Ca-alginate gels (D_{ox}=4.9%) than for unmodified gels. They attributed this to the lower molecular weight of oxidised alginate and to the ring opening caused by periodate oxidation, preventing oxidised G-residues from partaking in the ionic linkage. An increasing content of coupled MeOTyr also led to a decrease in Young's modulus.

Reductive amination was used for the coupling of tyrosine methyl ester, which may limit the formation of the gel network due to steric hindrance. This was viewed for gels containing 1:1 ratio of modified alginate, as POA (50%) gels presented slightly higher Young's modulus values than POA MeOTyr (50%) (figure 4.2). However, for POA (75%) and POA MeOTyr (75%) gels there were no significant differences in terms of Young's modulus values. This may be explained by the fact that POA (75%) underwent destruction instead of dissolution, and therefore the measured Young's modulus values were lower than expected.

The formation of a stable hydrogel is dependent on block affinity for divalent cations. By placing a calcium alginate gel in a sodium chloride bath, the calcium gelling ions will be replaced with sodium, resulting in the dissolution/destruction of the hydrogel [7], [12]. This ion exchange is translated as a successive decrease in Young's modulus for all tested gels. The saline bath was changed every 24 hours, leading to a renewal of sodium ions that would replace the gelling ions in the gel structure.

The reduction in Young's modulus caused by the saline treatments was proportional to the initial value measured for the control, POA (25%), POA (50%) and POA MeOTyr (50%), indicating that all were equally affected by the treatments. For POA (75%) and POA MeOTyr (75%) gels the decrease was less accentuated, but it is important to take into consideration that the initial values were substantially lower than for the other types of gels as this limited the number of measurements possible to perform. The number of saline treatments during which the gels maintained structural integrity also decreased with an increasing percentage of oxidised and coupled material. The average final measurement before loss of structural integrity was 1.9 ± 0.6 kPa.

The decrease in Young's modulus with saline treatment for the gels tested is in accordance with LeRoux *et al.* [51]. They verified that the Young's modulus of a calcium alginate gel produced with Macrocystitis pyrifera ($F_G = 0.39$; $N_{G>1} = 5$) also decreased when placed in a saline bath. Even though the alginate used during this thesis presented a higher G-content, the mechanisms of ion exchange are the same as for the work done by LeRoux.

For all conditions, the stiffness of the hydrogels decreased along time, as is shown by the decrease in storage modulus as a function of saline treatments. Such results indicated that the degradation of the hydrogels is possible to be tailored just by controlling the content of partially oxidised alginate. LeRoux *et al.* [51] described a decrease in complex modulus (|G*|) with saline treatment. This parameter is calculated through the storage modulus (E') and loss modulus (E"). Even though only the storage modulus was calculated for this work, a decrease in storage modulus would be an indicator of the decrease in complex modulus. The present findings would therefore be in accordance with the work of LeRoux.

For all hydrogels, it was observed that tan δ increased with the content of partially oxidised alginate thus presenting higher dissipative properties, which can be related to their lower stiffness.

5.1.2 Syneresis

External gelation is the second step for the production of the calcium alginate gels. It involves saturation of the gel with calcium, therefore leading to syneresis, which translates into gel shrinkage [31].

Periodate oxidation reportedly decreases the average G-block length, leading to enhanced chain flexibility [25]. Flexible segments are associated with higher degrees of syneresis and therefore gels mixed with modified alginate would be expected to present higher syneresis values than the control [30], [32], [33]. This was not observed and may be related to the decrease in crosslinking caused by the oxidation of G residues, which in turn may lead to a decrease in syneresis. Oxidation also leads to a reduction in molecular weight, which is associated with reduced syneresis [25], [30]. An increasing content of modified alginate led to a decrease in syneresis, which corroborates the theory that the decrease in crosslinking in conjunction with lower molecular weight may result in lower syneresis values.

5.2 Stability Characterisation

Swelling was either stable or decreased between the first and second saline treatments. There was a subsequent increase for all gels except for POA (75%). This is in agreement with Martinsen *et al.* [52], as they described an increase in swelling of calcium beads made of stipe alginate when placed in a saline bath. Swelling of the beads was observed to be higher when the bath contained a lower amount of $CaCl_2$. In this work, the saline bath did not contain $CaCl_2$, so swelling was expected to be higher than what they reported.

