Formation of Alginate Nanospheres

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For my parents

Contents

List of abbreviations

% (v/v)	Volume percentage
% (w/w)	Weight percentage
AES	Auger electron spectroscopy
CR	Congo red
Cryo-SEM	Cryo Scanning Electron Microscope
DLS	Dynamic light scattering
E/O mixture	Ethanol in Oil mixture
EDX	Energy-dispersive X-ray spectroscopy
G residues	α-L-guluronic acid residues
G'	Storage modules
G''	Loss modules
GDL	Glucono delta-lactone
m	molal
M residues	β-D-mannuronic acid residues
MCT oil	Medium Chain Triglyceride oil
MLS	Multiple Light Scattering
O/W	Oil in water emulsion
PGPR	Polyglycerol polyricinoleate
SEM	Scanning Electron Microscope
t_0	Starting point of an exponential increase in G' and G''
t_G	Crossover of G' and G'' is defined as gel point
W/O	Water in oil emulsion
W/O/W	Water in oil in water emulsion

Chapter I

General Introduction

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1.1 INTRODUCTION

This thesis describes food-grade techniques for the formation of micro- and nanoparticles, which could be used for trapping nutrients and functional food ingredients in their interior. In this introduction alginate and its purpose are shortly defined. Furthermore the outline and aim of this thesis are given.

1.2 ALGINATE

Alginate is a naturally occurring linear unbranched polysaccharide extracted from brown seaweed. Alginate consists of a varying proportion of (1,4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Figure 1.1). Alginate from different sources have varying composition and sequence of G and M residues and are therefore different in properties, but alginate can also be chemically modified to alter its properties. Alginate has been extensively reviewed with respect to its physical and chemical properties [1-3]. Due to its special properties, such as being non-toxic, biodegradable, low in costs and readily available, alginate is one of the most used polymers in (micro)particle formation [4-7]. However alginate is currently less commonly used in the formation of alginate nanoparticles [8]. Developing methods to produce such small particles would significantly increase the application range of alginate hydrogel system.
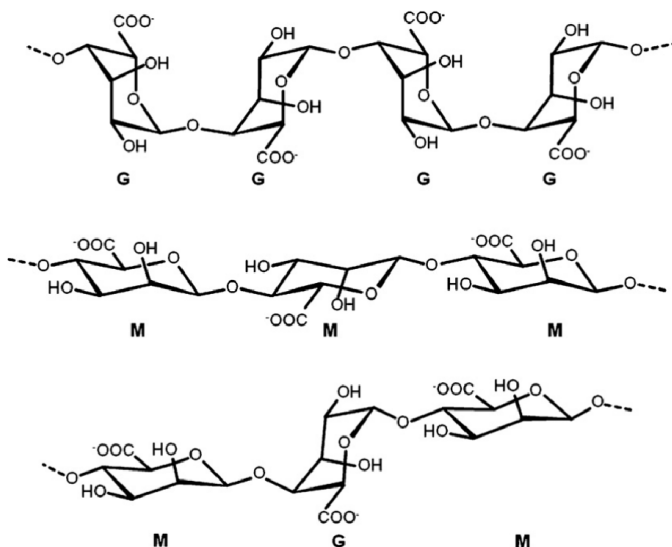

Figure 1.1 Chemical structures of G-block, M-block, and alternating block in alginate. [1]

1.3 ALGINATE PARTICLE FORMATION

Alginate can easily be gelled with multivalent cations under gentle conditions allowing formation of gelled beads. Gelation is obtained by the stacking of guluronic acid (G) blocks with the formation of "egg-box" calcium linked junctions (Figure 1.2). Although various cations can be used for gelation, and each with its specific affinity for alginate, calcium is most frequently used since it is clinically safe, readily available and low in cost [9-11]. Gelation of alginate occurs often through external gelation, the cations come from the exterior of the alginate system, or internal gelation, where the cations are released from the interior of the alginate system.
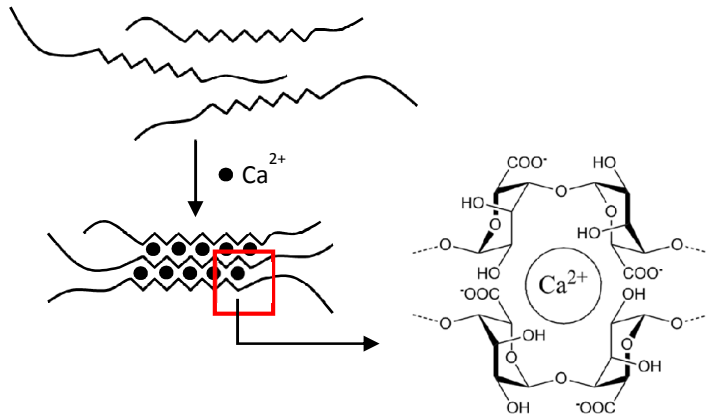

Figure 1.2 Formation of an alginate gel by calcium cations, resulting in "egg-box" calcium linked junctions.

Alginate particles of various sizes can be prepared and are classified in different size groups. Macro-particles can be easily seen with the naked eye, think of e.g. drug or vitamin tablets. Micro- particles range from a few microns to several millimeters in size. Particles that are smaller than 1 micrometer in diameter are classified as nano- particles. Nano-particles are currently less commonly available compared to micro-particles, but the need for smaller and smaller particles is increasing [7,12]. In food products, applying particles smaller than 25 micrometer has the advantage that they are undetectable by the palate of the consumer [13]. And in pharmaceutics nanoparticles containing drugs have shown several advantages over larger particles [8,14-22]. Therefore it is important to develop new and efficient (food-grade) techniques for the formation of micro- and especially nanoparticles.

The most common method for formation of alginate particles is by extruding an alginate solution through a needle dropwise in a bath with cations, such as in a $CaCl_2$ solution. The alginate particles are formed through external gelation and typically have a diameter of 500 – 5000 μm [23-27]. Several methods are based on this external gelation technique and by coaxial laminar air-flow at the nozzle, electrostatic fields and vibrating nozzles the alginate particle size could be significantly reduced and particles with a narrower size distribution could be obtained [10,23,28,29].

Alginate spheres can also be obtained through an emulsification-gelation method. First an alginate solution is emulsified in an oil phase followed by gelation of the alginate emulsion droplets, forming gelled spheres. Alginate emulsion droplets have been gelled through external gelation, whereby the emulsion is usually broken with a $CaCl_2$ solution, but the spheres are then often clustered, and multiple emulsion droplets (W/O/W) may form as a by-product. Internal gelation has also been used in gelation of alginate emulsion droplets and often an insoluble calcium source (usually $CaCO_3$) is added to the alginate phase prior to emulsification. Gelation is achieved by addition of an oil-soluble acid that causes calcium ion release, but usually results in alginate particles with an acidic pH which could be harmful to sensitive compounds inside the sphere. Mechanical emulsification and membrane emulsification have both been reported in formation of alginate particles [10,25,30-39]. Membrane emulsification has the advantage that it can produce alginate particles with a very narrow size distribution, but formation of particles is currently limited to the micro range (> 1 μm) and fouling of the membrane surface as well as feasibility for scaling up to industrial sizes remain a challenge. Mechanical emulsification is more feasible for industry and it can theoretically produce alginate particles in all size ranges, but the formed alginate particles have a relative wide size distribution. Although mechanical emulsification can produce alginate nanoparticles it is not yet very common.

The formation of alginate nanoparticles with a size of 250 – 850 nm was first reported by Rajaonarivony *et al*. in 1993 [40]. Since then other methods have also been reported for formation of alginate nanoparticles. Their formation and properties are further discussed in *Chapter 2*.

1.4 ENCAPSULATION

Encapsulation is the phenomenon where a component is shielded from its environment by enclosing it in another material, such as a core-shell microcapsule, or other colloidal systems like spherical and non-spherical hydrogel particles (Figure 1.3). In these colloidal hydrogel systems a component is trapped in their interior polymer network, and these particles may have an additional shell, giving extra protection from the environment, and additional mechanical stability. Such encapsulates must often withstand various temperatures, wide pH ranges and mechanical forces during product processing, storage and consumption. Alginate can easily be gelled with cations under gentle conditions making it ideal for entrapment of sensitive materials. The first alginate particles for encapsulation purposes were developed in 1980 [41]. In foods and pharmaceutics alginate has been used for encapsulation of functional ingredients like vitamins and probiotics [6,42], encapsulation of islet cells [41], for cell delivery and transplantation [22,43,44], for oral delivery of peptide or protein drugs [45], for sustained/controlled drug release [29,46], and to immobilize active cells [47] and enzymes.
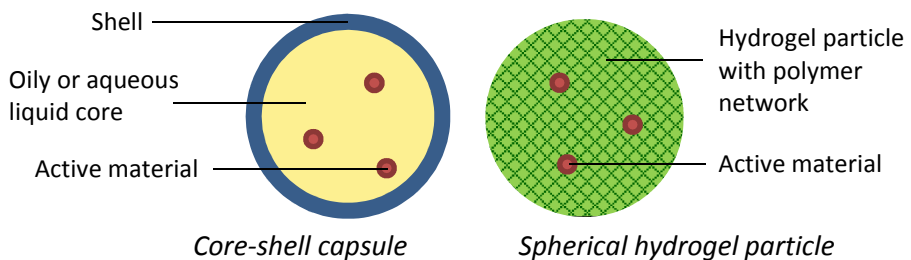

Figure 1.3 Schematic representation of a core-shell capsule and a spherical hydrogel particle. The capsule has an oily or aqueous liquid core, containing a certain compound, and has a shell around it. The hydrogel particle is a spherical matrix system with a component entrapped in their interior polymer network.

The application envisioned for the encapsulates determines their properties, and this requires simple and flexible production methods. In certain applications encapsulates must be very durable, rigid and impermeable to protect its vulnerable contents, but on the other hand the contents must be easily released at the target location in response to specific triggers. In other applications, such as cell or enzyme immobilization, encapsulates

are a type of carrier that enhance the mechanical stability of such compounds by embedding them in the interior of the particles and where transportation of valuable nutrients and oxygen across the encapsulate material is important [48]. The particle size is also determined by the envisioned application and determines whether macro, micro or nanoparticles are required.

Various methods can be used for encapsulation and common ones include mechanical techniques such as spray-drying/congealing, fluidized bed coating or extrusion. Furthermore physical-chemical techniques, such as (complex) coacervation, interfacial polymerization, solidification, gelation or evaporation, can be used for encapsulation.

1.5   AIM AND OUTLINE OF THIS THESIS

The aim of the research reported in this thesis was to develop new methods for the formation of microparticles with a diameter below 25 microns. These particles should be able to function as carriers. The pH conditions inside these particles should be sufficiently mild such that pH sensitive components can be encapsulated.

A graphical illustration of the short thesis outline is presented in Figure 1.4
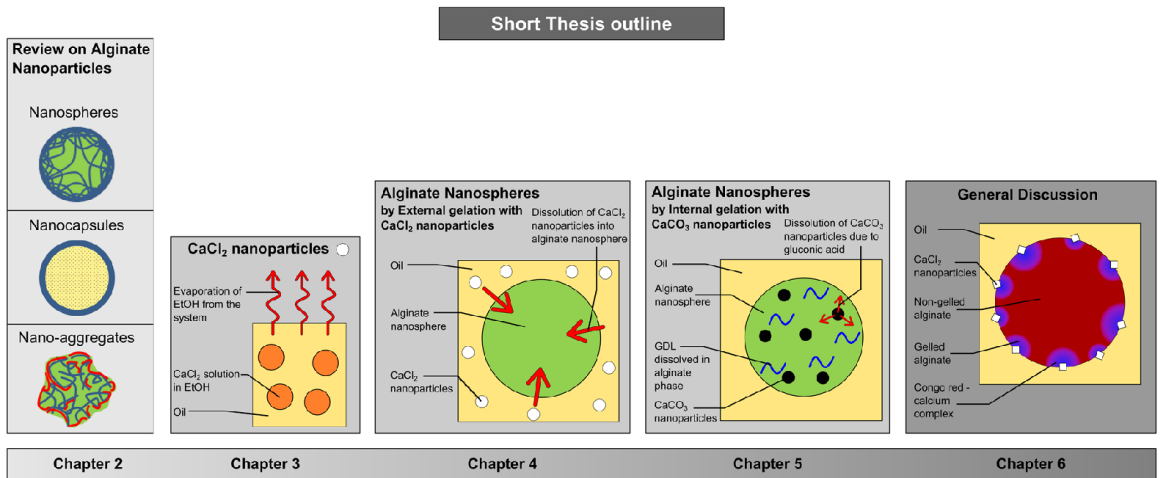

Figure 1.4 Graphical presentation of the thesis content.

In *Chapter 2*, we present a review which covers various existing techniques for the formation of alginate nanoparticles, and their properties.

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Chapter 3 presents a novel technique for the formation of food-grade salt nanoparticles using ethanol-in-oil emulsion. The method and particles were developed to achieve a new route for gelation of alginate nanoparticles.

Chapter 4 describes the formation and characterization of alginate nanospheres by external gelation using the food-grade salt nanoparticles described in *Chapter 3*.

Chapter 5 deals with the formation of alginate nanospheres by internal gelation utilizing $CaCO_3$ nanoparticles. Due to a different technique these alginate nanospheres have different properties, and therefore different potential applications, than the alginate nanospheres described in *Chapter 4.*

Finally, in *Chapter 6* a general discussion of the reported research is given, and future developments and challenges are discussed.

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Chapter II

Preparation Methods of Alginate Nanoparticles

2

ABSTRACT

This chapter reviews available methods for the formation of alginate nano-aggregates, nanocapsules and nanospheres. Primarily, alginate nanoparticles are being prepared by two methods. In the "complexation method", complex formation on the interface of an oil droplet is used to form alginate nanocapsules, and complex formation in an aqueous solution is used to form alginate nano-aggregates. In a second method w/o emulsification coupled with gelation of the alginate emulsion droplet can be used to form alginate nanospheres. We review advantages and disadvantages of these methods, and give an overview of the properties of the alginate particles produced with these methods.

2.1 INTRODUCTION

The first alginate particles for encapsulation purposes were developed in 1980 [1]. Since then much research has been performed on the development and application of alginate particles, resulting in alginate being one of the most commonly applied materials for the formation of hydrogel (micro) particles nowadays [2-5]. Alginate can easily be gelled with multivalent cations under gentle conditions, making it applicable for the entrapment of sensitive materials.

Most of the gelled alginate particles described in literature have a diameter larger than 100 µm. Alginate particles much smaller than that (< 1 µm) have several advantages over such larger alginate particles. Small particles have a higher mechanical strength and a larger specific surface area. They can easily flow through narrow nozzles and channels which would be blocked by larger particles. Especially in drug delivery, nanoparticles have attracted considerable attention and have several advantages over micron-sized particles [6-14]. However the formation of nanoparticles from alginate is less common, and synthetic polymers like poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(lactide-co-glycolide) (PLGA) have received significantly more attention in the literature [13].

In this review, the focus will be on the formation of alginate nanoparticles. First some general chemical and physical properties of alginate and different types of alginate bulk gels are discussed. This is followed with a brief overview of methods to prepare alginate *microparticles*. Then the different methods for the formation of alginate *nanoparticles* are discussed. Finally conclusions and a discussion of remaining challenges are given.

2.1.1 Alginate

Alginate has been extensively reviewed with respect to its physical and chemical properties and its use in the formation of (micro)particles and bulk gels [15-25]. Alginate is non-toxic, biodegradable, low in costs, and readily available, and has been found to be a mucoadhesive, biocompatible, and non-immunogenic substance. Alginate is an anionic polymer, found in brown algae and bacteria, and consists of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues, linearly linked by 1,4-glycosidic linkages. The composition and sequence of the G and M residues depend on the source of the used algae, and influence the properties of the alginate. Alginate can also be chemically modified to alter its properties[26,27]. Alginate can easily be gelled with multivalent cations under gentle

conditions making it ideal for the entrapment of sensitive materials. Various cations show different affinity for alginate, whereof calcium is most frequently used for alginate gelation, but calcium does not form the strongest binding with alginate [18,28,29]. The gelation of alginate solutions with cations can occur through so-called external gelation, internal gelation and gelation upon cooling [18].

For external gelation alginate is often dripped into a bath containing cations, such as a calcium chloride solution. The cations diffuse from the continuous phase into the interior of the alginate droplets and form a gelled alginate matrix from the outside migrating to the center of the alginate droplet [30-32]. Cationic polymers, such as chitosan, can be used to form a polyelectrolyte complex with alginate, and hence alginate capsules can also be obtained by dripping an alginate solution in a bath containing a cationic polymer [33,34].

Internal gelation makes use of a water insoluble calcium salt, like $CaCO_3$, which is mixed with the alginate solution. Calcium ions are subsequently released from the interior of the alginate phase by lowering the pH of the system, resulting in the formation of an alginate gel.

The third method uses an alginate solution with a calcium salt at elevated temperatures (90°C), which is then allowed to set through cooling [35]. The elevated temperature in this method makes it less gentle and is unsuitable for thermally labile material.

2.1.2 Alginate microparticles

Alginate is one of the most commonly used polymers for the formation of (micro)particles [2-5]. Several methods have been developed to form alginate microparticles of various size ranges (Figure 2.1). The majority of these methods is based on an external gelation method, where an alginate solution is added dropwise to a bath containing cation, such as in a $CaCl_2$ solution [36]. The material to be encapsulated is often mixed with the alginate solution prior to particle formation and gelation.

Typically an alginate solution is extruded through a needle to form alginate droplets with a diameter of 500 – 5000 µm [37-41]. More advanced droplet generation techniques have been developed as well, which have an improved yield, with smaller particles and a narrower size distribution [17,19,29,37]. Examples are systems where a coaxial laminar air-flow (AirJet) is used at the nozzle, which shears off the droplet, and yields droplets with a

diameter of 300 – 1000 µm [38,42-45]. Electrostatic fields have also been used to pull the droplet from the nozzle into the gelation bath, forming particles of 50 – 1000 µm [33,34,42,45-49]. In the vibrating nozzle technique a laminar flowing liquid jet from a nozzle breaks up into equal sized droplets by a superimposed vibration, resulting in particles of 150 – 2000 µm [38,42,45,50-52]. Another method mechanically cuts the continuous fluid jet which exits the nozzle into uniform segments, and forms beads (due to surface tension) in a range of 120 – 3000 µm (laminar jet break-up) [42,53-57]. All of the above described methods are syringe-based systems and have problems in large-scale production, because a large number of needles/nozzles are required, and operational problems such as needle blockage, cleaning and sanitation are significant [58]. Furthermore alginate microparticles obtained through dripping an alginate solution in a gelation bath may be deformed, and have a teardrop shape due to drag forces following the impact with the gelation bath [29].
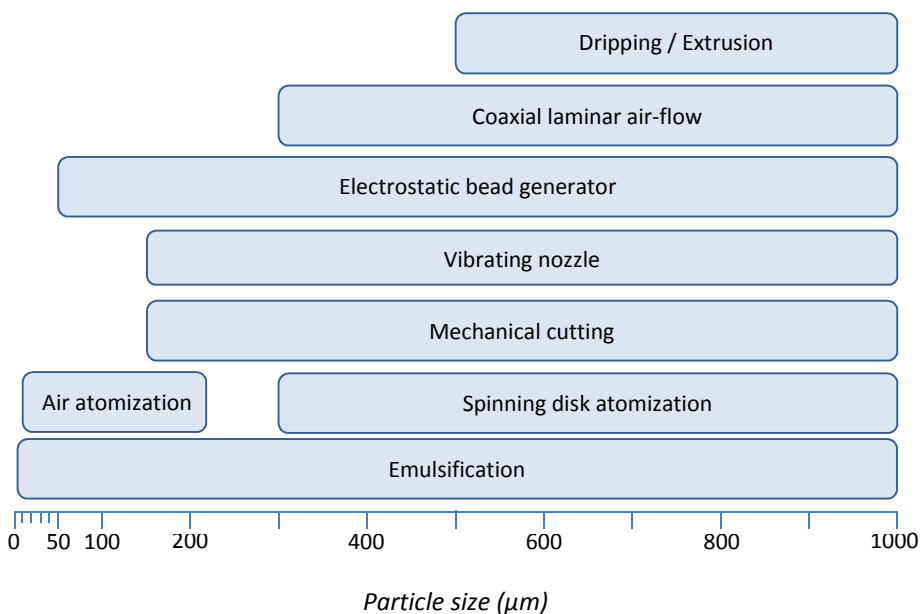
Figure 2.1 Methods for the formation of alginate microparticles, with the corresponding size range.

As an alternative to nozzle based methods, air atomization can be used for the formation of alginate particles, when coupled with external gelation, and yields particles of 5 – 200 µm [45,59,60]. Another external gelation method uses a spinning disk for droplet formation and results in alginate particles with a diameter of 300 – 3000 µm [61,62].

Mechanical emulsification and membrane emulsification have also been used for the formation of alginate microparticles [29,39,63-72]. An alginate solution is emulsified in an oil phase forming a w/o emulsion which is then followed by external gelation or internal gelation of the alginate droplets. By controlling the conditions under which the water-in-oil dispersion is produced, the particle size can be controlled from a few microns to millimeters in diameter [63].

2.2 ALGINATE NANOPARTICLES

Nanoaggregates, nanocapsules and nanospheres are nanosized systems with diameters generally ranging from 10 – 1000 nm in size. These systems can hold enzymes, drugs and other compounds by dissolving or entrapping them in, or attaching them to the particle's matrix. The method used to obtain the nanoparticles determines whether nano-aggregates, nanocapsules, nanospheres, or nanocapsules with a structured interior are obtained (Figure 2.2). Nano-aggregates can be described as nanosized colloidal systems in which the drug is physically dispersed, and can have different morphologies. Nanocapsules are vesicular systems in which the drug is confined to an oily or aqueous liquid core, surrounded by a polymeric membrane. Nanospheres are spherical particles with a gelled interior in which the entrapped component is physically dispersed [12,73-76]. Nanocapsules with a structured core have also been designed, and are basically a combination of a nanocapsule and a nanosphere. They are often produced by first preparing a nanosphere and subsequently an additional shell that is formed on the interface of the nanosphere.

Primarily alginate nanoparticle formation is based on two methods:

1. Complexation; complex formation can occur in an aqueous solution forming alginate nano-aggregates or on the interface of an oil droplet, forming alginate nanocapsules. A crosslinker such as calcium from calcium chloride is used for complexation of alginate. Complexation can also occur through mixing alginate with an oppositely charged polyelectrolyte such as poly-L-lysine.

2. Alginate-in-oil emulsification, coupled with external or internal gelation of the alginate emulsion droplet, forming alginate nanospheres.

Those methods are further discussed individually in the next sub-paragraphs. Table 2.1, 2.2 and 2.3 give an overview of alginate nano-aggregates, nanocapsules and nanospheres described in the literature.
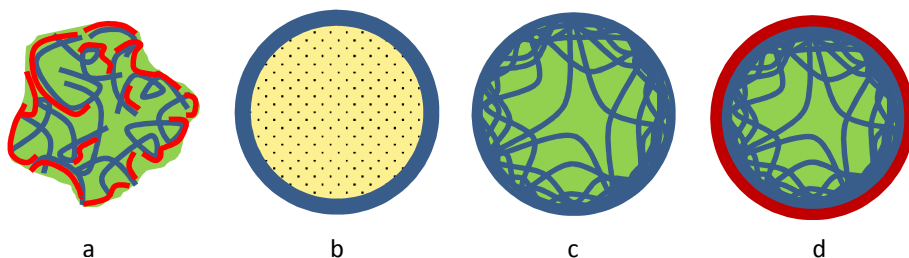
Figure 2.2 Schematic representation of a nano-aggregate (a), nanocapsule (b), nanosphere with structured interior (c) and nanocapsule with structured interior (d). The nanocapsule has an oily or aqueous liquid core with a shell around it, and nanospheres are spherical matrix systems. The nanocapsule with structured interior is a combination of a nanosphere and a nanocapsule.

2.2.1 Alginate nano-aggregate formation by self-assembly, and complexation

In 1993 Rajaonarivony *et al*. described a novel method for the formation of alginate nano-aggregates as drug carrier with sizes ranging from 250 – 850 nm [77]. The particles were formed in a sodium alginate solution by first adding calcium chloride and then poly-L-lysine.
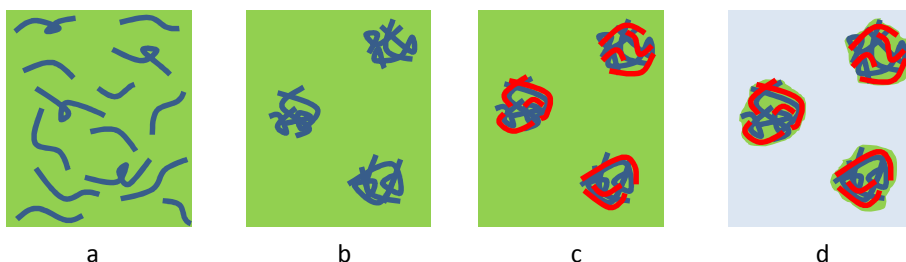
Figure 2.3 Schematic representation on formation of alginate nano-aggregates. Alginate solution (a). Upon addition of $CaCl_2$ the so called pre-gel state is formed consisting of nanosized aggregates dispersed in a water continuous phase (b). Addition of cationic polymer results in a polyelectrolyte complex coating of the nanoparticles (c). Isolated, washed, and redispersed nano-aggregates (d).

The concentrations of sodium alginate and calcium chloride were lower than those required for typical alginate gel formation. By mixing low concentrations of alginate and calcium chloride a so called pre-gel state was formed consisting of nanosized aggregates dispersed in a water continuous phase (Figure 2.3b). Addition of an aqueous polycationic

solution, such as poly-L-lysine, resulted in a polyelectrolyte complex coating of these alginate nanoparticles (Figure 2.3c).

