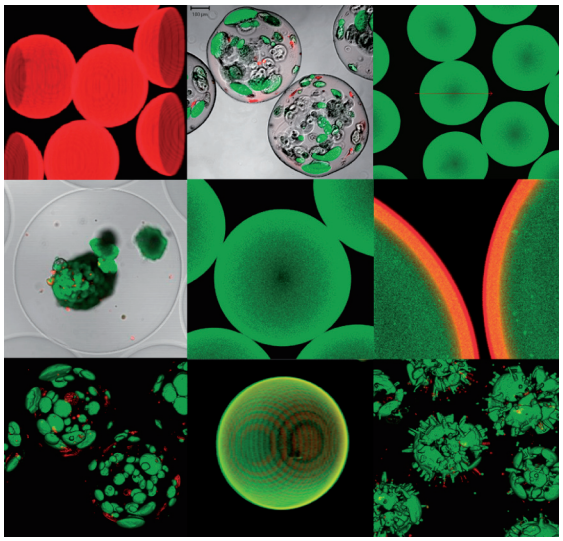
Ýrr A. Mørch

Novel Alginate Microcapsules for Cell Therapy

-A study of the structure-function relationships in native and structurally engineered alginates



Thesis for the degree of doktor ingeniør
Trondheim, February 2008

Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biotechnology

NTNU

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Preface

This thesis is submitted in partial fulfillment of the requirements for the academic title *doktor ingeniør* at the Norwegian University of Science and Technology (NTNU). The work was carried out at the Norwegian Biopolymer Laboratory (NOBIPOL), Department of Biotechnology, NTNU, Trondheim, under supervision of Professor dr.techn. Gudmund Skjåk-Bræk and co-supervised by dr.ing. Berit Løkensgard Strand. The work was financed through an individual doctoral fellowship by the Norwegian Research Council.

The thesis consists of a general introduction, aims of the study, and summary and general discussion of the six scientific papers given in the appendix.

Contributions to other publications not included in the thesis:

I. **Mørch, Y. A.**, Strand, B. L., Skjåk-Bræk, G. Alginate structure function relationships relevant to their use for encapsulation of cells. In The Bioartificial Endocrine Pancreas. Hallé, J-P., de Vos, P. Rosenberg, L. (eds.) Research Signpost. In press.

II. Maurstad, G., **Mørch, Y. A.,** Bausch, A. R., Stokke, B. T. (2008) Polyelectrolyte layer interpenetration and swelling of alginate–chitosan multilayers studied by dual wavelength reflection interference contrast microscopy. *Carbohydr. Polym.* **71** 672-681.

III. Brekken, C., **Mørch, Y. A**. A novel slow-delivery system for a CA in MRI. *Patent*. Patent number 320691. Registered 14.06.2004.

IV. Strand, B. L., **Mørch, Y. A.**, Skjåk-Bræk, G. (2000) Alginate as immobilization matrix for cells. Minerva Biotechnologica 12 (4) 223-233.

Summary

Alginate microcapsules have the potential as immune barriers for cell transplantation where the alginate gel protects the transplant from the host immune system. Microencapsulation can thus provide a way to overcome the need for immunosuppressive drugs. The successful use of alginates as immobilization material has, however, been hampered by their mechanical instability and high porosity. To overcome this problem, a polycation layer has traditionally been added to the alginate gels. However, polycations are toxic to cells and provoke inflammatory reactions. Hence, the main goal of the present work was to improve the functionality of alginate gels in order to omit the use of polycations. Alginate gel properties were found to depend strongly on many factors such as the choice of alginate material, choice of gelling ion and method of preparation, all which can be manipulated to form alginate gel beads with enhanced functionality.

In particular, specific enzymes (C-5 epimerases) that modify the alginate chain were used to form novel alginate materials, some with extreme composition not found in nature. Gels of enzymatically engineered alginate were found to be more elastic and compact, less permeable, and extremely stable under physiological conditions, offering significant advantages over native alginates. Hence, by controlling alginate nanostructure in a highly specific way, we were able to manipulate the macroscopic properties.

One of the most striking features of epimerization was the effect seen upon increasing the amount of alternating sequences in the alginate chains using the specific epimerase AlgE4. On the basis of a detailed study on the resulting products of AlgE4 epimerized alginate we hypothesized the direct involvement of the alternating sequences in junction formation.

The choice of gelling ions for alginate gel formation was also found to have significant effects on the final gel properties. Interestingly, the effect was highly dependent on the alginate material used. Application of alginates with extreme composition revealed that different block structures in the alginate bound the ions to different extent. Alginate beads with enhanced functionality could hence be formed using a proper combination of ions and alginate material.

Finally, confocal laser scanning microscopy (CLSM) was established as a versatile and easy method to visualize the macroscopic character of alginate microcapsules such as the polymer distribution in capsules. Further, effect of different encapsulation procedures on the final capsule characteristics was revealed.

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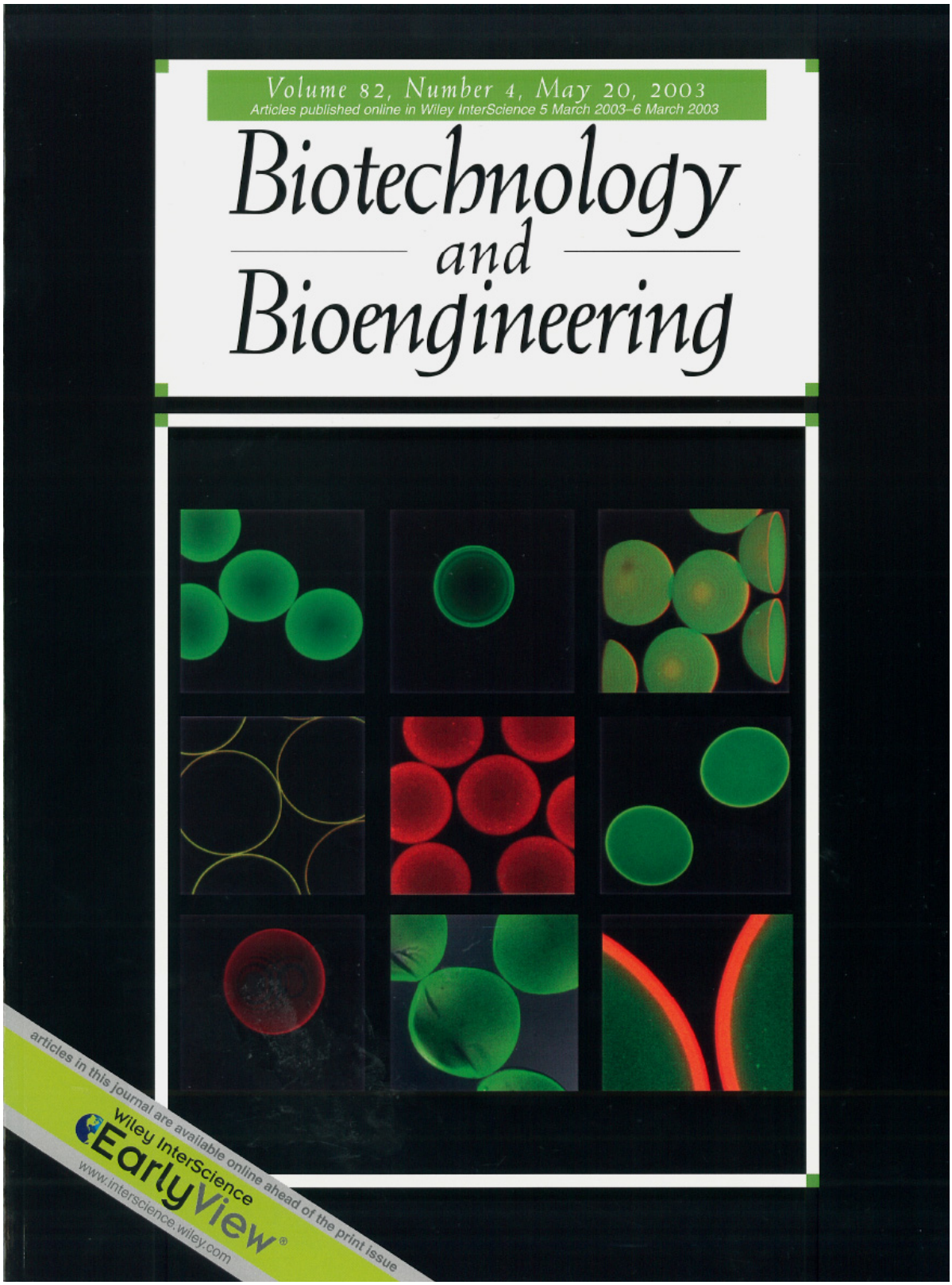

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The CLSM images of alginate microcapsules are taken by Yrr A. Mørch (Paper 1). The front page is reproduced with permission from the publisher.

List of appendix papers

1. Strand, B. L.[*], **Mørch, Y. A.**[*] , Espevik, T. and Skjåk-Bræk, G. (2003) Visualization of alginate-poly-L-lysine-alginate microcapsules by confocal laser scanning microscopy. *Biotechnol. Bioeng*. **82**, 386-394.

2. **Mørch, Y. A.**, Donati, I., Strand, B. L. and Skjåk-Bræk, G. (2006) Effect of Ca^{2+}, Ba^{2+} and Sr^{2+} on alginate microbeads. *Biomacromolecules* **7**, 1471-1480.

3. Strand, B. L., **Mørch, Y. A**., Syvertsen, K. R., Espevik, T. and Skjåk-Bræk, G. (2003) Microcapsules made by enzymatically tailored alginate. *J. Biomed. Mater. Res.* **64A**, 540-550.

4. Donati, I., Holtan, S., **Mørch, Y. A**., Borgogna, M., Dentini, M. and Skjåk-Bræk, G. (2005) New hypothesis on the role of alternating sequences in calcium-alginate gels. *Biomacromolecules* **6**, 1031-1040.

5. **Mørch, Y. A**., Donati, I., Strand, B. L. and Skjåk-Bræk, G. (2007) Molecular engineering as an approach to design new functional properties of alginate. *Biomacromolecules* **8**, 2809-2814.

6. **Mørch, Y. A**., Holtan, S., Donati, I., Strand, B. L. and Skjåk-Bræk, G. Mechanical properties of C-5 epimerized alginates. Manuscript.

* The authors contributed equally to this paper

1 INTRODUCTION

1.1 Motivation

Entrapment of living cells or other biologically active compounds is a well established method both for effective protection of the immobilized material and administration of low molecular weight compounds like drugs, proteins, food additives and chemicals. Although the potential uses of these systems in medicine, food industry and agriculture are numerous, much focus has been on the encapsulation of living cells for transplantation purposes. The far most studied system is the encapsulation of insulin producing cells (islets of Langerhans) into calcium-alginate microcapsules for transplantation into diabetic patients as an approach to treat Type 1 diabetes. The capsule acts to protect the transplanted cells from the host immune system by physical isolation of the graft.

In spite of extensive research in the field, there are still many problems connected to the use of alginate microcapsules as an immune barrier for transplantation purposes. One of the major obstacles preventing the use of alginate capsules *in vivo* has been overgrowth of immune cells on the capsule surface, which can cause failure of encapsulated cells. Cellular overgrowth has been attributed to an immune response to foreign cellular material leaking out through the capsule membrane, direct exposure of cells through capsule breakage or to the capsule material itself. Under physiological conditions the capsules are also exposed to a combination of destabilizing forces, leading to swelling, increased pore size, dissolution, and capsule rupture. To protect the cells from the host immune system, the capsule must therefore be carefully designed, especially with respect to stability and porosity. Traditionally, an outer polycation layer has been added to the capsules in order to decrease porosity and increase the long-term stability of alginate gel beads. Several studies have, however, indicated that the polycation provokes inflammatory reactions. Hence, one of the main goals and challenges of the present work has been to make alginate gel beads that are stable under physiological conditions over extended periods of time, without the use of polycations. The hope is that the great - and still growing - knowledge about alginate structure-function relationships can be used for this purpose.

With the high interest and expectations connected with the use of immobilized islets as treatment for Type 1 diabetes, the largest motivation for the present work has been to design an alginate bead for this specific application. However, upon learning more about these relations, other existing alginate technologies and new applications may be implemented.

1.2 Immunoisolation

Transplantation of living cells is a potential strategy for treatment of various diseases caused by the body's inability to produce required amounts of an essential molecule such as a hormone or an enzyme. Such cell therapy treatment involves the replacement or repair of damaged tissue or cells by means of transplantation of human or animal cells. As for whole organ transplants, patients undergoing cell therapy treatments run the risk of rejection, in which the body recognizes the cells as a foreign substance and seeks to destroy the transplant. As a consequence, a lifelong need for immunosuppressive drugs is required with the concomitant increased risk of tumor development [1], frequent infections and general toxicity [2]. In fact, the side-effects can be so severe that the disadvantages of such drugs outweigh the benefit of the transplant.

The general concept of immunoisolation is to prevent rejection by separating the transplanted cells from the hostile immunological environment in the host by a selectively permeable artificial membrane. The small pores of the membrane prevent the passage of high-molecular weight substances such as large antibodies and cytotoxic immune cells, at the same time allowing free passage of smaller molecules like nutrients, electrolytes, oxygen and biotherapeutic agents [3, 4] (Figure 1).

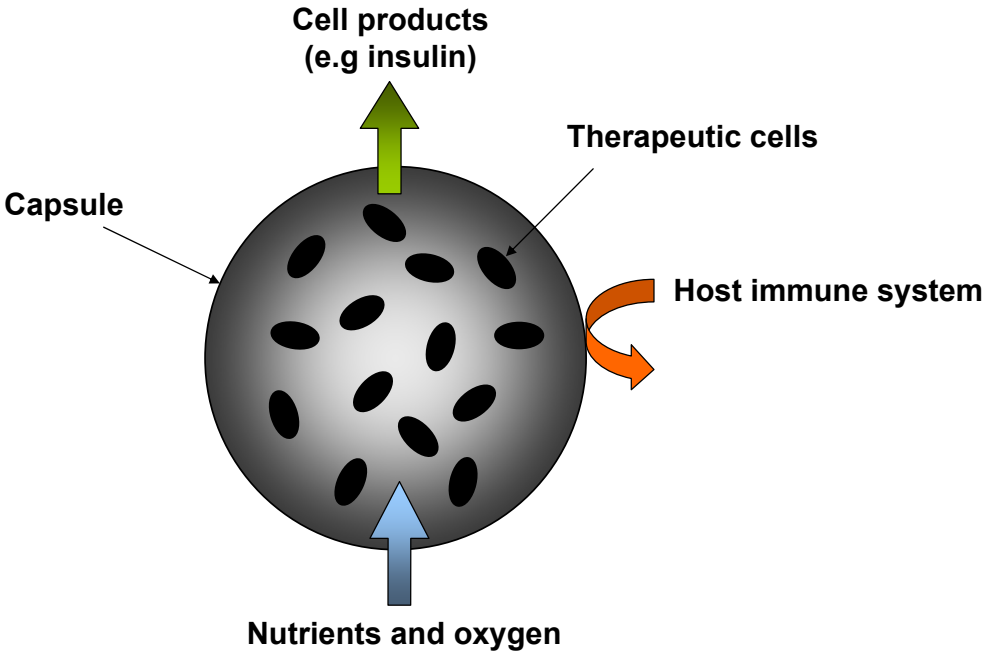

Figure 1. Immunoisolation exemplified by encapsulated therapeutic cells for transplantation purposes. The capsule material is typically a hydrogel matrix. Adapted and modified from [4].

Immunoisolation hence allows for transplantation of cells without the need for permanent immunosuppression. In addition, it may offer the possibility for successful transplantation of non-human tissue (xenotransplantation) in order to overcome the shortage of human organs [3, 5, 6]. The use of immunoisolation to transplant cells and tissues offers the opportunity to revolutionize current therapy for a wide variety of human diseases [5, 7-10] including the treatment of Parkinson's disease [11], liver failure [12], glioblastomas [13-15], hypocalcemia [16, 17], dwarfism [9] and diabetes mellitus [18].

Apart from immunoisolation, other major methods to avoid immunological responses include the use of immunosuppressive drugs, induction of tolerance to donor tissue in the host [19] or pretreatment of the graft to reduce immunogenicity [10, 20].

1.2.1 Host reactions to transplanted cells

Transplanted cells are naturally recognized as foreign material by the host immune system which as a result will seek to destroy the graft in a highly sophisticated manner. Immune rejection is the most important host response towards the cellular component being an allograft (same species) or xenograft (unlike species). The immune reaction against an allograft or a xenograft is complex and involves cells, antibodies, complement and cytokines which can cause cell damage. To understand how encapsulation may protect transplanted cells against these damaging components of the immune system, a brief presentation of the main host defense mechanisms are given below. These mechanisms can be divided in **cellular** and **humoral** immune responses. Figure 2 summarizes the challenges of an immunoprotective barrier.