The swelling behaviour of POA (75%) may indicate that the hydrogel suffered destruction and not dissolution. A decrease in swelling was observed between the second to last and last measurements for each batch of hydrogels, which may also be an indication of destruction instead of dissolution. The fact that the gels were greatly fractured during the last measurements supports this statement. Due to this, Young's modulus values for these instances may not be precise.

Saline treatment also affected the amount of material leached from the calcium alginate gels. It was not possible to conclude about the last saline bath for each sample as the gels were kept

there for a prolonged time after the last measurement, and this may have affected the amount of leaked material. For POA (25%) and POA MeOTyr (50%), the sum of the percentage of leaked material surpassed 100%, which may be attributed to salt content.

Nonetheless, stipe alginate was least affected by the presence of sodium ions as it presented the lowest amount of leaked material, ranging from 0.8 to 7% of the initial theoretical mass of alginate contained in each hydrogel, when disregarding the 32% leaked during the last saline treatment. Stokke et al. [16] found that leakage from 2% calcium alginate gels prepared by external gelling was in the order of 1 – 4 % of the starting material. Taking into consideration the fact that the alginate concentration used for this work was 1%, it is plausible that a higher percentage of leaked material would be obtained, due to inferior gel strength.

POA (75%) hydrogels released the highest amount of leaked material, but this may also be related to gel destruction and not dissolution, as mentioned previously. By disregarding the final saline treatment values, it is possible to conclude that the average last leakage value for POA gels was 15.7 ± 2.3 %, whilst for the POA MeOTyr gels it was 22.9 ± 0.8 %. This indicates that POA MeOTyr gels are generally more susceptive to leaching.

According to Martinsen *et al.* [52], molecular weight values below 240 kDa impact gel strength. The average molecular weight of the initial materials used ranged from 102 – 143 kDa. This could have influenced the results independently from the saline treatment.

SEC-MALS data allowed to conclude that mostly low molecular weight molecules were released first. The average molecular weight of the released molecules gradually increased as a function of the saline treatments. However, as polydispersity decreased as a function of the saline treatments, some high molecular weight molecules were also initially released.

The fact that F_G increased as a function of saline treatments indicates that alginate with a higher M-content leaked out first, followed by stipe alginate. Stokke *et al*. [16] demonstrated that the leached material from 1.5% calcium alginate beads placed in a 0.15 M NaCl solution presented higher M-content and MM-doublets. Consequently, G-content was lower in comparison with the initial material used, as was the G-doublets and triplets content, indicating an inferior average G-block length.

Periodate oxidation can decrease the average G-block length and average molecular weight [25], as is shown from the initial POA starting material. Lower values were therefore expected for POA and POA MeOTyr gels. This was observed regarding molecular weight for both POA and POA MeOTyr gels over the course of the saline treatments. On the contrary,

the average G-block length values of the final saline bath for POA MeOTyr gels were higher than the initial stipe alginate and POA MeOTyr values.

Initially high F_{MeOTyr} indicates that alginate coupled with MeOTyr was the first to leak out. The initial MeOTyr-content for the calcium alginate gels ($F_{MeOTyr} = 0.10$) was slightly higher than that of the start material ($F_{MeOTyr} = 0.07$). Since the degree of oxidation of the material is a mean value, and due to the fact that oxidation and subsequent reductive amination with coupling of MeOTyr occurs randomly, some molecules may present a higher grafting with MeOTyr than others. These molecules with a higher degree of oxidation and grafting are more likely to leak out first, contributing to the increased initial MeOTyr-content.

The molecular weight distribution and G-content of the saline bath are directly linked to the mechanical strength of the calcium alginate gels. The results from this work demonstrated that low molecular weight molecules and M-residues leak out first, which constitute the unbound chains from the gel network according to Stokke *et al*. [16]. The increase in molecular weight, G-content and average G-block length of the saline bath indicated that the sodium ions were replacing the gelling ions in the gel network, consequently decreasing the number of crosslinked chains. This successive substitution translated into a decreasing Young's modulus for all samples.