Since poly-L-lysine is toxic and immunogenic when injected into the human body, chitosan and Eudragit E100 have been used as an alternative cationic polymer [28,78-82]. Alginate aggregates have also been prepared by solely combining chitosan [83] or calcium ions [84,85] with a sodium alginate solution. The alginate and cationic polymer concentration, their molecular weight, the calcium chloride concentration, and the order of addition of calcium chloride and cationic polymer to the sodium alginate solution, were found to be of great influence on the size and properties of the obtained nanoparticles [28,77]. Subsequent high shearing conditions during nano-aggregate formation were also found to be of importance to obtain small nanosized alginate aggregates [83]. Chang *et al.* developed alginate nano-aggregates through self-assembly, that did not require calcium induced aggregation of alginate, or cationic polymers to form a polyelectrolyte complex with alginate. Instead they synthesized amphiphilic thiolated sodium alginate, and sonication facilitated an oxidation reaction of the thiol groups, inducing self-assembly of the alginate into nano-aggregates [86].

Alginate nano-aggregates have successfully been loaded with insulin [79,80], antisense oligonucleotide [87], and anti-cancer drugs, such as methotrexate [88] and 5-Fluorouracil [85]. The method of Rajaonarivony *et al.* has been adapted further resulting in alginate nanocomposites with intra-capsular formation of magnetic cobalt silicates that could be obtained by a spray-drying technique [89]. Recently supermagnetic alginate nano-aggregates were prepared for the immobilization of lipase, and were subsequently functionalized with oxidic poly-(ethylene glycol) [81].

2.2.2 Alginate nanocapsule formation by complex formation on the interface of emulsion droplets

One of the most commonly used methods for nanocapsule formation is the deposition of polymers on the interface of a template droplet, with subsequent solvent removal [90,91,92]. After the deposition of the polymers on the droplet interface, the polymer shell is stabilized by physical or covalent intermolecular crosslinking. Alginate has successfully been used in this way to produce nanocapsules of various sizes, and was used to

encapsulate testosterone[93], acyl derivatives[94], turmeric oil [75,78,91] and an ethanolic extract of *Phyllanthus amarus*[95].

The preparation of alginate nanocapsules consists of first mixing the drug or components that are to be encapsulated with an organic solvent, that will act as the interior oil phase of the capsules. The mixture is slowly added to an aqueous solution of alginate, containing an additional surfactant, such as Tween80. Subsequently, sonication is used to form an oil-in-water (o/w) emulsion (Figure 2.4b). Often calcium from a calcium chloride solution, is slowly added to the emulsion and alginate is deposited to form the nanoparticle membrane (Figure 2.4c). Finally, the nanocapsule aqueous suspension is allowed to equilibrate for a certain time prior to solvent removal (Figure 2.4d).
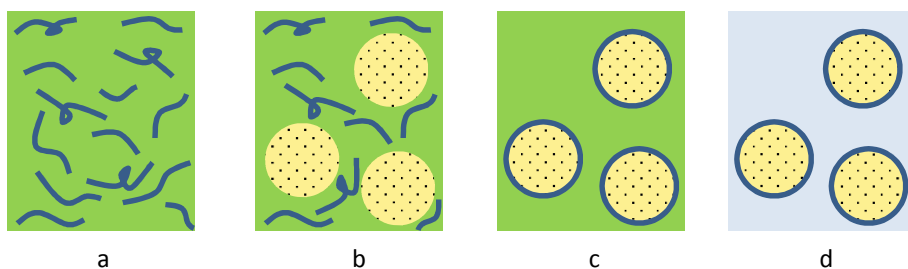

a b c d

Figure 2.4 Schematic representation on the formation of alginate nanocapsules. Alginate solution (a). Oil phase with dissolved drug is emulsified in alginate solution (b). Upon the addition of CaCl$_2$ the alginate deposits on the interface of the oil droplets (c). Solvent is removed from the nanocapsules and nanocapsules are isolated, washed and redispersed (d).

Chitosan has been used in addition to calcium chloride, resulting in a polyelectrolyte complex between alginate and chitosan that deposits on the interface, and it is believed that this improves the stability of the capsules and reduces the porosity of their membranes [75,78,94]. However the combined addition of chitosan and calcium chloride results in significantly larger nanocapsules (500-700 nm) compared to nanoparticles prepared without chitosan (150 nm) [75,78]. The order of the addition of chitosan and calcium chloride also influences the size of the nanoparticles, where the addition of chitosan after calcium chloride results in smaller particles then when chitosan is added before calcium chloride. Furthermore, the addition of Tween80 and/or sonication resulted in smaller nanoparticles than that of the samples prepared without Tween80 and/or

sonication [75,91]. It has also been demonstrated that the type of oil phase influences nanoparticle size and drug loading [91,93].

2.2.3 Alginate nanosphere formation from water-in-oil emulsions

Emulsion droplets are good templates for the formation of spherical particles, and the mechanical power input used to prepare the emulsion determines the size of the particles. Sonication, membrane emulsification and (self-assembled) microemulsions can also be used to form emulsion droplets [68-72,96-100]. Emulsions are relatively easy to produce and emulsion based methods for nanoparticle formation can therefore more easily be scaled up to industrial sizes than nozzle based methods. Alginate-in-oil (w/o) emulsions coupled with internal or external gelation have been used to prepare alginate micro- and nanospheres. The type of gelation used determines the properties of the formed alginate particle. Alginate gels prepared through internal gelation and external gelation differ in several properties, as matrix strength, stiffness, pore size and permeability [30-32]. Alginate particles prepared with external gelation tend to have a denser structure with smaller pores at the surface of the particle, compared to the interior of the particle, due to a concentration gradient of the cations (high concentrations at the surface and low at the core) (Figure 2.5d). Alginate particles prepared through internal gelation have a more even distribution of the cations throughout the particle, resulting in a more homogenous particle (Figure 2.6d). Since the properties of alginate particles prepared through external and internal gelation differ they are suitable for different applications.

Alginate nanospheres prepared through the emulsification coupled with *external* gelation are prepared through a 2 step procedure (Figure 2.5). First an alginate solution, containing a certain compound e.g. a drug or enzyme, is emulsified in an oil phase forming a w/o emulsion. Second a crosslinker, often calcium from a calcium chloride solution, is then added to the emulsion, resulting in the gelation of the alginate emulsion droplets, followed by the demixing of the emulsion. This method has successfully been used to prepare alginate nanospheres containing GFP-encoding plasmids[96], cytochrome-c as a marker[101], capsaicin as a hydrophobic model drug[102], and doxorubicin with carbon-coated iron nanoparticles to obtain magnetic nanospheres[97]. Oligochitosan has been used as alternative to calcium chloride to form a complex with low molecular weight alginate in alginate nanoparticle formation [98,99].
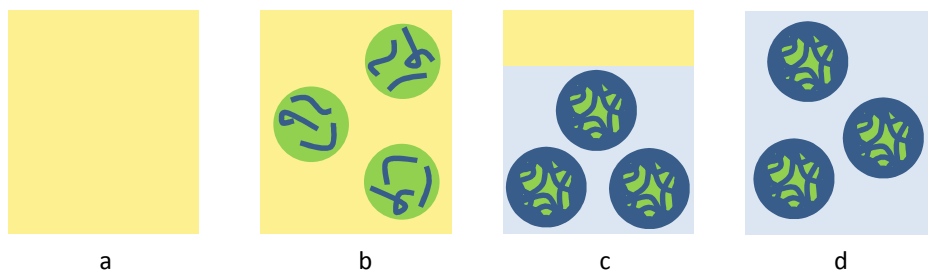
Figure 2.5 Schematic representation of the formation of alginate nanospheres by emulsification coupled with external gelation. Continuous oil phase (a). Emulsification of alginate solution in oil phase forming w/o emulsion (b). Addition of CaCl$_2$ solution to oil phase results in demixing of the emulsion and external gelation of the alginate nanospheres (c). Washed alginate nanospheres (d). The external gelation results in spheres with an inhomogeneous network that is denser and has smaller pores at the surface of the alginate sphere compared to the interior of the sphere.

Alginate composite nanoparticles have also been prepared though *water-in-water* emulsification by dispersing an alginate solution under a high shear directly in a calcium chloride solution[103]. The resulting particles appear more as random aggregates which are not entirely spherical and have significant surface roughness. Alginate nanospheres prepared from a w/o emulsion and demixing with a calcium chloride solution can often result in clustering of the alginate nanospheres[64], and multiple emulsion droplets (w/o/w) may form as a by-product.

Recently a novel method was developed to prepare alginate nanospheres from a w/o emulsion coupled with external gelation (Figure 2.6) [104]. The method does not include a demixing step with a calcium chloride solution, but instead food-grade calcium chloride nanoparticles[105] dispersed in the oil phase were used for the gelation of the alginate nanospheres, thereby minimizing the formation of clusters and multiple emulsion droplets. The gelled alginate nanospheres are obtained in the oil phase, but can be transferred to a water phase if desired.

Reis *et al*. demonstrated a two-step method for the formation of alginate nanospheres through the emulsification coupled with *internal* gelation [106]. First an alginate solution containing an insoluble calcium source, such as calcium carbonate, is emulsified in an oil phase forming a w/o emulsion. Then an oil-soluble acid is added to the oil phase, which lowers the pH inside the alginate particles, causing the release of Ca^{2+} from the insoluble

salt and thereby triggering *in situ* gelation of the alginate particles. The method was used to encapsulate insulin with high encapsulation efficiencies with and without additional coatings [29,106-109].
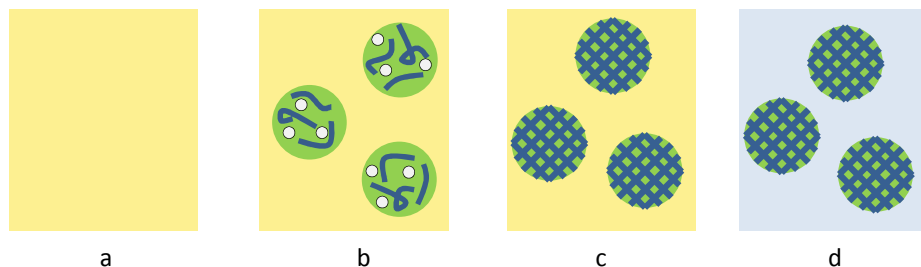

a   b   c   d

Figure 2.6 Schematic representation of the formation of alginate nanospheres by emulsification coupled with internal gelation. Continuous oil phase (a). Emulsification of alginate solution (containing insoluble calcium source) in oil phase forming w/o emulsion (b). Solubilizing calcium source by lowering pH inside spheres results in the internal gelation of the alginate nanospheres (c). Washed alginate nanospheres (d). The internal gelation results in alginate spheres with a very homogeneous alginate network throughout the entire sphere.

Recently a single step procedure for the formation of alginate nanospheres was developed based on the emulsification coupled with an internal gelation by Paques *et al* [110]. An alginate solution containing nanoparticles of calcium carbonate and glucono delta-lactone (GDL) was emulsified in an oil phase. The GDL hydrolyzes into gluconic acid and allows the dissolution of the calcium carbonate resulting in the gelation of the alginate particle. The concentrations of calcium carbonate and GDL were optimized such that a final pH around 6 was obtained inside the alginate nanospheres, and the shrinkage of the alginate gel was minimized. This makes these alginate nanospheres more feasible for pH sensitive compounds such as certain bacteria and enzymes, compared to the method of Reis *et al*. These typically had an internal pH around 4.5, and had much higher calcium concentration, which could cause the shrinkage of the alginate gel.

2.2.4 Nanocapsules with a structured interior

Alginate forms porous gels and the methods and materials used to prepare the alginate particles determine their porosity. A porous gel allows better transportation of nutrients and oxygen, especially in small particles, which have a larger specific surface

area. This is an important factor for e.g. immobilized enzymes and islets of Langerhans [111]. However for certain applications the entrapped compounds may leach out of the alginate particles, or the entrapped compounds are sensitive and need protection from other compounds like acids or salts, and permeability through the alginate gel must be minimized. Then additional shells must be applied to the alginate particles to reduce their permeability. The deposition of additional layers on an alginate nanosphere template results in the formation of a nanocapsule with structured interior.

Due to the anionic properties of alginate it is relatively easy to deposit a cationic polymer on the surface of alginate particle, to form a shell [19,23,37,112-115]. Poly-L-lysine and chitosan are often used to form a shell on alginate particles. But also other coatings such as silica have been used on alginate particles [89]. Alternating the deposition of cationic polymers with anionic polymers, allows the stacking of multiple shell layers on top of each other, a so called layer-by-layer assembly. Using multiple shell layers can significantly reduce the permeability of the entire shell and can also give additional mechanical strength to the capsule. Alginate particles can also be functionalized by adding shell layers, either by adsorbing functional compounds in the shell, or by modifying shell polymers by chemically attaching specific functional groups. Particle charge can also be modified, hence influencing the interaction with its environment. In target release it is important to design a particle with a shell that is sufficiently strong to protect its contents, but on the other hand it should easily release its contents at the target location.

2.3 CONCLUSION AND OUTLOOK

In this review the preparation methods of alginate nanoparticles have been summarized. Primarily alginate nanoparticle formation is based on two methods: the complexation method, and w/o emulsification coupled with the gelation of the alginate emulsion droplet. Alginate is a promising biodegradable polymer for the formation of nanoparticles for the application in the drug delivery and immobilization of enzymes since it is low in costs, readily available, non-toxic, and it can easily be gelled with cations. Using mild conditions and no organic solvents in the preparation of alginate nanoparticles make them applicable for the entrapment of sensitive materials and for use in pharmaceuticals and foods. The formation of shell layers and functionalizing the particle surface with certain groups or ligands are useful to obtain specific stability and functionality.

An important challenge that remains is the development of environmentally friendly processes for the formation of nanoparticles, with a narrow size distribution, high mechanical and chemical stability, and feasibility to scale up to industrial scale production volumes. Furthermore it is important to evaluate the cytotoxicity, immune response and biodegradability of such particles in their potential applications.

2.4 ACKNOWLEDGMENTS

2

Table 2.1 Overview of alginate nano-aggregates

Capsule	Active ingredient	Cross-linker	Size	Ref
Alginate / poly-l-lysine	Doxorubicin (model drug)	Calcium chloride and poly-l-lysine	250 – 850 nm	Rajaonarivony, 1993 [77]
Alginate / Poly-l-lysine	antisense oligonucleotide	Calcium chloride	300 nm	Aynié, 1999 [87]
Alginate / chitosan or Alginate / poly-l-lysine	-	Calcium chloride and chitosan or Calcium chloride and poly-l-lysine	282 – 656 nm (4)	De, 2003 [28]
Alginate / chitosan	DNA	Calcium chloride and chitosan	323 – 1600 nm	Douglas, 2005 [116]
Alginate / Poly-l-lysine	Methotrexate	Calcium chloride and Poly-l-lysine	186 - 622 nm (4)	Santhi, 2005 [88]
Alginate / Poly-l-lysine with silica[1]	Magnetic cobalt silicates	$CaCl_2$ $CoCl_2$	250 ± 150 nm	Boissière, 2006 [89]
Alginate / chitosan	DNA	Chitosan	157 nm	Douglas, 2006 [117]
Alginate / chitosan	Insulin	Calcium chloride and chitosan	423 – 850 nm	Sarmento, 2006 [118]
Alginate / chitosan	Insulin	Calcium chloride and chitosan	764 - 2209 nm	Sarmento, 2006 [80]
cisplatin – alginate complex / chitosan	Cisplatin	Chitosan	180 – 350 nm	Cafaggi, 2007 [119]
Alginate / chitosan	Insulin	Calcium chloride and chitosan	797 – 4895 nm	Sarmento, 2007 [120]
Alginate / chitosan	Insulin	Calcium chloride and chitosan	850 ± 88 nm	Sarmento, 2007 [121]

Table 2.1 Overview of alginate nano-aggregates

Capsule	Active ingredient	Cross-linker	Size	Ref
Alginate / chitosan	Insulin	Calcium chloride and chitosan	750 nm	Sarmento, 2007 [79]
Alginate / Eudragit E100	Gliclazide	Calcium chloride / Eudragit E100		Sonavane, 2007 [82]
Alginate / chitosan	Antibiotic gatifloxacin	Chitosan	205 – 572 nm	Motwani, 2008 [122]
Alginate / chitosan	-	Chitosan	-	Sæther, 2008 [83]
Alginate	5-Fluorouracil	Ca(OH)$_2$	480 – 1080 nm [4] 1200 – 3000 nm [4]	Yu, 2008 [85]
Alginate [2]	-	Ca(OH)$_2$	-	Yu, 2009 [84]
Amphiphilic thiolated sodium alginate	5-aminosalicylic acid	disulfide linkages	170 nm	Chang, 2012 [86]
Alginate / chitosan [3]	Fe$_3$O$_4$ nanoparticles / Candida rugosa lipase	Ca(OH)$_2$	60 nm	Liu, 2012 [81]

[1] By self-assembly and spray drying

[2] Self-assembly of nanosphere, vesicle, nanoparticle, and nanorod

[3] Self-assembly and layer-by-layer assembly

[4] Range depends on composition

Table 2.2 Overview of alginate nanocapsules

Capsule	Active ingredient	Cross-linker	Size	Ref
Alginate	testosterone	Calcium chloride	34.5 – 819.8 nm [4]	Bhowmik, 2006 [93]
Alginate	Turmeric oil	Calcium chloride	78 – 677 nm [4]	Lertsutthiwong, 2008 [91]
Alginate / chitosan	Turmeric oil	Calcium chloride and chitosan	522 - 667 nm [4]	Lertsutthiwong, 2009 [78]
Alginate / chitosan	acyl derivatives	Calcium chloride and chitosan	400 nm	Grebinişan, 2011 [94]
Alginate	Ethanolic extract of *Phyllanthus amarus*	Calcium chloride	213 ± 4.4 nm	Deepa, 2012 [95]

Table 2.3 Overview of alginate nanospheres

Capsule	Active ingredient	Cross-linker	Size	Ref
Alginate [1]	cytochrome-c	Calcium chloride	748 ± 280 nm	Monshipouri, 1995 [101]
Alginate and BSA [2]	5-Fluorouracil		166 ± 34 nm	Yi, 1999 [123]
Alginate [3]	GFP-encoding plasmids	Calcium chloride	55 – 100 nm	You, 2005 [96]
alginate-dextran [4]	Insulin	calcium carbonate	2604 ± 2141 nm	Reis, 2006 [107]
Alginate / chitosan [3]	plasmid DNA	Calcium chloride and chitosan	64 nm	You, 2006 [124]
Alginate / chitosan [5]	5-aminosalicylic acid	Calcium chloride and chitosan	< 10000 nm	Mladenovska, 2007 [125]
Alginate / chitosan [5]	5-aminosalicylic acid	Calcium chloride and chitosan	< 9000 nm	Mladenovska, 2007 [126]
Alginate-dextran [4]	Insulin	calcium carbonate	267 – 2760 nm	Reis, 2007 [106]
Alginate / oligochitosan [3]	-	Oligochitosan	136 nm	Wang, 2007 [98]
Alginate [6]	Hydroxyapatite and ferromagnetic nanoparticles	Calcium chloride	-	Yamada, 2007 [103]
Alginate-dextran with chitosan/albumin coat [4]	Insulin	calcium carbonate	< 564 nm (Aginate) < 1280 nm (Aginate/chitosan)	Reis, 2008 [108]
alginate-dextran sulphate core with chitosan-polyethylene glycol-albumin shell [4]	Insulin	calcium carbonate	< 1000 nm	Reis, 2008 [109]
Alginate [3]	Doxorubicin (superparamagnetic particles)	Calcium chloride	2670 nm	Liu, 2010 [127]

2

Capsule	Active ingredient	Cross-linker	Size	Ref
Alginate / oligochitosan (3)	BSA	Oligochitosan	136 nm	Wang, 2010 [99]
Alginate (3)	doxorubicin with carbon-coated iron nanoparticles	Calcium chloride	185 nm	Chen, 2012 [97]
Alginate (3)	-	CaCl$_2$ nanoparticles	200 – 1000 nm	Paques, 2013 [104]
Alginate (3)	Capsaicin as model hydrophobic drug	Calcium chloride	259 ± 27 nm	Tachaprutinun, 2013 [102]
Alginate (4)	-	calcium carbonate nanoparticles	200 – 2000 nm	Paques, 2014 [110]

[1] Liposomes with external gelation

[2] Emulsion solidification method

[3] water-in-oil emulsions with external gelation

[4] water-in-oil emulsions with internal gelation

[5] Spray-drying in calcium chloride / chitosan solution

[6] Dispersion in calcium chloride solution

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Chapter III

Food-grade Salt Nanoparticles

ABSTRACT

A simple method for preparing food grade particles in the nano range of ethanol soluble salts using ethanol-in-oil (E/O) mixtures is described. Salts $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ were dissolved in ethanol that subsequently was mixed with a medium chain triglyceride oil phase. It was found that type and concentration of salt have a significant influence on miscibility of ethanol and oil phase and on the stability of E/O mixtures. The ethanol phase was evaporated from the mixture at elevated temperatures, and salt particles with dimensions in the nano range (6 – 400 nm) remained suspended in the oil phase. It was found that the concentration of salt and volume fraction of ethanol in MCT oil have a significant influence on the size distribution of salt particles. The size of $CaCl_2$ and $MgCl_2$ nanoparticles was ascertained by scanning electron microscopy and dynamic light scattering.

3

3.1 INTRODUCTION

Nanoparticles have a wide range of applications. They can be used in medical applications [1-3], for analytics [4,5], in cosmetics [6,7], in food products [8-10], as templates [11-13], or optical and electronic applications [14-19].

Nanoparticles can find application in cases where microparticles cannot be used. For instance, membranes developed for emulsification can have very small pores and nanoparticles of salts could pass through these pores, whereas microparticles (5 – 20 µm) would block them. Also much research is done with glass microchannels [20-28] where different reactions occur within the small channels when substances are mixed (for example, alginate gelation [28]). Such nano salt particles could be ideal in this application since they will not block the channels and they allow development of even narrower channels. Chemical reactions and dissolution of the nano salt particles can occur faster as well compared to microparticles, which have a smaller surface-to-volume ratio.

Nano salt particles dispersed in oil may be used for alginate gelation. Tong *et al*. describe a method for formation of colloidosomes (430 µm) with alginate gel cores and shells of porous $CaCO_3$ microparticles [29].

Fortification of foods and beverages with mineral salts, such as calcium and magnesium, is an increasing common practice [30-39]. Addition of salts in a solubilized state can have disadvantages. Emulsion stability can be influenced by $CaCl_2$, which promotes droplet flocculation [40,41]. Salts can influence enzyme activity and react with other components in the food products, such as proteins [42-44] and flavor compounds [45]. $MgCl_2$ and $CaCl_2$ have a bitter and salty taste [30,46], which can be unwanted in a product. Encapsulation of mineral salts can be applied to improve their stability and reduce reactions with other components in the food products [31-33,36,47,48]. Dispersion of nano salt particles in oil is also an encapsulation method and results in a slow release system which may reduce their bitter off-taste and reactions with other components.

There are various methods to produce nanoparticles of salts, but many of these methods have shortcomings: most methods result in nonfood-grade particles, size control can be a problem, and redispering the particles may be difficult. In this paper a method is described for preparing food grade nano salt particles using ethanol-in-oil (E/O) mixtures.

The method results in particles in the nano size range that remain suspended in the oil phase, which allows instant use without the need of resuspending them [29,49].

A common method to produce nanoparticles from water soluble salts uses a w/o microemulsion, carrying a water soluble salt in the water phase. Subsequently, both phases (water and apolar solvent) are evaporated. By removing water from the micelles, salt/surfactant composites are formed, which can be resuspended in an apolar solvent [50,51]. The preparation of nanoparticles of water-soluble salts through evaporation of the liquid phases from the w/o microemulsion, has been described extensively in literature [50-57]. Typically water is used as a polar solvent, heptane as apolar solvent, and dioctyl sodium sulfosuccinate (AOT) is applied as surfactant. Heptane is also often used as apolar phase for redispersing the nanoparticles. The toxicity of solvents such as heptane makes the resulting nanoparticle method unpractical for applications in food. To produce food grade nanoparticles from water soluble salts, food grade solvents and surfactants should be used.

Water soluble food grade nanoparticles can also be obtained with spray drying techniques [58]. Although this method can result in food grade nanoparticles, the subsequent dispersing in suitable food-grade oil remains a challenging task. In our method this dispersion step is no longer necessary.

Our method uses only food grade materials and does not require evaporation of the continuous (apolar) phase. Medium Chain Triglyceride (MCT) oil is used as a non-volatile apolar continuous phase, and ethanol is used as dispersed phase instead of water. Ethanol is (partly) soluble in the MCT oil [59,60], and can be evaporated more easily than water, which also results in a faster method for obtaining nanoparticles. The nanoparticles remain suspended in the oil phase, which allows instant use without the need of resuspending them in a suitable (food-grade) oil phase.

3

3.2 MATERIALS AND METHODS

3.2.1 Materials

Calcium chloride dihydrate (GR for analyses, Merck, ≥99%), ethanol absolute (Merck, ≥99.2%), Grindsted polyglycerol polyricinoleate (PGPR) 90 kosher (Danisco), n-hexane (p.a., Merck, ≥99%), magnesium chloride hexahydrate (GR, Merck, ≥99%) and a medium chain triglyceride (MCT) oil (Miglyol 812 N, Sasol) were used as received.

3.2.2 Method for production of nanoparticles

Various molal solutions (0.1, 0.5 and 1.0 m) of the ethanol soluble salts ($CaCl_2.2H_2O$, $MgCl_2.6H_2O$) were prepared in ethanol. After complete dissolution of the salts the solutions were added, while stirring with a magnetic stirrer, in volume ratios up to 30% to a continuous phase of MCT oil, containing either 4% (w/w) PGPR or 6% (w/w) PGPR as surfactant. The samples were then sonicated using a Branson Sonifier 250 (output control level 6, duty cycle 60%) for 1 min, to form an E/O mixture. The ethanol was evaporated from the samples by heating them overnight while stirring. After all ethanol has been evaporated, samples were cooled down to room temperature in a desiccator containing nitrogen gas and a desiccant. Samples were kept in closed containers and stored in the desiccator until further analyses.

3.2.3 Stability of ethanol-in-oil mixtures

The stability of the created E/O mixtures was analyzed directly after sonication of the samples. Samples were visually examined, and their stability against creaming and coalescence was examined by Multiple Light Scattering (MLS) using a Turbiscan ma2000 (Formulaction SA, L'Union, France).

3.2.4 Size characterization of initial emulsion droplets

The size and size distribution of the emulsion droplets in the E/O mixtures were analyzed directly after sonication of the samples. Samples were therefore analyzed by Dynamic Light Scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) in a square glass cell (Malvern PCS1115).

