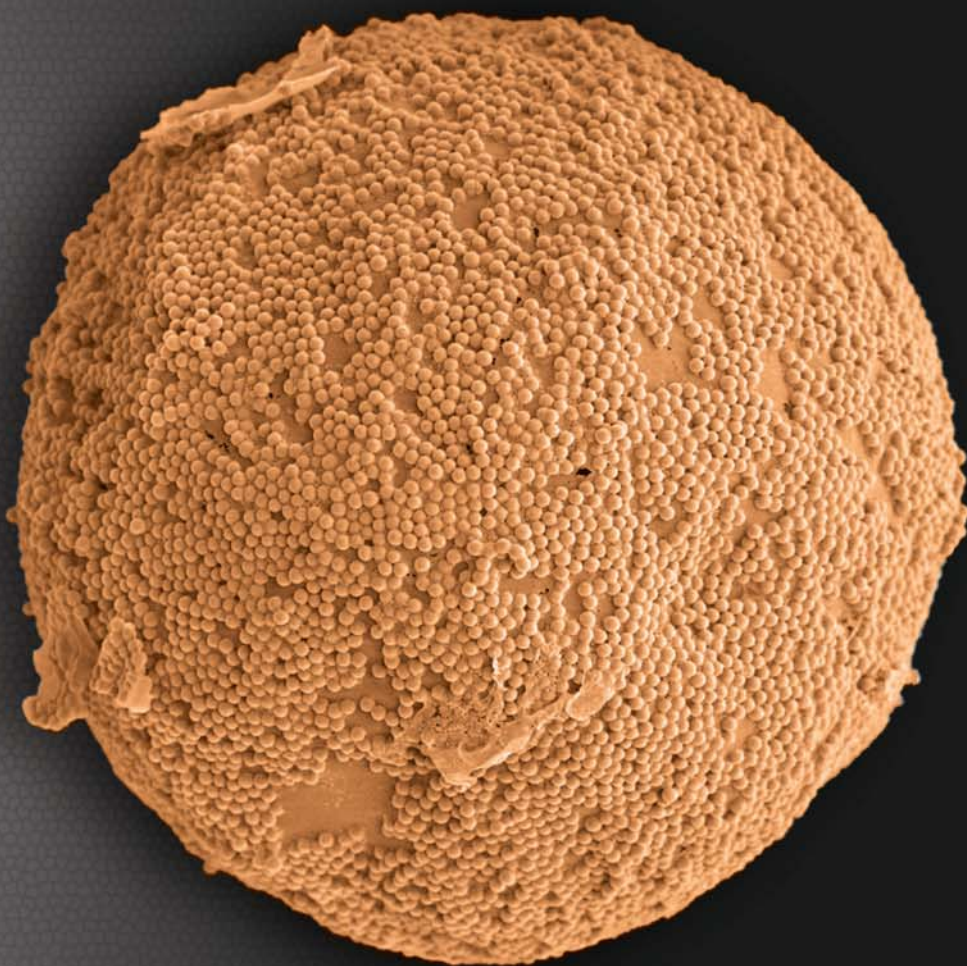


Colloidal-scale self-assembly of microcapsules for food



Francisco J. Rossier-Miranda

Propositions

1. Nested self-assembly, such as using self-assembled protein fibrils in the self-assembly of a microcapsule shell, represents a fundamentally new route into food structuring.

This thesis

2. The inclusion of protein fibrils in a pectin matrix renders a reinforcement comparable to chemical crosslinking in microcapsules assembled with a layer-by-layer strategy.

This thesis

3. The universe where we live is, at all scales, a place too intriguing to be left unexplored.
4. To solve a problem by combining two different strategies strengthens their strengths and weakens their weaknesses.
5. If everyone would accept self-assembly as one of the most fundamental mechanisms in nature, we would have totally different discussions about what life is and where it began.
6. Some travel while looking backwards; others travel while looking forward. They are bound to arrive to completely different destinations.
7. Since humans evolved language, music and research at about the same time, it would be a sad achievement of science to keep them apart in the laboratory.

Propositions belonging to the thesis entitled
“Colloidal-scale self assembly of food-grade microcapsules”

Francisco José Rossier-Miranda
Wageningen, 5 November 2010

Colloidal-scale self-assembly of microcapsules for food

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Colloidal-scale self-assembly of microcapsules for food

Thesis

Submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University

by the authority of the Rector Magnificus

Prof. dr. M.J. Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Friday 5 November 2010

at 1.30 p.m. in the Aula.

Francisco José Rossier-Miranda

Colloidal-scale self-assembly of microcapsules for food.

152 pages.

Thesis Wageningen University, Wageningen, NL (2010)

With references, with summaries in English, Dutch and Spanish

ISBN: 978-90-8585-785-3

to José Domingo

Contents

Chapter I	1
<i>Introduction</i>	
Chapter II	13
<i>Colloidosomes: Versatile Microcapsules in Perspective</i>	
Chapter III	35
<i>Polymer Microcapsules with a Fiber-Reinforced Nanocomposite Shell</i>	
Chapter IV	49
<i>Mechanical Characterization and Release Properties of Fiber Reinforced Microcapsules</i>	
Chapter V	75
<i>Encapsulation by a Hybrid Colloidosome-Layer-by-Layer Technique</i>	
Chapter VI	93
<i>Integrated Microfluidic Circuit for Electrostatic Layer-by-Layer Microcapsule Assembly</i>	
Chapter VII	107
<i>General Discussion and Outlook</i>	
Summary	123
Samenvatting	127
Resumen	133
Acknowledgement	141
About the author	147
Publications	149
Training Activities	151

Chapter I

Introduction

Introduction

This thesis aims at new insights and techniques for microencapsulation that can be used in food applications. In the first section of the introduction, microencapsulation and its purpose are defined in general terms, together with some illustrative examples. Next, various classic production techniques are shortly presented, together with the requirements that need to be met for their application in food products. In the last part of this chapter alternative methods are touched and placed into the perspective of the chapters of this thesis.

Microencapsulation

Microencapsulation is the term used for a collection of techniques that enable packing of active substances (solid, liquid, or gas), in closed, microscopic ‘containers’ that release their contents in response to specific triggers, at controlled rates. A microcapsule (Figure 1) is, ideally, a small monodisperse container composed of a core, which contains the active material, and a thin, semi permeable shell of well-defined thickness, which renders protection from the environment. This definition is so general because it has to accommodate a wide range of techniques, applications, microcapsule geometries and configurations, actives to be encapsulated, materials used as cores or shells, trigger and release mechanisms, and combinations thereof.

Depending on the application envisioned, the microcapsule normally has a size between a few and hundreds of micrometers, although more and more publications report on capsules in the submicron range. Since controlled delivery of active materials is the main goal of microencapsulation, size and monodispersity of the core are important, as is the integrity of the shell, which gives the microcapsule retention of the active component, and after a trigger, permeability. The self-assembly of structures with well-defined geometry, dimensions, and complexity is a lively field of research given the phenomena that arise when dealing with particles of colloidal dimensions.

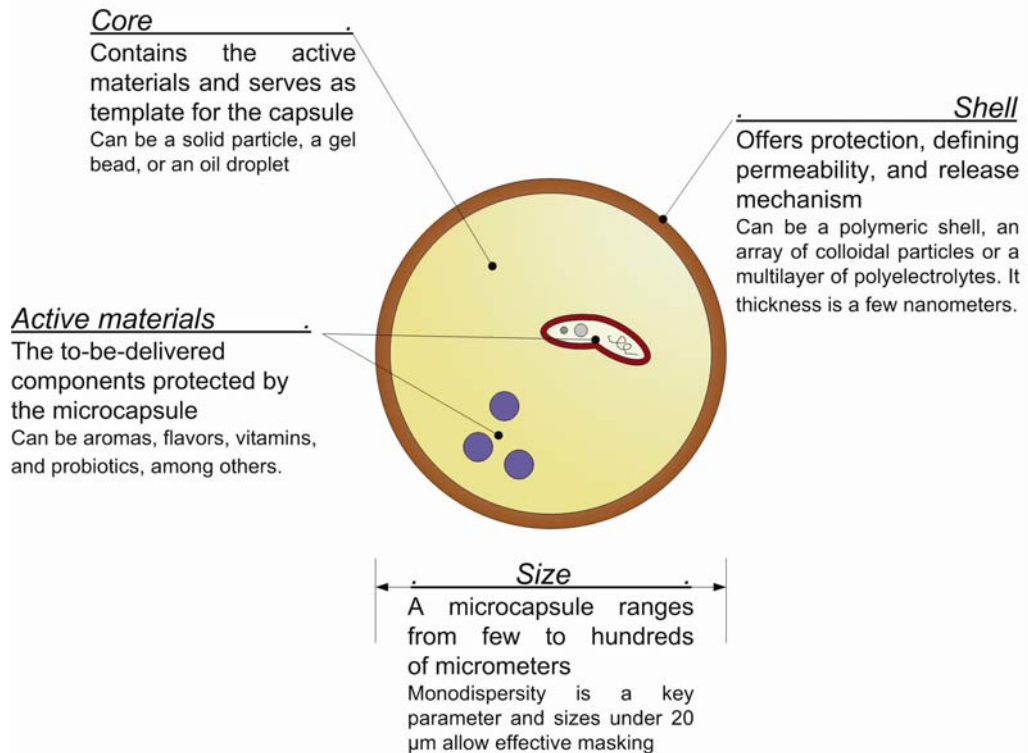


Figure 1: Schematic of a microcapsule

Microencapsulation is applied in a wide range of industries, besides the longer established applications in pharmaceutical and food products. It is for example of great importance in printing and electronics, with the encapsulation of pigments used in an inkjet printer¹ or in an electronic display². Another example is in the textile industry, where microcapsules in detergents slowly release the perfume after adsorption on the fabric³, which can also be kept clear of bacteria for longer time through encapsulation of antibacterials⁴, or can change color according to the body temperature by including microencapsulated thermosensitive inks⁵. In furniture polishing, creams with microencapsulated aromas will release them once the microcapsules break under shear.

Encapsulation methods

There are many techniques reported for microcapsule production. The most important ones from an industrial point of view (but by no means the only ones) are spray drying/congealing, fluidized bed coating, and extrusion^{6, 7}.

The techniques based on spray drying are cost effective, easily scalable, and make use of mature unit operations (e.g. spray dryers) and are nowadays routinely applied for encapsulation. For these techniques the active material that is to be encapsulated is dispersed in a polymer solution and subsequently sprayed through a nozzle. The droplets are dried (spray drying) or cooled (congealing), by which the matrix becomes glassy or otherwise immobile, by which the active is trapped into the polymeric matrix. This technology is widely applied to encapsulate organic and inorganic salts, enzymes, and flavors, among others. Disadvantages of these techniques are that part of a volatile encapsulated material can be lost through evaporation during the drying process, while congealing is only applicable with specific matrices that attain sufficient immobilization with cooling (e.g. oils and waxes). For both methods the maximum loading is often limited, as a large amount of matrix is necessary to envelop the active component.

Fluidized bed encapsulation involves spraying a liquid coating on fluidized particles that already contain the active material. Once the coating is applied, rapid evaporation takes place which transforms the liquid that was sprayed into a shell around the particles. It is a very flexible technique, since a large number of materials can be used as coating material. It can also be used in combination with freeze-drying. In this combined technique the extra coating layer around the polymeric matrix adds an extra level of protection to the particles. In general fluidized bed encapsulation is much more efficient in terms of loading, but may suffer from a significant loss of volatile components during the fluidization. The minimum particle size is relatively large, since the core has to be fluidized first - too small core particles would be blown away. The core material has to be solid, to enable fluidization without coalescence of the core particles.

In extrusion, a hot and concentrated polymeric mass is prepared in the starting section of the extruder. In a later zone in the extruder the mass is cooled down and the to-be-

encapsulated material is injected. Once the melt leaves the extruder it is dried and solidified (near-instantaneously by evaporative expansion and cooling) and milled. The shelf life of oxygen sensitive oils can be extended in this way, essentially capturing them in the resulting glassy starch matrix. Extrusion encapsulation can typically be used for components that are heat stable. The technique may lose some of its active material during the expansion after extrusion; the solidification is however quite fast. Milling will deliver smaller particles, but one is still limited to particles above around 50 μm , as milling to even smaller particles would induce too much damage.

When considering microencapsulation for application in the food industry the goals, challenges, and demands overlap at least partly with the ones for the pharmaceutical industry. Microcapsules designed to deliver components with health benefits for a consumer have to act inside the body and, therefore, have to be non-toxic, biodegradable, and especially have controlled and predictable release rates when submitted to well-defined triggers. Besides, for food low cost is important, as well as the food grade status of the materials employed.

Specifically in the food industry, an added benefit of encapsulation is that an encapsulated liquid can be treated as a solid, which leads to easier handling and formulation. Classical applications of microencapsulation in the food industry are encapsulated essential oils, masking of undesirable tastes, and protection of volatile or sensitive aromas and flavors⁸. Microencapsulation also opens a door to the design of new food products. The popular candies Pop Rocks, for example, release the encapsulated CO_2 only when the matrix that holds it captured melts in the mouth, creating a new sensorial experience. Clearly, encapsulation can lead to a new range of products that give different taste or experience during eating or preparation.

The most important targets for microencapsulation in the food industry are to extend the shelf life, enhance the encapsulation efficiency (percentage of the active component that ends up in the encapsulate) and to protect and target the delivery of nutraceuticals and probiotics⁹. For this the microcapsule needs to be stable during production, storage, and digestion, until its arrival to the targeted zone, where it should become unstable and

release its contents. For example, during digestion probiotics need to be protected from the acidity of the stomach, and be safely delivered in the more neutral environment of the small intestine. Before that, they also need to be protected from processing conditions and oxygen damage during manufacture, transportation, and storage. This specific application has received special attention in the last years, with promising results¹⁰.

The size of the microcapsules is especially important in the food industry since it will not only determine the microcapsule's behavior, but it will also be an important factor in the sensory perception by the consumer. It is generally accepted that sizes below 20 μm are under the threshold for consumer detection and are, therefore, suitable for masking. However, the techniques mentioned previously are unable to provide well-defined size distributions or coating characteristics, and although they are successfully used in industry, it is clear that there is room for improvement.

New routes: Colloidosomes and layer-by-layer microcapsules

The previously mentioned techniques are based on mechanical means to form the microcapsules. This implies that generally intensive force fields are used. Nowadays, it has been recognized that when we use local, smaller but well-directed forces already existing in nature, we can achieve better-controlled structures and processes. For example, it has been shown for emulsification that powerful colloid mills produce small but widely distributed emulsion droplets, while spontaneous snap-off of oil as a result of much smaller Laplace pressure differences, as applied in a micro-engineered channel, produces also small but highly monodisperse droplets¹¹. The same is true for microencapsulation, and during the last ten years active research has been carried out to find low-energy routes for microencapsulation based on self-assembly or taking advantage of other physical-chemical phenomena. For example, polylactide microcapsules of well defined characteristics can be produced, starting from emulsion droplets of a mixture of the polymer and a solvent in water, produced by membrane emulsification¹².

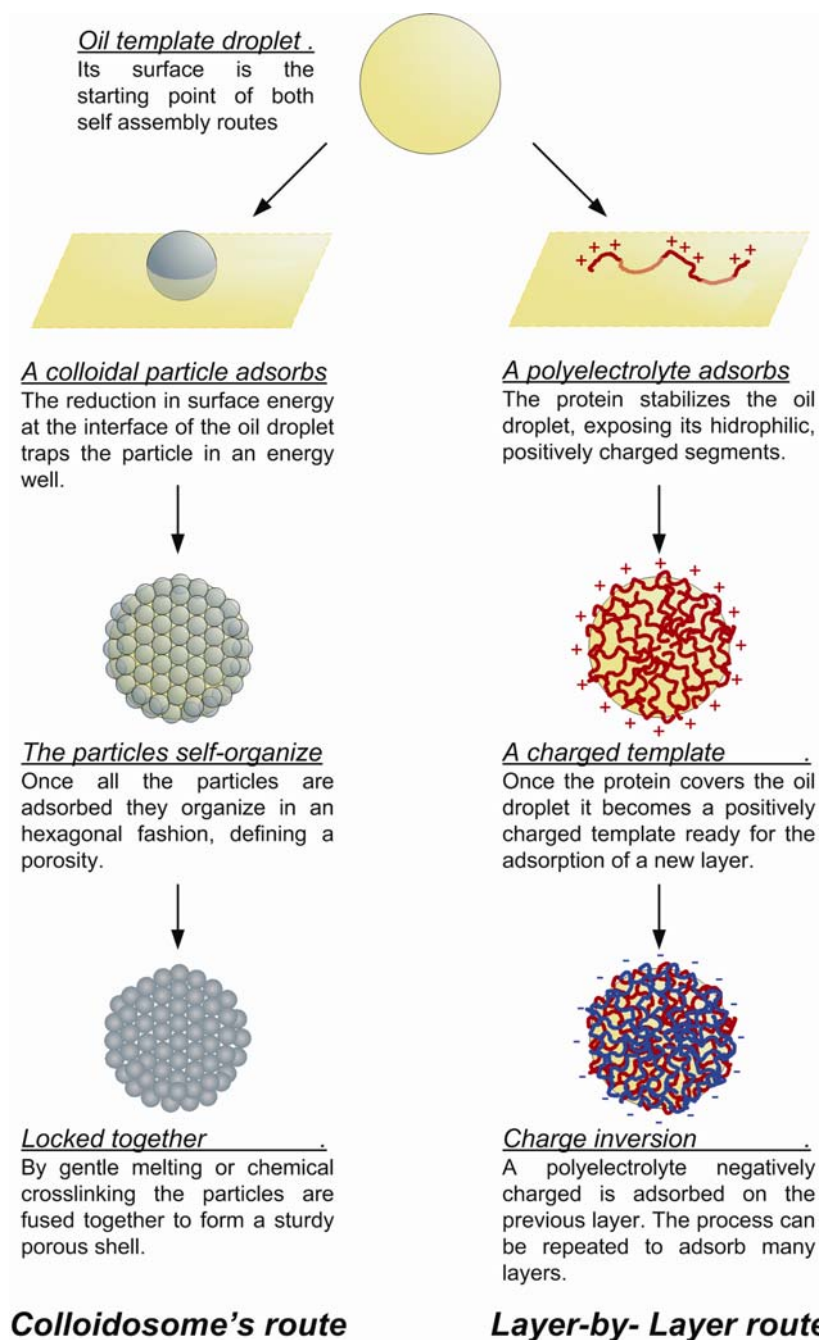


Figure 2: Two self-assembly routes for microencapsulation.

This thesis follows this route also for the preparation of the protective shell. It focuses on the combination of two approaches: the colloidosome route and the layer-by-layer route (Figure 2), and both make use of self-assembly at an interface, in this case an oil droplet, which acts as a template for the microcapsule and as a reservoir for the active material.

In the case of the colloidosomes (first introduced by Dinsmore¹³) colloidal particles are included during the emulsification of oil in water. The particles will be 'pushed' to the oil-water interface, and nest in it. This reduces the surface energy of the droplet and, for particles of colloidal size, this energy reduction is many orders of magnitude larger than the thermal energy that could remove the particle from the interface. The colloidal particles are, thus, irreversibly trapped at the oil droplet interface. If enough particles adsorb onto the oil interface, they will self-organize into an ordered array with defined pores in between the particles. The particles may then be locked together by a sintering or gluing step, forming a sturdy shell of a size defined by the oil droplet template, and with a porosity defined by the size of the colloidal particles used to construct the shell.

Layer-by-layer electrostatic assembly was originally used to construct films of nanometrically-controlled thickness on flat surfaces. Decher anticipated that this approach could be used for microcapsule construction¹⁴, although this remained for some time limited to the use of solid colloidal particles as templates for the layer-by-layer adsorption of inorganic materials¹⁵. Dickinson used multilayers of proteins and polysaccharides to increase the stability of emulsions¹⁶, giving a first step towards microencapsulation by layer-by-layer adsorption using food grade materials: an oil droplet stabilized by proteins in a solution of low pH provides a soft, positively charged template, on which another biopolymer that is negatively charged can be adsorbed. Positively and negatively charged polyelectrolytes can then be sequentially adsorbed to grow a shell around the oil droplet. Although the layer-by-layer method provides a tight control of the shell thickness, the fact that the layers are in the thickness range of molecular monolayers makes necessary to use a large number of adsorption cycles to obtain microcapsules with a mechanical strength high enough for practical applications, and obviously that leads to high processing cost.

Sometimes, new questions can be answered with old answers, and this work shows an example of it. Since the late Bronze Age, straw has been used to reinforce mud structures, resulting in a cheap, light, and strong material known as adobe. A similar method is used in modern times to reinforce polyester resin with glass fibers. Keeping the differences of scale and interactions involved, this is a good analogy of our approach to reinforce microencapsulation shells constructed by layer-by-layer assembly. Instead of using molecules of similar sizes, we included larger, stronger protein fibrils or silica particles (see figure 3a), to enhance the strength of the microcapsules at a reduced number of adsorption cycles. Besides the strength of the capsules and their release properties, the possibility to translate the technique to larger scale application is considered in this work. New production methods are therefore proposed, based on simple microfluidic devices that are purposely designed, and which can be used for production of capsules *on-chip* with as many as 10 layers (Figure 3b) in one step.

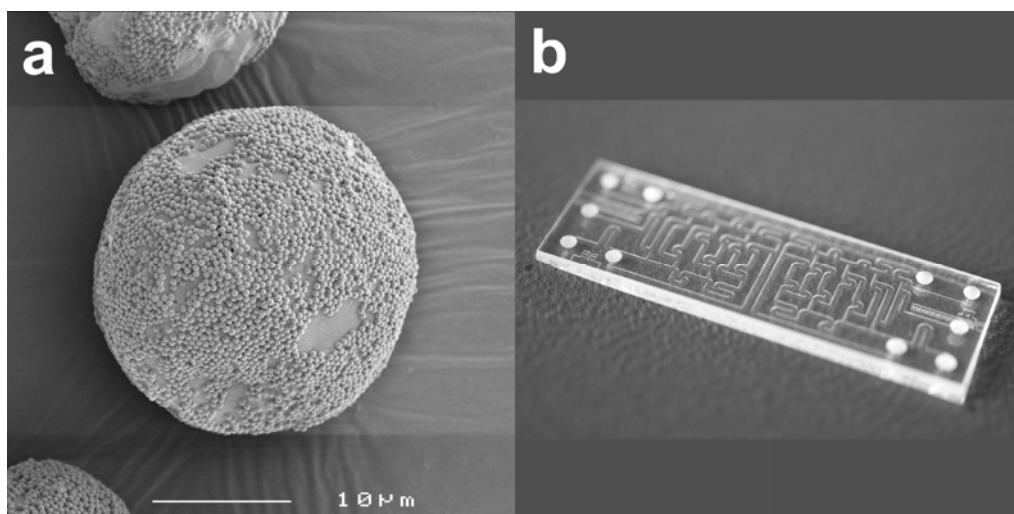


Figure 3: (a) A microcapsule assembled by the hybrid colloidosome/layer-by-layer route and (b) a microfluidic chip for the assembly of 10-layered microcapsules. The total dimensions of the microfluidic chip are 15 mm x 45 mm.

Aim and thesis outline

The goal of the research reported in this thesis was to identify combinations of food grade materials that could be used for the production of microcapsules using self-assembly strategies. These microcapsules should protect an eventual encapsulated material from low-pH conditions and deliver their contents in response to a change in pH, having in mind applications that would require delivery through the stomach into the small intestine.

In *Chapter 2*, we present a review which covers different materials, techniques, and applications of microcapsules known as colloidosomes.

Chapter 3 introduces a microencapsulation procedure in which sequential electrostatic adsorption of whey proteins, high methoxyl pectin, and whey protein fibrils is used to reinforce the structure.

Chapter 4 deals with the characterization of the fibril-reinforced microcapsules prepared according to the procedure discussed in chapter 3, for example with respect to their response to pH changes, but also gives valuable information on the assembly mechanism.

Chapter 5 presents procedures to prepare microencapsulates in which the inclusion of colloidal particles is used to make an easy-to-load open scaffold, of which the pores can be closed by subsequent adsorption of protein and pectin.

Chapter 6 shows a promising attempt to go from a batch process to a continuous one for the assembly of layer-by-layer reinforced microcapsules, with the help of a purposely-designed microfluidic device.

Finally, *Chapter 7* closes this thesis with a general discussions and an outlook on future developments in the area of microencapsulation.

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Chapter II

Colloidosomes: Versatile microcapsules in perspective

Abstract

Colloidal particles of different sizes and shapes can organize on suspended particles or emulsion droplets, forming hollow-porous microcapsules called colloidosomes. The potential of the colloidosomes to serve as targeted delivery/controlled release devices has been discussed many times in literature. However, obtaining well-defined colloidosomes at high yields is still an open challenge. We review and compare the different methods reported in literature to produce colloidosomes, not only to show the state of the art and the aspects requiring further development, but also to spot the possible future perspectives of research in this field.

This chapter has been published as: Rossier-Miranda, F. J.; Schroën, C. G. P. H.; Boom, R. M., Colloidosomes: Versatile microcapsules in perspective. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2009**, 343, 43-49.