5.3 Overview

The results obtained showed that Ca-alginate gels with chemically modified alginate are more prone to degradation than Ca-alginate gels with stipe alginate, when subjected to saline treatments. Residues that do not participate in the ionic linkage, namely M-residues, are released first from the gel network, in conjunction with low molecular weight molecules and methyl tyrosine ester.

Saline treatments result in the replacement of gelling ions in the gel network, consequently decreasing the degree of crosslinking. For all gels tested, an increase in the number of saline treatments led to an increase in G-content and average G-block length in the saline bath. Initially low G-content of the bath was caused by the leakage of alginate with a higher content of M. Due to the fact that G-content and G-block length are directly related to gel strength, their decrease in the gel network explains the consequent decrease in Young's modulus and storage modulus of the gels.

Furthermore, by varying the ratio of chemically modified alginate versus stipe alginate it was possible to tailor the degradation of the hydrogels. Partially oxidised alginate gels demonstrated differences regarding Young's modulus and storage modulus in accordance with the ratio of POA included in the gel. Differences in Young's modulus were also observed for both POA MeOTyr samples. However, between POA and POA MeOTyr samples containing the same ratio of POA or POA MeOTyr, Young's modulus values were not significantly altered by the incorporation of methyl tyrosine ester in the later. Therefore, even though the average molecular weight distributions and average G-block length were different for POA and POA MeOTyr gels with equal ratio of modified material, the effect of these on gel stability and consequent degradation was minimal. Additionally, a 3:1 ratio of modified alginate resulted in weaker gels that underwent faster degradation.

These findings allowed to achieve better insight into the degradation mechanism of calcium alginate gels when placed in a saline solution, which mimics physiological conditions. The desired degradation rate depends on the application in mind, as it should reflect the formation of new tissue [12]. Modified alginate thus has an advantage over stipe alginate due to its potential for the control of degradation. The ratios of modified alginate with stipe alginate studied may therefore have different applications depending on the required degradation rate.

5.4 Further work

Three types of calcium alginate hydrogels were studied during the course of the experimental work. Stipe alginate was used as a control; whilst stipe alginate mixed with partially oxidised alginate, and stipe alginate mixed with partially oxidised alginate coupled with methyl tyrosine ester were the main focus of the research. The aim was to analyse the stability of the hydrogels when subjected to saline treatment as a function of a decrease in gel strength and stiffness, as well as in regards to the composition of the degradation products.

The reduction in gel strength occurred due to the substitution of the gelling calcium ions with non-gelling sodium ions in the gel network. It would therefore be of interest to quantify this exchange in future work.

Methyl tyrosine ester was used as a model compound to mimic peptide coupling, and therefore further work should be done using the appropriate peptide for the application in mind. This would allow to confirm that the Young's modulus values obtained are valid for

that peptide. Additionally, these hydrogels could be seeded with cells and placed in a saline solution. This would permit to study cell migration and proliferation within the hydrogel, as well as the impact that cell incorporation might have on the degradation rate and gel strength.

6 Conclusion

The aim of this experimental work was to analyse the effects of saline treatment on the stability of ionically crosslinked chemically modified alginate gels. These effects were viewed as a variation in mechanical properties through measurement of Young's modulus, storage modulus and loss factor, as a function of the saline treatments. Syneresis and swelling values for the gels were obtained as a function of weight. Additionally, the leaked material was analysed to infer about the degradation products, more specifically in regards to their G and M-content, average G-block length, average molecular weight and polydispersity.

Ca-alginate gels with periodate oxidised alginate presented a higher degradability, which constitutes an advantage for its application in tissue engineering, where the desire is for a biodegradable structure. Oxidation followed by reduction also has advantages, such as peptide coupling for facile cell attachment to the hydrogel, consequently increasing cell viability. From the results it was shown that the incorporation of partially oxidised alginate into a calcium stipe alginate gel greatly influenced its dissolution. The ratio of POA or POA MeOTyr to stipe alginate was also studied, and it was found that an increasing amount of modified alginate led to a decrease in Young's modulus and storage modulus. This was caused by the interchange of gelling calcium ions with non-gelling sodium ions present in the saline solution. As a result, mixed-in partially oxidised alginate leaked out of the gel network, contributing to the reduction of strength and stiffness.