3.2.5 Size and size distribution of salt particles

Dynamic Light Scattering (DLS) was used to examine the size and size distributions of the salt particles, formed after evaporation of the ethanol phase, with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Undiluted as well as diluted samples (2 and 3 times, with MCT oil containing PGPR) were analyzed. All samples were analyzed in a square glass cell (Malvern PCS1115). Samples were measured at 20 °C, and a refractive index of 1.449 was used for MCT oil (product specifications). The refractive indices for the salts were set to 1.520 for $CaCl_2$ [61] and 1.59 for $MgCl_2$ [61].

3.2.6 Morphology of nanoparticles by SEM

MCT oil and surfactant were removed from the sample before characterizing the nanoparticles with the SEM. Washing was performed by adding hexane to the samples and centrifuging them at 4,000 g for 10 min in a Thermo Scientific centrifuge (Heraeus Multifuge X3R). Supernatant was removed and the pellet containing the nanoparticles was redispersed in hexane. This washing step was repeated 3 times. After the washing step the washed nano salt particles, dispersed in 5 ml hexane, were sonicated for 10 min in a sonication bath. A drop of this dispersion was put on a Nuclepore Track-Etched Membrane (Whatman, 13 mm circular, 5 µm pores) and transferred into a sputter coater (Balzers SCD 020) where it was dried under vacuum. It was subsequently sputter-coated with gold (3 min, 35 kA, 0.2 mBar Argon). Samples were analyzed at room temperature in a scanning electron microscope (Jeol JAMP-9500F, Wageningen, The Netherlands). Images were digitally recorded.

3.2.7 Morphology of nanoparticles by Cryo-SEM

MCT oil and surfactant were removed from the sample before characterizing the nanoparticles with cryo-SEM. Washing was performed by adding hexane to the samples and centrifuging them at 50,000 g for 20 min in a Beckman Coulter Avanti J-26 XP centrifuge. Supernatant was removed and the pellet containing the nanoparticles was redispersed in hexane. This washing step was repeated 3 times. Sample, dispersed in 5 ml hexane, was then sonicated for 10 min in a sonication bath. Sample was put on clean (plasma glowed) coverslips and air dried at room temperature. Coverslips (8 mm circular, Menzel, Brauschweig, Germany) were glued on a sample holder by carbon glue (Leit- C, Neubauer Chemicalien, Germany) and frozen at a liquid nitrogen (LN_2) cooled block. The

sample holder was transferred to a nondedicated cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto a sample stage at -93 °C. In this cryo-preparation chamber the samples were freeze dried for 2 min at -93 °C at 1.3×10^{-6} mbar to remove water vapor contamination. Sample was sputter coated with a layer of 5 nm Tungsten at the same temperature. Samples were cryo-shielded transferred into the field emission scanning microscope (Magellan 400, FEI, Eindhoven, The Netherlands) on the sample stage at -122 °C at 4×10^{-7} mbar. Analysis was performed at a working distance of 4 mm, with SE and BSE detection at 2 - 15 kV, 25 pA. All images were recorded digitally.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of ethanol-in-oil mixtures

3.3.2 Stability of ethanol-in-oil mixtures

Stability evaluated by visual observations and MLS. The stability of mixtures of ethanol, containing $CaCl_2.2H_2O$ (further named $CaCl_2$) or $MgCl_2.6H_2O$ (further named $MgCl_2$), in MCT oil containing PGPR as surfactant was examined for 8 hours. It was found that the volume percentage of ethanol added and the concentration of salt in the ethanol phase influenced the stability and appearance of the samples. An overview of visual observations and conclusions from the MLS measurements is given in Table 3.1. In Figure 3.1 and 3.2 some typical results from samples containing calcium and magnesium salts measured by MLS are presented.

Table 3.1 shows that depending on the salt concentration, and the volume percentage of ethanol added to the oil phase, either a transparent system was formed or an opaque. These systems appeared transparent or opaque directly after mixing the ethanol phase with MCT oil, and in several of the systems stability issues occurred during the 8 hour analyses. In general, it was found for both $CaCl_2$ and $MgCl_2$ that an increase in salt concentrations and/or increase in ethanol concentrations results in a decrease in stability of the mixture and can cause the mixture to become opaque. From Table 3.1 it can be seen that samples containing $MgCl_2$ have a less wide stability window than samples containing $CaCl_2$. When pure ethanol was added in different volume concentrations to the MCT oil containing PGPR it resulted in transparent and stable mixtures. This shows that the stability of the E/O mixtures is strongly affected by the salt concentration.

Table 3.1 Effect of salt concentration, ethanol concentration, and PGPR concentration on sample appearance and stability[a]

MCT	Ethanol (% v/v)	Salt concentration (in EtOH)		
		0.1 m	0.5 m	1.0 m
MCT + 4% (w/w) PGPR — $CaCl_2 \cdot 2H_2O$	5	T	T	T
		S	S	S
	10	T	O	O
		S	U (C)	U (C + P)
	20	T	O	O
		S	U (C + P)	U (C + P)
	30	T	O	O
		S	U (C + P)	U (C + P)
MCT + 6% (w/w) PGPR — $CaCl_2 \cdot 2H_2O$	5	T	T	T
		S	S	S
	10	T	O	O
		S	U (C + P)	U (C + P)
	20	T	O	O
		S	U (C + P)	U (C + P)
	30	T	O	O
		S	U (C + P)	U (C + P)
MCT + 4% (w/w) PGPR — $MgCl_2 \cdot 6H_2O$	5	T	O	O
		S	U (C)	U (C)
	10	T/O	O	O
		S	U (C)	U (P)
	20	O	O	O
		U (C + P)	U (C + P)	U (C + P)
	30	T	O	O
		S	U (C + P)	U (C + P)

MCT	Ethanol (% v/v)	Salt concentration (in EtOH)		
		0.1 m	0.5 m	1.0 m
	5	T	O	O
		S	U (C)	U (C)
	10	T/O	O	O
		S	U (P)	S
	20	O	O	O
		U (C + P)	U (C + P)	U (C + P)
	30	T	O	O
		S	U (C + P)	U (C + P)

(Left margin, rotated labels: MCT + 6% (w/w) PGPR; $MgCl_2.6H_2O$)

[a]Samples are examined for 8 hours by visual observation and MLS measurement. Samples are created with 0.1, 0.5 or 1.0 m $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ in ethanol, and mixed at respectively 5%, 10%, 20% or 30% (v/v) in MCT oil containing PGPR. Explanation of symbols; T = Transparent, O = Opaque, S = Stable, U = Unstable (with C = Coalescence and P = Phase separation).

From a chemical point of view it is known that ethanol can, due to its amphiphilic nature, dissolve to a certain extent in MCT oil [59,60]. However, the presence of water and impurities in the ethanol phase reduces its solubility in oil. As described above, it was found that the addition of different volume concentrations of pure ethanol to MCT oil resulted in transparent and stable mixtures (visual examination for 24 hours). When low concentrations of $CaCl_2$ or $MgCl_2$ were present in the ethanol phase transparent and stable mixtures were also obtained with MCT oil (Table 3.1). The PGPR seemed not required in these transparent mixtures since they remained visually stable during 24 hours. At sufficiently high salt concentrations phase separation appeared to occur, in which the system most likely separated into droplets of an ethanol rich phase in an oil rich continuous phase, and PGPR (at least partially) stabilizes the droplets. In the absence of PGPR, the unstable system phase separated almost instantly. The phase separation was influenced by temperature: a higher salt concentration was required for inducing phase separation at elevated temperature. The phase diagram of ethanol and MCT oil with the presence of $CaCl_2$ or $MgCl_2$ was not fully characterized, since this is outside the scope of the present study. Therefore, it is uncertain which amount of ethanol is mixed with the MCT oil and which amount is present as a separate phase.
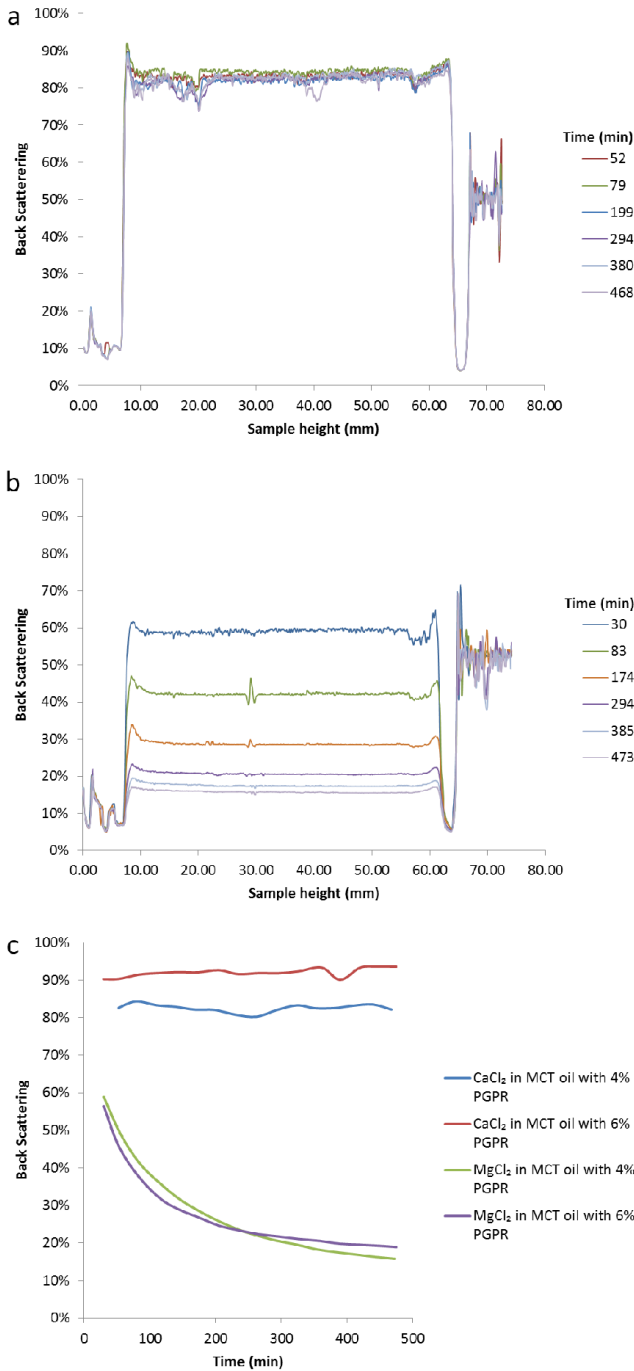
a

b

c

3

Figure 3.1 Typical MLS results from a stable sample and sample that changes over time. (**a**) Sample created with 1.0 *m* CaCl$_2$.2H$_2$O in ethanol and mixed at 5% (v/v) in MCT oil containing 4% (w/w) PGPR. Sample is monitored for 8 h. (**b**) Sample created with 1.0 *m* MgCl$_2$.6H$_2$O in ethanol and mixed at 5% (v/v) in MCT oil containing 4% (w/w) PGPR. (**c**) Backscattering at sample height of 30 mm for samples from Figure 3.1a and Figure 3.1b. The variations with 6% (w/w) of PGPR are presented here as well.

Figure 3.1 Typical MLS results from a stable sample and sample that changes over time.

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In Figure 3.1a data from the MLS measurements of samples created with 5% (v/v) 1.0 *m* CaCl$_2$.2H$_2$O in ethanol added to MCT oil is given. Each line represents the same sample measured at different times. The horizontal profile of the lines in Figure 3.1a are similar to each other, from which can be concluded that this sample is stable during 8 hours. In Figure 3.1b the data is given for a sample where MgCl$_2$.6H$_2$O is used instead. The lines in Figure 3.1b have a similar horizontal profile, but with different intensity. The back scattering percentage changes evenly over the entire sample height. It is interpreted that this change in back scattering is caused by a change in droplet size and indicates that the sample is not stable over time. After 24 hours it was also noticed that phase separation has occurred in the sample with MgCl$_2$. Looking at a certain sample height gives a better impression of the trend of the change in droplet size. In Figure 3.1c the data from Figure 3.1a and 3.1b are combined into one figure and results are shown only for the back scattering at a sample height of 30 mm. In order to observe the effect of PGPR concentration, samples containing 6% (w/w) of PGPR in MCT oil are presented here as well. For samples with CaCl$_2$, shown in Figure 3.1c, no change in droplet size occurs during 8 hours. Samples with MgCl$_2$ are unstable, as concluded from Figure 3.1b, and the change in droplet size results in a decrease of the back scattering signal. The change in droplet size in the sample with MgCl$_2$ is most likely caused by coalescence. But disproportionation (Ostwald ripening) may also play a large role here due to the solubility of ethanol in MCT oil. From Figure 3.1c no clear effect can be observed when increasing the PGPR concentration from 4% (w/w) to 6% (w/w) PGPR.

In Figure 3.2a the data from the MLS measurements of samples created with 20% (v/v) 1.0 *m* MgCl$_2$.6H$_2$O in ethanol added to MCT oil is given. From Figure 3.2a it can be seen that the sample is very unstable over time. The peak at about 10 mm sample height indicates formation of a transparent oil rich layer (bottom of sample), and at about 60 mm sample height (top of sample) a transparent ethanol rich layer is formed, clearly indicating the system is phase separating. Figure 3.2a also shows a partly horizontal profile (in between 11 – 40 mm sample height) where only the back scattering percentage changes evenly over time. This is an indication for a change in droplet size and the corresponding trend, represented by an ascending line, at a sample height of 30 mm is shown in Figure 3.2b. In order to see the effect of PGPR a similar sample produced in MCT oil containing 6% (w/w) of PGPR is shown here as well. Both samples give a comparable ascending line, which shows that the addition of extra PGPR to the sample did not affect the change in

droplet size. Also, for other samples no significant effect on the stabilization of the mixtures by an increased PGPR concentration could be observed.
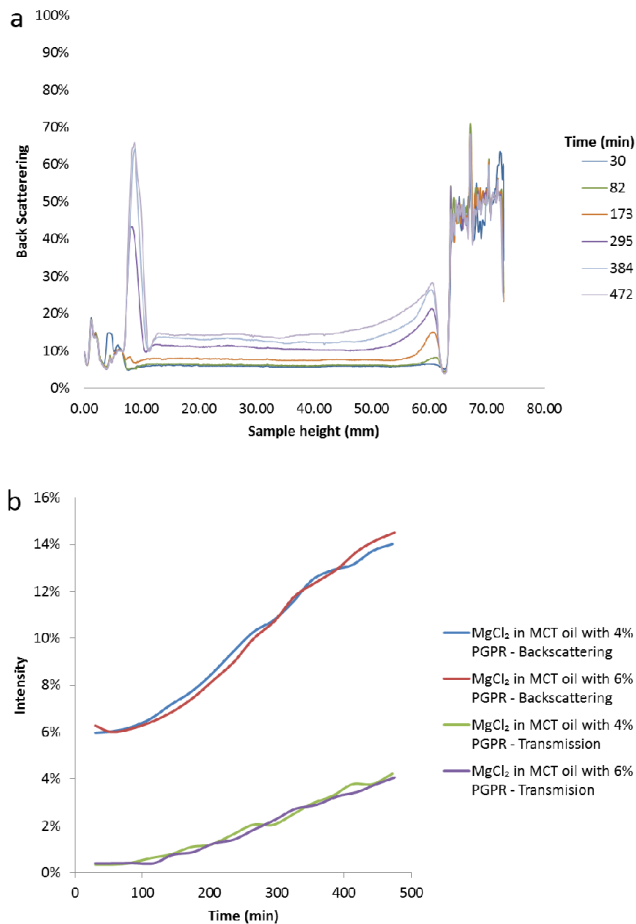

Figure 3.2 Typical MLS results from an unstable sample containing $MgCl_2.6H_2O$. (**a**) Sample created with 1.0 *m* $MgCl_2.6H_2O$ in ethanol and mixed at 20% (v/v) in MCT oil containing 4% (w/w) PGPR. Sample is monitored for 8 hours. (**b**) Backscattering and Transmission trend at sample height of 30 mm for the sample from Figure 3.2a. Variation with 6% (w/w) of PGPR is presented here as well.

3.3.3 Size characterization of initial emulsion droplets

E/O mixtures with $CaCl_2$ or $MgCl_2$ dissolved in the ethanol phase were analyzed by DLS. Samples that appeared as transparent showed droplets with a diameter in the range of 20 nm to 160 nm. Opaque mixtures contained droplets with a diameter in the range of 600 nm to 1400 nm. In the latter one the large emulsion droplets masked the smaller droplets. The instability of the opaque mixtures made DLS analyses less reliable for size characterization of the emulsion droplets in these samples.

3.3.4 Characterization of salt particles after evaporation of ethanol

3.3.5 Size and size distribution of salt particles

Samples containing salt particles were further analyzed with DLS to investigate the presence of nanoparticles. In Table 3.2 an overview is given from the DLS measurements. In all samples nanoparticles could be observed, but samples that appeared as opaque contain also many larger particles. This made the formulations used in these opaque mixtures less useful for production of salt nanoparticles.

DLS measurements showed the presence of nano salt particles in all samples prepared with $CaCl_2$ and $MgCl_2$. Samples appeared as transparent or opaque, as described in Table 3.2. The transparent samples contained mainly salt particles with an average below 300 nm, and only a negligible amount of larger salt particles were present. The opaque samples contained salt particles smaller than 400 nm but also a considerable amount of large particles, causing an opaque appearance. These large salt particles could be observed with a light microscope. The opaque samples were difficult to measure with DLS because of the wide size distribution of the salt particles. The larger salt particles masked the smaller salt particles. Therefore not all the particle sizes are listed in Table 3.2. In some of the samples, for instance 1.0 m $CaCl_2.2H_2O$ and 5% (v/v) of ethanol, salt crystals of 1-30 µm were found, but DLS measurements showed a peak average of 183 nm based on size distribution by volume.

It is remarkable that with 0.1 m $CaCl_2.2H_2O$ and 20% (v/v) of ethanol mainly particles with an average of 6 nm were found. A minor amount of large particles were found by DLS as well, which could also be observed by light microscopy. However, no clear peak around 200 nm could be observed with DLS. It is expected that the substantial amount of very small particles could be the result of Ostwald ripening in the sample. Since ethanol is

soluble in MCT oil, Ostwald ripening can easily take place and due to a higher content of ethanol in the sample it is likely to be more unstable compared to samples with a lower ethanol concentration. Ostwald ripening is the effect where larger droplets grow at the expense of the small ones, and the small droplets become even smaller. Evaporation of these smaller droplets could result then in smaller particles as well. This could be an explanation for a shift of the peak around 200 nm to the left and right, as could be observed in Figure 3.3. Particles around 10 nm were also found in other samples containing lower concentrations of ethanol, but these did not show up clearly with DLS.

Table 3.2 Overview of samples containing salt particles measured by DLS (using a Nanosizer)[a]

MCT	Ethanol (% v/v)	Salt concentration (in EtOH)		
		0.1 m	0.5 m	1.0 m
MCT + 4% (w/w) PGPR — $CaCl_2 \cdot 2H_2O$	5	Transparent 203.7 nm (100%)	Transparent 173.4 nm (80.2%) 1281.3 nm (19.9%)	Opaque 183.4 nm (100%)
	10	Transparent 231.8 nm (100%)	Opaque	Opaque
	20	Transparent 6.1 nm (97.4%) 895.4 nm (0.9%) 3549.5 nm (1.7%)	Opaque	Opaque
	30	Opaque	Opaque	Opaque
MCT + 4% (w/w) PGPR — $MgCl_2 \cdot 6H_2O$	5	Transparent 215.9 nm (100%)	Opaque 1175.3 nm (53.3%) 3083.4 nm (46.7%)	Opaque 90.2 nm (5.4%) 1156.7 nm (94.6%)
	10	Semi-Transparent 315.2 nm (100%)	Opaque	Opaque
	20	Opaque 93.3 nm (51.3%) 493.5 nm (48%) 3037.8 nm (0.3%)	Opaque	Opaque
	30	Opaque	Opaque	Opaque

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		Salt concentration (in EtOH)		
MCT	Ethanol (% v/v)	0.1 m	0.5 m	1.0 m
MCT + 6% (w/w) PGPR	5	Transparent 337.3 nm (100%)	Opaque 250.1 nm (100%)	Opaque 438.3 nm (92.4%) 3595.5 nm (7.6%)
	10	Semi-Transparent 341.2 nm (98.8%) 1397.5 nm (1.2%)	Opaque	Opaque
	20	Semi-Transparent 770.1 nm (88.6%) 4109.8 nm (11.5%)	Opaque	Opaque
	30	Opaque	Opaque	Opaque

Note: The leftmost column "MCT + 6% (w/w) PGPR" and the column "MgCl$_2$.6H$_2$O" span all data rows.

[a]Sample appearance, transparent or opaque, is given. Size represents the peak average based on size distribution by volume; the percentages represent the peak area. Samples are created with 0.1, 0.5 or 1.0 m CaCl$_2$.2H$_2$O and MgCl$_2$.6H$_2$O in ethanol, and are mixed at respectively 5%, 10%, 20% or 30% (v/v) in MCT oil containing PGPR. Ethanol phase has been evaporated at 60 °C, resulting in the formation of micro and nano salt particles.

From the E/O mixtures described in Table 3.1 and the salt particles described in Table 3.2 it can be concluded that only the transparent E/O mixtures were successful in producing salt nanoparticles with a minimal number of larger particles. The samples with 0.1 m salt and 30% (v/v) of ethanol resulted also in a transparent mixture; however, after evaporation of the ethanol phase the sample became opaque and large particles and crystals could be observed. The same holds for the sample with 1.0 m CaCl$_2$.2H$_2$O and 5% (v/v) of ethanol, which appeared transparent as mixture but became opaque after evaporation of the ethanol phase. During production of the particles, ethanol is removed from the system and the relative concentration of salt in ethanol is therefore increasing. If this relative salt concentration is too high the mixture becomes unstable. Full characterization of the phase diagram of ethanol and MCT oil with the presence of CaCl$_2$ or MgCl$_2$ can give better insight how the systems shift through the phase diagram during evaporation of ethanol, but this is outside the scope of the present study. Small emulsion droplets or even solutions of ethanol in MCT oil are useful for the formation of nanoparticles. However, having instable mixtures where coalescence and/or phase separation occurs reduces the chance to form nanoparticles and more large particles and crystals will be obtained.

In general it was found that increasing the volume percentage of ethanol and/or increasing the concentration of salt shows that the number of large particles increases and also the size of the large particles increases. An increase in volume percentage of ethanol and/or salt concentration results also in a more polydisperse size distribution of the salt particles. Samples prepared with $MgCl_2$ have a less wide concentration window for the formation of nanoparticles with a minimal amount of larger particles, compared to samples prepared with $CaCl_2$.

It was found that some of the transparent E/O mixtures were stable without the presence of PGPR. It was therefore investigated if some of these transparent mixtures were also suitable to form salt nanoparticles in absence of PGPR. Samples containing 0.1 *m* $CaCl_2.2H_2O$ with 5 till 20% (v/v) of ethanol in MCT oil and a sample containing 0.5 *m* $CaCl_2.2H_2O$ with 5% (v/v) of ethanol in MCT oil were prepared. None of these samples contained PGPR as surfactant and transparent mixtures were obtained. Ethanol was evaporated from the MCT oil phase at 60 °C during 14 hours, and samples were visually characterized and analyzed by a microscope. It was found that large crystals and salt particles were obtained, and particles also clustered together in samples prepared without PGPR. On the other hand, similar samples but with PGPR did result in nano salt particles. It can be concluded from this that a surfactant such as PGPR is required in the formation and stabilization of nano salt particles. Surfactant molecules, which are predominantly adsorbed on the surface of the nanoparticle, are expected to have an important role in controlling the nanoparticle size and stabilizing their suspension[50,51]. It was also observed that an increase in PGPR concentration reduced the amount of large particles that were formed. For instance, a sample with 0.1 *m* $MgCl_2.6H_2O$ and 20% (v/v) ethanol is more transparent when 6% (w/w) of PGPR is used than when 4% (w/w) PGPR is used. Light microscopy of the sample with 6% (w/w) PGPR revealed fewer large particles compared to the sample with 4% (w/w) PGPR.

Figure 3.3 shows the size distribution by volume, measured by DLS, of salt particles produced from 0.1 *m* salt and mixed at 5 and 20% (v/v) of ethanol in MCT oil containing 4% (w/w) PGPR. In Figure 3.3 only one peak around 200 nm can be observed for samples prepared with 5% (v/v) of ethanol. Sample prepared with 20% (v/v) of ethanol shows two peaks, one major peak around 6 nm and another minor peak around 4800 nm. As explained above it is expected that this shift is due to Ostwald ripening.
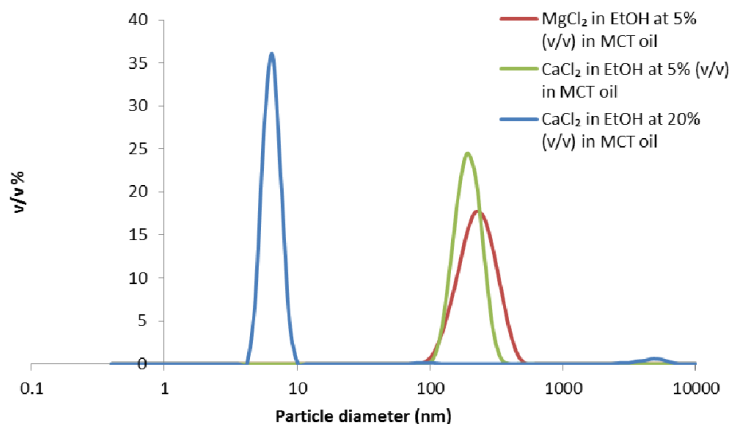
Figure 3.3 Size distribution by volume percentage, measured by DLS, of samples with 0.1 *m* salt and mixed at 5 and 20% (v/v) of ethanol in MCT oil containing 4% (w/w) of PGPR. Ethanol was evaporated at 60 °C.

3.3.6 Morphology of nanoparticles

The morphology of the formed salt nanoparticles was studied with SEM and Cryo-SEM. In Figure 3.4 micrographs from the SEM and Cryo-SEM of salt nanoparticles are shown.