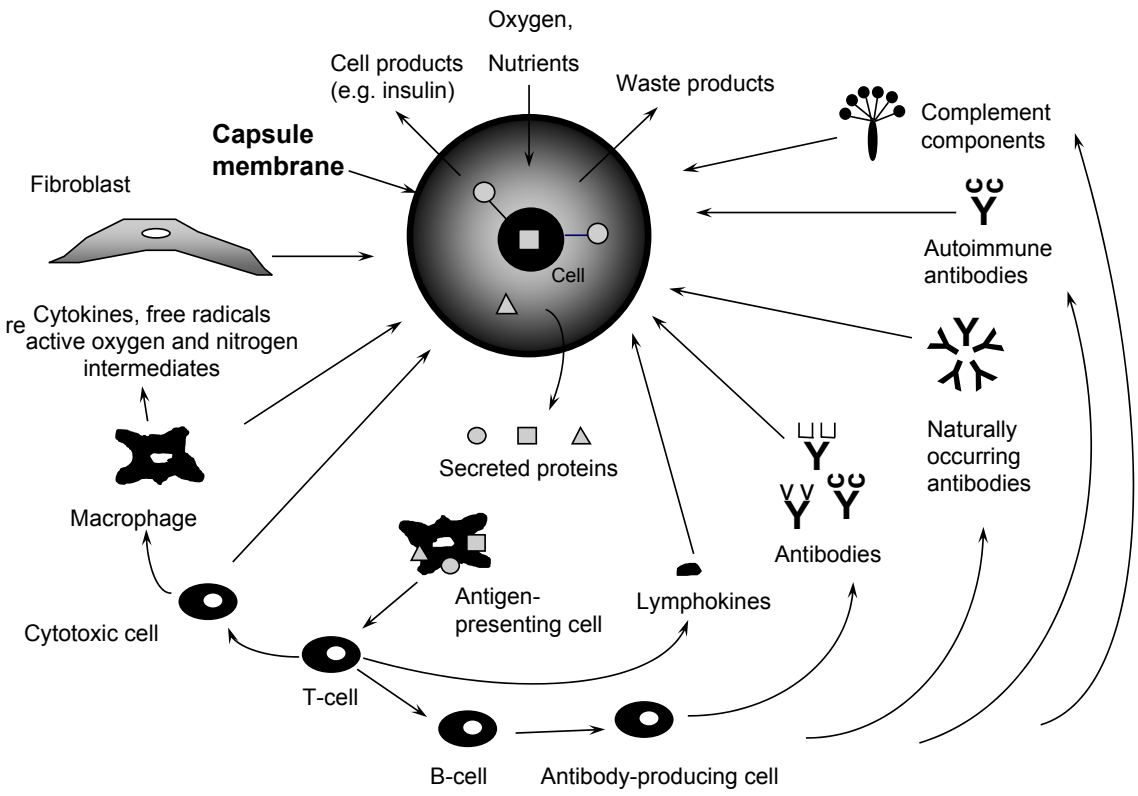

Figure 2. Simplified illustration of the challenges of an immunoisolating device. Adapted and modified from [21].

The **cellular responses** involve recognition of Class I major histocompatibility complex (MHC) on the donor graft by receptors on CD4+ or CD8+ T cells (direct pathway) leading to cytolytic killing of the donor cells. Immunoisolation of the graft can prevent the direct contact between grafted cells and T-cells, thus preventing the cytotoxicity. The direct pathway plays a dominant role in acute or early allograft rejection and may be less important in xenograft rejection which largely provokes the indirect pathway.

The indirect pathway involves recognition of antigens on the donor cell surface by antigen presenting cells (macrophages, B-cells, monocytes, dendritic cells) in association with Class II MHC molecules. The antigens are presented by these cells to the host CD4+ cells which secrete cytokines to provide necessary signals for growth, maturation and activation of cytotoxic CD8+ cells, B-cells, macrophages, leukocytes and endothelial cells. Although cytokines primarily act as a signaling system between immune cells, they are also capable of killing cells directly. Examples include interleukin-1β (IL-1β), tissue necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Various components leaking out from transplanted cells through

the artificial membrane (secreted proteins, cell surface antigens, phospholipids or DNA from dead cells) can act as antigens and thus initiate the indirect pathway.

While an immune barrier may prevent the direct contact between host and donor cells, the ingress of components of the **humoral response** may be a tougher challenge to meet. This involves production of naturally occurring antibodies against the graft (IgG, IgM) and complement activation. Binding of an antibody to the donor cell surface will normally not cause any damage. The cytotoxic events begin only if complement components pass through the membrane. Binding of the first complement component to IgM or IgG initiates a cascade ending in cell lysis [21, 22].

In addition to the cellular and humoral responses, macrophages and certain other immune cells can secrete low molecular weight reactive metabolites of oxygen and nitrogen including free radicals and nitric oxide which are toxic to cells in an nonspecific manner [21, 23]. As these components are smaller than the crucial nutrients and cell products that need to be able to diffuse freely through the immune barrier, their ingress cannot be prevented. However, although toxic to cells, their lifetime is short meaning that the damage they can cause will depend on how far they can diffuse before they disappear as a result of chemical reactions [21].

1.2.2 Immunoisolation devices

A large variety of immobilization devices of different geometries have been proposed for cell transplantation purposes. The primary encapsulation devices can be categorized by intravascular macrodevices, extravascular macrodevices and microcapsules (Figure 3) [3, 6, 22, 24-27].

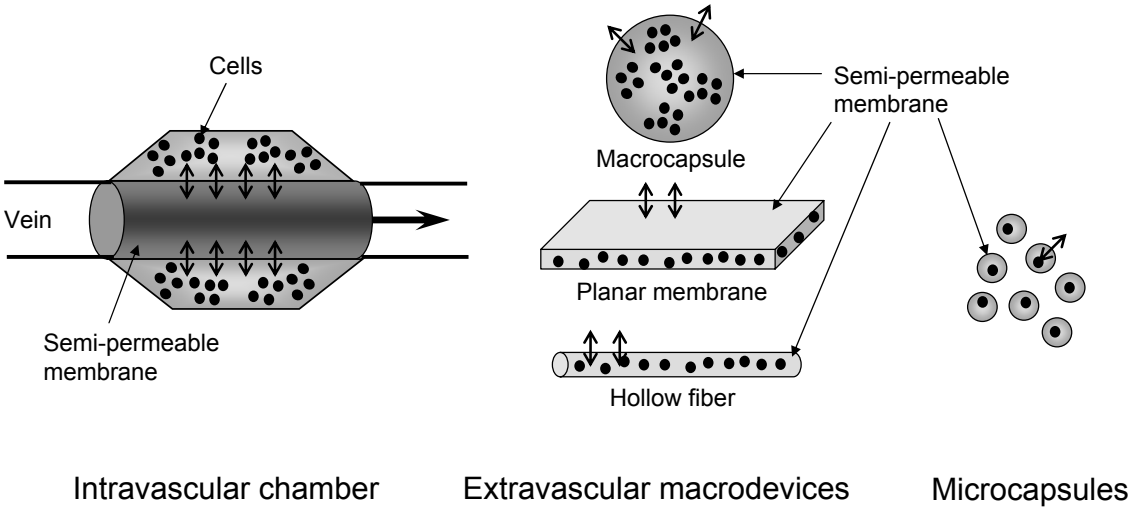

Figure 3. Immunoisolation devices. ↕ indicates transport of molecules over the immunoisolating membrane. Adapted and modified from [24].

Macrodevices are characterized by their large size (0.5-1.5 mm inner diameter, 1-10 cm in length) and internal capacity, containing thousands to millions of cells. The most common geometric configurations are intravascular chambers, hollow fibers and flat membranes (sheets) of different geometries. The intravascular macrodevices (chambers) are connected directly to the host's circulatory system. The blood flows through a microporous tube with a housing on the outside containing the implanted tissue [6, 25]. The graft is thus in close vicinity to the blood, allowing the graft to be well nourished. However, the risks connected with major surgery for implantation and retrieval as well as blood clotting has resulted in the lack of interest for this device over the last years [3, 24]. Extravascular devices are usually transplanted into the peritoneal cavity or subcutaneously and rely on the formation of new blood vessels. The major disadvantages of macrodevices have been their short life time due to breakage and fibrosis and the small surface to volume ratio. An advantage of these devices is the easiness of retrieval [24, 25].

Microencapsulation involves immobilizing cells in small spherical capsules or beads (0.2-1.0 mm in diameter). Most applications require thousands to hundreds of thousands of beads to provide the necessary cell number for a therapeutic dose. Microcapsules offer several distinct advantages over the use of macrocapsules, including greater surface to volume ratio, ease in manufacturing and a wider range of transplantation sites. In addition, implantation of microcapsules is usually by injection which removes the need for invasive surgery. Microcapsules are typically more durable than macrocapsules and more difficult to disrupt.

As only a few cells are encapsulated in each microcapsule, the whole graft is not lost if one capsule should brake. However, a disadvantage with the use of microcapsules can be the difficulty of retrieval if required, which typically is performed by lavage and needle aspiration [3, 24, 27]. To date, the limitation with the use of microcapsules has been fibrotic overgrowth and low stability.

1.2.3 Microcapsule materials

Finding a suitable material to construct an immunoisolating device is challenging: The capsule must be sufficiently permeable to allow nutrients to enter and therapeutic proteins to escape and it must be stable over extended time periods. In addition, the material must be non-toxic, cell-compatible and biocompatible. Ease of processing, sterilizability and mild encapsulation conditions are also of importance [25].

Numerous materials, both natural and synthetic, have been proposed for cell encapsulation including poly(glycolic acid) (PGA) [28], poly(lactic-*co*-glycolic acid) (PLGA) [29], agarose [30, 31], polyacrylates [25, 32], κ-carrageenan [33] and alginate [34]. Coating of cells with heparin [35], poly(ethylene glycol) (PEG) [28, 36], or various polycation nanolayers [37] has also been suggested. In addition, several combinations of materials forming multicomponent capsules have been proposed [30, 38] allowing independent control of different capsule parameters.

Due to their high water content and three-dimensional matrix structure thereby stabilizing biologically active substances, hydrogels of naturally occurring or synthetic polymers are the most commonly used materials for microencapsulation. Their hydrophilic nature reduces the friction between the capsule and surrounding fluids and tissues. In addition, hydrogels have a pliable consistency which prevents damage to surrounding tissues. Although some water-insoluble materials, due to their high stability, have been preferred by some groups for encapsulation of living cells, these are often limited by the need for organic solvents which may influence cell viability. Transparent hydrogels also allow easy visualization of the encapsulated cells [6, 25, 26].

Of all hydrogels used for microencapsulation, alginate has been and will be one of the most important immobilization materials [39, 40]. In addition to being heat stable, alginates possess the ability to form hydrogels rapidly and under very mild (physiological) conditions [41]. Moreover, no alginate-degrading enzymes have so far been reported in humans. Combined

with the fact that alginates are highly characterized and well understood both in the liquid and gel phase makes this biopolymer unique as an immobilization material.

1.3 Alginate

1.3.1 Source and applications

Alginate, first described by Stanford in 1881 [42], is a polysaccharide mainly occurring as a structural component in marine brown algae (Phaeophyta) [43]. A polymeric material resembling alginates from brown algae is, however, produced as a capsular component in soil bacteria such as *Azotobacter vinelandii* [44] and several species of *Pseudomonas* [45, 46]. In particular, the alginate-secreting bacterium *Pseudomonas aeruginosa* in cystic fibrosis patients [47] has gained much focus in recent years. In brown algae, alginate is the most abundant polysaccharide comprising up to 40% of the dry matter [48], mainly occurring in the cell walls and intercellular spaces where it consists of an insoluble mix of calcium, magnesium, sodium, and potassium salts [49]. Its main function is to contribute to the strength and flexibility of the seaweed plant, and the composition of the alginate varies both within different species of algae and within different parts of the plant [50]. Industrially, alginate has for many decades been used for its gelling, viscosifying and stabilizing properties and for its ability to retain water [51]. Alginates also have recognized potential in removal of toxic heavy metals from industrial waste by biosorption [52]. More recently, new biotechnological, biomedical and pharmaceutical applications have been added to the list, and some new alginate based materials have also emerged from extensive research in the field [4, 53].

Although present research holds many promises to the possibility of alginate production by microbial fermentation and post-polymerization modification of the molecule, all commercially available alginates are at present extracted from algae [4, 48]. The annual industrial production of alginate is about 30,000 metric tons. However, the sources of alginate may be regarded as unlimited [48].

1.3.2 Composition, sequence and molecular weight

Alginate is a family of linear binary copolymers of (1→4) glycosidically linked α-L-guluronic acid (G) and its C-5 epimere, the β-D-mannuronic acid (M) residue [54] (Figure 4a). The relative amount of the two uronic acid monomers as well as their sequential arrangement along the polymer chain differs widely, depending on the origin of the alginate. The uronic acid residues are distributed along the polymer chain in a pattern of blocks, where homopolymeric blocks of G residues (G-blocks), homopolymeric blocks of M residues (M-blocks) and blocks with alternating sequence of M and G units (MG-blocks) co-exist (Figure 4b) [55]. In M-blocks, diequatorial linkages connect the mannuronate residues forming a flat ribbon-like structure whereas in G-blocks, the linkage between two guluronate units is diaxial and hence shorter. G-blocks are therefore more buckled and less flexible, with junctions between the residues stabilized by intra-molecular hydrogen bonding. MG-blocks contain both equatorial-axial and axial-equatorial linkages, but the differing degrees of freedom of the two residues gives greater overall flexibility than the (1→4) linked β-D-mannuronate chains (Figure 4c). Hence, the stiffness of the chain blocks has been found to increase in the order MG < MM < GG [56-58].

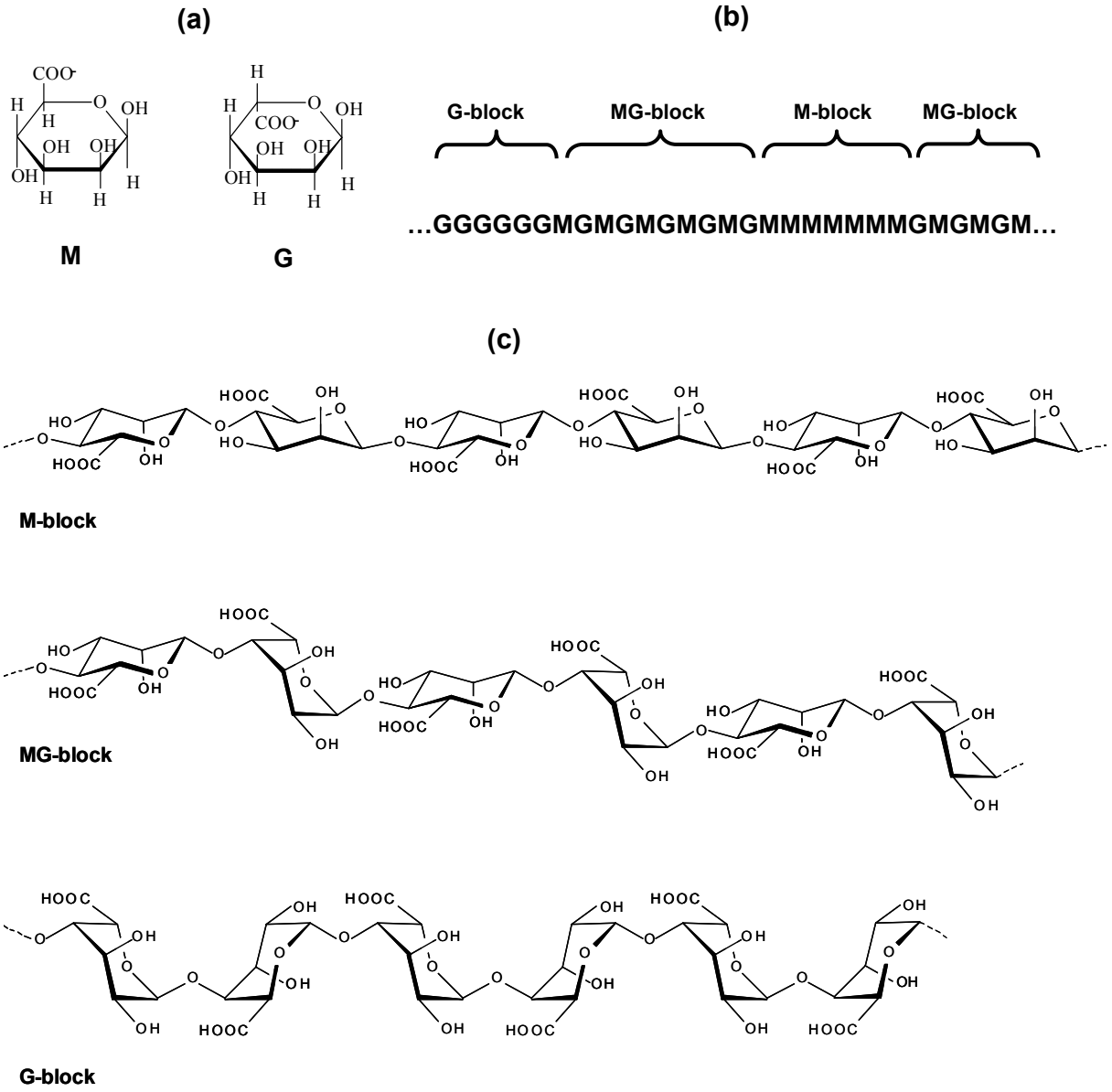

Figure 4. Alginate chemical structure. (a) Illustration of the Haworth formulas of the two monomers. M = β-D-mannuronic acid and G = α-L-guluronic acid. (b) Block composition in alginates. (c) Ring conformation in the alginate chain ($^4C_1$ for M and $^1C_4$ for G). Stretched ribbon-like structures are formed by equatorially linked poly-mannuronate chains (top) while axially linked poly-guluronate chains tend to assemble in folded ribbons (zigzag-shaped chain conformation, bottom). The cavities between each pair of guluronate residues present favorable binding sites for cations. Ring protons are not shown.

The composition and sequential structure of alginate is often a key functional attribute, and variations in the composition and/or the sequential structure may cause differences in performance of an alginate for a particular end use. As alginates are macromolecules of heterogeneous structure with no regular repeating unit [59] the alginate molecule cannot be described by the monomer composition alone. In stead, detailed information about the structure of alginates can be gained from high-resolution ^{1}H- and ^{13}C-nuclear magnetic

resonance spectroscopy (NMR) techniques [60-62]. Thus, the fraction of guluronic and mannuronic acid in an alginate sample (F_G and F_M, respectively) together with information about diad and triad frequencies and information about the average G-block length can be determined (see section 1.3.5).

In algal alginates F_G can vary in the range of 0.20 to 0.85 [63]. High contents of G is generally found in alginates prepared from stipes of old *Laminaria hyperborea* plants, whereas alginates from *Durvillea antartica*, *Ascophyllum nodosum*, *Laminaria digitata* and *Macrocystis pyrifera* are characterized by a low content of G-blocks [42]. Even more extreme composition can be found in alginates isolated from bacteria strains of *Azotobacter vinelandii* or *Pseudomonas* [63]. *Azotobacter* alginates resemble the seaweed material in that they are true block copolymers (F_G = 0.10-0.95), whereas *Pseudomonas* alginates invariably lack G-blocks (F_{GG} = 0). The main differences between the bacterial and algal alginates are however the presence of *O*-acetyl groups in the former which influence the swelling and water-binding potential of bacterial alginate gels [63, 64].