In sum, it was possible to alter the strength and degradation rate of calcium alginate hydrogels by incorporating chemically modified alginate. This made the hydrogel more susceptible to saline treatments and led to a more accelerated dissolution.

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Appendix A: Rheology Data

Mechanical strength of the gels was measured using Texture Analyser with a P/35 probe and a 5kg loading cell. Analysis was performed on Exponent 32. An exemplification of the calculation of Young's modulus and syneresis is shown for a sample of stipe alginate hydrogel before being subjected to saline treatments.

The gradient in N/m is obtained from compression testing. This parameter is used for the calculation of Young's modulus by taking into consideration the measured sample dimensions (Equation A.1).

$$E(Pa) = \frac{Gradient\ (N/m) \times Height\ (mm)}{Area\ (mm^2) \times 10^{-3}} \quad \textbf{(A. 1)}$$

$$E = \frac{484.4 \times 16.52}{155.6 \times 10^{-3}} = 51416\ Pa$$

However, the Young's modulus value calculated does not take into account the syneresis of the gel. This was done through the calculation of a correction factor for weight, which required the theoretical weight (w_0) of the gel based on the volume of the mould, admitting that gel density was equal to water density (0.001 g/mm^3) (Equation A.2).

$$w_0(g) = \pi \times \left(\frac{Diameter_{mould}}{2}\right)^2 \times Height_{mould} \times 10^{-3} \quad \textbf{(A. 2)}$$

$$w_0 = \pi \times \left(\frac{16}{2}\right)^2 \times 18 \times 10^{-3} = 3.62\ g$$

The correction factor was then calculated with the theoretical weight (w_0) and the sample weight (w) prior to compression through Equation A.3.

$$Correction\ factor_{Weight} = \frac{w_0}{w} \quad \textbf{(A. 3)}$$

$$Correction\ factor_{Weight} = \frac{3.62}{2.72} = 1.33$$

With the correction factor for weight, it is possible to calculate syneresis of the gel (Equation A.4).

$$Syneresis\ (\%) = 1 - \frac{1}{Correction\ factor_{weight}} \times 100 \quad \textbf{(A. 4)}$$

$$Syneresis = 1 - \frac{1}{1.33} \times 100 = 25\%$$

The corrected Young's modulus can also be calculated using the correction factor for weight (Equation A.5).

$$E_{corr}\ (Pa) = \frac{E}{\left(Correction\ factor_{weight}\right)^2} \quad \textbf{(A. 5)}$$

$$E_{corr} = \frac{51416}{(1.33)^2} = 29072\ Pa$$

Table A. 1 Data obtained from stipe alginate gels when subjected to saline treatments.

Stipe alginate	n	Height (mm)	Diameter (mm)	Weight (g)	Gradient (N/m)	Syneresis (%)	Young's modulus (corrected) (kPa)
	1	16.52	14.08	2.72	484.4	25	29.1
	2	16.62	14.24	2.75	492.1	24	29.7
Initial	3	16.52	14.30	2.76	512.0	24	30.7
	4	16.38	14.27	2.75	494.3	24	29.3
	5	16.33	13.99	2.73	474.4	25	28.7
Average		16.47	14.18	2.74	491.4	24	29.5
St. Dev.		0.12	0.13	0.02	13.9	0	0.8
	1	16.03	14.06	2.55	397.0	30	20.4
	2	15.92	13.68	2.58	366.0	29	20.2
1 saline treatment	3	15.96	13.56	2.62	378.2	28	21.9
	4	15.95	14.04	2.55	353.9	30	18.1
	5	15.98	14.02	2.58	376.0	29	19.8
Average		15.97	13.87	2.58	374.2	29	20.1
St. Dev.		0.04	0.23	0.03	16.0	1	1.4
	1	15.14	13.69	2.80	123.9	23	7.6
	2	16.02	13.95	2.76	138.2	24	8.4
2 saline treatments	3	15.72	13.87	2.74	113.9	24	6.8
	4	15.70	13.72	2.72	116.1	25	7.0
	5	16.04	13.54	2.72	102.8	25	6.5
Average		15.72	13.75	2.75	119.0	24	7.3
St. Dev.		0.36	0.16	0.03	13.1	1	0.8
	1	16.40	14.31	3.05	32.1	16	2.3
	2	16.64	14.44	3.08	45.3	15	3.3
3 saline treatments	3	16.51	14.74	3.07	29.9	15	2.1
	4	16.17	14.58	3.07	40.9	15	2.9
	5	16.83	14.71	3.08	47.6	15	3.4
Average		16.51	14.56	3.07	39.1	15	2.8
St. Dev.		0.25	0.18	0.01	7.9	0	0.6
	1	17.22	14.25	2.52	24.3	30	1.3
	2	16.57	14.98	2.59	17.7	28	0.9
4 saline treatments	3	16.30	13.67	2.48	28.8	31	1.5
	4	16.69	15.13	2.70	21.0	25	1.1
	5	17.55	13.63	2.61	19.9	28	1.2
Average		16.87	14.33	2.58	22.3	29	1.2
St. Dev.		0.51	0.71	0.09	4.3	2	0.2