Introduction

Recent advances in the field of microtechnology have made us aware of its potential for practically every area in human development. For example, nanometer to micrometer-sized particles with well-defined electrical and mechanical properties not only find applications in the field of electronics, but in the medical field as well. They are used in new contrast agents for imaging, encapsulation, and controlled release of drugs, and will be used in artificial organs. The size of these microparticles can be chosen to target specific tissues in the body. For instance, when particles between 5 and 15 μm are injected into the blood stream they can passively accumulate in the lungs because of entrapment into their capillaries. On the other hand, microcapsules of 1 - 5 μm could enable dosage through the air by inhalation¹. Particles under 0.2 μm can even access bone marrow². Microtechnology offers new opportunities to the food industry as well: it will offer a new palette of products with encapsulated aroma, flavors, vitamins, and other nutraceutical compounds, which are released under specific stimuli. Masking of active compounds can be achieved by using encapsulation particles under 30 μm which are, depending on the food matrix, under the sensorial perception limit of the consumer².

Different materials and techniques have been used in the fabrication of microcapsules, but it is still a challenge to combine biocompatibility, uniformity in size (monodispersity), and high rates of production with other specific properties for each application. An ideal microcapsule should have a well-defined, adjustable size and shape, should capture and retain the active material as efficiently as possible, and should have tunable physical and chemical stability under a wide range of conditions to release its content in response to a range of stimuli³.

A certain number of literature reviews are available for some microencapsulation systems like polymersomes⁴ and polyelectrolyte layer-by-layer microcapsules⁵. However, less attention has been dedicated to colloidosomes, which can be distinguished for its simple and flexible preparation principle and for the variety of microcapsules that can be made with this approach. The aim of this article is to review the different colloidosomes together with their production methods as reported in literature. To the knowledge of the

authors there is only one review that describes colloidosomes, among other systems⁶. In the current article we want to contribute giving guidelines for future design of colloidosomes, with special attention to their production process.

Colloidosomes

The first report about the production of colloidosomes is the pioneering work of Velev *et al.*⁷ in 1996, although the name was coined much later by Dinsmore *et al.* due to the analogy with liposomes⁸. The most general definition for a colloidosome is a microcapsule whose external shell is formed by colloidal particles. Colloidal particles typically have at least one of their characteristic dimensions in the nanometer or micrometer range (1 nm to 1 μm)⁹. Inside that range we can find cells and silica or latex particles along with many other materials. These components can only be effective for stabilizing colloidosomes when they are sufficiently mobile to adsorb to the colloidosome¹⁰. In addition, their surface characteristics and charge density are important for adherence to the surface².

The colloidal particles develop an ordered structure on the shell of the colloidosome by self-assembly. During self-assembly the colloidal particles organize themselves in a stable and regular structure according to the interactions determined by their shapes, electrical charges, mass, and surface properties¹¹. To form a colloidosome it is necessary to have a template that can give a defined shape and that provides a surface for the colloidal particles to adhere to. In principle solid particles could be used providing a “hard” template¹², as it has been done for the polyelectrolyte layer-by-layer microcapsules¹³. Such an approach, when used to produce colloidosomes, would inherit both its advantages and disadvantages. With a “hard” templating method is possible to obtain a remarkable monodispersity in the resulting microcapsules. However, the loading of such a colloidosome with an active compound would be cumbersome. If the colloidosomes are constructed first and then loaded by diffusion of the active compound, the loading process would be necessarily time-consuming, difficult to reproduce, and the maximum load would be limited to the maximum concentration of the active compound in the surrounding fluid. On the other hand, if the active compound is loaded prior to

colloidosome production within, e.g. a porous template particle, loading will be faster, but it has to be kept in mind that the active component can suffer degradation during the removal of the solid template¹⁴. Typically, emulsion droplets that provide a “soft” template are preferred in colloidosome production.

Soft-template method

Capsules synthesized by the soft-template method use emulsion droplets as templates for the particles’ self-assembly. The droplets of an emulsion adopt a spherical shape to reduce their interfacial area; hence these colloidosomes will always be spherical. Pickering¹⁵ observed more than 100 years ago that colloidal particles can stabilize emulsions. The interfacial energy of emulsion droplets will decrease after adsorption of a particle at the interface, if the interfacial tension between the two liquids is higher than the interfacial tension between the particle and the respective liquids. Generally, the adsorption energy of a particle in the micrometer range at an oil/water interface exceeds its thermal energy by many orders of magnitude, being for typical systems around 10^4 to 10^5 times larger¹⁶. This means that in the absence of other forces a particle adsorbed at the interface cannot leave the surface and is confined to the plane of the interface. Thus, spherical particles that slightly repel each other will organize in a hexagonal network at the interface, showing regular spaces (pores) between them¹⁷. The size of these pores can be adjusted by changing the size of the adsorbed particles¹⁸, or by using a combination of different particle sizes (in which the smaller ones fill the interstitial pores between the larger ones). However, the adsorption of the particles onto the surface is not trivial. Various barriers to adsorption, specific for each system, have to be overcome^{19, 20}, like the dehydration of charged functional groups present on the particle surface and the desorption of stabilizers from the template surface. But once a particle is adsorbed it is, in general, irreversibly adsorbed.

Usually, the soft-template method starts with the preparation of an emulsion (Figure 1). The energy necessary to overcome the barriers to the adsorption of the colloidal particles at the interface is given by the turbulence generated by agitation during homogenization. During the homogenization process the emulsion droplets are formed and the particles

accumulate at the interface. Subsequently, the particles are fixed in their positions. The template droplets can then finally be removed or dissolved. The starting template can be an oil-in-water or water-in-oil emulsion, different emulsification techniques can be used; the to-be-adsorbed particles can be dispersed initially in the dispersed or in the continuous phase; the particles can have different shapes and functionalities; and they can be locked in position by a number of techniques. The various options explored in literature are reviewed in the next section.

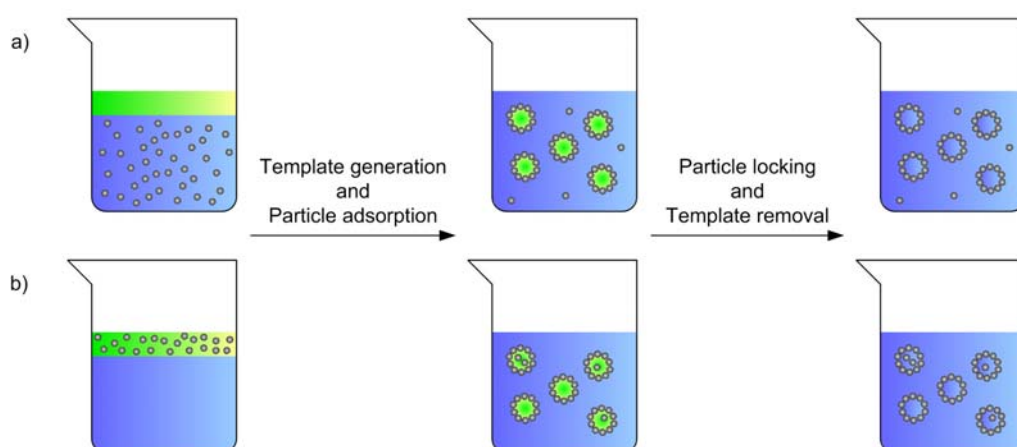


Figure 1: General methodology to produce colloidosomes. The colloidal particles can be dispersed in the continuous phase (a) or in the discontinuous phase (b) at the beginning.

Preparation, structure, and stability of colloidosomes

The first paper⁷ on the preparation of colloidosomes described the use of an emulsion of octanol in water as template. The colloidal particles, in this case polystyrene (PS) particles of 1 μm , were suspended in the aqueous phase. The emulsion was made using a homogenizer, therewith obtaining 10 - 100 μm colloidosomes. PS particles with positive or negative charge were tested. However, the charged particles did not adsorb naturally to the oil/water interface, since the dehydration of the charged groups needed for adsorption to the oil is an energetically unfavorable process. One approach to solve this was to use a surfactant; in that case, however, the adsorbed particles do not form an

ordered arrangement at the interface or were even desorbed from the interface. Adsorption could be achieved with pre-treatment of the particles, which reduced their electrostatic charge to a point low enough to be adsorbed on the oil droplet, and high enough to avoid flocculation in the aqueous phase. To lock the particles in place, bridging of adjacent particles by an adsorbed layer of casein was tested, but this was not sufficient to conserve the colloidosome's structure during dissolution of the template oil droplet. Coagulation of the adsorbed casein using HCl and CaCl_2 to form amore resistant shell was only partially successful in conserving the shell during the process of droplet dissolution. Chemically cross-linking the layer of casein with glutaraldehyde was more successful. However, even with these extra processing steps, the production yield was calculated to be only around 10%.

Croll and Stöver²¹ prepared colloidosomes of 200 μm stabilized with 0.6 μm poly(divinylbenzene-alt-maleic anhydride) particles, initially dispersed in the discontinuous organic phase. Various mixtures of solvents that can diffuse into water were tested as organic phase. The emulsions were made with a wrist shaker. The particles were locked into position with polyethylamines of different molecular weights which were high enough to avoid penetration of polyethylamines into the oil droplets. The rate of addition of the polyethylamines determined the characteristics of the colloidosome shell. Fast addition led to thick and porous shells, while slow addition gave thin and dense shells. In the latter case, the particles have more time to migrate to the interface and to arrange themselves. All colloidosomes constructed by addition of polyethylamines were not stable against removal of the template; they broke down after diffusion of the organic phase into the continuous phase²¹.

A similar approach was used by He *et al.*^{22, 23}. Colloidosomes of approximately 10 μm were produced by emulsification of a mixture of toluene and octanol in water, with particles of sulfonated PS (1 - 3 μm) suspended in the oil phase. Once the particles were at the surface, melamine-formaldehyde (MF) prepolymer was added, and rigid shells of PS/MF were formed (Figure 2a). The resulting colloidosomes were stable in aqueous solutions for long periods, and survived the removal of the oil core. The packing density

of the PS particles at the interface could be controlled by adjusting the ratio of toluene to octanol.

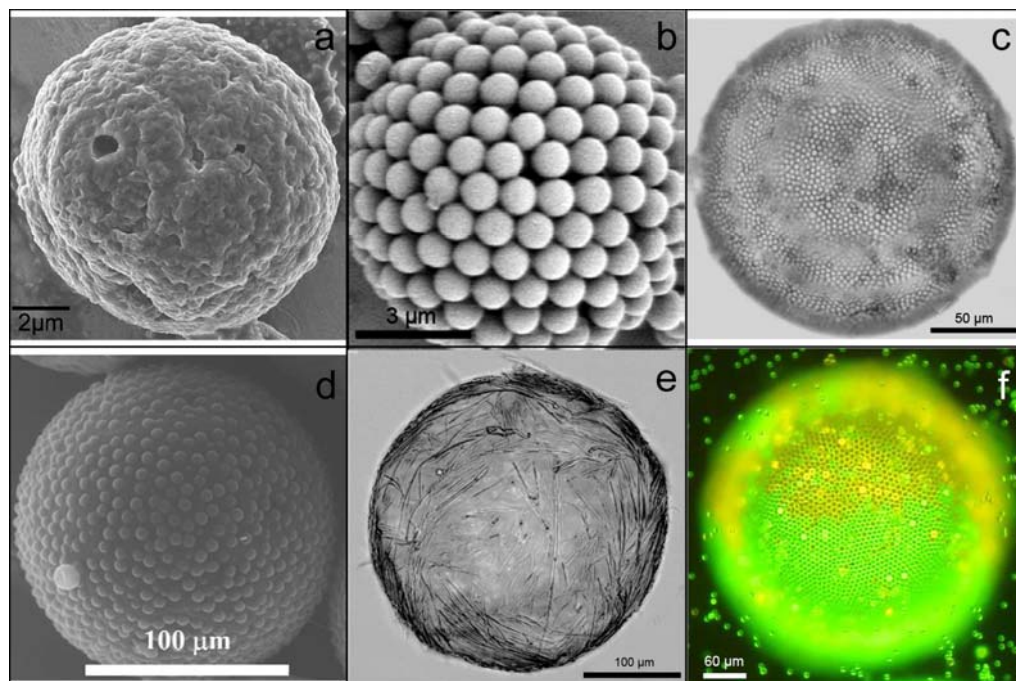


Figure 2: Different colloidosomes obtained with the soft template method: (a) polystyrene/melamine-formaldehyde composite assembled on an oil-in-water droplet²³. (b) Polystyrene particles assembled on a vegetable oil/toluene-in-water emulsion⁸. (c) Amine polystyrene particles assembled on a water-in-sunflower oil droplet²⁵. (d) Poly(DVB-55) porous particles assembled on a p-xylene-in-water droplet²⁶. (e) Polymeric microrods assembled on an agarose gel bead in tricaprylin²⁷. (f) PS particles assembled in a Janus arrangement: the upper half made with 4.9 μm particles and the lower half with 4 μm particles²⁸.

A simpler method for the assembly of colloidosomes without pre-treatment of the charged particles, or the addition of an extra reagent to lock the particles was proposed by Velev and Nagayama²⁴. The starting point is an emulsion of water in octanol with PS particles of 1 μm added to the aqueous phase. The 10 μm colloidosomes showed a

lower degree of polydispersity compared to earlier work. The higher viscosity of this system probably increases the efficiency of homogenization. However, a high concentration of particles is required in the dispersed phase to obtain a good yield: many free particles remained in the interior of the colloidosome. Further, these colloidosomes had a lower degree of order in their surface structure. Water diffused easily from the interior of the colloidosomes to the octanol, therewith simplifying template dissolution. The velocity at which the water is extracted into the continuous phase was found to be the dominant factor for ordering of the particles because it determines the time that the particles have to adhere to the interface and to assemble into a regular packing.

Dinsmore *et al.*⁸ investigated colloidosome production without pre-treatment of the particles. The presence of particles in the interior of the colloidosome could be avoided when a water-in-oil emulsion was used, with the particles suspended in the continuous organic phase. When the particles reached the water–oil interface, they became charged due to hydration of their surface groups, which induced ordering of the particles. One of the tested systems was an emulsion of water in a decahydronaphthalene suspension containing 0.7 μm poly-(methymethacrylate) particles with a stabilizing layer of poly-(hydroxystearic) acid. The particles adhered by van der Waals forces, and the colloidosomes could survive mild centrifugation. Another investigated system was an emulsion of water in a vegetable oil/toluene suspension with 0.9 μm PS particles modified with carboxylate. Two methods were used to immobilize the particles. One was to sinter the particles together by heating the colloidosomes over the glass transition temperature of PS. The pore size and the stiffness of the colloidosomes could be controlled by varying the sintering time. Figure 2b shows an example of the obtained structure. The second alternative was to include poly-L-lysine in the aqueous dispersed phase, therewith fixing the polystyrene particles from the inside. The resulting colloidosomes turned out to be flexible, deformable, and tough. Gordon *et al.*²⁹ used the same system, focusing on possibilities for release of the contents of colloidosomes under mechanical deformation using microcantilevers. The deformation was described with a finite differences model, which considered the shell not as a membrane formed by discrete PS particles, but as a homogeneous membrane of poly-L-lysine. It was found

that the mechanical properties of the colloidosome depended on the poly-l-lysine part of the shell and that the PS particles only played a stabilizing role; the colloidosomes could not be assembled without them. Besides, it was found that the colloidosomes were inflated by the osmotic pressure generated by the free poly-l-lysine in the interior. This suggested other possible mechanisms for controlled release of the load like changes in salt concentration, pH, and temperature.

Hsu *et al.*³⁰ observed that the stability of the particles in the oil influences the structure of the resulting layer and gives a direct method to lock the adsorbed particles. They worked with colloidosomes of sizes between 20 and 400 μm , assembled from water-in-oil and oil-in-water emulsions, with PS particles of 0.5, 1.1,

or 1.3 μm suspended in the organic phase, in this case toluene or chlorobenzene. Three situations can occur:

1. If the particles do not aggregate the emulsion droplet is covered with a layer of unlocked particles.
2. If the particles aggregate in the dispersed fluid an ordered and relatively rigid shell is formed.
3. If the particles aggregate in the continuous fluid a multilayer and disordered shell will be formed.

Cayre *et al.*²⁵ proposed a more direct method to improve the stability during transfer of colloidosomes to another fluid. Amine latex particles of 3.9 μm were dispersed in a continuous phase of tricaprylin and assembled at the interface with a hot agarose gel

solution, therewith obtaining colloidosomes with sizes between 60 and 180 μm (Figure 2c). Once cooled, the gel core solidified and gave additional structure to the colloidosome. As in Hsu and co-worker's study, these particles were unstable in tricaprylin, which promoted their coagulation. They proposed that this property could be used to control the pore size of the colloidosomes since this coagulation occurred at a very slow rate at room temperature. This kind of colloidosome could offer two levels of encapsulation: the first one via the gel matrix, which is controlled by the concentration of

agarose used, and the second one via the pore size of the colloidosome itself. Further, it was demonstrated that after the interlinking of the particles with glutaraldehyde the gel core can be removed by heating the suspension over the melting point of agarose, therewith also pointing to a method for release.

The integrity of the colloidosomes' shell after removal of the template can be improved by constructing them using microgels instead of solid particles. Croll and Stöver³¹ demonstrated this in their study with poly(divinylbenzene-alt-maleic anhydride) microgels of 0.15 and 0.65 μm , stabilized by polyamines. It was found that the resulting colloidosomes had flexible shells. Even though the shells collapsed after removal of the template oil droplet they did not break down. The release of the encapsulated material could be controlled by an on-off mechanism in response to changes in salinity, humidity, or pH, promoting the swelling and shrinking of the microgels and therewith opening and closing the pores defined between them. Unfortunately, the degree of ordering of the microgels at the interface was not as high as for solid particles.

As we can see, re-optimization is required for every colloidosome system, due to the complex interplay between particles and the organic and aqueous phases during the formation of colloidosomes and the fixation of the shell. Croll *et al.*^{26, 32} proposed to alleviate this by using permeable polymeric particles encrusted in a polymeric, impermeable structural shell. This would allow the diffusion to happen in a predictable way through the pores, which would be independent of the involved phases or the encapsulated material. This idea was tested using particles of poly-(divinylbenzene-55) with pores of exclusion size 500 Da embedded in a polyurea matrix (Figure 2d). It was found that the colloidosomes constructed in this way maintained the release of the test active component for a longer time than the colloidosomes constructed from polyurea and non-porous particles. The release can therefore be controlled by varying the load of particles in the shell of the colloidosome. It was also determined that the porosity of the particles improves their anchoring to the polyurea matrix. It was not possible to determine, however, if the release happened only through the pores or also through the particle–polyurea interface.

Noble *et al.*²⁷ reported on the assembly of hydrophobic polymeric microrods over droplets of agarose (Figure 2e). These microrods were polydisperse with approximate dimensions of 10 – 70 μm in length and 0.4 - 2.0 μm in diameter. The microrods gave an additional degree of steric repulsion to the “hairy” colloidosomes produced. The microrods could be interlinked with glutaraldehyde to increase the colloidosome’s strength.

Another option for improving control over the release rate is to deposit multiple layers of particles over the layer of fixed structural particles, in a way resembling the layer-by-layer deposition of polyelectrolytes over a hard template. Gordon *et al.*²⁹ reported the addition of a layer of submicron SiO_2 over colloidosomes made with poly-L-lysine stabilized PS. It was shown that an added layer improves the elastic modulus, the compatibility, and functionality of the shell. The internal polymer determines the response of internal pressure to changes in the surroundings, while the particles determine the colloidosome structure and the interlinking of the polymer.

Visualization of colloidosome assembly

The emulsification methods currently used to prepare the template emulsion do not allow visualization of the colloidosome formation process, while for comprehension of the involved mechanisms it would be very useful to be able to do so. A technique that allows the direct visualization of the particle assembly at the interface of the emulsion droplet, and the study of the colloidosomes’ properties as function of the number of adsorbed particles was proposed by Ashby *et al.*³³. A giant colloidosome between 1 and 2 mm was created at the tip of a capillary immersed in the organic phase, in this case n-decane or tricaprylin. An aqueous suspension of PS particles of 0.22 or 9.6 μm was injected into the organic phase through the capillary. Through repeated injection and withdrawal of the solution, the particles were given the time and energy needed to reach the interface. The colloidosomes were made in the oil phase, and transferred to the aqueous phase through the oil/water interface, forming a water-in-oil-in-water emulsion. The particles remained in the oil film therewith allowing their observation. Part of the experiment was to inflate the colloidosomes in the aqueous phase, ultimately reaching 13 times the

original size. It could be concluded that it is not necessary to have a densely packed monolayer to maintain the structure of the colloidosome. A possible explanation is that every particle acts as a spacer, therewith avoiding the coalescence of the two water–oil interfaces. This result is in agreement with the results from Midmore³⁴, and Vignati and Piazza³⁵. They reported stable emulsions with only 29% and 5% of the droplet surfaces covered by particles, respectively. Horozov and Binks³⁶ demonstrated recently that when an emulsion droplet is only partially covered with particles the steric repulsion is not the stabilization mechanism in the emulsion. There is stabilization through bridging because these particles form a dense monolayer at the contact zone, a result of the strong capillary attraction promoted by the meniscus surrounding them. However, even if stable in solution, a discontinuous monolayer cannot form a colloidosome strong enough to support itself.

Subramanian *et al.*²⁸ succeeded in the direct observation of the formation of colloidosomes. This was achieved by assembling the colloidosomes in a microfluidic device in which a droplet is formed at the same time as particles strike its interface and remain trapped at it. The curved interface is kept stationary to allow the adsorption of particles. In this way, silica particles were assembled over mineral oil droplets obtaining monodisperse colloidosomes with diameters around 200 μm and, therefore, the authors concluded that the main determining factor for the colloidosome size is the geometry of the outlet channel. They did not observe Brownian motion at the colloidosomes interface, which reveals that the reached packing is such that spontaneous jamming occurs; a liquid-solid transition despite the electrostatic repulsion between the particles. One of the most interesting results was the possibility to construct a so-called janus-armor with this system, that is, a colloidosome with two hemispheres produced from the ordered and separated packing of two different kinds of particles at each half of the shell (Figure 2f). Unfortunately, the removal of the inner core from the colloidosome was not reported, and therefore it cannot be stated if jamming is sufficient to create a stable colloidosome.

Future perspectives

The soft template approach benefits from the recent attention to emulsions stabilized by colloidal particles, which led to new theoretical and practical knowledge about how the size and hydrophobicity of particles, the type of oil and aqueous phases used, and the interrelation existing between all these factors influences the stability of the emulsion and ultimately that of the colloidosome^{19, 37-39}. However, insight on the interrelation between particles and solvents is not enough to have full control over the successful preparation of colloidosomes. As we have seen, the process employed to assemble the shells has a major influence on the obtained structures. Obtaining colloidosomes of a specific size with high monodispersity and yield, with a simple procedure, remains a challenge. In this section, we comment on how recent developments in related fields can improve the production of colloidosomes.

Figure 3 summarizes all the steps and techniques used in literature to obtain the colloidosomes presented previously in this article. From it we can observe that there are four parameters to be considered:

1. the kind of particle used to assemble the shell;
2. the applied emulsification technique;
3. the method used to lock the particles together into a solid shell;
4. the loading and release of active compounds from the colloidosomes.

These parameters are interrelated, which makes it difficult to judge them separately.

As discussed earlier the nature of the particles used will influence the self-assembly of the colloidosome and will influence the porosity of the obtained shell. The use of new and more complex materials may create functionalized shells with a more accurate targeting capacity and a more controlled content release. For example Duan *et al.*⁴⁰ prepared magnetic gelled-core colloidosomes from magnetite nanoparticles, which could be concentrated into specific tissues by applying an external magnetic field. The production of microgel-stabilized emulsions was also reported recently⁴¹. The microgels, made from poly-(N-isopropylacrylamide), can change their degree of swelling depending on the

temperature and the pH, therewith also offering different ways to trigger destabilization of colloidosomes. Kim *et al.*⁴² showed that not only the pores between the particles, but also the effective thickness of the shells, were completely controlled by the temperature response of the microgel that was used as a template to assemble the PS particles.

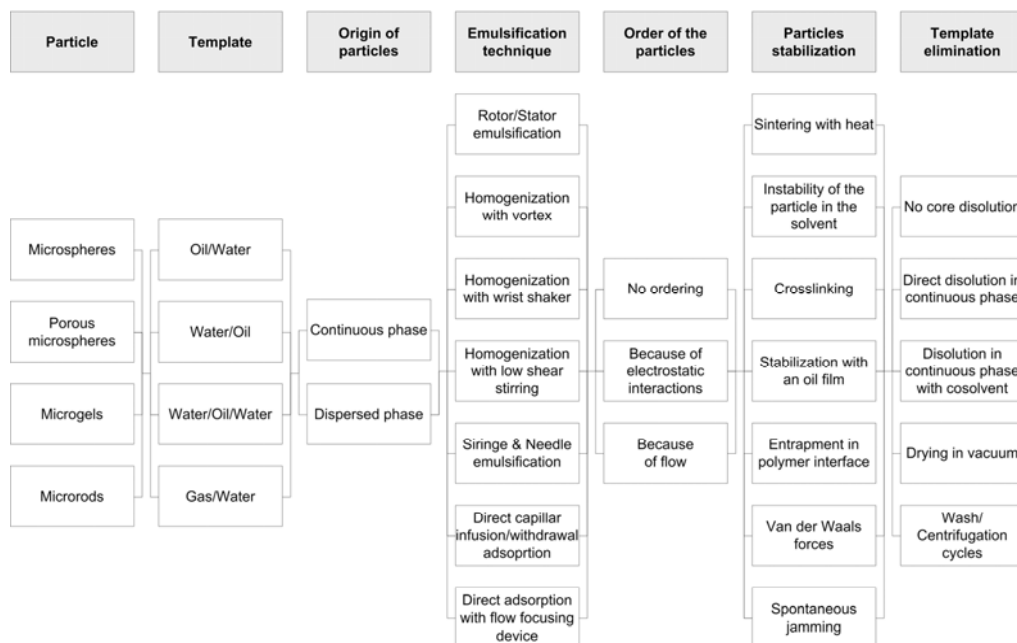


Figure 3: Overview of materials and methods used to produce colloidosomes.

