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Coating of alginate capsules

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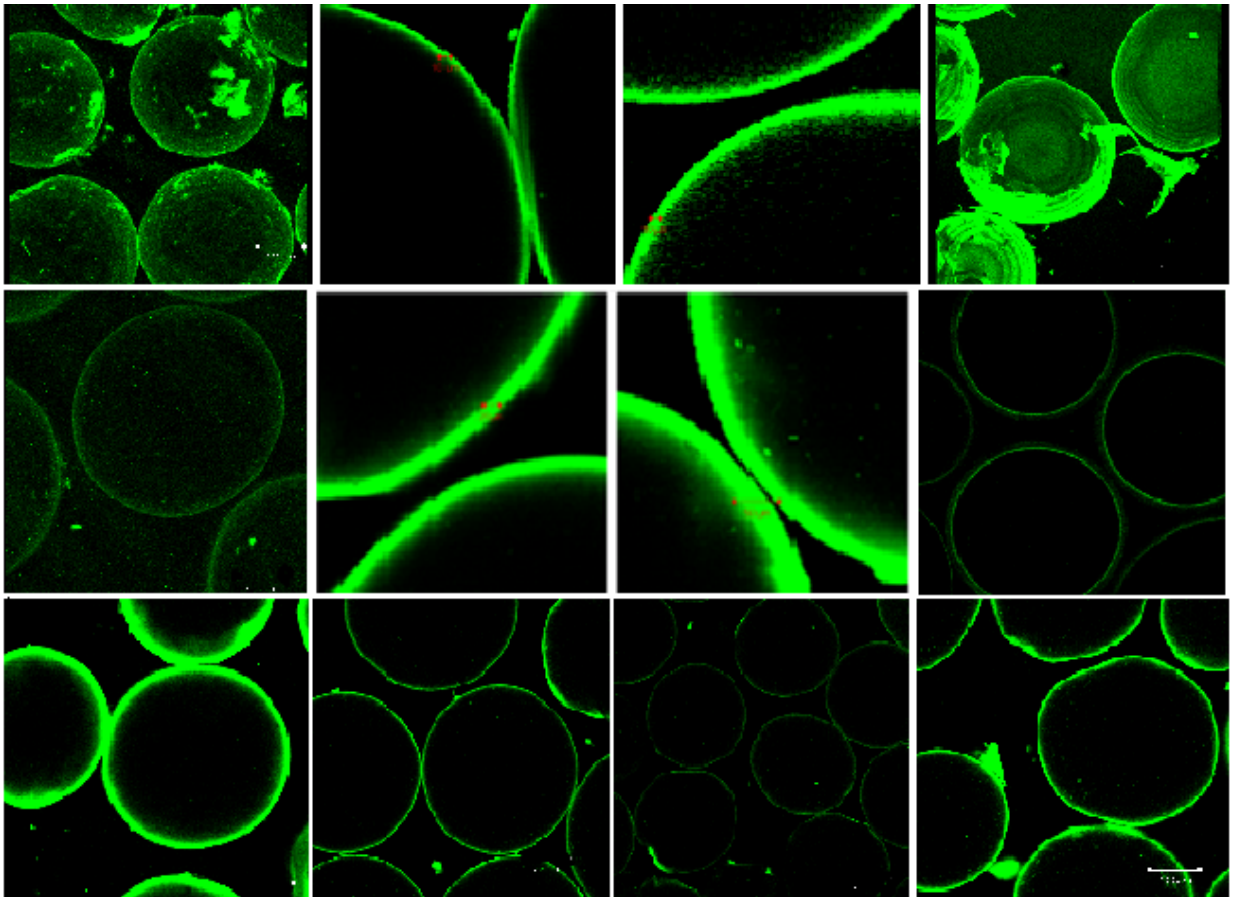

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Summary

Alginate is a popular candidate for encapsulation of cells due to the formation of gels with divalent ions under physiological conditions. Stable alginate gels can be formed by the selection of alginates with a high content of guluronic acid (G) and gelling in a mixture of calcium and barium. These alginate gels have been proposed as immune protective barriers for the transplantation of human pancreatic islets (insulin producing cells) for the treatment of type 1 diabetes 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. Although showing promising results in animal models, there are potential limitations of the Ca^{2+}/Ba^{2+}-beads concerning growth of host cells on the surface of capsules in primate models. Development of coating strategies for alginate based capsules could thus be beneficial for reducing the attachment of host cells.

Alginate microbeads/capsules were formed by electrostatic bead generator producing beads of 400μm. Afterward, the alginate beads were coated by fluoresceinamine labeled alginate that was visualized by confocal laser scanning microscopy (CLSM) and quantified by fluorescent spectroscopy. The binding of coating alginate to alginate-poly-L-lysine (PLL) capsules was also studied.

In this project, in the optimalisation of coating of alginate beads some parameters were studies such as concentration of coating alginate, different gelling ions both for core and coating alginate, exposure time for gelling solution for fixation of coating layer and different washing solution.

The long-term stability of coating layer of coated alginate beads was determined by measuring the fluorescent intensity of fluorescently labeled of coating alginate during a period of forty nine days. A stability study of alginate-alginate capsules revealed that Ca^{2+}/Ba^{2+} alginate coated with high-G alginate and washed with saline and used Ca^{2+} and Ba^{2+} with ratio 50:1 for fixation of coating layer were more stable coating than other capsules. The alginate beads coated with high-M or epimerized alginate were produced. It shows higher intensity of coating layer in both capsules coated with high-M or epimerized alginate than alginate beads coated with high-G alginate.

In continue of the study, the alginate-PLL capsules were coated with high-G, high-M, and epimerized and sulfated alginate. Alginate-PLL capsules coated with high-G, high-M and epimerized alginate shows no detective signal by confocal images and sulfated alginate

coating shows some signal of coating. The stability of coating for alginate-PLL-alginate capsules and alginate beads coated with epimerized or high-M alginate revealed that both kind of coating have high-stability over one week screening.

Three dimensional images of capsules, in confocal microscope, both epimerized and high-M coating alginates cover whole of capsules. However, in 3D images we have seen some fragment of coating gelling in the surround solution and attached to the capsules which can make disturbance in spectroscopy measurement. 3D images of alginate-PLL capsules coated with sulfated alginate show evenly distribution of coating.

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1 Introduction

Transplantation of Langerhans islets is a probable treatment of Type 1 diabetes which targets to replace insulin therapy and to have intimate glucose control. Encapsulation of living cells in calcium-alginate can improve the transplantation of immobilized insulin-producing cells in patients with diabetes since the cell will be protected from immune system by placing cells in alginate beads. An alginate-cell mixture is used to make alginate capsules where the Langerhans islets are fenced within the capsules [1].

However, there are still some obstacles related to the use of alginate capsules for transplantation. One major problem preventing the use of alginate capsules *in vivo* has been fibroblast overgrowth, which can cause failure of encapsulated Langerhans islets due to reduce diffusion of oxygen and nutrients to encapsulated cells and activated immune system damaging islets. The alginates used as a matrix for implanted cells in vivo, should not give rise to inflammatory reaction causing fibroblast over growth. Cellular overgrowth may be due to an implanted encapsulated cells immune response to cellular material leaking out through the capsule membrane, exposure of capsule components, cells or alginates through capsule breakage or to the alginate material itself [1]. To protect the cells from the body immune system, a capsule for cell immobilization and transplantation should be carefully designed. Under physiological conditions the capsules are also exposed to a combination of destabilizing forces, including the osmotic swelling of the core, disruption of the alginate-polycation complex and disruption due to shear-forces. Hence, one of the biggest challenges has been to create capsules that are stable under physiological conditions over long periods, ideally for several years.

Alginate capsules coated with poly-L-lysine (PLL) and alginate have been used for the microencapsulation of a variety of cell types such as parathyroid xenotransplantation [57] and may make cell transplantation promising without the use of immunosuppressive drugs. By binding PLL to the alginate core, a polyanion-polycation complex membrane will form, which moderates the permeability of the gel, and thus forms the immunoprotective barrier. In addition, PLL decreases osmotic swelling of the alginate capsules and therefore stabilizes the capsule. A last alginate layer is used to counteract the PLL with positive charge to reduce attachment of cells to the capsule. Though, it has been shown in many studies that the present of PLL increases the host reaction and is harmful to the encapsulated cells. However, King et al. are shown the improvement of the biocompatibility of alginate/poly-L-lysine/alginate microcapsules by the use of epimerized alginate as a coating layer [35], but still there are some challenges to

improving biocompatibility of microcapsules. Hence, in this project we have tried to make different kind of coating both on alginate beads and on alginate-PLL capsules using fluorescently labeled alginates for detection by fluorescent spectroscopy and CLSM.

1.1 Alginate

Alginate is naturally polysaccharide that was first defined by E.C.C. Stanford in 1881 (Moe et al., 1993). Since alginate can be found as a structural component in marine brown algae and as capsular polysaccharides in soil bacteria such as *Azotobacter vinelandii* [2] and several species of *Pseudomonas* [3], it is relatively widespread in nature. In brown algae, alginate is the most abundant polysaccharide containing up to 40% of the dry matter. The main function of alginate is believed to give strength and flexibility to the algal tissue. Industrially, alginate is used for its gelling, viscosifying and stabilizing properties and for its ability to retain water [4].

1.1.1 Chemistry

Alginate is a natural and linear block co-polymer. It is made of (1→4) linked β-D-mannuronic acid (M) and α-L guluronic acid (G). The relative amount of the monomers as well as sequential arrangement along the polymer chain differs widely from one alginate source to another [4]. The most probable ring conformations of the monomers are $^4C_1$ for M residues, and $^1C_4$ for G residues (Figure 1.1).

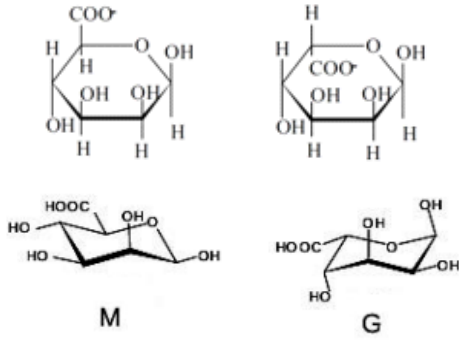


Figure 1.1: Top: Illustration of the Haworth formulas of β-D-mannuronic acid (M) and α-L guluronic acid (G) Bottom: The most probable ring conformation (chair conformations) of the alginate residues; $^4C_1$ for the M, and $^1C_4$ for the G

The uronic acids have pattern of blocks, homopolymeric blocks (G or M-blocks) in alternating structure and blocks with M and G units (MG-blocks), see Figure 1.2 [5]. The composition and sequence of the M and G residues can be determined by high-resolution ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR).

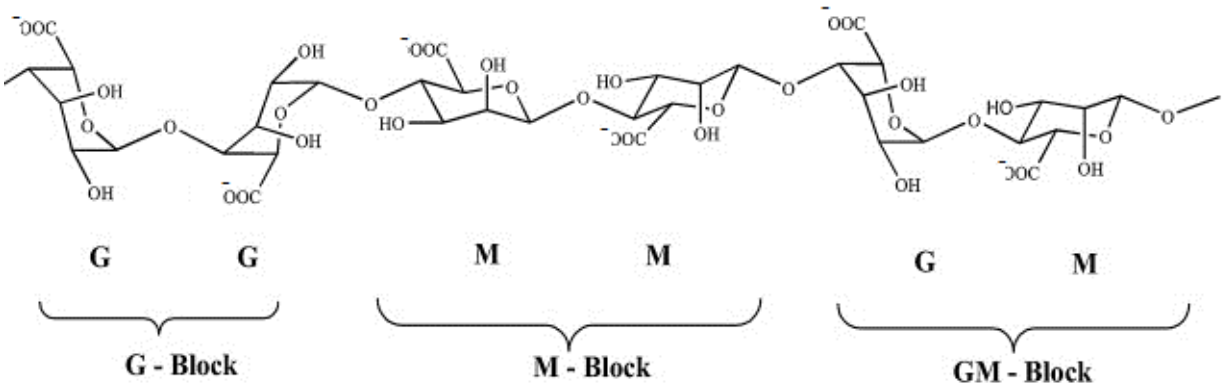


Figure 1.2 Block structure of alginate chain with its monomer M= β-D-mannouronic acid and G= α-L gulouronic acid [6].

The connection of two guluronate units is diaxial and therefore, it is shorter than M-blocks or MG-blocks. So, G-blocks are more folded and less elastic due to diaxial linkage which prevents free rotation around the glycosidic linkage. However, M-blocks have diequatorial linkages and longer chain and more elasticity. MG-blocks have axial-equatorial and equatorial-axial linkages; however, varying in degrees of free residues prepare larger elasticity in general than (1→4) β-D-mannuronate linkages in MM chains. Therefore, the relative flexibility of the glycosidic linkages in alginates decreased in the order MG < MM < GG [59, 7].

The alginate polymer is at risk of different depolymerization processes. The glycosidic linkages will be cleaved with exposure to acid, alkaline and free radicals. Alginate degradation is on its slowest at neutral pH and will increase at low pH and high pH. All the depolymerization reactions will increase with temperature [8].

1.1.2 Physical properties

Alginate has numerous valuable properties which are due to its macromolecular and polyelectrolyte character. The properties such as the ability to retain water, cation binding and gelling, and the ability to form viscous solutions at low concentration, cause alginate

to be a frequently used component in several applications such as thickeners, stabilizers, gel-forming and film-forming agents [9].

Alginate, similar to other polysaccharides, has a broad molecular weight distribution. The viscosity of an alginate solution is mostly related to the alginate concentration and the size of the alginate molecules. Draget et al showed alteration of M-blocks to MG-blocks by *in vitro* epimerization of gelling alginates cause distinct changes in the gelling behavior with calcium, mainly by increasing the degree of syneresis. This has been recognized to the higher flexibility of MG-blocks [60]. Hence, due to the structure of M and G blocks in alginate, the intrinsic flexibility of the alginate molecule has also shown to increase in order to GG < MM < MG. In addition, the intrinsic viscosity of alginate is linearly dependent on the ionic strength in the solution [58].

The alginate solution viscosity will mostly relate to the polymer concentration and molecular weight of sample. Alginate is a negatively charged polyelectrolyte; the electrostatic repulsive forces between the charged groups give high viscosity for alginate solution. High ionic strength can protect the charges and decrease chain extension. So, the viscosity (η) of an alginate solution is directly related to the ionic strength in the solution [10]. It has been shown that alginate solutions with extraordinarily high viscosity could decrease cell viability because of the high shear forces when mixing cells to these solutions [11].

Since the alginate's carboxylic groups are dissociated at physiological pH the alginate is a negatively charged molecule (a polyanion). The pK_a values of mannuronic and guluronic acid monomers are 3.38 and 3.65, respectively. Therefore, many of alginate's characteristics depend on the cations that neutralize the molecule. The majority of monovalent ions form soluble salts with alginate, whereas polyvalent ions (with exception of Mg^{2+} at low or very high concentrations) form gels or precipitates [12].

Alginates have the ability to bind divalent cations to form hydrogels. Ca^{2+} creates ionic interchain bridges between adjacent alginate chains of G-blocks, MG-blocks and G-and MG blocks which causing shape-stable hydrogels. Alginate hydrogels with high guluronic acid content (higher than 60%) present high strength, low shrinkage and high stability [13]. Since cells can be encapsulated in alginate under physiological conditions, it is a promising method for use extensively in medicine, such as cell therapy and tissue engineering.

The alginates affinity toward divalent ions is very much related to the alginate composition. It can be increased for divalent ions by following order: $Mg^{2+} << Ca^{2+} < Sr^{2+} < Ba^{2+}$ [14]. The probable chelation of ions by the GG-sequence is demonstrated in Figure 1.3.

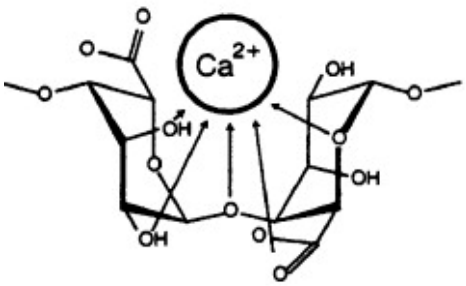


Figure1.3 The probable chelation of calcium ions by the GG-sequence in alginate; the calcium ion interacts with five different oxygen atoms of two adjacent G residues in the intrachain binding [15].

The Eggbox Model explains how G-blocks join together with calcium ion. It is the model for cross-linking of polymer molecules in alginate gels. The model name comes from the placed calcium ions in G-blocks similar to eggs in an eggbox (Figure 1.4) [16].

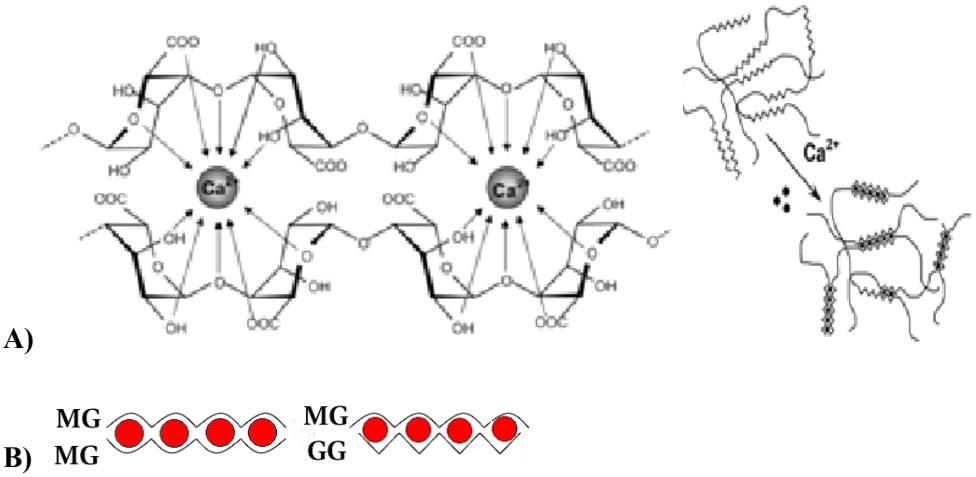


Figure 1.4: A) "The egg box model" for binding of Ca^{2+} to G-blocks. Two adjacent G- blocks make up a cavity where the Ca^{2+} binds, making a junction zone. B) Forming cross-links with Ca^{2+} (red circles) in the G- and MG-blocks [17].

The gelling properties of alginate are based on its ion-binding properties. The gel formation is a near-neighbor, auto-cooperative and reversible process, while cross-linking sequence are mostly made by stretches of G residues where is selectively bound by

divalent cations. After the first binding of divalent ion between two G-blocks, the binding of the next ion is entropically enhanced.

The lowest required size of adjacent G units for the creation of stable junctions has been described to range from about 8 to 20 G residues for Ca^{2+} [18]. Further, it has been found that the smallest length of G blocks essential for junction formation is reduced with enhancing the affinity of ions toward the alginate chain [19]. Hence, the G-blocks length is one of the great importance for the formation of stable junctions.

1.1.3 Biosynthesis

Contrary to other marine algal polysaccharides, for instance agar and carrageenans, the alginates are not limited to the marine algal, but are produced as exocellular material in some bacteria as well. The bacterial and algae alginate difference mostly is in the existing of O-acetyl groups on the second and/or third carbon(s) in M residues in bacterial alginates. *Azotobacter* alginates resemble the seaweed material which could be true block copolymers (FG = 0.10-0.95) with just additional the O-acetyl groups, whereas *Pseudomonas* species alginates consistently lack G-blocks (FGG = 0). O-acetyl groups influence the swelling and water-binding potential of bacterial alginate gels. Although *A. vinelandii* is a constant producer of alginate under various conditions, all commercial alginates are at present extracted from algal sources [20].

High contents of G-blocks 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 [21].

The alginates biosynthetic pathway was first explained in brown algae and discovered enzyme activities related to the alginate biosynthesis. The most exclusive feature of alginate biosynthesis is that the C-5 epimerase (step 7 in Figure 1.5) of β-D-mannuronic acid (M) into α-L-guluronic acid (G) occurs at the polymer level. This in-chain conversion of M to G residues is catalyzed by the mannuronan C-5-epimerases (Figure 1.5) [23].

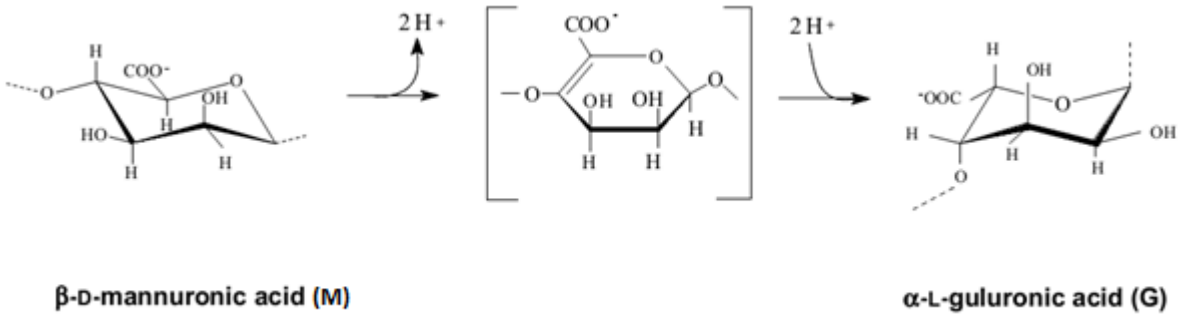


Figure 1-5. The anticipated mechanism for mannuronan C-5-epimerase. The result of the epimerization reaction is the conversion of M to G

Post-polymer epimerization of polysaccharides is a rather unusual incident in nature. The existence of C-5 epimerase was confirmed in both bacteria and brown alga. In the early studies, the enzyme was reported to produce single G or polymeric G-blocks. Studying on an *A. vinelandii* gene library has, on the other hand, shown the present of a family with seven mannuronan C-5-epimerase genes, the algE genes (algE1-algE7) which all have been sequenced and expressed in *Escherichia coli*. The AlgE epimerases produced by algE genes are different in size and activity. The activities of the enzymes are highly Ca^{2+}-dependent and every enzyme catalyzes the making of alginates with distinct monomer distribution patterns. The AlgE4 epimerase catalyzes the configuration of MG-blocks completely, whereas the other six enzymes bring in a mixture of stretches of G blocks and alternating sequences [31].

Since the epimerases alone determined the composition and distribution of M and G in the final alginate, the epimerization is the main reaction determining the ion-binding and gel-forming properties of alginates. Therefore, the epimerase gene family of *A.vinelandii* makes many potential for both basic and applied research. The enzymes can be used to change alginates giving rise to polymers with improved gelling properties in addition to a more consistent distribution of composition and block structure [22].

1.2 Application of alginate beads

Alginates have been used since 1980 as an encapsulation matrix for Langerhans islets [24]. There are several probable uses of encapsulated cells in industry, medicine, and agriculture. In industry and agriculture, this includes nitrification of domestic waste water [25], manufacturing of ethanol by yeast [26] and production of lactic acid by lactic acid bacteria [27]. In medicine, the use of encapsulated cells includes production of biological interferon from microencapsulated hybridoma cells [28] and entrapment of insulin-producing cells (microencapsulated islets) as a bioartificial endocrine pancreas [29].

1.2.1 Alginate as immobilization matrix

For fragile cells, such as animal cells, capsulation in alginate gels is attractive due to the option of encapsulate at close to physiological condition. The alginate microbeads formation steps comprise dispersing of cells in an aqueous sodium alginate solution (1-4 %), and dripping it into solution of divalent cations such as Ca^{2+},Sr^{2+} or Ba^{2+} (20–100 mM). Immediately hydrogel spheres will form and catch the cells in a network of ionic cross-linked polymer (< 5 min for beads of 500 μm [30]). The capsules size is dependent on the droplets size. The electrostatic bead generator is able to produce droplets with different sizes from about 150μm to 1 mm.

There are some challenges for finding an appropriate material to make capsules for immunoisolation. The capsule should be adequately permeable for permitting nutrients to go in and therapeutic proteins to go out. Moreover, it should be stable for required time periods. The material is required to be biocompatible, non-toxic and cell friendly [31].

From the hydrogels applied for microencapsulation, alginate is one of the most central immobilization materials. There are no alginate-degrading enzymes that up to now have been reported in humans. Also, alginates are much characterized and well understood in the gel and aqueous phase [31]. Alginate gel beads for immunoisolation should ideally have high mechanical and chemical stability, controllable swelling properties, a defined pore size, and a narrow pore-size distribution [1].