Table A. 2 Data obtained from POA (25%) gels when subjected to saline treatments.

POA (25%)	n	Height (mm)	Diameter (mm)	Weight (g)	Gradient (N/m)	Syneresis (%)	Young's modulus (corrected) (kPa)
	1	16.52	14.12	2.91	371.6	20	25.4
	2	16.52	13.84	2.89	379.3	20	26.6
Initial	3	16.77	14.49	2.95	413.6	18	28.0
	4	16.97	14.41	2.93	385.9	19	26.4
	5	16.78	14.39	2.93	359.4	19	24.3
Average		16.71	14.25	2.92	382.0	19	26.1
St. Dev		0.19	0.27	0.02	20.2	1	1.4
	1	16.31	14.13	2.88	256.6	20	16.9
	2	16.21	14.45	2.86	249.9	21	15.5
1 saline treatment	3	16.48	13.95	2.87	251.0	21	17.0
	4	16.17	13.92	2.83	238.9	22	15.5
	5	16.56	13.99	2.89	233.3	20	16.1
Average		16.35	14.09	2.87	246.0	21	16.2
St. Dev		0.17	0.22	0.02	9.5	1	0.8
	1	16.44	14.38	3.08	76.3	15	5.6
	2	16.68	14.06	3.07	60.8	15	4.7
2 saline treatments	3	15.95	14.05	3.07	70.8	15	5.2
	4	16.55	14.29	3.06	66.4	15	4.9
	5	16.19	13.90	3.02	49.8	17	3.7
Average		16.36	14.14	3.06	64.8	15	4.8
St. Dev		0.29	0.20	0.02	10.2	1	0.7
	1	16.62	14.84	3.22	38.7	11	2.9
	2	16.84	14.68	3.29	37.6	9	3.1
3 saline treatments	3	17.19	14.58	3.28	21.0	9	1.8
	4	17.18	15.33	3.31	27.6	8	2.2
	5	16.97	14.51	3.33	25.4	8	2.2
Average		16.96	14.79	3.29	30.1	9	2.4
St. Dev		0.24	0.33	0.04	7.8	1	0.6

Table A. 3 Data obtained from POA (50%) gels when subjected to saline treatments.

POA (50%)	n	Height (mm)	Diameter (mm)	Weight (g)	Gradient (N/m)	Syneresis (%)	Young's modulus (corrected) (kPa)
	1	16.59	14.26	3.03	232.3	16	16.9
	2	16.74	14.20	3.02	272.1	17	20.1
Initial	3	17.92	14.19	3.09	275.4	15	22.8
	4	16.86	14.73	3.13	287.6	13	21.3
	5	17.08	14.83	3.13	296.4	13	22.0
Average		17.04	14.44	3.08	272.7	15	20.6
St. Dev		0.52	0.31	0.05	24.6	1	2.3
	1	16.69	14.07	3.12	168.1	14	13.4
	2	17.01	14.51	3.13	171.4	13	13.2
1 saline treatment	3	16.69	14.31	3.07	181.4	15	13.6
	4	16.50	14.49	3.05	180.3	16	12.8
	5	16.69	14.08	3.12	174.7	14	13.9
Average		16.72	14.29	3.10	175.2	14	13.4
St. Dev		0.18	0.21	0.04	5.7	1	0.4
	1	16.25	15.81	3.22	36.5	11	2.4
	2	16.94	15.11	3.33	46.4	8	3.7
2 saline treatments	3	16.28	14.74	3.27	47.6	10	3.7
	4	17.03	15.05	3.30	37.6	9	3.0
	5	16.16	14.50	3.30	47.6	9	3.9
Average		16.53	15.04	3.28	43.1	9	3.3
St. Dev		0.42	0.49	0.04	5.6	1	0.6
	1	16.62	15.27	3.08	27.6	15	1.8
	2	17.44	15.48	2.99	34.3	17	2.2
3 saline treatments	3	16.94	15.16	3.04	36.5	16	2.4
	4	16.76	15.84	3.18	23.2	12	1.5
	5	17.48	15.34	2.89	21.0	20	1.3
Average		17.05	15.42	3.04	28.5	16	1.8
St. Dev		0.39	0.26	0.11	6.7	3	0.5