In Figure 3.4 it can clearly be seen that nano salt particles of various sizes below 400 nm were obtained. Figure 3.4a and 3.4b shows $CaCl_2$ nanoparticles of about 100 nm observed with the Auger/SEM. In Figure 3.4c and 3.4d a similar sample is represented but observed with the Cryo-SEM and a few large (270 nm) but also a considerable number of much smaller (20 - 60 nm) salt-particles were found. The washing step applied to the samples shown in Figure 3.4a and 3.4b and Figure 3.4c and 3.4d differed and is an explanation for the observed differences. The sample from Figure 3.4c and 3.4d was centrifuged at much higher speed (50,000 g for 30 min versus 4,000 g for 10 min for the sample in Figure 3.4a and 3.4b). Therefore more small $CaCl_2$ particles ended up in the pellet due to the higher centrifugation forces. Comparison of the results from $CaCl_2$ shows that the upper range (<342 nm) is quite comparable to what was found with SEM and Cryo-SEM (Figure 3.3). However, the lower range (<90 nm) is not really visible in Figure 3.3 for $CaCl_2$, whereas with Cryo-SEM several particles below 90 nm could clearly be seen. This could confirm that the larger particles mask the smaller ones, and therefore, the particles

below 90 nm are not observed with DLS measurements. It must be noted that the samples from Figure 3.3 were created with 4% (w/w) PGPR and the samples from Figure 3.4 were created with 6% (w/w) PGPR. Figure 3.4e and 3.4f shows nanoparticles of $MgCl_2$ which are in a size range of 300-400 nm. According to DLS results shown in Figure 3.3, much smaller particles must be present as well. It is expected that these smaller $MgCl_2$ particles were removed during the washing step, as it was also concluded for the sample in Figure 3.4a and 3.4b. All nano salt particles observed with SEM and Cryo-SEM seemed to be cubical, and this suggests that the particles have a cubic crystal structure.
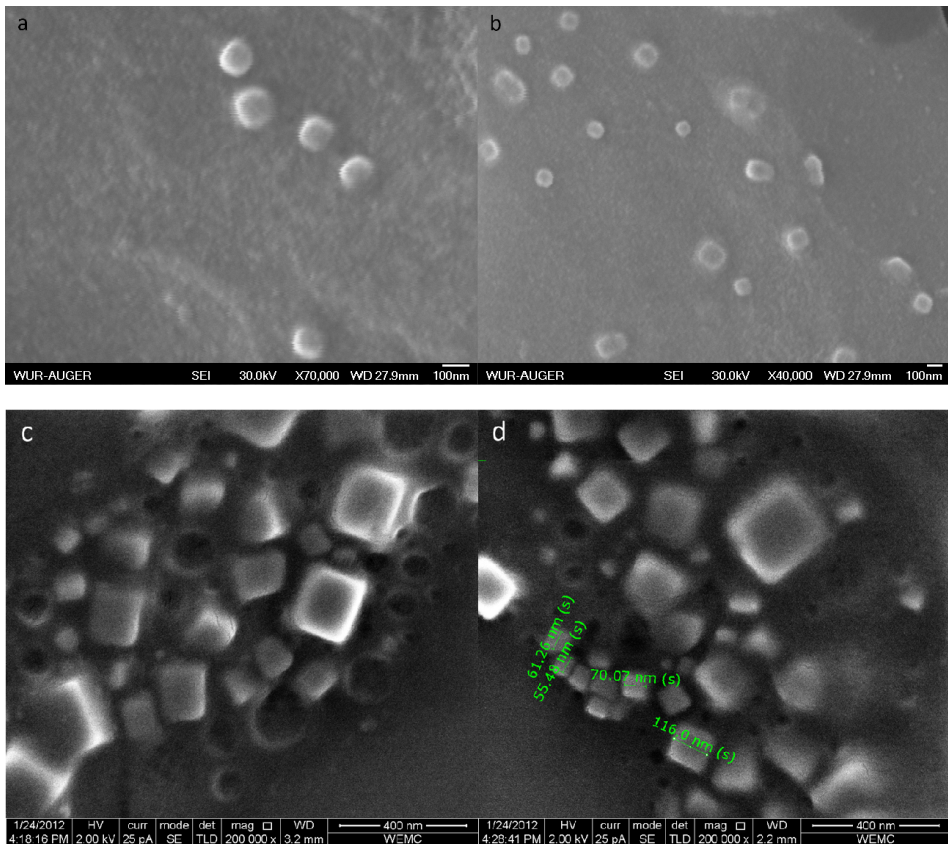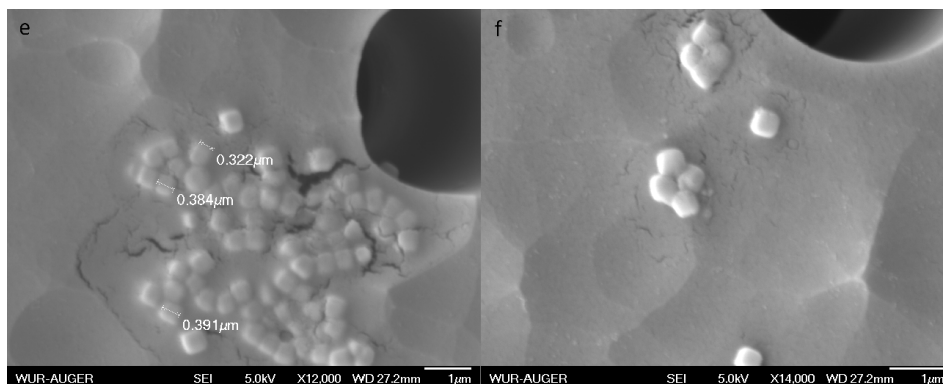
Figure 3.4 Nano salt particles analysed with SEM and Cryo-SEM. (**a** and **b**) CaCl₂ nano salt particles observed with Auger/SEM [Sample created with 0.1 *m* CaCl₂.2H₂O in ethanol and mixed at 5% (v/v) in MCT oil containing 6% (w/w) of PGPR. The ethanol was evaporated at 60 °C. Sample has been washed with hexane to remove oil and surfactant.] (**c** and **d**) CaCl₂ nano salt particles observed with Cryo-SEM [Sample created with 0.1 *m* CaCl₂.2H₂O in ethanol and mixed at 5% (v/v) in MCT oil containing 6% (w/w) of PGPR. The ethanol was evaporated at 60 °C. Sample has been washed with hexane to remove oil and surfactant.] (**e** and **f**) MgCl₂ nano salt particles observed with Auger/SEM [Sample created with 0.1 *m* MgCl₂.6H₂O in ethanol and mixed at 5% (v/v) in MCT oil containing 6% (w/w) of PGPR. The ethanol was evaporated at 60 °C. Sample has been washed with hexane to remove oil and surfactant.]

3.4 CONCLUSION

A simple method for preparing food grade salt particles in the nano range has successfully been developed. The salt particles were prepared from ethanol soluble salts using ethanol-in-oil (E/O) mixtures. It was found that increasing the salt concentration (CaCl₂ and MgCl₂) and ethanol concentration reduced the stability of the obtained mixtures. Also, the type of salt (CaCl₂ or MgCl₂) was influencing the stability of the mixture. MLS measurements showed that transparent mixtures remained stable for at least 8 hours and opaque mixtures were found to be unstable during these 8 hours. PGPR was required in these unstable systems to partly stabilize the droplets, whereas an absence of PGPR resulted in an almost instant phase separation in these systems. No significant effect on the stability of the mixtures could be observed when increasing the PGPR concentration from 4% (w/w) to 6% (w/w) PGPR.

It can also be concluded that salt particles with dimensions in the nano range (6 – 400 nm) were obtained in all samples after evaporation of the ethanol phase, but only the

samples that appeared as "transparent E/O mixture" were successful in forming salt nanoparticles with a minimum of larger salt particles present. The concentration of salt and volume fraction of ethanol was found to have a significant influence on the size distribution of salt particles. It was also found that PGPR was not only required to obtain more stable mixtures of ethanol and MCT oil but was also required in the formation and stabilization of nano salt particles.

The developed method is based on the use of food-grade materials, and the obtained nanoparticles are therefore regarded as food safe. This increases the range of applications and makes the application of these nanoparticles in food products possible, and thereby supports the development of new advanced food products.

3.5 ACKNOWLEDGEMENTS

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Chapter IV

Alginate Nanospheres Gelled with CaCl$_2$ Nanoparticles

ABSTRACT

A simple method for preparing gelled alginate spheres with a diameter smaller than 5 µm is described. A 1% alginate solution and a medium chain triglyceride (MCT) oil are used to prepare a water-in-oil (w/o) emulsion, stabilized by polyglycerol polyricinoleate. CaCl$_2$ nanoparticles with dimensions in the nano-range (6 – 400 nm), dispersed in MCT oil, are then added to the emulsion. Energy-dispersive X-ray spectroscopy (EDX) and Auger electron spectroscopy (AES) show that these nanoparticles migrate to the emulsion droplet interface, where they dissolve into the aqueous alginate phase and cause gelation, forming spheres. Gelation of the spheres was confirmed with a novel technique using Congo red as an indicator. A color change occurs upon the addition of CaCl$_2$ to a Congo red solution and we believe this is due to formation of a Congo red-calcium complex. Scanning electron microscopy shows that alginate spheres are mostly in a size range around 1 µm, but nanospheres as small as 200 nm and smaller were also found. Extending the size range of alginate spheres into the nano range, while maintaining relatively mild pH conditions in the interior of the sphere, will significantly extend the range of applications for this type of spheres.

4.1 INTRODUCTION

Alginate is a biopolymer commonly used for the preparation of hydrogel (micro) particles, and many other applications [1]. In pharmaceutics alginate matrices are used for the encapsulation of islet cells [2], and for cell delivery and transplantation [3-5]. Alginate is also used for oral delivery of peptide or protein drugs [6], for sustained/controlled drug release [7,8], and to immobilize active cells [9] and enzymes. In food it is applied as a stabilizer and thickener for a wide range of products, but also for encapsulation of functional ingredients like vitamins and probiotics [10,11].

Alginate hydrogel particles can enhance the mechanical stability of cells encapsulated in its interior, and protect its contents from immune responses, while remaining permeable for exchange of valuable components, such as nutrients. Most research on alginate hydrogel particles focusses on prevention of immune responses, and protection of encapsulated materials against harmful conditions, such as the acidity in the stomach. Many of these particles have additional shells to decrease the permeability of the particles [3,5,6,8,12-14]. However less research is invested in size control of the particles [11]. When large particles are added to food products they can negatively affect the structural and sensorial properties of these products. Humans can detect particles larger than around 25 μm by the palate [15]. When particles are added in products, particle size should be less than 25 μm to not negatively influence the textural properties of the product. For immobilization of enzymes the particle size can be an important factor as well, since the size of the particle affects the diffusion rates of reactants and reaction products.

There are various methods for preparation of alginate particles [8]. Most of the traditional methods produce particles with diameters much larger than 25 μm. In this paper a novel method for preparing much smaller alginate particles using water-in-oil (W/O) emulsions is described. After emulsification the alginate emulsion droplets are gelled with $CaCl_2$ nanoparticles, and remain suspended in the oil phase. Our method produces gelled alginate spheres with a diameter of about 1 μm and even nanospheres with a diameter below 200 nm can be obtained.

Traditional particle formation techniques by external gelation [16-18], such as dripping an alginate solution into a $CaCl_2$ solution, typically results in spheres of 500-1000 μm [12]. More advanced variations of this technique have been developed with improved yield, smaller spheres and narrower size distribution. In those methods single droplets are formed at the

tip of a nozzle, and a coaxial laminar air-flow or electric field is used to decrease droplet size. The droplets are collected in a $CaCl_2$ bath for gelation of the spheres. Droplets formed with these techniques are in a size range from 50 – 600 µm [19-21]. Other commercial techniques use vibrating nozzles and laminar jet break-up (JetCutter®). The JetCutter® forms droplets from about 120 µm and larger by cutting a solid jet of fluid by means of rotating cutting wires, into cylindrical segments, which then form beads due to surface tension. The droplets can be collected in a $CaCl_2$ bath for gelation into gel spheres.

Another external gelation technique uses a water-in-oil emulsion of an alginate solution in an apolar phase. The emulsion is then broken by addition of a $CaCl_2$ solution. The method can produce alginate spheres smaller than 100 µm, but particle size distributions tend to be very wide, spheres are often clustered, and multiple emulsion droplets (W/O/W) may form as a by-product.

Internal gelation of alginate spheres can be performed through an emulsion technique in which an alginate emulsion, containing $CaCO_3$ particles, is emulsified in an apolar phase [22]. The gelation is achieved by addition of an oil-soluble acid that causes calcium ion release. Alternatively Glucono delta-lactone (GDL) can be added to the alginate phase which slowly lowers the pH inside the alginate spheres [17,23-25]. This lowering in pH can be harmful to sensitive compounds inside the sphere. External gelation usually maintains a mild pH. Besides a lower pH inside the alginate sphere the internal gelation process also results in spheres with a different matrix strength, stiffness, pore size and permeability compared to spheres prepared with external gelation [16-18].

Tong *et al.* [26,27] describe a method where an aqueous solution of alginate is emulsified in oil containing $CaCO_3$ microparticles (5 µm) to produce a water-in-oil emulsion. A self-assembled layer of $CaCO_3$ microparticles is formed at the liquid-liquid interface and acts as a stabilizer. GDL, present in the aqueous phase, gradually lowers the pH in the alginate spheres and as result Ca^{2+} is released from the $CaCO_3$ and allows for gelation of the alginate sphere. The spheres have an average diameter of 430 µm. In our method gelled alginate spheres can be obtained with a diameter of about 1 µm and even nanospheres with diameters below 200 nm can be obtained. Our method uses $CaCl_2$ nanoparticles dispersed in the oil phase and the pH in the spheres remains at neutral pH. The alginate nanospheres can remain dispersed in the oil phase. If alginate nanospheres are required in

a water continuous phase they can be harvested from the oil phase without the formation of multiple emulsion droplets (w/o/w) and without the clustering of droplets.

Extending the size range of alginate spheres into the nano range, while maintaining relatively mild pH conditions in the interior of the sphere, will significantly extend the range of applications for this type of spheres.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Buffer solution Boric acid / potassium chloride / sodium hydroxide (Merck, pH 10), buffer solution di-sodium hydrogen phosphate / potassium dihydrogen phosphate (Merck, pH 7), buffer solution citrate / hydrochloric acid (Merck, pH 4), calcium chloride dihydrate (GR for analyses, Merck, ≥99%), Congo red (Sigma, ~40%), ethanol absolute (Merck, ≥99.2%), Grindsted polyglycerol polyricinoleate (PGPR) 90 kosher (Danisco), n-hexane (p.a., Merck, ≥99%), medium chain triglyceride (MCT) oil (Miglyol 812 N, Sasol), and sodium alginate (Fischer Scientific, General purpose grade) were used as received.

4.2.2 Method for production $CaCl_2$ nanoparticles

The $CaCl_2$ nanoparticles were prepared using a method described elsewhere [28]. A volume ratio of 5% of a 0.1 molal solution of $CaCl_2.2H_2O$ in pure ethanol was added to MCT oil containing 6% (w/w) of PGPR. The mixture was sonicated for 1 min and alcohol was evaporated from the mixture by heating them overnight at 60 °C, while stirring on a magnetic stirrer. After all alcohol has been evaporated the sample was cooled down to room temperature before being used.

4.2.3 Method for production of alginate nanospheres

An alginate solution was prepared on a magnetic stirrer by adding 1 or 2% (w/w) of alginate (based on 100% water) to demineralized water. After complete dissolution of the alginate, the alginate solution was added, while stirring with a magnetic stirrer, in volume ratios up to 15% to a continuous phase of MCT oil, containing 6% (w/w) polyglycerol polyricinoleate (PGPR) as surfactant. Emulsification is then performed with an Ultra Turrax (Ika® T25 digital) at variable speed ranging from 5,000 to 10,000 rpm for 10 min, forming a stock emulsion of alginate in MCT oil. The stock emulsion was then diluted, while stirring

on a magnetic stirrer, with MCT oil containing $CaCl_2$ nanoparticles and 6 % (w/w) PGPR. The final systems were kept overnight while stirring to allow gelation of the alginate emulsion droplets. For comparison of the gelled alginate spheres with non-gelled alginate droplets the stock emulsion was also diluted with MCT oil containing only 6 % (w/w) PGPR. The MCT oil used for this dilution was treated with ethanol using a similar method as for the production of $CaCl_2$ nanoparticles, but no $CaCl_2.2H_2O$ has been dissolved in the ethanol. All samples were kept in closed containers and stored in a refrigerator until further analyses.

4.2.4 Analysis of alginate nanospheres by Cryo-SEM and EDX analyses on chemical elements

The sample containing the alginate nanospheres was glued on copper hollow rivets and directly frozen in liquid ethane. The copper rivets were placed in a cryo-sample holder in liquid nitrogen (LN_2). The sample holder was transferred to a non-dedicated cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto a sample stage at -93˚ C. In this cryo-preparation chamber the samples were fractured and freeze dried for 3 min at -93 °C at 1.3×10^{-6} mbar to remove water vapor contamination. The sample was sputter coated with a layer of 5 nm Tungsten at the same temperature. The samples were cryo-shielded transferred into the field emission scanning microscope (Magellan 400, FEI, Eindhoven, The Netherlands) on the sample stage at -122 °C at 4×10^{-7} mbar. The analysis was performed at a working distance of 3 – 4.5 mm, with SE and BSE detection at 2 - 15 kV, and 13 pA. All images were recorded digitally.

EDX analyses were accomplished in the same field emission scanning electron microscope at -122 °C by a X-Max/AZtec X-ray analyser (Oxford Instruments Analytical, High Wycombe, England) at an acceleration voltage of 15 kV, 200pA, WD 4mm.

4.2.5 Analysis of alginate nanospheres by SEM and AES analyses on chemical elements

MCT oil and surfactant were removed from the sample before characterizing the alginate nanospheres with the SEM. Washing was performed by adding hexane to the samples and centrifuging them at 500 g and 1,000 g for 30 min in a Thermo Scientific centrifuge (Heraeus Multifuge X3R). The supernatant was removed and the pellet, containing the alginate nanospheres, was redispersed in hexane. This washing step was

repeated 3 times. After the last washing step the washed alginate nanospheres were dispersed in 5 ml hexane. A drop of this dispersion was put on a Nuclepore Track-Etched Membrane (Whatman, 13 mm circular, 5 µm pores) and transferred into a sputter coater (Balzers SCD 020) where it was dried under vacuum. It was subsequently sputter-coated with gold (3 min, 35 kA, 0.2 mBar Argon). Samples were analyzed at room temperature in a scanning electron microscope (Jeol JAMP-9500F, Wageningen, The Netherlands). All images were recorded digitally.

Auger electron spectroscopy (AES, Jeol JAMP-9500F, Wageningen, The Netherlands) was used to determine the chemical elements present in the sample at an electron beam energy of 10 keV. In order to perform Auger Electron Spectroscopy analyses the washed sample was put on a silicon nitride wafer and dried under vacuum. No sputter coating was applied to the sample.

4.2.6 Analyses of Congo red – CaCl₂ solutions

A Congo red stock solution of 0.1% (w/w) was prepared with MilliQ water. The stock solution was then diluted with MilliQ water or buffer solution (buffer pH 4, pH 7 and pH 10) to prepare samples containing respectively 0.01% and 0.001% Congo red. Also a 0.01% and 0.001% Congo red solution in MilliQ water with $CaCl_2$ was prepared. pH values were determined using a digital pH meter. The absorbance spectra of the samples were then measured using a spectrophotometer (Cary 50 UV-Visible spectrophotometer).

4.2.7 Analyses of Congo red in alginate solutions

5 ml of a 1 % (w/w) alginate solution containing 0.01% (w/w) of Congo red was added in a test tube. Then 2 ml of a $CaCl_2$ solution (respectively 0.00%, 0.05%, 0.50% or 1.00% (w/w) $CaCl_2$) was carefully poured on top of the alginate solution. Samples were visually observed over time.

4.2.8 Analyses of Congo red in alginate emulsions

A 1 % (w/w) alginate solution was prepared containing 0.01% (w/w) of Congo red and emulsified at 15 % (v/v) in MCT oil containing 6 % (w/w) PGPR, using an Ultra Turrax. This stock emulsion was then diluted, while stirring on a magnetic stirrer, with MCT oil containing $CaCl_2$ nanoparticles and 6 % (w/w) PGPR, into an emulsion containing 3% (v/v) of alginate solution. For comparison of the gelled alginate spheres with non-gelled

alginate droplets the stock emulsion was also diluted with MCT oil containing only 6 % (w/w) PGPR. The MCT oil used for this dilution was treated with ethanol using a similar method as for the production of $CaCl_2$ nanoparticles, but no $CaCl_2.2H_2O$ has been dissolved in the ethanol. Samples were then visually analyzed at different time intervals.

4.3 RESULTS AND DISCUSSION

4.3.1 Alginate nanospheres analyzed by Cryo-SEM

4.3.2 Morphology of gelled alginate nanospheres by Cryo-SEM

A 1% (w/w) solution of alginate was prepared and used to prepare a stock emulsion with MCT oil as continuous phase. The stock emulsion consisted of 3% (v/v) alginate solution in MCT oil with 6% (w/w) PGPR as surfactant. $CaCl_2$ nanoparticles were dispersed in the MCT oil phase to induce gelation. Light microscopy showed the presence of separate alginate spheres in all samples and no clusters were found. In Figure 4.1 micrographs from the Cryo-SEM of alginate nanospheres in an MCT oil matrix are shown.

In Figure 4.1 it can clearly be seen that alginate spheres of various sizes below 5 μm were obtained. Figure 4.1a and 4.1b shows some typical alginate nanospheres prepared with an alginate – $CaCl_2.2H_2O$ weight ratio of 8:13. In the sample a few relative large alginate spheres (1-5 μm) were found, but also many alginate spheres in the nano range and even spheres with sizes below 200nm. Figure 4.1c and 4.1d shows some typical alginate nanospheres prepared with an alginate – $CaCl_2.2H_2O$ weight ratio of 7:54. These alginate nanospheres visually did not differ much from the sample in Figure 4.1a and 4.1b which had a lower $CaCl_2$ concentration. No $CaCl_2$ nanoparticles in the MCT oil phase were found, which could be an indication that the $CaCl_2$ particles have dissolved into the alginate nanospheres. Energy dispersive X-ray spectrometer (X-Max/AZtec X-ray analyzer, Oxford Instruments Analytical) of the Cryo-SEM was used to determine elements present in the spheres. In Figure 4.2 typical results of the X-ray spectrometer are shown.
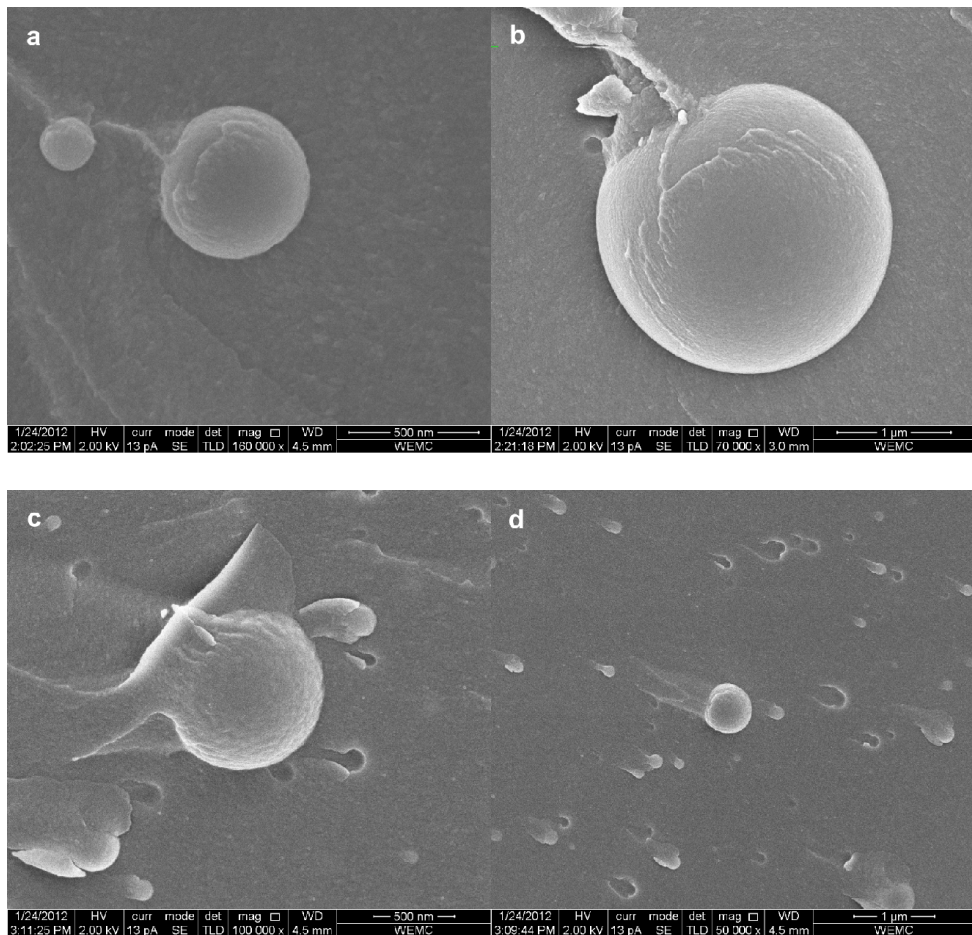
4

Figure 4.1 Alginate nanospheres in a matrix of MCT oil gelled with $CaCl_2$ nanoparticles. (**a** and **b)** Alginate – $CaCl_2.2H_2O$ weight ratio = 8:13. (**c** and **d)** Alginate – $CaCl_2.2H_2O$ weight ratio = 7:54.