If the content of α-L-guluronic acid (G) increases, it provides mechanically stronger gels which show high porosity, low shrinkage during gel-formation, and low swelling. By increasing the content of β-D-mannuronic acid (M), the gels become softer and more

elastic, with the related shrinkage and decrease of porosity during gel-formation, but with high swelling and following reduction in stability and increasing porosity.

1.2.2 Microcapsule as immune-barriers

Transplantation of living cells is a possible approach for treatment of various diseases caused by the body's failure to produce required amounts of a crucial molecule such as a hormone or an enzyme. In organ and cell transplantation patients have the risk of rejection because the body distinguishes the cells as an unfamiliar substance and looks for ways to destroy the transplant. Therefore, transplanted patients have the lifetime need for immunosuppressive drugs that can cause the increase in risk of tumor development, many infections and common toxicity [5]. Actually, the side-effects of using these drugs can be worse than the benefit of the transplant.

The idea of immunoisolation is to avoid rejection through separating the transplanted cells from the destructive immunological surroundings in the host by a selectively permeable membrane. The small pores of the membrane avoid the passage of substances with high molecular weight like large antibodies and immune cells. Simultaneously they allow the passage of smaller molecules like nutrients, electrolytes and oxygen (Figure 1.6) [7].

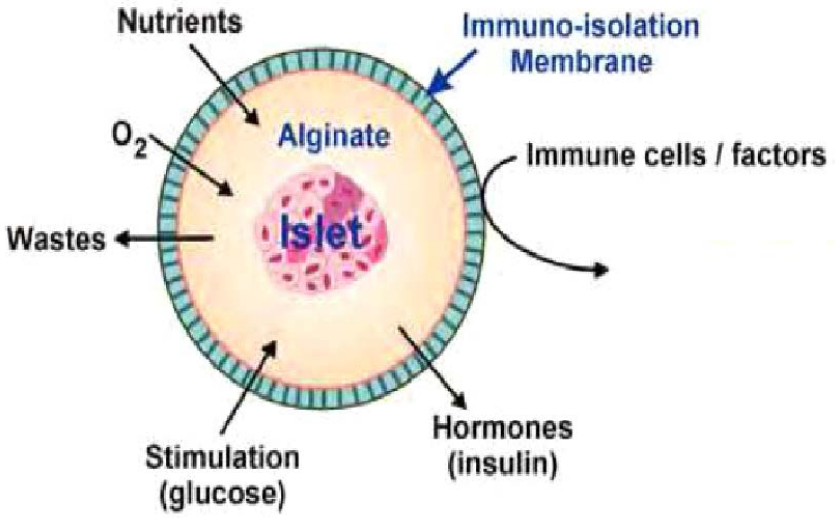


Figure 1.6 Immunoisolation of insulin producing cells (Langerhans islets) in an alginate microcapsule [32].

Thus Immunoisolation permits the transplantation of cells without requirement of immunosuppression. Immunoisolation of transplanted cells and tissue could thus alter present therapy for a large range of human diseases [8].

1.3 Capsule physical properties

Alginate beads for immune-protection should be characterized by: 1) Having high stability, both mechanical and chemical 2) Low swelling 3) Toxic content and immunogenic contaminants should be in low levels 4) Pore size should be defined [33].

Alginate microcapsules for transplantation should have enough mechanical resistance to hold up the forces such as compression applied on *in vivo*. High mechanical strength is needed to avoid capsules breakage and the exposure of encapsulated cells. The mechanical strength of alginate gels can be enhanced by increasing the guluronic acid content or by exchanging Ca^{2+} with cross-linking ions of higher affinity. However, this will often change other capsule parameter. Increasing G blocks content in the alginate increase the permeability of microbeads [34].

At physiological condition, Ca-alginate beads have a tendency to swell and dissolve, and it is a probable severe problem in immune-isolation systems. The sensitivity of the gel network towards chelating compounds like citrate, or non-gelling cations like sodium can cause the swelling. First, swelling could increase beads porosity. After that, gel interruption causes the transplanted cells to disclosure.

The effect of different alginates and cross-linking ions on important microcapsule properties has been described by Y.A. Mørch et al. The stability and gel strength was enhanced with high-G alginate gels when replacing the usual Ca^{2+} ions with Ba^{2+}. By using strontium, gels with characteristics between calcium and barium were obtained [14]. The Ca-alginate gel stability at physiological conditions is related to polymer concentration, alginate composition and molecular weight (MW). Calcium ions can exchange with non-gelling sodium ions, the network junctions will be dissolved and therefore reducing the gel strength. A membrane of polyanion-polycation composite will stabilize against swelling both by partially discharging the polymer network and by increasing the elastic forces [35].

2. 4 Coating of alginate microcapsules

Both alginate microbeads and alginate-polycation microcapsules have been used for immunoisolation. The most generally and widely non-coated alginate beads which are studied are the barium-cross-linked alginate microbeads. The stability of alginate beads increased by replacing calcium with barium as cross-linking agent [14,36].

2.4.1 Alginate-polycation microcapsules

Alginate based hydrogel beads can be deficient in long-term stability, in view of the fact that the hydrogel rests upon the existence of the cross-linking ion. This is of significant in biological fluids, where competing ions (sodium) and divalent cations chelators (citrate) are present. When using polycation for coating (e.g. poly-L-lysine (PLL)), intermediate-G or low-G alginates have been shown to form more firm connections between the polycation and alginate than high-G alginates [37].

King et al. and Strand et al showed that binding of epimerized or poly-MG alginate as coating layer on alginate-PLL capsules can improve the bio-compatibility of capsules, but still cannot entirely eliminate the harmful effects of PLL on the biocompatibility of the capsules [35, 68].

The polycation increase the mechanical stability restricts the permeability of alginate microcapsules. Calcium beads are coated with the polycation by suspending the beads in polycation solutions such as PLL. During this step, polycations bind to the alginate gel and induces the formation of complexes at the capsule surface. The presence of these complexes decreases the porosity of the membrane [38, 61]. Strand et al, has shown alginate and PLL overlaid by using CLSM (Figure 1.7). The most commonly used alginate-based capsules are formed by the alginate-PLL system, but also other polycations such as poly-L-ornithine (PLO) and poly-D-lysine have been used [70].

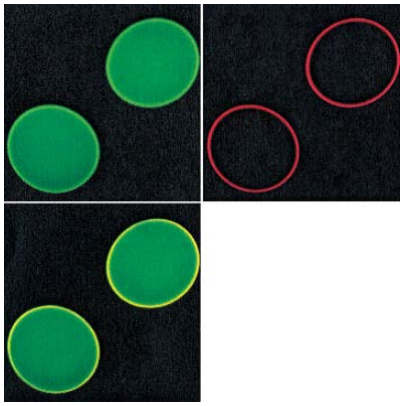


Figure 1-7: ***Micrographs are optical sections taken through the equator of the capsules. The upper left micrograph shows the alginate (green), the upper right micrograph shows the PLL (red), and the lower left micrograph shows alginate and PLL overlaid*** **[39].**

Darrabie and colleagues showed that PLO as coating biomaterial for providing perm-selectivity is less immunogenic than PLL. Also, they reported that PLO coated alginate microcapsules are mechanically stronger than PLL-coated microcapsules [40].

PLL is toxic to different kind of cell lines, it stimulates complement and macrophages to produce interleukin-1 (IL-1) and it is better to use a second alginate as the coating layer to shield the toxic PLL [61, 62].

2.4.2 Other coating of alginate beads

There are not much published about coating of alginate with other materials than polycation. July et al have studied the persistence, proliferation, and function of porcine hepatocytes in both primary cultures without entrapping in alginate and after encapsulation in alginate beads. The beads were coated with a membrane made by propylene glycol alginate and human serum albumin. Their data showed that a large number of hepatocytes in coated alginate beads stayed functional and were inaccessible to immunoglobulin. This structure was shown as a capable device for an extracorporeal bioartificial liver, including xenogeneic hepatocytes, to treat severe liver disease in humans [41].

Outokesh et al. prepared stable alginate microcapsules by coating of alginate microcapsules with chitosan in the potential of the selective removal of heavy metal ions from dilute solutions [65].

2 Theory

2.1 Formation of alginate capsules

Among various anticipated technologies for cell encapsulation, the use of alginate gel micro-beads is the most promising and adaptable method [1]. The method of encapsulation is by itself easy since the immobilization process has one step procedure of bead formation upon dripping alginate into a solution of divalent ions and does not need a profound skill in polymer chemistry, hydrogels and membranes [42]. However as there is a strong correlation between composition of alginate and physical properties, changing bead properties requires knowledge in alginate physical /chemical properties [5].

Micro-bead diameters of 200 μm to 1 mm can be produced by using an electrostatic potential (1-10 kV). Droplets of alginate solution are pulled from a needle tip into a gelling bath. A voltage is applied between the needle feeding the alginate solution and an electro-conductive bath of divalent ions. The voltage forces the droplets to fall off the needle tip before it has grown to the point where it falls off due to its own weight. The beads are formed when droplets fall into the bath. The high voltage has been confirmed not to damage the encapsulated cells. Size of needle, voltage and viscosity of alginate solution regulates size of droplet. In addition, the alginate solution run speed (flow) and the distance between the needle and the gelling solution can have effect on the bead size and shape (Figure 2.1) [43].

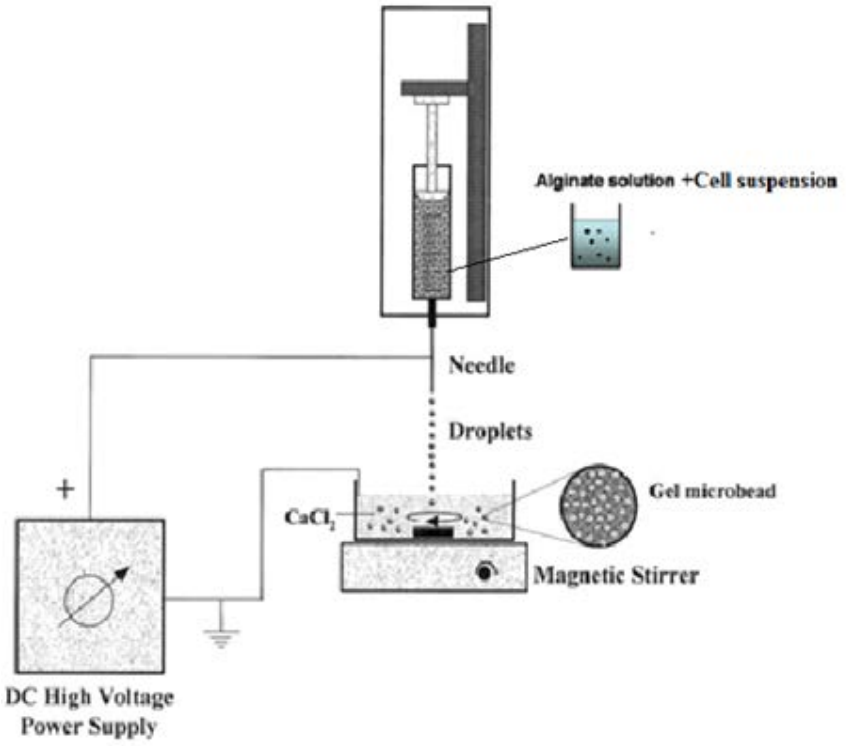
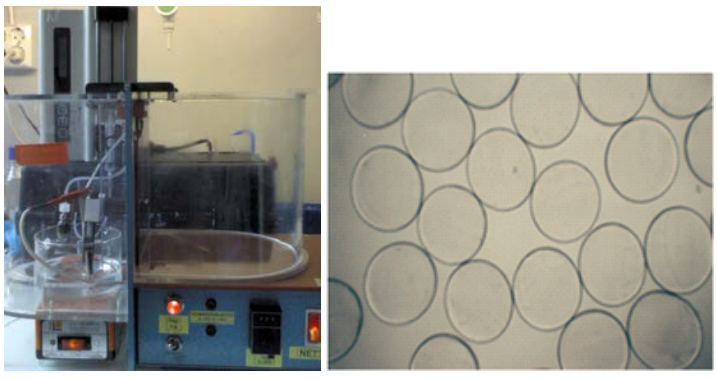


Figure 2.1: Using an electrostatic bead generator for encapsulation of cells. Cell suspension mixed with alginate solution injected with a syringe pump to the gelling bath ($CaCl_2$ or other divalent cation solution). The voltage is fixed to the gelling bath and the alginate syringe-needle to earth [44].

The electrostatic bead generator used in this project was built by the mechanical workshop at the Department of physics at Norwegian University of Science and Technology in Trondheim (Figure 2.2).



(A) **(B)**

Figure 2.2 (A) The electrostatic capsule generator used in this project. The voltage coupled to the gelling bath and the alginate feeding-needle can be adjusted. The alginate solution is fed by a syringe by a syringe pump. (B) A capsule population made by the electrostatic capsule generator. In this example, the capsule diameter is approximately 0.5 mm.

The coated alginate capsule, which was developed in this project, has two main components: A capsule core material of cation (calcium or barium) alginate gel (made with the electrostatic capsule generator) enclosed with a fluorescent labeled alginate membrane. A schematic description of the coated capsule formation is given in Figure 2.3. The capsule components, its functions and characteristics are summarized in table 2.1.

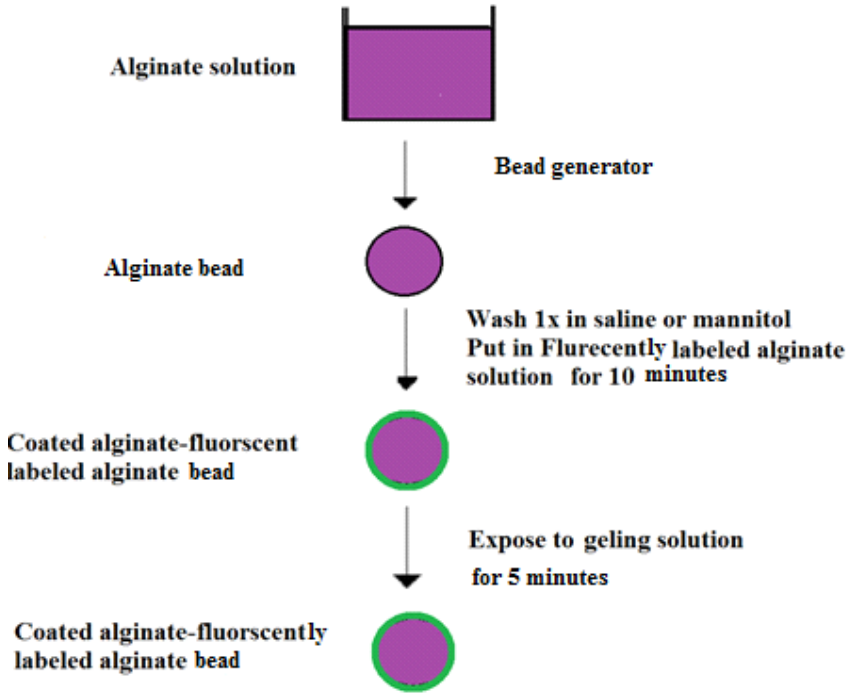


Figure 2.3 A schematic description of the formation of alginate-fluorescently labeled alginate microbeads.

Table 2.1 Function and characteristics of capsule materials [46].

Material	Function	Characteristics
Capsule core material	Entrapment of cells in spherical beads under mild conditions. Form a template for binding of the coating layer.	Rapid gel former. Mechanically and chemically stabile. Non-toxic, free from immunogenic contaminants, but some alginate materials have themselves shown to be immunogenic.
Coating	Change surface properties of the beads, e.g. elasticity, topography and biocompatibility	Non-toxic, non-biodegradable. free from immunogenic contaminants

In addition another coating strategy was used where alginate-PLL were coated with different kinds of alginate such as sulfated, high-G, high-M and epimerized alginate which they were labeled by fluoresceinamine.

The alginate beads used in this project were made from a 1.8% w/v solution of sodium alginate from the *Laminaria hyperborea* stipe (F_G=0.64, [Π]=980 ml/g) in 0.3 M mannitol. The coating alginate was the same alginate as core alginate labeled by fluorescence and dissolved in 0.9% NaCl (w/v) to different concentrations (0.5, 0.2 and 0.1% (w/v)).

2.2 Fluorescence

Fluorescence is the result of excitation, excited-state lifetime and fluorescence emission stages process in definite molecules called fluorophores or fluorescent dyes. Figure 2.4 shows the three stage process for the fluorescence of fluorophores explained by the electronic-state diagram (Jablonski diagram). In excitation stage (step 1) a photon of energy ($h\nu_{EX}$ in Figure 2.4) is provided by an outward source such as an luminescent lamp or a laser and absorbed through the fluorophore, making an excited electronic single state (S_1'). In second stage (step 2), excited-state lifetime, a finite time (usually 1–10 nanoseconds), the fluorophore undertakes conformational changes of likely interactions with its molecular environment. These processes have some main consequence where the energy of S_1' is partly degenerated, causing a relaxed single excited state (S_1) from which emitted fluorescence is created. In last stage (step 3), fluorescence emission, a photon of energy ($h\nu_{EM}$) is emitted and the fluorophore goes back to its ground state S_0. As a result of energy emission through the excited-state lifetime, the energy of this photon is lower and it has longer wavelength than the excitation photon $h\nu_{EX}$. The energy or wavelength differences demonstrated by ($h\nu_{EX} - h\nu_{EM}$) are called the Stokes shift. The Stokes shift is important to the sensitivity of fluorescence techniques since it lets emission photons to be sensed in contrast to a low background, remote from excitation photons [46].

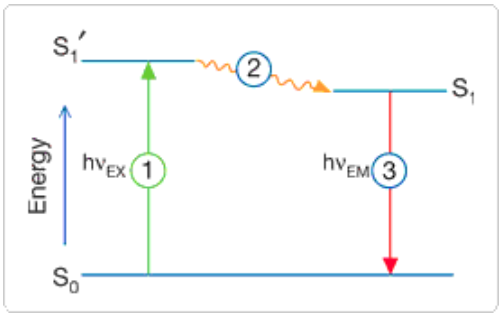


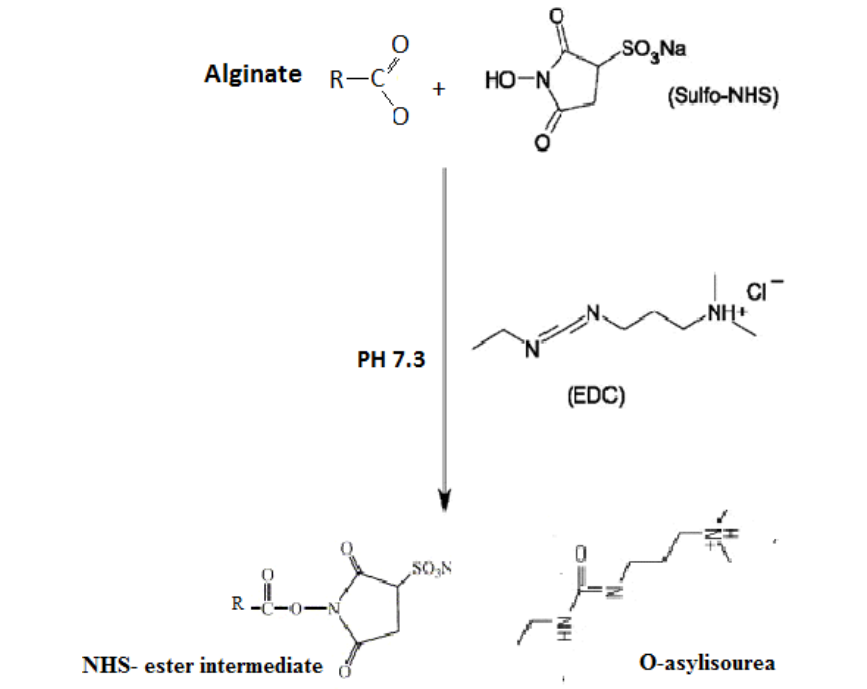
Figure 2.4 Jablonski diagram demonstrating the processes of the formation of an excited electronic singlet state by optical absorption and emission of light. The labeled stages 1, 2 and 3 are explained in the text [46].

The confocal laser scanning microscope (CLSM) is a useful device for gaining comprehensive information about polymers in a 3D network. Different phases as well as gradients of concentration of the polymer can be detectable in the CLSM. Since polymers typically do not have fluorescent constituents, which are described by a highly delocalized π-electron system, the polymers need to be labeled with fluorochromes. The fluorescent light intensity will be related to the concentration of fluorescence labeled components [47].

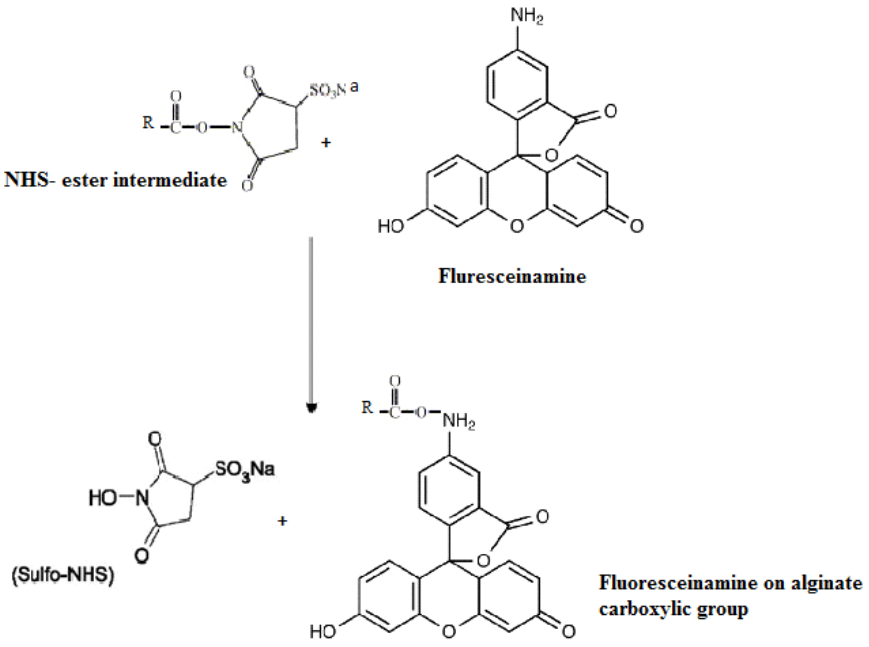
Qualitative binding of different polymers can be identified in the confocal microscope by labeling the polymers with different fluorochromes. The fluorochromes will emit light with different wavelengths, so different polymer giving rise to different colors that could easily separate from each other in the microscope.

2.3 Fluorescence labeling of alginate

Alginate can be fluorescently labeled by covalent binding of the amino groups of fluorescent molecules to the carboxylic groups of the alginate. To have easer formation of amide bonds between the carboxylic group and amine, carbodiimides can be used. The water-soluble carbodiimide EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] is an extensively used molecule for this purpose. It rejoins with a carboxylic group to form a very reactive intermediate. The adding of N-Hydroxysulfosuccinimide (Sulfo-NHS) to the carbodiimide reaction results in the formation of the intermediate Sulfo-NHS ester (Figure 2.5 A). This intermediate is less susceptible to hydrolysis than the EDC ester precursor made without Sulfo-NHS, hence giving more yields in the reaction with amino-containing ligands. This Sulfo-NHS intermediate reacts with a primary amine, which in this case is the primary amine of the flourochrome fluoresceinamine [48] (Figure 2.5 B). The result is a fluorescent alginate molecule with absorption and fluorescence emission maxima of approximately 490 nm and 520nm, respectively, hence emitting green light in the CLSM.



(A)



(B)

Figure 2.5 Covalent coupling of fluoresceinamine to alginate using carbodiimid chemistry. A) To enhance the amide bond formation, the use of the water-soluble carbodiimide EDC, together with sulfo-NHS produces an active sulfo-NHS ester intermediate. B) The activated carboxylic group of the sulfo-NHS ester intermediate reacts with the primary amine of the fluorescent component, fluoresceinamine [48].

2.4 Spectrophotometry

Spectrophotometry is a technical method based on the light absorption of a substance. Spectrophometry is a device to quantity light intensity in a part of the spectrum (Figure 2.6), as transmitted by specific substances.