Bearing in mind the techniques used for obtaining the “janus” colloidosomes, we may consider the creation of a colloidosome not by using particles with multiple functionalities but by mixing different kinds of particles in the same shell, some enhancing the targeting ability and others controlling the rate of release. This is especially important in the case of responsive drug carriers in medicine.

The size distribution of the template emulsion directly influences the size distribution of the colloidosomes, and therefore new emulsification techniques offering better monodispersity are relevant. Two promising techniques are membrane emulsification and emulsification with microdevices. The current membrane of choice is made of Shirasu Porous Glass, with which it is possible to produce emulsions of oil-in-water,

water-in-oil or double emulsions⁴³. Advantages of membrane emulsification are the relative monodispersity, the high production rates, and the low energy consumption compared to other methods. However, stabilization of emulsions using colloidal particles in these systems is not straightforward, since the colloidal particles migrate to the interface rather slowly, while droplet formation is fast.

In case of microdevices, there is a range of different options. To name a few we can consider the work of Yobas *et al.*⁴⁴ who presented what they called a high-performance flow-focusing geometry device to produce both oil-in-water and water-in-oil emulsions with high generation frequency and reproducibility, or the work of Utada *et al.*⁴⁵ based on a microcapillary device to produce various and complex double emulsions albeit with very large droplet sizes. Richer gel cored colloidosomes as discussed in previous sections could be produced in microfluidic devices as shown in the work of Amici *et al.*⁴⁶ in which alginate gel beads are produced with microfluidic systems. Xu *et al.*⁴⁷ prepared emulsions with the microchannel emulsification method using silica nanoparticles aggregates as stabilizing agents. They found that monodisperse emulsion droplets were formed, and that the emulsions prepared in this way were more stable than the ones prepared by conventional emulsification methods.

It was indicated that one of the main drawbacks of the colloidosomes is the risk of incomplete surface coverage with colloidal particles, which can lead to leakage and core contamination. Even with a perfect coverage, colloidosomes prepared with just colloidal particles would never be able to encapsulate low molecular weight drugs, aromas or flavors in a controlled way, because of the large pores defined by the interparticle space. As discussed, polymers can be used to stabilize the emulsion and to adsorb and cross-link the colloidal particles. Bon *et al.*⁴⁸ carried out a polymerization reaction inside the colloidosome resulting in poly-(methyl methacrylate) microgels embedded in a solid polymeric shell. However, extreme conditions were needed to perform the polymerization process. Methodologies like layer-by-layer deposition that can be carried out at ambient conditions, seem more suited to achieve stability, especially when including layers of

smaller particles or polymers to reduce the permeability of the colloidosomes, and layers of bigger particles to contribute to the mechanical stability.

It was proposed that hydrophobic components can be loaded into colloidosomes by starting with oil-in-water emulsions, while hydrophilic components can be loaded by starting with water-in-oil emulsions. In both cases, the dispersed phase should contain the active compound in high concentrations. Actually, hydrophilic and hydrophobic compounds could, in principle, be loaded at the same time by using double emulsions as templates. However, many proposed methods for the assembly of the shells did not include a loading step. Loading an empty, and presumably low permeability shell after its preparation is not an easy task. An elegant option for loading a colloidosome with charged components was proposed by Zhu *et al.*⁴⁹. They used an alginate gel as template for the assembly of polyelectrolyte multilayer microcapsules. The core of the capsule, consisting of the negatively charged biopolymer can act as an “electrostatic sponge” adsorbing a model enzyme and macromolecule with high efficiency, even when the concentration of the molecule in the bulk solution was low. However, the capsule has to be closed afterwards to ensure that release does not take place prematurely.

A stimuli-responsive capsule will imitate the action of biological systems, such as the release of a hormone from a natural organ. The change of pH is an attractive trigger for drug delivery, considering that, for example, differences in pH inside tumors or inflamed wounded tissue have been reported⁵⁰. In addition, using the change of pH in the gastrointestinal tract as trigger seems logical for food related applications. For both types of application, the used materials have to be non-toxic, non-immunogenic, food grade and need to be safe in all the stages of the drug delivery or consumption process, including excretion. Further, they mostly need to be water-soluble. If non-degradable, its size needs to be under the renal limit, to avoid accumulation in the body⁵⁰.

It is clear that many interesting options become available, slowly but surely, to influence those parameters that are of utmost importance for the production and performance of colloidosomes. Ultimately, a combination of these parameters is expected to lead to a new range of colloidosomes for various applications.

Conclusion

Colloidosomes are new particulates with promising capacities for microencapsulation and controlled release in medical or food related applications. The methods for preparation are more flexible and simpler than other microencapsulation techniques. The variety of materials that can be used to assemble the colloidosomes could result in a wider range of mechanisms to target specific areas (in the body) and to trigger the release of their contents. The use of new responsive materials might even improve these mechanisms further. New emulsification techniques will enable preparation of highly monodisperse colloidosomes at high production yields. Production and visualization of colloidosomes under well-defined and controlled conditions in microdevices will give valuable information to describe the process and the involved time scales. Finally, a field in direct need of exploration is the characterization of the colloidosomes in relation to encapsulation and release of compounds.

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Chapter III

Polymer microcapsules with a fibril-reinforced nanocomposite shell

Abstract

Polymer microcapsules can be used as controlled release systems in drugs or in foods. Using layer-by-layer adsorption of common food proteins and polysaccharides we produced a new type of microcapsule with tunable strength and permeability. The shell consists of alternating layers of pectin and whey protein fibrils, yielding a fibril-reinforced nanocomposite shell. The strength can be tightly controlled by varying the number of layers or the density and length of the fibrils in the protein layers. The mechanical stability of these microcapsules appears to be superior to that of currently available multilayer capsules. The method involves only standard unit operations and has the potential for scaling up to industrial production volumes.

This chapter has been published as: Sagis, L. M. C.; de Ruiter, R.; Rossier-Miranda, F. J.; de Ruiter, J.; Schroën, K.; van Aelst, A. C.; Kieft, H.; Boom, R.; van der Linden, E., Polymer Microcapsules with a Fiber-Reinforced Nanocomposite Shell. *Langmuir* **2008**, 24, 1608-1612.

Introduction

Microcapsules have been used in various medical applications, such as the controlled delivery of anticancer drugs, vaccines, antibacterial agents, and contrast agents for in vivo diagnostics¹. They could also play an important role in disease prevention. By incorporating them into a new generation of low-fat and low-calorie functional foods, they could contribute to solve the growing problem of obesity in the modern industrial world.

Polymer microcapsules can be formed by the self-assembly of amphiphilic block copolymers²⁻⁵, extrusion methods^{6, 7}, or electrostatic layer-by-layer adsorption⁸⁻¹⁵. Depending on the application, the demands on the properties of the shell of the microcapsules and the production method may vary widely. When used for the encapsulation of small components such as volatile flavors and low-molecular-weight pharmaceuticals the interface should have a low permeability. Currently available encapsulation systems can retard the release of such components^{10, 12, 13} but for most applications, especially for products with an appreciable shelf life, the release is still too fast. For the slow release of vitamins or other functional ingredients in the lower gastrointestinal tract an interface is needed with low permeability under the acidic conditions of the stomach and high permeability in the approximately neutral pH environment of the gastrointestinal tract¹⁶. Those microcapsules should have not only significant mechanical stability but also substantial stability against acidic or enzymatic digestion. Even more important, for applications in food products the capsules would obviously need to be foodgrade and preferably inexpensive, putting substantial limitations on the materials that can be used. Besides, the production method should involve available unit operations so that the process can easily be scaled up to produce large quantities of the capsules at low cost.

Here we have investigated a method of producing fibril-reinforced capsules based on electrostatic layer-by-layer adsorption on emulsion droplets using combinations of oppositely charged proteins and polysaccharides. Microcapsules prepared with synthetic polymers are generally produced using solid nano and microparticles as templates^{8-10, 12}. Layer-by-layer adsorption of proteins and polysaccharides onto oil droplets has been

applied only to enhance the stability of emulsions^{17, 18} but not yet to produce microcapsules. Liquid templates have been used to produce microcapsules from pure proteins¹⁹ or alternating layers of proteins and phospholipids^{11, 15}. Here, alternating layers of positively charged whey protein isolate (WPI) fibrils (Figure 1a) and negatively charged high methoxyl pectin (HMP) were adsorbed onto oil droplets dispersed in an aqueous phase at a pH of 3.5. Using alternating layers of WPI-fibrils and HMP gave a shell with a structure of a fibril-reinforced nanocomposite. The oil phase can be removed by dispersing the capsules in an appropriate solvent for the oil and freeze drying or by critical point drying. Here we opted for freeze drying. After the removal of the oil, the capsules can be inflated by dispersing them in an aqueous solution containing the component to be encapsulated²⁰.

For capsules with a shell with low permeability, this process would be too slow. Alternatively, hydrophilic components may be encapsulated directly using water-in-oil-in-water emulsions as a template, where the internal water phase contains the hydrophilic components to encapsulate.

Using emulsion droplets as a template allows the size and size distribution to be tightly controlled simply by the energy input in the initial homogenization step and the concentration of the primary emulsifier (here, unheated WPI). The strength of the shell can be influenced by varying the number of layers, the size of the capsules, the length and concentrations of fibrils, and the HMP concentration and by fine tuning the attractive electrostatic interactions between the fibrils and HMP (*i.e.* by varying the pH and ionic strength or by using a polyelectrolyte with a different charge density). Recent studies on polyelectrolyte multilayer capsules have shown that the permeability of these systems can be tuned by varying the number of layers^{10, 12, 13}. Further studies of the permeability are needed to confirm this for the new capsules.

Experimental

WPI is a by-product of cheese manufacturing and consists mainly of the protein β -lactoglobulin (β -lg) and minor components such as α -lactalbumin, bovine serum albumin,

and immunoglobulins. HMP is a polysaccharide isolated from plants, with an α -(1,4)-linked d-galacturonic acid backbone.

Protein and pectin solutions are prepared by dissolving 0.1% w/w WPI (BiPRO, Davisco Foods International) or HMP (Pectin JMH-6, CP Kelco ApS, degree of methoxylation 69.8%) in a 25 mM sodium chloride solution at pH 3.5. The solutions are centrifuged for 30 min at 14000 rpm in a J2-MC centrifuge (rotor JA-14, Beckman) and filtered through 0.45 mm cellulose acetate syringe filters (FP 30/0,45 CA-S, Schleicher & Schuell). A fibril solution is prepared by holding a 2% w/w WPI solution in a 20 mL glass vial at 80 °C for 16 h under continuous stirring^{21, 22}. After heating, 44% of the protein monomers are incorporated into the fibrils. The remaining 56% is present in solution as denatured, partially hydrolyzed, non-aggregated material. The fibril solution is diluted to a total protein concentration of 0.58% w/w.

The length distribution of the fibrils is obtained from birefringence decay in stopped flow experiments. The birefringence experiments are performed on a strain-controlled ARES rheometer (Rheometric Scientific) that is equipped with an optical analysis module. The rheo-optical technique and the determination of the length distribution are described by Rogers *et al.*²³. Samples are subjected to steady shear at a shear rate of 25 s⁻¹ for 10 s, and the decay of birefringence is measured after stopping the flow. Three to six measurements are performed on each sample. The length distributions show a peak at about 1.5 μ m.

The HMP is characterized by means of size-exclusion chromatography coupled to multiangle laser light scattering (SEC-MALLS). The separation is executed with an Äkta Purifier system (Amersham Pharmacia Biotech) using a mobile phase of 0.2 M sodium nitrate solution at pH 3.5. The MALLS detector is a DAWN EOS (Wyatt Technology Corporation) with a laser of wavelength 685 nm, and it is coupled to a refractive index detector, a Spectra System RI-150 (Thermo Separation Products). The data are analyzed using Astra version 4.90.07 (Wyatt Technology Corporation), resulting in an HMP molecular weight of 2.7×10^3 kDa and a radius of gyration of 46 nm.

A 1% w/w emulsion of hexadecane in unheated WPI solution is produced using a homogenizer with a rotor-stator dispersion tool (Ultra Turrax T25 Basic, Ika-Werke) using a setting of 9500 rpm for 1 min. Because the proteins are below their isoelectric point, the emulsion droplets have a positive charge. To avoid interactions between the non-adsorbed WPI and the biopolymer of the next layer, the droplets are separated from the serum by means of centrifugation. After the isolation, the droplets are dispersed into a solution of HMP. The HMP is negatively charged at the chosen pH of 3.5. The bilayered droplets can be isolated again and dispersed in a fibril solution to deposit a third layer of a positively charged mixture of WPI-fibrils and non-aggregated WPI. Subsequently, additional layers of HMP and WPI-fibrils can be deposited by repeating the same procedures. No additional washing steps were performed after each centrifugation step. As a result, a small amount of non-adsorbed material is transferred to the next solution.

To determine the size distribution and ζ -potential, the emulsion droplets are isolated from the serum and dispersed in 25 mM NaCl solution at pH 3.5 to a final droplet concentration of about 0.01% w/w. The droplet size distributions of the emulsions are determined using a MasterSizer 2000 (Malvern Instruments). Three measurements are performed on each sample. The ζ -potential of the emulsion droplets is determined with a ZetaSizer 2000 (Malvern Instruments). On each sample, five to eight measurements are performed.

To investigate the coverage of the emulsion droplets with protein fibrils, the fluorescent dye thioflavin T is bound to the protein fibrils, and the emulsion droplets are observed with confocal scanning laser microscopy. The samples are observed using a Zeiss LSM5 Pascal confocal system mounted on an inverted microscope (Zeiss Axiovert 200). The 458 nm line of an argon laser was used to excite the samples, and the emission fluorescence was observed after passing a 475 nm LP filter. To examine the microcapsules with SEM, they were freeze dried, fixed onto double-stick carbon tape, placed in a dedicated preparation chamber (Oxford Instruments CT 1500 HF, Eynsham, England), and sputter coated with 5 nm of platinum. Specimens were analyzed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at room

temperature at a working distance of 8 mm with SE detection at 3.5-5 kV. All images were recorded digitally (Orion, 6 E.L.I. sprl., Belgium) at a scan rate of 100 s (full frame) at a size of 2528 x 2030, 8 bit. The images were optimized and resized with Adobe Photoshop CS.

Results and Discussion

In our study, we based our choice of concentrations on the stability maps proposed by McClements²⁴. The concentrations of fibrils and HMP need to be high enough to ensure complete coverage of the droplets and prevent bridging flocculation, but they must not exceed a critical concentration, above which depletion interactions will cause the droplets to aggregate²⁴. The opposite charges of the HMP and the WPI-fibrils cause the ζ -potential of the microcapsules to alternate between a value of -9 mV when HMP is the outer layer and a value of +28 mV when WPI-fibrils form the outer layer. These values for the ζ -potential remain constant, independent of the layer number, allowing a seemingly indefinite number of layers to be deposited on the colloidal template (Figure 2a). The size of the microcapsules remains approximately constant after each adsorption cycle (Figure 2b) and is equal to the size of the original emulsion droplets, which shows that the ζ -potential of the alternating layers is sufficiently high to maintain a stable emulsion during the deposition and separation steps.

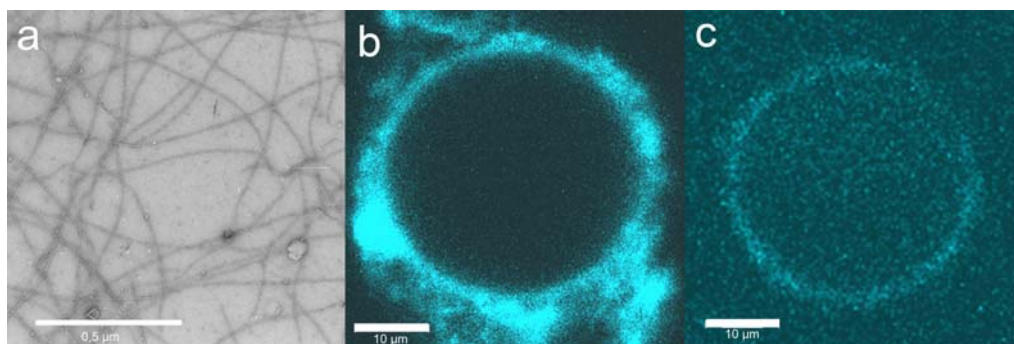


Figure 1: WPI-fibril deposition in three- and four-layer microcapsule. (a) TEM picture of WPI-fibrils. (b) CSLM picture showing the WPI-fibril deposition after the adsorption of the third layer. (c) CSLM picture showing the fibril conformation on the third layer after the HMP adsorption in the fourth layer.

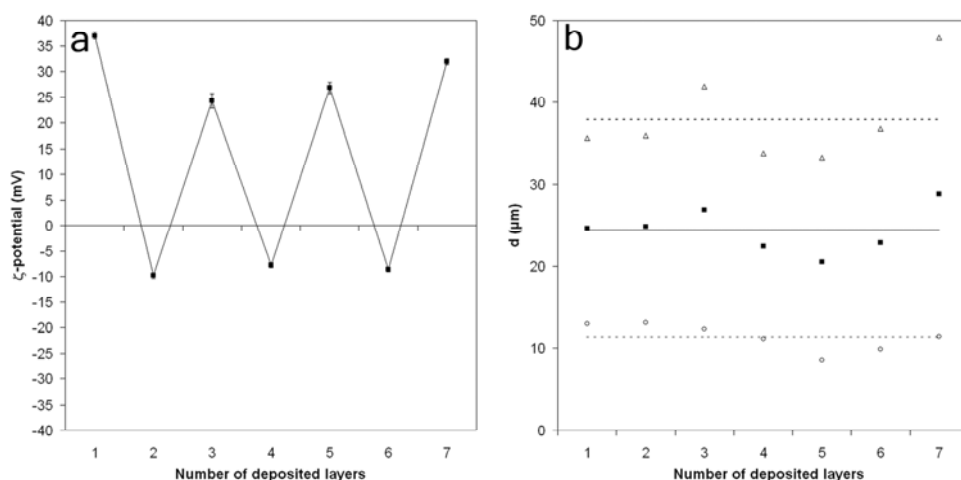


Figure 2: Size and ζ -potential of multilayer microcapsules. (a) ζ -potential after the deposition of each layer. (b) Size after the deposition of each layer. (\blacksquare) $D_{3,2}$ is the surface area mean diameter, and \circ $d(0.1)$ and \triangle $d(0.9)$ are the diameters below which 10% and 90% of the droplets lie, respectively. The straight lines show the average value for each parameter.

The inclusion of WPI-fibrils (Figure 1a) in the layers gives the shell a structure of a fibril-reinforced nanocomposite. When the outer layer of the microcapsules consists of WPI-fibrils, we observe a “hairy” structure with confocal scanning laser microscopy (CSLM) (Figure 1b), showing that the adsorbed layer is not a monolayer. The charge of the fibrils causes them to extend into the surrounding solution and not (yet) collapse on the microcapsule. This morphology may increase the steric repulsion between microcapsules²⁵, enhancing the stability needed to add the next layer. The hairy structure appears to be partially removed or compacted during the next deposition step, as can be seen from the smooth fluorescent ring shown in Figure 1c.

After the removal of the oil by freeze drying, microcapsules with different numbers of layers were studied using scanning electron microscopy (SEM). These observations allowed us to qualitatively determine the relation between the number of layers and the strength of the microcapsules. As an example, we compared microcapsules made of

three layers (Figure 3a) with those made of seven layers (Figure 3b). Capsules with three layers have more defects in the shell and have shrunk and buckled, showing that the shell is not strong enough to resist the capillary forces during the removal of the oil or external mechanical forces once it is hollow. We also observed a dimpling of four-layer microcapsules caused by focusing the electron beam of the SEM on a particular area on the surface of the microcapsule (Figure 4b). When subjected to the same treatment, microcapsules with seven layers maintain their size and spherical shape. For the three- and four-layer systems, we see that only capsules with a diameter smaller than about 10 μm are relatively stable. For the seven-layer systems, we also observe stable particles with a diameter larger than 10 μm . Figure 3c presents a magnification of a crack present in one of the microcapsules of Figure 3b, showing that the wall thickness of a seven-layer shell is around 300 nm. Because the WPI-fibrils have a thickness of about 4 nm and the HMP has a radius of gyration of 46 nm, the absorbed layers are not monolayers, which is in agreement with the CSLM observations. The layer thickness obtained for the seven-layer WPI/HMP system is significantly higher than the thickness of the shells made by layer-by-layer adsorption of other polyelectrolytes^{10-13, 26, 27}. The latter systems have a thickness per layer of polyelectrolyte on the order of 1-3 nm, which implies that even in systems with up to 20 layers¹⁰⁻¹³ the total layer thickness does not exceed a value of about 50 nm. The WPI/HMP system therefore appears to be far more effective for creating microcapsules with high mechanical stability. Most polyelectrolyte multilayer microcapsules tend to collapse after drying^{10, 12-15}, whereas WPI/HMP capsules with similar numbers of layers remain spherical after drying. The extreme thickness of the layers is a result of the high stiffness and charge of the fibrils, which lead to the formation of layers with a hairy structure (Figure 1b) instead of monolayers. Figure 4c shows a four-layer system where the WPI-fibrils are replaced by unheated globular WPI. In this system, we observe only relatively small microcapsules ($<5 \mu\text{m}$), and all capsules have completely collapsed upon drying. These systems are considerably weaker than the four-layer system in Figure 4a,b. This shows conclusively that the fibrils indeed reinforce the shell of the microcapsules. An exact quantification of the reinforcing effect is currently not

available but could possibly be obtained either by atomic force microscopy or osmotic stress experiments.

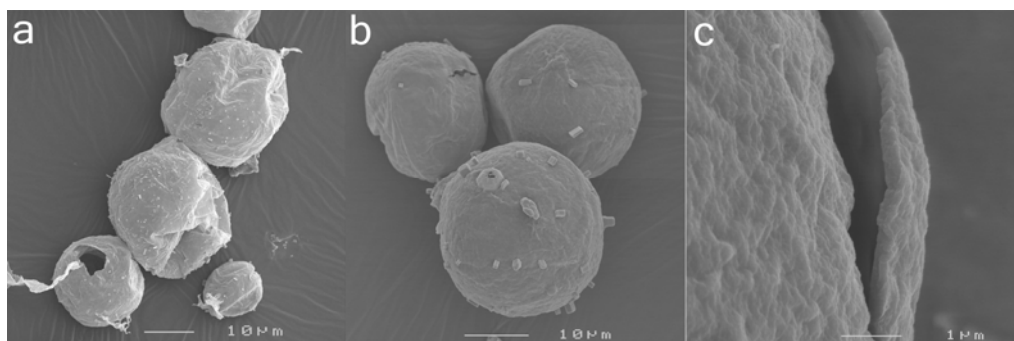


Figure 3: Microcapsule strength with three and seven layers. (a) SEM picture of microcapsules produced with three layers. (b) SEM picture of microcapsules produced with seven layers. (c) SEM picture showing the thickness of the microcapsule wall constructed with seven layers.

Figure 4d shows a detailed view of the surface of a seven layer microcapsule in which fibrous structures can be observed. This corresponds to a network of bundles of WPI-fibrils in which the empty spaces are filled with a mixture of nonaggregated, partially hydrolyzed WPI and HMP. This structure is basically a composite material where fibrils are the reinforcing phase, giving the material its tensile strength. For the interior WPI layers, this structure is wedged between two isotropic HMP layers that act as a glue for the individual fibril layers. Because the layer-by-layer process we have used here involves only standard unit operations and inexpensive shell materials, it has the potential for scaling up to industrial volumes. A limiting factor for scaling up is the low volume fractions of droplets and low concentrations of fibrils and HMP used in the process, which increases the cost of separation significantly. Process volumes could be reduced by reducing the droplet size (this increases the maximum volume fraction of oil droplets that can be used²⁴) or by limiting the number of layers of the microcapsules.