4.3.3 Energy dispersive X-ray spectrometer analyses of gelled alginate nanospheres

Creating an X-ray map image with the energy dispersive X-ray spectrometer gives a good insight in the location of the calcium. In Figure 4.2b it can clearly be seen that calcium is mainly present in the alginate spheres and a minimal amount of calcium can be found in the continuous phase of MCT oil. From the performed line scan, given in Figure 4.2c, it can also be concluded that the calcium concentration in the alginate spheres is higher than in the surrounding MCT oil. This confirms the earlier hypothesis that the

calcium nanoparticles, which were initially dispersed in the MCT oil phase, have been dissolved into the alginate nanospheres, explaining they could not be detected in the cryo-SEM pictures. This shows it is feasible to use CaCl$_2$ nanoparticles dispersed in an apolar phase for the gelation of alginate nanospheres. Note that the spatial resolution of the EDX is insufficient to study spatial variations of the calcium concentrations in the interior of the spheres. Such variations could lead to spatial variations in rheological properties in the spheres. Similar variations in calcium distribution have been observed in other studies [16-18].
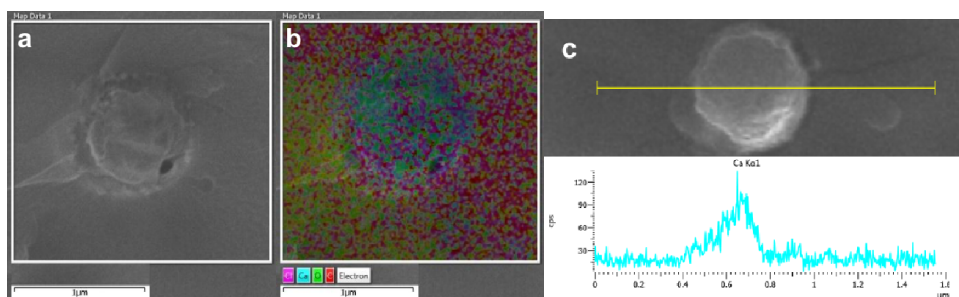
Figure 4.2 Alginate nanosphere in a MCT oil phase gelled with CaCl$_2$ nanoparticles and examined with energy dispersive X-ray spectrometer [Alginate – CaCl$_2$.2H$_2$O weight ratio = 7:54]. (**a**) Scanned area for X-ray image mapping in the sample. (**b**) X-ray image mapping of chlorine (purple), calcium (blue), oxygen (green) and carbon (red) for Figure 4.2a. (**c**) Line scan of an alginate nanosphere in a MCT oil phase gelled with CaCl$_2$ nanoparticles with intensity for calcium.

4.3.4 Alginate nanospheres analyzed by SEM

4.3.5 Morphology of gelled alginate nanospheres by SEM

A solution of respectively 1 and 2% (w/w) alginate was prepared and used to prepare an emulsion with MCT oil as continuous phase. The alginate in MCT oil emulsion was then mixed with CaCl$_2$ nanoparticles dispersed in an MCT oil phase, forming a final system consisting of 5% (v/v) alginate nanospheres in MCT oil with 6% (w/w) PGPR as surfactant. Light microscopy showed the presence of separate alginate nanospheres and no clusters were found. After multiple washing steps with hexane the samples were studied with SEM. These washing steps could result in loss of the smaller alginate spheres. Typical micrographs are shown in Figure 4.3.

Alginate nanospheres are clearly visible from the SEM micrographs in Figure 4.3. Spheres of about 1 µm could be observed but also nanospheres below 300 nm were found. The application of SEM for analyses requires dry nanospheres and therefore the liquid phase has been removed, which could result in shrinkage of the nanospheres. However Cryo-SEM analyses showed nanospheres in similar size ranges compared to the dry nanospheres analyzed with SEM.
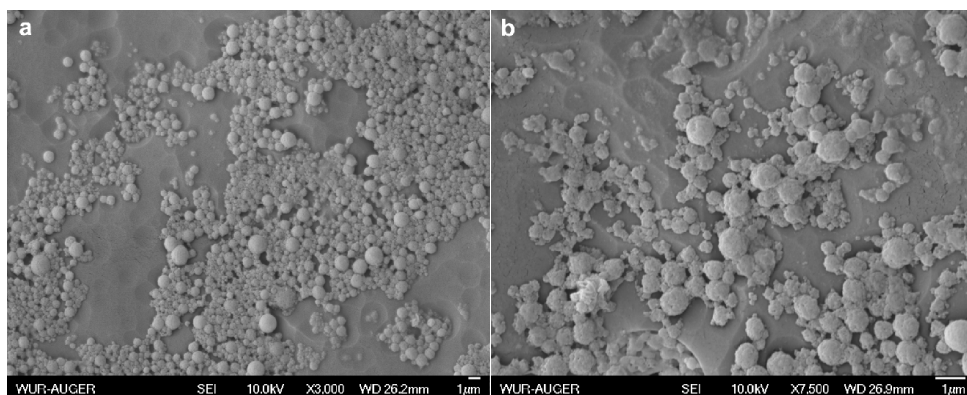

Figure 4.3 Alginate nanospheres gelled with CaCl₂ nanoparticles. For gelation of the alginate droplets CaCl₂ nanoparticles dispersed in MCT oil were mixed with an alginate in oil stock emulsion forming a system with 5% (v/v) alginate nanospheres in MCT oil emulsion containing 6% (w/w) of PGPR. (**a**) A 1% (w/w) alginate solution was used to prepare the droplets [Alginate – CaCl₂.2H₂O weight ratio = 7:9]. (**b**) A 2% (w/w) alginate solution was used to prepare the droplets [Alginate – CaCl₂.2H₂O weight ratio = 3:2].

MCT oil and surfactant were removed from the nanospheres for SEM analyses. Even without a surfactant present the alginate nanospheres could be re-suspended in hexane after centrifugation. This confirms that the nanospheres must be in a gelled state and CaCl₂ nanoparticles migrate to the emulsion droplet interface, where they dissolve into the aqueous alginate phase and cause gelation (if the nanospheres would still be in a liquid state the alginate nanospheres would coalesce in the absence of a surfactant, especially during centrifugation, and no separate alginate nanospheres would be observed with SEM). Auger Electron Spectroscopy was also used to determine if the elements calcium and chlorine were present in the nanospheres. Typical results from Auger Electron Spectroscopy are listed in Figure 4.4 and Figure 4.5.

4.3.6 Analyses on elements of gelled alginate nanospheres by Auger Electron Spectroscopy

The map images of calcium and chlorine in Figure 4.4 and Figure 4.5 clearly overlap the pattern of the alginate nanospheres. The map image of calcium also includes carbon since carbon and calcium give a peak at a kinetic energy (eV) level close to each other. The map image of chlorine is more reliable since chlorine shows a peak at a lower kinetic energy (eV) level compared to carbon. These results confirm that the $CaCl_2$ nanoparticles migrate to the emulsion droplet interface, where they dissolve into the aqueous alginate phase and cause gelation of the nanospheres.
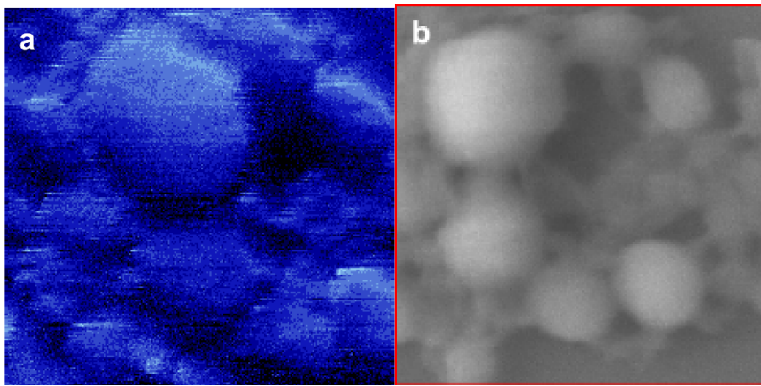
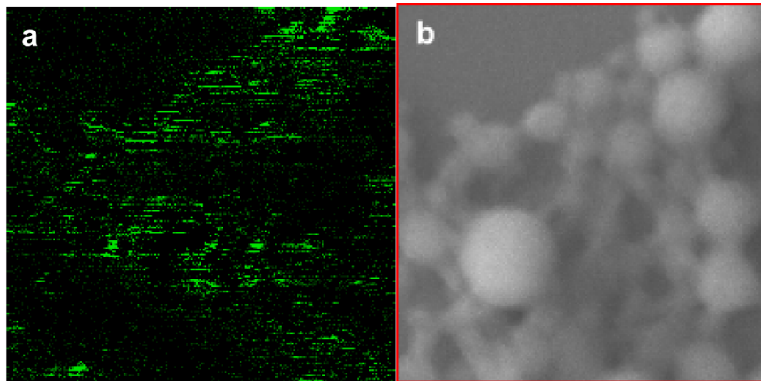**Figure 4.4** Auger map of calcium (**a**) for sample presented in (**b**)

Figure 4.5 Auger map of chlorine (**a**) for sample presented in (**b**)

4.3.7 Congo red as indicator of CaCl₂ and as tool to determine gelation of alginate

4.3.8 Influence of CaCl₂ on pH and absorbance spectrum of Congo red solution

It is observed that a Congo red (CR) solution changes color from red to purple/blue upon addition of CaCl₂, which we believe is due to formation of a Congo red-calcium complex. CR is normally used as pH indicator and changes its color from red to blue at pH 3.0-5.2. Several experiments were conducted to examine the effect of CaCl₂ on CR, to establish whether CR could be used to determine the gelation of alginate. CR solutions were prepared and their pH values and absorbance spectra (Figure 4.7) were measured. Pictures of these CR solutions are shown in Figure 4.6.
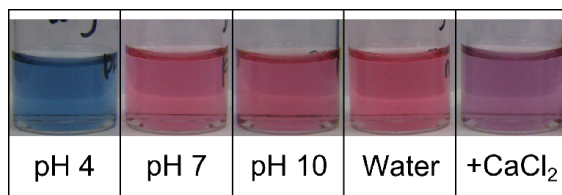

Figure 4.6 Variations in color of different 0.001% Congo red solutions. Samples are prepared in buffer solutions (pH 4, pH 7, pH 10), in MilliQ water and in MilliQ water with CaCl₂.2H₂O.
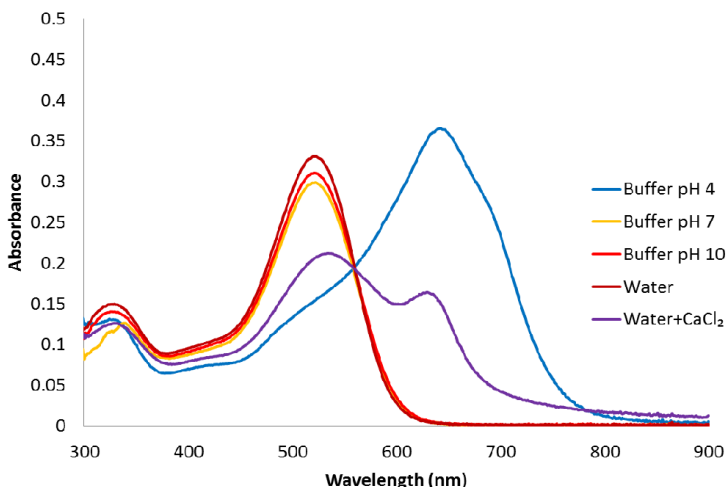

Figure 4.7 Absorbance spectra of 0.001% (w/w) Congo red samples. Samples are prepared in buffer solutions (pH 4, pH 7, pH 10), in MilliQ water and in MilliQ water with CaCl₂.2H₂O.

CR solutions in water, buffer pH 7 and buffer pH 10 result in red colored solutions (Figure 4.6). When CaCl₂.2H₂O is added to the CR solution in water a purple/blue solution is obtained, but the pH remains far above the transition pH range. A CR solution prepared with buffer pH 4 results in a clear blue solution. Measuring the absorbance spectra of these samples shows that samples prepared with water, buffer pH 7 and buffer pH 10 have a maximum absorbance around 522 nm. The sample prepared with buffer pH 4 has a maximum absorbance around 642 nm. The sample prepared with water and CaCl₂.2H₂O shows a bimodal distribution, with a maximum absorbance around 534 nm and 629 nm. Both peaks are a bit shifted compared to the other samples. The bimodal distribution could be an indication for formation of a Congo red-calcium complex. For other salts (NaCl, KCl, CaSO₄, Ca₃(PO₄)₂) a similar color change from red to purple/blue, as with CaCl₂, was observed. In literature it is reported that a Congo red-copper complex can be formed [29].

The color changes in CR solutions induced by addition of CaCl₂ as discussed above makes CR a suitable indicator for presence of Ca²⁺. This property makes it an interesting tool to determine the gelation state of alginate when being gelled by Ca²⁺.

4.3.9 Congo red as tool to determine gelation state of alginate

Figure 4.8 shows samples were a CaCl₂ solution was carefully added on top of a 1% alginate solution containing CR. The concentration of CaCl₂ solutions was varied among the samples from 0%, 0.05%, 0.5% and 1% CaCl₂ (2 ml CaCl₂ solution added to 5 ml alginate solution). Over time it can clearly be seen that the CaCl₂ solution (and its ions) is migrating into the alginate solution and thereby changing the color from red to blue/purple, while gelling the alginate. With a higher concentration of CaCl₂ could be observed that a larger part of the sample has turned blue compared to samples with lower concentrations of CaCl₂. It was found that the alginate is gelled slightly into the red section of the sample. Apparently the threshold value to induce a color change of CR by calcium is not reached at this section or the calcium has a higher affinity for the alginate and no free calcium is available to form a Congo red-calcium complex. The color changes in the samples of Figure 4.8 also illustrate clearly that the CaCl₂ solution and its ions migrates easily through the alginate gel. It is therefore important to realize that additional shells, with lower permeability, may be required when alginate is used for encapsulation. Figure 4.8 shows clearly that the alginate gels swell in presence of the CaCl₂ solution. In bulk

systems and at a macroscopic scale CR seems useful for indicating the state of gelation in alginate systems where $CaCl_2$ is used.
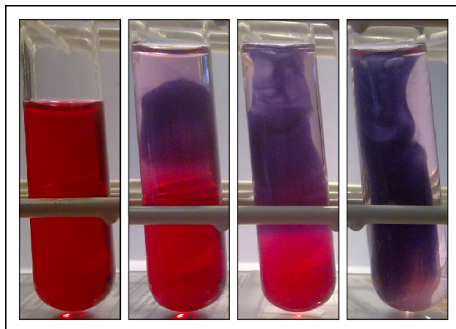

Figure 4.8 Color changes of alginate solution, containing 0.01% (w/w) Congo red, triggered by calcium from a $CaCl_2$ solution. The $CaCl_2$ concentration is increasing in the samples from left to right.

In order to see if CR is also applicable as indicator for the state of gelation of small alginate nanospheres an alginate in oil dispersion, with CR dissolved in the alginate phase, was prepared. One sample was used without further modification and for another sample $CaCl_2$ nanoparticles were added for the gelation of the alginate nanospheres. Both samples were visually observed over time for color changes. For the sample containing the alginate in oil dispersion with CR dissolved in the alginate phase no significant changes could be observed over a period of 22.5 days. In the sample that contained the additional $CaCl_2$ nanoparticles color changes could be observe over time. Within several minutes after addition of the $CaCl_2$ nanoparticles a color change of the sample was already visible.

Figure 4.9 Alginate nanospheres containing 0.001% Congo red dispersed in MCT oil. Left sample is an emulsion of alginate solution in oil (no $CaCl_2$ added) and in the right sample $CaCl_2$ nanoparticles have been added.

Figure 4.9 shows at the left a regular alginate in oil dispersion with CR dissolved in the alginate phase and at the right a similar sample, but with added CaCl₂ nanoparticles. The sample at the left is more pink-like than the sample at the right, which is darker and more purple-like. Due to scattering of the light by the small spheres, color differences in the alginate in oil dispersion are less obvious than when observing the color changes of CR in the bulk phase, illustrated in Figure 4.8. The color changes that take place in the dispersion containing the CaCl₂ nanoparticles makes it possible to see that the sample is changing over time; the CaCl₂ nanoparticles are migrating and dissolving into the alginate nanospheres and a Congo red-calcium complex can form resulting in a color change of the sample. This color change shows indirectly that the alginate nanospheres must be in a gelled state due to the presence of calcium inside the sphere, as was also confirmed by EDX and AES analyses.

4.4 CONCLUSION

The newly developed method for the formation of gelled alginate spheres with sizes below 5 μm is found successful. Cryo-SEM and SEM analyses show that many of the alginate spheres are in a size range around 1 μm, but even alginate spheres below 200 nm were found. The size of the alginate spheres can easily be tuned by adjusting the mechanical forces in the initial emulsification step.

Calcium and chlorine were found in the alginate nanospheres using EDX and AES analyses. This confirms that the CaCl₂ nanoparticles migrate to the emulsion droplet interface, where they dissolve into the aqueous alginate phase, forming gelled spheres. SEM images confirmed the gelling of the alginate spheres. Furthermore it was found that addition of CaCl₂ to a CR solution results in a color change of the CR solution which is believed to be the result of the formation of a Congo red-calcium complex. This property of CR was successfully used to visualize gelation of alginate spheres with CaCl₂ nanoparticles.

Extending the size range of alginate spheres into the nano range, while maintaining relatively mild pH conditions in the interior of the sphere, will significantly extend the range of applications for this type of spheres.

4

4.5 ACKNOWLEDGEMENTS

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4

Chapter V

Alginate Nanospheres Gelled with CaCO₃ Nanoparticles

ABSTRACT

Gelled nanospheres of alginate are prepared through a single step technique involving emulsification and gelation. $CaCO_3$ nanoparticles, together with glucono delta-lactone (GDL), are dispersed in an alginate solution, which is subsequently dispersed in an oil phase. Gelation of the alginate sphere is induced by calcium cations. $CaCO_3$ is used as calcium source where GDL, hydrolyzed into gluconic acid, allows its dissolution. A $CaCO_3$/alginate mass ratio of 0.1/1 was found optimal with respect to gel properties and a GDL/$CaCO_3$ molar ratio of 1.98/1 results in a final pH around 6. It was found that nanoparticles of $CaCO_3$ result in smaller alginate spheres and reduces the gelation time significantly, compared to microparticles of $CaCO_3$. pH time dependence and rheology were determined during gelation of macroscopic alginate gels. Cryo-SEM and light microscopy confirmed formation of gelled alginate spheres below 10 μm with majority of spheres smaller than 2 μm and even spheres below 200 nm were observed.

Application of nanoparticles of $CaCO_3$ for gelation of alginate spheres extends the size range of alginate spheres into the nano range and it significantly reduces the gelation time. The extension into the nano range, the reduction in gelation time, and the possibility of a final internal pH around 6 all significantly extend the range of applications for these alginate nanospheres.

5

5.1 INTRODUCTION

Alginate is one of the most commonly applied materials for the formation of hydrogel (micro) particles [1-4], because it is easy to gel, low in costs, readily available and non-toxic. Alginate particles for encapsulation purposes have been developed since 1980 [5] and much research has been performed ever since on further development and applications [6-13]. Alginate is used in encapsulation of islet cells, for controlled release of drugs, and immobilization of active cells [5,13-15].

Typically alginate spheres are prepared by dripping an alginate solution into a calcium chloride bath, allowing the spheres to gel [16]. This method is referred to as external gelation and results in spheres of 500 μm and larger [14,17]. Other cations than calcium, such as barium and zinc, have been studied as well for gelation of alginate [1,14,18]. Alternatively, spheres can be prepared through a water-in-oil emulsification method whereby an alginate solution is emulsified in an oil phase. The emulsion is then "broken" by addition of a $CaCl_2$ solution, which allows the droplets to gel. Although the method can yield spheres much smaller than the usual 500 μm [14], the droplets often stick together and multiple emulsions (w/o/w) can be formed.

Another method for preparation of alginate spheres, also based on a water-in-oil emulsification method, uses an internal source of calcium [19]. For this so-called internal gelation method $CaCO_3$ particles are dispersed in the alginate phase prior to the emulsification step. The alginate solution is then emulsified in an oil phase and an oil-soluble acid or glacial acetic acid is added to the oil phase. The acid migrates through the oil-water interface into the alginate droplets where it solubilizes the $CaCO_3$, allowing the formation of gelled spheres. The alginate spheres are gelled before they are transferred into the water phase, which makes them more robust for transfer compared to the external gelation method that uses $CaCl_2$ solution to break the emulsion. Since an acid is used for dissolution of the $CaCO_3$ particles the final pH inside the spheres is often in the acidic range. This acidic pH can be harmful to sensitive compounds, such as probiotics, when they are encapsulated in the alginate spheres.

Most gelled alginate particles described in literature are larger than 100 μm, but smaller gelled alginate particles can have several advantages over the larger alginate particles. Small particles have a higher mechanical strength, a larger specific surface area and better transportation of nutrients and oxygen, which is an important factor *e.g.* for

immobilized enzymes and islets [20]. Gelled alginate particles in the sub-micron and nano range can find many applications where particles in the micron range, prepared with microparticles of $CaCO_3$, are too large. Literature also mentions that the need for particles below 100 μm is increasing [4,21]. For application in food it is important to realize that particles below 25 μm will not be detected by the human palate when they are consumed [22]. Most alginate particles described in literature are much larger than these 25 μm and will therefore negatively affect the textural and sensorial properties of products they are applied in.

Recently we have published a novel method for the formation of gelled alginate spheres in the nano range using an alginate in oil emulsion and external gelation with $CaCl_2$ nanoparticles [23]. The food-grade $CaCl_2$ nanoparticles [24] were used as calcium source and were dispersed in the oil continuous phase of the alginate-in-oil emulsion. The $CaCl_2$ nanoparticles dissolved in the interface of the alginate emulsion droplets and induced gelation.

Alginate spheres gelled with external sources of calcium differ in several properties from alginate spheres gelled with internal sources of calcium [25-27]. Differences can be found in matrix strength, stiffness, pore size and permeability. Variations on these properties were found to be related to the spatial variations of the calcium concentrations between external versus internal calcium sources [25-27]. In external gelation a concentration gradient of calcium is present, from a high value at the surface to low one at the core. This results in alginate spheres with varying properties between the surface and core of the sphere [28]. In alginate spheres prepared by internal gelation the calcium is more evenly distributed, resulting in spheres with more uniform properties throughout the entire sphere.

In this chapter we describe a method for the formation of nanospheres of alginate through w/o emulsification and internal gelation by nanoparticles of $CaCO_3$. The method described here differs from other acid induced internal gelation methods in that the pH inside the alginate spheres remains close to neutral, making the spheres more applicable for sensitive compounds. Glucono delta-lactone (GDL) is used inside the alginate spheres and is added to the alginate solution prior to the emulsification step, instead of glacial acetic acid, which is normally added to the oil phase. GDL slowly hydrolyses into gluconic acid, which allows dissolution of the $CaCO_3$ particles followed by gelation of alginate.

Furthermore the gelled alginate spheres are prepared with nanoparticles of $CaCO_3$ instead of microparticles, which allow formation of smaller alginate spheres and results in faster gelation of the spheres. Using nanoparticles of $CaCO_3$ instead of microparticles for gelation of alginate spheres will also result in even smaller spatial variations of the calcium concentrations. It is therefore expected that even more uniform properties throughout the entire sphere are obtained compared to alginate spheres prepared with $CaCO_3$ microparticles.

In this chapter various $GDL/CaCO_3$ molar ratios and their effect on pH in macroscopic systems have been described. The effect of different $CaCO_3/alginate$ mass ratios on shrinkage and syneresis of alginate gels has been studied. The gel strength during gelation has also been studied as well as the effect of size of the $CaCO_3$ particles, temperature and alginate concentration. Based on the bulk studies an optimal formulation has been selected and was used for formation of alginate nanospheres, which have been characterized by Cryo-SEM.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Calcium carbonate (GR for analyses, Merck), calcium carbonate nanoparticles ($CaCO_3$, 97.5%, 15-40nm; SkySpring Nanomaterials, Inc. Houston, USA), glucono delta-lactone (GDL) (Fluka Chemika), grindsted polyglycerol polyricinoleate (PGPR) 90 kosher (Danisco), n-hexane (p.a., Merck, ≥99%), medium chain triglyceride (MCT) oil (Miglyol 812 N, Sasol), and sodium alginate (Algin) Texturas (El Bulli, Spain) were used as received.

5.2.2 Influence of $GDL/CaCO_3$ molar ratio on pH in bulk systems

A 0.1% (w/w) dispersion of $CaCO_3$ particles in water was prepared. The amount of GDL added to the sample was varied among different samples, using $GDL/CaCO_3$ mol ratios of 1.5/1 – 3.5/1. The samples were kept for 24 hours to allow full hydrolyses of GDL and pH was recorded using a pH-meter connected with a recorder. The non-dissolved $CaCO_3$ was removed from the sample by vacuum filtration (Whatman, filter paper 1). The filter paper was subsequently dried at elevated temperature and non-dissolved amount of $CaCO_3$ was determined by weighing.

Furthermore the pH time dependence was determined for samples containing alginate. $CaCO_3$ nanoparticles or microparticles (with a $CaCO_3$/alginate mass ratio of 0.1/1) were dispersed in water. In case $CaCO_3$ nanoparticles were used the sample was sonicated for 10 minutes using a Branson Sonifier 250 (output control level 6, duty cycle 55%), to allow complete dispersion of the nanoparticles. Sodium alginate was added to obtain a ratio of 1% (w/v) and the sample was stirred using a magnetic stirrer. After complete dissolution of the alginate, GDL was added (using various GDL/$CaCO_3$ ratios of 1.5/1 – 3.5/1) and the sample was stirred for 1 more minute. The sample was allowed to gel and pH was recorded for 15 hours using a pH-meter connected with a recorder.

5.2.3 Influence of $CaCO_3$/alginate mass ratio, alginate concentration, $CaCO_3$ particle size and temperature on gel strength in bulk systems

$CaCO_3$ nanoparticles or microparticles (with a $CaCO_3$/alginate mass ratios ranging from 0.05/1 – 0.4/1) were dispersed in water. In case $CaCO_3$ nanoparticles were used the sample was sonicated for 10 minutes using a Branson Sonifier 250 (output control level 6, duty cycle 55%), to allow complete dispersion of the nanoparticles. Sodium alginate was added to obtain a ratio of 1 or 2% (w/v) and the sample was stirred using a magnetic stirrer. After complete dissolution of the alginate, GDL was added (GDL/$CaCO_3$ mol ratio of 1.98/1) and the sample was stirred for 1 more minute. The sample was transferred into a rheometer (Anton Paar 501 with cc17 geometry) for analyses of the gelation process. The measurement was started within 5 min after addition of GDL to the sample. Samples were measured at different gelation temperatures (10 – 40ºC) using a 1% strain and a frequency of 1Hz, and data points were recorded every 30 seconds.