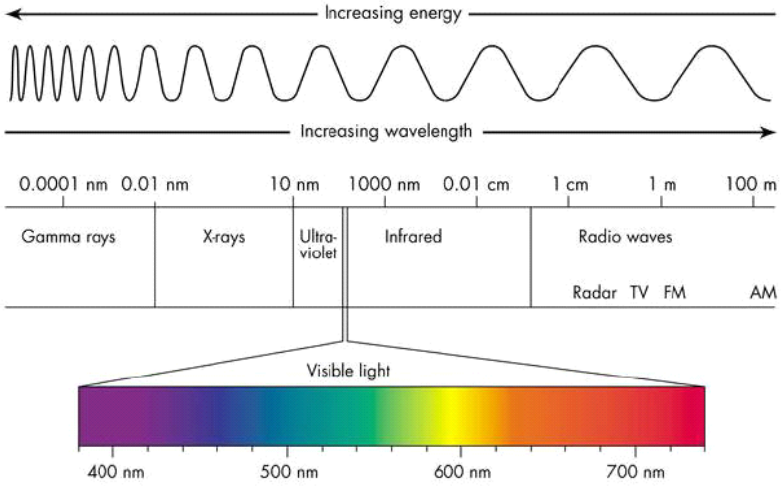


Figure 2.6 The electromagnetic spectrum; There are range of spectrophotometers which operate in the UV, visible and near infrared section of the electromagnetic spectrum [49].

2.4.1 Fluorescence spectrophotometry

Fluorescence spectrophotometry is measuring the quantity of fluorescence, such as photoluminescence, released by a substance whereas it is exposed to ultraviolet, visible, or other electromagnetic radiation. Light emitted intensity produced by a fluorescent molecule is a function of the solute concentration and consequently it can be used for analysis. Hence, here in our experiment by measuring the fluorescent intensity, it is possible to measure the quantity of coated alginate. Measurements are generally prepared by reference to dilutions of reference substance (Standard fluorescence intensity curve). Thus, the overall scheme for fluorescence spectroscopy is to excite by radiation at the maximum wavelength of absorption, and to measure or compare the intensity of the emitted light with reference solution [50].

Fluorescence intensity can be measured by spectrofluorometers which consists of a radiation or light source, a primary filter (monochromators), a sample chamber, a secondary filter (optical), and a fluorescence detection system (Figure 2.7) [51].

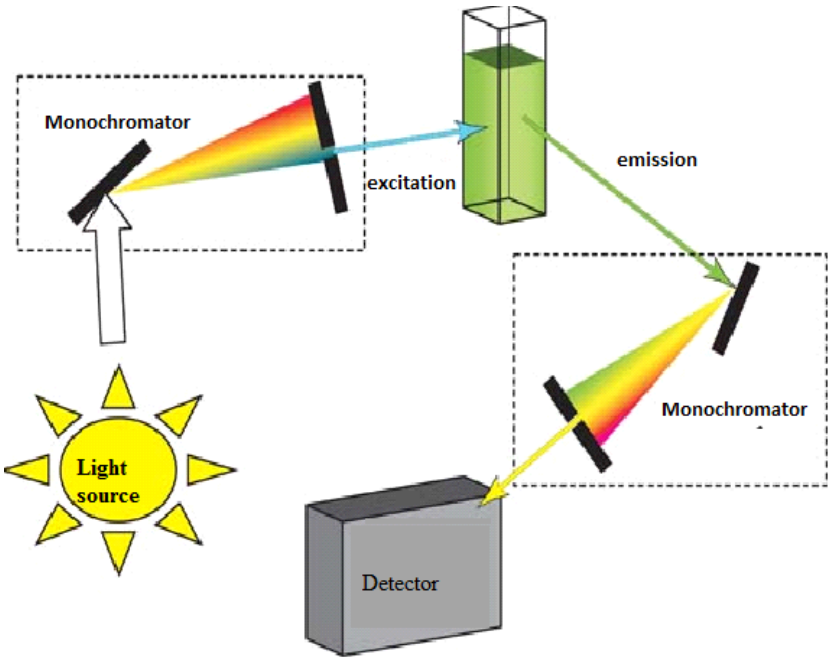


Figure 2.7 General schematic representation of fluorescence spectrometer [51].

Several kind of radiation sources are used in fluorescence spectrophotometry. The high pressure xenon arc lamp is frequently used in fluorescence spectrophotometry since it has an energy range covering from the ultraviolet into the infrared specter of light. Filters are used to remove unwanted scatter. The primary filter picks short wavelength radiation which can cause excitation on the test substance, whereas the secondary filter is usually allows the longer wavelength fluorescence to transmit but blocks the distributed excited radiation [50].

In this experiment, the *Infinite™ 200 series Quad-4 Monochromator™ microplate readers* (spectrophotometers) manufactured by Tecan Group Ltd in Switzerland is used to measure the fluorescent intensity of fluorescent labeled alginate in coated capsules.

2.5 Confocal scanning laser microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) has become a frequently used technique for studying structure in biological and medical research. It is possible to have high-resolution optical images with high selectivity by using CLSM [53]. In CLSM, the resolution of a usual light microscope is really enhanced by setting up a pinhole in the back-focal plane and the use of laser allows the excitation of fluorescence [47].

Specimens must be very thin and translucent to find figure on their inner structure by usual transmitted-light microscopy; otherwise image resolution will be reduced. In various cases it is a difficult to fulfill these requirements. The main feature of confocal microscopy is a facility to improvement in-focus images from selected depths, which the development called an optical sectioning. In ordinary microscopes the both magnifying lens (objectives) are illuminated and make available a view of a specimen. As the objective focuses light on planes below the specimen surface, the image quickly becomes inconceivable. The light is reflected by a lot of small particles, so that differentiating the object from its environment is difficult. In a CLSM, this is overcome by the collection of light that is reflected straight and only from the plane of interest [54].The light reflected, at the focus of the high-Numerical aperture objective is planned onto an adjustable pinhole diaphragm. Undesirable light coming from other specimen areas is concentrated outside the pinhole, which permits only a small fraction of it to pass, so that it really improves the limiting depth of focus usually existing in optical microscopes. Smaller pinhole causes a smaller amount of unwanted light or fluorescence from out-of-focus areas to be recognized on the photo-detector, so consequence is a very sharp image of a single plane in the specimen. The most common setup of a CLSM is depicted in Figure 2.8 [54].

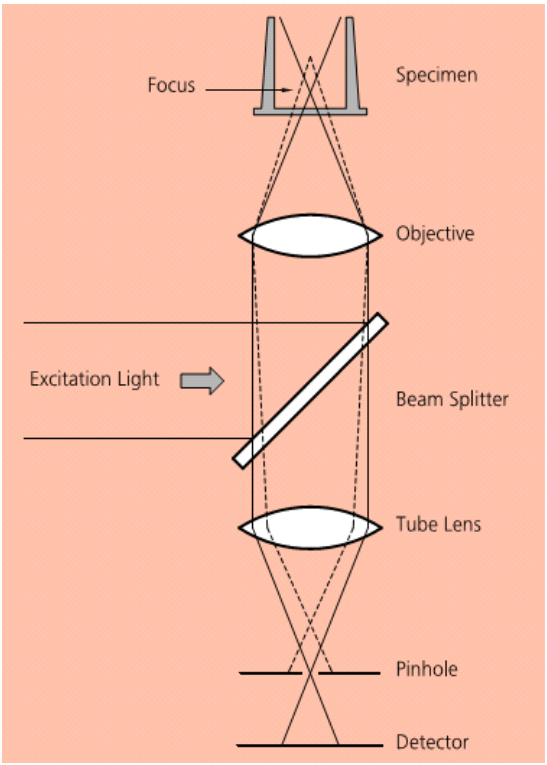


Figure 2.8 Setup of confocal laser scanning microscope [54].

To have an image of whole selected object plane, the object plane has to scan in a point-by-point, line-by-line raster by an XY light deflection system. In a CLSM, there are high resolution and the facility to produce 3D images. Sample complexes can be imagined via their fluorescence spectra by fluorescence labeling. By a distinct focusing (Z axis) program it is possible to combining 2D optical slides scans to 3D by z-stack (the z-axis of the sample) (Figure. 2.9) [54]. The depth resolution is determined by the wavelength of the light source (the lasers), in addition to the pinhole size. Common laser Ar laser (Argon) with λ=544 and 488 nm, the He-Ne laser (Helium-Neon) with a wavelength (λ) of 1152 nm (IR) or 632.8 nm, and a short wavelength He-Ne laser with λ=344 nm, can be used for reflection and fluorescent studies. The use of the fluorescent mode of the confocal microscope is the most used for imaging biological objects of various types [47].

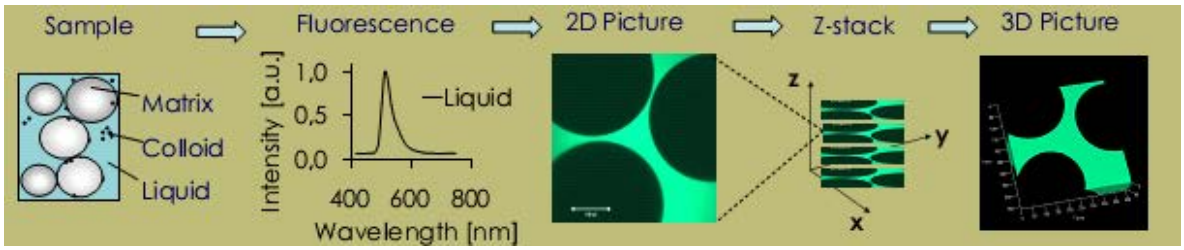


Figure 2.9 Usage of fluorescence spectra of sample compounds to create 2D and 3D images [62]

3. Aims

This project aims at exploring the coating of various alginate capsules (e.g. alginate beads and alginate-polycation capsules). Alginates gels cross-linked with different divalent ions will be explored for coating using ions leaking from the gel as well as a secondary exposure to gelling ions after the coating step. Gelling ions, washing solutions and alginate composition will be varied. Further will alginate-polycation capsules be tested for adhesion of various alginate coats and in particular interesting for coating with sulfated alginates. Fluorescently labeled alginates will be used for characterization of the coating with CLSM, and quantification of bound coating and stability of the coating will also be measured.

Despite many studies with confident results showing the successful use of alginate capsules as carriers of Langerhans islets *in vivo*, there is still room for capsule optimalisation in particular regarding biocompatibility.

The project aims at exploring the coating of both alginate microbeads and alginate-PLL microbeads with polymers. Fluorescently labeled polymer will be used for characterization of coating. Morphology of the coated alginate beads will be measured and further quantification of bound coating polymer and stability of coating layer will be evaluated using fluorescent spectroscopy and CLSM.

Objectives of the project are listed below:
- Fluorescently label alginate and sulfated alginates for use in capsule characterization
- Develop method for quantification of coating using fluorescent spectroscopy
- Use CLSM and fluorescent spectroscopy to analyses the coating of beads; both qualitatively and quantitatively
- Optimize coating (bound polymer and stability of coating):
A) For alginate microbeads coated with alginate
B) For alginate-PLL microcapsules

4. Experimental

4.1 Alginates

The composition, molecular weight (M_w) and intrinsic viscosity of the alginate used in this project is listed in Table 4.1.

Table 4.1 Chemical composition, molecular weight (M_w) and intrinsic viscosity [η] of alginate used in the project. F assigns the fraction of monomers and monomer sequences found in the sample and $N_{G>1}$ is the average length of G-blocks larger than 1.

Alginate Source	***L. hyperborea* stipe.**	***M. pyrifera* stipe**	**Mannuronic epimerized by AlgE4 and AlgE6 [*a]**	**Mannuronic epimerized by AlgE4 to poly MG[*b]**
Alginate type	High-G core and coating alginate	High-M coating alginate	Epimerized coating alginate	Sulfated coating alginate
F_G	0.63	0.43	0.55	0.46
F_{GG}	0.52	0.22	0.18	0
F_{MM}	0.26	0.36	0.08	0
F_{GGG}	0.48	0.16	0.15	0
F_{MGM}	0.067	0.18	0.35	1
$N_{G>1}$	12.6	4	7	0
M_w ($\times 10^{-3}$) [g/mole]	277	210	-	143
[η] [ml/g]	980	879	640	-

***a: The epimerized alginate made by Yrr.B.Mørch in 2007**

***b: The sulfated alginate made by Øystein Arlov in 2012**

4.2 Fluorescence labeling of alginate

To be able to examine the coating in the CLSM, the coating alginate had to be labeled with fluorescent molecules.

In this project, four type of alginate (high-G, high-M, epimerized (poly-MG) and sulfated alginate) were labeled with fluoresceinamin and used as coating layer. The same protocol of labeling were used for all four coating alginate.

The coating alginate was fluorescently labeled with the fluorochrome fluoresceinamine by slight modification of method previously described [43].

2.05 g of alginate were dissolved in 60 ml (half of total volume) distilled water and waiting for completely dissolving by stirring.

Then, 50 ml double concentration of MES buffer (0.2M MES, 0.6M NaCl pH-5.5) was added to the dissolved alginate. Here, to have MES with pH-5.5, we had to add 120 droplets of NaOH 1M.

EDC powder (0.208 g) (1-Ethyl-3-(3-dimethylaminopropyl) carbodiamide Hydrochloride, SIGMA®) was added to 9mM in 3 ml MES. Then it was added to alginate solution while stirring.

Immidiatly after adding EDC, Sulfo NHS powder (0.235 g) (N-Hydroxysulfosuccinimide sodium salt, from FLUKA) to 9mM was dissolved in 3 ml MES was added to alginate solution. The solution was kept at room temperature with stirring for 30 minutes.

The Fluoresceinamine powder (0.188 g) (C_{20} H_{13} NO_3, mixed isomers, SIGMA®) was dispersed in 4 ml MES and after that was added to a concentration of 4.5mM to the alginate solution. The reaction mixture was stirred at room temperature for 18 hours by magnetic stirrer (protected from light). The amount of fluoresceinamine theoretically corresponds to labeling 1/20 of the carboxylic groups.

To remove unreacted fluorescent molecules, the solution was transferred to dialysis membranes (from *Spectro/Por*, MWCO 12-14000) with 20.4 mm diameter. It was dialyzed against ion free water (three shifts) and then, was left over night at 4°C. Day two; it was dialyzed against 1 M NaCl for 24 hours (three shifts) and finally against ion free water until the water loosed its brown to yellow color (approx. 5 shifts). After that, the conductivity of surrounding solution was measured and the final conductivity in solution was 15 µs (Microsiemens). The final concentration for EDC, Sulfo-NHS, alginate and

Fluoresceinamine in the reaction mixture was 1.73, 1.95, 16.66 and 1.56 mM respectively. During dialysis, the solutions were protected from light.

The solution was filtered with Glass Micro Fiber Filter (Whatman) 2.7 µm, 1.6 µm, 0.7 µm and finally 0.22 µm pore size.

The pH of the alginate solutions were adjusted to 7.2-7.4 before the samples were freeze-dried (protected from light) on the vacuum freeze-dryer (type ALPHA 1-4 LD from CHRIST®) over night. The freeze-dried fluorescence labeled alginate was kept at room temperature protected from light until use [39].

4.3 Capsule formation

To find the best-suited parameters for the formation of alginate coat on alginate beads, different concentration of alginate for coating, with various gelling solutions ($CaCl_2$,$BaCl_2$ and $CaCl_2$ + $BaCl_2$) and with varying treatment in washing and second gelling solution were made by the electrostatic bead generator. The resulting capsules were evaluated in the CLSM and by fluorescence spectroscopy.

For core of alginate beads, about 1ml of 1.8% alginate solution from *Laminaria hyperborea* stipe high-G content (SF60: F_G=0.64, [η]=980 ml/g) (Table 4.1) in 0.3 M mannitol was filled in 5 ml syringe. A beaker below the needle was filled with different gelling solution (50 mM $CaCl_2$, 50 mM $CaCl_2$+ 1 mM $BaCl_2$ or 10 mM $BaCl_2$ in de-ionized water) in order to make different alginate beads. A magnet was placed in the beaker, and stirring turned on. The following conditions were used for microbeads formation:

- Needle size: 0.35 mm in outer diameter
- Flow: 10 ml/hr
- Voltage 7 kv
- Distance from needle tip to gelling bath 2 cm

Ca^{2+}-alginate beads were made by letting the alginate solution drip into 50 mM $CaCl_2$ solution in water while stirring. Ca^{2+}/Ba^{2+}-alginate beads were made by dripping alginate into 1 mM $BaCl_2$+ 50 mM $CaCl_2$ in 0.3 M mannitol solution. Ba^{2+}-alginate beads were made by using 10 mM $BaCl_2$ in 0.3 M mannitol solution as gelling solution. The droplet

size was controlled using above parameter that resulted capsules of approximately 400-500 μm in diameter. The beads were left in the gelling bath with stirring for at least 5 minutes after the last droplet and before they were transferred onto a filter.

The core of coated beads was coated by high-G fluorescence-labeled alginate with different concentration (0.5%, 0.2% and 0.1%) of the fluorescent labeled high-G alginate (FG=0.64, [η]=980 ml/g) in 0.9% saline (NaCl), to see effect of various concentration of coating alginate. To reduce the extra gelling florescent in coated beads solution, instead of using filter, the coating was down in tube in the following step:

1. The beads were washed 1x in 15 ml 0.9% NaCl or 15 ml 0.3 M mannitol, for 1 minute.

2. The beads washing solution were put in 15 ml tube to sediment the beads, for 2 minute.

3. The washing solution was removed and the beads exposed to fluorescent labeled alginate (in 0.9% NaCl), put on turn-over for 10 minute.

4. The beads were put on filter to remove fluorescent labeled alginate. A paper is used under filter to remove extra and unbound fluorescent labeled alginate around beads.

5. The beads were exposed to 10 ml different second gelling solution (50 mM $CaCl_2$, 50 mM $CaCl_2$+ 1 mM $BaCl_2$ and also 10 mM $BaCl_2$ in de-ionized water) in turn –over, for 10 or 5 minute.

6. The beads were stored 1x in 10 ml 0.3M mannitol.

The parameters tested in this screening procedure were:

- Concentration of coating alginate(high-G fluorescently labeled alginate)
- Different gelling ions both for core of alginate beads first (for core of alginate beads) and second gelling solution (for coating layer)
- Exposure time in second gelling solution
- Different washing solution (saline (NaCl) and mannitol) before coating

The screening was performed by varying the parameters above, one at a time, followed by an examination of the capsules in the CLSM.

In addition, the alginate-PLL capsules were coated in the following step:

1. 1 ml Calcium-alginate beads (used 1.8 % SF60 (high-G) alginate in 0.3 M mannitol) gelled in 50mM CaCl2 were made by using electrostatic capsule generator with 0.35 mm needle, 7kV and 10 ml/hr flow, ca. 2 cm between needle and gelling solution

2. The beads were washed 3x in 0.9 NaCl solution on filter, ca 1 min per wash, 10 ml saline per wash

3. The beads were exposed to PLL (0.06% PLL (HBr) in 0.9% NaCl, 10 ml) for 5 min (still on filter), smoothly shaking. The concentration of PLL-HBr is given relative to the concentration of PLL-HCl, so that 0.06% PLL-HBr in the text corresponds to 0.05% PLL-HCl.

4. The beads were washed 3 times in 0.9 % NaCl solution on filter, ca 1 min per wash, 10 ml saline per wash.

5. The beads were exposed to coating solution (0.1% coating, fluorescently labeled, in 0.9% NaCl, 10 ml) for 10 minutes

6. The beads were washed 3x in 0.9 NaCl solution on filter, ca 1 min per wash, 10 ml saline per wash

7. The capsules were stored in 0.3M mannitol

8. The capsules were quantified for bound coating alginate by fluorescent spectroscopy and CLSM, for 1, 3, 5 and 7 days after the capsules were made.

Fluorescently labeled coating alginates which used to make Alginate-PLL-Alginate were:

- Sulfated alginate
- High M alginate
- High G alginate
- Epimerized alginate

4.3 Long- term stability of coating layer

The long –term stability of alginate-fluorescent labeled alginate with various treatments was tested by measuring the amount of fluorescent on the coating layer over time (days) in coated capsules by fluorescent spectrophotometry. Six different types of alginate-fluorescent labeled alginate capsules were examined. The capsule parameters are given in table 4.2.

Table 4.2 the six different capsules were made for measuring long- term stability of coating layer

Capsule no.	Gelling ion	Core alginate	Washing solution	Coating alginate	Second Gelling ion
1	Ca^{2+} and Ba^{2+}	High-G	NaCl (Saline)	High-G fluorescent labeled alg.	Ca^{2+} and Ba^{2+}
2	Ca^{2+} and Ba^{2+}	High-G	Mannitol	High-G fluorescent labeled alg.	Ca^{2+} and Ba^{2+}
3	Ca^{2+} and Ba^{2+}	High-G	NaCl (Saline)	High-G fluorescent labeled alg.	Ba^{2+}
4	Ca^{2+} and Ba^{2+}	High-G	Mannitol	High-G fluorescent labeled alg.	Ba^{2+}
5	Ca^{2+}	High-G	NaCl (Saline)	High-G fluorescent labeled alg.	Ca^{2+} and Ba^{2+}
6	Ca^{2+}	High-G	NaCl (Saline)	High-G fluorescent labeled alg.	Ba^{2+}

Capsule without alginate coating layer (Ca^{2+}/Ba^{2+} –alginate capsule) were also made from high-G core alginate as control capsules. In addition, a standard curve for fluorescent intensity for concentrations from 0-50 mg/ml was prepared.

The coated capsules were prepared as described before in 4.2 section and then prepared for measuring fluorescent intensity by spectrophotometer.

4.3.1 Fluorescent spectroscopy

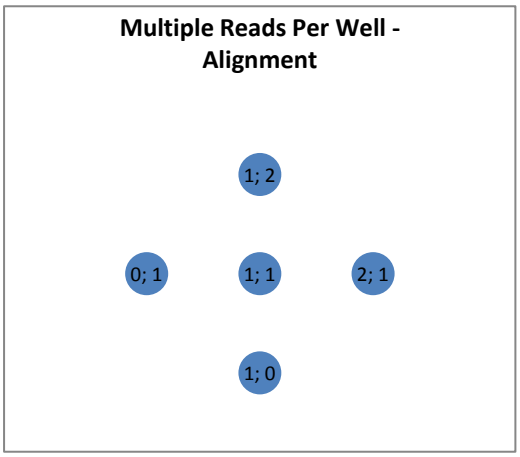
The concentration of the fluorescently labeled alginate on coated bead was monitored by reading the visible light at λ = 535 nm in the coated capsules. The spectrophotometer used was of the type *Infinite™ TecanUv-visible recording spectrophotometer.*

To prepare the samples for measuring the fluorescent intensity of coating capsules the following step is done:

- The capsules soaked in second gelling solution (fixation of coating alginate solution) washed with mannitol and kept in 15 ml tube at 4°C.
- Removed the extra mannitol solution above sedimented coated capsules until to have ratio of 1 to 5 of capsule to mannitol solution (here it was about 0.5 to 2.5).
- Mixed the sediment capsules with Mannitol by pipetting and then 100 µl of capsule solution by using the cut off tip pipet.
- 100 µl of 0.3M Mannitol added to 3 wells of *Nunclon 96 Flat Bottom Black Polystyrol LumiNunc FluoroNunc* for each different coated capsules

The parameter used in the fluorescent intensity measurements by spectrophotometry was as follows:

- Mode: Fluorescence Top Reading
- Multiple Reads per Well (Circle (filled)): 3 x 3
- Multiple Reads per Well (Border): 750 µm
- Excitation wave-length: 485 nm
- Emission wave-length: 535 nm
- Excitation Bandwidth: 9 nm
- Emission Bandwidth: 20 nm
- Gain: 100 Manual
- Number of flashes: 25
- Integration Time: 20 µs
- Z-Position (Manual): 18370 µm (Optimized)



3.5 Visualization of Capsules in the CLSM

The capsules were examined in a *LSM 510 confocal laser scanning microscope* by Carl Zeiss. All the settings for the confocal microscope and the imaging of capsules were computer-controlled. The settings used for imaging of the coated alginate capsules with fluorescent labeled alginate are listed below.

- **Computer:** Siemens SCENIC with two 21" screens
- **Computer Program:** LSM 510 by Carl Zeiss
- **Ocular:** Plan-NeoFluar 10×/3W
- **Laser:** 488nm: Argon 458, 488, 514
- **Laser power:** 81.2
- **Filters:** 505–530 bypass (BP)
- **Excitation**: 20%
- **Pinhole:** 199
- **Optical slice:** <30.7µm
- **Stack size:** 512×512
- **Pixel depth:** 8 bit
- **Detector gain:** 771
- **Amplifier gain**: 1

In order to obtain an image of the capsules' core center, all capsules were examined by scanning through an equatorial slice of the capsules. Eight scans were performed, and the mean was used to reduce the noise. The same settings including laser power were used on the all result in order to obtain comparable intensities.