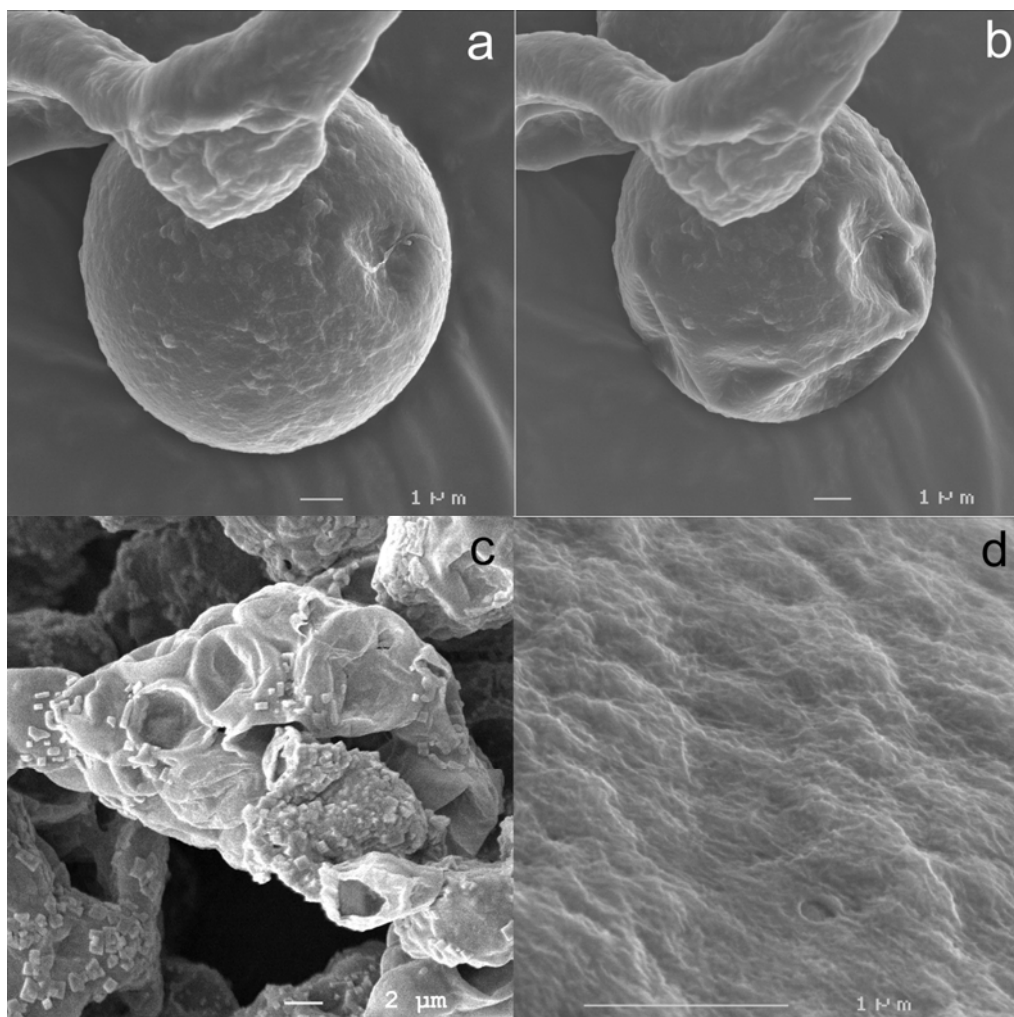


Figure 4: Effect of fibrils on the strength of the capsules. (a) A microcapsule constructed with four layers. (b) Buckling of its surface after the electron beam was focused on a specific point on its surface. (c) Four-layer system prepared with unheated globular WPI. (d) SEM picture showing the surface of a microcapsule with seven layers.

Conclusions

We have demonstrated a flexible method of producing multilayer fibril-reinforced microcapsules from food-grade components using standard unit operations in which the size of the microcapsules can be controlled by the emulsification method used for the

template and their strength can be controlled by varying the number of layers. It is expected that the number of layers also strongly affects the permeability of the microcapsules^{10, 12, 13}, although this still needs to be confirmed by permeability measurements. The method can produce a wide range of microcapsule sizes to fully cover the size spectrum needed for different pharmaceutical and food-related applications. It can be easily adapted to include other biopolymers, such as carrageenans, alginates, and charged starches. Other fiber sources may also be used, such as lysozyme fibrils and fibers of cellulose derivatives. These fibril-reinforced systems represent an entirely new class of microcapsules, with mechanical stability that appears to be superior to that of currently available multilayer systems.

Acknowledgments

The authors thank Shen Yi for preparing the HMP/WPI capsules.

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Chapter IV

Mechanical characterization and release properties of fibril-reinforced microcapsules

Abstract

Despite the fair number of microencapsulation principles that have been developed, the actual protection and smart delivery of sensitive ingredients remains a challenge in the food industry. A suitable technique should use food grade and inexpensive materials, and ensure tight control over the capsule size and release trigger mechanism. For example, encapsulates may need to survive the low pH of the stomach to release their contents in the neutral environment of the small intestine. In this chapter we present layer-by-layer (LbL) microcapsules assembled from whey protein isolate (WPI), high-methoxyl pectin (HMP) and WPI-fibrils. The narrow size distribution of these capsules is determined by the oil-in-water droplets used as templates, and their mechanical properties and pH response can be tuned by the number of layers adsorbed. Capsules with > 8 layers have a mechanical strength comparable to chemically cross-linked polymer capsules, because of the reinforcement by the WPI-fibrils in combination with the shell completion. Typically, capsules with 5 layers survive pH 2 for more than 2 hr, but dissolve within 30 minutes at pH 7. At higher number of layers, the capsules are even more stable. Contrary to other encapsulates, these capsules can be dried and are suitable for application in dry products.

This chapter has been submitted as: Rossier-Miranda, F. J., Schroën, K., Boom, R. Mechanical Characterization and Release Properties of Fibril-Reinforced Microcapsules prepared by Layer-by-Layer Adsorption.

Introduction

Techniques such as spray drying, fluidized bed coating, and extrusion¹⁻³, are frequently used for microencapsulation in the pharmaceutical industry, but do not totally satisfy the demands for microencapsulation in the food sector. Besides the obvious need to use food grade inexpensive materials, microencapsulation should also ideally aim at smart delivery. This implies that the delivery system should offer enough protection for the encapsulate during production, transport, and storage and, most importantly, present a well defined trigger mechanism to release its contents⁴. For this, a well defined size distribution, ideally monodisperse and under 20 μm , is desired to avoid detection by the consumer (gritty taste).

An illustrative example is the encapsulation of probiotics; the capsule should protect the bacteria from oxygen during storage, from the acid conditions of the stomach, and from proteases during passage of the gastro intestinal track, to finally release them in the more neutral environment of the small intestine. Some examples are known from literature, mainly for liquid dairy products; e.g. the encapsulation of bifidobacteria in a matrix of protein/pectin/alginate was proven to give adequate protection in a simulated stomach environment⁵. However, these encapsulates should ideally be stored in a dry state, and it is not expected that the current techniques can be used for this; since cracks usually occur during processes like spray drying⁶. Other approaches like complex coacervation of casein and pectin⁷, or preparation of capsules of casein, fructo-oligosaccharides, and starch that are used to protect an oil-in-water emulsion loaded with probiotics⁸, could be used to improve encapsulation efficiency and release.

In general, the mechanical stability of encapsulates is relatively unexplored, though it is very relevant for the ease of processing of the product to which the encapsulate is added. We therefore have systematically evaluated the dependence of shell thickness and integrity on the mechanical strength and release characteristics in response to pH changes.

In previous work, we found that larger building blocks such as colloidal particles and protein fibrils, can greatly contribute to the integrity of capsules. For example, in colloidosomes^{9, 10} colloidal particles may be fused together on the surface of oil or water droplets to form a sturdy shell. We will show in *Chapter 5* that it is possible to use a combination of LbL adsorption and particle deposition to form capsules that enable loading after preparation of a scaffold, after which the capsules can be closed. Also other colloidal particles such as WPI-fibrils can be used in the assembly of microcapsules by LbL electrostatic adsorption¹¹. Although the proof of principle of the shell formation processes is known, details on the mechanical strength and release properties of the resulting encapsulates are missing, and this is essential in the evaluation of such capsules for application in e.g. foods.

In this work, we investigate microcapsules assembled by LbL electrostatic adsorption¹² using WPI, HMP and WPI-fibrils on oil droplets. Capsules with a range of numbers of layers were prepared. They were compared with respect to the thickness of the shell as determined by SEM, the roughness and shell integrity of the capsules (AFM and SEM), and the mechanical strength of the resulting shell (AFM), along with the response of the obtained microcapsules to changes in pH (image analysis). The formation of the multilayers and their stability was followed closely by reflectometry which allowed us to gain better insight in the formation mechanism and link this to the obtained shell thicknesses, degree of shell completion and linked to that, mechanical strength, and shell stability.

Materials and Methods

Materials

The following materials were purchased from Merck: n-hexadecane (synthesis), sodium chloride (analysis), hydrochloric acid (analysis), sodium hydroxide (analysis), acetic acid (analysis), and sodium dihydrogen phosphate (analysis). From Sigma-Aldrich, we obtained formic acid (reagent), ethanolamine (reagent), and hyperbranched polyethylenimine (PEI, average Mw ~25,000). Phosphoric acid (analysis) was purchased

from Riedel-de Haën, and dimethyldichlorosilane (reagent) from BDH. The whey protein isolate used was from Davisco Food International, and the high methoxyl pectin was from CP Kelco. All materials were used as received.

Biopolymer solutions

WPI and HMP solutions were prepared dissolving 2% w/w of WPI or HMP in 10 mM formate buffer at pH 3.5. The solutions were centrifuged during 30 minutes at 14000 rpm (Beckman J2-MC, rotor JA-14, RCF 18879), and the supernatant was filtered upon dilution before utilization (Millipore Millex HP, PES, pore size 0.45 μm). If needed, the pH of the solutions was further adjusted to 3.5 with 1M HCl or 1M NaOH.

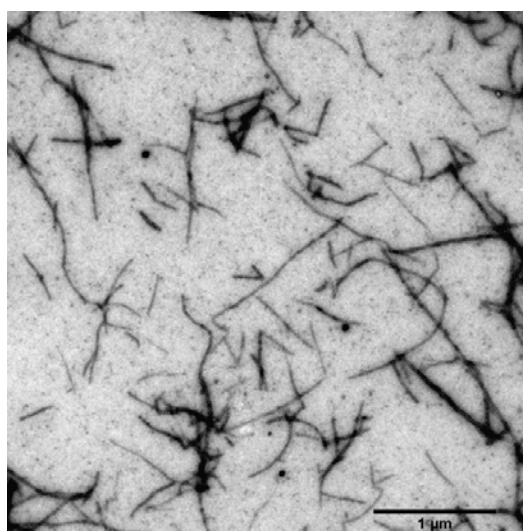


Figure 1: WPI-fibrils obtained by heating and stirring of a 2% w/w WPI solution. The scale bar represents 1 μm .

Protein fibrilization

WPI-fibrils were prepared with a modified version of the methodology developed by Bolder et al¹³. The pH of a 2% w/w WPI in MilliQ water solution was adjusted to 2 using HCl 6 M. The solution was then heated to 80 °C under continuous stirring at 600 rpm during 10 hours. Figure 1 shows the obtained WPI-fibrils, having a typical length of

around 1 μm and a width of around 10 nm. Before use in the LbL assembly, the WPI-fibril solution was diluted in buffer formate 10 mM (pH 3.5).

Electrostatic LbL-microcapsule assembly

Figure 2 depicts the LbL assembly. First, a soft colloidal template is prepared by emulsifying 1wt% hexadecane in a 0.3% w/w WPI solution at pH 3.5. A coarse emulsion was prepared with a rotor stator homogenizer operated for 5 minutes at 24000 rpm (IKA Ultra-Turrax T18 basic, with a S18N-19G dispersion tool). This coarse emulsion was loaded in a pressurized vessel to be further emulsified using the pre-mix emulsification technique¹⁴. The loaded emulsion was passed 10 times through a filter with a pore size of 5 μm (Millipore Millex-SV, PVDF Durapore) under 1 bar of pressure of nitrogen. The droplets have positive charge due to the WPI that stabilizes them. A concentrated cream, consisting of positively charged oil droplets, was separated from the solution by mild centrifugation (1 hour at 70 RCF in a centrifuge Beckman Coulter Allegra X-22R, rotor SX4250). Any remaining free WPI was washed out by a three-fold redispersion/centrifugation cycle with buffer formate at pH 3.5. The clean, concentrated cream was then redispersed in a HMP solution. The HMP, being negatively charged at pH 3.5, adsorbs onto the WPI layer. The rinsing procedure was repeated to remove any excess HMP. The same cycle was applied with the solution of WPI-fibrils, which inherits the positive charge of WPI at pH 3.5. The sequential adsorption of WPI-fibrils and HMP may be repeated at will to grow a shell around the oil droplet. Since it is inevitable that some fraction of the smaller microcapsules is lost in each centrifugation step, the concentrations of the polymer solutions were calculated for each adsorption step according to the number and size of microcapsules, such that they be enough to avoid bridging and depletion flocculation¹⁵ for each adsorption step. The concentrations used were equivalent to target surface loads of 0.3 g/m^2 for the first two layers, and 0.5 g/m^2 for the following ones. Once the target number of layers was adsorbed, the oil template was removed by freeze drying, following the methodology described in the work of Sawalha et al¹⁶.

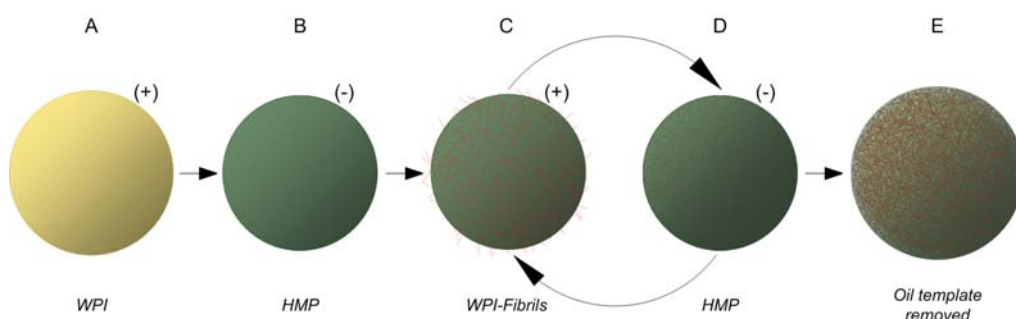


Figure 2: Electrostatic LbL assembly. (A) An emulsion droplet is stabilized by a WPI layer, yielding a positively charged template. (B) After removing the excess WPI, a layer of HMP is adsorbed, yielding a negative charge. (C) A layer of WPI-fibrils is adsorbed on the HMP layer, reinforcing the microcapsule, and yielding a positive charge. (D) A layer of HMP adsorbing on the WPI-fibrils layer removes weakly adsorbed fibrils, and compacts the rest, smoothing the surface. Steps (C) and (D) can be repeated at will to grow a thicker shell around the oil droplet. (E) The template oil droplet is removed by freeze-drying.

Characterization

Size and charge distribution

After each adsorption cycle, the size distribution of the microcapsules was determined by light scattering, using a Malvern Mastersizer 2000. Samples with a total concentration of 1% w/w hexadecane were loaded to the sample dispersion unit (Hydro SM small volume, Malvern) prepared with 50 ml of 10 mM formate buffer (pH 3.5), until an obscuration of 20 units was reached. To check the stability of the samples each sample was measured 3 times with pauses of 15 second between each measurement. If the sample was stable, an average size distribution was calculated from the three measurements. If a change in size distribution was detected the sample was checked with light microscopy, to determine if the change was caused by clustering, which could be corrected during the next adsorption step.

Similarly, the ζ -potential of the sample was also determined after each adsorption cycle by light scattering, using a Malvern ZetaSizer Nano. The average ζ -potential was

calculated from 5 measurements on a sample. Typical ζ -potential values for HMP layers are around -13 mV, for WPI layers around +38 mV, and for WPI-fibril layers around +23 mV. A complete change in the ζ -potential to the values found as standard for each adsorbed layer was taken as confirmation of a successful adsorption cycle.

Sample preparation for SEM and shell thickness determination

Freeze dried microcapsules were placed on the sticky side of transparent “household” single-sided sticky tape. The microcapsules were pushed firmly on the sticky layer with compressed air. The transparent tape loaded with the microcapsules was then placed onto brass holders prepared with double-sided sticky carbon tape (EMS, Washington, U.S.A.). By peeling off the transparent tape, a number of microcapsules on the carbon tape became fractured. Intact, non fractured samples were loaded on a second double-sided sticky carbon tape. The specimen holders with the microcapsules were sputter coated with 10 nm platinum in a dedicated preparation chamber (CT 1500 HF, Oxford Instruments, Oxford UK). The samples were imaged with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at room temperature, at a working distance of 8 mm, with SE detection at 3.5 kV. Images were digitally recorded (Orion 6 PCI, E.L.I. sprl. Belgium) at a scan rate of 100 seconds for a full frame of size 2855 x 2154, 8 bit. The average shell thickness was calculated from at least 5 capsules per sample, measured in at least 10 different positions at the shell of each capsule, using the software ImageJ 1.40g.

Mechanical Strength and Roughness (sample preparation and AFM)

Clean silicon oxide strips were immersed in a 1mg/mL PEI solution for 15 minutes, rinsed with MilliQ water, and subsequently submerged in a dispersion of microcapsules, containing 5 to 10 layers, at a concentration of 0.5% w/w. The capsules were allowed to adsorb on these modified stripes during 15 minutes. After that, the samples were frozen at -80 °C for one hour, after which they were freeze dried. The excess of sample not adsorbed to the silicon strip was removed with compressed air. The mechanical response of the microcapsules to load was registered using an Atomic Force Microscope

(AFM, Nanoscope II, Digital Instruments) equipped with a Multimode PicoForce probe (Veeco) utilizing a silicon cantilever with spring constant $k=40$ N/m (ACTA-W, tip radius 6 nm, opening angle $<15^\circ$, Applied Nanostructures). Between 8 and 18 microcapsules were studied for each sample, collecting 10 force curves from each microcapsule. The data from the force curves was fitted to a model based on Hertz model, describing the deformation caused by a cylindrical cone indenting a soft material with Poisson ratio equal to 0.5, as in the work of Radmacher, Fritz and Hansma¹⁷.

The AFM was also used to assess the roughness of surfaces with multiple adsorbed layers. Clean silicon oxide stripes, with an oxide layer thickness of approximately 30 nm, were hydrophobized with dimethyldichlorosilane, as in the work of Schroën *et al.*¹⁸, to emulate the oil/water interface. These stripes were submerged in solutions of 0.1% w/w WPI, WPI-fibrils and HMP, in the same order as would be the case for the corresponding capsules. The stripes were submerged for 15 minutes in solution to ensure sufficient surface coverage, after which they were extensively rinsed with 10 mM buffer formate pH 3.5, and submerged in the next solution. After the pre-determined layers were established, the samples were freeze dried. From these samples, areas of $5 \times 5 \mu\text{m}$ were scanned with AFM in tapping mode, and the surface analyzed with the NanoScope 6.13 software. The calculated skewness, a measure of the symmetry of the surface data around a mean value, is reported as the roughness. A strongly non-zero skewness value suggests the presence of sharp spikes or pits on an otherwise perfectly flat surface.

Adsorption – Reflectometry

The adsorption experiments were performed in a reflectometer as described in the work of Dijt *et al.*¹⁹. As discussed in the mechanical strength section, clean silicon oxide stripes were hydrophobized with dimethyldichlorosilane to emulate an oil/water interface. Solutions of 0.1% w/w WPI, WPI-fibrils and HMP were sequentially pumped at a flow rate of 2 ml/min into the stagnation cell to follow the adsorption on the modified silicon oxide stripes. Following each adsorption step there was a washing step with 10 mM formate buffer. Both the adsorption and the washing time were fixed at 15 minutes each, within which the adsorbed amount reached its maximum value. The change in signal (ΔS) from

the reflectometer can be linearly related to the adsorbed mass per unit area if the refractive index increments of the materials are known. However, following Kovacevic *et al.*²⁰, we report only the raw ΔS because the refractive index increments of WPI, HMP and WPI-fibrils are slightly different, and the composition of the formed multilayer structure is not exactly known (exchange is expected to take place). The ΔS data thus gives semi quantitative information about adsorption and desorption, knowing that one ΔS unit roughly corresponds to 2.2 mg/m².

pH response

To test the pH response of the microcapsules, first appropriate support surfaces needed to be constructed. For this, glass slides were used; on them either a single layer of WPI, or a double layer of WPI/HMP was adsorbed at pH 3.5, depending on the charge of the microcapsules to be adsorbed. The WPI and HMP concentrations were 0.1% w/w, and the surfaces were exposed to the solutions for 15 minutes after which they were rinsed with 10 mM formate buffer of pH 3.5. Finally, the slides were dried under vacuum at 40 °C. This provided enough surface charge to adsorb microcapsules of the opposite charge. The test surfaces were then submerged in a solution containing 0.5% w/w microcapsules for 30 minutes; samples with an external layer of HMP were adsorbed on slides prepared with WPI, and samples with an external layer of WPI-fibrils were adsorbed on slides prepared with WPI/HMP. Again, non adsorbed microcapsules were removed by rinsing with buffer formate. The glass slides with the adsorbed microcapsules were frozen and freeze dried as described in section 2.4.4. Once freeze dried, the samples were exposed to different buffers (pH 2-11) and the response of the microcapsules was monitored by light microscopy (Carl Zeiss MicroImaging, Inc., Axiovert 200) and recorded during 8 hours (frame rate 0.5 fps, Axio Vision Real 4.5). While standard light microscopy gave information on the number of intact, gas-filled microcapsules during the pH response experiments, the use of a circular polarizer filter helped detecting the presence of deflated, non-dissolved shells.

Results

The emulsification method chosen to prepare the oil droplets yielded a size distribution with a $d(0.5)$ of around 6 μm and a span of 1.5. This size was chosen as the target size for the ease of the characterization experiments (visibility in the light microscope and working distance of the AFM); the size of the template can be tuned by changing the membrane used in the premix emulsification, or by changing the emulsification technique. We have easily produced batches of 20, 15, 12, 8, and 6 μm using rotor-stator homogenizers, cross-flow emulsification with Shirazu porous glass membranes, and premix emulsification.

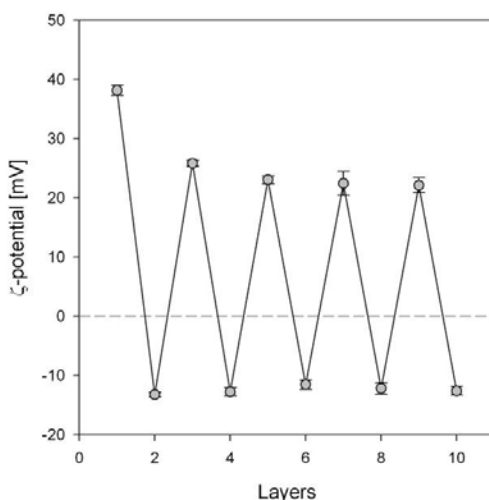


Figure 3: ζ -potential during LbL adsorption, the first layer is prepared with whey protein isolate (WPI), the second with high methoxyl pectin (HMP), after which the odd numbers correspond to layers made with WPI-fibrils, and the even numbers to layers with HMP.

Figure 3 shows the typical evolution of the ζ -potential during the LbL assembly, starting from +38 mV for the WPI layer, followed by -13 mV for the HMP layer, and subsequently alternating between values of around +23 mV for the WPI-fibril layer, and around -13 mV for the next HMP layer. Although the ζ -potential difference between positively and negatively charged layers is smaller than values reported in other works²¹⁻²⁴ (typically

between 60 and 80 mV), the charge inversion in our system provides a potential difference that is sufficient to ensure the adsorption of a next layer. Although charge inversion is not a prerequisite for LbL assembly²⁵, in these systems its important to keep the ζ -potential larger than ± 11 mV, which is generally recognized as the agglomeration threshold for colloidal systems²⁶. The steadiness in the ζ -potential difference during the LbL assembly of our system suggests that the consecutive adsorption cycles of HMP and WPI-fibrils can be extended to an indefinite number of layers. This obviously helps in fine tuning of the shell's thickness. In this study, we have used up to 10 layers in total.

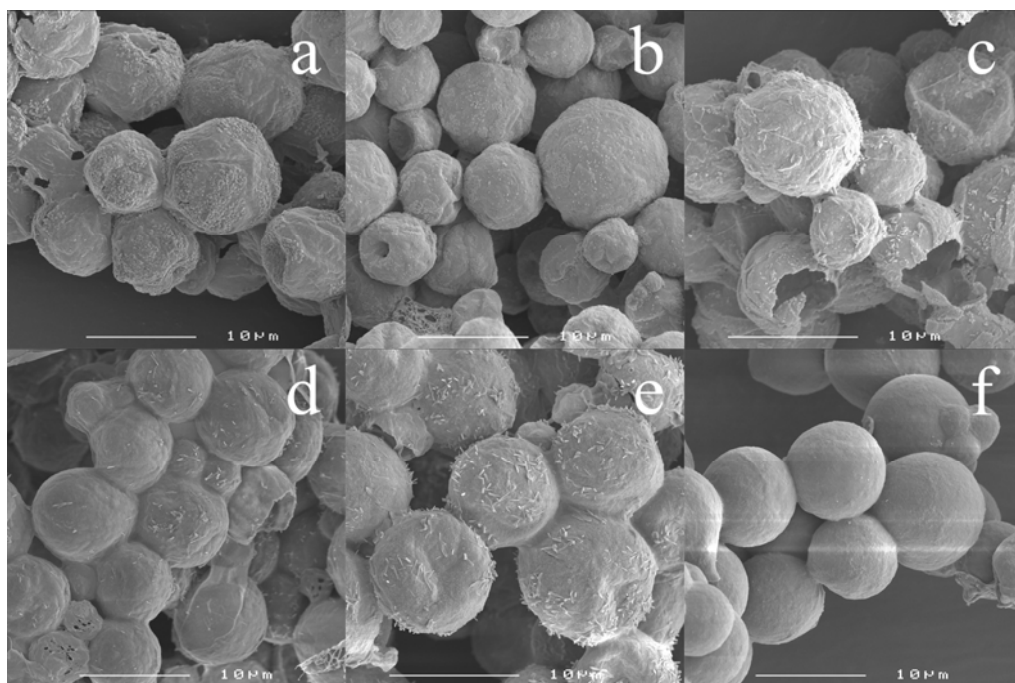


Figure 4: SEM images of microcapsules with 5 (a), 6 (b), 7 (c), 8 (d), 9 (e) and 10 (f) layers. As the number of adsorbed layers increases the number of defects in the shell reduces, and the outer topography of the microcapsules smoothes.