Alginates are polydisperse with respect to molecular weight, and because of this the molecular weight of the polymer is an average of the whole distribution of molecular weights. Depending on the end use and the sensitivity of the application to the molecular mass, the presence of a wide range of alginate fractions may be an issue. In such cases, calculation of the polydispersity will be important, and in general, a narrow molecular weight distribution is preferred [42].

In its native form, the polymer chains of alginate are rather long. However, being a single stranded polymer alginate is susceptible to a variety of depolymerization processes. The glycosidic linkages are cleaved by both acid and alkaline degradation mechanisms and by oxidation with free radicals. As a function of pH, degradation of alginate is at its minimum at neutrality and increases at low pH due to proton catalyzed hydrolysis [65] and at high pH due to β-alkoxy-elimination [66]. Degradation by free radicals is mainly due to oxidative-reductive depolymerization reactions caused by contamination of reducing agents like polyphenols from the brown algae [53]. All these depolymerization reactions increase with temperature. During processing, the molecular weight will hence decrease and is rarely higher than 500,000 g/mole for commercial alginates.

1.3.3 Physical properties

The industrially important properties of alginates are mainly a result of the macromolecular and polyelectrolytic character of the molecule. As there is a strong correlation between structure and function of alginates, the chemical composition and molecular weight of the molecule will determine alginate's applicability for commercial uses.

Viscosity

The viscosity of an alginate solution will mainly depend on the molecular weight and concentration of the polymer sample. However, as the intrinsic flexibility of the chain blocks varies, the composition might also affect the final viscosity of the solution. Since alginate is a highly negatively charged polyelectrolyte, the electrostatic repulsion between the charged groups will further contribute to viscosity by forcing the molecule into a very extended random coil conformation [67]. Consequently, the ionic strength of the solution will be of importance in this aspect as high ionic strength will lead to shielding of the charges and hence reduced chain extension. It has been shown that the intrinsic viscosity, [η], of an alginate solution is linearly dependent on the ionic strength in the solution [42, 68].

High molecular weight has been shown to be beneficial for several applications of alginate. However, the use of very high molecular weight materials is limited by their high viscosity, e.g. making sterile filtration and general handling of the solution difficult. In addition, it has been reported that very high viscosity alginate solutions may reduce cell viability due to the high shear-forces required to mix cells with these solutions [69].

Solubility

Sodium alginate and most other alginates of monovalent metals are soluble in water and give solutions of high viscosity. However, the pH of the solvent is an essential parameter determining the solubility of alginates in water as it will influence the presence of electrostatic charges on the uronic acid residues [70]. As a result of this, alginic acid, the protonized form of alginate, is generally insoluble in water [68] forming either gels or precipitates [71, 72]. The pK_a values of the two monomers have been determined as 3.38 for M and 3.65 for G (in 0.1M NaCl), and consequently the pK_a of the polymer increases with increasing G content [73]. The solubility of an alginate sample at low pH is, however, not necessarily a direct effect of the pK_a [70]. High amounts of polyalternating structure in an alginate sample has been shown to increase its solubility at low pH [74]. In fact, a sample of pure polyalternating

structure was demonstrated to be completely soluble over the whole pH range at short equilibration times [70]. The reason for this was suggested to be structural, based on the irregularities of the axial/equatorial linkages in the alternating sequences [70].
In addition to the pH being important for alginate solubility in water, the ionic strength of the solute and the presence of divalent ions play important roles due to salting out effects and gelling, respectively [75].

Ion binding

Present and future applications of alginates are mainly linked to the most striking feature of the alginate molecule – the formation of an ionotropic gel in the presence of certain multivalent cations like Ca^{2+}, Ba^{2+} and Sr^{2+}. The binding of ions is highly selective and the affinity strongly depends on the alginate composition [54]. The affinity of alginates for alkaline earth metals has been shown to increase in the order $Mg^{2+} << Ca^{2+} < Sr^{2+} < Ba^{2+}$ [76]. More specifically, from experiments involving equilibrium dialysis of alginate [54, 76, 77] it was found that the selectivity for alkaline earth metals increased markedly with increasing G content, but that MM and MG blocks were almost without selectivity. Smidsrød [54] reported that the binding strength of divalent ions to the three alginate fragments is as follows:

GG-blocks: Ba **>** Sr **>** Ca **>>** Mg
MM-blocks: Ba **>** Sr **~** Ca **~** Mg
MG-blocks: Ba **~** Sr **~** Ca **~** Mg

The high selectivity between similar ions indicates that interactions between alginates and these ions are not purely electrostatic. Rather, a complexing of ions related to the structure of G-blocks is taking place [41]. The mechanism has been explained by an “eggbox” model [78-81] where the diaxially linked G residues form cavities which function as binding sites for ions, like eggs in an eggbox (Figure 5). When sequences of G-blocks form bonds with similar sequences on the same or other molecules junctions are formed as illustrated in Figure 5, creating a gel network. More recent work suggests lateral association far beyond a pure dimerization of chains which increases with increasing [Ca^{2+}] and G content (Figure 5b) [82, 83].

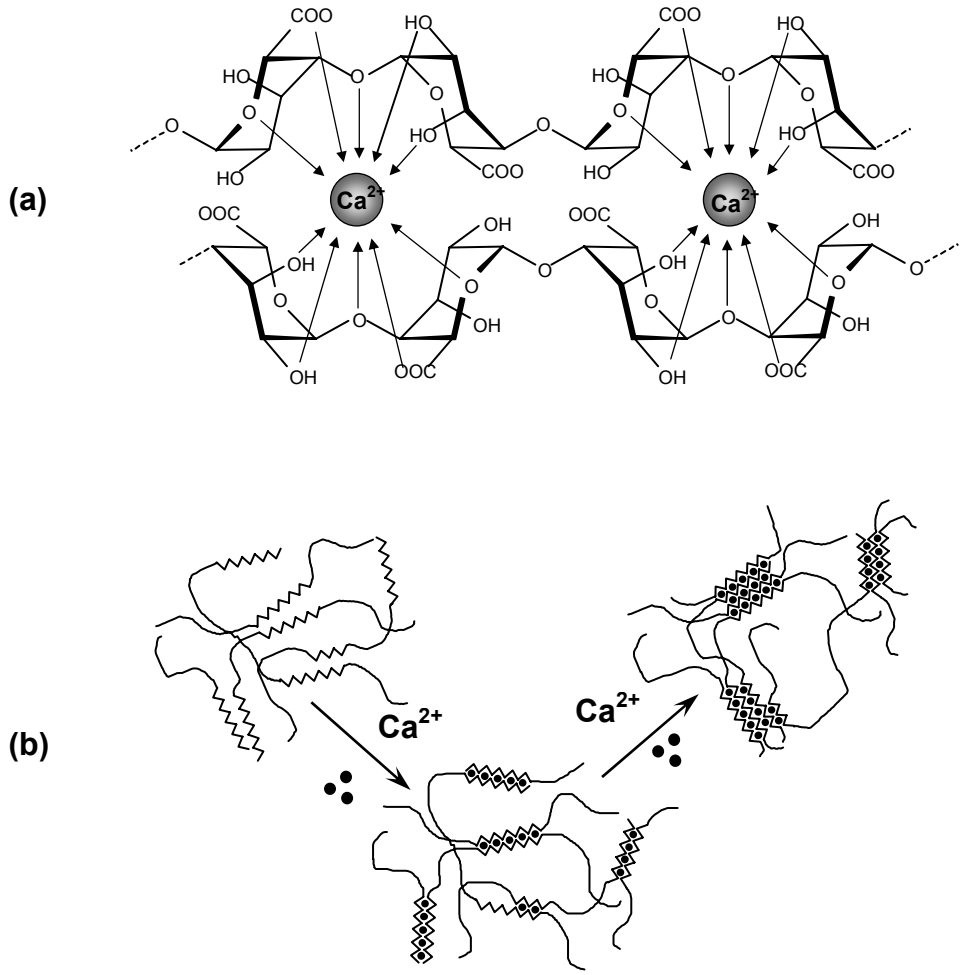

Figure 5. Binding of divalent ions to alginate – the eggbox model. (a) Chelation of divalent cations. (b) Lateral association of chains.

The gel formation is irreversible and autocooperative, physically explained by the entropically unfavorable binding of the first divalent ion between two G-blocks and the more favorable binding of the following ions (zipper mechanism) [75]. The minimum required size of adjacent G units for formation of stable junctions has been reported to range from approximately 8 to 20 G residues for Ca^{2+} [84, 85]. Further, it has been reported that the minimum length of G blocks necessary for junction formation decreases with increasing affinity of ions toward the alginate chain [86]. Hence, the *length* of G-blocks is of great importance for the formation of stable junctions.

1.3.4 Alginate gels

An alginate gel, like many other biopolymer gels, is a continuous biopolymeric network swollen with water where the network is bound together by non-covalent crosslinks - a so-

called “physical gel” [87]. The properties of alginate gels are largely governed by factors such as chemical composition, sequence, molecular weight and molecular weight distribution of the polymer [53].

Formation of gels

When a solution of sodium alginate comes into contact with calcium or other divalent cations, a rapid, strong and irreversible formation of a gel takes place. The formation of alginate gels is special in the sense that it is independent of temperature, and the resulting gels are heat stable [41].

There are two fundamentally different methods for preparing alginate gels by ionic crosslinking: The diffusion method and the internal gelation method. The methods differ in the way the crosslinking ion is introduced [41, 75] and hence the gelling kinetics of the two methods is very different.

The diffusion method is characterized by allowing calcium (or other divalent ions) diffuse from an outer reservoir into the alginate solution (Figure 6). Although this method is widely used in food industry [51], it has also become a very popular method as an immobilization technique. The method is very rapid and will result in an inhomogeneous distribution of alginate as discussed in the section covering alginate beads.

The internal gelation method is based on the mixing of an inactive form of the crosslinking ion with alginate solution (Figure 6). For calcium the insoluble $CaCO_3$ or Ca^{2+} ions complexed with chelating agents like EDTA or citrate can be used. The Ca^{2+} ions are released by lowering the pH adding organic acids or slowly hydrolyzing lactones. This method allows for a slow and controlled formation of gels and will almost always result in a homogenous distribution of alginate within the gel. Using a system of alginate mixed with $CaCO_3$ and GDL (D-glucono-δ-lactone) the gelling kinetics can be controlled by the calcium carbonate particle size. In addition, the pH of the final gel may be easily set by adjusting the relative proportions of calcium salt and GDL [88]. The gel strength and breaking strength of internally set gels will also in general be lower compared to diffusion gels as the former usually are calcium limited [41, 89].

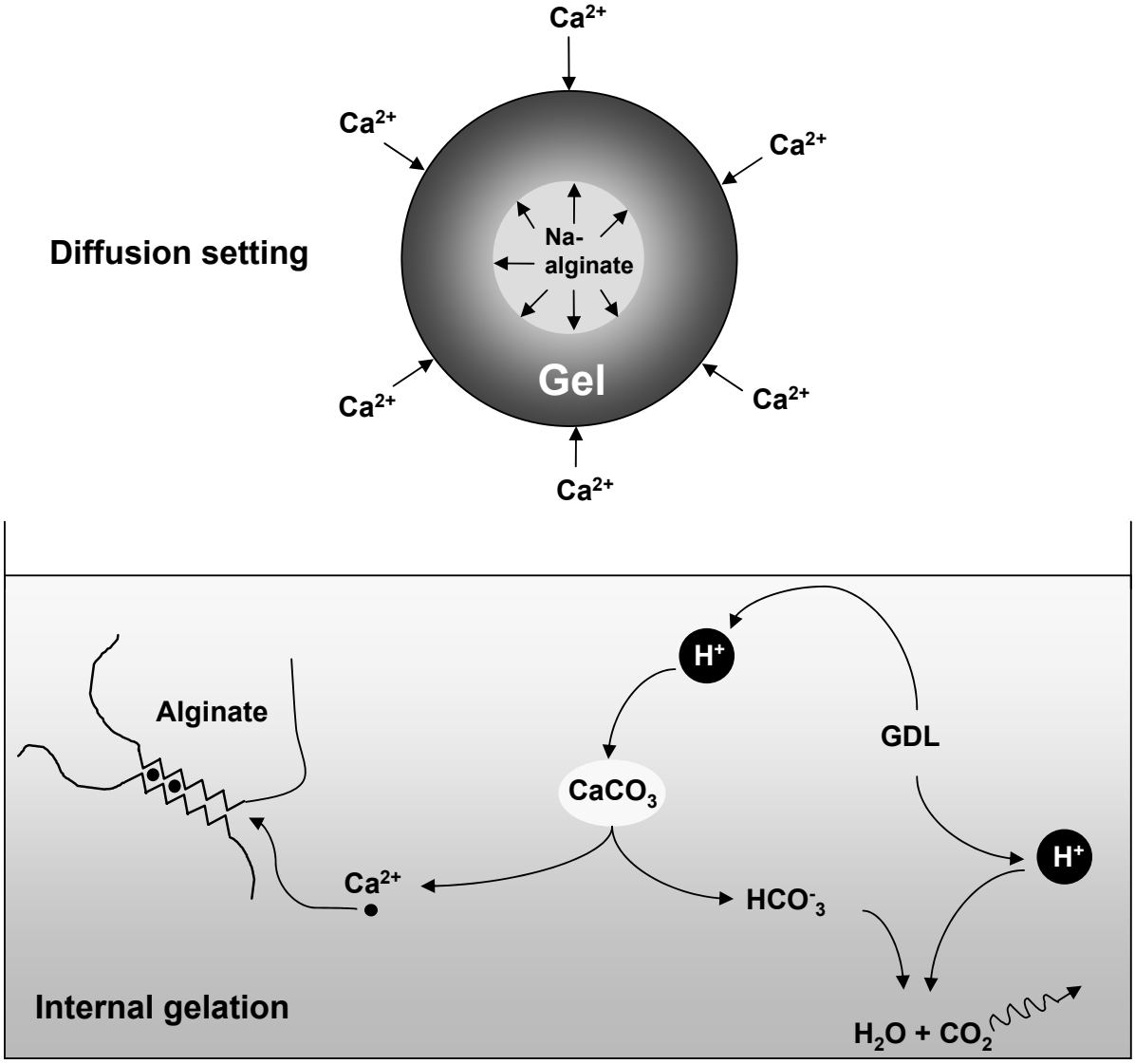

Figure 6. Principal differences between the diffusion method (exemplified by a capsule) (top) and the internal gelation method (bottom). Adapted and modified from [41].

Syneresis

Binding of multivalent cations to a binding site on alginate is rapid, and probably results in a certain degree of sub-optimal crosslinking in alginate gels [50]. Building a network structure of perfectly aligned blocks to form the maximum possible number of interguluronate sites is limited by diffusion of the polymer chains. To get a higher degree of overlap it is necessary for a series of slow dissociation/association steps to occur after the initial crosslinking, and alginate gels are hence said to be non-equilibrium gels [90].

The shrinkage of hydrogels with the concomitant release of water is referred to as syneresis. The result of syneresis is a smaller gel with a higher total concentration of alginate. Syneresis is highly dependent on alginate composition. Alginates containing long G-blocks will shrink

less during gel formation than alginate with shorter G-blocks due to the formation of strong irreversible junctions in the former, hindering reorganization of the network structure [91]. Alginates containing high amounts of alternating structures will, however, undergo great syneresis due to the larger flexibility of the MG-blocks, allowing denser packing and thus further intra- and intermolecular crosslinking [91-93]. Up to a certain point, syneresis also increases with increasing [Ca^{2+}] [91], increasing gelling time [91] and increasing molecular weight [93].

Application of small-angle X-ray scattering techniques (SAXS) has provided information about the lateral thickness of junction zones in alginate gels [82, 94, 95]. In these studies, an increase in syneresis is illustrated upon lateral aggregation of junction zones.

Mechanical rigidity of alginate gels

The elasticity of a gel is a measure of its deformation under stress and is often described by rubber elasticity theory. This theory is based on rubber-like (entropic) networks which are crosslinked by stable, covalent bonds where the crosslinks are point-like and allowed to move freely in the elastic body under the elastic influence of the chains. Upon deformation of the gel, the free energy change is caused by the decrease in entropy, solely [96, 97]. In an alginate gel network, however, the crosslinks are not point-like, but rather composed of long, non-permanent junctions where the chains between junctions are very restrictive in their movements. In fact, it has been shown [98, 99] that upon deformation the entropy of alginate gels increases rather than decreases as should be expected from the theory of rubber elasticity. This has been explained by the rupture and movement of junctions upon deformation, meaning that the elasticity of alginate gels is energetic rather than entropic in origin [98-100].

The elastic modulus (or Young's modulus) is frequently used as a measure of gel strength for alginate gels. In general, the elastic modulus will depend on the number and strength of crosslinks as well as on the length and stiffness of the chains between crosslinks [75]. Hence, the gel strength has been shown to be a function of composition, molecular weight, polymer concentration and type of ions used for crosslinking.

Increased ion binding and hence also enhanced mechanical rigidity is found with alginates rich in G residues [101]. In general, G-rich alginates form stronger gels than alginates high in mannuronic acid. Specifically, the mechanical strength increases with the *length* of G-blocks stemming from association of long G-blocks and a shortening of the elastic segments [88, 102, 103].

As the gel strength increases with the affinity between polymer and crosslinking ion, the modulus depends on the type of divalent ion used for gelation [54]. It has been reported that the minimum length of G blocks necessary for junction formation decreases with increasing affinity of ions toward the alginate chain [86], probably resulting in an increased number of elastic chains per unit volume. The increase in mechanical strength with ion affinity may thus be due to a combination of higher strength or higher number of junctions [90].
For Ca-saturated alginate gels made by diffusion the modulus increases with molecular weight up to a certain point. The gel strength becomes constant when intrinsic viscosity exceeds approximately 600 ml/g [91]. The modulus of Ca-limited gels is, however, more molecular weight sensitive [89].
Young's modulus has been shown to be proportional to the square of alginate concentration [102]. As alginates undergo syneresis to various extents, the concentration differences have to be taken into account when calculating the Young's modulus [91].