5. Results

5.1 Optimalisation of coating

In the procedure of making alginate microbeads coated with alginate, different parameters for the formation of coating was optimized including type of first and second gelling solution, washing steps, concentration and exposure time of coat and coat solvent. A summary of all optimalisation step are given in Table 5.1. We were interested to see if the treatments affected the binding and distribution of high-G coating alginate in coated capsule. The coated alginate capsules were stored in mannitol solution and studied in the CLSM. To reduce the noise and increasing the clarity of images eight scans were performed and the mean was used. The same settings including laser power were used on all result in order to obtain comparable intensities. The images were constructed from optical cross-sections through equator of all beads sample by scanning through an equatorial slice of the capsules (optical slice < 30.7 μm).

5.1.1 Coating capsules with different washing

Ca^{2+}-capsules were examined for effect of washing in mannitol solution before exposure to fluorescent labeled alginate. Without washing, the capsules were trapped in fluorescent labeled alginate that was forming a gel due to excess of divalent cations around the capsules. Lot of fluorescent labeled alginate was lost and was also difficult to see the coating layer (Appendix A Figure A.1).

Further, dissolving the coating alginate in 0.9% NaCl (w/v) could make better fluorescent coated beads than labeled alginate dissolved in 0.3M mannitol (Appendix A Figure A.2).

To see if there were any visible differences in the coated capsules washed with saline or mannitol solution before exposure to coating solution, calcium/barium capsules (1.8%) coated with fluorescent labeled alginate (0.1%) , and $BaCl_2$ (10mM) used as second gelling solution, were prepared. The resulting Ca^{2+}/Ba^{2+} coated capsules (washed with saline (0.9% NaCl (w/v)) or mannitol solution) were compared in CLSM. The resulting images are given in Figure 4.1.

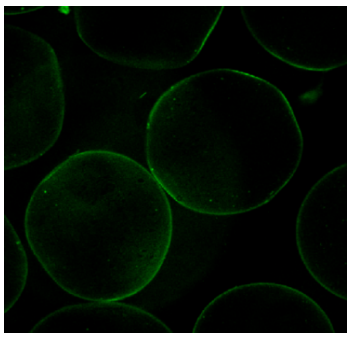
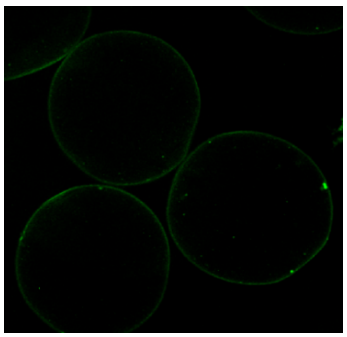
A)  B) 

Figure 4.1: Ca^{2+}/Ba^{2+}capsules coated 0.1% fluorescent labeled high-G alginate dissolved in NaCl, second gelling solution: $BaCl_2$ (10mM) , A) Washed with saline B) Washed with Mannitol solution.

The images in Figure 4.1 show that capsules washed with saline (A) have higher intensity of coating layer than washed with mannitol solution (B). In addition, the same effect of washing was seen for capsules with different gelling ions in first and second gelling bath (Figure 4.3).

The 1.8% (w/v) high-G alginate capsules were coated with different concentration (0.5%, 0.2% and 0.1% (w/v)) of high-G fluorescent labeled alginate dissolved in 0.9% NaCl to see if concentration had some effect on distribution of coating alginate. Using high concentration of fluorescent labeled alginate (0.5% and 0.2%) caused gelling in the solution of fluorescent alginate when beads were added. By contrast, by using lower concentration of fluorescent labeled alginate 0.1%, gellation in the coating solution was avoided (Table 4.1).

Exposure time for second gelling solution, which was used after exposure to coating alginate solution for fixation of coating layer, was examined to see the effect on alginate coating. The result of CLSM images shows that there are no visible differences as effect of different exposure time (10 and 5 minute) for second gelling solution (Appendix A Figure A.3).

The affinity of alginate for different divalent cations varies with the alginate and the cation [14]. We wanted to see how the higher affinity of alginate to Ba^{2+} than to Ca^{2+} influenced the coated microcapsule structure. Therefore, different divalent cations (Ca^{2+}/Ba^{2+}, Ca^{2+} and Ba^{2+}) capsules washed with mannitol solution before exposure to coating solution were examined to see the effect on the coating layer. In addition, uncoated alginate was used as control to be sure that background fluorescence did not influence the CLSM images. Figure 4.2 shows the control Ca^{2+}/Ba^{2+} capsules and different divalent cation-capsules coated with 0.1% fluorescent labeled alginate washed with mannitol and same first and second gelling solution. The thicknesses of coating layer

in alginate capsules with different divalent cation were in following order: Ca^{2+}/Ba^{2+} capsules > Ca^{2+}capsules > Ba^{2+} capsules.

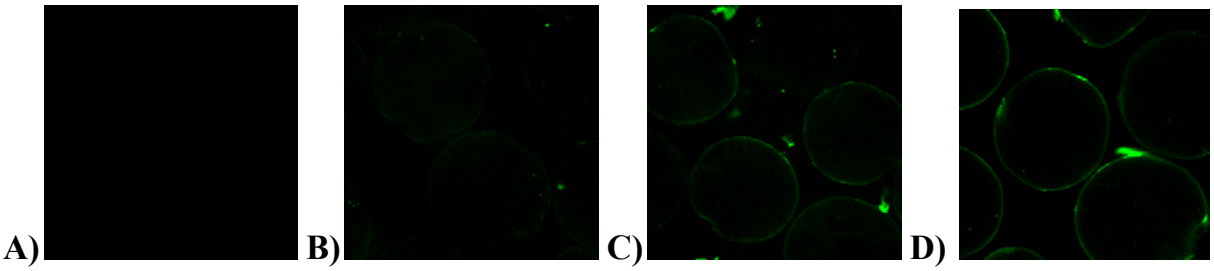


Figure 4.2: Coated beads with 0.1% fluorescent labeled high-G alginate dissolved in NaCl, washed with mannitol solution, A) Control Calcium-Barium beads without Coating Fluorescent labeled alginate B) Barium beads, 2end gelling solution: $BaCl_2$ C) Calcium beads, 2end gelling solution: $CaCl_2$ D) Calcium-Barium beads, 2end gelling solution: $CaCl_2$ + $BaCl_2$

In continuation to see effect of different treatments six coated capsules with different washing and different gelling solution (first and second gelling solution) were examined by CLSM. As we can see in Figure 4.3, the images illustrate that washing with saline make higher intensity of coating layer than capsules washed with mannitol solution. In addition, calcium beads (Calcium as first gelling solution) had thinner coating layer when gelled in calcium/barium.

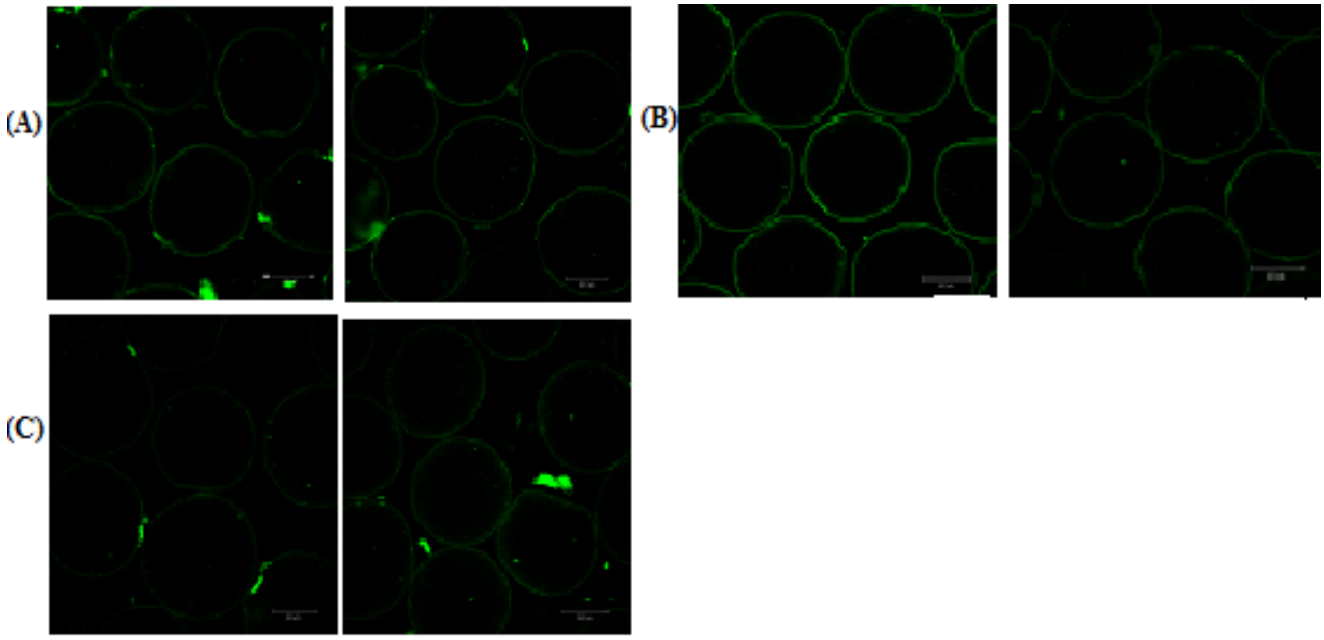


Figure 4.3 Coated calcium/barium (A and B) and calcium (C) beads with 0.1% fluorescent labeled high-G alginate dissolved in 0.9% (w/v) NaCl A) Washed with saline (Left) and mannitol solution (Right), $CaCl_2$ + $BaCl_2$ used as second gelling solution (B) Washed with saline (Left) and mannitol (Right), $BaCl_2$ used as second gelling solution (C) Both capsules washed with saline (left and right), $CaCl_2$ + $BaCl_2$ (left) and $BaCl_2$ (right) used as second gelling solution.

Table 4.1The various capsules made from different gelling solution (first and second), different concentration of high-G fluorescent labeled coating alginate, different exposure time and different coating solvent.

Sample	1st Gelling solution	1st washing solution	Fluorescent alginate coating	2nd Gelling solution	2nd washing solution	Comments
1	Ca^{2+}	Mannitol	0.5% in saline	Ca^{2+} , 5min	Mannitol	High gel formation in the coating solution.
2	Ca^{2+}	Mannitol	0.2% in saline	Ca^{2+}, 5min	Mannitol	Lower gel formation in the coating solution.
3	Ca^{2+}	Mannitol	0.1% in saline	Ca^{2+}, 5min	Mannitol	Little gel formation in the coating solution. The coating was better than Ba^{2+}-beads.
1	Ca^{2+} and Ba^{2+}	Mannitol	0.5% in saline	Ca^{2+} and Ba^{2+}, 5min	Mannitol	High gel formation in the coating solution.
2	Ca^{2+} and Ba^{2+}	Mannitol	0.2% in saline	Ca^{2+} and Ba^{2+}, 5min	Mannitol	Lower gel formation in the coating solution.
3	Ca^{2+} and Ba^{2+}	Mannitol	0.1% in saline	Ca^{2+} and Ba^{2+}, 5min	Mannitol	Little fluorescent label alginate was gelling in coated beads solution. The coating was better than for Ca^{2+}-beads and Ba^{2+}-beads.
1	Ba^{2+}	Mannitol	0.5% in saline	Ba^{2+}, 5min	Mannitol	High gel formation in the coating solution.
2	Ba^{2+}	Mannitol	0.2% in saline	Ba^{2+}, 5min	Mannitol	Lower gel formation in the coating solution.
3	Ba^{2+}	Mannitol	0.1% in saline	Ba^{2+}, 5min	Mannitol	Little fluorescent label alginate was gelling in coated beads solution. The coating was less coating than for other gelling solutions
1	Ca^{2+} and Ba^{2+}	Saline	0.1% in saline	Ca^{2+} and Ba^{2+}, 5min	Mannitol	The coating was best on the surface of beads.
2	Ca^{2+} and Ba^{2+}	Mannitol	0.1% in saline	Ba^{2+}, 5min	Mannitol	The coating was weaker when using saline for washing.
3	Ca^{2+} and Ba^{2+}	Saline	0.1% in saline	Ba^{2+}, 5min	Mannitol	The coating was good but there was some fluorescent leakage from the surface to core of beads.
1	Ca^{2+}	Mannitol	0.5% (w/v) in mannitol	Ca^{2+}, 5min	Mannitol	The coating was lower than coated capsules with coating alginate dissolved in saline. The coating was better than Ba^{2+}-beads.
2	Ca^{2+} and Ba^{2+}	Mannitol	0.5% (w/v) in mannitol	Ca^{2+} and Ba^{2+}, 5min	Mannitol	The coating was lower than coated capsules with coating alginate dissolved in saline. The coating was better than ca-beads.
3	Ba^{2+}	Mannitol	0.5% (w/v) in mannitol	Ba^{2+}, 5min	Mannitol	The coating was lower than coated capsules with coating alginate dissolved in saline. The coating was less coating than for other gelling solutions.
1	Ca^{2+}	Mannitol	0.5% (w/v) in saline	Ca^{2+}, 5min	Mannitol	The coating was better than coated capsules with coating alginate dissolved in mannitol.
2	Ca^{2+} and Ba^{2+}	Mannitol	0.5% (w/v) in saline	Ca^{2+} and Ba^{2+}, 5min	Mannitol	The coating was better than coated capsules with coating alginate dissolved in mannitol.
3	Ba^{2+}	Mannitol	0.5% (w/v) in saline	Ba^{2+}, 5min	Mannitol	The coating was better than coated capsules with coating alginate dissolved in mannitol.
1	Ca^{2+} and Ba^{2+}	Saline	0.1% (w/v) in saline	Ca^{2+} and Ba^{2+}, 5min	Mannitol	There was no effect on different exposure time in coating binding.
2	Ca^{2+} and Ba^{2+}	Saline	0.1% (w/v) in saline	Ca^{2+} and Ba^{2+}, 10 min	Mannitol	There was no effect on different exposure time in coating binding.

4.2 Binding and long-term stability of coating layer

4.2.1 Fluorescent spectroscopy

The long-term stability of the coating layer in various capsules with different treatments was tested by measuring the fluorescent intensity of coated capsules over time. The long-term stability of six different types of alginate beads coated with fluorescent labeled alginate, made of high-G alginate for both core and coated layer, was examined. Capsules were made from 1.8% (w/v) alginate solutions and washed with saline (0.9% (w/v) NaCl) or Mannitol 0.3M before they were coated with a high-G fluorescent labeled alginate (0.1% w/v). Capsules were made with either calcium (50 mM $CaCl_2$) or with both calcium and barium (50mM $CaCl_2$ and 1mM $BaCl_2$) in the gelling solution. After coating the second gelling solution consisted of either high concentration of barium (10mM $BaCl_2$) or a mixture of calcium and barium (50mM $CaCl_2$ and 1mM $BaCl_2$).

The results from long-term stability of coating for the calcium/barium and calcium alginate coated capsules with different second gelling solution and washing solution is presented in Figure 4.4. The fluorescent intensity varied between 450 and 800 for corresponding amounts of control beads (Ca^{2+}/Ba^{2+}-alginate beads without fluorescent coating) (Appendix C, Figure C.1). The fluorescent intensity was measured every one or two days for the first two weeks then every a week until forty nine days.

All capsules were stored at 4°C, in darkness. The concentration of fluorescent labeled alginate on the coated capsules was monitored by reading the fluorescent coated capsules.

A standard curve for fluorescent intensity of fluorescent labeled alginate and the raw data from the spectrophotometery measurements are given in Appendix B and Appendix C, respectively.

Figure 4.4 illustrate that Ca^{2+}/Ba^{2+}-alginate beads coated with fluorescent labeled alginate and washed with saline had highest fluorescent intensity both initially and over time in storage solution. Therefore, the capsules exposed to Ba^{2+} in 2nd gelling solution lost some of the initially bound coating during first two weeks resulting in less fluorescent intensity during long time storage (15-49 days).

A similar trend was observed for the Ca^{2+}-beads. In Figure 4.4, we can see also that calcium beads with different second gelling solution have lower fluorescent intensity than Ca^{2+}/Ba^{2+}-beads, as we have seen previously (Figure 4.2). However, the use of barium as fixation for coating layer in calcium-beads resulted in higher coating than calcium

capsules fixed by calcium and barium. However reduced intensity over the first two week, intensity resulted in similar intensities during long term storage.

Figure4.4 illustrates that in some initial days all coated capsules loss of the coating layer. However, the first two weeks for the capsules exposed to Ba^{2+}/Ca^{2+} second gelling solution, the coating remained stable throughout the time of measurements, 49 days, as we can see the fluorescent intensity is not decreasing over time.

As we have seen before (Figure 4.1), in Figure 4.5 also show that the two Ca^{2+}/Ba^{2+} coated capsules washed with mannitol solution has lower initial intensity as well as intensity over time than capsules washed with saline (Figure 4.4). In this case a loss of intensity was observed for the first 3 days when Ba^{2+} was used as 2^{nd} gelling solution, whereas loss of intensity was observed over the first two weeks for Ca^{2+}/Ba^{2+} as 2^{nd} gelling solution. Figure 4.5 and 4.4 shows that the long term stability of coating is the same as washed with saline and both has high stability of coating.

Figure 4.6 illustrate the relative concentration of coating layer (fluorescently labeled High G alginate) for capsules washed with saline calculated from standard fluorescent labeled alginate curve (Appendix B). The calculation is given in Appendix B. Figure 4.6 shows the concentration of coating layer in Ca^{2+}/Ba^{2+}-beads coated with high-G alginate and since the same coating alginate was used for all samples, Figure 4.6 reflected Figure 4.4.

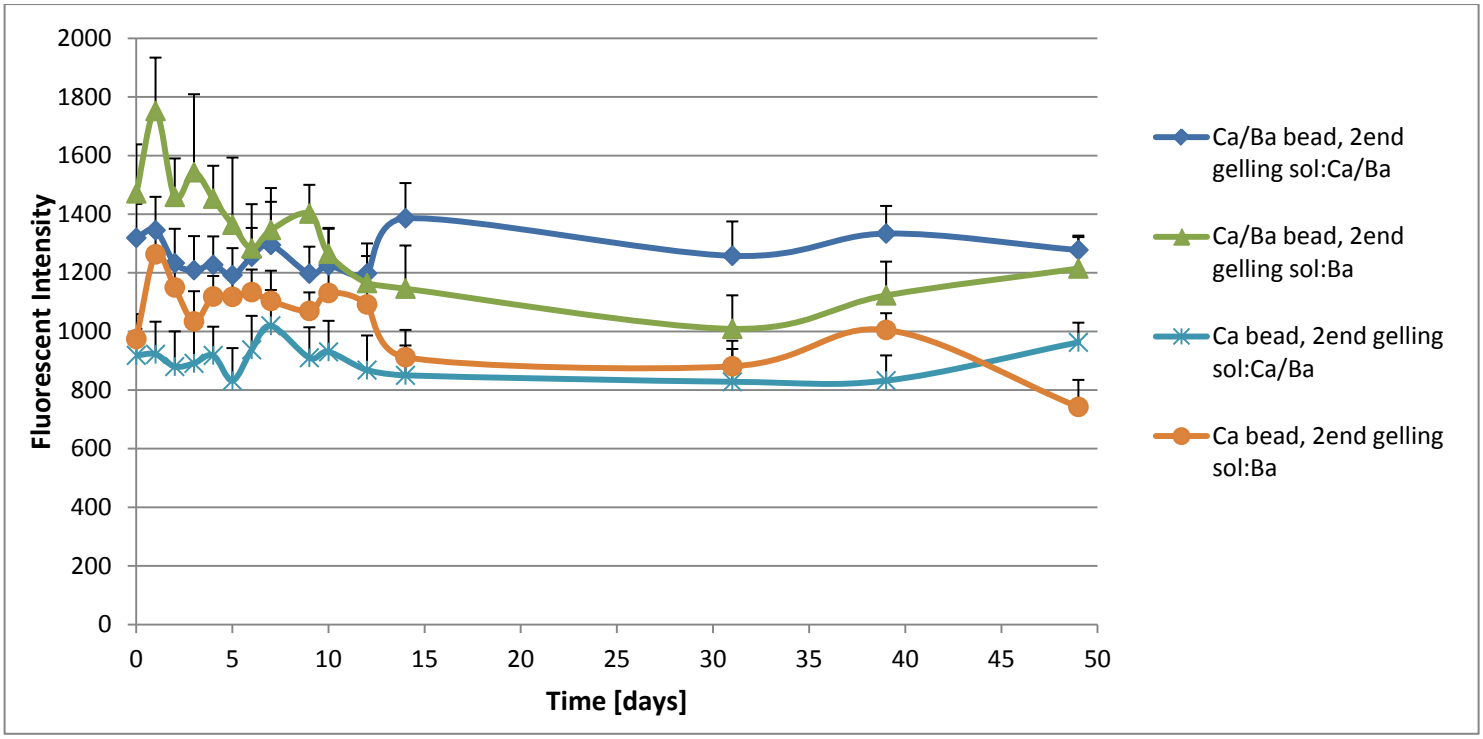


Figure 4.4 Long-term stability of coating layer, measured as the fluorescent intensity capsules as a function of time for all four different coated capsules where all capsules were washed with saline (0.9 % (w/v) NaCl).

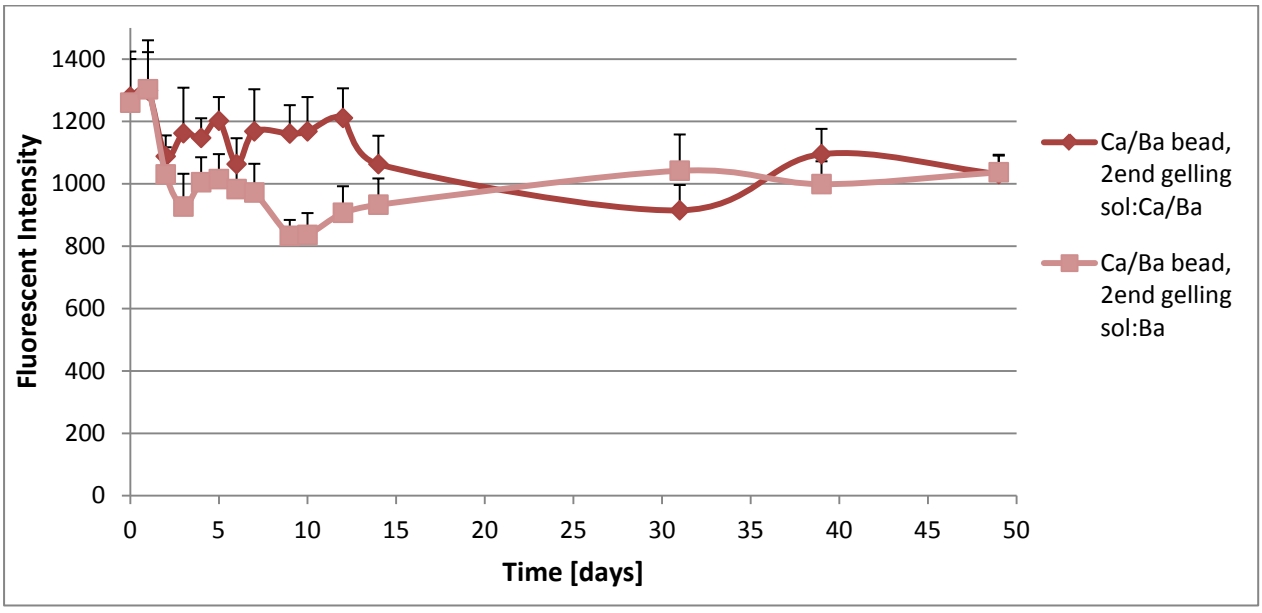


Figure 4.5 Long-term stability of coating layer, measured as the fluorescent intensity of coated layer in capsules as a function of time for two calcium/barium coated capsules where capsules were washed with mannitol. Used two different second gelling solution were used for fixation of coating layer: $BaCl_2$ (10mM) or $CaCl_2$ (50mM)+ $BaCl_2$ (1mM).