Table A. 4 Data obtained from POA (75%) gels when subjected to saline treatments.

POA (75%)	n	Height (mm)	Diameter (mm)	Weight (g)	Gradient (N/m)	Syneresis (%)	Young's modulus (corrected) (kPa)
	1	17.13	14.82	3.27	89.6	10	7.3
	2	16.69	14.59	3.23	102.8	11	8.2
Initial	3	16.98	14.61	3.26	104.0	10	8.6
	4	16.83	14.36	3.18	88.5	12	7.1
	5	17.31	14.51	3.27	98.4	10	8.4
Average		16.99	14.58	3.24	96.7	10	7.9
St. Dev		0.24	0.17	0.04	7.3	1	0.7
	1	16.43	13.71	2.69	44.2	26	2.7
	2	16.66	14.56	3.10	56.4	14	4.1
1 saline treatment	3	17.42	14.27	3.00	56.4	17	4.2
	4	17.30	14.70	3.19	64.1	12	5.1
	5	16.32	14.88	3.08	55.3	15	3.8
Average		16.83	14.42	3.01	55.3	17	4.0
St. Dev		0.50	0.46	0.19	7.1	5	0.9
	1	16.50	15.16	2.57	29.9	29	1.4
	2	15.76	15.00	2.46	35.4	32	1.5
2 saline treatments	3	16.80	14.15	2.76	18.8	24	1.2
	4	16.62	15.03	2.23	15.5	38	0.6
	5	14.14	16.00	2.32	34.3	36	1.0
Average		15.96	15.07	2.47	26.8	32	1.1
St. Dev		1.09	0.66	0.21	9.1	6	0.4

Table A. 5 Data obtained from POA MeOTyr (50%) gels when subjected to saline treatments.

POA MeOTyr (50%)	n	Height (mm)	Diameter (mm)	Weight (g)	Gradient (N/m)	Syneresis (%)	Young's modulus (corrected) (kPa)
Initial	1	16.80	14.43	3.03	245.5	16	17.7
	2	17.04	14.78	3.01	242.2	17	16.7
	3	16.69	14.34	3.03	233.4	16	16.9
	4	16.91	14.28	3.06	219.0	15	16.6
	5	16.80	14.26	3.07	234.5	15	17.8
Average		16.85	14.42	3.04	234.9	16	17.1
St. Dev		0.13	0.21	0.02	10.3	1	0.6
1 saline treatment	1	16.34	14.30	3.01	171.4	17	12.1
	2	16.44	14.44	3.03	165.9	16	11.7
	3	16.65	14.78	3.06	148.2	15	10.3
	4	16.51	14.53	3.01	153.7	17	10.6
	5	15.91	14.63	3.04	158.1	16	10.6
Average		16.37	14.54	3.03	159.5	16	11.0
St. Dev		0.28	0.18	0.02	9.3	1	0.8
2 saline treatments	1	15.75	14.20	3.22	48.7	11	3.8
	2	15.96	14.30	3.21	49.8	11	3.9
	3	15.98	14.89	3.14	44.2	13	3.1
	4	16.34	14.55	3.24	55.3	10	4.4
	5	16.11	14.49	3.23	61.9	11	4.8
Average		16.03	14.49	3.21	52.0	11	4.0
St. Dev		0.22	0.27	0.04	6.8	1	0.7
3 saline treatments	1	17.76	15.55	2.93	42.0	19	2.6
	2	17.34	15.76	3.31	25.4	8	1.9
	3	17.74	15.84	3.17	36.5	12	2.5
	4	17.63	15.55	3.1	47.6	14	3.2
	5	17.56	15.92	3.26	33.2	10	2.4
Average		17.61	15.72	3.15	36.9	13	2.5
St. Dev		0.17	0.17	0.15	8.4	4	0.5

Table A. 6 Data obtained from POA MeOTyr (75%) gels when subjected to saline treatments.