The force needed to rupture a gel can also be used for describing the mechanical properties of alginates gels. Whereas the elastic modulus is determined at small deformation, the rupture strength gives information about the gel behavior at large deformation. As both the modulus and rupture strength will be correlated to the number of crosslinks in the network, it might be expected that in general the rupture strength will increase with the apparent modulus. However, rupture strength is not necessarily related to its elastic modulus [104, 105]. A low-modulus M-rich alginate will typically show high rupture strength (high elasticity), whereas a high-modulus G-rich alginate will show lower rupture strength (brittle consistency) [75, 91]. For a G-rich alginate the shorter, stiffer polymer chains between junctions transmit more energy to the junction zones upon compression, thus facilitating rupture. A junction carrying the highest stress, normally the shortest one in length, fractures first and the energy released from the fractured junction will transfer to the neighboring chains, accelerating the break of other junctions [106].
Rupture strength, like the modulus, is very dependent on molecular weight, increasing with increasing MW, but does not become constant above a certain value [75].

The elastic modulus and rupture strength can be determined by compression measurements of gel cylinders to rupture. Young's modulus (E) is calculated from the slope of the initial zone of the resulting stress-strain curve, representing the behavior of an elastic spring obeying Hook's law [107]. E is defined as [108]:

$$F/A = E \cdot (\Delta l/l) \quad (1\text{-}1)$$

where F is the force needed to compress a material with a contact surface A a length Δl of the total length l.

Alginic acid gels

Under controlled conditions alginates form acid gels rather than participates at pH below the pK_a value of the uronic acid residues. The gels have been proposed to be stabilized by intermolecular hydrogen bonds. As for ionically crosslinked gels, the chemical composition and monomer sequence is important for the physical properties of acidic gels. However, in contrast to ionically cross-linked gels, alginic acid gels are equilibrium gels [41, 72]. With the exception of some pharmaceutical uses, the number of application for alginic acid gels is so far limited [48].

1.3.5 Physical and chemical characterization of alginate

One of the main advantages of using alginate for immobilization purposes is that they have been extensively studied over many decades. The characterization of alginates is feasible through numerous well-known methods. The most important methods used for characterization of alginate in the present study are presented shortly in the following.

Chemical composition and sequence

Alginate protons or carbons will absorb energy at various frequencies depending on their position in the alginate, and on the neighboring monomers. The four diad (nearest neighbor) frequencies, F_{GG}, F_{GM}, F_{MG} and F_{MM}, and the eight possible triad (next nearest neighbor) frequencies, F_{GGG}, F_{GGM}, F_{MGG}, F_{MGM}, F_{MMM}, F_{MMG}, F_{GMM} and F_{GMG} can thus be determined by high-resolution ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR) techniques [60-62]. An example of a typical ^{1}H-NMR spectrum is shown in Figure 7.

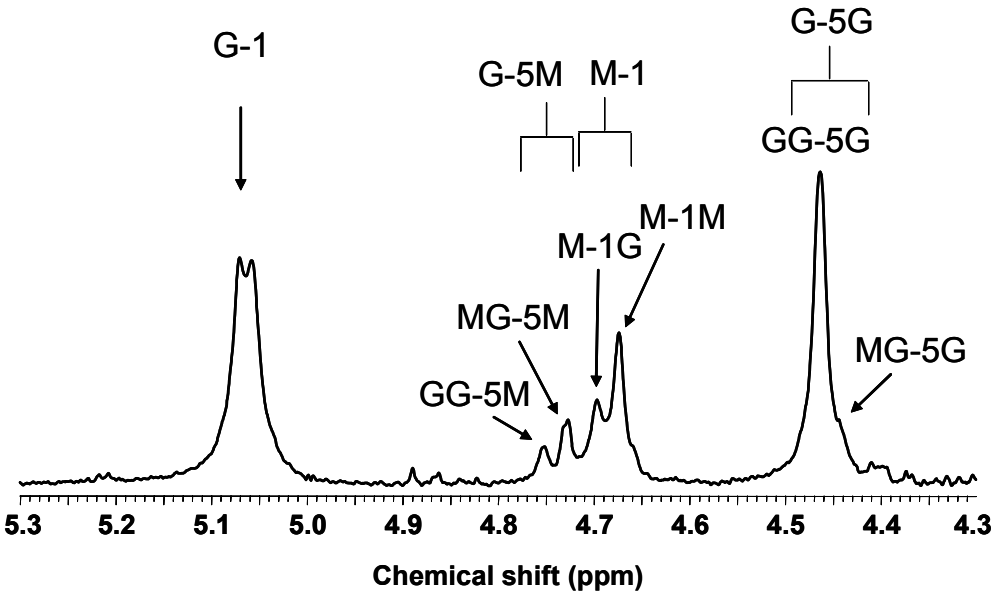

Figure 7. ^{1}H-NMR spectrum of a *Laminaria hyperborea* stipe alginate (F_G>0.7).

The molar fractions can be calculated from integration of characteristic signals and the following relations:

$$F_{GG} + F_{MG} = F_G \quad (1\text{-}2)$$

$$F_{MM} + F_{MG} = F_M \quad (1\text{-}3)$$

$$F_{GG} + F_{MM} + F_{GM} + F_{MG} = 1 \quad (1\text{-}4)$$

For long chains, where the contribution from the end-groups can be neglected, $F_{MG} = F_{GM}$. From this, the average length of G-blocks (larger than 1 unit), which has been shown to correlate well with gelling properties, can be calculated:

$$\overline{N}_{G>1} = \frac{F_G - F_{MGM}}{F_{GGM}} \quad (1\text{-}5)$$

Although NMR technology is a very powerful tool, it has been limited to giving information on the average length and distribution of blocks only. Recent development in the field of polysaccharide sequencing allows for a more detailed characterization. The true reconstruction of the block structure of alginates can be assessed by the use of specific alginate degrading enzymes (lyases) and subsequent analysis of the digest. After fractionation of the digest by size exclusion chromatography (SEC), the composition and molecular mass

of each fraction is analyzed by NMR and ESI-MS (low molecular weight fractions) or HPAEC-PAD/MALDI-TOF-MS for the high molecular mass fractions [109].

Molecular weight and molecular weight distribution

Commercial alginates are polydisperse with respect to molecular weight. Hence, the molecular weight is expressed as averages over the whole distribution of molecular weights either as the number average ($\overline{M}_n$) or the weight average ($\overline{M}_w$). In a polydisperse molecule population, the relation $\overline{M}_w > \overline{M}_n$ is always valid, while for a monodisperse molecule population we have $\overline{M}_w = \overline{M}_n$. For a randomly degraded polymer, $\overline{M}_w \approx 2\overline{M}_n$ [110]. The polydispersity index is given by the fraction $\overline{M}_w / \overline{M}_n$ gives information about the molecular weight distribution in an alginate sample. Values have been reported for alginates ranging from 1.4 to 6.0 [48], but are typically between 1.5 and 3.0 for commercial alginates.

$\overline{M}_n$ and $\overline{M}_w$ may be determined e.g. by size exclusion chromatography with multiple angle laser light scattering detection (SEC-MALLS). Measurements of the intrinsic viscosity can also give information about the molecular weight through the Mark-Houwink-Sakurada equation [111] (see below).

Intrinsic viscosity

The intrinsic viscosity [η] describes a polymer's ability to form viscous solutions in water under specified solvent and temperature conditions. It is determined by measuring the efflux time of a polymer solution at a series of concentrations in a capillary viscometer.

The intrinsic viscosity is directly related to the molecular weight of a polymer through the Mark-Houwink-Sakurada (MHS) equation [97]:

$$[\eta] = K \cdot \overline{M}_v^a \tag{1-6}$$

where K is a constant, $\overline{M}_v$ is the viscosity derived average molecular weight, and a is an empirical constant describing the conformation of the polymer and increases with increasing chain extension. For alginate, the exponent (a) is close to unity at an ionic strength of 0.1 (for example, 0.1 M NaCl). For $a = 1$, $\overline{M}_v = \overline{M}_w$. For $a < 1$, $\overline{M}_n < \overline{M}_v < \overline{M}_w$ [97]. By determining the intrinsic viscosity, the viscosity average molecular weight can be determined if K and a are accurately known for the sample. However, as a and K will by highly affected

by the stiffness of the chains, their values will greatly depend on the composition and sequential structures in alginate [111].

1.4 C-5 Epimerases

In brown algae as well as in the alginate producing bacteria, alginate is initially produced as polymannuronic acid (F_M = 1.0) [112]. The final step in alginate biosynthesis, however, involves a unique process: The extracellular C-5 epimerization of mannuronic acid to guluronic acid at the polymer level [113, 114]. This in-chain conversion of M to G residues is catalyzed by the mannuronan C-5 epimerases (Figure 8), without breaking the glycosidic linkages. Post-polymer epimerization of polysaccharides is a relatively rare event which in nature is, in addition to alginate biosynthesis, only known to take place in the biosynthesis of the glycosaminoglycans heparin/heparin sulphate [115] and dermatan sulphate [116] in the conversion of D-glucuronic acid into L-iduronic acid [117].

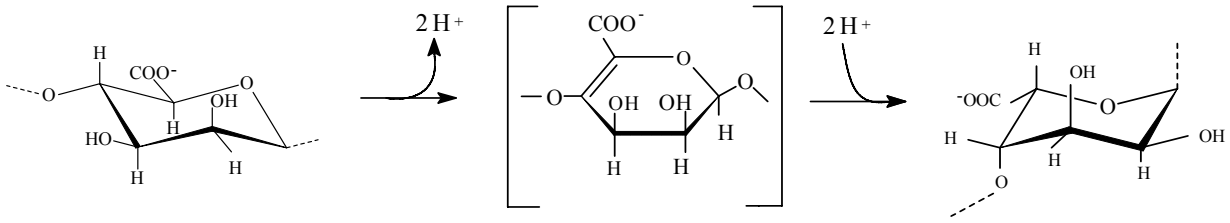

Figure 8. The proposed mechanism for mannuronan C-5-epimerase [118]. The result of the epimerization reaction is the conversion of mannuronic acid to guluronic acid with the subsequent change in ring conformation from $^4C_1$ (in ManA) to $^1C_4$ (in GulA), maintaining the large, bulky carboxyl group in the equatorial position.

The presence of C-5 epimerase was first demonstrated in *A. vinelandii* [119, 120] and has now also been reported in *Pseudomonas aeruginosa* [114] and *Pseudomonas syringae* [121] as well as in a wide range of brown algae [122]. In the early investigations, the enzyme was reported to introduce single G residues or homopolymeric blocks of G, depending on the concentration of calcium ions [120, 123]. Screening of an *A. vinelandii* gene library has, however, proved the existence of a family of seven mannuronan C-5-epimerase genes, the *algE* genes (algE1-algE7) which all have been sequenced and expressed in *Escherichia coli* [124-126]. The protein products of the *alg*E genes, the AlgE epimerases, vary in both size and activity. The activities of the enzymes are highly Ca^{2+}-dependent, but do not require co-

factors like NAD^+/NADH [120]. Based on the epimerases studied so far, it is clear that each enzyme catalyzes the production of alginates with distinct monomer distribution patterns. The AlgE4 epimerase catalyzes the formation of MG-blocks exclusively, while the remaining six enzymes introduce a mixture of continuous stretches of G residues and alternating sequences [127].

1.4.1 Structure and function of C-5 epimerases

The seven C-5 epimerases (AlgE1-AlgE7) all contain repeats of two types of structural units, designated A and R. Both modules are highly conserved at the amino acid sequence level among the members of the protein family. Due to the variability in the number of A and R repeats in the *algE* genes, the molecular weights of the AlgE epimerases vary in the range of 57.7-191 kDa [128] (Figure 9).

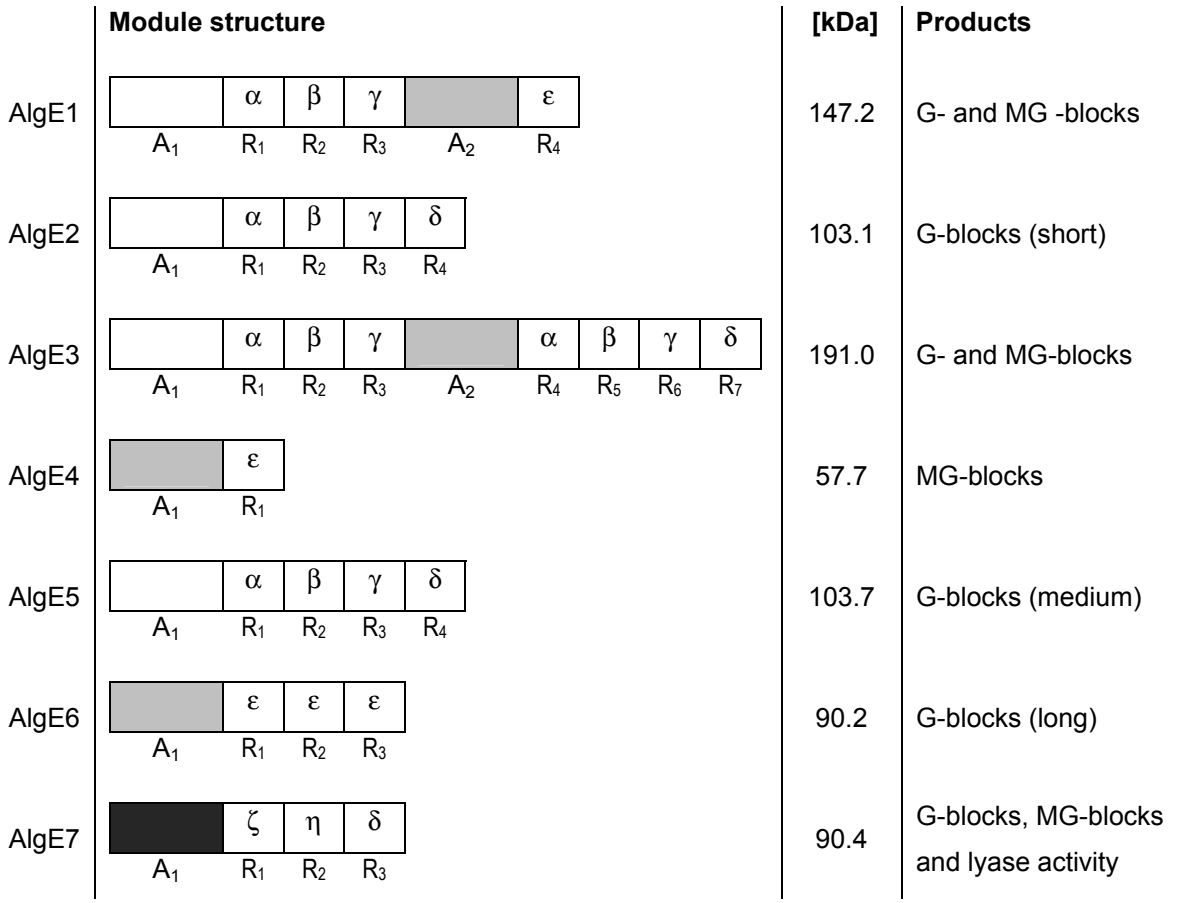

Figure 9. The modular structure, molecular mass and enzyme activities of the extracellular mannuronan C-5 epimerases AlgE1-AlgE7 from *A. vinelandii*. Each mannuronan C-5 epimerase is composed of different numbers of A modules (385 amino acids) and R modules (153 amino acids), designated A_1-A_2 and R_1-R_7. Closely related A-modules are indicated with identical shading. Closely related R-modules are indicated with identical Greek letter (α, β, γ, δ, ε, ζ, η) [42].

The A modules are present in 1-2 copies in each epimerase [127] and have shown to contain the substrate-binding site [129]. Their function is to catalyze the epimerization reaction, and hence determine the final distribution of M and G in the end product. The R modules are present in 1-7 copies [124, 125] and probably modulate the enzyme-substrate binding strength [129]. The A and R modules both contain Ca^{2+} binding motifs [130].

Since all alginates found in nature are produced by the C-5 conversion of homopolymeric mannuronan, the structural variability found in the polysaccharide originates from the different catalytic properties of the various epimerases [126]. While AlgE2 [131] and AlgE5 [109] make relatively short G-blocks, AlgE1 and AlgE6 make longer G-blocks [109]. In addition to G-blocks, AlgE1 and AlgE3 can introduce alternating structure in the polymer chain [109, 127, 132]. However, AlgE4 produces strictly alternating MG sequences [128, 133, 134]. AlgE5 and AlgE7 are combined epimerase and alginate lyases [109, 135]. As none of the AlgE epimerases are capable of epimerizing acetylated mannuronan residues found in bacterial alginates, acetylation may play a part in the epimerization pattern in the final product [63].

The biological function of all the epimerases is not yet fully understood. However, since *A. vinelandii* requires alginates of varying composition under different environmental conditions and different stages of its life cycle, it seems possible that the organism can control the expression of its epimerase genes as a response to certain environmental signals. It is believed that Ca^{2+} levels determine the relative activity of the enzymes as the epimerases have different activity at different calcium concentrations [63, 126, 136]
Until recently, there was limited knowledge about the epimerases in algae. However, epimerases from *Laminaria digitata* have now been isolated, with structural similarities to the bacterial C5-epimerases [137]. Depending on the season, age of the plant and tissue type, different epimerases are likely required in order to tailor the relative contents and distributions of G-, M- and MG-blocks in the polymer chains. It is reasonable to believe that this way of controlling the physical properties of alginates is widespread in nature [63, 126].

1.4.2 Large scale production of recombinant epimerases and bacterial alginate

As the mannuronan C-5 epimerases are not yet commercially available they have to be expressed recombinantly and purified. The production of epimerases is performed by

intracellular over-expression using different *E.coli* strains containing plasmids encoding the epimerase of interest. The enzymes are purified by sonication of the cells, centrifugation, filtration and application on an ion-exchange column before the fractions are analyzed for activity [132, 138]. The purified enzymes can then be used for modification of alginate *in vitro* (see below) and the outcome of the epimerization reaction determined by NMR spectroscopy of the alginate prior to and after epimerization [138].