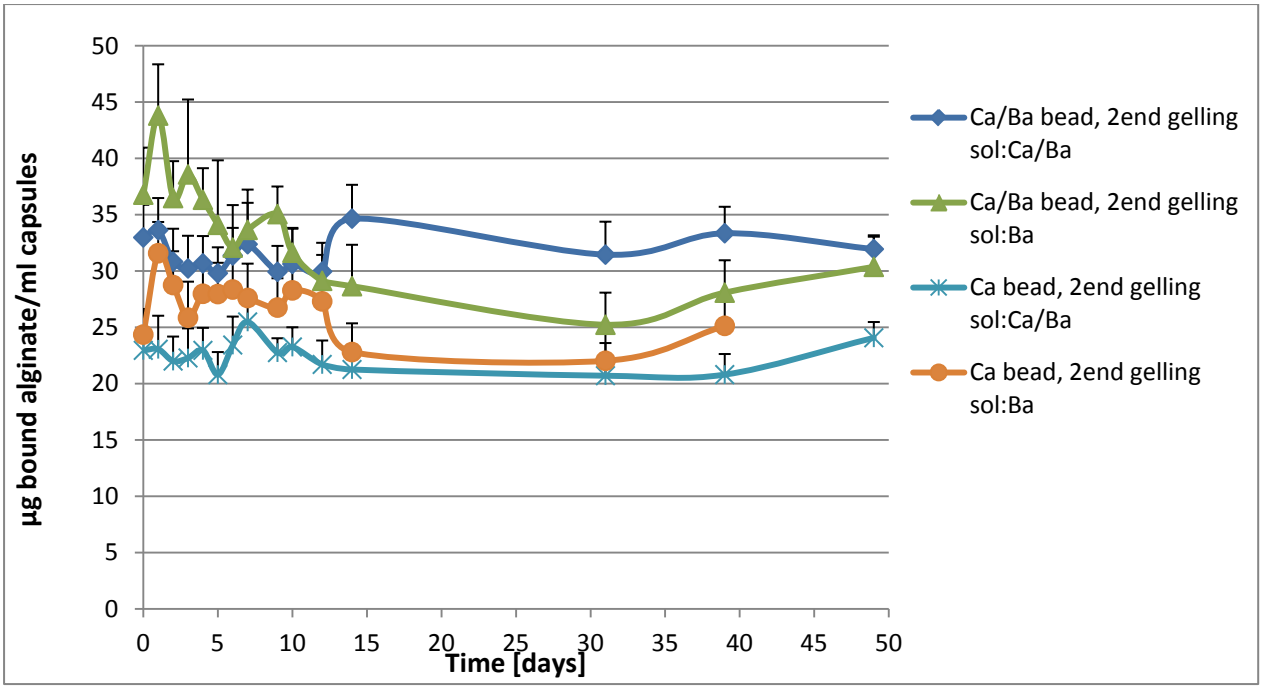


Figure 4.6 Long-term stability of coating layer, calculated by using standard curve as the fluorescent concentration of coated layer in capsules as a function of time for four calcium/barium and calcium coated capsules where capsules washed with saline.

4.2.2 Visualization of capsules for long-term stability by CLSM

The stability assay showed that calcium/barium capsules were a better template for coating with fluorescent labeled alginate than calcium-capsules. In addition, it was shown that washing capsules before exposure to coating solution with saline (0.9% NaCl (w/v)) has more effect on initial binding and stability of coating layer than when washed with mannitol solution. Therefore, it was of interest to see if there were any *visible* differences and capsules prepared to the fluorescence spectroscopy measurements were also visualized in a confocal laser scanning microscope (CLSM).

Images of the capsules were made by scanning through the capsule equator, giving a section of the beads. Images were taken almost every day for first and second week, then one a week for the next 2 weeks. The capsules stored in 4°C in darkness.

The CLSM images for the calcium-barium and calcium alginate beads coated with fluorescently labeled alginate capsules are presented in Figures 4.7-4.10 and Figures 4.11, respectively.

Figure 4.7 illustrates how the coating alginate layer (high G fluorescently labeled alginate (green)) covered the core of calcium/barium alginate (without fluorescent labeling) washed with saline and $CaCl_2$ + $BaCl_2$ as second gelling solution over time in storage solution. It can be seen from Figure 4.7 that the coated beads had high stability which corresponds to the observations from the fluorescence spectroscopy (Figure 4.4) and the fluorescent intensity (coated layer) was not reduced very much over time. Some variation in the fluorescent intensity of the coating was observed over time without any apparent explanation.

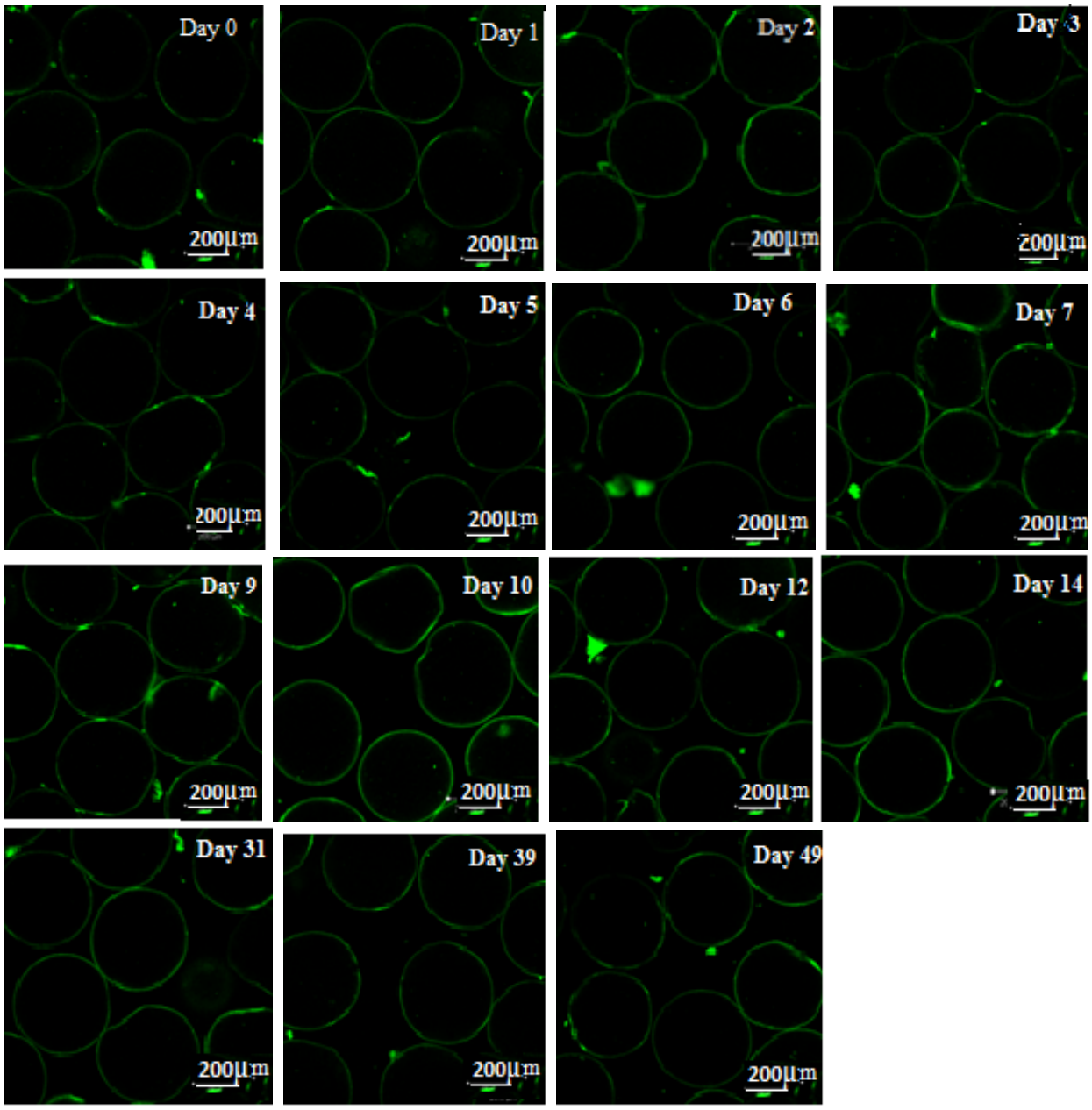


Figure 4.7 Confocal microscopy images of Ca^{2+}/Ba^{2+} capsule washed with 0.9% NaCl (w/v) before coating with fluorescent labeled high-G alginate. Second gelling solution: $CaCl_2$ and $BaCl_2$. Scale bar represented 200µm.

Figure 4.8 illustrates the confocal images of the Ca^{2+}/Ba^{2+} capsule washed with mannitol solution and $CaCl_2$(50mM) + $BaCl_2$ (1mM) as second gelling solution over time in storage solution. It can be seen from Figure 4.8 that the coated beads washed with mannitol had high stability which corresponds to the observations from the fluorescence spectroscopy (Figure 4.4) and the fluorescent intensity (coated layer) was not reduced very much over time. Lower intensity of coating layer was seen in the coated beads washed with mannitol than coated beads washed with saline (Figure 4.7).

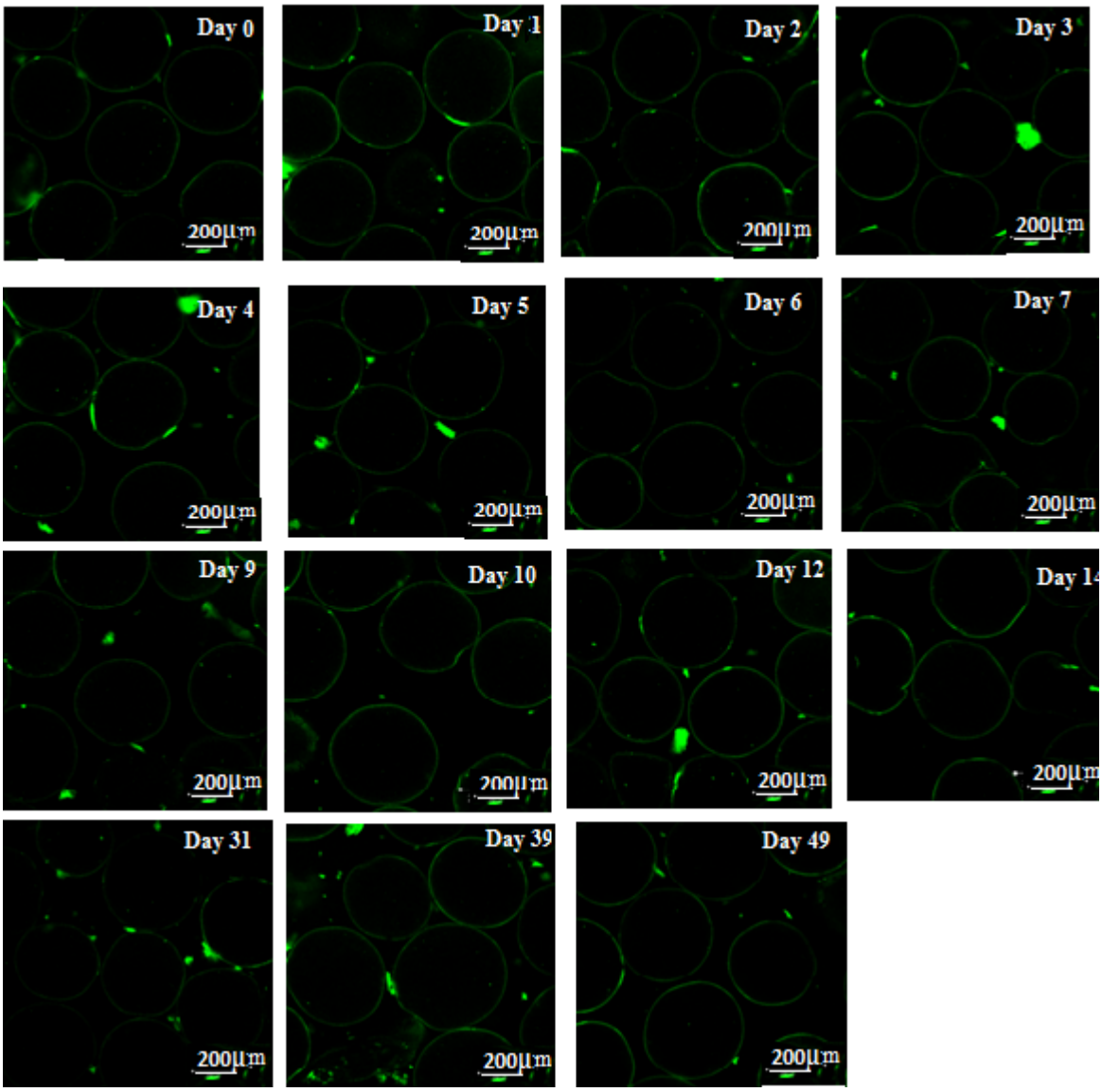


Figure 4.8 Confocal microscopy images of Ca^{2+}/Ba^{2+} capsule washed with Mannitol solution before coating with fluorescent labeled high-G alginate. Second gelling solution: $CaCl_2$ and $BaCl_2$. Scale bar represented 200µm.

Figure 4.9 shows CLSM images of how the coating alginate forms a thin membrane (green) on top of Ca^{2+}/Ba^{2+} alginate capsule washed with saline and $BaCl_2$ (10mM) as second gelling solution to stabilize the coating layer. It can be seen from Figure 4.9 that the Ca^{2+}/Ba^{2+} coated beads washed with NaCl and fixed with barium have highest initial intensity of coating layer between the all six capsules which corresponds to the observations from the fluorescence spectroscopy (Figure 4.4). However, gradually over time the coating layer was reduced. From day 4, reduced intensity of the coating layer can be observed.

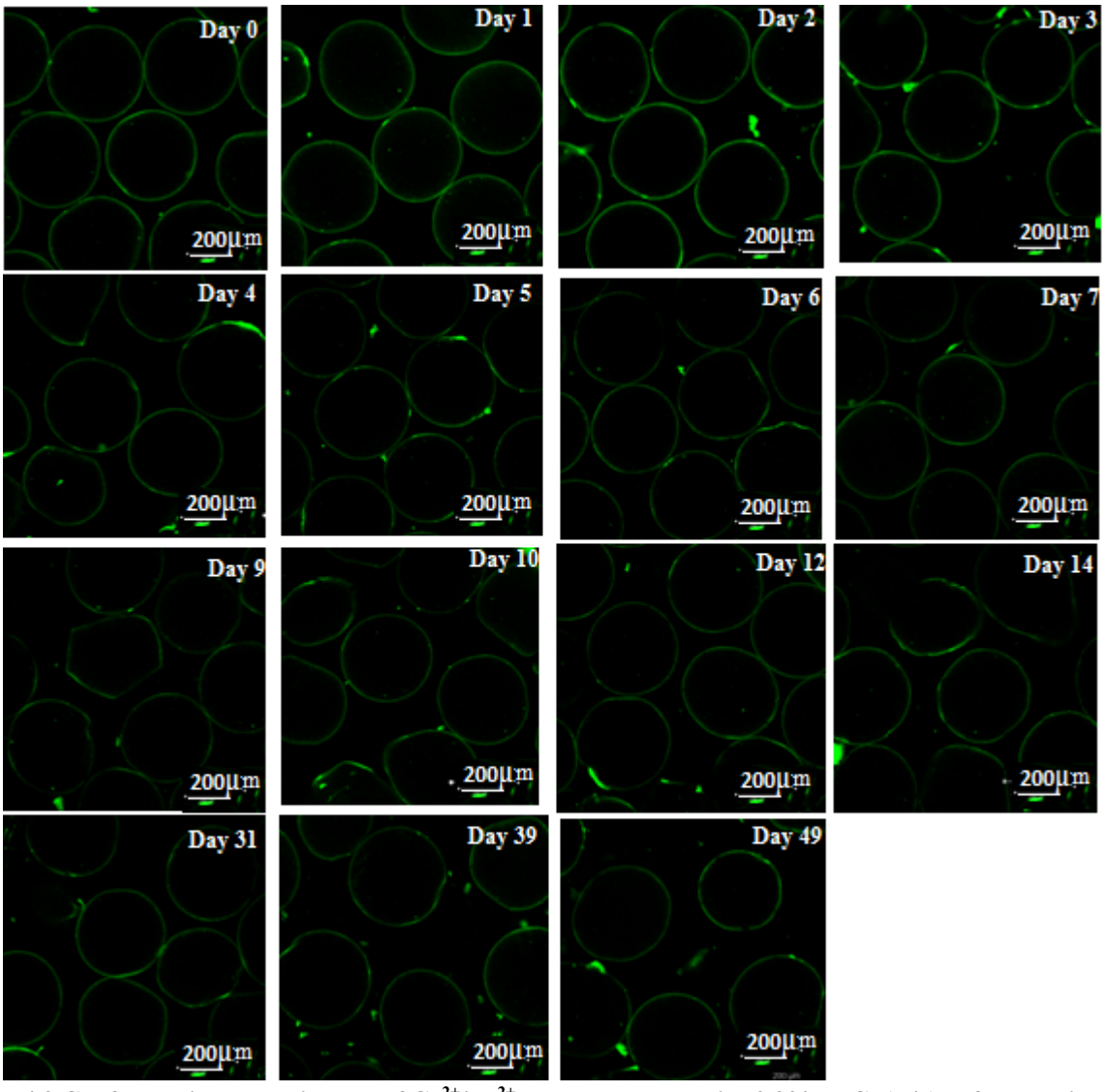


Figure 4.9 Confocal microscopy images of Ca^{2+}/Ba^{2+} capsule washed with 0.9% NaCl (w/v) before coating with fluorescent labeled high-G alginate. Second gelling solution: BaCl2. Scale bar represented 200µm.

In Figure 4.10, Ca^{2+}/Ba^{2+} alginate capsule with high G fluorescent labeled alginate washed with mannitol solution and $BaCl_2$ (10mM) as second gelling solution is shown. It can be seen from Figure 4.10 that the Ca^{2+}/Ba^{2+} beads washed with mannitol have lower intensity of coating layer than beads washed with 0.9% NaCl (w/v) which corresponds to the observations from the fluorescence spectroscopy (Figure 4.4). By comparing the images of first and last day in Figure 4.10, it can be seen that coating is stable throughout the time of the experiment.

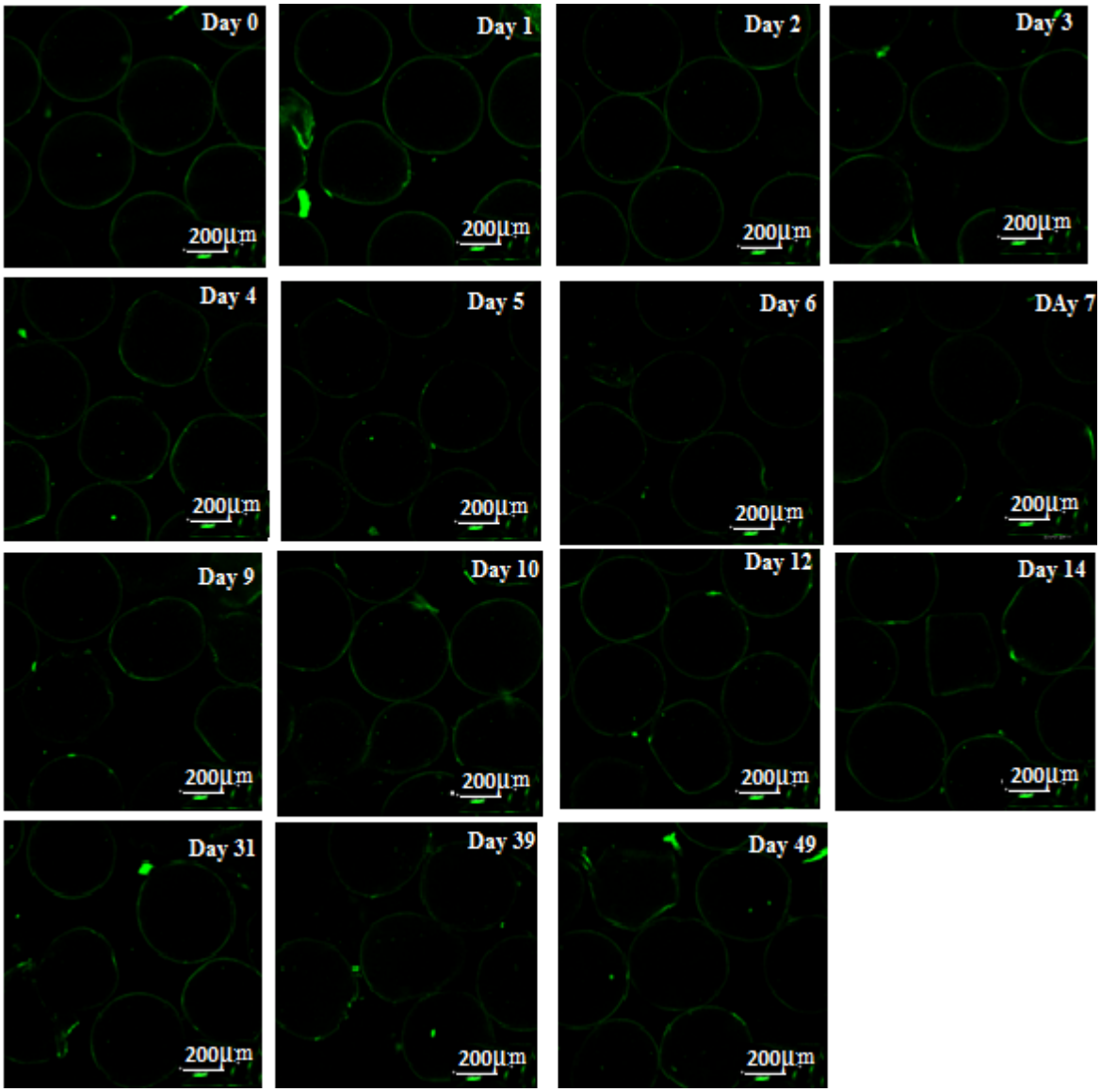


Figure 4.10 Confocal microscopy images of Ca2+/Ba2+ capsule washed with Mannitol solution before coating with fluorescent labeled high-G alginate. Second gelling solution: BaCl2. Scale bar represented 200µm.

The CLSM images in Figure 4.11 are of calcium capsules coated with fluorescent labeled high-G alginate, using $CaCl_2$ (50mM) + $BaCl_2$ (1mM) or $BaCl_2$ (10mM), after exposure to coating solution. Both were washed with saline. Calcium beads (Figure 4.11) have less coating than calcium/barium beads (Figure 4.7 and 4.9) with same washing solution (saline) in either fixed capsules with $CaCl_2$ (50mM) + $BaCl_2$ (1mM) or $BaCl_2$ (1mM). In fact, the lowest coating between the six made capsules with different treatment could be seen in calcium capsules washed with saline either fixed capsules with $CaCl_2$ (50mM) + $BaCl_2$ (1mM) or $BaCl_2$ (1mM). Some days out of 49 days is shown in Figure 4.11 because it was the same images from day 0 to day 49 where hardly any coating could be detected. However, calcium coated capsules used $BaCl_2$ (10mM) as second gelling solution shows higher intensity of fluorescent in spectrophotometer measurement (Figure 4.4).

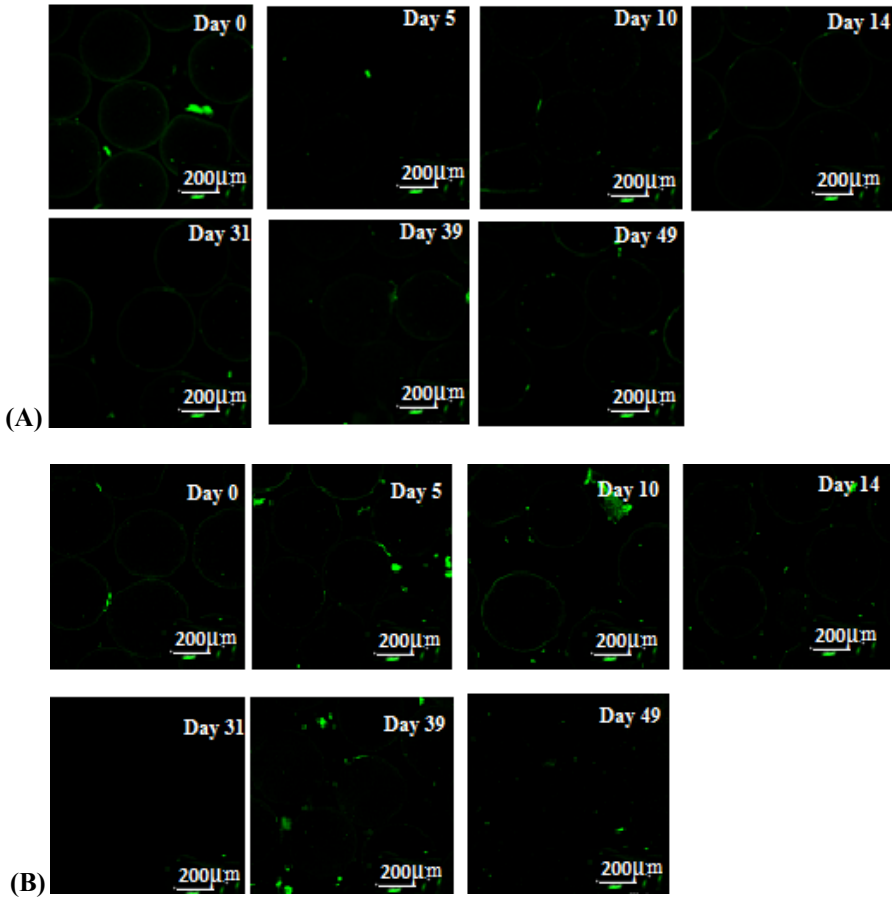


Figure 4.11 Confocal microscopy images of Ca^{2+} capsule washed with 0.9% NaCl (w/v) before coating with fluorescent labeled high-G alginate. Second gelling solution: A) $CaCl_2$ and $BaCl_2$, B) $BaCl_2$. Scale bar represented 200µm.