POA MeOTyr (75%)	n	Height (mm)	Diameter (mm)	Weight (g)	Gradient (N/m)	Syneresis (%)	Young's modulus (corrected) (kPa)
Initial	1	17.32	14.68	3.05	115.0	16	8.4
	2	16.62	14.39	3.06	100.6	15	7.4
	3	17.18	14.41	3.09	110.6	15	8.5
	4	16.67	14.49	3.13	106.2	13	8.0
	5	16.89	14.39	3.11	110.6	14	8.5
Average		16.94	14.47	3.09	108.6	15	8.2
St. Dev		0.31	0.12	0.03	5.4	1	0.5
1 saline treatment	1	16.59	14.53	3.10	60.8	14	4.5
	2	16.42	14.30	3.05	35.4	16	2.6
	3	16.79	14.54	3.18	66.4	12	5.2
	4	16.75	15.11	3.15	69.7	13	4.9
	5	16.04	14.84	3.18	71.9	12	5.2
Average		16.52	14.66	3.13	60.8	13	4.5
St. Dev		0.30	0.31	0.06	14.8	2	1.1
2 saline treatments	1	16.74	15.60	3.26	24.3	10	1.7
	2	17.55	15.96	3.26	28.8	10	2.0
	3	17.90	15.12	3.14	25.4	13	1.9
	4	17.53	15.18	3.27	43.1	10	3.4
Average		17.43	15.47	3.23	30.4	11	2.3
St. Dev		0.49	0.39	0.06	8.7	2	0.8

Appendix B: Calcium Alginate Stability Data

Table B. 1 Average molecular weight, polydispersity, guluronate and mannuronate content, average G-block length larger than one and MeOTyr content values obtained due to leakage from calcium alginate hydrogels when these were placed in successive NaCl baths with gentle stirring. Increasing percentages of partially oxidised alginate and partially oxidised alginate coupled with methyl tyrosine ester were analysed. Stipe alginate was used as a reference sample. Partially oxidised alginate (POA) and partially oxidised alginate coupled with methyl tyrosine ester (POA MeOTyr) samples were analysed. MeoTyr content was not applicable for stipe alginate and POA (-). An average between the values obtained from the repetition of the fourth saline treatment and the fifth saline treatment for stipe alginate is presented (*).

Sample/ Number of saline treatments		M_w (kDa)	M_w/M_n	F_G	F_M	$N_{G>1}$	F_{MeOTyr}
Stipe alginate	1	42	2.4	-	-	-	-
	2	56	2.6	0.69	0.31	16	-
	3	81	2.2	0.68	0.32	12	-
	4	118	2.3	0.71	0.29	16	-
	5*	138	1.9	0.68	0.32	15	-
POA (25%)	1	59	2.4	0.42	0.58	4	-
	2	60	2.3	0.58	0.42	6	-
	3	102	2.0	0.69	0.31	13	-
	4	132	1.8	0.67	0.33	10	-
POA (50%)	1	32	3.6	0.43	0.57	3	-
	2	51	2.5	0.60	0.40	6	-
	3	95	2.0	0.68	0.32	10	-
	4	101	2.0	0.68	0.32	11	-
POA (75%)	1	87	2.7	0.52	0.48	7	-
	2	93	2.1	0.64	0.36	8	-
	3	110	1.9	0.67	0.33	9	-
POA MeOTyr (50%)	1	46	2.1	0.37	0.63	5	0.11
	2	71	2.3	0.62	0.38	9	0.07
	3	107	1.9	0.70	0.30	15	0.05
	4	123	1.9	0.69	0.31	16	0.04
POA MeOTyr (75%)	1	79	2.4	0.51	0.49	7	0.10
	2	90	2.0	0.66	0.34	12	0.07
	3	103	1.9	0.69	0.31	16	0.06