The use of mannuronan as substrate for *in vitro* epimerization opens up the possibility for production of perfectly tailored alginates not found in nature. In addition, the availability of mannuronan is important for the extensive studies of the AlgE epimerases. It has been shown that *P. aeruginosa* synthesizes an epimerase encoded by *algG* and that epimerase defective mutants of AlgG produce polymannuronic acid [114]. In fact, this organism served as the first source of mannuronan. However, as *P. aeruginosa* is an opportunistic human pathogen [136], the recent availability of large quantities of mannuronan (F_M = 1.0) stems from the non-pathogenic epimerase negative $AlgG^-$ strain of *P. fluorescens* [139]. High molecular weight mannuronan is produced by fermentation using fructose as carbon source [138].

1.4.3 In vitro epimerization for biotechnological applications

Since the epimerases alone decide the composition of M and G in the final alginate, the epimerization is the key reaction affecting the alginate structure and thus the ion-binding and gel-forming properties of the alginates. For this reason, the epimerase gene family presents many possibilities for both basic and applied research. It has previously been shown that the enzymes can be used to modify alginates giving rise to polymers with enhanced gelling properties [92, 103]. Another interesting prospect is the use of *in vitro* C-5 epimerization to reduce the compositional heterogeneity in an alginate sample. As algal alginates may vary from plant to plant and from one tissue to another, commercial alginates are mixtures of subpopulations of molecules with great compositional and structural heterogeneity. Treatment of commercial alginates with C-5 epimerases may result in a more uniform distribution of composition and block structure thereby improving their physical properties [53, 63]. For example, as it has been shown that M-blocks are potent stimulators of the immune system [140], homogeneous alginates with few M-blocks produced by selected epimerases may be better suited for implantation in humans than the heterogeneous commercially available

alginates. However, as the available epimerases are produced in bacteria, the requirements for extensive purification of the *in vitro* epimerized alginate products will be high.
Due to their ability to form strong gels, alginates rich in G-blocks are particularly in demand. So far, these alginates have exclusively been obtained from *Laminaria hyperborea* stipes, which represent a limited source. As AlgE1 and AlgE6 have shown to epimerize polyM- and polyMG-alginate to a G-content of 90 and 97 % respectively [109], these epimerases may become valuable tools for tailoring G-block alginates in future products. Hence, recombinant epimerases have great potential as tools for producing unique alginates - for new applications, as well as in traditional industrial products.

Recently, with the use of a combination of various high-tech tools [134, 141-144], more knowledge has been gained on the mode of action and substrate specificity of the C-5 epimerases. In these studies, AlgE5 was for instance shown to prefer polyMG as substrate whereas AlgE1 and AlgE6 prefer mannuronan or substrates containing G-blocks [109]. The difference between AlgE1 and AlgE6 with regard to length and distribution of the G-blocks they introduce was also illustrated and explained by their different degree of processivity [109]. Knowledge about the epimerization patterns and substrate specificities opens up the possibilities for an even more specific tailoring of alginates. By this means, the length and distribution of blocks in the alginate can be controlled, further improving the performances in a number of applications [109].

Complete knowledge about the structure-function relationships of all the modules hold promise to the future creation of new epimerases with desired technical properties. This could be done by site-specific mutagenesis or by exchanging modules or parts of modules between different epimerase genes. The resulting epimerases could then be utilized to epimerize alginate *in vitro* or *in vivo* [136]. However, the obvious drawback of *in vivo* epimerization would be the lack of control of the end product compared to *in vitro* modifications [127].

The AlgE4 epimerase

Of all the C-5 epimerases isolated from *Azotobacter vinelandii*, AlgE4 is probably the most studied enzyme [58, 70, 92, 93, 128, 129, 133, 134, 143-146]. In particular, the very recent availability of a pure polyalternating sample, made by AlgE4 epimerization of mannuronan, can facilitate the study of the role of alternating structures in solution and in the gel state. It has been shown that the alginate product of AlgE4 epimerization possesses some rheological

qualities that can be advantageous in certain applications. Introducing alternating sequences in calcium alginate gels leads to a faster sol/gel transition and a considerably larger syneresis. The gel strength has also shown to increase slightly by epimerization [92]. The effects have been explained by the reduction of the stiffness of the polysaccharide chain due to the intrinsically higher flexibility of MG compared to GG and MM diads.

On a polymannuronic backbone, the AlgE4 introduces G entirely in an alternating matter, to a total G-content of 45-47%. A theoretical value of 50% conversion is not achieved as the epimerase does not convert the last few M residues on chain ends [143]. It has been found that in alginates already containing G-blocks, a few G residues can be introduced next to a pre-exciting G, thus contributing to an elongation of the G-blocks [92, 128].

During AlgE4 epimerization the fraction of GMG blocks increases linearly as a function of the total fraction of G residues and comparably much faster than that of MMG blocks. This suggests that the enzyme epimerizes alginate by a non-random mechanism either by sliding along the alginate chain (processive mechanism) or by recognizing a pre-existing G residue as a preferred substrate in its repeated attacks [128]. More recent studies using time-resolved NMR spectroscopy [134], mathematical modeling [133] and atomic force microscopy [144] suggested that AlgE4 is processive. Further, it has been demonstrated that on average 10 residues are epimerized for each enzyme-substrate encounter and that a minimum size of 6 residues is required for AlgE4 activity [143].

1.5 Alginate microcapsules

1.5.1 Formation of alginate microbeads/capsules

Of the many proposed technologies for encapsulation of cells, the use of alginate gel microbeads stands out so far as one of the most promising and versatile method [4, 24, 147]. One of the main reasons for this is the simplicity of the method where the immobilization procedure can be carried out in a single step process and does not require a deep expertise in related fields of polymer chemistry, membranes, hydrogels and networks. However, it should be kept in mind that oversimplification over the past years has been shown to result in irreproducible results and information noise [24] which in some cases could have been avoided with more knowledge on the biopolymer and capsule technology.

The entrapment is carried out by mixing an aqueous solution of sodium alginate (1-4 %) with cells, and dripping the mixture into a solution containing divalent cations such as Ca^{2+}, Sr^{2+} or Ba^{2+} (20–100 mM). The divalent ions will diffuse into the sodium alginate droplets, instantaneously forming hydrogel spheres entrapping the cells within a three-dimensional lattice of ionically crosslinked polymer (< 5 min for beads of 500 μm [148]). The size of the resulting capsules is determined by the size of the droplets. Simple devices can be used to prepare gel beads in sizes ranging from a few microns to >5 mm [4]. Evenly sized microbeads with diameters below 1 mm and down to < 200 μm can be formed by establishing an electrostatic potential of 1-20 kV between the needle feeding the alginate solution and the gelling bath using an electrostatic bead generator (Figure 10). The electrostatic potential pulls the droplets from the needle tip into a gelling bath. The potential can be static [149, 150]. In addition to needle size, adjusting the voltage magnitude easily controls the droplet size. The viscosity and flow rate of the alginate solution will also influence the capsule size [151].

Other encapsulation techniques include [152]:

Extrusion through a needle: Alginate solution is dripped from a syringe with appropriate diameter needle directly into a gelling bath. While this method does not require any instrumentation, the size and size distribution of the produced beads are difficult to control.

Coaxial air or liquid flow: The coaxial air jet system is a simple way of generating small beads (down to around 500 μm), although the size distribution will normally be larger as compared to an electrostatic system. A coaxial air stream is used to pull droplets from a needle tip into the gelling bath.

Vibrating jet breakage: A vibrating nozzle generates drops from a pressurized vessel.

Rotating jet breakage: Bead generation is achieved by cutting a solid jet of fluid coming out of a nozzle by means of a rotating cutting device. The fluid is cut into cylindrical segments that then form beads due to surface tension while falling into a gelling bath.

Emulsification methods: An emulsion of alginate in oil can be added to a $CaCl_2$ solution, leading to bead formation. However, the particle size cannot be easily controlled and the capsules tend to coagulate into large masses before hardening properly [153].

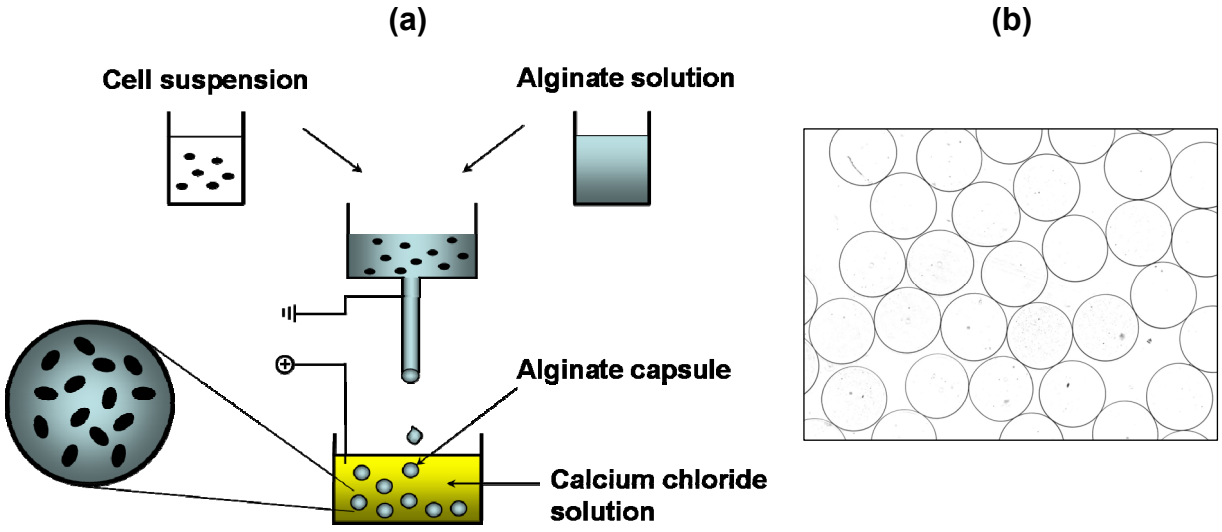

Figure 10. (a) Encapsulation of cells using an electrostatic capsule generator. Cells are mixed with an alginate solution and fed by a syringe pump (not shown). The voltage is coupled to the gelling bath and the alginate feeding-needle to earth. The distance between the needle and the gelling solution can be adjusted. (b) A representative capsule population of empty capsules made with the electrostatic capsule generator. The capsule diameter is approximately 0.5 mm.

As alginates are negatively charged polymers, they form strong complexes with polycations such as polysaccharides (e.g. chitosan) [154-156], polypeptides (e.g. poly(L-lysine) [34, 157], poly(L-ornithine) [158]) or synthetic polymers (e.g. poly(methylene-*co*-guanidine) [159, 160] and poly(ethylene imine) [161, 162]). As these complexes do not dissolve in the presence of non-gelling cations or calcium chelators they have been extensively used to stabilize the gel and reduce the porosity of Ca-alginate beads. Of all the polycations used for this purpose, poly(L-lysine) is the most extensively studied since it was first proposed in the original paper by Lim and Sun [34] describing the encapsulation of islets of Langerhans. In fact, it has been estimated that approximately 90% of all publications on cell encapsulations have involved the use of PLL with or without modifications of the original capsule [163].

The variations in the procedures of forming an alginate/polycation membrane are wide. The most common method is to use a three-step procedure (Figure 11) where alginate beads are first produced by gelling of alginate droplets in calcium chloride. After gelling, the second step is to transfer the beads into a solution of polycation, forming a capsule with a polycation/alginate complex membrane around the beads. Polycations like poly(L-lysine) are cytotoxic and immunogenic and their positive charge will also lead to attachment of cells to the surface. Hence, for transplantation purposes, it is necessary to apply an additional layer of alginate or other coatings to neutralize any excess of positive charges on the capsule surface [164, 165].

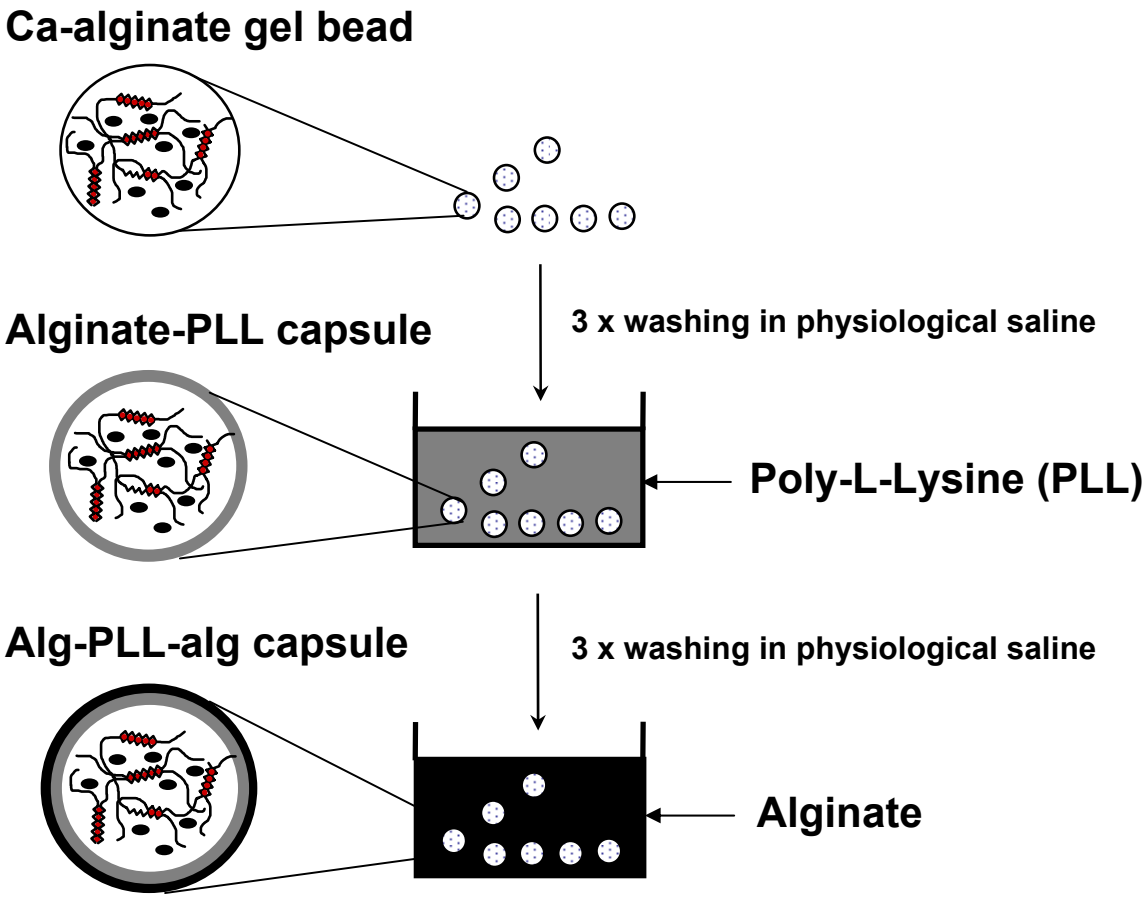

Figure 11. Schematic description of the treatment of alginate *beads* to form alginate-polycation *capsules*. The method is exemplified with poly(L-lysine) as polycation followed by coating with an outer alginate layer to shield the positive charges on the alginate-PLL capsule. In reality the polycation forms complexes with both the alginate core and coating [166], resulting in two layers (alginate core and alginate-polycation complex) rather than the three separate layers shown in the figure.

The original capsule designed for immobilization of islets [34] was based on a liquid core capsule. In this system, the crosslinking ions (Ca^{2+}) are exchanged with non-gelling Na^{+} ions

by sequestering agents like citrate, dissolving the gel core. This will give a polyanion-polycation complex that behaves as a semi-permeable membrane. Since that time, many groups have utilized this system and some authors claim improved cell viability in these capsules [167]. However, the obvious drawback is an increased osmotic pressure and less elastic strength inside the liquid capsule, decreasing the mechanical stability of the capsules [167, 168]. Many modifications of the original alginate microcapsule have emerged over the past years including the use of other polycations [160, 169], formation of multilayer capsules [170], addition of an outer alginate gel (mic/mac) [171], surface modification on the alginate/polylysine membrane [172] and exclusion of the polycation layer forming a simple Ca- or Ba-alginate gel bead [173-176].

1.5.2 Alginate microcapsules for treatment of Type 1 diabetes

The possible uses of alginate for immobilization of living cells in food industry, medicine and agriculture are numerous, ranging from ethanol and beer production by yeast cells [177, 178], to the production of pharmaceuticals such as antibiotics and steroids [179]. On an industrial scale, immobilized cells are much easier to handle than free cells [180]. In addition, higher viability, stability, productivity and proliferation have been reported for some immobilized cell systems [181-183]. However, of all the cells used in alginate immobilization systems, major interest has been devoted to insulin producing cells for the treatment of Type 1 diabetes [34, 184] which is the main focus in the following sections.

Insulin is a hormone essential for normal glucose homeostasis, secreted by the β-cells within cell clusters known as islets of Langerhans in the pancreas [185]. Diabetes is characterized by disordered metabolism and inappropriately high blood sugar (hyperglycemia) resulting from either low levels of insulin or from abnormal resistance to insulin's effects. The disease is one of the leading causes of morbidity and mortality worldwide [186] and is currently considered as an epidemic disease due to the fast increasing cases globally. In fact, the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [187].

Diabetes is classified into Type 1 (insulin-dependent) and Type 2 (insulin-independent) diabetes. Type 1 diabetes results from loss of the insulin-producing β-cells due to an autoimmune destruction of the cells, leading to absolute or impaired insulin deficiency and the life-long need of exogeneous insulin [188]. Although Type 1 diabetes to a certain degree

can be controlled with exogenous insulin, the current administration methods including insulin injections, pumps and inhaled insulin [189] do not prevent hypo- and hyperglycemic fluctuations. As a result, there is a risk of acute complications like hypoglycemia and ketoacidosis as well as secondary complications like cardiovascular diseases, stroke, kidney failure, blindness, neuropathy, ulcerations, amputations and premature death [185, 190]. This has prompted the search for a diabetes "cure" [27].