4.3 Coating alginate beads with alginates of different composition

4.3.1 Coating alginate beads with epimerized and high-M alginates

To see if there are visible differences between binding of epimerized (high-MG) and non-epimerized (high-G) coating alginate to core alginate beads in the confocal microscope, high-G coating alginate (F_G=0.68), epimerized (high-MG) alginate (F_G=0.55) and high-M alginate (F_G=0.43) were labeled with fluoresceinamine and used as coating materials.

The calcium/barium-beads of 1.8% (w/v) high-G alginate dissolved in mannitol solution were coated by 0.1% (w/v) fluorescence labeled epimerized alginate dissolved in 0.9% (w/v) saline. Two different second gelling solutions ($CaCl_2$ (50mM) + $BaCl_2$ (1mM) or $BaCl_2$ (10mM)) were used and capsules were washed with saline before exposure to coating solution. The coated beads were screened for stability of coating for seven days. The resulting images of capsules as seen in the CLSM are given in Figure 4.12. The thickness images are made by gradually increasing magnification by using LSM510 computer program.

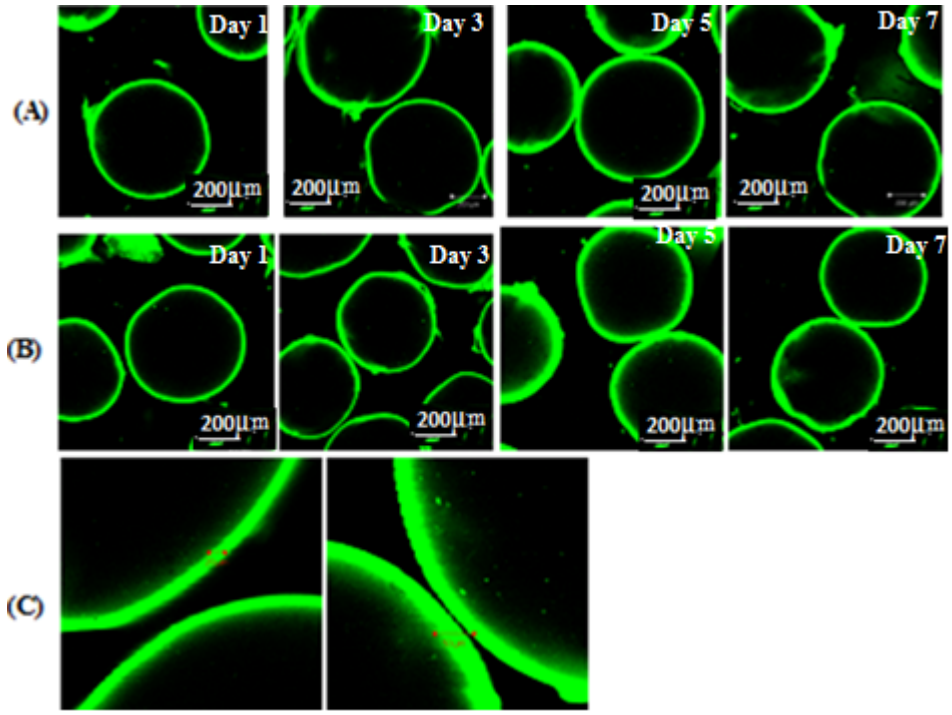


Figure 4.12 Confocal microscopy images of Ca^{2+}/Ba^{2+} - beads coated with 0.1% (w/v) fluorescently labeled epimerized alginate washed with saline. (A) Used $CaCl_2$ (50mM) + $BaCl_2$ (1mM) as gelling solution for coating layer; (B) Used $BaCl_2$ (10mM) as gelling solution for coating layer; (C) The left images shows the thickness of coating layer in capsules fixed by Ca^{2+}/Ba^{2+} and it was around 20 µm and right images: for capsules fixed by Ba^{2+} was around 40 µm. Scale bar represented 200µm.

Figure 4.12 illustrates how the epimerized alginate binds to the non-epimerized high-G alginate beads forming an outer membrane. Further, the images illustrate how the capsules have more coating alginate using epimerized alginate than used non-epimerized high-G alginate as coating layer (Figure 4.9). In addition, Figure 4.12 shows that after one week, it seems that thickness of coating layer has not changed either in capsules fixed by $CaCl_2$ (50mM) + $BaCl_2$ (10mM) or fixed by $BaCl_2$ (1mM) solution. The thickness of capsules fixed by $BaCl_2$ (10mM) (40µm) seems to be two times higher than for capsules fixed by $CaCl_2$ (50mM) + $BaCl_2$ (1mM) (20µm) (Figure 4.12 C). However, it seems from the Figure 4.12 that the thickness of coating layer for capsules fixed by $BaCl_2$ was not homogeneous over the whole surface of capsules.

Calcium/barium capsules of non-labeled high-G alginate coated with fluorescence labeled high-M alginate were prepared and examined in confocal microscope. To see the affinity of barium to make stronger and higher binding to alginate the barium ions as well as Ca^{2+}/Ba^{2+} were used to fixation of coating layer as second gelling solution and were screened over seven days. The resulting images are given in Figure 4.13.

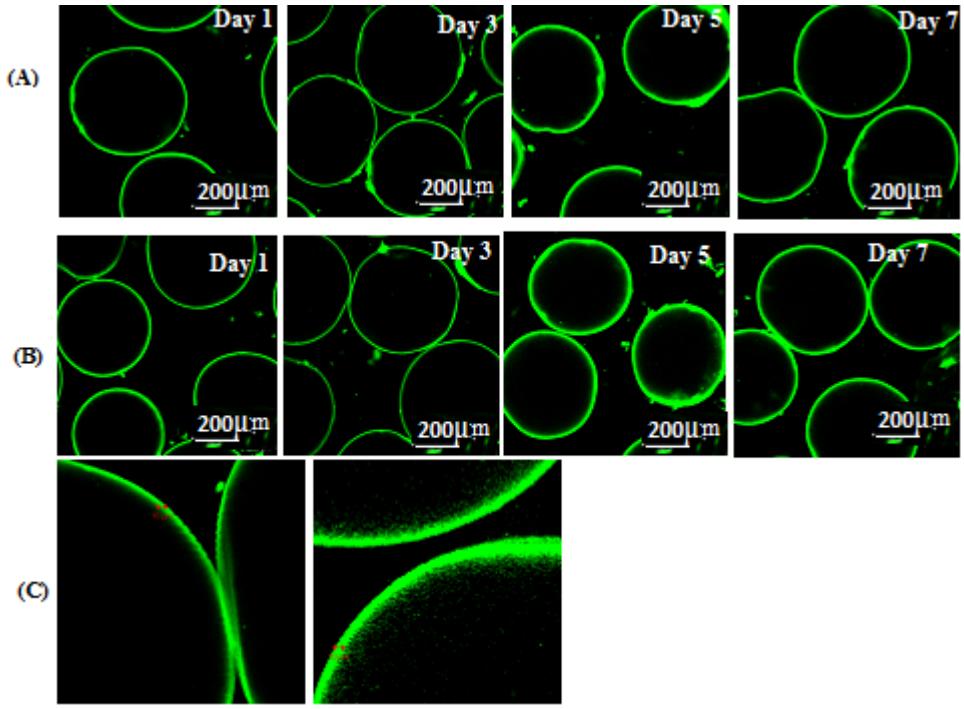


Figure 4.13 Images of Ca^{2+}/Ba^{2+} - high-G alginate beads coated with 0.1% (w/v) fluorescently labeled high-M alginate washed with saline. (A) Used $CaCl_2$ (50mM) + $BaCl_2$ (1mM) as 2nd gelling solution for coating layer; (B) Used $BaCl_2$ (10mM) as gelling solution for coating layer, (C) The images shows the thickness of coating layer (green layer) which for both capsules fixed by Ca^{2+}/Ba^{2+} (left) and Ba^{2+} (right) were 10 µm.

The images in Figure 4.13 of calcium/barium alginate beads coated with high-M alginate shows the thickness and quality of binding of high-M coating alginate to the high-G core alginate beads. The images show that the high-M alginate has covered the capsules well. However, in comparison with epimerized alginate as coating alginate, the thickness of high-M coating alginate was almost five times less than thickness of epimerized coating alginate (Figure 4.12 C and 4.13 C). It seems from Figure 4.13 that the stability of high-M coating alginate in capsules is good since after one week, the thickness of coating layer has not changed. The thickness of capsules fixed by $BaCl_2$ (10mM) seems to be the same as capsules fixed by $CaCl_2$ (50mM) + $BaCl_2$ (1mM) (10µm) (Figure 4.13 C).

To see if the fluorescent labeled coating alginate layer covered the whole capsule, a three-dimensional scan through high-G non-epimerized beads coated with epimerized alginate or coated with high-M alginate was performed using the CLSM. The resulting images are given in Figure 4.14.

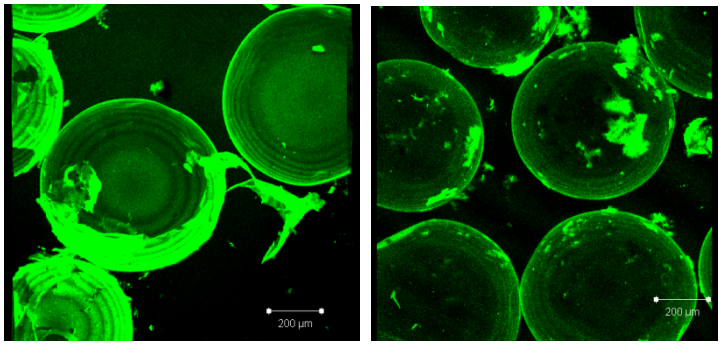


Figure 4.14 The three dimension projection of coated Ca^{2+}/Ba^{2+} -capsules with fluorescently labeled of Epimerized (MG-blocks) (left) and High-M (right) alginate. Both capsules types were fixed with barium (second gelling solution) and washed with saline.

As 3D projection images in Figure 4.14 illustrates, the capsules coated with epimerized alginate covered the whole capsules. However, there is high coating gelling particles attached to capsules coated with epimerized alginate. 3D images of capsules coated with high-M shows also covering of whole capsules with high-M alginate but with lower intensity and lower gelling particle around and attached to capsules than capsules coated with epimerized alginate.

In general, there are visible differences of coating thickness between capsules coated with high-M and epimerized alginate where the thickness of high-M coating layer is lower than epimerized alginate. In addition, a comparison of Ca^{2+}/Ba^{2+} capsules coated with high-G (Figure 4.9) and high-M (Figure 4.13) alginate revels that the capsules coated with high-M alginate have higher intensity of coating than capsules coated with high-G alginate.

Different labeling of the coating alginates slope of standard curve was being 40, 50, and 87 for high-G, high-M and epimerized alginates, respectively. Hence quantification of the bound alginate using fluorescent spectroscopy based on the standard curve (Appendix B) was performed.

The fluorescent intensity and quantity of coating alginate in alginate beads coated with epimerized (high-MG) and high-M alginate were quantified by fluorescent spectroscopy for 1, 3, 5 and 7 days after the capsules were made (Figure 4.15).

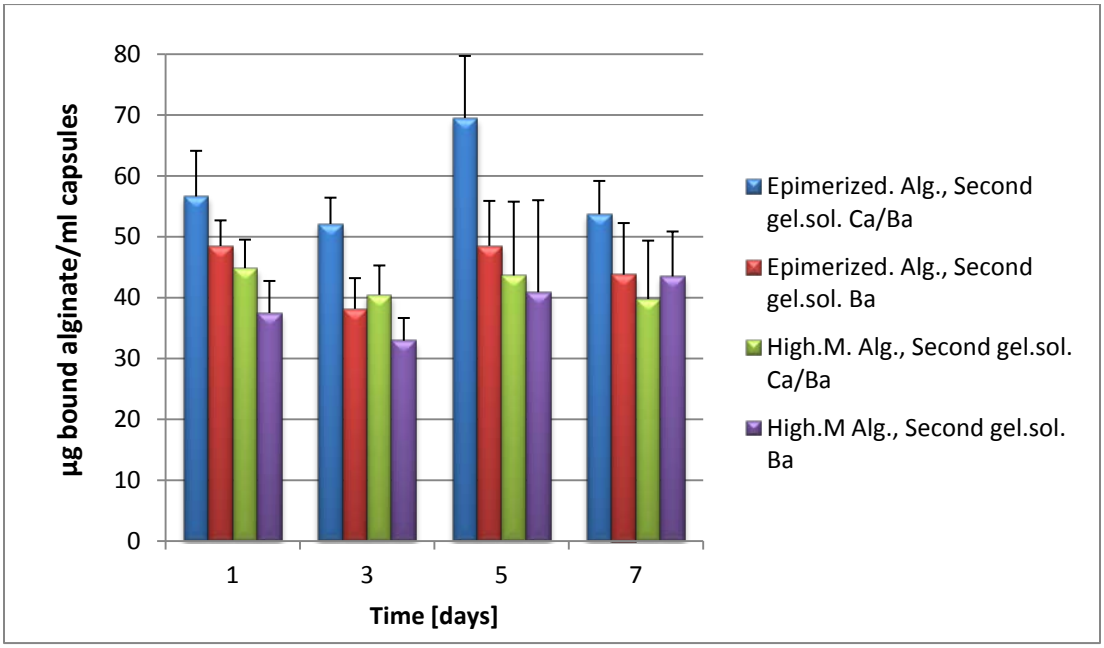


Figure 4.15 Quantity and stability of coating layer in alginate-alginate capsules, calculated by using standard curve as the fluorescent concentration of coated layer in capsules as a function of time for 7 days.

As spectrophotometry measurements of concentration of coating alginate, Figure 4.15, shows the quantity of coating alginate binding in capsules coated with epimerized alginate are higher than coated capsules with high-M alginate. Also this result was confirmed by CLSM images (Figure 4.12 and 4.13). However, as we can see the standard deviation of the intensity of bounding for both high-M and epimerized coating alginate was high. The capsules fixed with calcium/barium shows almost same or higher concentration of coating than fixed capsules with barium in spectrophotometry measurements. In addition, Figure 4.13 illustrate that both high-M and epimerized alginate as coating alginate have high stability of binding in coated capsules.

4.3.2 Coating of alginate-PLL capsules

We have seen that a epimerized alginate binds better to high-G alginate than dose a high-G and high-M coating alginate. In addition, there is some interest to find a good coating for alginate-PLL capsules to improve biocompatibility of capsules.

The high-M, high-G alginate and epimerized alginates used in the previous experiments as coating of the alginate beads were now used as coating of the alginate-PLL capsules. In

addition, a sulfated poly-MG alginate (from Øystein Arlov) were labeled with fluoresceinamin and used as coating alginate in alginate-PLL-alginate capsules. Figure 4.16, 4.17 and 4.18 shows the confocal images of alginate-PLL capsules coated by sulfated poly-MG, high-G and high-M , epimerized alginate respectively. Optical slicing shows how the coating alginate over alginate-PLL capsules a green color, forming an outer layer. The optical slicing, three-dimensional (3D) image and the thickness of alginate-PLL-sulfated alginate are given in Figure 4.16.

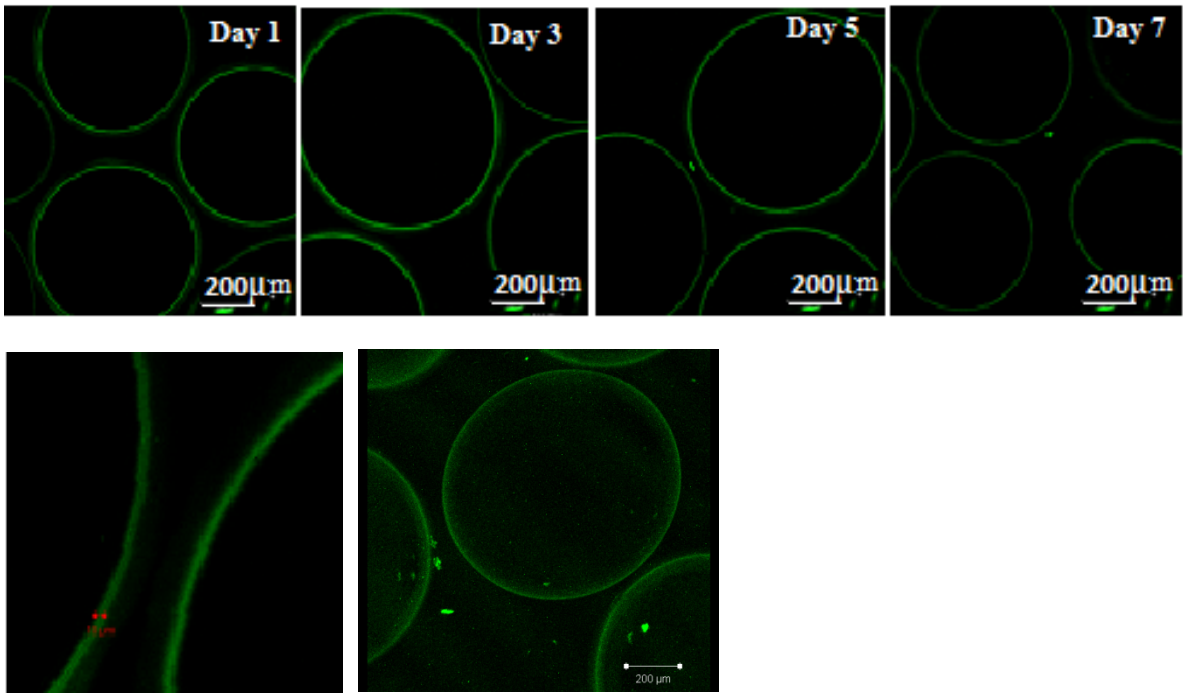


Figure 4.16 Confocal microscopy poly images of Ca^{2+}alginate-PLL capsules coated with 0.1% (w/v) fluorescently labeled sulfated (high-MG) alginate. Upper images: optical slice of capsules; Bottom left images are made by gradually increasing magnification by using LSM510 computer program and shows the thickness of coating layer (green layer) which was 10 µm; Bottom right: three-dimensional images of capsules.

The confocal images of coated alginate-PLL capsules with sulfated alginate (highly negative charge) shows good binding of sulfated alginate on alginate-PLL capsules (Figure 4.16). The thickness of coating layer for sulfated alginate coating layer was around 10µm.

The 3D image can show that the coating alginate covered the whole alginate–PLL capsule or not. Sometimes the covering could be uneven over the capsule surface. The 3D images of alginate-PLL- sulfated alginate capsules were shown in Figure 4.16. It shows that the sulfated alginate covered whole alginate–PLL capsule.

Figure 4.17 illustrate confocal images of the fluorescently labeled alginate–PLL- high-M or high-G alginate capsule. High-M and high-G alginate could not be detected on the alginate-PLL capsules.

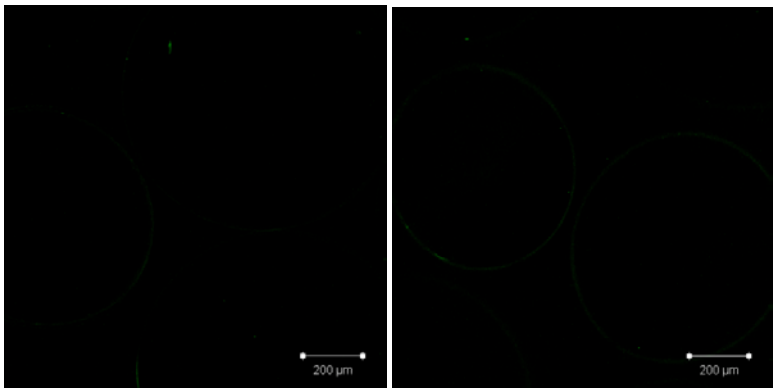


Figure 4.17 Confocal microscopy images of Ca^{2+}alginate-PLL capsules coated with 0.1% (w/v) fluorescently labeled high-M (left) and high-G (right) alginate.

Figure 4.18 shows very low signal of binding of epimerized alginate to alginate-PLL capsules. In compare to high-G and high-M alginate, it seems that epimerized alginate can bind a bit better than high-G and high-M to alginate-PLL capsules. However, it could be consider that the intensity of standard curve for epimerized alginate was around two times higher than other coating alginate. That means there are more fluoresceinamine bound to the epimerized alginate than the other alginates.

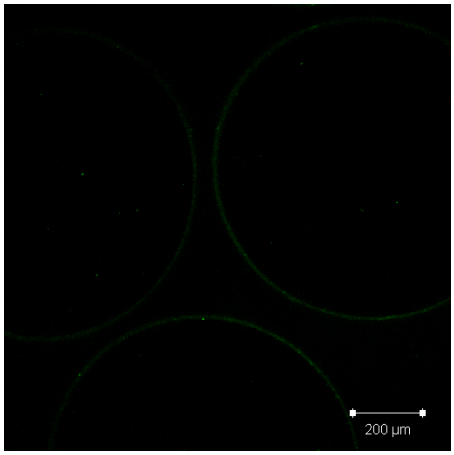


Figure 4.18 Confocal microscopy images of Ca^{2+}alginate-PLL capsules coated with 0.1% (w/v) fluorescently labeled epimerized alginate.

Figure 4.19 illustrates the fluorescent intensity and quantity of coating alginate (sulfated, high-M, high-G and epimerized (high-MG)) in alginate-PLL capsules were quantified by fluorescent spectroscopy for 7 days after the capsules were made.

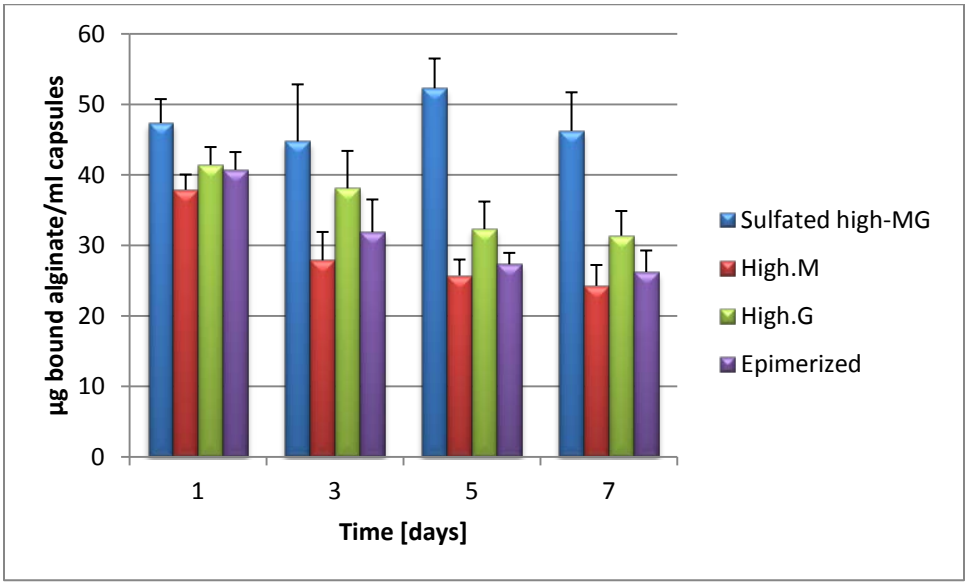


Figure 4.19 Quantity and stability of coating layer (sulfated, high-M, high-G and epimerized alginate) in alginate-PLL capsules, calculated by using standard curve as the fluorescent concentration of coated layer in capsules as a function of time for 7 days.

As Figure 4.19 shows, sulfated coating alginate (highly negative charge) was bound in higher amounts to alginate-PLL capsules than the other coating alginates. The intensity of coating in high-M, high-G and epimerized high-MG alginate are close to each other with low differences in intensity. However the high-G alginate binding was slightly better to the capsules than epimerized and high-M alginate. It was seen the stability for sulfated alginate coat since the concentration of coating in spectrophotometry measurement does not change in 7 day, and the small decline of coating intensity for the other coating alginate.