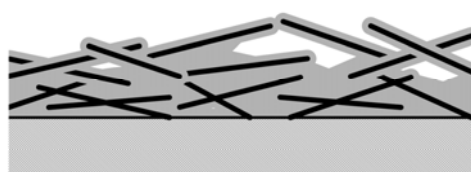
System with WPI fibrils plus HMP



Fibrils adsorbed on WPI/HMP covered interface



HMP adsorbs on surface and around fibrils. With more layers of fibrils, holes in between fibrils are filled



Additional layers: HMP adsorbs on surface and around fibrils. With more layers of fibrils, holes in between fibrils are filled

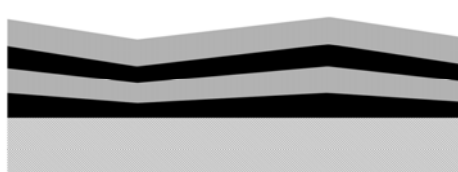
System with two molecularly dissolved polymers



Molecular layer of polymer adsorbed



Second layer adsorbs; roughness is slightly increased



Additional layers: roughness slowly increases as layers adsorb slight more on protruding patches

Figure 5: Comparison between film assembly by LbL adsorption from HMP and WPI-fibrils (left) and two molecularly dissolved polymers (right).

Figure 4 shows the change in the external topography of the microcapsules as the number of adsorbed layers increases from 5 to 10. As more layers adsorb, the surface of the capsules becomes smoother and defects (pinholes, fractures) will become scarcer. The roughness introduced with the adsorption of each WPI-fibril layer (Figure 4 a, c and e) is thus reduced by the following adsorption of a HMP layer. This suggests that, while the WPI-fibrils help building up a more or less open structural frame on the microcapsule by non-ideal stacking of the fibrils on the surface, the HMP adsorbs in the gaps between the fibrils, therewith healing the defects present in the early stages of the shell formation (also schematically depicted in Figure 5). This observation is confirmed by AFM measurements of the same materials adsorbed on hydrophobized silicon oxide flat surfaces (Figure 6). One can see also here a reduction in the overall roughness of the

surface, which indicates that there is a reduction in the number of defects in the capsule layer, as was also visible in the SEM images.

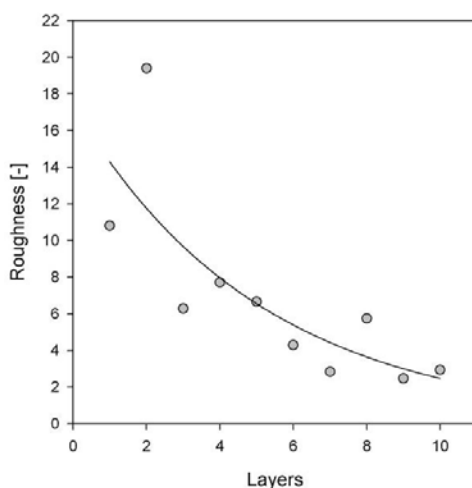


Figure 6: Evolution of surface roughness during the LbL adsorption process, as obtained from AFM measurements. The first layer corresponds to WPI, the rest of the even layers corresponds to HMP, and the rest of the odd layers to WPI-fibrils.

Although one cannot directly compare our results with other systems, there are some similar results reported in literature. Fery *et al.*²⁷, using the system poly(acrylic acid)/poly(allylamine), also found a reduction in roughness with increasing number of layers, however in their system the roughness approached an asymptote after the adsorption of 10 layers. This reduction in roughness is not general for LbL systems: for the system poly(vinylpyrrolidone)/ poly(methacrylic acid) a roughness increment of 5 nm was found with every two additional layers²⁸. Considering the envisioned encapsulation applications the roughness reduction with the WPI-fibrils/HMP system is advantageous since the capsules will become more concise and less penetrable with increasing number of layers. Given the healing of defects that may occur with additional layers, we expect that the pH response and the mechanical stability of the capsules will be non-linear with the number of layers.

Even though the SEM pictures of the microcapsules do indicate defects, the actual thickness of the capsules grows linearly as depicted in Figure 7 for capsules of 8 μm and 12 μm . Given the characteristic (smallest) dimensions of HMP (46 nm) and WPI-fibrils (10 nm), this is remarkable and indicative of high amounts of deposited material. The thickness we obtained is also high when compared with other LbL systems, like the 22 nm obtained for 14 layers of polyallylamine hydrochloride/ poly(sterenesulfonate)²¹ or 250 nm obtained for 40 layers of the system PAH/i-carrageenan²⁹. It seems to be a characteristic of LbL systems to show exponential increase of the shell thickness with the first few layers^{30, 31}, followed by linear increase. This might be related to the manner of adsorption as depicted schematically in Figure 5, where the fibrils stack irregularly on the interface. HMP then adsorbs in this irregular stack. In the next cycle, the fibrils cannot diffuse into the layer as HMP can do, due to their large dimensions; thus, the next layer will be relatively porous. With subsequent adsorption cycles, the voids are gradually filled in, and the adsorption process becomes more linear. It has been reported that exponential growth takes place during the first 4-6 adsorption cycles³². The transition from exponential to linear may therefore be related, for our system, to a transition from an open, porous shell with many defects, to a closed shell that has progressively fewer defects with more layers. In contrast, in a system with two polymers that were molecularly dissolved prior to adsorption, the exponential growing is caused by the diffusion of the newly added polyelectrolyte into the growing shell^{33, 34}.

The variability observed in the shell thickness arises from the random adsorption mechanism of these biopolymers/colloidal particles, as shown by simulations³⁵. In each step, as more and more material adsorbs, the overall charge of the microcapsule changes, which after a certain threshold results in a barrier to adsorption, leaving uncovered areas on the surface of the microcapsule. Those uncovered patches will still locally present the charge of the previous adsorption step and, therefore, will not be available for the next one, resulting in a thinner patch, to be further completed with the adsorption of more layers.

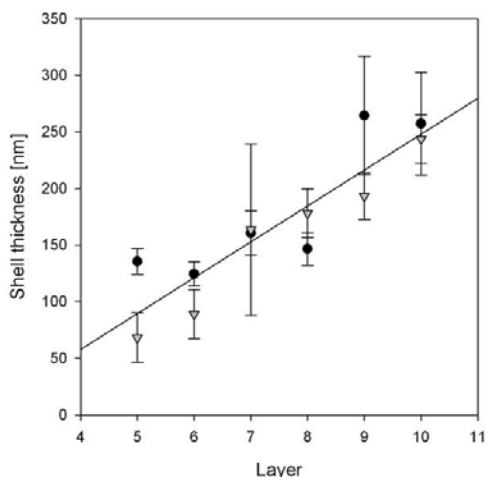


Figure 7: Shell thickness increment during LbL adsorption. Circles are capsules from a batch of 12 μm diameter, and inverted triangles are capsules from a batch of 8 μm . The solid line represents the linear regression of the data, provided to guide the eye.

The mechanical strength of the capsules was investigated by AFM; the maximum indentation for each case is similar to the shell thickness measured with SEM (Figure 7), which ensures that the measurements were made in the linear elastic response regime³⁶. The results for the Young modulus (E) as function of the indentation depth and the number of layers adsorbed on the microcapsules (Figure 8) is in agreement with the qualitative results from Figure 4. Capsules with 5, 6, and 7 layers are much weaker than the ones with 8 or more layers. The remarkable change in the quality of the microcapsules from 7 to 8 layers is also reflected here, where, instead of showing a steady, linear increase in the capsules' strength, capsules with 8 or more layers are 2 to 3 times stronger than the capsules with fewer layers. The maximum values of E are high and comparable to the ones reported elsewhere for 14 layers of cross-linked poly(vinylpyrrolidone)/poly(methacrylic acid)²⁸ (with a shell thickness of around 28 nm), or for 11 layers of poly(allylamine hydrochloride)/poly(styrenesulfonate)³⁷ (with a shell thickness around 24 nm). When compared to poly(diallyldimethylammonium chloride)/poly(styrenesulfonate)³⁸ (with a shell thickness around 28 nm), our system presents E values that are around 6 times higher, although it should be mentioned that

strict comparisons are difficult given the different experimental conditions and models used in each work. It is however clear that the capsules prepared here are very strong, and comparable to chemically cross-linked systems.

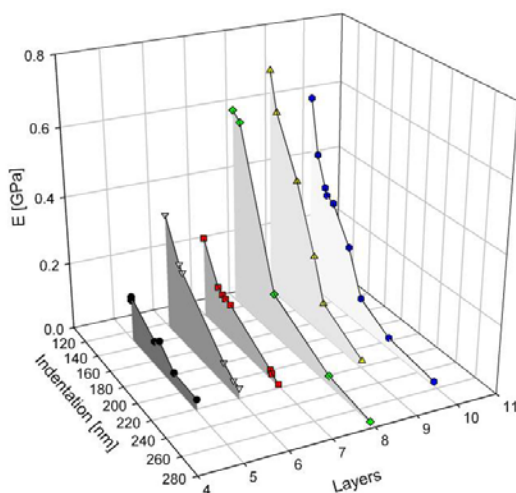


Figure 8: Effect of the number of adsorbed layers on the mechanical response of microcapsules.

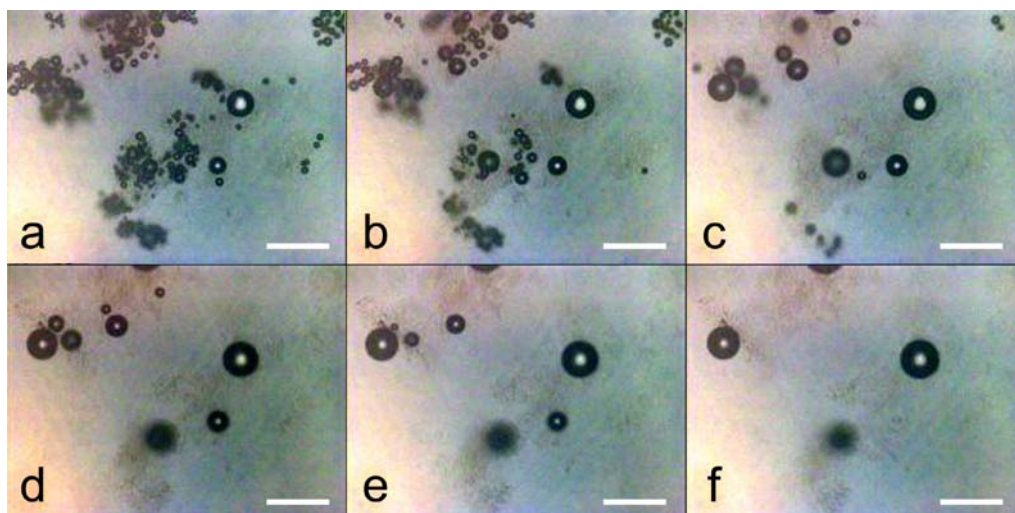


Figure 9: Selected frames during the stability tests of LbL-microcapsules with 5 layers at pH 7. a) 0 min, b) 1 min, c) 5 min, d) 10 min, e) 15 min, f) 30 min. Scale bar represents 50 μm.

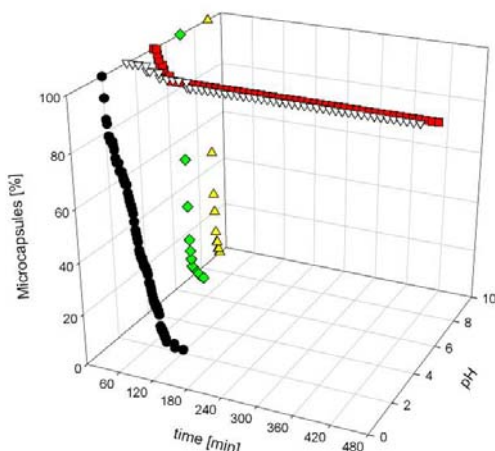


Figure 10: Response of LbL-microcapsules with 5 layers to various pH's as function of time.

Figure 9, shows a time sequence from a pH response experiment with 5-layered capsules at pH 7.0. It is clear that at this pH the capsules dissolve rapidly. The capsules were tested at various pH conditions, which are summarized in Figure 10. The pH values were chosen such that the results could be linked to the expected response of the microcapsules during digestion. All the buffers had a constant ionic strength of 10 mM, therewith allowing us to focus on only the effect of the pH. When starting from our base case, pH 3.5, which is the pH at which the microcapsules are assembled, the decay is very slow and stops after 2 hours, with almost 95% of the capsules remaining intact after 8 hours of exposure. It was expected that at this pH, the capsules would be very stable. The loss of 5% of the microcapsules corresponds most probably to capsules damaged during freeze drying. When increasing the pH to 5.2, which is the isoelectric point of β -lactoglobulin, the major component of WPI and WPI-fibrils, still 89% of the capsules remain intact during 8 hours at that pH. Most probably, positive patches in the WPI-fibrils that may have become more compacted during drying hold the structure of the shell together. At pH 2, the HMP has lost its negative charge, therewith causing the almost total dissolution of the microcapsules after 2 hours. However, dissolution of capsules is

much more marked at neutral or basic pH-values, where both β -lactoglobulin and HMP are positively charged, and thus repel each other. For capsules with 5 adsorbed layers, total dissolution happens after 30 minutes at pH 7, and 12 minutes at pH 9.

Since the capsules are very unstable at pH 7, we decided to take this pH as a starting point for the evaluation of the effect of the number of layers (see Figure 11 for the results of 5 - 10 layered capsules). Capsules with 5, 6, and 7 layers totally fill with buffer after 30 minutes, while capsules with 8, 9, and 10 layers seem to be unaffected. This shows that, at least for pH 7 and an ionic strength of 10 mM, the defects in the shell are more important for the capsule stability than the desorption of HMP and WPI-fibrils. This is not only the case for the pH stability, but also for the mechanical strength as discussed previously.

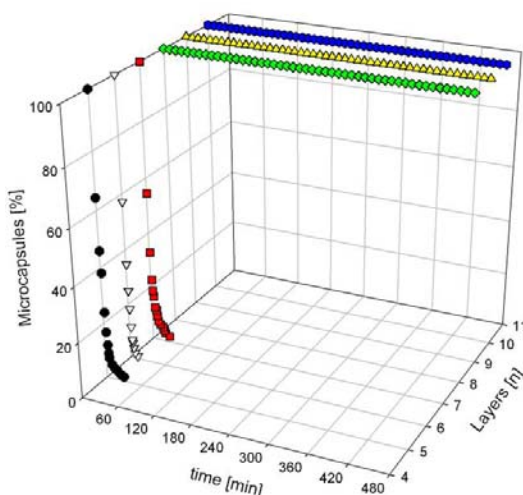


Figure 11: Response of LbL-microcapsules with various numbers of layers as function of time at pH 7.

The charges distributed over the long WPI-fibrils provide many simultaneous ‘anchoring’ points for the same fibril, therewith making it difficult for them to desorb. Besides, the drying of the capsules will compact and possibly reinforce the shells. All these effects seem to be stronger for capsules with more layers. In literature, cracks in microcapsules shells have been mentioned as one of the causes for failure to protect e.g. probiotics in

dry products⁶. In our system, the number of cracks and pinholes is reduced drastically after the adsorption of 8 layers, suggesting that these capsules offer better protection in dried products.

When investigating the dissolution behavior of the capsules in detail with the help of polarized light, we could confirm that even if the capsules 'collapsed' because of intruding liquid, the shell did not dissolve, not even when the buffer had totally penetrated the capsules. Only when using more extreme conditions such as pH 12 with an ionic strength of 30 mM, the shells could be truly dissolved (data not shown). We can therefore conclude that the capsules show great internal integrity.

To obtain better understanding of the microcapsules' response to a pH change we emulated the adsorption/desorption process adsorbing the WPI, HMP and WPI-fibrils on a hydrophobized flat silicon oxide surface, and we followed the process by reflectometry (Figure 12). The first two adsorption cycles give a relatively small effect on the signal, while the effect on ΔS becomes larger for consecutive layers. This may be because more adsorbed material provides more anchoring points for the next layer, and better intertwining of the layers; however, since a change in ΔS is a result of all the components present this cannot be concluded with certainty. We can conclude that the 10 layer system shows a very different behavior when exposed to different pH-values. At pH 3.5, as expected, the system was very stable, as was the case in the tests with the microcapsules. A change to pH 7 quickly causes desorption of all the material, except for the layer directly adsorbed to the template surface. This is in agreement with our observations on the capsules that disintegrate fast. At pH 2, desorption is much slower, with the mass equivalent to more than 4 layers still adsorbed after 45 minutes. We expect that slowly peeling off the outer layers by desorption could eventually expose the defects of the inner layers, as was the case for the 5-layered capsules presented in Figure 10 (pH 2). The results obtained by reflectometry are generally in very good agreement with the pH stability tests done on dried microcapsules.

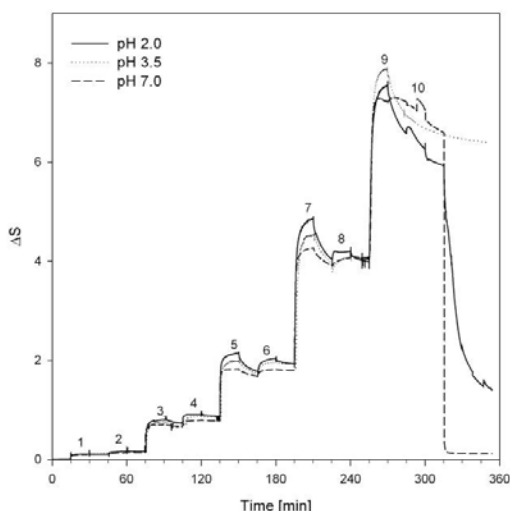


Figure 12: LbL adsorption of a 10-layered film of WPI (1), HMP (2 and even numbered layers) and WPI-fibrils (3 and higher odd numbered layers), and consecutive effect of pH on desorption as followed by reflectometry.

Conclusions

The LbL adsorption of WPI, HMP, and WPI-fibrils on the surface of oil droplets offers a flexible route to assemble microcapsules. The use of oil droplets as colloidal templates ensures the control of the size distribution of the resulting microcapsules, while the LbL scheme delivers control on the capsules' shell thickness with nanometric precision. The inclusion of WPI-fibrils reinforces the capsules, obtaining a denser, thicker, and stronger shell compared to other LbL systems present in literature. The adsorption of each layer not only increases the shell thickness, but also heals defects that may be present in the previous layers, presenting a non-linear transition in surface roughness and elastic modulus when going from capsules with 7 or less adsorbed layers, to capsules with 8 or more layers. The capsules with 8 or more layers are very stable at various pH values.

In summary, the LbL method presented here, that only uses food grade components, allows us to tune the properties of mechanically strong capsules to a desired response

as function of pH through the number of layers applied on the template oil droplet of which the size can be chosen at will.

Acknowledgements

The authors thank Adriaan van Aelst for his help with the SEM analyses, Remko Fokkink for his help with the reflectometry experiments, Mike Kleijn for her help with the AFM measurements, and Xiaoli Zhao and Shuyan Shi for experimental work.

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Chapter V

Encapsulation by a hybrid colloidosome/layer-by-layer technique

Abstract

Although many different methods for microencapsulation are known, the mechanical strength of the capsules and the costs related to their production prevent large scale application. We present here a method to produce microcapsules combining the assembly of colloidal particles at interfaces with electrostatic layer-by-layer (LbL) adsorption using a soft template. A strong encapsulate with porous walls is obtained that can be used as an easy-to load scaffold. The pores in the walls can be closed and healed through subsequent adsorption of more layers. All ingredients used in the assembly with the LbL-colloidosomes are food-grade and inexpensive.

This chapter has been submitted as: Rossier-Miranda, F.J., Schroën, K., Boom, R., Encapsulation by a hybrid colloidosome/layer-by-layer technique

Introduction

It is undeniable that microencapsulation has been a hot topic in recent years. An abundant and always increasing number of techniques can be found in literature¹, usually with claims about applicability in various industries²⁻⁴, including food and pharma. However, many of these techniques can not be applied in the food industry, since they involve non-food grade materials or materials that are simply too expensive to be commercially interesting for low-added value products like foods.

Two routes for microencapsulation have attracted special attention, namely assembly by layer-by-layer adsorption⁵ (from now on called LbL) and assembly from Pickering emulsions; the resulting capsules are called colloidosomes⁶.

LbL microcapsules are assembled from pairs of polymers that can interact with each other, usually by electrostatic interaction. These polymers are adsorbed sequentially in layers on a template, which can be a solid particle or a soft body such as an oil droplet. The size distribution of the resulting microcapsules is determined by the size and monodispersity of the template used. The shell thickness, along with its permeability⁷ and mechanical properties⁸, is determined by the number of adsorbed layers.

Colloidosomes are assembled from colloidal particles which are locked at the interface of oil-in-water or water-in-oil emulsion droplets. Generally, it is easier to assemble colloidosomes from water-in-oil emulsions⁹, as most colloidal suspensions are aqueous. The template (e.g. the oil droplet) once more determines the size of the resulting colloidosomes; however in contrast to the LbL systems the resulting shell has distinctive pores between the particles in the shell. The pores define the colloidosome's permeability¹⁰; further the shape and nature of the colloidal particles co-determine the mechanical properties of the shell¹¹⁻¹³.

Two aspects are important for colloidosome assembly. The first aspect is the wettability of the particles to be used: ideally, to seat efficiently in the interface, they have to be more or less equally wetted by both fluids. The second aspect is the mechanism used to lock the particles together. To tune the wettability, the particles can be chemically

modified; as locking mechanism, particles can be fused together by heating the system near the melting or glass temperature of the colloidal particles to induce sintering, by introducing a second phase that glues the particles together, or by cross-linking with e.g. glutaraldehyde¹⁴ or polylysine¹⁵.

An issue common to any encapsulation method is to establish a route to load the active component into the microcapsule¹⁶. With colloidosomes, loading can take place through the droplet that is used as a template: oil droplets may be pre-loaded with hydrophobic components, or with water droplets holding hydrophilic substances, or both. For microcapsules formed by layer-by-layer deposition, the active component should be inside the template before the deposition process is completed.

In this chapter, we take a different approach to make microcapsules: we modified the classic route to assemble colloidosomes and complemented it with LbL deposition. The presence of (non-porous) particles in an LbL system drastically reduces the permeability of the shell, and increases the mechanical stability without the need for numerous adsorption steps, as was noted earlier with the assembly of protein fibrils as reinforcement material¹⁷. In the current work, we created first an open capsule that can be loaded with various components after creation of the wall, which can be completed and closed later through the deposition of more layers. Since we target applications in food, we limit ourselves to food grade and cost effective ingredients only.

Experimental

Materials

The following materials were used as received: n-hexadecane (Merck, synthesis grade), sodium chloride (Merck, analytical grade), hydrochloric acid (Merck, analytical grade), sodium hydroxide (Merck, analytical grade), formic acid (Aldrich, reagent grade), whey protein isolate (WPI, Davisco Food International), and high methoxyl pectin (HMP, CP Kelco). Two kinds of silica particles were obtained from Microparticles Berlin GmbH, one modified with amino groups, with a diameter of 0.5 μm (SiO_2NH_2), and one modified with carboxyl groups, with a diameter of 0.6 μm (SiO_2COOH).

Methods

LbL-colloidosomes preparation

The microcapsules were prepared following the layer-by-layer electrostatic adsorption method, schematized in Figure 1. A soft template was prepared by emulsifying *n*-hexadecane in a solution of WPI 0.1% w/w in formate buffer at pH 3.5 and 10 mM NaCl, using an Ultra-Turrax homogenizer (IKA T18 basic with a S18N-19G dispersion tool) at 24000 rpm during 5 minutes. The template oil droplets were separated from the serum by mild centrifugation, at 94 RCF during 1 hour, and draining. The recovered cream, made from concentrated oil droplets covered with WPI, was washed three times with buffer to remove any excess of non-adsorbed WPI. The positively charged concentrated cream was then ready for addition of the next layer. Since at the set pH WPI and SiO_2NH_2 are positively charged, and HMP and SiO_2COOH are negatively charged, it is possible to adsorb them sequentially following the same described procedure. In this way various hybrid LbL-colloidosomes were assembled with 2 layers (WPI- SiO_2COOH), 3 layers (WPI/HMP- SiO_2NH_2), 4 layers (WPI/HMP- SiO_2NH_2 -HMP), 5 layers ($2\times(\text{WPI/HMP})$ - SiO_2NH_2), and 7 layers ($2\times(\text{WPI/HMP})$ - SiO_2NH_2 -WPI/HMP), and the degree of closing and position of the components were investigated. The concentrations used in each layer were determined from the size distribution and number of templates available in each adsorption cycle, in order to avoid bridging flocculation. Once the capsule was complete, the template oil droplet was removed by freeze drying in a Christ Epsilon 2-6D (Osterode, Germany) under the same conditions described in the work of Sawalha et al¹⁸.

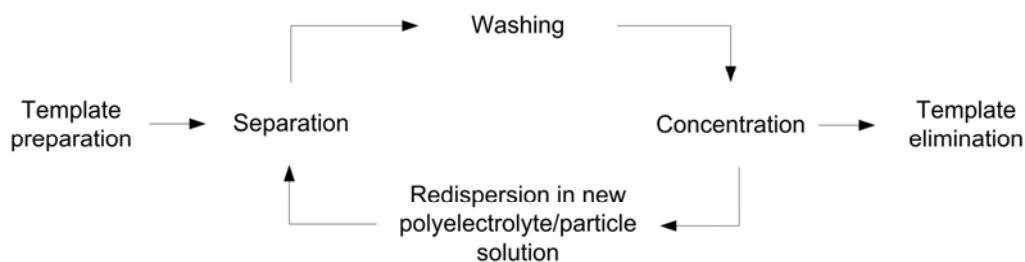


Figure 1: Scheme for the layer-by-layer adsorption method.