Islet transplantation

To date, whole pancreas transplantation or transplantation of islets of Langerhans from deceased human donors is the only treatment for Type 1 diabetes that can induce an insulin-independent normoglycemic state. Whole pancreas transplantation, however, requires major surgery and lifelong immunosuppression. Hence, most pancreas transplantations are done in diabetic patients with severe late-stage complications, undergoing simultaneous kidney transplantation. As a result, pancreas transplantation is not available to the vast majority of diabetic patients [27, 191].

Islet transplantation, on the other hand, is much less invasive where functional islets, isolated from the pancreas of cadaveric donors, are injected into the portal vein. Before the year 2000, few centers performing islet transplantation achieved high rates of sustainable insulin independence after this procedure among patients with Type 1 diabetes. In 2000, Shapiro et al. [192] reported up to a year of follow-up in seven patients treated with steroid-free immunosuppressive therapy combined with infusion of an adequate mass of freshly prepared islets from two or more pancreases. In all seven patients, insulin independence was achieved and the treatment became known as the Edmonton protocol [193]. In spite of the high success rate, the most recent update [193] shows that insulin independence is usually not sustainable, with only 10% of patients maintaining insulin independence 5 years after transplantation, and many patients still suffer from immunosuppressive-related events.

Hence, immunoisolation of insulin-producing cells still remains the most promising solution for a cure of Type 1 diabetes. The principle of microencapsulation of islets of Langerhans in alginate-polycation microcapsules has been widely studied by numerous research groups since it was first introduced by Lim and Sun in 1980 [34]. Allotransplantation has been shown to work routinely in diabetic rodents and in a limited number of large animals [194]. Recently, the first allotransplantations in humans with encapsulated pancreatic islets without immunosuppression were successfully performed [195]. Xenografts have also been shown to

reverse diabetes in rodents [196, 197] and non-human primates [198]. A Phase I/II clinical trial using neonatal pig islets encapsulated in alginate microcapsules without immunosuppression is currently ongoing [199, 200].

A more recent approach has been to omit the polycation layer using simple Ca- or Ba-alginate beads. This has given promising results in allotransplantation as well as in some xenotransplantation studies in animals [174, 175, 201-206].

Overcoming the limited islet supply

Donor islets are extremely scarce relative to potential demand, presenting an impending problem which must be overcome if immobilized islets are to become a widely applied treatment. Xenotransplantation, regeneration therapy (generation of pancreatic β-cells from pre-existing β-cells or from stem cells) and development of insulin-producing cell-lines are all methods that are being extensively studied to overcome this problem [27, 207, 208].

Although many species have been evaluated as potential islet donors, the pig has been considered the best suited, primarily due to the similarity of the porcine insulin to human insulin [185]. The major barriers for using xenografts are the risks of cross-species infections in addition to ethical aspects [27, 209, 210]. While the development of relatively non-toxic immunosuppressive or tolerance-inducing regimens will be required to justify clinical trials using pig organs, recent advances in the understanding of the biology of xenograft rejection and zoonotic infections, and the generation of α1,3-gal-deficient pigs have moved this approach closer to clinical application [211].

The use of insulin-producing cell lines may still not be as efficient as using intact islets because of the intricate intra-islet relationship between the cells that govern the overall glucose homeostasis in the body [27]. In addition, the control of growth remains a major obstacle. Despite the great effort and resources put into research in the field, expansion of islets or development of β-cells from stem cells is still in very early experimental phase and many questions and problems still remain unsolved.

In addition to immunoisolation, recent progress in the induction of donor specific tolerance [19, 27] and the development of pancreatic β-cells from human stem cells [207] gives hope for transplantation without immunosuppression. However, as diabetes is an autoimmune disease, the need for protection of the cells from the immune system may still be needed in order to avoid immune reaction to the insulin-producing β-cells.

1.6 Capsule properties

Although many animal studies have given promising results, the successful use of alginate/polycation capsules as carriers for insulin producing cells *in vivo* has been hampered by the capsules' bioincompatibility and mechanical instability. The major hurdles have been their sensitivity to calcium sequestrants and non-gelling ions such as sodium, the osmotic swelling under physiological conditions, the high porosity and wide pore size distribution, and the presence of uncoated polycations which are the major contributors to fibrotic reaction towards the capsule. All in all, these shortcomings make controlled insulin release difficult.
To overcome some of these problems it is crucial to realize some of the limitations due to the nature of the alginate molecule itself as a biodegradable polydisperse material and to the nature of the gel as a reversible ionic network. To date, it has been difficult to find an ideal alginate that fulfils the multitude of requirements as an immobilization matrix for islet cells. However, alginate gel beads should ideally be characterized by [4, 180, 212]:
1) High mechanical and chemical stability; 2) Controllable swelling properties; 3) Low content of toxic, pyrogenic and immunogenic contaminants and 4) Defined pore size and a narrow pore size distribution.

1.6.1 Stability and swelling

Ca-alginate beads tend to swell and dissolve at physiological conditions. The swelling is caused by the gel network sensitivity towards chelating compounds such as phosphate, citrate and lactate, or non-gelling cations such as sodium and magnesium. Swelling and dissolution of the gel network represents a potential serious problem in immunoisolation systems. First of all, swelling leads to increased porosity and the loss of control of pore size. Secondly, disruption of the gel results in exposure of the transplanted cells.

Ionically linked alginate gels can be looked upon as osmotic swelling systems where the gel surface functions as a semi-permeable membrane through which the polymer molecules cannot diffuse out. The swelling force resulting from the presence of the fixed charges on alginate may be identified with the osmotic pressure across the semi-permeable membrane in a typical Donnan equilibrium [97]. Due to the attracting power of the fixed charges in the alginate network and requirements of electroneutrality, the concentration of *mobile* ions will always be greater in the gel than outside. Consequently, the osmotic pressure of the solution inside will exceed that of the external solution.

Generally, the osmotic contribution to swelling of an ionic network is composed of two terms; The polymer-solvent mixing term ($\Delta\mu_{mix}$) and the chemical potential difference of water due to uneven distribution of ions between the inside and the outside of the gel, ($\Delta\mu_{ion}$). In an alginate gel the ionic term is dominating and the contribution comes from the non-cooperatively bound counter ions. The swelling is opposed by the physical crosslinks, leading to an elastic network retraction force [97, 108, 213, 214] (Figure 12).

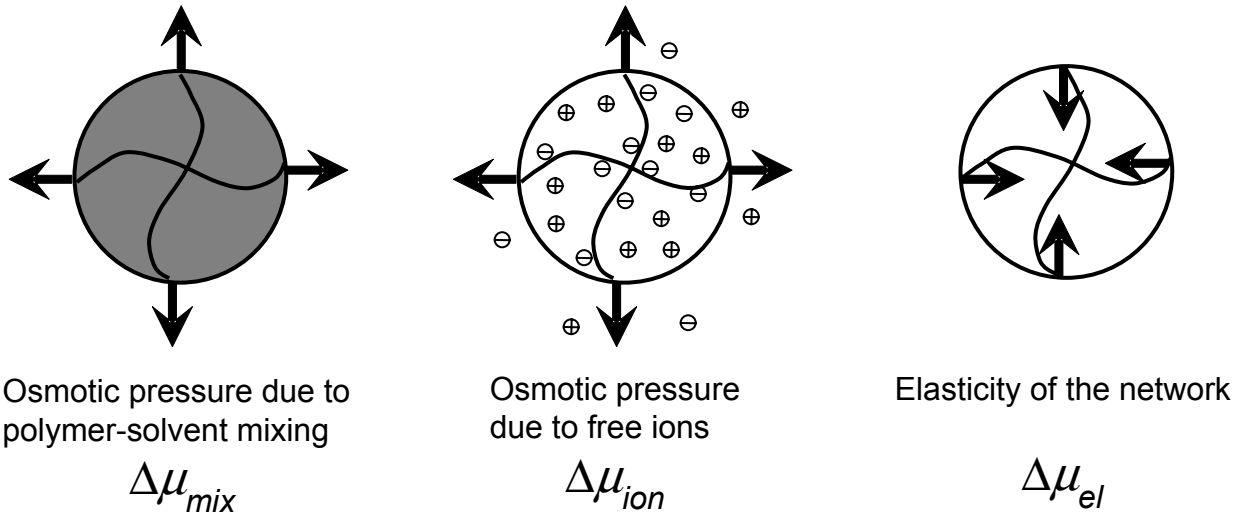

Figure 12. Factors influencing the swelling of a gel network.

At equilibrium the volume of the gel is stabilized by the elastic forces of the gel network:

$$\Delta\mu_{mix} + \Delta\mu_{ion} + \Delta\mu_{ela} = 0 \qquad (1\text{-}7)$$

The polymer-solvent mixing term will mostly depend on the interactions between the polymer and the solvent (hydration of the polymer) [214], whereas the elastic term is highly dependent on the number and strength of the crosslinks in the polymer network. The ionic term will depend on the salt concentration outside the gel, the valence of the counter ion and the effective charge density of the polymer [213].

The swelling of Ca-alginate gels under physiological conditions depends on the alginate composition and polymer concentration. Calcium ions will be exchanged with non-gelling sodium ions, dissolving the junctions in the network, consequently reducing the elastic term. At the same time the number of dissociable counter ions increases as - given the different valence of Ca^{2+} and Na^{+} - each calcium ion will be replaced with two sodium ions (enhancing

the ionic term). Both these effects contribute to swelling of the hydrogel network. Swelling can be reduced by using alginate with a high content of long guluronic acid blocks [91, 168] or by replacing calcium ions with stronger binding ions such as Sr^{2+} or Ba^{2+} [39, 168, 215].

A polyanion-polycation complex membrane will stabilize against swelling both by increasing the elastic forces and by partly decharging the polymer network [216]. For small capsules where the surface to volume ratio is high this decharging can be so effective that the capsules collapse [151]. Increasing concentration and exposure time to poly(L-lysine) and decreasing molecular weight of the polypeptide increases the stability of capsules. In addition, poly(L-lysine) binds to a higher extent to high-M alginate than to high-G alginate, and higher concentration of polymer at the surface will enhance the binding of polycations to alginate beads [6, 157, 168, 216, 217].

Another approach for stabilizing alginate gels is by covalent crosslinking. Various techniques have been applied, including direct crosslinking of the carboxyl groups [218] or covalent grafting of alginate with synthetic polymers [219]. A combination of covalent and ionic crosslinking has also been proposed [220] as well as covalent crosslinking of the polylysine to the alginate [221]. A drawback using some of these methods has been the lack of selectivity characterizing chemical modifications that generally occur both on guluronic acid (G) and mannuronic acid (M) residues, hampering the ability of the modified alginate to form instantaneous calcium gels. A new approach has recently shown to be able to overcome this limit by using a combination of mannuronan C5-epimerases and chemical modification [222].

1.6.2 Biocompatibility

The two major components of an immunoisolation device are the transplanted cells and the immobilization material. The interconnection between the host responses to the capsule material and the transplanted cells determines the biocompatibility of the device. The inflammatory response towards the capsule material itself can impact the immune response towards transplanted cells and vice versa [10, 166]. Moreover, it is important to realize that the concept of a biocompatible material is not synonymous with the material being inert, as implantation of any material to the body evokes a certain immune reaction [24].
The biocompatibility of the capsule material will depend on many factors, such as chemical composition, surface charge, porosity, surface roughness, implant site and shape [10].

Leakage of capsule material out of the device may also affect its biocompatibility [140]. A central consequence of the inflammatory response to the capsule material is the activation of macrophages resulting in the release of cytokines, growth factors, proteolytic enzymes, and reactive oxygen and nitrogen intermediates. The inflammatory response eventually leads to formation of a fibrotic tissue on the capsule surface composed largely of collagen, macrophages, fibroblasts and few, if any, capillaries [10, 22, 223, 224]. Some speculate that overgrowth can increase vascularization and thereby increase the *in vivo* function. However, the general view is that fibrosis should be minimized as it has been demonstrated that islets in capsules without overgrowth provide better functionality [212, 225]. A thick fibrous tissue surrounding the implanted microcapsule prevents proper nutrient and oxygen diffusion to the encapsulated cells, quickly leading to massive loss of cell viability and functionality [10, 22], which unfortunately has been a repeating trend in animal studies with encapsulated islets [226, 227].

Biocompatibility of alginate

Although alginates fulfil the requirements as additives in food and pharmaceuticals, as a natural material they contain various amount of other biological compounds such as endotoxins (LPS), proteins, complex polysaccharides and polyphenols which might be harmful to sensitive cells, or have impact on the biocompatibility of the capsules [228, 229]. In the recent years, there has been much focus on the necessity of purification of the alginate material with regard to biocompatibility concerns [6, 39, 229] as some authors have claimed that alginate purity is the main factor determining the biocompatibility [40, 230]. Several procedures for purifying alginates are found in the literature [228, 229, 231], and ultrapure grades of alginates approved for implantation purposes are available commercially [232]. However, evidence of overgrowth on capsules of highly purified alginate [233] suggests that there probably are additional factors influencing biocompatibility. Presence of residual contaminants in ultrapure commercial samples has very recently been revealed [229, 231], thus illustrating the difficulties connected with evaluations of possible bioincompatibility causes.

It is controversially under discussion whether alginate with low or high content of G is advantageous, or if the molar mass has an impact on alginate biocompatibility. Alginates with a high content of M have shown to stimulate human monocytes to produce TNF-α, IL-1 and IL-6 [140]. The cytokine-inducing effect [234] and fibrosis [235] has shown to increase with increasing molecular weight. Others report that the biocompatibility is independent of

mannuronic/guluronic acid ratio and molar mass of the alginate [236]. Due to the low cytokine response, alginates with a guluronic acid content higher than 50% has been recommended for transplantation purposes [237]. In a diabetic patient transplanted with encapsulated islets, antibodies against alginate were found 1-2 weeks after transplantation. The antibody response was, however, found to be transient [237].

As calcium does not form crosslinks with M-blocks, low MW high-M material has been predicted to leak out of the capsules [86] and evoke an immune response [140]. This has been, at least partly, linked to the β(1→4) glycosidic linkages between the mannuronic residues since also other homopolymeric diequatorial polyuronates, like D-glucuronic acid (C6-oxidized cellulose), also exhibit this feature [4].

Biocompatibility of alginate/polycation microcapsules

Fibrosis to encapsulated islets has in general been attributed to exposure of donor cell through capsule rupture or protruding cells or to cell material leaking out of the capsule [6, 166, 238]. Nevertheless, empty capsules are routinely found with fibrotic overgrowth [164, 165, 224, 239, 240].

The fibrotic overgrowth on empty alginate/polylysine capsules has in numerous studies been connected to the poly(L-lysine) [164, 216, 233, 236, 241, 242]. Alginate/polylysine capsules have been shown to activate complement [243] and macrophages [240, 244] and the polypeptide has been reported to be toxic to numerous cell lines [241]. Since poly(L-lysine) is a positively charged molecule it also favors the adherence of cells which tend to have a netto negative surface charge [212]. Reducing the amount of poly(L-lysine) improves the biocompatibility of the capsules [241]. The importance of alginate composition on biocompatibility of alginate/polylysine capsules is controversial. Some investigators have reported increased fibrosis with high-M alginates [164, 240, 245], whereas others report that high-G alginates induce fibrosis to a larger extent [242, 246, 247]. It has been suggested that these differences may be due to differences in alginate purity [248].

Although some believe that poly(L-lysine) polycation has a low probability of success as a result of its poor biocompatibility [237], others have obtained promising *in vivo* results replacing PLL with poly(L-ornithine) [195] or poly(methylene-*co*-guanidine) hydrochloride [38]. However, this topic is still debatable as others have shown that alginate/polyornithine capsules induce fibrosis to a higher degree compared to alginate/polylysine capsules [227, 249].

The importance of coating the polycation to shield excess negative charges has also been acknowledged [164]. Low molecular weight alginate seems to be favorable in binding to the polycation [157] and enzymatically modified alginate using the AlgE4 epimerase has been shown to increase the biocompatibility [216]. A more efficient coating has been promoted using polyacrylic acid [165]. In connection with the latter study and other studies [40, 250], the surface roughness of capsules was thought to be an important factor for biocompatibility as rough surfaces present suitable targets for the attachment and growth of anchor-dependent cells like monocytes, macrophages and fibroblasts. Surface charge has likewise been suggested to play a role in biocompatibility [251, 252]. The influence of implantation site [166, 202, 253] and host species [242, 254] on the biocompatibility of alginate capsules has also been reported.

All in all, although intensely studied for more than a decade, no general agreement exists concerning alginate's biocompatibility in the scientific community yet.

1.6.3 Permeability

Permeability is a major factor determining the functionality of alginate capsules, particularly for cell transplantation applications. Maintenance of cell viability and function requires both protection against the host immune system and sufficient supply of nutrients and oxygen. At the same time, the necessity of almost unrestricted diffusion of catabolic products like insulin out of the capsule should be met. Preventing immune cells from entering the capsule membrane is a relatively easy challenge to meet. However, these cells can secrete low molecular weight molecules that may be deleterious to cells. The challenges of keeping these outside the capsule membrane are more serious, since many of these components are smaller or similar in size to essential cell nutrients or products [21]. Hence, it is a difficult, if not impossible task to design a capsule meeting all these permeability requirements. Nevertheless, certain molecules of the immune system, like cytokines, immunoglobulins and elements of the complement system should ideally be completely, or at least partly, prevented from entering the capsule membrane to avoid or minimize the host reaction to the implant.

The permeability of capsules is determined by both the **molecular weight cut-off** (size-based exclusion) and the rate of **diffusion** of the molecules.

The driving force in a **diffusion** process is the chemical potential gradient (or concentration gradient) of molecules in the solution. The movement results from random motion of particles, causing them to move from an area of high concentration to an area of low concentration [110]. In hydrogels, the diffusion of solutes is determined by many factors [255]. The first is the obstruction caused by the presence of impenetrable slowly moving polymer chains that increase the path length for diffusion. The second process is the hydrodynamic drag on the moving solute at the polymer-solvent interface. The third is the different extend of heterogeneity of the membrane material with fluctuation of diffusion properties across the membrane material. Finally, residual charges, presence of counter ions, hydrogen bonds, polar and hydrophobic interactions of the membrane material will affect the diffusion of solute, especially essential in diffusion of biological molecules.