For visual determination samples were prepared using a similar method, but a GDL/$CaCO_3$ molar ratio of 3.5/1 was used instead. The samples were kept in a beaker glass and were visually examined after gelation.

5.2.4 Method for production of alginate spheres

The alginate spheres were prepared through an emulsification/internal gelation technique based on a method described elsewhere [19]. Dry $CaCO_3$ nanoparticles (with a $CaCO_3$/alginate mass ratio of 0.1/1) were dispersed in water and sonicated for 10 minutes using a Branson Sonifier 450 (output control level 5, duty cycle 50%), to allow complete dispersion of the nanoparticles. Sodium alginate was added to obtain a ratio of 1 or 2%

(w/v) and the sample was stirred using a magnetic stirrer. After complete dissolution of the alginate, GDL was added (GDL/CaCO$_3$ mol ratio of 1.5/1 or 1.98/1) and the sample was stirred for 1 more minute. The mixture was then emulsified in volume ratios up to 30% in a continuous phase of medium chain triglyceride (MCT) oil, containing 2% (w/w) PGPR as surfactant. Emulsification was performed with an Ultra Turrax (Ika® T25 digital) at 10,000 rpm for 10 minutes. The samples were mildly agitated for 8 hours using a magnetic stirrer to prevent sedimentation and clustering of the alginate droplets during gelation. All samples were kept in closed containers and stored in a refrigerator until further analyses.

5.2.5 Analysis of alginate spheres by Cryo-SEM

A small droplet of the sample containing the alginate nanospheres was put on copper hollow rivets and directly frozen in liquid ethane. The copper rivets were placed in a cryo-sample holder in liquid nitrogen (LN$_2$). The sample holder was transferred to a non-dedicated cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto a sample stage at -93˚ C. In this cryo-preparation chamber the samples were fractured and freeze dried for 3 minutes at -93°C at 1.3×10^{-6} mbar. The sample was sputter coated with a layer of 5 nm Tungsten at the same temperature. The samples were cryo-shielded transferred into the field emission scanning microscope (Magellan 400, FEI, Eindhoven, The Netherlands) on the sample stage at -121°C at 2.1×10^{-6} mbar. The analysis was performed at a working distance of 4– 4.5 mm, with SE detection at 2 kV, and 6.3 and 13 pA. All images were recorded digitally.

5.3 RESULTS AND DISCUSSION

5.3.1 Influence of GDL/CaCO$_3$ molar ratio on pH in bulk systems

To optimize the final pH in the alginate spheres first the influence of various GDL/CaCO$_3$ molar ratios on pH during the alginate gelation process was studied in bulk gels. The molar ratios of GDL/CaCO$_3$ ranged from 1.5/1 till 3.5/1. In Table 5.1 and Figure 5.1 the influence of the molar ratio of GDL/CaCO$_3$ on pH is shown. Theoretically 2 mol of GDL is required to dissolve 1 mol of CaCO$_3$ [28,29]. Experimentally no undissolved CaCO$_3$ was found in the sample at a GDL/CaCO$_3$ molar ratio of 1.88/1. This experimental ratio differs slightly from the theoretical value, which might be due to minimal loss of CaCO$_3$ during the experimental analyses.

Table 5.1 shows the final pH of different GDL/CaCO$_3$ molar ratios. As can be concluded from Table 5.1 samples prepared with GDL/CaCO$_3$ molar ratios below 2.0/1 will have an excess of CaCO$_3$ and the final pH in the sample will be around neutral. Increasing the GDL concentration above this molar ratio of 2.0/1 will result in an excess of GDL and a sample with a final pH in the acidic range.

Table 5.1 Effect of various GDL/CaCO$_3$ molar ratios on final pH in aqueous systems *

GDL / CaCO$_3$ molar ratio	1.79/1	1.88/1	1.98/1	2.07/1
pH	7.7	7.5	7.8	5

*A CaCO$_3$ concentration of 0.1% (w/w) was used to prepare the samples. The samples are prepared with CaCO$_3$ (micro)particles. pH was measured after 24 hours.
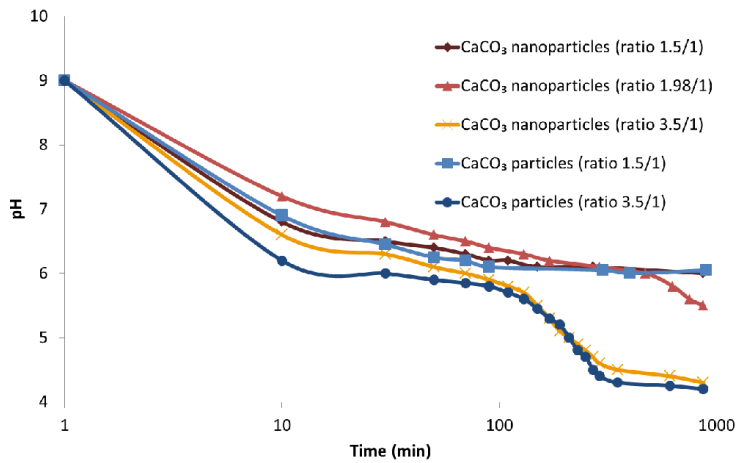

Figure 5.1 Decrease in pH over time for different molar ratios of GDL/CaCO$_3$ and influence of CaCO$_3$ particle size determined in 1% (w/w) alginate systems. A CaCO$_3$ concentration of 0.1% (w/w) is used.

Figure 5.1 shows the pH time dependence for samples with different molar ratios of GDL/CaCO$_3$ measured in an alginate system of 1%. Nanoparticles of CaCO$_3$ or microparticles were used to observe the effect of the CaCO$_3$ particle size on the pH time dependence. In literature GDL/CaCO$_3$ molar ratios of 1.5/1 and 3.5/1 are used [21], and we have tested the same ratios here. Comparing the result from Table 5.1 and Figure 5.1 it is

possible to see some differences in pH values. It must be noted that the samples in Table 5.1 are measured in aqueous system whereas the samples from Figure 5.1 are measured in alginate systems. Samples in Figure 5.1 prepared with a GDL/CaCO$_3$ molar ratio of 1.5/1 have a slightly lower pH compared to results from Table 5.1, but there is sufficient CaCO$_3$ to obtain a final pH around neutral. Samples prepared with a GDL/CaCO$_3$ molar ratio of 3.5/1 had a final pH around pH 4.2 and illustrate that an excess of GDL is present resulting in the acid pH. All samples presented in Figure 5.1 were gelled, illustrating that CaCO$_3$ dissolves even at neutral pH values, which has been described in literature as well [28]. Only a slightly more gradual decrease in pH time dependence was observed in samples prepared with nanoparticles of CaCO$_3$ compared to microparticles. Ström *et al.* demonstrated that release of calcium via the controlled dissolution of CaCO$_3$ by GDL can be rate limited either by GDL hydrolyses or by CaCO$_3$ dissolution, dependent on particle size [30]. If CaCO$_3$ dissolution would occur at a higher rate in samples prepared with nanoparticles of CaCO$_3$ the final (stable) pH would be reached at an earlier stage compared to samples prepared with microparticles. In our results the final (stable) pH is reached for both CaCO$_3$ particle sizes at similar times and no significant effect of CaCO$_3$ particle size on the pH time dependence was observed. This demonstrates that the CaCO$_3$ dissolution occurs at similar rate for both CaCO$_3$ particle sizes; suggesting that the rate of GDL hydrolysis is the rate limiting process.

5.3.2 Influence of CaCO$_3$/alginate mass ratio, alginate concentration, CaCO$_3$ particle size and temperature on gel properties in bulk systems

To optimize the alginate gelation process, first rheological experiments on bulk gels were performed. The effect of CaCO$_3$/alginate ratio was studied at a constant GDL/CaCO$_3$ ratio of 3.5/1. In Figure 5.2 and Figure 5.3 the effect of different CaCO$_3$/alginate mass ratios can be observed.
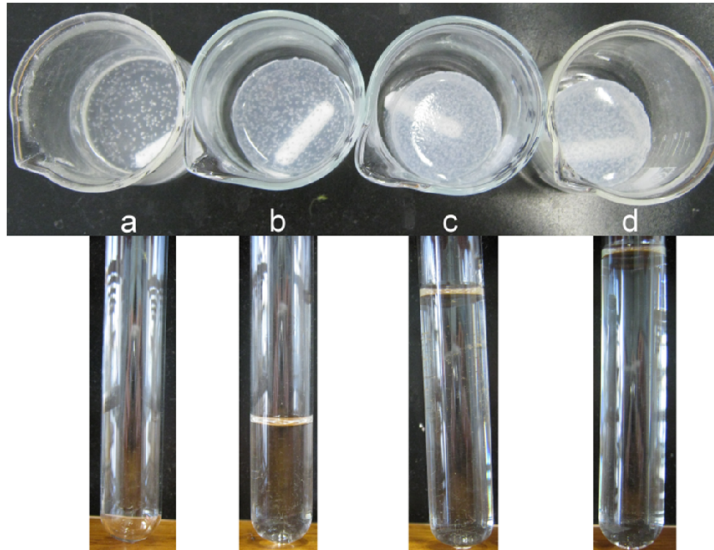
Figure 5.2 Shrinkage of alginate gels in bulk systems. **Top:** Alginate gels and the effect of different concentrations of $CaCO_3$ on gel shrinkage. **Bottom:** The amount of fluid in test tubes that was excreted due to syneresis in the alginate gels above. The $CaCO_3$/alginate mass ratios are 0.1/1; 0.2/1; 0.3/1; 0.4/1 for a, b, c, d respectively.

From experiments it was observed that samples prepared with 0.05% (w/w) $CaCO_3$ and 1% (w/w) of alginate did not gel completely and a more viscous lumpy sample was obtained. These concentrations of $CaCO_3$ and alginate are therefore considered too low for preparation of gelled alginate spheres. Samples prepared with 0.1% (w/w) $CaCO_3$ and 1% (w/w) of alginate did form a firm gel and no shrinkage and syneresis could be observed. Firm gels were also obtained at $CaCO_3$ concentrations of 0.2% (w/w) and above, while maintaining a 1% (w/w) alginate concentration, but shrinkage and syneresis occurred in these samples. The higher the concentration of $CaCO_3$ the more shrinkage and syneresis occurred. A $CaCO_3$/Alginate concentration ratio of 0.1/1 was concluded most suitable because no shrinkage or syneresis occurred. This value of 0.1% $CaCO_3$ and 1% alginate is much lower than described in literature where 5% $CaCO_3$ and 1% alginate is used [21,31].
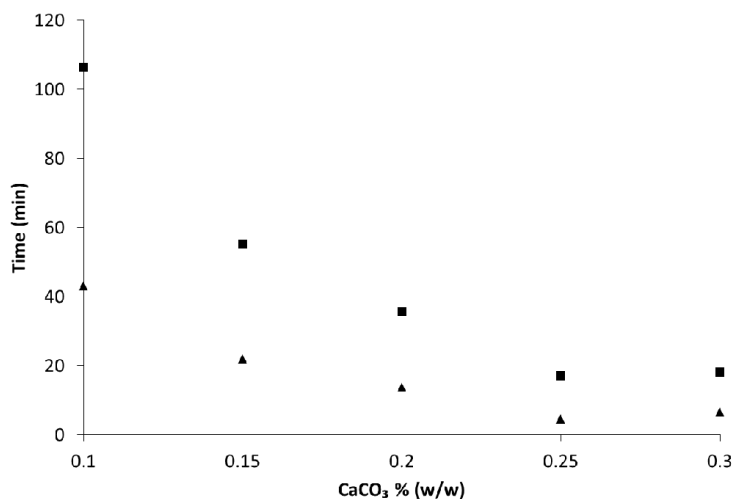
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Figure 5.3 t_0 (▲) t_G (■) in 1% (w/w) alginate systems with different concentrations of calcium and a GDL/CaCO$_3$ mol ratio of 1.98/1. t_0 is defined as the starting point of an exponential increase in storage modules (G') and loss modules (G''). The crossover of G' and G'' is defined as gel point (t_G).

From rheological analyses, represented in Figure 5.3, can be observed that an increase in CaCO$_3$ concentration results in a decrease in time of t_0 and t_G. t_0 is defined as the starting point of an exponential increase in storage modules (G') and loss modules (G'') and t_G as the crossover of G' and G''. An increase in calcium concentrations while maintaining a constant alginate concentration and GDL/CaCO$_3$ ratio will result in more release of calcium ions in the same time span. The more calcium ions are available the more linked alginate strands can be formed. Therefore faster gelation in samples with higher calcium concentrations can occur and the t_G will be reached earlier compared to samples with lower calcium concentrations. This effect can clearly be observed in Figure 5.3. Higher CaCO$_3$ concentration have also a closer packing of particles in the system, therefore less permeation of calcium ions is required to link alginate strands together and t_G can be reached at an earlier stage compared to lower CaCO$_3$ concentration.

The gel strength of two concentrations of alginate, 1 and 2%, were analyzed. Also the influence of CaCO$_3$ particle size and temperature during gelation on the rheological properties was studied. Typical results are shown in Figure 5.4 and Figure 5.5.
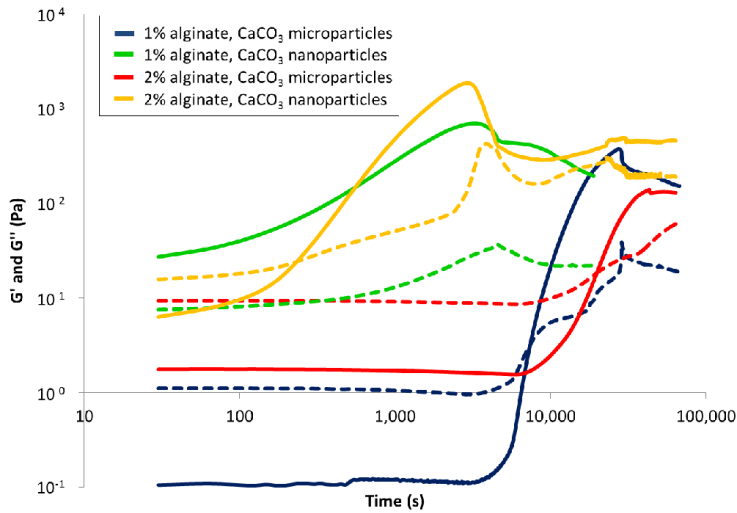
Figure 5.4 G' (solid line) and G'' (dashed line) during gelation of alginate gels. The measurement was started within 5 min after addition of GDL to the samples. Gels prepared with 1% (w/w) alginate and calcium source of 0.1% (w/w) $CaCO_3$ microparticles (in blue) or 0.1% (w/w) $CaCO_3$ nanoparticles (in green). Gels prepared with 2% (w/w) alginate and calcium source of 0.1% (w/w) $CaCO_3$ microparticles (in red) or 0.2% (w/w) $CaCO_3$ nanoparticles (in yellow). A GDL/$CaCO_3$ mol ratio of 1.98/1 was used in all samples.

From Figure 5.4 it can clearly be seen that t_0 starts significantly earlier in samples prepared with nanoparticles of $CaCO_3$ and t_G is also reached at an earlier stage compared to samples prepared with microparticles. The t_G in the sample with 1% alginate and 0.1% of $CaCO_3$ nanoparticles has already been reached within the 5 minutes required to add the GDL to the sample and the measurement in the rheometer is started. The particle size has a significant effect on gel time, but not a significant effect on the pH time dependence. As it was concluded from Figure 5.1 the GDL hydrolyses and $CaCO_3$ dissolution occur at comparable rates for both nanoparticles of $CaCO_3$ and microparticles and could therefore not explain the significant faster gel time in samples prepared with $CaCO_3$ nanoparticles. The faster gel time could be the result of $CaCO_3$ nanoparticles being more homogeneously distributed throughout the alginate system compared to $CaCO_3$ microparticles. Having a more homogenous distribution requires less permeation of calcium ions through the alginate system for linking alginate strands together. In Figure 5.4 also an effect of alginate concentration on gel time can be observed: increasing the alginate concentration

postpones the moment when the t_G is reached. This effect has also been described in another study [32]. The decrease in G' and G'' after the G_{max} was reached (Figure 5.4), is most probably the effect of the high strain (1%) used for the analyses. Due to this high strain the network in the gel could be weakened resulting in the decrease of G' and G''. The effect is most pronounced in the sample prepared with 2% alginate and $CaCO_3$ nanoparticles, because this is the strongest, thus most brittle, gel.
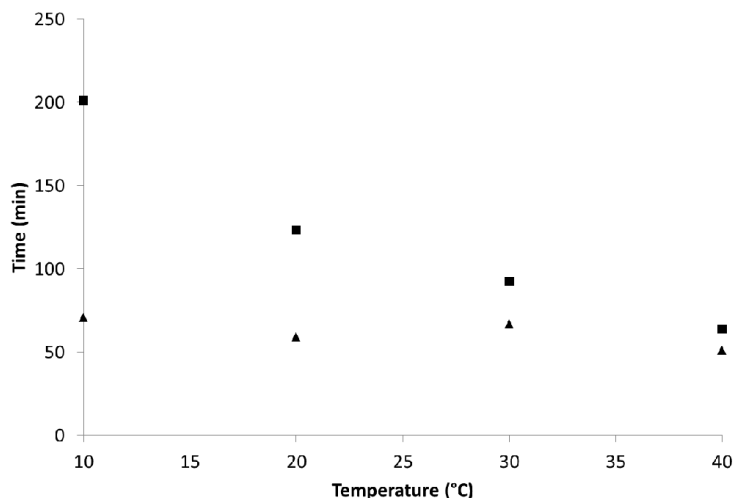

Figure 5.5 Influence of temperature on t_0 (▲) and t_G (■) in 1% (w/w) alginate systems prepared with $CaCO_3$ microparticles. A $GDL/CaCO_3$ mol ratio of 1.98/1 is used in all samples.

In Figure 5.5 the effect of temperature on t_0 and t_G is represented for alginate gels prepared with $CaCO_3$ microparticles. With increasing temperature t_G decreases in time, but t_0 remains about constant. It is known that the rate of hydrolyses of GDL is increased by heat [33]. This allows faster GDL hydrolyses and $CaCO_3$ dissolution at higher temperatures and more calcium ions are released in a shorter time span compared to samples prepared at lower temperatures.

5.3.3 Morphology of gelled alginate spheres by Cryo-SEM

A 1% (w/w) alginate solution was prepared for formation of alginate spheres. Prior to dissolution of the alginate $CaCO_3$ nanoparticles were dispersed in the water phase using

an ultra-sonic probe. Finally GDL was added to the mixture to allow dissolution of $CaCO_3$ nanoparticles. The mixture was then emulsified, before gelation of the alginate mixture could set in, with an ultra turrax in an MCT oil phase containing PGPR as surfactant. The sample was kept overnight to allow complete gelation of the alginate spheres before Cryo-SEM analyses (Figure 5.6).

From Figure 5.6 can be seen that gelled alginate spheres of various sizes are obtained. The majority of the spheres showed a diameter below 2 µm as also can be seen in the micrograph obtained from light microscopy (Figure 5.7). A minor number of alginate spheres appeared to have a diameter between 2 – 10 µm, which is still much smaller than most alginate spheres described elsewhere [15,17]. We also found many alginate spheres around 300 nm and even some alginate spheres with a diameter below 200 nm were found.
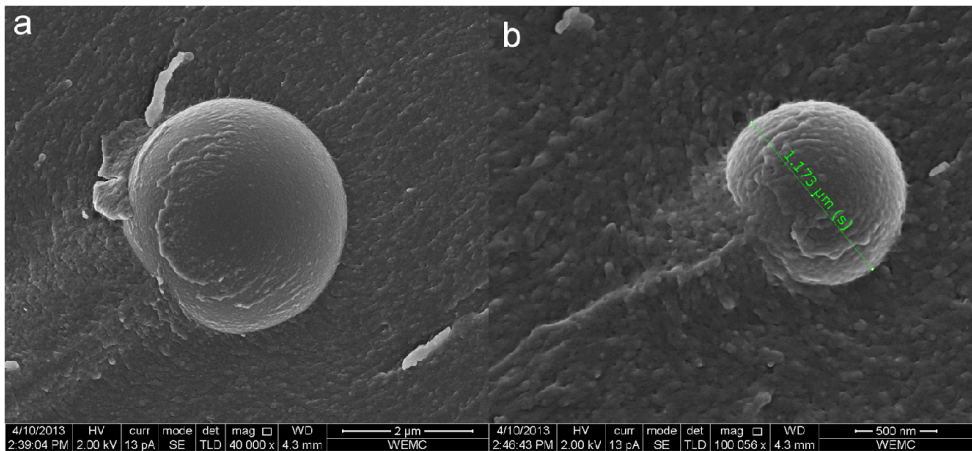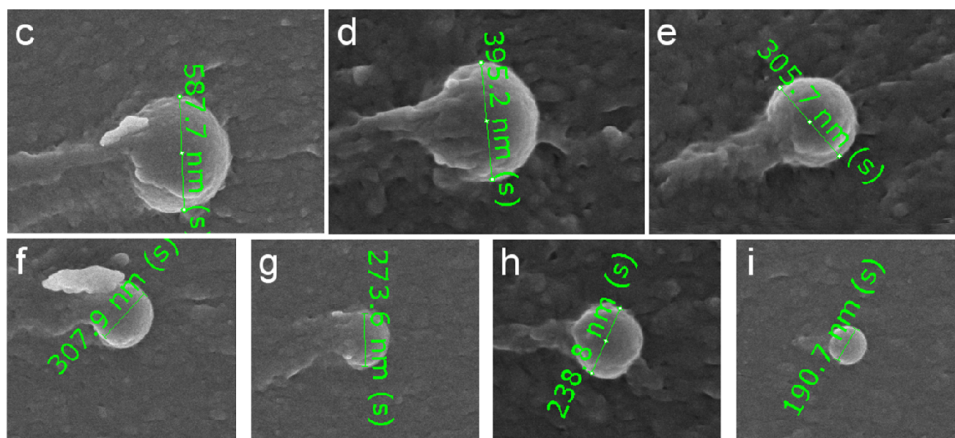
Figure 5.6 Cryo-SEM micrograph of alginate spheres of various sizes in a matrix of MCT oil and gelled with calcium from $CaCO_3$ nanoparticles. All spheres are prepared with 1% alginate (w/w), $CaCO_3$/alginate mass ratios are 0.1/1 and a GDL/$CaCO_3$ mol ratio of 1.98/1.

Unfortunately no fractured spheres were found during Cryo-SEM analyses, which would enable us to get better insight in the interior of the spheres. It would be very interesting then to study the effect of $CaCO_3$ particle size on the spatial variations of the calcium concentrations in the interior of the spheres and its effects on the internal structure of the spheres. As discussed above it is expected that alginate spheres produced with nanoparticles of $CaCO_3$ have a more homogenous distribution of the calcium within the sphere compared to spheres prepared with microparticles. This more homogenous distribution of the calcium could be an explanation for reaching t_G in an earlier stage (as shown in Figure 5.4) compared to $CaCO_3$ microparticles, which cause larger spatial variations of the calcium concentrations. Other studies have already shown that spatial variations of the calcium concentrations could lead to spatial variations in rheological properties in the spheres [25-27].
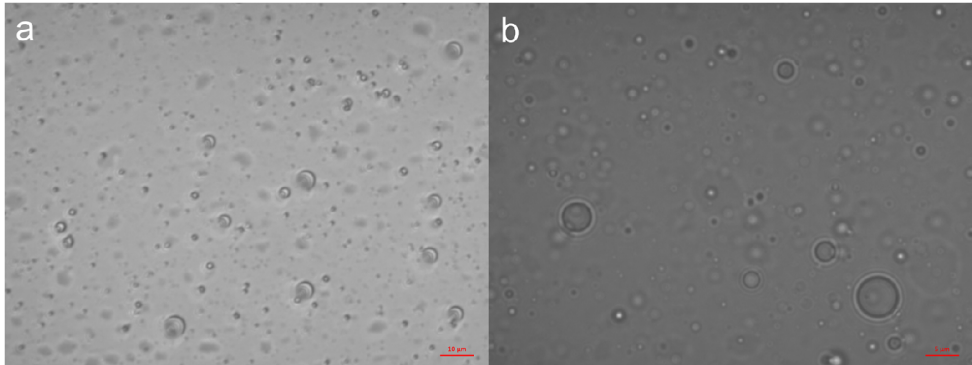
Figure 5.7 Light microscopy micorgraphs of alginate spheres of various sizes in a matrix of MCT oil and gelled with calcium from $CaCO_3$ nanoparticles. All spheres are prepared with 1% alginate (w/w), $CaCO_3$/alginate mass ratios are 0.1/1 and a GDL/$CaCO_3$ mol ratio of 1.98/1. **a.** magnification 50x (scale bar 10 μm). **b.** magnification 100x (scale bar 5 μm)

5.4 CONCLUSION

Application of nanoparticles of $CaCO_3$ for the gelation of alginate spheres extends the size range of alginate spheres to the nano range. Cryo-SEM and light microscopy confirmed formation of gelled alginate spheres below 10 μm with majority of spheres smaller than 2 μm and even spheres below 200 nm were observed. This is significantly smaller than when using the traditional external gelation method with $CaCl_2$, which typically yields spheres of 500 μm and larger [14,17]. Rheological analyses in bulk systems showed that the gelation time was significantly reduced when nanoparticles of $CaCO_3$ were applied instead of microparticles. It was also found that an increase in alginate concentration postponed the gel point of the alginate gel. Using bulk analyses the formulation for alginate gels was optimized and a $CaCO_3$/alginate mass ratio of 0.1/1 was found optimal with respect to gel properties and a GDL/$CaCO_3$ molar ratio of 1.98/1 resulted in a final pH around 6. The extension into the nano range, the reduction in gelation time, and the possibility of a final internal pH around 6, all significantly extend the range of applications for these alginate nanospheres.