Characterization of LbL-colloidosomes

After each adsorption cycle, the size distribution and the ζ -potential of the LbL-colloidosomes were determined by light scattering using a Malvern Mastersizer 2000 and a Malvern ZetaSizer Nano, respectively. Light microscopy (Zeiss Axioplan microscope equipped with a MotionPro HSC system) was used to check for cluster formation. For SEM, freeze-dried microcapsules were placed onto brass holders with double-sided sticky carbon tape (EMS, Washington, U.S.A.). The microcapsules were attached to the sticky layer with pressurized air. The specimen holders with the LbL-colloidosomes were sputter coated with 10 nm Platinum in a dedicated preparation chamber (CT 1500 HF, Oxford Instruments, Oxford UK). The analysis was performed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at room temperature at a working distance of 8 mm, with SE detection at 3.5 kV. Images were digitally recorded (Orion 6 PCI, E.L.I. sprl. Belgium) and optimized and resized by Photoshop CS.

Reflectometry experiments

Adsorption experiments were performed in a stagnation flow cell (a.k.a. reflectometer as described in the work of Dijt *et al.*¹⁹), with 0.1% w/w solutions of WPI or HMP. Silicon oxide stripes, with an oxide layer thickness of 80 nm, were prepared and hydrophobized with dimethyldichlorosilane as in the work of Schroën *et al.*²⁰ Following each adsorption step there was a washing step with formate buffer. Both the adsorption and the washing time were fixed at 15 minutes each, within which the adsorbed amount reached its maximum value. The refractive index increments used were 0.15 for the HMP²¹ and 0.18 for the WPI²².

Results and Discussion

Hybrid LbL-colloidosomes were assembled from SiO₂ particles adsorbed on oil droplets that were previously covered with WPI and HMP layers or combinations thereof. Figure 2a shows a typical micrograph of LbL-colloidosomes assembled from SiO₂ microparticles adsorbed on a single layer of WPI. These LbL-colloidosomes are stable in solution, provided that the particle concentration is above the saturation concentration, and below

the depletion concentration²³. However, they are not stable enough to survive the template removal; Figure 2b shows a colloidosome that has collapsed after freeze drying. Nevertheless, the close-up in Figure 2c shows that a single layer of WPI seems to 'glue' some of the SiO₂ microparticles together.

The amount of protein adsorbed at the oil/water interface generally corresponds approximately to monolayer coverage²⁴, after equilibrium has been established. Protein that is not adsorbed properly is most likely removed during the washing steps²⁵. The amount of material that can be adsorbed in a single adsorption cycle is therefore limited, and not sufficient to stabilize the colloidosome during freeze drying. We thus decided to use the layer-by-layer technique to increase the amount of material in the interface.

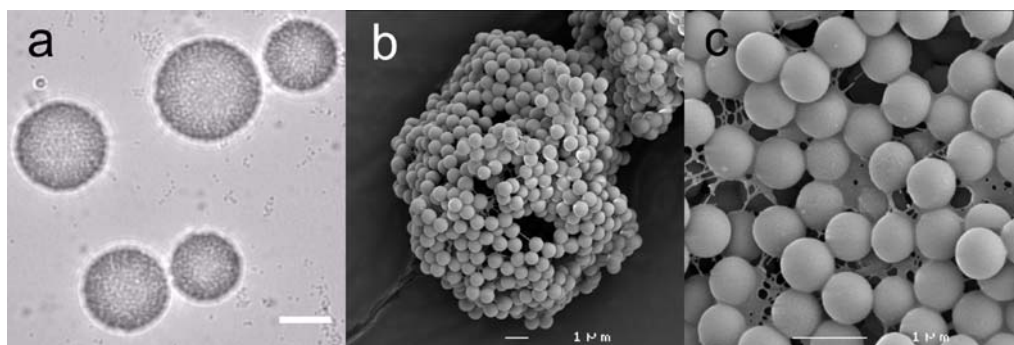


Figure 2: LbL-colloidosomes assembled from SiO₂ particles adsorbed on a single layer of WPI, (a) Micrograph of microcapsules as prepared in liquid, without removal of the oil template (scale bar represents 10 μm) (b) SEM picture after removal of the oil template (c) Detail of SiO₂ particles glued together by a layer of WPI.

Figure 3 shows a typical charge inversion plot found for the investigated LbL systems. When the charge inversion is complete, we observe rather constant ζ -potentials around +56 mV for a SiO₂-NH₂ layer, and -14 mV for a HMP layer. It is interesting to note that the ζ -potential of a layer of WPI adsorbed on an oil droplet (+39 mV) differs significantly from that of a WPI layer adsorbed on a HMP layer (around +7 mV), and from the ζ -potential of WPI dissolved in the formate buffer (around +17 mV). At pH 3.5 all the main proteins of WPI (β -lactoglobulin, α -lactalbumin, and albumin) are well under their isoelectric points, and therefore positively charged. When in solution, WPI proteins are in

the globular form and expose only part of their charged groups; however, when adsorbed on the oil interface they are expected to reorganize and expose their most hydrophobic portions to the oil, and the most hydrophilic (and therefore charged ones) to the continuous phase²⁶, therewith causing this difference in ζ -potential.

When adsorbed on a HMP layer, part of the charge of WPI is compensated by the electrostatic interaction with HMP. Although the remaining charge is not as high as that of a WPI layer on the interface of an oil droplet, it is sufficient to adsorb the next layer of HMP without neutralizing the charge of the LbL-colloidosome. We also found that adsorption of a layer of SiO_2COOH on a layer of WPI is not enough to cause charge inversion, probably due to a low surface coverage. This should however not be a problem for adsorption of subsequent layers, since charge inversion is not a prerequisite for assembly of LbL systems²⁷.

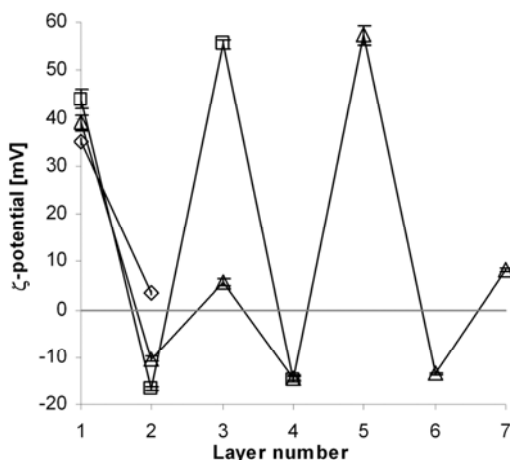


Figure 3: ζ -potential of adsorbed layers. SiO_2 on a single layer of WPI (◇), SiO_2 on a WPI/HMP sequence and covered with a single layer of HMP (□), and SiO_2 on a double WPI/HMP sequence and covered with a HMP/WPI sequence (Δ).

Figure 4 shows typical examples of LbL colloidosomes prepared from oil droplets covered by one (a) or two (b) WPI/HMP sequential layers. In both cases, the SiO_2 particles are sufficiently locked together to maintain the spherical shape after template

removal, but the coverage is much higher with a double WPI/HMP layer. It is expected that the low surface coverage of the single layered system was caused by the template removal by freeze drying. Since the oil has to be removed as a gas at low pressure, much volume has to escape from the capsule; this may have created buildup of internal pressure, which ruptured part of the wall. The system with two layers is more robust and withstood the pressure buildup. Detailed images of the walls of both capsules are shown in Figure 5.

The interstitial phase obtained after a single WPI/HMP sequence with SiO_2 particles (figure 5a) seems considerably stronger than that by a single WPI layer (Figure 2c) but many open spaces remain between the particles (Figure 5a). The available WPI/HMP amount is apparently not sufficient to close the capsule. A double WPI/HMP sequence forms a continuous membrane that deforms without breaking upon adsorption of the particles, which remain embedded in this matrix (Figure 5b).

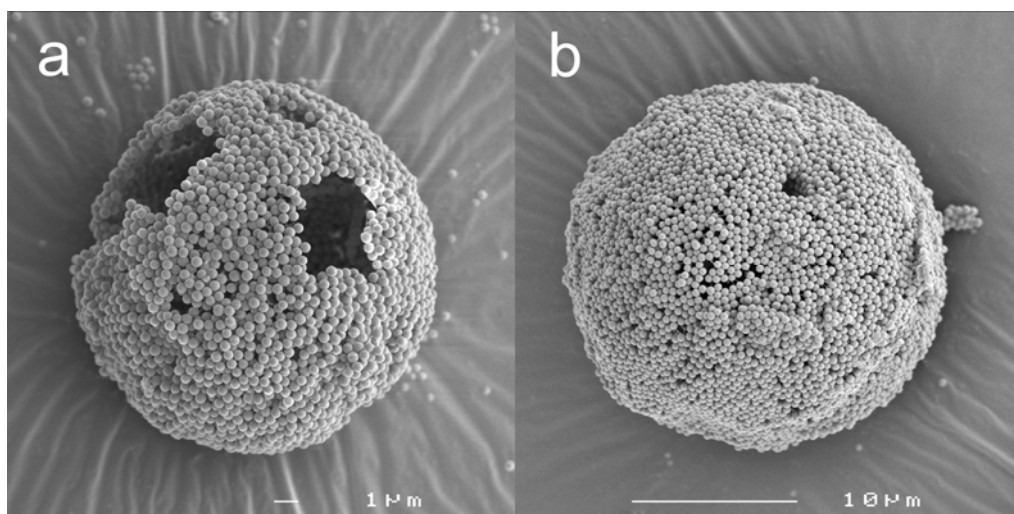


Figure 4: LbL-Colloidosomes. (a) SiO_2 microparticles adsorbed on a single (WPI/HMP) sequence. (b) SiO_2 microparticles adsorbed on a double (WPI/HMP) sequence.

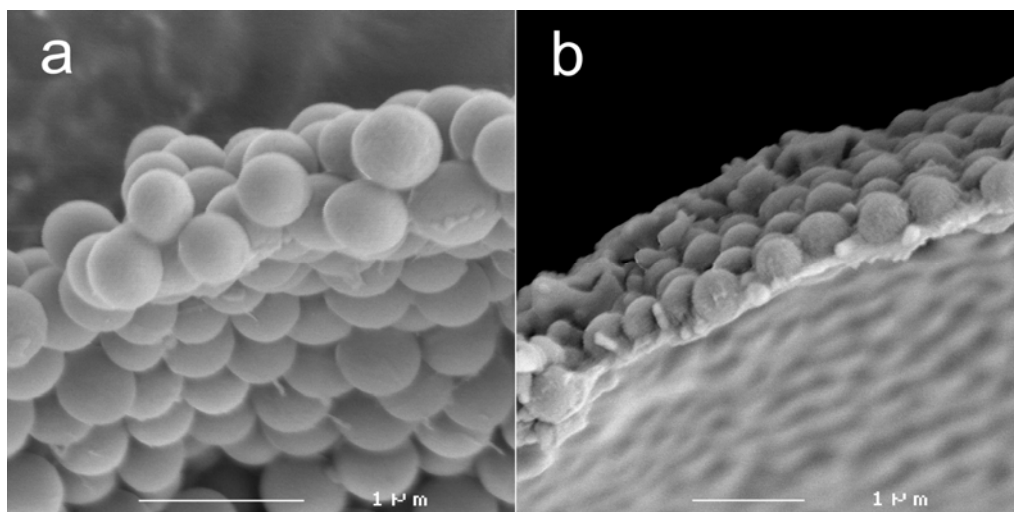


Figure 5: Details of SiO₂ particles and supporting WPI/HMP sequences. (a) SiO₂ microparticles adsorbed on a single WPI/HMP sequence. (b) SiO₂ microparticles adsorbed/embedded on/in a double WPI/HMP sequence.

Since the deposition of the interstitial (cement) layer is of great influence on the construction of the capsules, we decided to emulate this part of the capsule formation, by adsorbing HMP and WPI sequentially on a flat, hydrophobized silicon oxide surface. The adsorbed mass of polymers in each step was followed by reflectometry. Figure 6 shows a typical curve for the adsorption of two WPI/HMP sequences. The amount of adsorbed WPI in the first layer (1.4 mg/m^2) is in the normal range for proteins like β -lactoglobulin at oil/water interfaces²⁵, and the adsorbed amount of HMP in the second layer is also close to values of pectin adsorbed on proteins at an interface, as reported elsewhere²⁸. However, the mass adsorbed in the following layers showed different behavior, the mass of WPI adsorbed in the third layer is more than 6 times higher than the first one, and the adsorbed amount in the fourth layer is 6 times higher compared with the second one. This additional adsorbed mass in the third and fourth layers explains, at least in part, the big differences that were observed in stability of the capsules with the single layer, and the single and double sequence of WPI/HMP. The non-linear deposition of polyelectrolyte has been observed both experimentally and theoretically²⁹⁻³⁰, especially when biopolymers are used. When one layer is adsorbed, the polyelectrolyte can diffuse

into the previous layer, (depending on polyelectrolyte size, stiffness, and charge density) and form contacts in and on the growing layer, therewith increasing the total space available for adsorption.

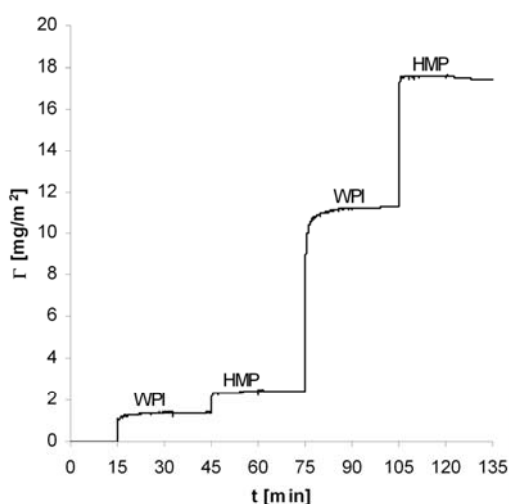


Figure 6: Adsorbed amount during multilayer formation on hydrophobized silicon oxide by the sequential electrostatic LbL adsorption of WPI and HMP.

Apart from the adsorption of WPI and HMP, the deposition of the colloidal particles is important. As can be seen in Figure 2c, the SiO_2 particles that are adsorbed on a single WPI layer seem to be glued by their equatorial area, instead of adsorbed by their polar areas, and the WPI between particles forms strands that seems to be stretched. We expect that this is caused by the excess adsorption of the SiO_2 particles. In figure 2c, only a small amount of WPI is present in a very thin film on the droplet. After the first particles have adsorbed, new particles may have only limited contact with the WPI film, due to steric hindrance of the previously adsorbed particles. There is thus a balance between hydrodynamic forces dragging the particles away, and the elastic force from the WPI, attaching it to the capsule. For some particles the drag will be too large, while others will be retained. At the end, the capsule is covered by those particles that were retained with just enough WPI^{31,32}. This explains the stretched strands between the particles, and the roughness of the capsule.

In the system with a single WPI/HMP sequence (figure 5a) much more WPI and HMP is present, as is shown by figure 6. The amount of biopolymer present to fill the interstitial spaces between the particles is much larger. At the same time, the surface charge is not higher (Figure 3), since the WPI and HMP partially compensate each other's charge. As a result, the amount of biopolymer relative to the amount of charge is higher. The amount of particles that adsorbs is determined by the overall surface ζ -potential: the surface charge is neutralized and then reverted to its equilibrium charge (due to the charge of the particle that are not covered by the polyelectrolytes; Figure 3). Thus, there is more WPI/HMP phase present to fill the interstitial space between the particles, and the layer is denser than with a single WPI layer.

In case of the system with a double WPI/HMP bilayer (Figure 5b), there is even more WPI/HMP present, while the surface has the same surface ζ -potential. Therefore, while a similar amount of particles will adsorb (dictated by the overall surface potential), there is more WPI/HMP phase present to fill the interstitial spaces; therefore these walls are less porous.

Even though the systems with the double WPI/HMP sequence retain their shape better, they are still somewhat porous. This enables us to load the capsules with active components, after their assembly. A prerequisite is that one needs to be able to close the remaining pores after the loading. As a proof of principle, we attempted to close the scaffolds with extra layers of WPI/HMP (Figure 7). It is clear that a single HMP layer is not enough to close a capsule having one WPI/HMP bilayer, but when starting from a system with a double WPI/HMP bilayer, the adsorption of an extra HMP/WPI sequence can close the microcapsule pores, as shown in Figure 7b. Thus, it can be concluded that closure of the porous scaffolds is possible as long as the primary capsule has sufficiently low porosity.

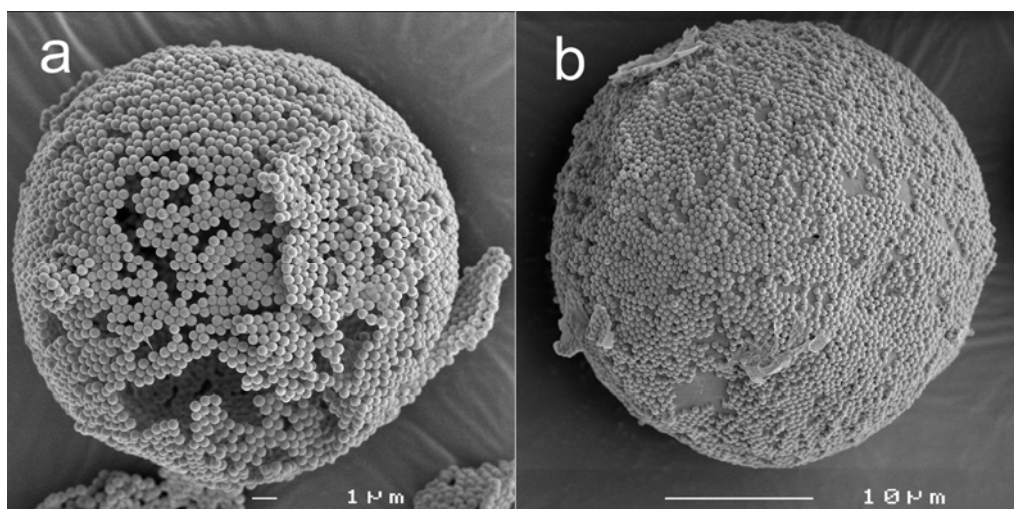


Figure 7: LbL-Colloidosomes. (a) SiO₂ microparticles adsorbed on a single WPI/HMP sequence and covered with a single HMP layer. (b) SiO₂ microparticles adsorbed on a double WPI/HMP sequence and covered with a HMP/WPI sequence.

Conclusions

A hybrid method to prepare microcapsules was presented, combining layer-by-layer adsorption of whey protein isolate (WPI) and high methoxyl pectin (HMP), and deposition of SiO₂ colloidal particles for reinforcement. The stability and porosity of the capsules can be altered by adsorbing more layers, but even after deposition of 2 layers of WPI and 2 layers of HMP and a SiO₂ layer, the capsules were still somewhat porous. This creates the possibility to load the capsules after their assembly with an active component. It was shown that the remaining pores can be closed by an additional HMP/WPI sequence.

In summary, the presented method allows the creation of a primary capsule that can be loaded and then closed at will, using inexpensive food-grade materials, while all process steps are operated at room temperature. We therefore expect this method to be of interest for application in the food and pharma industries.

Acknowledgment

The authors thank Adriaan van Aelst for his help with the SEM analyses, Remko Fokkink for his help with the reflectometry experiments, and Jolet and Riëlle de Ruiter for preliminary experiments.

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Chapter VI

*Integrated microfluidic circuit for
electrostatic layer-by-layer
microcapsule assembly*

This chapter has been submitted as: Rossier-Miranda, F.J., Schroën, K., Boom, R.,
Integrated microfluidic circuit for electrostatic layer-by-layer microcapsule assembly

Introduction

Microcapsules, which can be used for encapsulation of active ingredients, can be prepared by various methods. The properties of the capsules, which determine where and when the content will be released, obviously depend on the preparation method and, frequently, the conditions during preparation cannot be controlled such that all capsules have the same properties.

An improvement to this is the system we recently presented; we prepared microcapsules of controlled size, with high, tunable mechanical strength, from food grade materials, using electrostatic layer-by-layer (LbL) adsorption¹ (inset of Figure 1). Oil droplets containing the active ingredient are stabilized with whey protein isolate (WPI) at low pH, creating a positively charged template that is used as a starting point for the sequential adsorption of negatively charged high methoxyl pectin (HMP) and positively charged WPI-fibrils. Although this methodology is flexible and accurate, it is a time consuming batch process. This is mainly because each adsorption stage needs to be carried out with an excess of polyelectrolyte in solution, to avoid bridging of the microcapsules. After this, the non-adsorbed polyelectrolyte has to be removed before the next adsorption step takes place to avoid complexation with the other polyelectrolyte.

In e.g. stirred vessels, the polyelectrolyte concentration and stirring conditions should be chosen such that the chance of two capsules meeting before completion of the layer is negligible² and mostly this is 'solved' by vigorous agitation or sonication in combination with a low template particle concentration (typically under 5% v/v). Besides, polyelectrolyte concentrations are chosen higher than the saturation concentration, of which the excess needs to be removed in a later stage. If the capsules could be kept mechanically separated and the polyelectrolyte is allowed sufficient time to cover the surface the process could be carried out at much lower concentrations, which would also make the rinsing steps superfluous. In this note, we present a system that is operated at low concentrations which are just enough to cover the available surface, therewith eliminating the need for removal of polyelectrolyte excess. At the same time, we aim to

keep the particles separated sufficiently long by having the particles in purely laminar flow.

To our knowledge only one approach has been used for continuous assembly of LbL microcapsules³. With the help of microfiltration membranes and continuous agitation, the microcapsules could be kept in suspension, while being rinsed with different polyelectrolytes solutions, to adsorb up to 21 layers. However, intermediate washing steps were still needed to remove excess material. This approach is suitable for components that can pass the membrane pores but unfortunately this is not the case for bigger colloidal particles such as our WPI-fibrils, contributing to the necessity for a new approach. In addition, any process that will be applied on larger scales has to be efficient in terms of ingredients and water use and waste generation.

Microfluidics have been used to synthesize microcapsules by polymerization⁴⁻⁸, solvent diffusion^{9, 10}, and crosslinking¹¹⁻¹⁵, although in most cases the microfluidic device was used only as an emulsification tool to ensure accurate control over the microcapsule size. In those cases, even though the preparation takes place in the chip, the actual cross linking or solvent evaporation step is carried *off-chip*. To the best of our knowledge, the only reported microfluidic device used for assembly of microcapsules by LbL adsorption *on-chip* can be found in the work of Priest and collaborators¹⁶. They assembled a three-layered microcapsule, of poly(methacrylic acid) and poly(N-vinylpyrrolidone) on a template droplet of liquid crystal. Their system included a separate rinsing stage, in which the excess of polymer was removed with a comb withdrawal channel, which acts as a concentration step. The authors also pointed out that to go to a higher number of adsorption cycles, the system would become much more complex, as would the pumping infrastructure. Often, the infrastructure around a microfluidic device is much larger than the device itself and is responsible for most of the volume and costs of the system. In the work presented here we focused on the preparation of LbL capsules in a relatively simple system that does *not* require complex pumping or valves to control the flow in the microchip.

The main challenge of this work is to keep the microcapsules separated and continuously flowing in laminar flow, while using very low polyelectrolyte concentration, thus eliminating the need for washing steps. This was done by adjusting the length, *i.e.* the hydraulic resistance, of different sections of the microfluidic circuit to control the different flow rates and residence times needed for the electrostatic LbL assembly, and we will show the proof of principle here.

Experimental

The designed microchips consist of three main channels; one for the template emulsion droplets, one for a solution of high methoxyl pectin (purchased from CP Kelco), and one for a solution of WPI- fibrils. The template emulsion was prepared by emulsifying 1% w/w hexadecane in a 0.3% w/w WPI solution at pH 3.5. A coarse emulsion was prepared with a rotor stator homogenizer operated for 5 minutes at 24000 rpm (IKA Ultra-Turrax T18 basic, with a S18N-19G dispersion tool). This coarse emulsion was loaded in a pressurized vessel to be further emulsified using the pre-mix emulsification technique¹⁷. The loaded emulsion was passed 10 times through a filter with a pore size of 5 μm (Millipore Millex-SV, PVDF Durapore) under 1 bar of pressure of nitrogen. This results in oil droplets with a diameter of 5 μm , positively charged due to the WPI that stabilizes them. A concentrated cream was separated from the solution by mild centrifugation (1 hour at 70 RCF in a centrifuge Beckman Coulter Allegra X-22R, rotor SX4250). Any remaining free WPI was washed out by a three-fold redispersion/centrifugation cycle with 10 mM formate buffer at pH 3.5. The clean, concentrated cream was then redispersed in the formate buffer to give the 5% v/v solution used in the experiments. WPI-fibrils were prepared with a modified version of the methodology developed by Bolder et al¹⁸. Shortly, the pH of a 2% w/w WPI in MilliQ water solution was adjusted to 2 using 6 M HCl. The solution was then heated to 80 °C under continuous stirring at 600 rpm during 10 hours, obtaining WPI-fibrils with typical dimensions of around 1 μm in length and 10 nm in thickness. Both, the WPI-fibrils and the HMP were diluted with 10 mM formate buffer before their use in the microfluidic LbL assembly.

Microfluidic circuit design

97

bringing one of the two polyelectrolytes in contact with the oil template sequentially. In each of these contact points the template emulsion droplets in the main stream are diluted, and the resulting volumetric flow rate in the main channel increases. This means that when the solution merges with the emulsion at a junction the distance of the droplets increases by a factor equal to the flow rate of the solution divided by the flow rate of the emulsion.