Diffusion of small molecules seems to be very little affected by the alginate gel matrix. The diffusion of glucose and ethanol has been reported to be as high as 90% of the diffusion rate in water [256, 257]. The diffusion rate, however, decreases with increasing cell load [258]. For larger molecules such as proteins, diffusional resistance occurs, although even large proteins with molecular weights > $3 \cdot 10^5$ Da will leak out of the gel beads with a rate depending on their molecular size, shape and pH of the solution [91, 257, 259].

Since the alginate gel matrix is negatively charged, electrostatic forces between the gel network and ionic substrates must also be considered. Hence, in addition to size, the diffusion of proteins over the alginate membrane will also depend on the total net charge of the protein molecule [260]. The negative charges of the alginate matrix are also responsible for the observed difference between protein ingress and egress. At neutral pH, most proteins are negatively charged and will therefore not easily diffuse into the matrix. When such proteins are immobilized in a gel, the repulsive forces result in an efflux that is greater than their free molecular diffusion rate [147].

In addition to diffusion characteristics, the **molecular weight cut off** (MWCO), pore-size and pore size distribution may be essential parameters in an immobilization system. Electron microscopy and inverse size exclusion chromatography has been used to study pore size and molecular weight cut-off of alginate gels and capsules [54, 260-263]. It has been estimated that the size distribution of pores in an alginate gel from *L.hyp* stipe (high-G alginate) is wide, ranging from 5-150 nm [50].

The MWCO and the rate of diffusion of solute are obviously connected and of equal importance for quantification of the capsule permeability characteristics. Nevertheless, the

vast majority of studies only characterize the MWCO to quantify the diffusion properties. However, this does not adequately predict the diffusion properties of a certain solute in hydrogels where there are large variations in membrane chemical composition, density, interactions with solutes as well as non-uniformity in pore sizes [264].

It is important to emphasize that MWCO is determined by size and shape of the considered molecule rather than its molar mass. Hence, the molecular weight of a protein does not necessarily indicate whether it will be able to penetrate the capsule membrane or not. Rather, the radius of gyration (R_G) gives a more direct measurement of the spatial requirements of a protein in solution [110] (Figure 13).

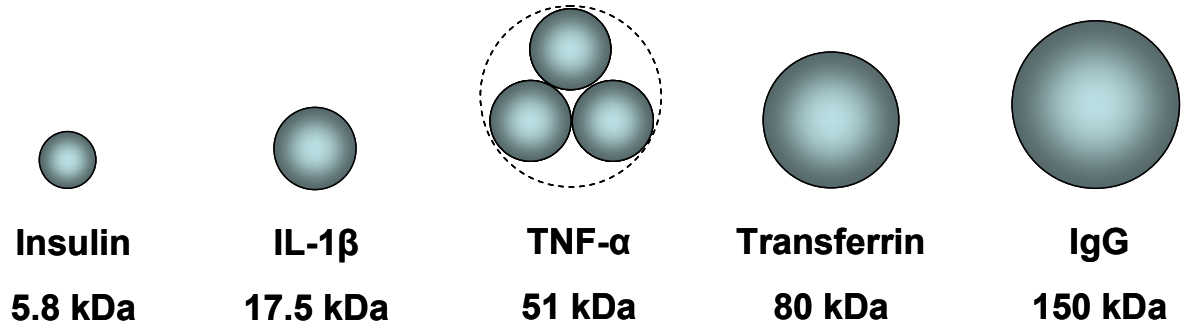

Figure 13. Size comparison of some globular proteins. The radius of gyration (R_G) of a globular protein is proportional to the cubic root of the molecular weight [110]: $R_G \propto M_w^{1/3}$.

Both the alginate concentration and composition will all affect the permeability of the gel. Increasing polymer concentration decreases the permeability [256, 260]. The highest diffusion rates of proteins, indicating the most open pore structure, are found in beads made from high G-alginates [91], in particular those containing long G-blocks [82, 94]. This has been explained by the stiff and static network of these gels compared to the more dynamic and entangled network structure of the low-G gels with their relative long elastic segments. Lastly, the Ca^{2+} concentration in the gelling solution will further influence the porosity of the Ca-alginate gel as high concentrations of calcium will result in lateral association of junctions. This will leave more space in between the junction zones and thus make a more porous gel [155].

In many gel systems syneresis is considered a problem. However, for immunoisolation purposes, syneresis might be an advantage by creating gels with reduced porosity. Since the alginate concentration is inversely proportional to the volume of the beads, the linear dimension of the pores in the gel-network is expected to be roughly proportional to the

inverse cube root of the concentration of alginate provided that the alginate molecules are uniformly distributed throughout the gel-bead. Finally, a concentration gradient of polymer will have an impact on permeability, where a higher concentration at the surface of the beads (see section 1.6.5) may reduce the porosity at the surface [260].

Ca-alginate gel beads have been shown to be permeable to immunoglobulin G (IgG, 150 kDa) [151, 265] and complement [266]. The high porosity of the alginate network has promoted the development of coating techniques, and several of the procedures for stabilizing the alginate gels mentioned in section 1.6.1 will also have some influence on the porosity of the gel. Formation of polyanion-polycation membranes with polypeptides [151, 217, 263, 265, 267] or chitosan [268] has been used to prevent diffusion of antibodies through the capsule membrane, and the capsules can be made impermeable to TNF (51 kDa) using higher concentrations or exposure times in poly(L-lysine) [265, 268]. Molecules as small as insulin can be retained after using poly(D-lysine) as polycation [265].

1.6.4 Capsule strength

An alginate microcapsule to be used for transplantation should have sufficient mechanical resistance to withstand the forces, including compression and shear stress, exerted on the capsule *in vivo*. Increasing the strength and resistance of capsules will increase the durability of the transplant and prevent the exposure of encapsulated cells. Although it is a rather simple task to increase the mechanical strength of alginate gels, e.g. by increasing the guluronic acid content or replacing Ca^{2+} with stronger binding ions (see section 1.3.4), this will often represent drawbacks on other capsule parameters. As an example, Ca-alginate beads with the highest mechanical strength are generally made from alginate with a content of guluronic acid higher than 70% and an average G-block length of > 15 [91]. Increasing the G-content will, however, simultaneously lead to higher permeability [91] and a more brittle consistency [269].

The required mechanical stability for capsules containing therapeutic cells depends on the proposed site of implantation. Lacik et al reported a few to tens of grams per capsule for intraperitoneal application [24]. Others have estimated a threshold of approximately 50 g per capsule for their general safe handling [163]. The cell load will probably influence the mechanical properties in a negative fashion [270] and the same will apply for capsules with a dissolved core [167, 168].

1.6.5 Polymer distribution in gel beads

Gelling kinetics can have a dramatic effect on functional properties of alginate gels. It has previously been demonstrated that calcium alginate gels prepared by dialysis often exhibit a concentration inhomogeneity characterized by a high concentration of polymer at the calcium-alginate interface and decreasing concentration towards the center of the gel (Figure 14) [148, 271, 272]. This may be a preferred structure in microcapsules due to lower porosity at the surface [260] and higher resistance against swelling [168]. The inhomogeneity has been explained as a result of a rapid and virtually irreversible site binding of divalent metal ions as they diffuse into an alginate solution, producing an inwardly moving gelling zone. The steepness of the gradient has been assumed to be governed by the relative diffusion rate between calcium ions and polymer molecules. The diffusion of alginate towards the gelling zone, leading to a depletion of alginate in the center (Figure 6 and 14), is faster than the self-diffusion of the macromolecules due to the coupled diffusion of the sodium ions.

The polymer gradient of the gel can thus be controlled by a careful selection of the gelling conditions. In general, low molecular weight alginate, low concentration of gelling ions and absence of non-gelling ions give the highest inhomogeneity [271].

Inhomogeneity can be suppressed by letting gel formation take place in the presence of non-gelling salt such as sodium chloride, a salt that is frequently used as osmolyte when handling animal cells. The presence of sodium ions influences the gelling process by disrupting the diffusion of sodium alginate towards the gelling zone [271]. The result will be a capsule with a homogenous distribution of alginate molecules in the gel core (Figure 14). Thus by replacing sodium chloride as osmolyte with a neutral compound such as mannitol or glycerol inhomogeneous gels are obtained.

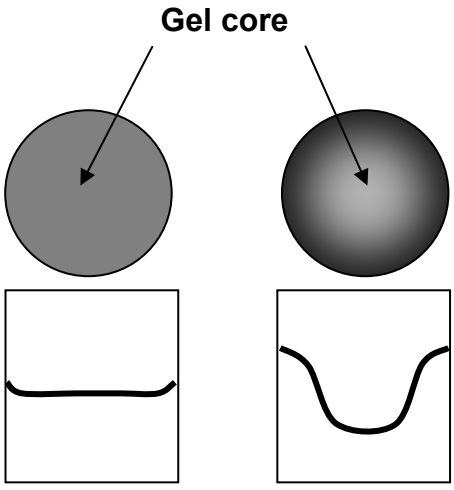

Figure 14. Schematic illustration of polymer gradients in alginate gels: Homogeneous (left) and inhomogeneous (right) gel beads. Equatorial sections of the capsules are shown.

1.6.6 Size

Bead size is an additional important parameter of alginate gel beads and capsules in the immunoisolation of islets. The use of smaller capsules increases the surface to volume ratio, leading to improved insulin kinetics [273] and enhanced oxygen [21] and nutrition supply [263]. As the volume of a sphere has a cubic relation with its diameter, size reduction has a dramatic effect on transplantation volume. This also raises potential for new implantation sites [150, 263]

However, the appropriate size will often be a compromise. Due to their larger surface to volume ratio, smaller capsules are more prone to osmotic stress and subsequent swelling than larger capsules [151, 212]. When very small beads are coated with polycation, the surface tends to collapse due to decharging of the network [151]. The bead itself must also be large enough to cover the islets which can range in size from 50-400 μm. From a practical point of view, larger beads may also be easier to handle. In some studies, increasing bead size has shown to increase the biocompatibility [204, 205], whereas other studies have shown the opposite trend [274].

2 AIMS

The overall scope of these studies has been to enhance the functional properties of alginate gels intended for use as immunoisolating material for living cells, with focus on islets of Langerhans.

The specific aims were to:

1) Study alginate capsules using confocal laser scanning microscopy in order to elucidate how various gelling ions, encapsulation procedures and gelling kinetics affect the macroscopic structure of capsules.

2) Increase the stability and reduce permeability of the alginate gel, with the aim to reduce or omit the polycation layer, thereby increasing the biocompatibility and functionality of the microcapsules. The goal was to evaluate two different approaches:

- **i.** Exchange Ca^{2+} as gelling ion with ions of higher affinity to alginate
- **ii.** Enzymatic modification of alginate

3) Illuminate the role of alternating sequences in alginate gel formation in order to get a better understanding of the manipulations that can be done to enhance capsule properties by enzymatic modification.

3 SUMMARY OF PAPERS

Paper 1

In Paper 1 we evaluated confocal laser scanning microscopy (CLSM) as a tool for investigating the distribution of polymers in various capsules and beads using fluorescent probes covalently linked to alginate and poly(L-lysine) (PLL). CLSM was shown to provide a novel and simple method for studying the distribution of fluorescent-labeled molecules in an optical slice of capsules.

CLSM studies of Ca-alginate gel beads revealed a more inhomogeneous distribution of alginate in beads formed in the absence of non-gelling ions. In the formation of alginate/PLL capsules the polymer gradient was destabilized due to the presence of non-gelling ions in the washing step and in the PLL solution. The inhomogeneous structure of Ca-alginate gels was preserved upon removing non-gelling ions (Na^+) from these solutions. By exchanging Ca^{2+} with lower concentrations of Ba^{2+}, extremely inhomogeneous gel beads were formed that preserved their structure during the washing and exposure to PLL in saline.

PLL was shown to bind at the very surface of the alginate core, forming a shell-like membrane. The thickness of the PLL-layer increased by approximately 100% during two weeks of storage, but no further increase was seen after two years of storage. The coating alginate was shown to overlap the PLL layer. CLSM imaging did however not give information about differences in binding among coating alginates of different composition.

Paper 2

In this study, we wanted to characterize the effect of different alginates and crosslinking ions on important microcapsule properties. Great effects were seen on the selection of type of gelling ions on both the swelling properties and gel strength of high-G alginate and high-M alginate. Both the dimensional stability and gel strength increased for high-G alginate gels when exchanging the traditional Ca^{2+} ions with Ba^{2+}. The use of Ba^{2+} decreased the size of alginate beads and reduced the permeability to immunoglobulin G. Strontium gave gels with characteristics lying between calcium and barium. Interestingly, high-M alginate showed an opposite

behavior in combination with barium and strontium as these beads were larger than beads of calcium-alginate and tended to swell more, also resulting in increased permeability. The recent availability of pure polyM and polyMG samples made ion binding studies on these samples possible. Circular dichroism studies revealed that different block structures in the alginate bind the ions to a different extent. More specifically, Ca^{2+} was found to bind to G- and MG-blocks, Ba^{2+} to G- and M-blocks, and Sr^{2+} to G-blocks solely.

Paper 3

In Paper 3 we investigated how capsule properties such as stability and permeability were affected by increasing the number of alternating blocks in the alginate chains using the AlgE4 epimerase. Inhomogeneous calcium-alginate gel beads were made with original and AlgE4-epimerized alginates of different origin. The epimerized alginates formed initially smaller alginate gels that showed increased resistance to osmotic swelling compared with the original unmodified alginate samples. The permeability, measured as diffusion of immunoglobulin G (IgG) into Ca/Ba-alginate gel beads, was reduced by epimerization and further reduced by addition of poly(L-lysine) (PLL). The osmotic stability of alginate/poly(D-lysine)/alginate capsules was enhanced by the use of epimerized alginate. Indeed, stable capsules with low permeability to tumor necrosis factor (TNF) could be made with low PDL exposures. Finally, alginate with an alternating structure was found to interact more strongly with the alginate-PLL capsule than did alginate with a high content of M blocks or G blocks or than an alginate consisting mainly of M.

Paper 4

The striking effects observed in Paper 3, upon introducing alternating blocks into the alginate backbone of seaweed alginate, lead us to investigate the specific role of the alternating sequences in the Ca-alginate gel network. To date, the general assumption has been that alternating sequences do not contribute to gelling *per se*, but rather contribute to the gels overall functionality by reducing the stiffness of the polysaccharide chain. In Paper 4 we show that G blocks in fact are not the only sequences involved in junction formation, and propose the direct involvement of long

alternating sequences in the gel network. The new hypothesis was based on experimental data from alginates enzymatically enriched in alternating sequences. More specifically, results from ^{1}H NMR and circular dichroism studies indicated for the first time the formation of mixed junctions between G and MG blocks. This was supported by the analysis of the Young's modulus of hydrogels from natural and epimerized samples obtained at low calcium concentrations. Furthermore, the "zipping" of long alternating sequences in secondary MG/MG junctions was suggested to account for the shrinking (syneresis) of alginate gels in view of its dependence on the length of the MG blocks. As a consequence, a partial network collapse, macroscopically revealed by a decrease in the Young's modulus, occurred as the calcium concentration in the gel was increased.

The effect of the presence of secondary MG/MG junctions on the viscoelastic properties of the gels was investigated by measuring their creep compliance, which suggested an ion mediated mechanism preventing slipping within the network of the gel.

Paper 5

Here we report the application of a set of mannuronan C-5 epimerases for the preparation of novel alginates with highly improved functional properties essential for numerous applications as gel matrices. The final polymers are designed to optimize calcium binding and to enhance flexibility in the interjunction regions.

The structural modification of alginate is based on a two-step process starting with a homopolymeric mannuronan which was epimerized with the C-5 epimerase AlgE4, converting the entire polysaccharide backbone into strictly polyalternating sequences. The resulting polyMG alginate was further processed by a second C-5 epimerase, AlgE1, converting M residues interspersed by G or by condensing G blocks. Thus the most important structural features distinguishing these engineered polymers from seaweed or bacterial alginates are the *absence* of M blocks and the high content of long G blocks. The total guluronic acid content in the product was controlled by epimerization time, ending up with various samples of different composition.

Ca-alginate gels of the engineered alginates were found to be more elastic and compact, less permeable, and extremely stable under physiological conditions, offering significant advantages over native alginates. Moreover, the properties were

highly dependent on the total G content. The observed effects were believed to be a result of the combination of the very strong binding of Ca^{2+} to both long GG and MG blocks. This study hence showed that by controlling alginate nanostructure its macroscopic properties can be highly controlled.

Paper 6

In Paper 6 we further investigated the mechanical properties of enzymatically modified alginates. In particular, differences in the mechanical properties of alginates epimerized with AlgE4, introducing alternating structure, and two G-block forming epimerases (AlgE1 and AlgE6) were studied. Both well-characterized algal polymers and pure mannuronan were epimerized with the bacterial enzymes.

Composition and sequential structure of controls and epimerized alginates were analyzed by ^{1}H-NMR spectroscopy. Mechanical properties of Ca-alginate gels were further examined by uniaxial compression measurements giving Young's modulus, syneresis, rupture strength and elasticity of the gels. Both Ca-saturated and Ca-limited gels were examined.

The G-block forming epimerases were found to introduce long homologous G-blocks, indicating a processive mode of action. Differences in epimerization pattern depending on the alginate backbone were also shown. The mechanical properties of Ca-alginate gels were highly dependent on the length of both GG and MG sequences.

As expected, Young's modulus (E) increased with increasing amount of guluronic acid in Ca-saturated samples. Epimerization of seaweed alginates led in all cases to increased E compared to control, samples epimerized with AlgE4 resulting in highest gel strength values.

Surprisingly, a pure polyalternating sample gave gels of extremely high rupture strength compared to all other samples. However, increasing the amount of G in the form of G-blocks by only 2% using AlgE1, the rupture strength was reduced by over 60%. The high rupture strength of the polyMG sample was explained by a possible mechanism involving "sliding" of MG-MG junctions.