5. Discussion

5.1 Optimalisation of coating

In this project, the possibility of coating alginate beads with alginate based on the leakage of gelling ions from the core alginate was performed. A variety of parameters such as different concentration of coating alginate, variety in exposure time in second gelling solution, different gelling ions (both for core of alginate beads first and second gelling solution (for coating layer)) and different washing solution (mannitol or saline), to see if this treatment affected the binding and distribution of coating alginate in coated capsules.

Using high concentration of fluorescent labeled alginate (0.5% and 0.2%) caused gelling in the solution of fluorescent alginate when beads were added. This makes sense since high concentration of coating alginate will cross-link with leaking gelling ions and make the coating alginate gel. Therefore, when the lower concentration of coating alginate was used, it was shown better coating on beads rather than clump in surrounding solution.

The exposure time in second gelling solution were then examined. The second gelling solutions were used to fix the coating layer that may be loosely bound to the alginate core. After exposing the beads to coating alginate, the beads were exposed to five and ten minutes of different gelling solutions. The results do not vary between the two capsules with different exposure time (Appendix A, Figure A.4), probably because 5 minute exposure is enough to form a stable gel.

As we can see in Appendix A Figure A.1, shows the washed and non-washed capsules before exposure to coating solution, non-washed capsules seems to have more coating which is make sense since the divalent cation were not removed and they could cross-link with the alginate coating solution. However, it is almost impossible to recognize the coating layer in non-washed coated capsules due to gelling of the fluorescent around beads in the solution. Hence, the capsules were washed with mannitol or saline solutions.

The capsules washed with mannitol shows less intensity of coating layer than capsules washed with saline either with $CaCl_2$ (50mM) +$BaCl_2$ (1mM) or $BaCl_2$ (10mM) used as gelling solution for coating layer (Figure 4.1 and 4.3). Mannitol is ion free solution and has no effect of exchanging ions in alginate beads and just help to remove gelling solution (divalent ions) around the beads. Therefore, by removing the divalent ions around beads, there are no divalent ions around core capsule for cross-linking with G or M-blocks in coating alginate solution. Hence, in this way, the possible of to have a high intensity of

coating in washed capsules with mannitol became low. Whereas the capsules were washed with saline has high intensity of coating alginate. This could be explained by exchanging the two sodium ions in saline with calcium in core of coated capsules which leads to increase cross-linking of coating solution with calcium ions come out from core of capsules.

Divalent cations such as Ca^{2+} and Ba^{2+} can bind between the G blocks of adjacent alginate chains, creating ionic interchain bridges resulting in hydrogels from soluble alginate molecules. The alginates affinity toward divalent ions has been shown to increase for alkaline metal ions by following order: $Mg^{2+} << Ca^{2+} < Sr^{2+} < Ba^{2+}$. Under physiological conditions, calcium ions are exchanged with the non-gelling sodium ions (two Na^{+} for each Ca^{2+}), which leads to an increased osmotic swelling of the capsules. Barium ions, on the other hand, which have a much higher affinity for the alginate, will be too tightly bound to the alginate for the Na^{+} to take their place. This phenomenon is confirmed for coating strategy in barium capsules. Barium capsules did not bind the fluorescently labeled coating alginate due to tight binding of Ba^{2+} in the alginate core and not releasing any divalent cations to make cross-link with coating gelling solution to make fluorescent labeled alginate coating layer (Figure 4.2 B).

Thu et al.(1996) and Mørch et al (2006) found that a Ca^{2+}/Ba^{2+} ratio of 50:1 in the gelling solution was enough to stabilize the capsules significantly. In Figure 4.2 C, it is make sense that the most intensity of coating layer were in Ca^{2+}/Ba^{2+} coated capsules, since the Ca^{2+} ions could be easier released from the core than Ba^{2+} ions. So far, $Ca^{2+/}Ba^{2+}$ coated capsules could create ionic interchain bridges with coating alginate solution and to make the thick coated layer. Uncoated alginate was used as control in order to compare the coated capsules in degree of coating with same settings of imaging as coated beads.

Mørch et al (2012) have found that the amount of binding of divalent ions to the alginate beads are in following order $Ba^{2+} > Ca^{2+}/Ba^{2+} > Ca^{2+}$ with ratio of divalent Ion: (mol/mol) 0.404, 0.316 and 0.181 respectively [69]. Hence it is make sense since the calcium coated capsules, as shown in Figure 4.2, shows lower coating than calcium/barium coated capsules due to higher binding of divalent ions in Ca/Ba core beads than calcium core beads resulting in less leaking of the ion of calcium beads and less binding of coating alginate to ions than Ca/Ba core beads. In addition, the Ba^{2+} ions binding to alginate uronic acid have shown the highest binding (0.404), but because of tight cross-linking to core alginate blocks it is hard to leakage ions to surface of beads and resulting probable of binding of coating alginate is low (Figure 4.2 B).

5.2 Binding and long-term stability of coating layer

The initial binding and stability of coating layer in coated capsules at physical conditions are one of the most important factors for capsule integrity. Six type of alginate-labeled alginate capsules were examined with respect to long-term stability by spectrophotometer. In addition, in parallel with spectrophotometer measurements of fluorescent intensity of coating layer was done by CLSM.

Figure 4.4 and Figure 4.7-11, the spectroscopy measurements and CLSM images of long term stability of coating layer, revealed that calcium/barium-alginate beads have more ability to bind to high-G alginate (fluorescently labeled) than calcium-alginate beads. The reason for that as explained in section 5.1 could be due to higher binding of divalent ions in Ca/Ba core alginate. In addition, the spectroscopy measurements and CLSM images for the six different capsules shows that as we have explained previously in section 5.1 that the washing capsules with saline could make higher binding of coating layer than washing with mannitol.

It has been known that by using Ba^{2+} as alginate gelling solution we can have strong gellation and cross-linked between alginate G or MG-blocks [14].The Ca/Ba capsules were washed with saline which coating layer fixed by barium ions has shown (both in spectroscopy measurement and CLSM images) higher intensity of coating than coated capsules fixed by ratio 50: 1 of calcium/barium ions (Figure 4.4). This could be because of higher affinity of ions to M and G-blocks of core alginate and making stronger cross-linking between G-blocks. However, there was a loss of coating from the barium fixed coating over the first 2 weeks resulting in about the same intensity over time as Ca/Ba fixed coating. The reason for that could be due to accumulation of barium from Ca/Ba solution make Ca/Ba and Ba fixed coating not so different [69].

The stability of coating in all six beads was rather good since the fluorescent intensity of coating layer were not so reduced after 49 days. This was seen both in the fluorescent spectroscopy as well as in CLSM (Figure 4.4, 4.5 and 4.7-11). However, between six coated capsules the calcium/barium beads washed with saline and $CaCl_2$ (50mM) + $BaCl_2$ (1mM) as second gelling solution shows highest stability of the coating compared to other capsules. Although little is known about the reasons of that, it has been known that washing with saline could replace calcium divalent with two sodium monovalant ions ions in calcium/barium-core destabilize gel network. It may lead to a decreased cross-linked density on core beads making a better integration of the coating alginate with the core.

The highest level of binding of coating layer (fluorescent intensity) between all six different coated capsules was in the calcium/barium beads washed with saline and $BaCl_2$ (10mM) as second gelling solution.

There are many sources of error in the long-term stability tests; the most important is the uncertainty of the measurements due to pipetting technique of capsules to ensure same volume of capsules each time. As it can be seen from the long-term stability curves (Figure 4.4 and 4.5), the uncertainty fluorescence measurements are high, as for example, the absorption measurement in day 0 is lower than day1and measurements have high standard deviations. This has to be taken into account when analyzing Figures 4.4 and 4.5. However, CLSM images and fluorescent spectroscopy measurements was shown corresponds.

Although confocal microscopy is a valuable method for qualitative evaluations of capsules, it is not a very suitable tool for quantitative measurements of polymer concentrations. Confocal imaging of the different capsules revealed that there are many variations, not only between batches, but also among different capsules from the same batch. Small changes in the setting for the confocal microscope were also enough to alter the confocal images of capsules drastically. As an example, increasing the intensity of fluorescent light in low amount could change the apparent in thickness and background of images. Hence, every time the same settings were used.

During the laser scanning of capsules in the confocal microscope, it was registered that the brightness of the fluoresceinamine molecules decreased somewhat after 3-4 scans. Hence, a different and less light-sensitive fluorochrome could be tested for future work with the laser scanning confocal microscope.

CLSM images (Figure 4.8 and 4.10) and spectroscopy measurements (Figure 4.5) reveals that capsules of fixed coating layer with calcium and barium seem to have same stability as capsules of fixed coating layer with only barium while both were washed with mannitol. It does not make sense since the coated capsules fixed with barium seem could have higher stability than capsules fixed with calcium and barium due to firm cross-linking of barium with coating layer alginate.

Figure 4.11 illustrates that there are not visible differences between the calcium coated capsules fixed with calcium /barium and fixed with only barium in confocal imaging. However, there are some differences of intensity of coating layer for them in spectrophotometer measurements which barium fixed coated capsules were shown more stability and higher intensity of coating layer than capsules fixed with calcium/barium. It

seem right for the reason that barium has more interest to make firm bound with coating alginate and made strong coating layer than calcium ions. In general, as explained previously calcium ions in calcium beads are lower than ions in Ca/Ba beads and, therefore, there are low leakage and so it makes sense since we have seen lower coating in calcium capsules.

5.3 Coating alginate beads with alginates of different composition

Increasing the number of alternating sequences in the alginate chains using the AlgE4 epimerase, increase the stability and reduce the permeability of alginate beads [31]. In this work, we wanted to study the effect of alginate composition in the coating alginate on the initial binding of the coating in the coating alginate and the stability of the coating layer. A high-M alginate (F_G=0.43) and an epimerized alginate consisting of G- and MG-blocks (F_G=0.55) were used to compare to the high-G alginate (F_G=0.64) used in the previous sections.

The capsules coated with epimerized alginate showed highest thickness of coating layer compare to other coating alginate (high-G or high-M). Confocal images of coated capsules were showing that the use of barium as fixations of coating resulted in higher thickness of coating than capsules fixed with calcium and barium ratio of 50:1. This higher intensity of coating with epimerized alginate was also seen by spectrophotometry measurement (Figure 4.15). Increasing flexibility of the alginate chain is known in intrinsically higher flexibility of MG compared to GG and MM blocks, and hence the epimerized alginate (MG-blocks) can easily bind to divalent ions than high-G or high-M alginate. Therefore, this makes sense that higher coating was seen in epimerized alginate than other coating alginate in alginate capsules coated with other alginate. Another possibility to have the higher intensity of coating in epimerized alginate can be due to lower molecular weight and viscosity of epimerized alginate than high-G or high-M alginate.

As it can be seen from standard curve of fluorescently labeled coating alginates (Appendix B), high-G high-M and epimerized alginate, the standard curve for epimerized alginate shows almost two times higher than high-G and high-M alginate which it means that the amount of fluoresceinamine covalently linked to the epimerized alginate was higher than high-G and high-M. Therefore, the higher coating of capsules coated with epimerized alginate in images of CLSM could be due to higher specific intensity (slop of standard curves) of the epimerized alginate and this should be consider in the results.

High-M alginate (F_G=0.43) were labeled by fluoresceinamin and used as coating layer on high-G alginate to make another coated capsules to see how the high-M alginate can bind to Ca/Ba- alginate beads. The high-M coated alginate capsules two times were tested for using barium as second gelling solution and may be needed repetition since it was showing the capsules treated with barium had the same thickness of coating as coated capsules fixed by calcium and barium with ratio 50:1. This is not make sense due to the strong cross-linking of barium to alginate blocks the higher intensity of coating is expected from capsules fixed by barium than fixed by calcium/barium.

5.4 Coating of Alginate-PLL capsules

The main purpose of coating alginate-polylysine capsules with an outer alginate layer is to bind unreacted positive charges of the capsules surface in order to shield polylysine which has shown to cause host reactions [66].

The binding of PLL depended on the washing procedure. Washing with saline before exposure to PLL will remove extra gelling solution and somewhat dissolve the gel by exchange of the calcium ions bound to mannuronic acid residues as well as to the short G-blocks. This would later lead to an enhanced binding of PLL with a concomitant destabilization of the gels. After exposure to PLL capsules are again washed with saline to remove the unbound PLL. And at the end, after exposure to coating solution, the capsules washed with saline to remove unbounded coating solutions [43].

A poly-MG alginate is sulfated with chlorosulfonic acid (from Øystein Arlov), which it made very highly negative charge, were used for coating of alginate-PLL capsules. Figure 4.16 show that the sulfated coating alginate formed a thin membrane. The sulfated alginate is probably highly bound to PLL due to absorbency of highly negative charge of sulfate with very positive charge of PLL and making the good coating in alginate-PLL capsules. The three- dimensional image in Figure 4.16 also shows that the sulfated coating alginate evenly distributed on the capsule surface. However it is possible that the coating does not always cover the whole capsules, therefore it would be interesting to examine the effectiveness of the coating by measuring the positive charge on the coated surface. Another interesting approach could be to increase either the coating alginate concentration or the exposure time in coating and examine the resulting capsules in CLSM to see if the binding was improved.

High-M alginate duo to higher intrinsic flexibility of molecule than high-G alginate can cross-link easier to PLL than High-G. Earlier studies show that a high-M alginate gel binds PLL better than a high-G gel [37], and Strand et al [68] shows that the poly-MG alginate (epimerized) poly-M (F_G=0.06) alginate block alginate of M-blocks and G-blocks (F_G=0.45) can bind to alginate-PLL in following order: high-MG > block alginate >high-M. However, in this study, in spectroscopy measurements, the binding to alginate-PLL was in following order: high-G > epimerized (high-MG and G) >high-M (Figure 4.19). Since it is very difficult to take exactly the same number of capsules for measuring the intensity of coating, hence, it is possible to have some error in spectroscopy measurement. However, as CLSM images does not give much information (Figure 4.17) and there are not any differences in binding of coating alginate to capsules and both high-M and high-G alginate very thin coating bind to alginate-PLL capsules. We have seen that using the carbodiimid chemistry for linking primary amines to the carboxylic groups of the alginate gives around 0.2% labeling (meaning that 0.2% of the carboxylic groups are linked to the fluoresceinamine) (personal communication, Berit L. Strand). It is possible to find the labeling efficiency by measuring the standard curve of fluoresceinamine by itself to calculate the specific binding that will be a measure of how many fluoresceinamine units there is per carboxylic group and hence the labeling efficiency.

Strand et al [39] shows that high-G alginate can make good coating on alginate-PLL capsules in CLSM with the same degree of labeling, while in our study the high-G alginate shows no signal of coating in alginate-PLL capsules. There is no explanation for this; however it could be due to degree of solubility of fluoresceinamin in the process of labeling of alginate.

The binding efficiency of coating alginates to alginate-polylysine capsules is dependent on molecular weight of coating alginate, increasing with decreasing weight [37]. Since the molecular weight of the epimerized alginate is lower than high-M and high-G, this should further improve the binding. The very thin coating layer is probably the reason why there are no visible differences between the two coating materials.

5.5 Future work

For future work, the alginate multilayer could be produced with difference type of composition, labeling and using different type of cells or proteins to increase more biocompatibility of alginate microbeads. Further work on sulfated alginate coating by using different concentration and using different degree of sulfation with different molecular weight can be studied.

In this study, we have used mannitol as storage which is a ion free solution and so there are no effect on exchanging ions with divalent ion in alginate capsules. By using NaCl (at physiological condition) as storage solution bead, it is possible to see the effect on the initial coating and long term stability of the coated capsules. It is possible to study on some changing in the procedure of making coating of alginate-PLL capsules, as for example, no washing or washing with mannitol after exposure to PLL and after exposure to coating solution can be done in further work. In addition, it can work on removing the coating gelling fragments that were seen in surrounding solution and bounded to capsules for example by washing or reducing concentration of coating alginate. This fragment can make disturbance in interpretation of results.

Conclusions

In this project it was found that alginate could bind to alginate beads making outer membrane on alginate beads that may be important for improvement of biocompatibility. In the process of optimalisation of coating, different parameter was examined and consequently best beads formulation was performed. The parameters which consider for optimalisation were: 1) The use of high concentration of high-G coating alginate (0.5% and 0.2%) shows gelling in the coating solution and unevenly binding of coating alginate in coated capsules. 2) The exposure time for second gelling solution, used for fixation of coating layer, was examined and the result shows not relevant significant differences between the two capsules with different exposure time. 3) The thicknesses of coating layer in alginate capsules with different divalent cations were shown in the following order: Ca^{2+}/Ba^{2+} capsules > Ca^{2+}capsules > Ba^{2+} capsules. 4) The alginate coated with high-G alginate washed with saline before exposure to coating solution shows higher intensity of coating layer than washed with mannitol. 5) In addition, we have seen that the labeled alginate dissolved in 0.9% NaCl could make better coating than alginate dissolved in 0.3M mannitol.

Long-term stability study of coating layer for calcium or calcium/barium-alginate beads coated with high-G alginate revealed that calcium/barium coated capsules washed with NaCl and stabilized with $CaCl_2$ (50mM) + $BaCl_2$ (1mM) had the most stable coating layer in comparison to other alginate-high-G alginate capsules. The highest initial binding of coating layer among alginate beads coated with high-G alginate was calcium/barium capsules washed with NaCl and stabilized with barium.

The calcium/barium alginate beads coated with high-M alginate shows that the high-M alginate could make better coating than high-G, however, in comparison with epimerized alginate as coating alginate, the high-M coating alginate had less coating. Hence the result shows that different composition can influence the intensity of coating. Epimerized and high-M coating alginate was shown to be stable for one week of experimental follow up. The capsules coated with epimerized and high-M alginate covered whole of capsules.

The alginate-PLL capsules coated with sulfated alginate (highly negative charge) shows good coating. The epimerized alginate same as high-G and high-M alginate shows low signal of binding to alginate-PLL capsules. 3D images of alginate-PLL capsules coated with sulfated alginate show evenly distribution of coating.

The most parameter for optimalisation of alginate coating is discussed here in this project, however, it is still need some work on removing the coating gelling fragments. Sulfated alginate shows promising result for coating polycation capsules and it may need some future work to optimization of alginate polycation capsules.

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Appendix

Appendix A: Optimalisation of coating

The 1.8% high G alginate capsules were coated by 0.5 % fluorescent labeled alginate dissolved in 0.9% NaCl. The beads without and with washing (mannitol) before expose to fluorescent labeled alginate for Ca-beads are shown in Fig A.1. Without washing, the beads were trapped in labeled alginate gelling particle due to excise of divalent cation around beads. Lot of fluorescent labeled alginate was lost and it also was difficult to see the coating layer.

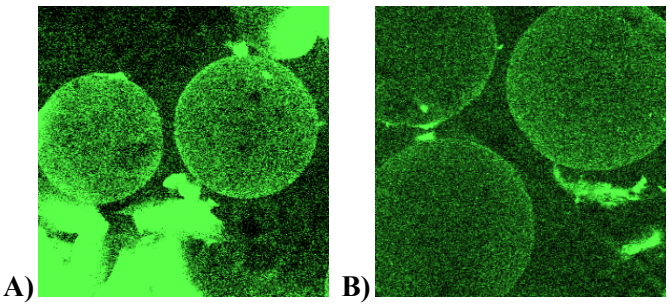

Figure A.1 Ca^{2+}-capsules coated with fluorescent labeled alginate 0.5% solved in NaCl 0.9%, A) Without washing(mannitol) B) With washing(mannitol) before expose to fluorescent labeled alginate by filter way.

Figure A.2, shows coated capsules with the fluorescent labeled alginate as coating layer which dissolved in either saline or 0.3 M mannitol (ion-free solution). We were interested to see if this treatment affected the distribution of coating alginate. As we can see from Figure A.2, it seems that the labeled alginate dissolved in 0.9% saline could make better fluorescent coated beads than labeled alginate dissolved in 0.3M mannitol.

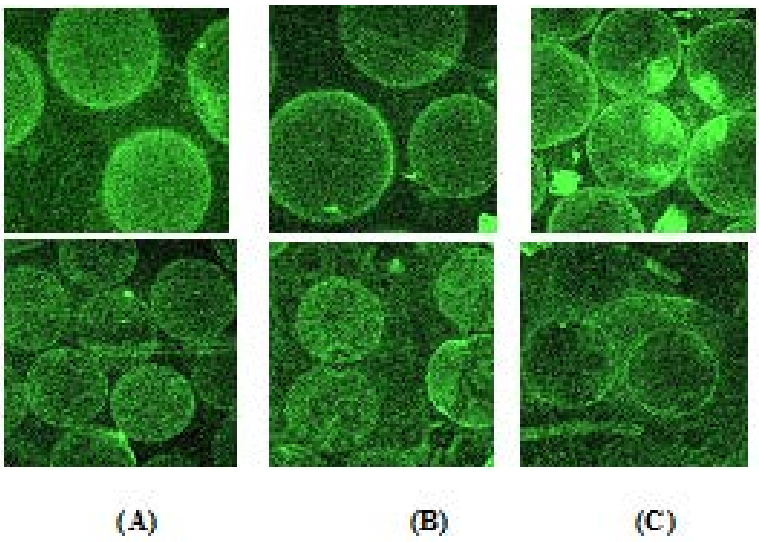

Figure A.2: Coated calcium beads washed with mannitol before expose to fluorescent labeled alginate (coating solution). Fluorescent labeled alginate 0.5% dissolved in NaCl 0.9% (upper images) and in 0.3M mannitol (bottom images) A) Ca^{2+}-beads B) Ca^{2+}/Ba^{2+}-beads C) Ba^{2+}-beads

Exposure time for second gelling solution used for fixation of coating layer, in Ca/Ba beads coated with high-G alginate, was examined to see the effect on alginate coating. CLSM images show that there are no visible differences as effect of different exposure time (10 and 5 minute) for second gelling solution (Figure A.3).

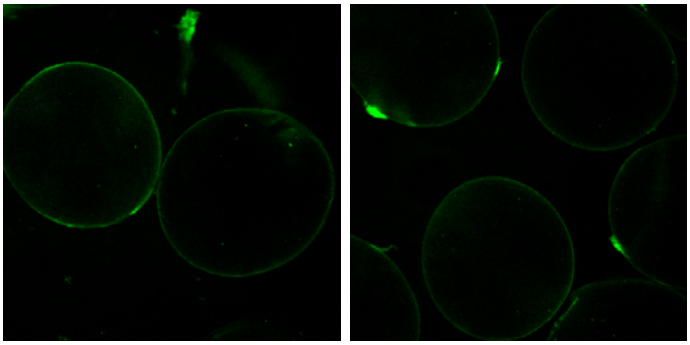

Figure A.3: Coated Ca/Ba beads washed with saline before expose to 0.1% fluorescent labeled alginate dissolved in 0.9% NaCl (coating solution). Exposure time for second gelling solution ($CaCl_2$ (50mM) + BaCl2(1mM)) A) 5 minute B) 10 minute

Appendix B: Standard Curve for Fluoresceinamin in Fluorescent Labeled Alginate

A standard curves for fluoresceinamin labeled alginates was prepared, making it possible to have related fluorescence intensity measurements to different concentration of 0.1% fluorescent labeled alginate dissolved in 0.9% NaCl. The excitation at 485 nm was measured at known concentrations of 0.1% fluorescent labeled alginate in saline. The samples were made with the emission intensity value 0 to 50 at 535nm. For each coating solution, fluorescently labeled high-G, high-M, epimerized and sulfated alginate, a standard curve were prepared. The raw data of high-G, high-M, epimerized and sulfated alginate, is presented in Table B.1, B.2, B.3 and B.4 respectively. And also, the resulting standard curve for high-G, high-M, epimerized and sulfated alginate are presented in Figure B.1, B.2, B.3 and B.4 respectively. To remove effect of solvent (saline), the emission intensity value of zero concentration (only saline) is withdrawn from all value.

Table B.1 The data from the emission intensity measurement for known concentration of 0.1% High-G fluorescently labeled alginate. The values were used to make a standard curve for 0.1% fluorescently labeled of High-G alginate.

0.1% fluorescent labeled High-G alginate concentration [mg/ml]	Measured emission intensity at λ = 535 nm			Average value	Calculated absorption value
	Parallel 1	Parallel 2	Parallel 3		
0	350	368	402	353	0
5	564	569	580	564	201
10	774	776	770	784	408
20	1190	1180	1169	1181	812
25	1398	1391	1391	1385	1023
50	2541	2524	2515	2516	2156

Table B.2 The data from the emission intensity measurement for known concentration of 0.1% High – M fluorescently labeled alginate. The values were used to make a standard curve for 0.1% fluorescently labeled of High-M alginate.