To keep the residence time constant in each stage, the length of each section at the main channel needs to be adjusted:

$$L_{m,n} = L_{m,n-1} \cdot \frac{Q_{m,n}}{Q_{m,n-1}} \quad (1)$$

Where L_m is the length of the main channel that carries the emulsion, and Q_m its volumetric flow rate. Due to the dilution, less surface area will be available for adsorption after each junction. Since we want to use only one inlet for HMP and one for WPI-fibrils (that is, we do not want to tailor the concentration of polymer to be dosed at each junction), the volumetric flow rates at each connection channel have to be chosen accordingly:

$$Q_{c,n} = Q_{c,n-2} \cdot \frac{Q_{m,n-2}}{Q_{m,n}} \quad (2)$$

Where Q_c is the volumetric flow at the connection channels.

To control the volumetric flow rates of the connection channels it is necessary to adjust the hydraulic resistances of the distribution channels for pectin and fibril, as was proposed by Kim *et al.*¹⁹ for their serial dilution microchip. For construction simplicity, we chose to work with channels that have the same width and depth. We use an analogous approach to those known for electric circuits (Figure 2). In this analogy the relation between pressure drop, hydraulic resistance and volumetric flow rate can be treated as the relation between voltage, electric resistance and electric current²⁰.

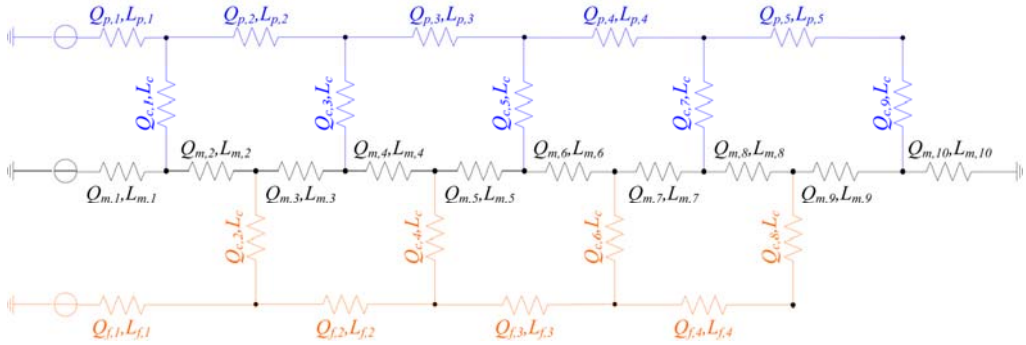


Figure 2: Electric circuit network analogy for the design of a microfluidic chip that can be used to assemble 10-layered microcapsules.

The network can be solved using Kirchhoff's voltage and current law. For example, for the first loops including the second connection channels of HMP and WPI-fibrils we have, according to Kirchhoff's voltage law:

$$\begin{aligned} Q_{p,2} \cdot R_{p,2} + Q_{c,3} \cdot R_c - Q_{m,3} \cdot R_{m,3} - Q_{m,2} \cdot R_{m,2} - Q_{c,1} \cdot R_c &= 0 \\ Q_{f,2} \cdot R_{f,2} + Q_{c,4} \cdot R_c - Q_{m,4} \cdot R_{m,4} - Q_{m,3} \cdot R_{m,3} - Q_{c,2} \cdot R_c &= 0 \end{aligned} \quad (3)$$

The equations for the other loops can be constructed in a similar way. Once the lengths needed in the distribution microchannels were determined, we designed microfluidic devices for the LbL assembly of microcapsules with 6, 8, and 10 layers, which implies that the outer layer consists of HMP and will be negatively charged. Figure 3 shows the circuit for the assembly of 10-layered microcapsules schematically and in real.

The designed microfluidic devices were produced in borosilicate glass by Micronit Microfluidics BV (Enschede, the Netherlands). The microfluidic device consists of a lower plate, in which the channels are (chemically) etched, annealed to a top plate with inlets. After enclosure, the main and distribution microchannels have a uniform depth of 50 μm and width of 600 μm , which implies that the channels are flat (much wider than deep). The dimensions of the connection channels were 300 μm in width and 50 μm in depth.

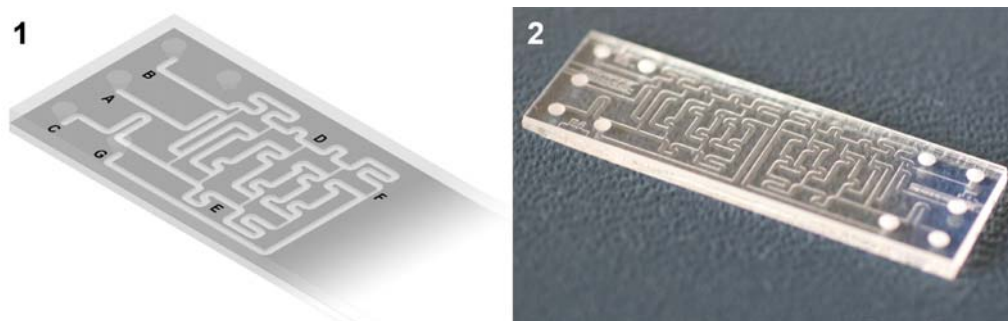


Figure 3: 1. Design of the microfluidic circuit for the assembly of 10-layered microcapsules. (A) template emulsion inlet, (B) pectin inlet, (C) fibrils inlet, (D) Distribution channel for pectin, (E) distribution channel for fibrils, (F) connection channel, (G) outlet. 2. Glass microfluidic chip for the assembly of LbL microcapsules with 10 layers featuring two of the circuits depicted in (1).

Results

The flows in the microchips were first checked without using the full assembly set-up. Latex tracer particles (5 μm) were injected in the chip, and through particle tracking we could confirm the volumetric flows in the different sections of the circuit. When injecting the polyelectrolyte solutions in excess, in absence of the template emulsion, we could ratify that the different connection channels dosed each polyelectrolyte at the right location in the circuit. This is illustrated in Figure 4a: the concentration of the formed complex increases after contact with each of the connection microchannels.

Next, we used the circuit to adsorb HMP and WPI-fibrils on 5 μm hexadecane droplets stabilized by WPI (Figure 4b). The microcapsules were collected at the outlet and were washed by the three-fold redispersion/centrifugation procedure described above, to determine their ζ -potential (Malvern ZetaSizer Nano, for which the average ζ -potential was calculated from 5 measurements on a sample). We could thus confirm that the droplets, with an original ζ -potential of around +17 mV changed to a ζ -potential of around -12 mV (chip for 6 layers), -10 mV (8 layers), and -8 mV (10 layers). These values are very close to the standard ζ -potential value found for microcapsules with an outer layer of HMP (around -13 mV) when prepared by our batch method.

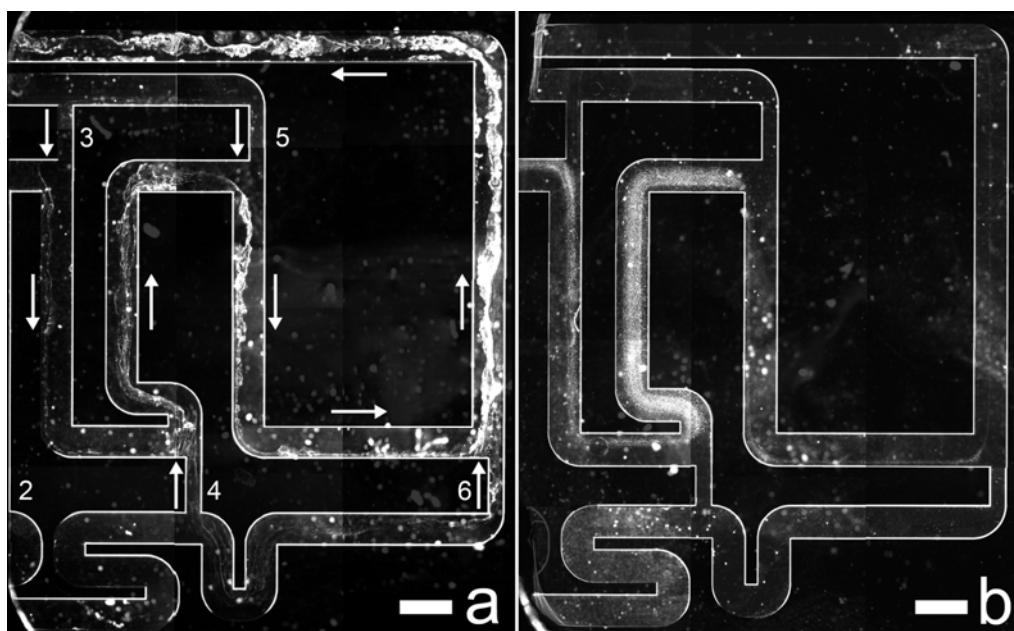


Figure 4: Polarized light microscopy image of the microfluidic circuit for the assembly of 6-layered microcapsules. (a) HMP and WPI-fibrils are dosed in excess at the distribution channels, confirming the increase in concentration of the HMP/WPI-fibril complex after contact with each connection channel. The numbers indicate the number of the layer being dosed, and the arrows the flow direction. (b) Template emulsion droplets flowing in the main channel, receiving the next polyelectrolyte and diluting after contact with each connection channel. The scale bar represents 1 mm.

Conclusions

The system that we have introduced here is simple; it only uses three ingoing flows, and the capsules can be obtained from the outlet without the need to remove any excess polyelectrolyte material. Increasing the number of layers only requires chips that are somewhat more complex, however the number of ingoing flows and thus the complexity of the integration of the device with the outside world does not change. A drawback could be that the capsules are diluted on their way through the chip; however this effectively shields the capsules from flocculation as well, and standard concentration methods can be used to concentrate the assembled capsules. Since the microfluidic chip is specifically designed for this purpose, and given its fixed set of hydraulic resistances, the circuit can

handle different sets of flow rates, as long as the proportionality between them is constant and equal to the one considered during the design. This allows for flexibility in the residence time desired for each adsorption step.

We believe that using this kind of microfluidic circuits can simplify the assembly of capsules by the layer-by-layer technique, and lead to better control over the assembly process, not just for the current system, but also for other combinations of biopolymers.

Acknowledgements

The authors thank Miguel Suárez for experimental work.

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Chapter VII

General discussion and outlook

This last chapter summarizes the findings in earlier chapters, and gives a perspective on future developments in the field of microencapsulation. The number of publications in the area that appear per year has multiplied in the last two decades (Figure 1a), and this is also the case for the techniques that are used in this thesis, such as self assembly and microfluidics (Figure 1b). However, the combination of microencapsulation and microfluidics has not yet gained much momentum and we see in this genuine opportunities.

A wide variety of materials and microencapsulation systems are reported in literature. In spite of this variety, only a few preparation methods are applied on industrial scale. Most of these methods have a physical-mechanical basis (see Figure 2). These methods allow poor control over the encapsulate size and their stability, processability, and release properties. Interfacial self-assembly, the approach taken in this thesis, should allow the assembly of encapsulates with significantly better defined properties.

In Chapter 2 we reviewed the production of colloidosomes, a method for microencapsulation exploiting self-assembly. We showed that well defined encapsulates could be obtained taking advantage of the natural interactions present among its building blocks. With this route porous and remarkably strong microcapsules can be obtained in few steps, given the use of rather 'large' building blocks of colloidal size. That inspired in this work the search for a methodology for microencapsulation using interfacial self-assembly using building blocks that are themselves either colloidal or, even more interesting, self-assembled in their own right.

Chapter 4 presented microcapsules assembled exclusively from common food grade materials (whey protein isolate and pectin) via another self-assembly method, *i.e.* electrostatic layer-by-layer adsorption. When using only molecularly dissolved materials, the layer-by-layer assembly produces microcapsules with very thin shells. The methodology presented here, however, used novel protein fibrils to reinforce the shells. These protein fibrils are, in turn, also product of the self-assembly of peptides ordered under shear¹. With the adsorption of each new layer the shell thickness and mechanical strength of the microcapsules could be adapted to specific requirements. This illustrates

that the application of a two-stage self-assembly strategy may well yield a range of new possibilities for the preparation of functional materials. In the same direction, Chapter 5 shows that microcapsules built with the layer-by-layer method could be greatly reinforced with the inclusion of colloidal particles, such as silica particles, in an hybrid colloidosome/layer-by-layer methodology.

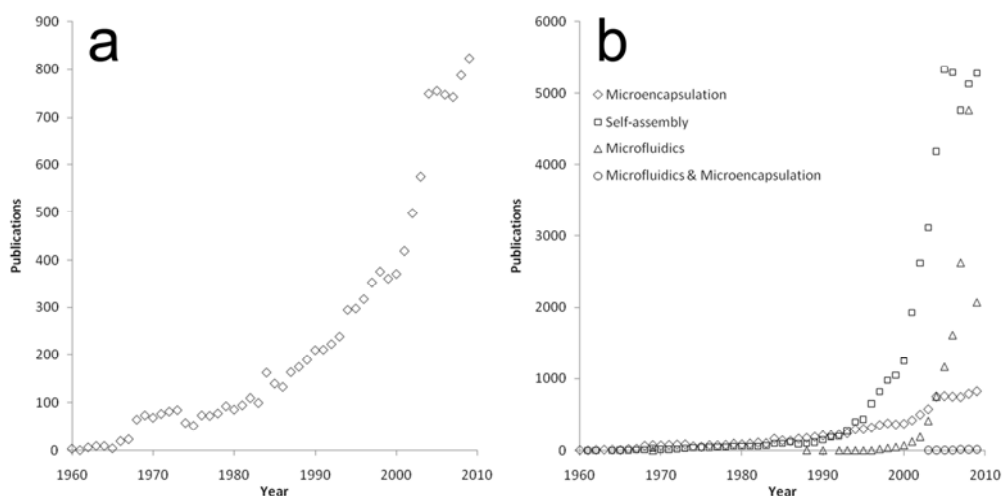


Figure 1: (a) Publications about microencapsulation during the last 50 years and (b) comparison with the publications on related topics such as self-assembly and microfluidics.

Chapters 3-5 show just a few of many possible ingredient combinations and process conditions that can be used to prepare microcapsules. New types of protein fibrils are becoming available, such as the ones assembled from hen egg white lysozyme³, from ovalbumin⁴, or bovine serum albumin. Apart from those that can be prepared directly by self-assembly from naturally occurring proteins others, genetically engineered for self-assembly (e.g. recombinant gelatin-like and collagen-like proteins) have already been used for controlled release from hydrogels^{5, 6} that may be reinforced with carbon nanotubes⁷. Obviously, not all these materials can be used in food, but it is clear that the range of components that can be used is continuously increasing. Apart from the protein fibrils, the pectin used in this study as negatively charged polyelectrolyte may itself carry

prebiotic effects⁸. By careful choice of the components, systems may be created that have synergistic function; not only with the physical functionality as building block⁹, but also as nutritionally functional component.

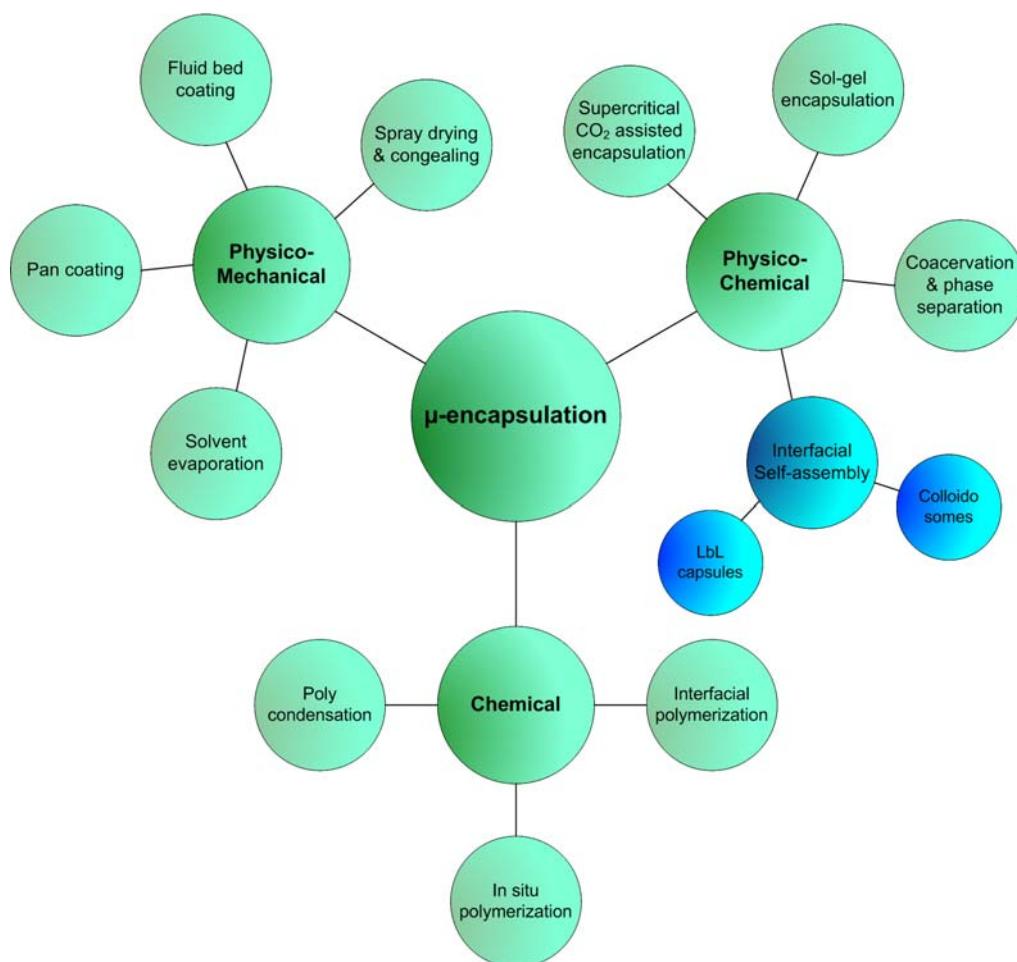


Figure 2: Microencapsulation methods, adapted from Venkata et al.² to include interfacial self-assembly.

Regarding the loading of the microcapsules one can make use of the fact that the capsules are only gradually built up, as shown in Chapter 5. By first applying only e.g., 5 layers, one can first prepare an encapsulate that is still open. The capsule could then be

loaded and subsequently apply a few more layers which will close the pores. Regarding release, the functionality may be expanded from a pH trigger as was shown in Chapter 4. By using thermoresponsive materials¹⁰ in the shell, one might trigger the release of the load by localized heating, perhaps during product preparation, during consumption, or in medical applications by using infrared light. For this last category of applications one may also envisage the use of magnetic particles¹¹ in the shell, which would enable the accumulation of the encapsulates at specific locations inside the body by applying a magnetic field.

In Chapter 4, reflectometry is used to investigate *in-situ* layer-by-layer adsorption on a model surface. The adsorption on an emulsion droplet may well be somewhat different, given the different nature of the interface and its curvature, but the method did give us a good indication of the amounts and dynamics of the layer-by-layer process. Recently, a technique based on holographic optical tweezers was used to determine the size of milk fat globules with nanometric precision, and their refractive indexes¹². This technique might be used to follow the adsorption of polyelectrolytes on the droplets. By comparing these results with the (better defined) results on the model flat surfaces, more information may be obtained about the actual adsorption mechanisms.

It was further demonstrated that the strength of the composite protein fibrils/pectin obtained from layer-by-layer adsorption was comparable to systems with chemical crosslinking. Despite the large number of publications about microencapsulation, only a few deal with determination of the mechanical characteristics, which is indicative of the challenges in measuring these properties. While the strength has to be measured on the scale of the encapsulate, there is a risk of measuring only local properties that are not representative of the whole microcapsule. Our choice was to test the elasticity of a large number of microencapsulates using an atomic force microscope, to minimize the risk of only measuring local properties. The alternative, to observe the deformation of the microcapsules under changes of osmotic pressure¹³, demands fluorescent labeling that could affect the interaction between the building blocks of the microcapsule and is not suitable for dry samples.

Although the use of colloidal-scale building blocks such as protein fibrils and silica particles was shown to lead to better properties with fewer layers, the preparation is very laborious. Industrial application would require a quick and simpler production method. In Chapter 6 it was shown that microfluidics, a rapidly developing area in its own right (Figure 1b), may contribute to better microencapsulation methods. Using a system with small dimensions ensures that the encapsulates will remain separated from each other as they are being formed, due to the Stokes flow and due to their alignment in the centre of the microchannels. This suppresses their flocculation, which is difficult to avoid otherwise without extensive washing and rinsing. Of the thousand publications on microfluidics per year only a dozen relate to microencapsulation and, in most cases, it is only used to generate the template for the microcapsule¹⁴; *i.e.* an emulsion droplet. In Chapter 6, we introduced a microfluidic device intended to truly accomplish layer-by-layer assembly *on-chip*, without the need to use excess peripheral infrastructure.

The use of microfluidic circuits like the proposed may well offer a better control and simplify the assembly of capsules by the layer-by-layer technique, opening new opportunities for faster screening of candidate combinations of templates, polyelectrolytes, and colloidal particles for microencapsulation. Static mixing structures, and/or sections for emulsification may be included for the generation of monodisperse droplets *on-chip*, by means of T-junctions¹⁵, Y-junctions¹⁶, flow focusing¹⁷ or the recent spontaneous droplet generator system coined as EDGE¹⁸. Figure 3 shows an integrated design, that includes a plateau for EDGE emulsification plus the channel circuit necessary for the layer-by-layer deposition sequence, in a standard 10-hole borosilicate glass microfluidic chip.

While such a microfluidic device would deliver one-step encapsulate preparation, it would do so only on small scales. This would be useful for screening purposes but it is still far from the scale required for production in industry. We can however make a rough estimation of what the required size of a microfluidic device would be for commercial scale production of microcapsules. The recommended concentration for the probiotic bifidobacteria in dairy products is 10^7 cfu/ml¹⁹. If we want to produce enough

microencapsulated probiotics to enrich 1 m³ per hour of a product we need then to encapsulate 10¹³ cfu.

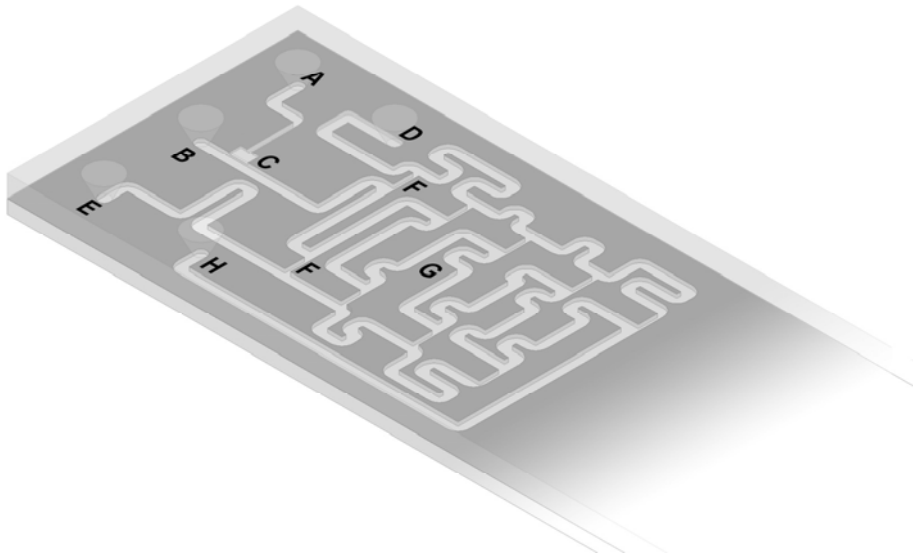


Figure 3: Concept for microfluidic chip for droplet generation and LbL assembly on a standard 10-hole borosilicate chip. The oil that enters in (A) spontaneously generates monodisperse templates on the plateau at (C), where it meets the continuous phase injected in (B). Pectin injected through (D) and protein fibrils injected through (E) are dosed at the connection channels (F) according to the hydraulic resistances of the circuit. The layer-by-layer assembly occurs in the main channel (G) at the end of which the multilayered microcapsules leave the circuit at the outlet (H).

The typical thickness of the encapsulate walls synthesized with the layer-by-layer technique is 100-200 nm. If we use a microcapsule of 20 µm this would imply that 97% of the volume is available for the probiotics. If we use 90% of this available volume the amount of microorganisms per microcapsule would be 1.2x10³ cfu. We would therefore need to produce around 8.3x10¹⁰ microcapsules per hour.

To avoid clustering we need to keep the microcapsules at a relatively low concentration of 5 % v/v (they may be later concentrated for application in a product). Table I shows

the volume of possible production units to produce 8.3×10^{10} microcapsules per hour for different scenarios, given current technology for bonding up to 20 wafers of borosilicate in a stack, to form one mass-parallelized microfluidic device. With the current design a unit of 61 liters would be needed (Case 1), but this can be easily improved if the layout of the microfluidic circuit is optimized and the maximum surface area of the wafers is used without affecting the bonding (Case 3). The second case illustrates the effect of a reduced packing of the microorganisms to 50% (implying a shell thickness of $4.1 \mu\text{m}$ for the same total microcapsule size of $20 \mu\text{m}$) of the volume of the microcapsule.

The best possible scenario is Case 4 which combines efficient system design with high encapsulate loading, plus a volume fraction of 10% v/v during synthesis, which may become possible by further optimization of the microchannel system. The system then needs to be just 20 liters.

Table I: Different scenarios for the evaluation of a microfluidic unit for layer-by-layer assembly

Case	Microfluidic design	Probiotics packing	Conc. at processing	Unit volume
	(% area microchannels)	(%v/v)	(%v/v)	(L)
1	33	90	5	61
2	50	50	5	73
3	50	90	5	40
4	50	90	10	20

The production of the template is not considered in this evaluation, but Van Dijke estimated that with a unit of 270 liters it was feasible to produce $1 \text{ m}^3/\text{h}$ of a 4% v/v emulsion¹⁸, and this is very close to the template volumes needed as the starting point for our layer-by-layer assembly. One could either generate the templates in a separated

first step (following Van Dijke's system), or integrate the droplet formation inside the chip, as is shown in Figure 3.