Results from this work led to better understanding of the relationships between polymer structure and mechanical properties in alginates and the role of long alternating sequences in this respect. Further, the results highlighted the importance of

choosing an appropriate method to describe the rheological properties of the gel, depending on its intended application.

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4 GENERAL DISCUSSION

4.1 What determines the final capsule properties?

A large portion of the work in this thesis has been attributed to relating alginate composition to the final properties of alginate gels in a search for a better suited capsule for immunoisolation purposes. It was found that the final gel properties depend on many factors, such as the **choice of alginate material** (Paper 1, 2, 3, 5 and 6), **choice of gelling ion** (Paper 1, 2 and 3) and **method of preparation** (Paper 1).

4.1.1 Choice of alginate material

Alginate composition and sequence has in numerous studies shown to be a major determinant for the final gel properties. Alginates containing high amounts of mannuronic acid and alternating sequences will form compact, but softer gels as compared to materials containing long G-block sequences which form a more open and stiff gel network [91, 269]. Present and previous studies have shown that the composition also affects both the binding of alginate to divalent ions (Paper 2) and the binding of polycations to the alginate gel (Paper 1 and 3).

A controversy is that alginates of high mechanical stability give the most permeable gels [91] while the initially least permeable gels have a lower stability. Hence, epimerization of high-G alginates with AlgE4, allowing a denser structure by increased syneresis and further stabilization due to enhanced calcium binding (Paper 3-5) might combine these two properties which both are important in ensuring good immune protection. The steeper polymer concentration gradient in gel beads of epimerized alginates (Paper 5) contributing to lower permeability is also preserved to a larger degree compared to controls (unpublished data).

King and coworkers [216] showed that AlgE4 epimerization of a high-G seaweed alginate was not sufficient to completely prevent the swelling of alginate beads. In contrast, extremely stable gels could be formed by a two-step epimerization of mannuronan (Paper 5). Further, this demonstrated the possibility of fine tuning of the hydrogel properties by using molecular engineering to design alginates with the ideal composition for a given application (Paper 5 and 6). Lastly, as algal alginates show great compositional and structural heterogeneity, the use of epimerases as tools in

polysaccharide engineering can provide well-defined alginates with a much more homogenous composition (Paper 5), better suited in pharmaceutical and biomedical applications.

4.1.2 Choice of gelling ion

The alginate composition was in Paper 2 also shown to be an essential factor for increased stabilization of the gel network by means of barium and strontium ions. Barium was shown to bind to G-blocks and M-blocks, while the binding to alternating sequences was limited. Strontium, on the other hand, bound to G-blocks solely. This will have a large impact on the final functionality of the capsules, further illustrated by the low stability of both Ba-alginate and Sr-alginate gels containing high portions of MG sequences (Paper 2 and unpublished data).

When replacing calcium for barium in order to enhance stability, the potential toxicity of barium ions must be taken into consideration. Thus, the concentration of barium should be minimized and the alginate material chosen according to the highest possible binding. In Paper 2 and 3 we demonstrated that low concentrations of barium (Ca:Ba proportions of 50:1) were enough to stabilize the gel network considerably and thus higher barium concentrations would be unnecessary for this purpose. This may be of importance as many researches have started to replace the alginate/polycation capsule with a pure Ba-alginate bead, sometimes using concentrations as high as 25 mM Ba^{2+} [176]. This leaves barium as the only counter ion, also for alginate chains not bound in junctions, and these free barium ions will be easily exchanged with sodium *in vivo*. Thu et al have shown that when using low amounts of barium the leakage of Ba^{2+} ions from alginate/polylysine capsules of high-G alginate materials is very limited [168]. Ongoing *in vitro* studies on Ba-alginate and Ba/Ca-alginate beads show that the long-term leakage of barium is up to 25 times higher from a pure Ba-alginate bead compared to beads of Ba/Ca alginate (unpublished data).

4.1.3 Method of preparation

Alginate gels prepared by dialysis have earlier been shown to exhibit a concentration inhomogeneity where the polymer concentration is considerably lower in the center of

the gel than at the surface [148, 271, 272]. Further, the steepness of the concentration gradient has been shown to be dependent on the method of preparation, in particular on the concentration of gelling ions and the presence of non-gelling ions (Na^+). In Paper 1 and 2 we were able to study this phenomenon by means of fluorescence labeled alginate and confocal laser scanning microscopy. The concentration of gelling ions was shown to be of greater importance than their affinity upon inducing a concentration gradient (Paper 2). However, ions of higher affinity were less prone to destabilization of the gel network during washing in saline (Paper 1).
Further evidence for the importance of the method of preparation are results from measurements of syneresis showing that alginate gels made first in the presence of calcium and then dialyzed against Ba^{2+} or Sr^{2+} showed a higher degree of syneresis compared to gels made in the presence of mixtures of Ba/Ca or Sr/Ca (unpublished results). This observed hysteresis adds to the knowledge that alginate gels are of non-equilibrium nature [90, 102], their properties being dependent on the history of formation in addition to the environment to which the gels are placed.

An obvious observation and important message which can be drawn from the above mentioned papers is that changing a single capsule parameter will have effects on many of the other parameters determining the final functionality of the gel. Unfortunately, the connections are not always straightforward. This is one of the reasons for the major discrepancies found among the different scientific results in the field of alginate capsule research. Hence, when choosing a system for cell encapsulation, all the above mentioned factors need to be taken into consideration.

4.2 Role of alternating sequences on alginate gel properties

The recent availability of the C-5 epimerases has allowed for an extremely efficient tuning of both composition and physicochemical properties of the alginate. In particular, the epimerase AlgE4 which enables the conversion of M blocks into alternating sequences has provided new alginates with interesting properties. Results from Papers 3, 4, 5 and 6 all illustrate this by the striking effects seen upon introducing MG blocks into the alginate chains. The higher degree of both syneresis, stability and elastic modulus observed for these samples were in previous studies [92,

93] and in Paper 3 attributed to the intrinsically higher flexibility of MG compared to GG and MM diads [56] solely.

Although the calcium binding ability of alternating sequences has been suggested [79, 102, 275], the undoubtedly lower affinity toward the cation has confined MG blocks to a mere part of the elastically active chain in the gel network, without any direct involvement in the formation of the junction zones. In Paper 2 and 4 we showed that a pure alternating sample in fact could form a solid Ca-alginate gel. Further, in Paper 4 we hypothesized the direct involvement of long alternating sequences in the formation of a gel network by means of both pure MG-MG and mixed MG-GG junctions. Examination of the rheological properties of epimerized samples containing long polyalternating sequences (Paper 6) further suggested the "sliding" of junctions upon deformation as opposed to the unwinding of the junctions, explaining the strikingly high rupture strength of a polyalternating sample. In sum, the availability of epimerases has allowed us to get a better understanding of the structure-function relationships of alginate gels.

The binding of alternating sequences to other divalent ions (Paper 2) has also given us new insight into the gel properties upon replacing Ca^{2+} with ions of higher affinity to alginate (Ba^{2+} and Sr^{2+}). The studies revealed that Ba^{2+} and Sr^{2+} in fact did not bind to alternating structures to the same extent as Ca^{2+}, probably related to their different ionic radius (increasing in the order $Ca^{2+} < Sr^{2+} < Ba^{2+}$) [276] and the size of cavities formed by MG diads. The different diad pairs presumably exhibit specific molecular geometry making them variously suitable to bind the different divalent cations [76, 277].

While many features of the alternating sequences have been elucidated in present work, some questions still remain unanswered. The temperature dependence on Young's modulus (E) for a polyalternating sample would, for instance, possibly help determine whether the gel network of a polyalternating sample is of an enthalpic or entropic (rubber like) nature.

The role of alternating sequences on the different functional properties of alginate gels, as interpret from the results from Paper 3-6, can be summarized as shown in Table 1.

Table 1. Summary of the role of alternating sequences on functional properties of alginate gels

Functional property	Effect of increasing MG sequences	Possible reason for the observed effect
Syneresis	↑ Increased	• Partial network collapse due to zipping of long alternating sequences • More flexible junctions allowing for more crosslinks to form
Porosity	↓ Reduced	• More compact polymer gel network due to syneresis • Less swelling
Stability against swelling	↑ Increased	• More compact network leading to increased number of crosslinks and lateral association • Reduced number of mobile counter ions • Possible cooperative binding of Ca^{2+} to MG sequences
Inhomogeneous polymer distribution	↑ Increased	• Reduced size due to higher degree of syneresis
Rupture strength	↑ Increased	• Increased polymer concentration due to high degree of syneresis • Increased number of junctions which need to be unwound before rupture
Young's modulus	↑ Increased	• Elongating alternating sequences leads to formation of new MG-MG and mixed MG-GG junctions

4.3 Capsules or beads?

One of the mail goals of Paper 1-3 and Paper 5 was to optimize the stability of the alginate gel core in order to be able to reduce or even omit the polycation layer, which in general is believed to be the main contributor to the bioincompatibility of capsules *in vivo*. As the polycation is added to reduce permeability and increase the stability of capsules (Paper 1 and 3), omitting the polycation layer implies making alginate gel beads with lower permeability and improved stability.

4.3.1 Permeability

Many groups have shown that a simple Ca-alginate gel bead protects from direct cell-to-cell contact [201, 225, 266, 278, 279]. However, smaller molecules have been shown to traverse the membrane to various extents. These include cytokines as well as preformed antibodies or antibodies to antigens which have leaked across the barrier.
In spite of all the research on the field, the exact permeability requirements for complete immune protection are still not known as there are large discrepancies between the results from different studies [24, 253]. The cytotoxic events of antibodies and complement begin only if complement components pass through the membrane. Binding of the first complement component (C1q) to IgM or IgG initiates a cascade ending in cell lysis. However, IgM (750 kDa) and C1q (400 kDa) are larger than IgG (150 kDa). Thus, it has been suggested that if passage of IgM and C1q across the barrier is prevented, a specific antibody-mediated attack on the islet cells should be prevented [21]. Numerous studies indicate that prevention of host-donor cell contact may be enough to hinder the activation of the cellular responses. Lanza and coworkers showed that Ca-alginate gel beads permeable to IgG and complement protected islet xenografts against destruction in mice [266] and more recently Dufrane et al [201] showed that xenografts in non-human primates were partly protected in spite of the ingress of both IgG and IgM. In general, encapsulation of xenograft will require more stringent permeability control as the host response to xenogeneic tissue will be stronger than to allogeneic tissue where the shed antigens in the former case quickly will be recognized as foreign [26, 212]. Finally, the transplanted cells may need to be protected against antibodies produced during the preexisting autoimmune

disease, in case of Type 1 diabetes, which might bind to surface antigens on transplanted cells [21].

In Paper 2 we showed that Ba-alginate beads were on the limit of excluding IgG, although confocal images of fluorescence labeled IgG revealed that IgG was able to diffuse into the bead center. Results from Paper 3 pointed to a reduction in permeability of Ca-alginate gel beads by increasing the amount of alternating structure in the alginate backbone using the C-5 epimerase AlgE4, and in Paper 5 the permeability was further reduced by making novel alginate materials with G-blocks intersperced by long alternating sequences using a combination of epimerases. In all these cases, the reduced permeability compared to controls (Ca-alginate gel beads) can probably be attributed to the increased stability preventing the swelling of beads which would lead to increased porosity. For epimerized samples the high degree of syneresis, particularly profound for samples in Paper 5, likely led to further reduction in permeability due to the more compact gel network. Lastly, in Paper 1 and 5 the inhomogeneous polymer distribution in beads of Ba-alginate and epimerized alginate, respectively, might have contributed further to lowering the porosity for these gels.

Control of pore sizes in the tens of nanometers has been suggested as the most realistic way to achieve immunoisolation [38, 280]. In one study pore size of 18 nm and lower was shown to be required to keep IgG out of a semi-permeable membrane [189]. However, choosing the "ideal" pore size will always be a compromise. Keeping out TNF-α (51 kDa) may for instance also prevent the ingress of the iron-transporting protein transferrin (80 kDa), important for cell function [265] whereas protecting cells from the detrimental effects of IL-1β [281] may be difficult without concomitantly hampering the egress of insulin (see Figure 13).

4.3.2 Biocompatibility

A host response to capsules has been shown to start immediately after implantation, initiated by the inflammation caused by the implantation of the capsules. Since this is difficult to avoid, especially without the use of immunosuppressives, it is apparent that that manipulation of the capsule properties in order to reduce host reactions would be a favorable course of action [212]. In recent years the method of using

simple Ba-alginate beads in order to avoid the polycation layer, has been exploited by many researchers giving promising results both in allo- and xenograft transplantation [174-176, 205, 206, 236, 282-285] as well as in biocompatibility studies on empty beads [236]. In Paper 2 we demonstrated the increased stability and decreased permeability for Ba-alginate gels, factors which might contribute to the high success rate of this system. In addition to this, the observed binding of Ba^{2+} ions to mannuronic acid residues (Paper 2) may be a positive contributor to the biocompatibility of Ba-alginate beads. The binding of barium, although weak, might prevent low MW alginate oligomers enriched in mannuronic acid to leak out of the beads evoking an immune response [140]. Moreover, this may be completely avoided by using a two-step epimerization of mannuronan in which the product contains MG and GG blocks solely (Paper 5).

The feasibility of manipulating specific capsule properties using mannuronan C-5 epimerases was demonstrated in present work (Paper 3, 5 and 6), possibly bringing us one step closer to making a biocompatible alginate bead with improved functionality. However, the functionality and biocompatibility of these beads with islets *in vivo* still remains to be studied. So far, no differences in biocompatibility were found comparing empty Ca/Ba alginate beads of epimerized and non-epimerized high-G seaweed alginate [216].

While several factors, some of which are not identified [239], are likely to influence biocompatibility, numerous studies have demonstrated that achieving a suitable level of biocompatibility requires, at minimum, a highly purified alginate [239, 286, 287]. The main factor hampering the use of the novel epimerized materials *in vivo* has so far been their high content of proteins (epimerases) and LPS, the latter stemming from the bacterial production of both the mannuronan alginate and epimerases (section 1.4.2). The production of epimerases in GRAS[i] organisms is currently being tested, allowing for endotoxin free alginate products.

Although sample purity has been acknowledged as an important factor in biocompatibility issues, having viable cells inside the capsule might be of equal importance. Damaged or hypoxic islets may produce stress proteins [212], and components of dying cells may leak out of the capsule causing an immune response. In addition, as topography has shown to have impact on biocompatibility [40, 250],

[i] Generally Recognized As Safe

the surface roughness of alginate beads as compared to capsules might need to be evaluated [40].

Enhanced biocompatibility of alginate/polycation microcapsules can also be achieved by reducing the amount of polycation bound and/or increasing the binding of the outer alginate coating for a more efficient shielding of the positive charges [216, 241]. The implications of the results in Paper 3 are that using a core of alginate epimerized with AlgE4 produces a more stable and less porous capsule than using the native alginate. Because the increase in alternating sequence stabilizes the gel, lower amount of polycation is required to produce small and stable capsules, at the same time retaining the porosity characteristics with regard to ingress of IgG and TNF. Also, using an epimerized alginate with polyalternating structure as coating material is believed to increase the biocompatibility due to better coating of the toxic polycation layer [216].

It may be that ultimately the simplest system of them all, the alginate bead, will be sufficient to provide immune protection in the case of allogeneic models, whereas the development of microcapsule materials for xenogeneic models will remain a challenge. In this respect, results from the present work have given more insight into the possibilities for improving the stability and permeability characteristics of gel beads.

So far, the major pitfalls making it difficult to interpret whether the alginate gel beads will be suited for islet transplantation have been the large discrepancies in results from animal studies from the various groups working with alginate encapsulated islets. A system working in one animal model may not work in another slightly different model, depending on the capsule properties, animal model and transplantation site. In general, the lack of standardization between laboratories has been a major contributor to this. Ongoing preclinical trials in non-human primates, and pending clinical trials in humans on Ca/Ba alginate beads containing human islets [288], will hopefully provide better understanding on this topic in the near future.

Table 2 summarizes the major advantages and disadvantages of using alginate gel beads as an immunoprotective membrane for cell transplantation.

Table 2. Advantages (+) and disadvantages (÷) for using alginate gel beads as compared to alginate-polycation capsules for immunoisolation.

+	÷
• **Increased biocompatibility** • **More open network** - allows easier diffusion of nutrients and cell products which may be hampered by the polycation layer [265, 289] • **Non-toxic** • **One-step process** – mild conditions for cells and easier to produce • **Protrution of cells less likely to occur** [290] • **Negative surface charges -** may exclude immune cells [278]	• **Lower stability** • **Larger porosity** – allows easier access for immune components

5 CONCLUDING REMARKS

In spite of extensive research in the field of immunoisolation over the recent years, optimization of alginate capsules is still needed for them to become useful in the clinic as an immobilization device. The present work aimed at optimizing the alginate encapsulation system for cell therapy purposes, with particular focus on the treatment of Type 1 diabetes.

In this respect, we have shown that added knowledge about the alginate structure-function relationships is of great importance, as careful selection and manipulation of both capsule materials and encapsulation procedures made it possible to improve the functional properties of alginate microcapsules.

In particular it was demonstrated that:

- The encapsulation procedure influenced the final capsule structure and properties, and that CLSM proved to be a convenient tool to study the effect of different factors during capsule formation as well as the final characterization of alginate microcapsules.

- A proper combination of gelling ion and alginate material was of great importance in order to improve and control the functional properties of alginate gel beads. Large differences were seen on the selection of type of gelling ions on both the swelling properties and gel strength of high-G alginate and high-M alginate.

- Increasing the amount of alternating structure in seaweed alginates enhanced the functional properties of alginate beads and capsules with regard to stability and permeability.

- G blocks in fact are not the only sequences involved in junction formation, but that long alternating sequences also play an important part in the in the alginate gel network by forming MG-MG and mixed MG-GG junctions.

- Utilization of specific epimerases made possible the modification of polymer nanostructure, resulting in completely new alginate materials with highly improved functional properties.

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