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5.5 ACKNOWLEDGEMENTS

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Chapter VI

General Discussion

ABSTRACT

The aim of the work described in this thesis was to develop new methods for the formation of (nano)particles with a diameter below 25 microns. These particles should function as carriers for functional ingredients. After a general introduction the thesis starts with a review on existing preparation methods of alginate nanoparticles. In the following chapter (*chapter 3*) a novel method for the formation of food-grade salt nanoparticles is described. *Chapter 4* utilizes the method described in *chapter 3* for the formation of $CaCl_2$ nanoparticles, which are then used for external gelation of alginate nanospheres. *Chapter 5* describes the formation of alginate nanospheres by internal gelation with $CaCO_3$ nanoparticles. In this last chapter the findings of these earlier chapters are summarized and discussed. We evaluate the success and limitations of the new methods and their resulting particles. Furthermore challenges and suggestions for future research with respect to this new encapsulation process are discussed.

6

6.1 INTRODUCTION

Alginate is a natural polysaccharide from brown seaweed and has been used for many applications in industry [1]. It is readily available, non-toxic, biodegradable and low in cost. Alginate can easily be gelled with cations under gentle conditions making it ideal for entrapment of sensitive materials. Due to the special properties of alginate it is one of the most commonly applied materials for the formation of hydrogel (micro) particles [2-5]. Alginate particles for encapsulation purposes have been developed since 1980 [6] and much research has been performed ever since on further development and applications [1,7-13]. Currently the need for smaller and smaller particles is increasing, especially particles in the nano-range since they have several advantages over the larger ones.

In this thesis, the formation of alginate nanospheres through emulsification coupled with gelation was investigated. The developed methods may find application as system for embedding (sensitive) compounds through a simple and flexible method. In Figure 6.1, the content of the thesis is summarized in a graphical presentation. In the first part of the thesis, chapter 2, we present a review which covers various existing techniques for the formation of alginate nanoparticles, and their properties. In the second part, the formation of alginate nanospheres and their properties and function are studied. This second part starts with the preparation of food-grade salt nanoparticles (chapter 3), which are used in the following chapter 4 for the formation of alginate nanospheres by external gelation. Alginate nanospheres were characterized by SEM and Cryo-SEM. Furthermore Energy-dispersive X-ray spectroscopy (EDX) and Auger electron spectroscopy (AES) were used to confirm presence of calcium ions in the alginate spheres, indicating the spheres were in a gelled state. Gelation of the spheres was confirmed with a novel technique using Congo red as an indicator, which shows a color change upon the addition of $CaCl_2$ to the system. We conclude this second part of the thesis with the formation of alginate nanospheres by internal gelation with $CaCO_3$ nanoparticles (chapter 5). Rheology and pH time dependence were determined during gelation of macroscopic alginate gels. The results were used to optimize $CaCO_3$/alginate mass ratio and GDL/$CaCO_3$ molar ratio in the recipe for formation of the alginate spheres.

In this last chapter, we summarize and discuss the findings of the previous chapters. Firstly, the type of particles and various existing methods are discussed together with the limitations and future challenges. We will then focus on formation of alginate

nanospheres and their properties and function. This section starts with a discussion on the formation of food-grade salt nanoparticles and suggestions for future research. This is followed by a discussion on alginate nanospheres prepared through w/o emulsification and gelation with $CaCl_2$ or $CaCO_3$ nanoparticles. Furthermore we discuss the current limitations and future challenges of the new methods for formation of alginate nanospheres.
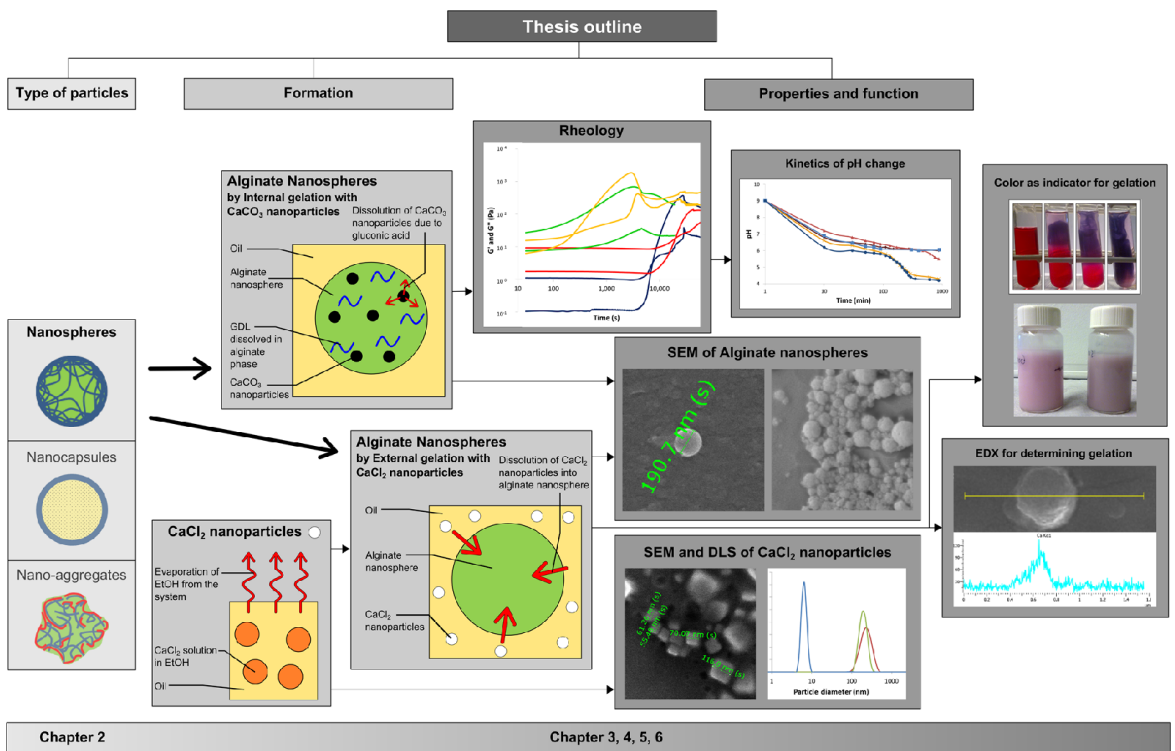

Figure 6.1 Graphical presentation of the thesis content.

6.2 TYPE OF PARTICLES

In *chapter 2* we reviewed the preparation methods of alginate nanoparticles. The formation of alginate nanoaggregates, nanocapsules and nanospheres is primarily based on two methods. In one the "complexation method" is used to form nanoaggregates or nanocapsules by complex formation in an aqueous solution or on the surface of oil

droplets. In a second method w/o emulsification coupled with gelation of the alginate emulsion droplets can be used to form alginate nanospheres.

Alginate is one of the most commonly applied materials for the formation of hydrogel (micro) particles nowadays and it has shown promising results [2-5], but studies on alginate nanoparticles are less common. Smaller alginate particles, especially nanoparticles, have several advantages over larger alginate particles: small particles have higher mechanical strength, a larger specific surface area and can flow more easily through narrow nozzles and channels, which would otherwise be blocked by larger particles. In drug delivery systems nanoparticles can easier be absorbed by the human body, migrate through tissue and flow through veins in the human body, and allows administration of lower drug doses, resulting in less adverse effects caused by the drug [14-22]. In food applications, particles below 25 μm are not detectable by the human palate, allowing addition of alginate nanoparticles containing functional ingredients to food products, without negatively influencing their textural and sensorial properties [23]. Nanoparticles may be used in flavor encapsulation, since the large surface area of the nanoparticles allows fast release of the flavor compounds in the consumer's mouth. The application of alginate nanoparticles can result in development of food products with novel properties. Using mild conditions and no toxic solvents in the preparation of alginate nanoparticles makes them applicable for entrapment of sensitive materials and for use in pharmaceuticals and foods. Additional shells can be formed on the alginate nanoparticles surface to control permeability and stability, and certain groups and ligands may be attached to the particles service to obtain additional functionality [8,9,11,12,24-26].

The review in *chapter 2* shows that several important challenges remain for the production of small alginate particles. The particles should be obtained by environmentally friendly processes, and should result in nanoparticles with a narrow size distribution, high mechanical strength and chemical stability. Furthermore the method should have feasibility to be scaled up to industrial scale production volumes. Before alginate nanoparticles can be applied in their potential applications it is important to evaluate their cytotoxicity, immune response and biodegradability.

6.3 FORMATION, PROPERTIES AND FUNCTION

Chapter 3 describes a method for the formation of food-grade salt nanoparticles. The salts $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ are used for formation of salt nanoparticles and are dissolved in ethanol. The ethanol phase is subsequently mixed with a medium chain triglycerides oil phase. The resulting ethanol-in-oil (E/O) mixture is then heated to evaporate the ethanol phase, and salt particles are formed with dimensions in the nano range (6 – 400 nm). We found that the concentration of salt and volume fraction of ethanol in the MCT oil have significant influence on the size distribution of the salt particles. Increasing the concentration of salts and/or volume fraction of ethanol above a certain level resulted in the formation of salt *microparticles* (1 – 25 µm) with various morphologies, such as butterflies, needles, spheres and cuboids (Figure 6.2).
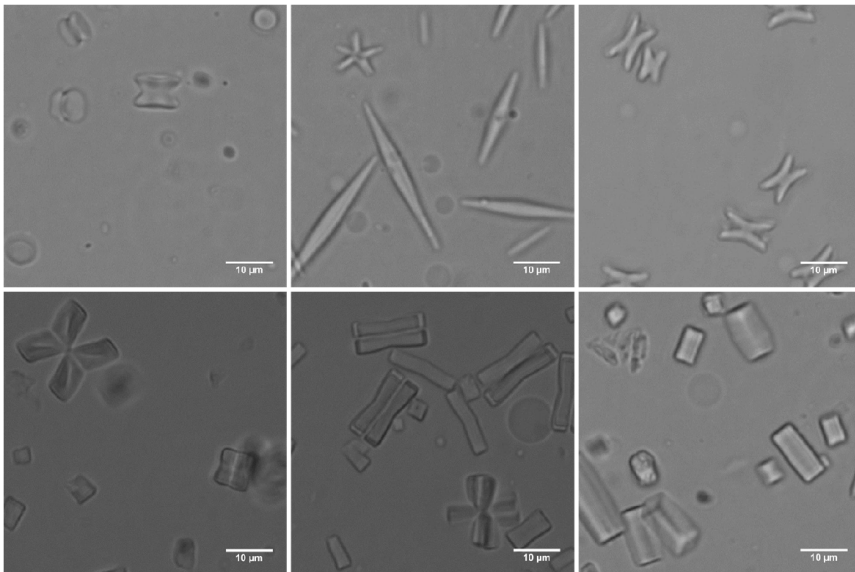

Figure 6.2 Various morphologies of magnesium salt microparticles. Microparticles are formed at concentrations of salts and/or volume fraction of ethanol higher than those levels typically used in nanoparticle formation.

For the E/O mixtures it was also found that type and concentration of salt have significant influence on the miscibility of the ethanol and oil phase, and on the stability of the E/O mixtures. Mixing pure ethanol and MCT oil at variable ratios resulted in transparent and stable mixtures. Transparent and stable mixtures were also obtained

when low concentrations of salt were present in the ethanol phase. At sufficiently high salt concentrations phase separation appeared to occur and a surfactant, such as PGPR, was required to (partly) stabilize the mixture. At elevated temperatures, higher salt concentrations were needed to induce phase separation. The stability of the E/O mixtures with salt appeared important for the formation of salt nanoparticles since unstable mixtures resulted in formation of salt nanoparticles and microparticles, whereas stable mixtures only resulted in formation of salt nanoparticles. Although a surfactant was not required in some of the E/O mixtures with salt, the surfactant was required for formation and stabilization of the salt nanoparticles. For future research and optimization of the method it would be useful to fully characterize the phase diagram of ethanol and MCT oil in the presence of salts. This will give better insight how the system shifts through the phase diagram during evaporation of ethanol and allows better tuning of the concentrations for formation of salt nanoparticles. Furthermore it will be interesting to study the influence of high shear forces or sonication during evaporation of the ethanol phase on the particle size distribution.

In *chapter 4* the formation of alginate nanospheres through W/O emulsification and external gelation with $CaCl_2$ nanoparticles is described. A 1% alginate solution was emulsified in MCT oil forming a W/O emulsion, stabilized by PGPR. The alginate emulsion droplets were gelled through a novel procedure by dispersing $CaCl_2$ nanoparticles in the MCT oil, which dissolve into the aqueous alginate phase and cause gelation. The $CaCl_2$ nanoparticles were obtained through the method described in *chapter 3*. From Energy-dispersive X-ray spectroscopy (EDX) and Auger electron spectroscopy (AES) analyses we concluded that the $CaCl_2$ nanoparticles migrated from the MCT oil into the alginate emulsion droplets, where they dissolved, resulting in gelled alginate spheres. Gelation of the spheres was confirmed with a novel technique using Congo red as an indicator. A color change of the sample could be observed if Congo red was present in the alginate spheres upon dissolution of $CaCl_2$ nanoparticles in these alginate spheres, due to formation of a Congo red – calcium complex. Using Congo red in alginate spheres during external gelation prepared by extruding an alginate solution in a $CaCl_2$ bath, resulted in a nice visual gradient of calcium permeating from the alginate sphere surface towards the core (figure 6.3).
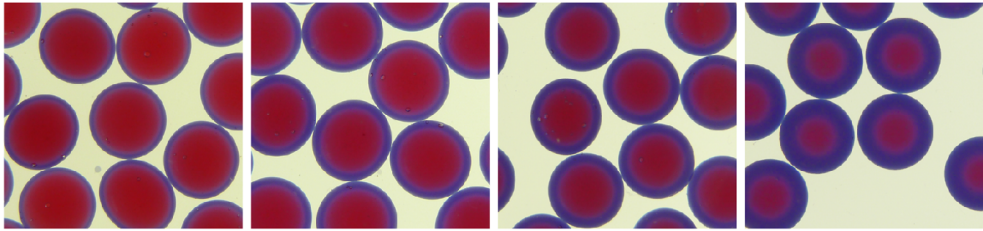
Figure 6.3 Photographs of alginate spheres containing Congo red at different stages of gelation in a CaCl$_2$ solution (gelation time is increasing from left to right). An alginate solution containing Congo red has been added dropwise to a CaCl$_2$ solution forming spheres of 2 – 3 mm in diameter. Calcium migrates from the surface of the alginate spheres towards the core, forming gelled spheres. This effect is nicely visualized by a change in color of Congo red from red to blue, due to formation of a Congo red – calcium complex.

The alginate spheres described in *chapter 4* were washed in order to remove MCT oil and surfactant. SEM analysis showed separate alginate spheres, indicating that the spheres must be in a gelled state. SEM and Cryo-SEM show that alginate spheres are mostly in a size range around 1 µm, but nanospheres as small as 200 nm and smaller were also found. This allows formation of significantly smaller alginate spheres, compared to traditional methods where formation of alginate spheres by external gelation are based on a dripping/extrusion technique. The method described in *chapter 4* has the advantage that alginate spheres are not clustered and no multiple emulsion droplets (W/O/W) are formed, which typically is a problem in the external gelation method when an alginate-in-oil emulsion is broken with a CaCl$_2$ solution.

Chapter 5 also describes the formation of alginate nanospheres through W/O emulsification and uses internal gelation with CaCO$_3$ nanoparticles instead of the external gelation process described in *chapter 4*. We dispersed CaCO$_3$ nanoparticles, together with glucono delta-lactone (GDL), in an alginate solution, which was subsequently dispersed in an oil phase. The CaCO$_3$ nanoparticles were used as calcium source and GDL, hydrolyzed into gluconic acid, allows their dissolution. Analysis of macroscopic alginate gels allowed optimization of the calcium/alginate ratio and GDL/CaCO$_3$ ratio, resulting in alginate gels with minimal shrinkage and syneresis, and a final pH around 6. Cryo-SEM and light microscopy confirmed that most alginate spheres were smaller than 2 µm and even spheres below 200 nm were observed. Due to utilization of CaCO$_3$ nanoparticles, instead

of microparticles, the size range of alginate spheres could be extended into the nano range and gelation time of the alginate nanoparticles was significant reduced.

In both *chapters 4 and 5* a W/O emulsification method is used to prepare alginate spheres. By using emulsion droplets as template for formation of these alginate spheres, perfectly round spheres with a smooth surface can be obtained. By controlling the power input used during emulsification, the size of the alginate spheres can easily be controlled and microspheres as well as nanospheres can be obtained. Using emulsion droplets as template for formation of alginate spheres allows high encapsulation efficiencies. It also has as advantages that water-soluble compounds, captured in their interior, cannot easily leach out into the MCT oil phase during formation of the alginate spheres. An emulsification method to prepare alginate nanospheres has feasibility for industrial scale up. Sonication can be used for emulsification instead of mechanical agitation and may allow formation of even smaller alginate nanospheres. However sonication might be harmful to certain compounds, such as bacteria, captured in the alginate nanospheres. An important challenge that remains is formation of monodisperse alginate spheres. Microfluidic devices and membranes can yield monodisperse emulsion droplets, but formation of droplets in the nano-range remains a challenge using such devices as well as industrial scale up. The effects of ethanol described in *chapter 3* may be used as an approach for formation of monodisperse alginate nanospheres using membrane emulsification. Alginate solutions could be diluted with ethanol followed by membrane emulsification of the mixture in an oil phase, resulting in alginate emulsion droplets of a few microns. Due to the solubility of ethanol in oil, it can dissolve to a certain extent in the oil phase. Ethanol can then relatively easy be evaporated from the emulsion, resulting in shrinkage of the alginate emulsion droplets into the nano range. The obtained alginate emulsion droplets can then be gelled through external gelation with $CaCl_2$ nanoparticles to form monodisperse alginate nanospheres. Alternatively internal gelation may be used, but gelation of the alginate emulsion droplets must be postponed to prevent blocking of the membrane pores. This could be achieved by preparing the emulsion droplets at elevated temperatures, which are then allowed to set through cooling, or replacing the GDL with glacial acetic acid, which can be added to the oil phase after emulsification. An initial challenge of this approach will be to prevent fouling of the membrane surface. A precondition is that the encapsulated content is not sensitive to ethanol.

In *chapter 4* external gelation and in *chapter 5* internal gelation is used for the formation of alginate nanospheres. The type of gelation that is used significantly influences the properties of the alginate spheres. Differences can be found in matrix strength, stiffness, pore size and permeability. Variations on these properties were found to be related to the spatial variations of the calcium concentrations between external versus internal calcium sources [27-29]. In external gelation a concentration gradient of calcium is present, from a high value at the surface to low one at the core. This results in alginate spheres with varying properties between the surface and core of the sphere [30]. In alginate spheres prepared by internal gelation the calcium is more evenly distributed, resulting in spheres with more uniform properties throughout the entire sphere. Using $CaCO_3$ nanoparticles for internal gelation of the alginate spheres, instead of $CaCO_3$ microparticles, should result in even smaller spatial variations of the calcium concentrations, and therefore, it is expected that also more uniform properties throughout the entire sphere are obtained. In general alginate spheres prepared through external gelation have smaller pores at the surface than at the interior of the alginate particle. Using $CaCl_2$ nanoparticles for external gelation of alginate spheres could result in a different concentration gradient of calcium through the sphere compared to alginate spheres obtained through external gelation with a $CaCl_2$ solution (figure 6.4).

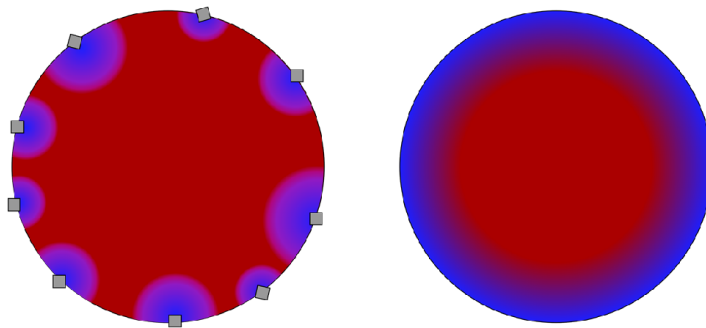**Figure 6.4** Schematic representation of concentration gradients of calcium during external gelation of alginate spheres. External gelation with $CaCl_2$ nanoparticles (left) and external gelation with $CaCl_2$ solution (right).

The $CaCl_2$ (nano)particles, used in formation of such alginate spheres, could result in high and low calcium concentration regions randomly distributed on the surface of the alginate

spheres, and thereby result in regions with different properties such as smaller pores and larger pores. This might result in alginate spheres with unique properties. The size and number of $CaCl_2$ (nano)particles used for gelation will influence the distribution of calcium on the particle surface and therefore influence the properties of the alginate spheres. It would be very interesting to study the variations in properties of alginate spheres gelled with $CaCl_2$ nanoparticles of various sizes and compare the results with alginate spheres gelled with a $CaCl_2$ solution. Furthermore it would be interesting to study how these externally gelled alginate spheres differ compared to alginate spheres prepared by internal gelation with $CaCO_3$ nanoparticles or $CaCO_3$ nanoparticles. SEM analyses could give better insight in morphology and surface topography of the various alginate spheres. Diffusion rates and mechanical properties may be studied in bulk systems. Furthermore properties such as drug content, drug release and solubility of the alginate spheres will be relevant for determining which method is most suitable for certain applications.

6.4 CONCLUDING REMARKS

In this thesis we successfully developed methods for the formation of alginate spheres below 25 μm in diameter. The use of mild conditions and no organic solvents in the preparation of alginate nanoparticles makes them applicable for entrapment of sensitive materials. The developed methods use food-grade materials making the resulting particles suitable for application in foods and pharmaceuticals. Most of the produced alginate spheres had a diameter below 2 microns and even alginate spheres below 200 nm were obtained. The size of the alginate spheres can be tuned by controlling the power input used during emulsification. The type of gelation used, internal or external, determines the properties of the alginate spheres. Methods using internal gelation, as described in *chapter 5*, are relevant for applications requiring homogenous properties throughout the entire particle. Applications requiring particles with varying properties between the surface and core of the sphere should use methods based on external gelation, as described in *chapter 4*.

For future research it will be interesting to further characterize the properties (such as morphology, matrix strength, stiffness, pore size, permeability, drug content and drug release) of the alginate spheres described in this thesis, and compare them with traditional alginate spheres obtained through external gelation in a $CaCl_2$ solution.

In literature various methods for formation of additional shells on alginate particles are already described. Application of shells on the particle surface reduces the particle permeability and allows tuning of the release from the particle, e.g. in the human body, to a certain trigger. Attaching additional functional groups and ligands to the particle surface can add specific functionality and stability. The alginate spheres described in this thesis seem feasible for formation of additional shells on their surface, but this should be further developed to obtain specific properties.

The formation of alginate spheres described in this thesis may significantly extend the range of applications for this type of spheres. Further precise tuning of the particle size of the alginate spheres will make their application even more versatile. Membrane emulsification may be used in formation of monodisperse alginate spheres in the nano range, but will impose various challenges.

While many different methods and materials are available for encapsulation, the methods described in this thesis will contribute to the development of novel, healthier, smarter and functional products in foods and pharmaceutics.

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Summary

Alginate is a naturally occurring linear unbranched polysaccharide extracted from brown seaweed. Depending on the source and subsequent chemical modification the properties of the alginate are determined. Alginate can easily be gelled with multivalent cations under gentle conditions allowing formation of gelled beads. This makes it ideal for entrapment of sensitive materials or other compounds. Alginate is one of the most used polymers in (micro)particle formation, but formation of alginate nanoparticles is less common. Alginate particles can be used for encapsulation of compounds by enclosing them in its interior. Such particles, moreover encapsulates, can give extra protection from the environment and improve the mechanical stability of the entrapped compounds. In foods and pharmaceutical products have nanoparticles, containing compounds, several advantages over larger particles. Each particle has specific requirements for its application and simple and flexible production methods are required. Therefore it is important to develop new and efficient (food-grade) techniques for the formation of micro- and especially nano-particles.

In this thesis we describe various methods for the formation of alginate nanoparticles. We aim for formation of particles with a diameter below 25 µm that can be used as encapsulate for application in food products. The pH conditions inside these particles should be sufficiently mild such that pH sensitive components can be encapsulated.

Chapter 2 presents a review and covers the various techniques for the formation of alginate nanoparticles and their properties. Primarily, alginate nanoparticles are being prepared by two methods. In the "complexation method", complex formation on the interface of an oil droplet is used to form alginate nanocapsules. Or complex formation in an aqueous solution is used to form alginate nano-aggregates. In a second method w/o emulsification coupled with gelation of the alginate emulsion droplet can be used to form alginate nanospheres. We review advantages and disadvantages of these methods, and give an overview of the properties of the alginate particles produced with these methods.

In chapter 3 we present a novel simple technique for the formation of food-grade salt nanoparticles from ethanol-in-oil emulsions. Salts $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ are dissolved in ethanol that subsequently is mixed with a medium chain triglyceride (MCT) oil

phase. It is found that type and concentration of salt have a significant influence on miscibility of the ethanol and oil phase and on the stability of E/O mixtures. The ethanol phase is evaporated from the mixture at elevated temperatures, and salt particles with dimensions in the nano range (6 – 400 nm) remain suspended in the oil phase. It is found that the concentration of salt and volume fraction of ethanol in MCT oil have a significant influence on the size distribution of salt particles. The size of $CaCl_2$ and $MgCl_2$ nanoparticles is ascertained by scanning electron microscopy and dynamic light scattering.

Chapter 4 describes the formation and characterization of alginate nanospheres by external gelation using the food-grade salt nanoparticles described in *Chapter 3*. A 1% alginate solution and a MCT oil are used to prepare a water-in-oil (w/o) emulsion, stabilized by polyglycerol polyricinoleate. $CaCl_2$ nanoparticles with dimensions in the nano-range (6 – 400 nm), dispersed in MCT oil, are then added to the emulsion. Energy-dispersive X-ray spectroscopy (EDX) and Auger electron spectroscopy (AES) show that these nanoparticles migrate to the emulsion droplet interface, where they dissolve into the aqueous alginate phase and cause gelation, forming gelled spheres. Gelation of the spheres is confirmed with a novel technique using Congo red as an indicator. A color change occurs upon the addition of $CaCl_2$ to a Congo red solution and we believe this is due to formation of a Congo red-calcium complex. Scanning electron microscopy shows that alginate spheres are mostly in a size range around 1 µm, but nanospheres as small as 200 nm and smaller are also found. Extending the size range of alginate spheres into the nano range, while maintaining relatively mild pH conditions in the interior of the sphere, will significantly extend the range of applications for this type of spheres.