0.1% fluorescent labeled High-M alginate concentration [mg/ml]	Measured emission intensity at λ = 535 nm			Average value	Calculated absorption value
	Parallel 1	Parallel 2	Parallel 3		
0	342	341	336	340	0
5	741	729	738	736	396
10	898	907	885	897	557
20	1371	1364	1358	1364	1024
25	1602	1587	1581	1590	1250
50	2702	2697	2683	2694	2354

Table B.3 The data from the emission intensity measurement for known concentration of 0.1% epimerized fluorescently labeled alginate. The values were used to make a standard curve for 0.1% fluorescently labeled of epimerized alginate.

0.1% fluorescent labeled Epimerized alginate concentration [mg/ml]	Measured emission intensity at λ = 535 nm			Average value	Calculated absorption value
	Parallel 1	Parallel 2	Parallel 3		
0	340	339	408	362	0
5	867	843	836	849	486
10	1446	1412	1403	1420	1058
20	2216	2180	2143	2180	1818
25	2611	2564	2531	2569	2207
50	4330	4300	4273	4301	3939

Table B.4 The data from the emission intensity measurement for known concentration of 0.1% sulfated high MG blocks fluorescently labeled alginate. The values were used to make a standard curve for 0.1% fluorescently labeled of epimerized alginate.

0.1% fluorescent labeled Epimerized alginate concentration [mg/ml]	Measured emission intensity at λ = 535 nm			Average value	Calculated absorption value
	Parallel 1	Parallel 2	Parallel 3		
0	354	362	348	355	0
5	695	691	705	697	342
10	958	956	930	948	593
20	1336	1363	1332	1344	989
25	1519	1540	1537	1532	1177
50	2367	2337	2353	2352	1997

The equation for the regression line for standard curve of fluorescent labeled high-G, high-M, epimerized (high-MG) and sulfated high-MG alginate was calculated as F-1, F-2, F-3, F-4:

$$\mathbf{Int = 40x.C_{fluorescent\ labeled\ high\text{-}G\ alginate}} \quad \text{(F-1)}$$

$$\mathbf{Int = 50x.C_{fluorescent\ labeled\ high\text{-}M\ alginate}} \quad \text{(F-2)}$$

$$\mathbf{Int = 85x.C_{fluorescent\ labeled\ epimerized\ alginate}} \quad \text{(F-3)}$$

$$\mathbf{Int = 45x.C_{fluorescent\ labeled\ sulfated\ alginate}} \quad \text{(F-3)}$$

Where Int is the fluorescent intensity value and $C_{\text{fluorescent labeled alginate}}$ is the fluorescent labeled alginate concentration [μg/ml].

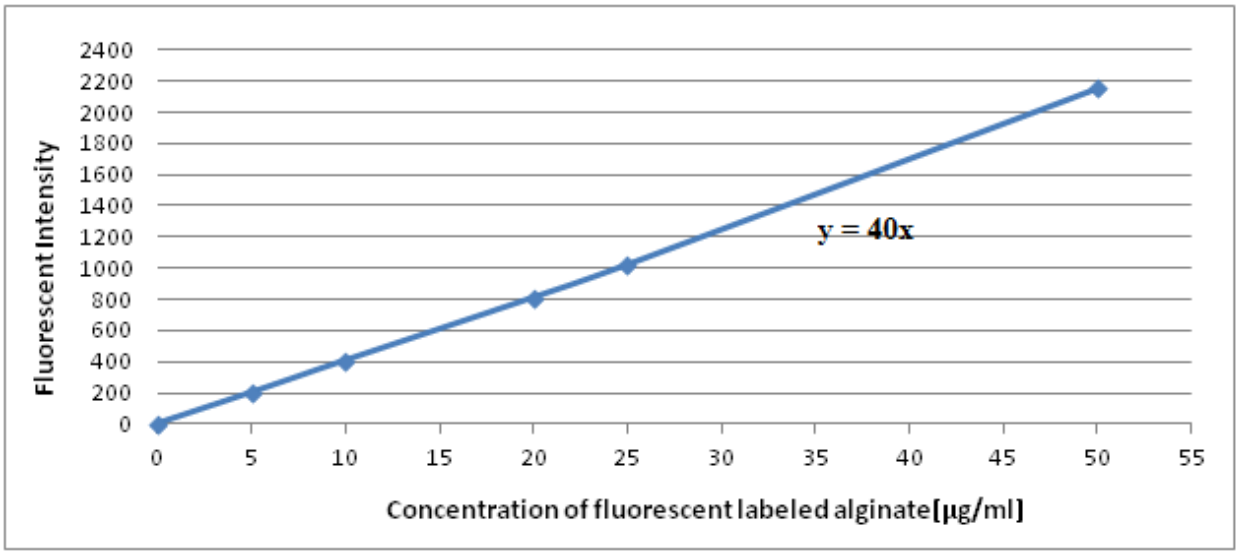

Figure B.1 Standard curve for the determination of fluorescent labeled high-G alginate concentrations from emission intensity at λ = 535 nm.

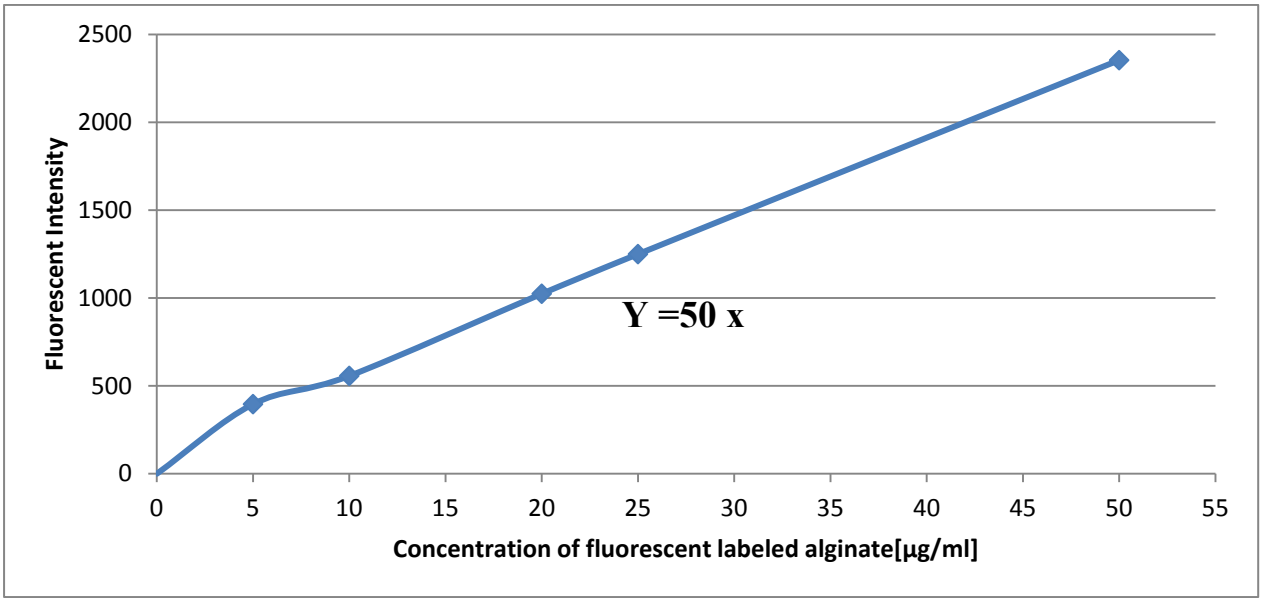

Figure B.2 Standard curve for the determination of fluorescent labeled high-M alginate concentrations from emission intensity at λ = 535 nm.

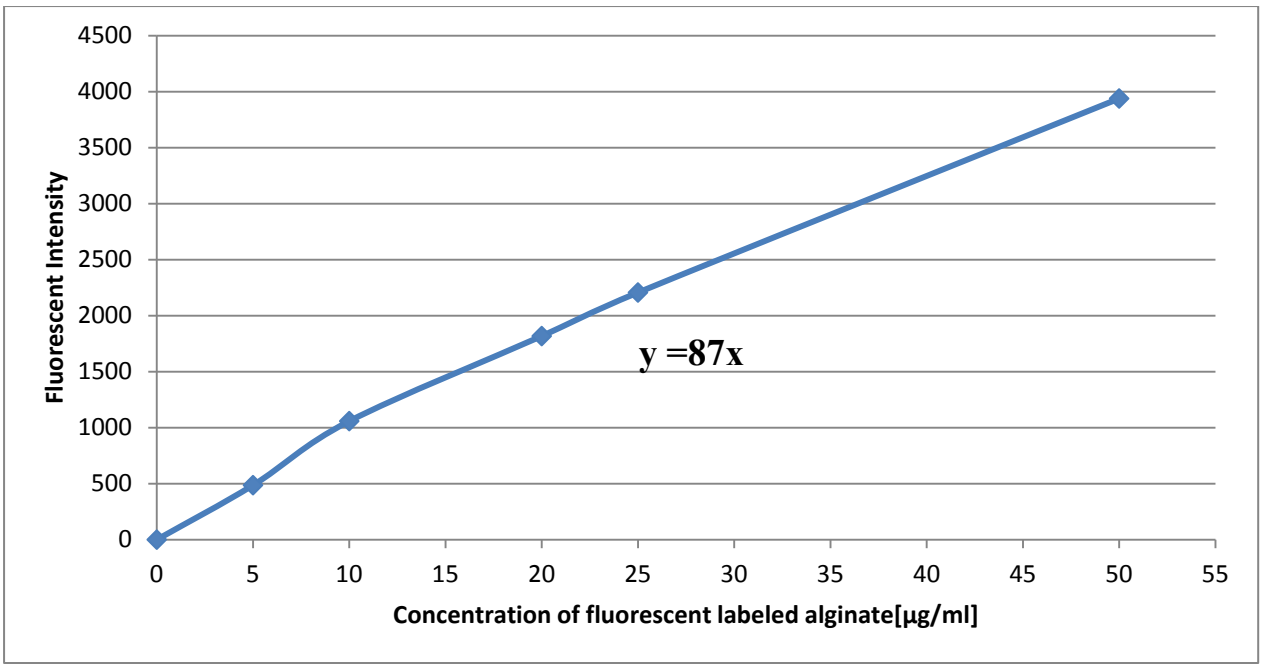

Figure B.3 Standard curve for the determination of fluorescent labeled epimerized alginate concentrations from emission intensity at λ = 535 nm.

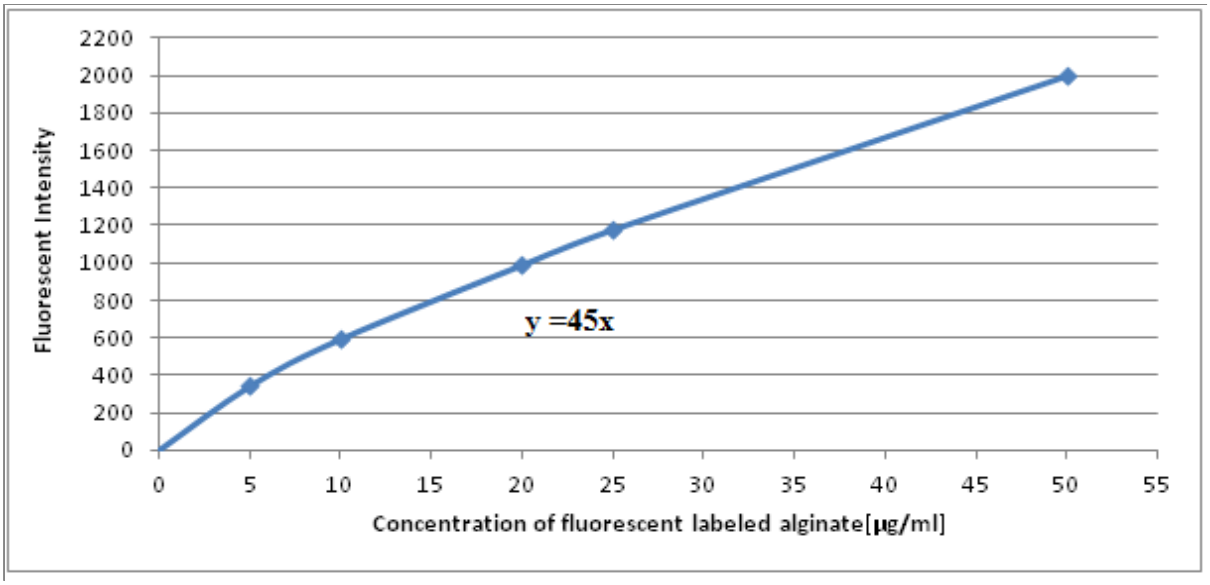

Figure B.4 Standard curve for the determination of fluorescent labeled sulfated high MG-alginate concentrations from emission intensity at λ = 535 nm

Appendix C: Long-Term Stability Measurements

To determine the long-term stability of different coated capsules, the fluorescent intensity of capsules with fluorescent labeled alginate coating was measured over time in four types of calcium/barium- alginate capsules and two types of calcium-alginate capsules. The concentration of fluorescent intensity will then be proportional to the stability of coated layer on top of capsules in a sample.

The emission intensity of the fluorescent coating layer of capsules was measured with spectrophotometer at λ=535 nm. For each measurement three parallel (wells) and multiple reads per well (Circle filled (3 x 3)) were used. In addition, in each measurment, the control beads (without coating (fluorescent)) are measured to see the intensity of background of beads (not fluorescent). The capsules were kept in mannitol at 4°C.The results from the measurements are listed in Table C.1-C.6.

Table C.1 Emission intensity measurements for calcium coated alginate capsules at λ=535 nm. Washed with 0.9% NaCl (w/v) before exposure to coating solution; $CaCl_2$ (50mM) + $BaBl_2$ (1mM) as second gelling solution.

Time [days]	Emission intensity at λ = 535 nm					
	Parallel 1	Parallel 2	Parallel 3	Average	StDev	Control
0	1330	1307	1101*	1318	116	526
1	1346	1210	1479	1346	121	678
2	1076	1257	1209	1233	117	474
3	1215	1254	1157	1209	116	533
4	1310	1254	1117	1227	97	577
5	1151	1208	1216	1192	92	534
6	1151	1363	1251	1255	98	542
7	1257	1450*	1177	1217	148	561
9	1115*	1227	1250	1238	92	629
10	1224	1207	1245	1225	128	643
12	1086	1504*	1309	1198	102	622
14	1239	1309	1610*	1274	96	708
31	1288	1228	1258	1258	117	828
39	1313	1364	1325	1334	94	757
49	1336	1284	1213	1278	44	655

* Neglected value

Table C.2 Emission intensity measurements for calcium coated alginate capsules at λ=535 nm. Washed with Mannitol before exposure to coating solution; $CaCl_2$(50mM)+$BaBl_2$(1mM) as second gelling solution.

Time [days]	Emission intensity at λ = 535 nm					
	Parallel 1	Parallel 2	Parallel 3	Average	StDev	Control
0	1035*	1266	1292	1279	145	526
1	1222	1436*	1238	1230	114	678
2	1064	1163	1037	1088	67	474
3	1103	1196	1186	1162	146	533
4	1144	1058	1239	1147	63	577
5	1192	1190	1224	1202	76	534
6	991	1067	1131	1063	83	542
7	1121	1185	1197	1168	136	561
9	1160	1255	1069	1161	91	629
10	1174	1162	1318*	1168	110	643
12	1262	1160	1302*	1211	95	622
14	1151	1157	882*	1154	91	708
31	980	932	832	915	81	828
39	1070	1063	1153	1095	81	757
49	1001	1064	1029	1031	59	655

* Neglected values

Table C.3 Emission intensity measurements for calcium coated alginate capsules at λ=535 nm. Washed with 0.9% NaCl (w/v) before exposure to coating solution; $BaBl_2$(10mM) as second gelling solution.

Time [days]	Emission intensity at λ = 535 nm					
	Parallel 1	Parallel 2	Parallel 3	Average	StDev	Control
0	1479	1545	1390	1471	167	526
1	1825	1721	1782	1776	192	678
2	1545	1321	1374	1413	131	474
3	1523	1562	1739*	1543	206	533
4	1334*	1503	1521	1512	112	577
5	1437	1641	1013*	1539	229	534
6	1516	1210*	1355	1436	151	542
7	1162*	1538	1338	1438	143	561
9	1338	1395	1474	1402	98	629
10	1251	1293	1251	1265	85	643
12	1173	1239	1084	1165	92	622
14	1066	1187	1186	1125	147	708
31	988	1047	992	1009	114	828
39	1117	1213*	1130	1123	115	757
49	1218	1131	1295	1215	112	655

* Neglected values

Table C.4 Emission intensity measurements for calcium coated alginate capsules at λ=535 nm. Washed with mannitol before exposure to coating solution; $BaBl_2$(10mM) as second gelling solution.

Time [days]	Emission intensity at λ = 535 nm					
	Parallel 1	Parallel 2	Parallel 3	Average	StDev	Control
0	1135	1397	1249	1260	140	526
1	1229	1455*	1226	1227	119	678
2	1124	992	975	1030	87	474
3	999	930	851	927	105	533
4	866	1270*	880	873	80	577
5	1050	909	1087	1015	80	534
6	879	907	1164*	893	101	542
7	1022	1012	883	972	93	561
9	766	924	810	833	51	629
10	803	1089	868	920	70	643
12	955	954	860	923	85	622
14	1003	867	929	933	84	708
31	937	971	1219*	954	109	828
39	969	990	1039	999	81	757
49	881	1054	1020	1037	56	655

* Neglected values

Table C.5 Emission intensity measurements for calcium coated alginate capsules at λ=535 nm. Washed with 0.9% NaCl (w/v) before exposure to coating solution; $CaCl_2$(50mM)+$BaBl_2$(1mM) as second gelling solution.

Time [days]	Emission intensity at λ = 535 nm					
	Parallel 1	Parallel 2	Parallel 3	Average	StDev	Control
0	844	893	1025*	867	85	526
1	876	1021*	868	872	70	678
2	893	923	823	880	79	474
3	850	1488*	932	891	146	533
4	872	950	932	918	70	577
5	841	816	838	832	83	534
6	988	920	902	937	77	542
7	1055	1057	944	1001	103	561
9	954	849	927	910	63	629
10	1025	869	990	961	85	643
12	824	911	868	868	93	622
14	878	823	849	850	93	708
31	732	907	846	828	87	828
39	839	824	917	832	57	757
49	1018	939	931	963	92	655

* Neglected values

Table C.6 Emission intensity measurements for calcium coated alginate capsules at λ=535 nm. washed with 0.9% NaCl (w/v) before exposure to coating solution; $BaBl_2$(10mM) as second gelling solution.

Time [days]	Emission intensity at λ = 535 nm					
	Parallel 1	Parallel 2	Parallel 3	Average	StDev	Control
0	1025	988	908	974	91	526
1	1290	1279	1219	1263	111	678
2	1291	1201	1098	1197	120	474
3	989	984	1130	1034	128	533
4	1139	1185	1034	1119	98	577
5	1081	1207	1066	1118	111	534
6	1111	1149	1243*	1130	116	542
7	1105	1135	1072	1104	120	561
9	1059	1111	1040	1070	104	629
10	1144	1132	1117	1131	106	643
12	1029	1155	1239*	1141	118	622
14	898	886	953	912	102	708
31	904	842	896	881	112	828
39	913	1097	1262*	1005	86	757
49	779	706	1049	743	67	655

* Neglected values

The fluorescent intensity varied between 450 and 800 for corresponding amounts of control beads (Ca/Ba-alginate beads without fluorescent coating) (Figure C.1). Therefore emission intensity of control bead is not subtracted from intensity of coated capsules.

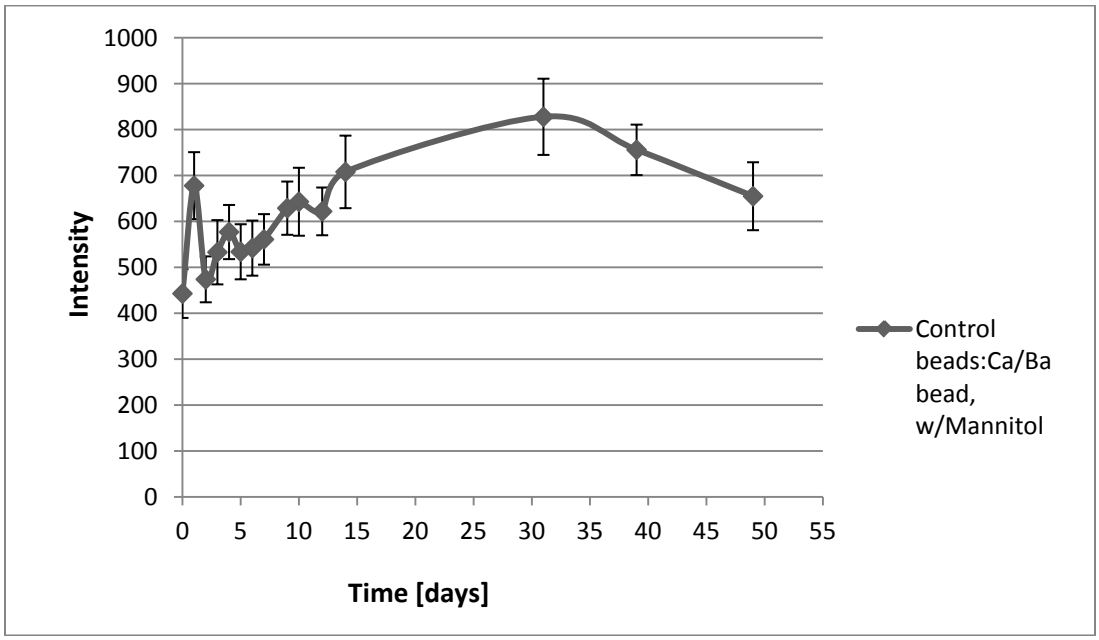

Figure C.1 The long-term measurement of intensity of control beads (Ca/Ba-beads without coating) measured as the fluorescent intensity of beads as a function of time by spectrophotometry at λ=535 nm.

Appendix D: Stability Measurements of coating of alginate-PLL capsules

To see the stability of coating layer in Ca-alginate-PLL-alginate capsules, the fluorescent intensity of coating alginate which fluorescently labeled was measured over time (some days) in capsules with four different coating alginates. The concentration of fluorescent intensity will then be proportional to the stability of coated layer on top of capsules in a sample.

The emission intensity of the fluorescent coating layer of capsules was measured with spectrophotometer at λ=485 nm. For each measurement three parallel (wells) and multiple reads per well (Circle filled (3 x 3)) were used. The capsules were kept in mannitol at 4°C.The result from the measurements are listed in Table C.1-C.6

Table D.1 Emission intensity measurements for calcium alginate-PLL-alginate capsules at λ = 535 nm. The fluorescently labeled sulfated alginate was used as coating layer on the alginate-PLL capsules.

Time [days]	Emission intensity at λ = 535 nm				
	Parallel 1	Parallel 2	Parallel 3	Average	StDev
1	1890	1780	2722	2131	364
3	1975	2087	1978	2013	103
5	2268	2499	2291	2353	190
7	1897	2196	2150	2081	246

Table D.2 Emission intensity measurements for calcium alginate-PLL-alginate capsules at λ = 535 nm. The fluorescently labeled High-G alginate was used as coating layer on the alginate-PLL capsules.

Time [days]	Emission intensity at λ = 535 nm				
	Parallel 1	Parallel 2	Parallel 3	Average	StDev
1	2076	1712	2346*	1894	201
3	1401	1372	1409	1394	109
5	1303	1284	1271	1286	114
7	1181	1127	1333	1214	148

*Neglected value

Table D.3 Emission intensity measurements for calcium alginate-PLL-alginate capsules at λ = 535 nm.The fluorescently labeled High-M alginate was used as coating layer on the alginate-PLL capsules.

Time [days]	Emission intensity at λ = 535 nm				
	Parallel 1	Parallel 2	Parallel 3	Average	StDev
1	1788	1376	1808	1657	101
3	1464	1537	1570	1524	212
5	1271	1304	1255	1293	87
7	1141	1274	1350	1255	140

Table D.4 Emission intensity measurements for calcium alginate-PLL-alginate capsules at λ = 535 nm. The fluorescently labeled epimerized (high-MG) alginate was used as coating layer on the alginate-PLL capsules.

Time [days]	Emission intensity at λ = 535 nm				
	Parallel 1	Parallel 2	Parallel 3	Average	StDev
1	3882	3002	3742	3542	220
3	2726	2767	2827	2773	403
5	2280	2251	2599	2377	141
7	2634	2001	2211	2282	265