Chapter 5 deals with the formation of alginate nanospheres by internal gelation utilizing $CaCO_3$ nanoparticles. $CaCO_3$ nanoparticles, together with glucono delta-lactone (GDL), are dispersed in an alginate solution, which is subsequently emulsified in an oil phase. Gelation of the alginate sphere is induced by calcium cations. $CaCO_3$ is used as calcium source where GDL, hydrolyzed into gluconic acid, allows its dissolution. A $CaCO_3$/alginate mass ratio of 0.1/1 is found optimal with respect to gel properties and a GDL/$CaCO_3$ molar ratio of 1.98/1 results in a final pH around 6. It is found that nanoparticles of $CaCO_3$ result in smaller alginate spheres and reduce the gelation time significantly, compared to microparticles of $CaCO_3$. The pH evolution as a function of time and rheology are determined during gelation of macroscopic alginate gels. Cryo-SEM and

light microscopy confirm formation of gelled alginate spheres below 10 µm with majority of spheres smaller than 2 µm and even spheres below 200 nm are observed. Application of nanoparticles of $CaCO_3$ for gelation of alginate spheres extends the size range of alginate spheres into the nano range and it significantly reduces the gelation time. In alginate spheres prepared by internal gelation calcium is more evenly distributed compared to alginate spheres gelled by external gelation, resulting in spheres with more uniform properties throughout the entire sphere, and therefore different potential applications. The extension into the nano range, the reduction in gelation time, and the possibility of creating a final internal pH around 6 all significantly extend the range of applications for these alginate nanospheres.

Finally, in *Chapter 6* a general discussion of the reported research is given. An overview on future developments and challenges is discussed.

Samenvatting

Alginaat is een natuurlijk voorkomende lineair onvertakte polysacharide welke wordt verkregen uit bruin zeewier. De eigenschappen van het alginaat zijn afhankelijk van de oorsprong van het zeewier en van eventuele chemische modificaties. Alginaat kan eenvoudig worden gegeleerd met multivalente cationen onder milde omstandigheden en is erg geschikt voor het maken van gegeleerde bolletjes. Hierdoor is het zeer geschikt voor het insluiten van gevoelige materialen of andere componenten. Alginaat is een van de meest gebruikte polymeren voor de vorming van (micro)deeltjes, maar de vorming van alginaat nanodeeltjes is minder gebruikelijk. Alginaatdeeltjes kunnen worden gebruikt voor het encapsuleren van componenten door ze in te sluiten in de binnenkant van de alginaatdeeltjes. Zulke deeltje, ofwel encapsulaten, geven extra bescherming tegen de omgeving en verbeteren de mechanische stabiliteit van de ingesloten componenten. In voedingsmiddelen en in farmaceutische producten hebben nanodeeltjes verschillende voordelen ten opzichte van grotere deeltjes. Elke toepassing stelt specifieke eisen aan de deeltjes voor een toepassing en hiervoor zijn eenvoudige en flexibele productiemethoden nodig. Daarom is het belangrijk om nieuwe en efficiënte (voedselveilige) technieken te ontwikkelen voor de vorming van micro- en voornamelijk nano-deeltjes.

In dit proefschrift beschrijven wij verschillende methoden om alginaat nanodeeltjes te bereiden. Onze doelstelling is om deeltjes te ontwikkelen met een diameter kleiner dan 25 μm en deze dienen gebruikt te kunnen worden als encapsulaat in voedingsmiddelen. De pH condities in deze deeltjes moet voldoende neutraal zijn zodat ze ook kunnen worden gebruikt voor het encapsuleren van pH gevoelige componenten.

Hoofdstuk 2 presenteert een recensie en behandelt verscheidene technieken voor het bereiden van alginaat nano deeltjes en hun eigenschappen. Primair worden alginaat nanodeeltjes bereid volgens twee methoden. In de "complexatie methoden" wordt complex vorming op het interface van olie druppeltje gebruikt om alginaat nano capsules te bereiden. Daarnaast wordt complex vorming in een waterige oplossing gebruikt voor de vorming van alginaat nano-aggregaten. In een tweede methode wordt w/o emulsificatie gekoppeld met gelering van de alginaat emulsie druppel en gebruikt voor de vorming van alginaat nano bolletjes. We beoordelen voor en nadelen van deze methodes en geven een

overzicht van de eigenschappen van de met deze methodes geproduceerde alginaat deeltjes.

In hoofdstuk 3 presenteren we een nieuwe methode voor de vorming van voedselveilige zout nano deeltjes uit ethanol-in-olie emulsies. De zouten $CaCl_2.2H_2O$ en $MgCl_2.6H_2O$ worden opgelost in ethanol, dat vervolgens word gemixt met middellange ketens van een triglyceriden (MCT) olie fase. Er wordt vastgesteld dat type en concentratie van het zout significante invloed heeft op de mengbaarheid van ethanol met de olie fase en de stabiliteit van E/O mengsels. De ethanol fase wordt uit het mengsel verdampt bij hogere temperaturen, en zoutdeeltjes met afmetingen in het nanogebied (6 - 400 nm) blijven gesuspendeerd achter in de olie fase. Er wordt vastgesteld dat zoutconcentratie en volume fractie van ethanol in MCT olie een significante invloed hebben op de grote verdeling van de zout deeltjes. De grote van $CaCl_2$ en $MgCl_2$ nanodeeltjes is vastgesteld met elektronenmicroscopie en dynamische licht verstrooiing.

Hoofdstuk 4 beschrijft de vorming en karakterisering van alginaat nanobolletjes door externe gelering met de voedsel veilige zout nanodeeltjes zoals beschreven in hoofdstuk 3. Een 1% alginaat oplossing en een medium chain triglyceride (MCT) olie zijn gebruikt om een water-in-olie (W/O) emulsie te maken gestabiliseerd door polyglycerol polyricinoleate. $CaCl_2$ nanodeeltjes met dimensies in het nano gebied (6 - 400 nm), gedispergeerd in MCT olie, worden vervolgens toegevoegd aan de emulsie. Energie-dispersieve X-ray spectroscopy (EDX) en Auger elektronen spectroscopie (AES) demonstreren dat deze nanodeeltjes migreren naar het interface van de emulsie druppel, waar ze oplossen in de waterige alginaat fase en gelering veroorzaken, waardoor geleerde bolletjes ontstaan. Gelering van de bolletjes is vastgesteld met een nieuwe techniek waarbij Congo rood als indicator is gebruikt. Een kleur omslag ontstaat na toevoeging van $CaCl_2$ aan de Congo rood oplossing en wij vermoeden dat dit door de vorming van een Congo rood-calcium complex komt. Elektronenmicroscopie demonstreert dat alginaat bolletjes voornamelijk een afmeting hebben rond 1 μm, maar nanodeeltjes van 200 nm en kleiner zijn ook vastgesteld. Het uitbreiden van de grootte verdeling van alginaat bolletjes in het nanogebied, terwijl een relatief neutrale pH in de bolletjes gehandhaafd blijft, zal significant bijdragen aan het aanbod van toepassingen voor dit type bolletjes.

Hoofdstuk 5 gaat over de vorming van alginaat nanobolletjes door interne gelering gebruik makend van $CaCO_3$ nanodeeltjes. $CaCO_3$ nanodeeltjes, samen met glucono delta-

lacton (GDL), zijn gedispergeerd in een alginaat oplossing, welke vervolgens is geëmulsifeerd in een olie fase. Gelering van de alginaat bolletjes wordt geïnitieerd door calcium cationen. $CaCO_3$ is gebruikt als calcium bron, terwijl GDL, gehydrolizeerd tot gluconzuur, zorgt voor het oplossen van $CaCO_3$. Een $CaCO_3$/alginaat massaratio van 0.1/1 blijkt optimaal met betrekking tot gel eigenschappen en een GDL/$CaCO_3$ molverhouding van 1.98/1 leidend tot een eind-pH rond de 6. Er is vastgesteld dat nanodeeltjes van $CaCO_3$ resulteren in kleinere alginaatbolletjes en een significante vermindering in geleringstijd, in vergelijking met $CaCO_3$ microdeeltjes. De pH evaluatie als functie van de tijd en reologie zijn vastgesteld tijdens gelering van macroscopische alginaat gelen. Cryo-SEM en licht microscopie bevestigt de vorming van geleerde alginaat bolletjes kleiner dan 10 µm waarvan het merendeel van de bolletjes kleiner is dan 2 µm, en zelfs bolletjes kleiner dan 200 nm zijn gevonden. Toepassing van $CaCO_3$ nanodeeltjes voor gelering van alginaat bolletjes breidt de grootte uit in het nanogebied en reduceert significant de geleringstijd. In alginaat bolletjes vervaardigt via interne gelering is calcium gelijker verdeeld in vergelijking tot alginaat bolletjes geleerd via externe gelering, wat resulteert in bolletjes met homogenere eigenschappen in het hele bolletje waardoor er mogelijk ook andere toepassingen zijn. De uitbreiding van alginaat bolletjes naar het nanogebied, zal significant toedragen aan het aanbod van toepassingen voor dit type bolletjes.

Tenslotte, in hoofdstuk 6 wordt een algemene discussie gegeven van het verrichte onderzoek. Een overzicht van toekomstige probleemstellingen en uitdagingen is opgenomen.

Résumé

L'alginate est un polysaccharide naturel linéaire, non lié, extrait d'algues brunes. Ses propriétés sont déterminées selon la source et les modifications chimiques qu'on lui applique. Dans des conditions douces, l'alginate peut être facilement gélifié avec des cations polyvalents, cela permet alors la formation de bille gélifiée. Celles-ci seront idéales pour l'encapsulation de matériaux sensibles ou autres composants. L'alginate est l'un des polymères le plus utilisé pour la formation de (micro)particule. Cependant la fabrication de nanoparticule d'alginate est moins commune. Les particules d'alginate peuvent être utilisées pour l'encapsulation de certains éléments. De telles particules, en outre sous forme bille, permettent de protéger les composants vis-à-vis de l'environnement, et confère une meilleur stabilité mécanique à l'émulsion. Dans l'industrie agro-alimentaire et des cosmétiques, les nanoparticules contenant des composants ont de nombreux avantages par rapport à des particules millimétriques (=plus grosses). Chaque type de particule a des exigences spécifiques selon son utilisation, des méthodes de productions simples et adaptables sont donc nécessaires. Pour cette raison, il est important de développer des techniques (adaptées à l'alimentaire) nouvelles et efficaces pour la production de micro et plus particulièrement nano-particules.

Dans cette thèse, nous décrivons différentes méthodes de préparation de nanoparticules d'alginate. Notre objectif est d'obtenir des particules d'un diamètre inférieur à 25 µm qui peuvent être utilisés comme capsule dans des produits à usage alimentaire. Les conditions de pH à l'intérieur de ces particules doivent être suffisamment proches du neutre afin que les composants sensibles au pH puissent être encapsulés.

Le chapitre 2 présente une étude bibliographique et détaille les différentes techniques de formation de nanoparticules à base d'alginate et leurs propriétés. Tout d'abord, les nanoparticules d'alginate sont préparées selon deux méthodes. Dans une première méthode appelée « méthode de formation de complexes », la formation d'un complexe à la surface d'une gouttelette d'huile est utilisé pour obtenir des nano-capsules d'alginates. La formation de complexes dans une solution aqueuse est quant à elle utilisée pour obtenir des nano-agrégats d'alginate. Dans une seconde méthode une émulsion eau-dans-

huile (E/H) couplée à la gélification des gouttelettes d'alginate est utilisée pour former des nanosphères à base d'alginate.

Dans le chapitre 3, nous présentons une technique nouvelle et simple pour la formation de nanoparticules de sel alimentaire à partir d'émulsions éthanol-dans-huile. Les sels de $CaCl_2.2H_2O$ et de $MgCl_2.6H_2O$ sont tout d'abord dissouts dans de l'éthanol puis ensuite mélangés à une huile de triglycéride à chaîne moyenne (TCM). Nous observons alors que le type de sels et leurs concentrations ont une influence significative sur la miscibilité de la phase éthanol et de la phase huile, mais aussi sur la stabilité des mélanges E/H. La phase éthanol s'évapore à des températures élevées et les particules de sel ayant des dimensions nanométriques (6-400 nm) restent en suspension dans l'huile. Nous constatons alors que la concentration de sel et le volume de la fraction d'éthanol dans l'huile à TCM ont une influence significative sur la distribution de la taille des particules de sel. La taille des nanoparticules de $CaCl_2$ et $MgCl_2$ est vérifiée par microscopie électronique à balayage et diffusion dynamique de la lumière.

Le chapitre 4 décrit la formation et la caractérisation des nanosphères d'alginate par gélification externe utilisant les nanoparticules de sel (alimentaire) décrit au chapitre 3. Une solution d'alginate à 1% et une huile à triglycéride à chaîne moyenne (TCM) sont utilisées pour préparer une émulsion eau-dans-huile (E/H) stabilisée par du polyricinoléate de polyglycérol. Des nanoparticules de $CaCl_2$, de diamètre entre 6-400 nm, dispersées dans l'huile à TCM, sont ensuite ajoutées à l'émulsion. Une spectroscopie X à dispersion d'énergie (EDX) et une spectroscopie d'électrons Auger (AES), montrent que ces nanoparticules migrent vers l'interface des gouttelettes de l'émulsion, où elles se dissolvent ensuite dans la phase aqueuse d'alginate et provoquent la gélification, formant ainsi des sphères gélifiées. La gélification des sphères est confirmée grâce à une nouvelle technique utilisant comme indicateur le rouge Congo. Lors de l'ajout de $CaCl_2$ à une solution de rouge Congo, un changement de couleur se produit. Nous pensons que cela est dû à la formation d'un complexe rouge Congo - calcium. L'examen à microscopie électronique à balayage (MEB) montre que les sphères d'alginate ont pour la plupart une taille d'environ 1 pm, cependant des nanosphères de la taille de 200 nm et encore plus petites sont également observées. Obtenir des sphères d'alginate d'une taille nanométrique tout en maintenant des conditions de pH relativement douces à l'intérieur

de la sphère permet d'augmenter considérablement la gamme d'usage de ce type de sphères.

Le chapitre 5 traite de la formation des nanosphères d'alginate par gélification interne utilisant des nanoparticules de CaCO3. Les nanoparticules de CaCO3 sont dispersées avec de la glucono-delta-lactone (GDL) dans une solution d'alginate, puis le tout est émulsifié dans une phase huile. La gélification de la sphère d'alginate est induite par des cations calcium. Le CaCO3 est utilisé en tant que source de calcium dans laquelle la GDL, hydrolysée en acide gluconique, permet sa dissolution. Un rapport de masse CaCO3/alginate de 0,1 / 1 a été déterminé comme optimal par rapport aux propriétés de gel, de plus un rapport molaire GDL/ CaCO3 de 1,98 / 1 résulte en un pH final d'environ 6. Il est constaté que par rapport à des microparticules de CaCO3, les nanoparticules de CaCO3 permettent d'obtenir des sphères d'alginates plus petites et elles diminuent le temps de gélification de manière significative. La rhéologie et l'évolution du pH en fonction du temps sont déterminées au cours de la gélification de gels macroscopiques d'alginate. Une Cryo -SEM et un examen de microscopie optique confirment la formation de sphères d'alginate gélifiées inférieures à 10 µm, la majorité des sphères étaient de taille inférieure à 2 µm et des sphères plus petites que 200 nm sont même observées. L'utilisation de nanoparticules de CaCO3 pour la gélification des sphères/billes d'alginate permet d'obtenir des sphères d'alginate de l'ordre du nanomètre, et elle permet de réduire considérablement le temps de gélification. Dans les sphères d'alginate préparées par gélification interne, le calcium est mieux réparti que dans des sphères d'alginate gélifié par gélification externe. Les sphères ont alors leurs propriétés réparties dans toute la sphère d'une manière plus uniforme ce qui offre divers usages potentiels. L'extension à l'échelle nanométrique, la réduction du temps de gélification, et la possibilité de créer un pH final interne autour de 6 augmentent de manière significative la gamme d'utilisation de ces nanosphères d'alginate.

Enfin, au chapitre 6 une discussion générale de la recherche présentée est donnée. Un aperçu sur les développements et les défis de l'avenir sont discutés.

Acknowledgement

As child I was already fascinated by scientists as Newton and Einstein, and dreamt of being an inventor one day myself. After graduating from my master study I asked myself "a PhD or not?"; I enjoyed my master study at Wageningen University, was interested in more scientific research and the subject of the PhD position offered matched perfectly with ideas I have had during my master study. The answer to my question may be clear by now. Doing a PhD is not always that easy and you definitely do not get a PhD degree for free, however I can look back with pleasure and have learned a lot, both in my profession and about myself. Here I would like to take the opportunity to personally thank some people that contributed to the success of my PhD. The list is by far not complete, for which I express my apologies. The success of finalizing my PhD is due to the support and contribution of many people for whom I am truly thankful!

First of all I would like to express my gratitude to my co-supervisor: Leonard, I could not wish for a better supervisor and really appreciate your dedication. You were always there for me, had patience, and gave encouragement and great ideas. Every time I entered your office and was puzzled with a problem you found something positive in my results, managed to motivate me again and I could leave again with a relaxed feeling sometimes even wondering what was all the fuzz about. I am also very happy for sharing our non-scientific interests, like whiskies.

I am very grateful to my supervisors, Erik and Cees. Erik, I am very thankful for the fruitful discussions and your enthusiasm. You were always positive, had creative ideas and I very much enjoyed the discussions with you, both about my project and other topics. You gave me new insights, energy and motivation for my project. I am also grateful for hosting me in your department after my contract ended. Cees, I still remember our first meeting in Zutphen and I am very grateful for offering me this chance. The first 1.5 years you helped me to discover the wonders in membrane emulsification, after which we continued more and more to the formation of alginate nanospheres. You gave me lot of freedom to work and we had interesting discussions. Thanks for your scientific insights.

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Working in two groups, Organic Chemistry and Food Physics, resulted in many nice colleagues and I often wished I could split myself in two and enjoy the best from both of them. During the first two years I was mainly situated at Organic Chemistry. Willem, Nagesh, Ai and Tin as we were all PhD students from Cees we shared 2 offices at the upper floor at ORC and of course the microfluidics lab. I should definitely include Rokus here as well. I would like to thank you all for the social interactions and interesting discussions we had. Willem, thanks for being a colleague and a friend. I always enjoyed the discussions, dinners, movies and hikes together. Tin, thanks for being my roommate; I also enjoyed playing badminton with you at the sport center. Nagesh thanks for the collaboration in the project and it was great going together to the summer school in Switzerland. Alexandre and Dani, I really appreciated your warm company and the hikes in nature. I enjoyed the WE-days, volleyball and labuitjes with the ORC group. The PhD trip to China was a very instructive experience and made the PhD's a closer group. Also the extended stay in Beijing with Willem, Nagesh, Anke, Yessie and Jacinthe was great! I very much enjoyed and learned many things during the discussions as PhD representative in the Daily Board meetings at ORC. Ronald thanks for the nice talks and of course the chemicals and equipment. Elly and Aleida are thanked for their help with all the paperwork. And I also like to thank all other PhD's, staff members and technicians of ORC for their help en great moments!

After two years my project became more and more related to the Food Physics group. A complete different group, with different people and I was very happy to be a part of them. There I shared room 211a most of my time with Elisabete, Silvia and Hassan. You were great room-mates, we had nice discussions and I will always remember the nice

atmosphere and laughs. Hassan, I learned a lot from you and your culture and one day I would love to visit you in Palestine. Later also Pauline, Jacob and Min joined 211a, which was also very enjoyable to share the room with. One floor up was the other PhD room with Ardy, Nam-Phoung, Dilek and Yul, who were always very welcoming and willing to help. Els, it was always very nice to walk into your office for a chat, advice and you were always ready to help with booking conferences, paperwork etc. Harry, you are a great person! Thanks for all the nice moments, your help in the lab and off course your magic touch with equipment. I would also like to thank Elke and Paul for the nice discussions we had and of course the informal moments. A few floors up were the TIFN PhD's and researchers that populated the 7th floor and who were a great addition to the Food Physics family. We had many nice activities, from drinks in de Vlaam, to labuitjes, lunches in Arboretum and 6-kamp at WE-day! The PhD-trip to Japan was an amazing experience and for me it was also very interesting to see the differences with China were I had been the year before with ORC. I really enjoyed the program with the group and off course joining the dinners/drinks in the evening with Erik, Paul, Leonard and Elke. And not to forget our extended stay with Yul, Michel, Elisabete and Miguel was awesome. Thanks to all the persons in the Food Physics group for their help and great time. I also like to thank the researchers, technicians and PhD-students of the FQD group and it was great to share things from coffee breaks to 'korfbal' competitions with you!

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Besides working as PhD-student I was weekly active as scout leader at Scouting Thorheim Doorn. The leader teams I was part of were really great and I always enjoyed all the scout activities with the kids that boosted me with new energy. Also the other activities with the group are really nice and I'm still happy being part of the group with many great persons. I'm very grateful to one of my best friends Florens de Haan for being able to share this hobby and many other things together. I was also very happy to join you and your group at summer camp in Switzerland.

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First I lived at "Huize Hemazicht" in de Hoogstraat with great house mates who made me feel at home. Later I moved to the Asterstraat with Joost and Monique with whom I very much enjoyed living with. Joost, you are a good friend to me and I'm grateful you are willing to be one of my paranymphs. Currently I live in Epe where I wrote an important part of my thesis while being accompanied with the cat Lala sleeping on my lap. While working at home I was very happy to share the coffee breaks and informal moments at the Bolster B.V.

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Thanks to all and please keep in touch,

Jerome

About the author

Jerome Philippe Paques was born in De Bilt, The Netherlands, on November 16th, 1981. After graduating from secondary school, Revius Lyceum Doorn, he started his Bachelor (HBO) study Food Technology at the Van Hall instituut in Leeuwarden in 2001. In 2003 he went for his first internship to the Institute of Food, Nutrition and Human Health at Massey University in New Zealand, where he worked on "The structure, rheology and sensory properties of dairy foods". For his second internship he went to Numico Research in Wageningen in 2004 and worked on the "Chemical, physical and rheological properties of carbohydrates". In 2005 he completed his BSc study as Food Technologist with a thesis carried out at Dr. Oetker Leeuwarden and focused on the development of a new working method to improve the efficiency of the production capacity. After his BSc study he continued in the same year with a MSc study at Wageningen University on Food science and technology and a specialization on product functionality. For his MSc thesis he submitted one of his own ideas as project proposal, which was then carried out at the Physics and physical chemistry of foods group of Wageningen University. In this project he studied the formation of microbubbles with ultrasound and application of these microbubbles in food products in order to replace fat droplets. After his MSc graduation in 2007 he started in the same year his PhD-project at the Laboratory of Organic Chemistry, and the Physics and physical chemistry of foods group of Wageningen University, The Netherlands. The project, under supervision of Dr. Leonard Sagis, Prof Erik van der Linden and Prof Cees van Rijn was part of larger project and aimed for encapsulation of probiotics. The results of his research are presented in this thesis.

Aside from his professional education, Jerome is qualified as rock climbing instructor and gives bushcraft workshops. He also enjoys scouting, mountaineering, skiing, hiking, cooking and webdesign.

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List of Publications

PEER REVIEWED JOURNALS

Paques, J. P.; Van Der Linden, E.; Sagis, L. M. C.; Van Rijn, C. J. M., Food-grade submicrometer particles from salts prepared using ethanol-in-oil mixtures. *J. Agric. Food. Chem.* **2012,** *60*, 8501-8509.

Paques, J. P.; van der Linden, E.; van Rijn, C. J. M.; Sagis, L. M. C., Alginate submicron beads prepared through w/o emulsification and gelation with $CaCl_2$ nanoparticles. *Food Hydrocolloids* **2013,** *31*, 428-434.

Paques, J. P.; Sagis, L. M. C.; van Rijn, C. J. M.; van der Linden, E., Nanospheres of alginate prepared through w/o emulsification and internal gelation with nanoparticles of $CaCO_3$. *Food Hydrocolloids* **2014**, 40, 182-188.

Paques, J. P.; van der Linden, E.; van Rijn, C. J. M.; Sagis, L. M. C., Preparation methods of alginate nanoparticles. *Adv. Colloid Interface Sci.* **2014**, 209, 163-171.

PROCEEDINGS

6th International Symposium on Food Rheology and Structure (ISFRS), **2012**, Zurich, Switzerland

PATENTS

Paques, J. P.; Koning, A.; Peters, J.; Luyten, H. Hard and Semi-Hard Cheese Comprising Water Retaining Particles, a Method for Preparing Such Hard or Semi-Hard Cheese, and Use of Such Particles in the Preparation of Cheese. **2013**. N2011654

Overview of completed training activities

DISCIPLINE SPECIFIC ACTIVITIES

Courses

Food Hydrocolloids (VLAG course, 2009)
Advanced Organic Chemistry (VLAG course, 2009 – 2010)
Summerschool Bioencapsulation (COST865 course, 2009, Anzere, Switzerland)
Surfactants and polymers in aqueous solution (ECIS training course, 2011, Potsdam, Germany)

Conferences

Annual NWO conference, Organic Chemistry (2007 and 2008, Lunteren, The Netherlands)
MicroNano Conference (2007 - 2010, The Netherlands)
25th European Colloid and Interface Society (ECIS) Conference (2011, Berlin, Germany
IX International Conference on Bioencapsulation (2011, Amboise, France)
6th International Symposium on Food Rheology and Structure, ISFRS (2012, Zurich, Switzerland)

GENERAL COURSES

19th VLAG PhD week (VLAG course, 2008)
Techniques for writing and presenting a scientific paper (VLAG course, 2010)
Career perspectives course (Advice agency Meijer en Meijaard - WGS course, 2010)
Mini-symposium "How to write a world class paper" (Wageningen UR Library, 2011)

OPTIONALS

Preparing PhD research proposal
Group meetings Laboratory of Organic Chemistry (2007 – 2009)
Group meetings Physics and Physical Chemistry of Food (2009 – 2011)
PhD study trip, Laboratory of Organic Chemistry (2009, China)
PhD study trip, Laboratory of Physics and Physical Chemistry of Food (2010, Japan)