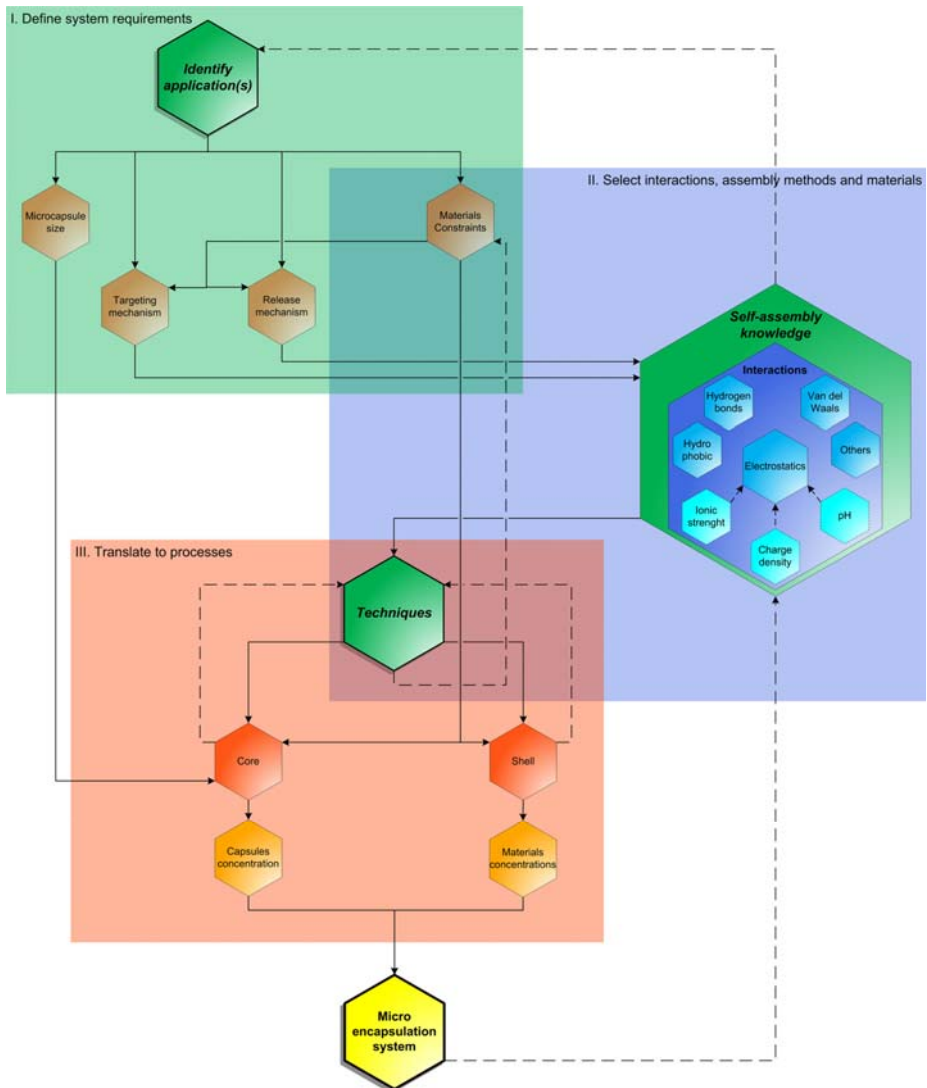


Figure 4: Diagram depicting the methodology for design of new microencapsulation systems. Dashed lines indicate feedback in the research scheme.

As stated throughout this chapter, integration of different aspects is needed for the successful design of new microencapsulation systems based on self-assembly. Figure 4 aims to summarize this, showing the path followed during this thesis, starting from a clear application for microencapsulation which narrowed the design of microcapsules down to the main parameters, and resulted in a working methodology. For example, for microencapsulation of probiotics, the size of the microcapsule should be 20 μm or less to avoid grittiness, the microorganisms should be released in the small intestine as a result of pH change, and obviously all materials used should be food grade and preferably inexpensive.

Once the trigger for release is defined, the right interaction for self-assembly can be identified. In our case, a change in pH pointed towards electrostatic adsorption, but there are others interactions that could be exploited as well, as shown in Figure 4. The size and uniformity are important for the uniformity of the release. This is an important factor in the choice of techniques for template production (membrane pre-mix emulsification) and the methodology for the assembly (sequential adsorption of polyelectrolytes). In our example the choice of the right methods to produce the core is a critical step, since the survival of probiotics during production is obviously affected by the emulsification technique used²⁰.

A microencapsulate is a complex assembly of several materials with complex behavior. Its design should be improved continuously using the experience gained during the screening for materials to polish the methodologies. Eventually, this can also inspire the creation new techniques, as indicated by the dashed lines in Figure 4.

In conclusion, microencapsulation may become an indispensable part of the food industry toolbox to create new, more attractive, smarter, and healthier functional products, when sufficiently robust and diverse encapsulation methods are available. This thesis has reported on the development of encapsulates using layer-by-layer adsorption. While other methods may be just as important, it may serve as an example of how the use of different types components may lead to better properties, but only when one combines understanding of the physico-chemical aspects of the assembly processes,

microfluidics for better control of microcapsule synthesis, and process engineering to scale up towards production volumes.

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Summary

Microencapsulation is the technique of enclosing valuable or delicate materials in small containers for targeted delivery. These containers may consist of a core and a thin shell surrounding it. The core, with a size ranging from a few to hundreds of micrometers, contains the active material, and serves as template for the microcapsule. The shell, with a thickness of no more than a few hundred nanometers, lends mechanical, physical, or chemical protection, and the means to release the active materials in response to a well defined trigger.

Microencapsulation is used in a number of industries, *e.g.* pharmaceuticals and food. In the case of medicines, microencapsulation is important for the delivery of drugs at the correct location, in the right dose, and at the right moment, which otherwise would have to be overdosed to reach the desired therapeutic effects. In the case of food, microencapsulation may mask ingredients that would cause off-taste and protect sensitive or volatile materials like flavors, aromas, and antioxidants. In addition, microencapsulation will enable new product concepts, such as products with new sensory experiences (fizzy products from encapsulated CO₂) and, more importantly, healthier foods including nutraceuticals or probiotics.

The microencapsulation methods that are used nowadays in industry on a fairly large scale allow limited control over the uniformity of the microcapsule size and geometry, and the thickness of the shell, while the harsh operation conditions pose restrictions on the type of ingredients that can be used. This poses a challenge to improve production methods and materials. Besides, specifically for food, the materials should additionally be food grade and inexpensive.

In this thesis, microcapsules (1-20 micron) are constructed via self-assembly; *i.e.* using driving forces already available in nature. From the routes using self-assembly proposed in literature for microencapsulation two called our special attention, both starting from an oil droplet as template which determines the size of the microcapsule. The first route is that of colloidosome preparation, in which colloidal particles adsorb irreversibly and

organize at the oil-water interface, creating a sturdy shell with pores defined by the interstices between the particles. The second route is electrostatic layer-by-layer adsorption: polyelectrolytes of opposite charge are sequentially adsorbed on a charged template, creating a thin film of which the thickness can be controlled with precision in the order of few nanometers. We have combined the two methods by using larger building blocks to construct stronger shells with better defined characteristics, using less adsorption cycles and based on food grade materials. These microcapsules are designed to protect an eventual encapsulated material from low-pH conditions and deliver their contents in response to a change in pH, having in mind applications that would require delivery through the stomach into the small intestine.

In *Chapter 2*, the available techniques to produce colloidosomes are reviewed. Microcapsules can be assembled from both, oil-in-water or water-in-oil emulsions, which translates in the potential ability to encapsulate hydrophilic and hydrophobic materials. Since the size of the microcapsules is defined by the droplet used as template the construction of almost perfectly monodisperse microcapsules is within reach, given the current developments in emulsification technology. The choice of size, geometry, and origin of the colloidal particles to be used to assemble the shell, and the means to lock them together, gives control over the targeting and release behavior of the colloidosomes. The requirement that always needs to be met is that particles should have affinity for the both oil and water at the interface, imposing a restriction on the choice of the to-be-used materials. Further, the methods usually applied to lock the colloidal particles to form the shell (e.g. heat up the microcapsule to sinter particles together) were seen as a drawback for the encapsulation of delicate materials such as foods, for which many active components are heat sensitive or volatile. Without significant sintering of the particles the capsule does not have protective properties. The next chapters are therefore devoted to new microencapsulation techniques that complement the colloidosome and the layer-by-layer adsorption routes.

In *Chapter 3*, the first results are presented on microcapsules prepared by sequential electrostatic adsorption of protein fibrils, with approximate dimensions of 1 μm in length

and 4 nm in width, and high methoxyl pectin. The encapsulation procedure was carried out at low pH (3.5), which allowed us to use positively charged oil droplets stabilized with whey protein isolate as templates. Confocal scanning laser microscopy showed that the fibrils adsorbed as an open structure on a layer of pectin in quantities much larger than a monolayer. This was confirmed from the shell thickness determined by scanning electron microscopy. The fibrils remained trapped at the surface after the adsorption of another layer of pectin. It could be shown indirectly that the inclusion of fibrils in the shell had a tremendous impact on the mechanical strength when compared to microcapsules made of layers of protein and pectin only. Since the microcapsules were assembled at low pH, under the action of pH- and ionic strength-dependent electrostatic interactions, we envision that these capsules would survive low pH, and increase their permeability, or totally disorganize, when exposed to a neutral pH. This is further elucidated in the next chapter.

In *Chapter 4* extensive characterization of the fibril-reinforced microcapsules is reported. Each layer (of fibrils or pectin) added approximately 30 nm to the total thickness, which is considerably more than monolayer coverage, as measured by reflectometry. The microcapsules keep their integrity when exposed to pH below 5.2 (showing slow dissolution at pH 2), but disintegrate at pH 7 or higher. The response is non linear for increasing number of layers, as it was the case for the mechanical strength of the microcapsules. While microcapsules with 7 or less layers had a similar Young modulus, microcapsules with 8 or more layers had twice that strength, around 0.6 GPa, comparable with the strength of polymeric microcapsules that are chemically cross-linked. This was related to the defects present in the shells as observed with scanning electron microscope: capsules with 8 or more layers had smooth and defect-free shells, which resulted in high pH stability.

The available food grade materials allowed the definition of a second system (to be assembled also at low pH) presented in *Chapter 5*. A single layer of charged silica particles was adsorbed on sequential layers of whey protein and high methoxyl pectin, therewith reducing the number of adsorption steps. Those pre-adsorbed layers stabilized

the adsorbed hydrophilic silica particles that, otherwise, would make a poor colloidosome structure. In this way a highly porous but strong structure that could be easily loaded was obtained. After loading the pores can be closed through the adsorption of additional layers of protein and pectin.

The drawback of the two microencapsulation systems described above is that polyelectrolytes needed to be used in excess, and intermediate washing steps to rinse out the non-adsorbed materials were essential. To overcome this we went a step ahead in *Chapter 6* using a microfluidic device to carry out layer-by-layer adsorption of up to ten layers of protein fibrils and pectin in continuous mode. The design of the chip is simple and does not require complex infrastructure around it, since it relies only on the right balance of the hydraulic resistances of different sections of the microfluidic circuit to control the dose of the materials for the microcapsules' assembly. Although issues like surface modification of the chip for long-run operation, and the scale-up of the process to industrially-interesting volumes are still a challenge, we feel that this is an important step forward toward controlled microcapsule formation.

The integration of knowledge on self-assembly, of which some examples can be found in this thesis, combined with the search for new food-grade materials that can act synergistically to assemble a smarter and multifunctional shell, and better design of microfluidics for tight control of this process are key to mature microcapsule formation into a real tool for the food industry. *Chapter 7* discusses further requirements for the production of reinforced layer-by-layer microcapsules using microfluidics, and closes this thesis with a general discussion of the results, in the light of possible future developments in the area of microencapsulation.

Samenvatting

Microencapsulatie is de techniek om waardevolle of delicate materialen in kleine capsules in te sluiten voor gerichte afgifte. Deze capsules kunnen uit een kern en een dun omhulsel bestaan. De kern, met een grootte die kan variëren van enkele micrometers tot enkele honderden van micrometers, bevat het actieve materiaal, and dient als mal voor de microcapsule. Het omhulsel, met een dikte van niet meer dan een paar honderd nanometers, biedt mechanische, fysieke, of chemische bescherming, en de mogelijkheid om actieve materialen vrij te geven naar aanleiding van een geschikte trigger.

Microencapsulatie wordt in een aantal industrieën gebruikt, bijvoorbeeld in de farmaceutische en levensmiddelenindustrie. In het geval van geneesmiddelen, is microencapsulatie belangrijk voor de afgifte van medicijnen op de juiste plaats, in de juiste dosis, en op het juiste ogenblik, die zouden moeten worden overgedoseerd om de gewenste therapeutische resultaten te bereiken. In het geval van voedsel, kan microencapsulatie ingrediënten, die een afwijkende smaak veroorzaken, maskeren en gevoelige of vluchtige materialen, zoals smaakstoffen, aroma's, en antioxidanten, beschermen. Bovendien zal microencapsulatie nieuwe productconcepten, zoals producten met nieuwe sensorische ervaringen (bruisende producten door geïncapsuleerd CO₂) en, nog belangrijker, gezonder voedsel inclusief supplementen (nutraceuticals) of probiotica mogelijk maken.

De microencapsulatie technieken die tegenwoordig in de industrie op vrij grote schaal worden gebruikt staan slechts beperkte controle toe over de uniformiteit van de microcapsule grootte en geometrie, en de dikte van het omhulsel, terwijl de extreme proces omstandigheden het type ingrediënt dat kan worden gebruikt beperkt. Dit vormt een uitdaging om productiemethoden en -materialen te verbeteren. Daar komt nog bij dat specifiek voor voedsel, de materialen geschikt moeten zijn voor voedingsmiddelen (food grade) en goedkoop.

In dit proefschrift worden microcapsules (1-20 micrometer) via zelf-assemblage geconstrueerd; d.w.z. door middel van drijvende krachten die al in de natuur voorkomen. Van alle routes die voor microencapsulatie in de literatuur voorgesteld worden en die zelfassemblage gebruiken, trokken twee onze speciale aandacht. Beide routes beginnen met een oliedruppel als mal die de grootte van de microcapsule bepaalt. De eerste route is die van colloïdosoom bereiding, waarin colloïdale deeltjes irreversibel aan het oliewater grensvlak adsorberen en zich organiseren, waardoor een stevig omhulsel met poriën, die door de ruimtes tussen de deeltjes worden gedefinieerd, wordt gevormd. De tweede route is elektrostatistische laag-voor-laag adsorptie: polyelectrolyten met tegengestelde lading worden opeenvolgend op een geladen modelvorm geadsorbeerd, waardoor een dunne laag, waarvan de dikte met een nauwkeurigheid in de orde van enkelen nanometers kan worden gecontroleerd, wordt gevormd. Wij hebben de twee methoden gecombineerd door grotere bouwstenen te gebruiken om sterkere omhulsels te construeren met beter gedefinieerde kenmerken, gebruikmakend van minder adsorptiecycli en gebaseerd op materialen die geschikt zijn voor levensmiddelen. Deze microcapsules zijn ontworpen om uiteindelijk ingekapselde materialen tegen lage pH waarden te beschermen en hun inhoud af te geven als de pH verandert, met in het achterhoofd toepassingen die passage van de maag en afgifte in de dunne darm zouden vereisen.

In Hoofdstuk 2 worden de beschikbare technieken om colloïdosomen te produceren besproken. Microcapsules kunnen uit zowel olie-in-water of water-in-olie emulsies worden geassembleerd zodat zowel hydrofiele als hydrophobe materialen geïncapsuleerd kunnen worden. Aangezien de grootte van de microcapsules door de druppel, die als mal wordt gebruikt, wordt bepaald, is de constructie van bijna perfecte monodisperse microcapsules haalbaar, gezien de huidige ontwikkelingen in emulgeertechnieken. De keus wat betreft de grootte, geometrie, en oorsprong van de colloïdale deeltjes die worden gebruikt om het omhulsel te assembleren, en de middelen om ze aan elkaar aan te binden, geeft controle over het te kiezen doelwit en het afgiftegedrag van de colloïdosomen. Een vereiste, waaraan altijd moet worden voldaan, is dat de deeltjes een affiniteit voor zowel de olie en het water aan het grensvlak moeten

hebben. Dit beperkt de keuze van de te gebruiken materialen. Verder, werden de methodes, die doorgaans worden toegepast om de colloïdale deeltjes aan elkaar aan te sluiten om het omhulsel te vormen (b.v. opwarming van de microcapsule om de deeltjes te sinteren), gezien als nadeel voor het inkapselen van delicate materialen zoals voedsel, waarvan vele actieve componenten warmtegevoelig of vluchtig zijn. Zonder voldoende sintering van de deeltjes heeft de capsule geen beschermende eigenschappen. De volgende hoofdstukken zijn daarom toegewijd aan nieuwe microencapsulatie technieken die de colloïdosoom en laag-voor-laag adsorptie routes complementeren.

In hoofdstuk 3 worden de eerste resultaten van microcapsules gepresenteerd, die door opeenvolgende elektrostatische adsorptie van eiwitfibrillen van ongeveer 1 μm in lengte en 4 nm breedte en pectine met een hoog methoxyl gehalte, geprepareerd zijn. De inkapselingsprocedure werd bij lage pH (3.5) uitgevoerd, zodat, als mal, positief geladen oliedruppeltjes gestabiliseerd met wei-eiwit isolaat gebruikt konden worden. Confocale laser scanning microscopie toonde aan dat de fibrillen in hoeveelheden die veel groter waren dan een monolaag adsorbeerden, en een open structuur vormden op een laag pectine. Dit werd door de dikte van het omhulsel, die door scanning elektronen microscopie werd bepaald, bevestigd. De fibrillen bleven aan het oppervlak zitten na de adsorptie van een volgende laag pectine. Indirect kon worden aangetoond dat het gebruik van fibrillen in het omhulsel een enorme invloed had op de mechanische sterkte, vergeleken met microcapsules die alleen van lagen eiwit en pectine werden gemaakt. Aangezien de microcapsules bij lage pH geassembleerd werden door de werking van pH en ion sterkte-afhankelijke elektrostatische interacties, voorzagen wij dat deze capsules een lage pH zouden overleven, en doordringbaar zouden worden of totaal zouden desorganiseren wanneer ze aan neutrale pH zouden worden blootgesteld. Dit wordt verder toegelicht in het volgende hoofdstuk.

In hoofdstuk 4 wordt de uitgebreide karakterisering van de fibril-versterkte microcapsules gerapporteerd. Elke laag (van fibrillen of pectine) voegde ongeveer 30 nm toe aan de totale dikte, wat aanzienlijk meer is dan de bedekking van een monolaag, zoals gemeten

door reflectometrie. De microcapsules hielden hun integriteit wanneer ze aan een pH lager dan 5.2 werden blootgesteld (langzame ontbinding bij pH 2 wordt aangetoond), maar de microcapsules desintegreerden bij pH 7 of hoger. De respons is niet lineair voor een toenemend aantal lagen, wat wel het geval was voor de mechanische sterkte van de microcapsules. Terwijl de microcapsules met 7 of minder lagen een gelijkwaardige Young modulus hadden, hadden de microcapsules met 8 of meer lagen tweemaal die sterkte, rond 0.6 GPa, vergelijkbaar met de sterkte van polymere microcapsules die chemisch zijn gecrosslinked. Dit werd geweten aan de mankementen in de omhulsels zoals waargenomen met scanning elektronen microscopie: de capsules met 8 of meer lagen hadden gladde omhulsels zonder mankementen, die in hoge pH stabiliteit resulteerden.

De beschikbare materialen die geschikt zijn voor voedingsmiddelen, stonden de definitie van een tweede systeem (dat ook bij lage pH kan worden geassembleerd) toe dat in hoofdstuk 5 wordt voorgesteld. Een enkele laag van geladen silica deeltjes werd op opeenvolgende lagen van wei-eiwit and hoge methoxyl pectine geadsorbeerd. Daarmee werd het aantal adsorptiestappen verminderd. Deze pre-geadsorbeerde lagen stabiliseerden de geadsorbeerde hydrofiele silica deeltjes, die anders een slechte colloïdosoom structuur zouden vormen. Op deze wijze werd een hoogst poreuze maar sterke structuur, die gemakkelijk zou kunnen worden geladen, verkregen. Na het laden kunnen de poriën door de adsorptie van extra lagen van eiwit en pectine worden gesloten.

Het nadeel van de twee, hierboven beschreven, micro-encapsulatie systemen is dat er een overvloedige hoeveelheid polyelectrolyten gebruikt moest worden en dat de tussengelegen wasstappen, om die niet-geadsorbeerde materialen weg te spoelen, essentieel waren. Om dit te overwinnen gingen we een stap verder in hoofdstuk 6, waar we een geïntegreerd microfluidisch systeem gebruikten om laag-voor-laag adsorptie op ononderbroken wijze, uit te voeren met maximaal tien lagen eiwitfibrillen en pectine. Het ontwerp van de chip is eenvoudig en vereist geen complexe infrastructuur, aangezien het gebaseerd is op slechts de juiste balans van de hydraulische weerstanden van de

verschillende secties van de microfluidisch circuit om de concentratie van materialen voor de assemblage van de microcapsules te beheersen. Hoewel kwesties zoals oppervlaktemodificatie van de chip voor langdurig gebruik, en het opschalen van het proces tot aan industriële volumes nog een uitdaging zijn, zijn wij van mening dat dit een belangrijke stap vooruit kan zijn voor gecontroleerde microcapsule vorming.

De integratie van kennis van de zelf-assemblage, waarvan sommige voorbeelden in dit proefschrift kunnen worden gevonden, gecombineerd met het onderzoek naar nieuwe materialen geschikt voor voedingsmiddelen, die synergetisch kunnen werken om slimmere en multifunctionele omhulsels te assembleren, en een beter ontwerp van microsystemen voor een strakke controle van dit proces, zijn zeer belangrijk voor de verdere ontwikkeling van microcapsulevorming tot een echt hulpmiddel voor de voedselindustrie. Hoofdstuk 7 bespreekt verdere eisen ten aanzien van de productie van versterkte laag-voor-laag microcapsules, gebruikmakend van microfluidische systemen, en sluit dit proefschrift met een algemene bespreking van de resultaten, in het licht van mogelijke toekomstige ontwikkelingen op het gebied van microencapsulatie, af.

Resumen

Microencapsulación es la técnica que permite encerrar materiales valiosos o delicados en pequeños contenedores para una dosificación dirigida. Estos contenedores pueden estar constituidos por un núcleo y, rodeándolo, un delgado revestimiento. El núcleo, de un tamaño de varios a cientos de micrómetros, contiene el compuesto activo y sirve de plantilla para la microcápsula. El revestimiento, de un espesor de no más de unos cientos de nanómetros, otorga protección mecánica, física o química, y el mecanismo para liberar el compuesto activo en respuesta a un estímulo bien definido.

La microencapsulación es usada en diferentes industrias, como por ejemplo en la farmacéutica y en la alimentaria. En el caso de la medicina, la microencapsulación es importante para la dosificación de medicamentos en el lugar correcto, en la dosis adecuada y en el momento justo. De otro modo, estas drogas tendrían que ser sobre-administradas para alcanzar los efectos terapéuticos deseados. En el caso de los alimentos, la microencapsulación puede enmascarar ingredientes que causen sabores indeseados y proteger materiales sensibles o volátiles, como sabores, aromas y antioxidantes. Además, la microencapsulación hará posible la conceptualización de nuevos productos, tales como artículos que otorguen nuevas experiencias sensoriales (por ejemplo CO₂ encapsulado en productos efervescentes) y, más importante, alimentos más saludables que contengan nutraceuticos o probióticos.

Los métodos usados hoy en día para microencapsulación en escala industrial solo permiten un control limitado de la geometría y tamaño de las microcápsulas, así como del espesor del revestimiento, mientras que condiciones de producción extremas imponen restricciones sobre los tipos de ingredientes que pueden ser utilizados. Esto plantea el desafío de mejorar los materiales y métodos productivos. Concretamente en el caso de los alimentos, los materiales deberían ser además de grado alimentario y económicos.

En esta tesis se construyeron microcápsulas (1–20 micrones) mediante auto-ensamblaje, es decir, usando las fuerzas motrices disponibles en la naturaleza. Dos de

las rutas propuestas en la literatura para el auto-ensamblaje llamaron nuestra atención. La primera ruta es la usada para preparar coloidosomas, en la que partículas coloidales se adsorben irreversiblemente y se organizan en la interfase agua-aceite, creando una cáscara sólida con poros definidos por los intersticios entre las partículas. La segunda ruta es la adsorción electrostática capa-a-capas: polielectrolitos de carga opuesta son adsorbidos secuencialmente sobre una plantilla cargada, creando una película delgada cuyo espesor puede ser controlado con precisión en el orden de unos pocos nanómetros. En este trabajo combinamos los dos métodos usando unidades estructurales más grandes para construir revestimientos más resistentes, con mejores características, usando menos ciclos de adsorción, y basados en materiales de grado alimentario. Estas microcápsulas están diseñadas para proteger al material encapsulado frente a condiciones de bajo pH, y para liberar su contenido en respuesta a un cambio de pH, teniendo en mente aplicaciones que requerirían dosificación en el intestino delgado.

El Capítulo 2 reseña las técnicas disponibles para producir coloidosomas. Estas microcápsulas pueden ser ensambladas tanto a partir de emulsiones de aceite-en-agua como de agua-en-aceite lo que se traduce, en principio, en la habilidad para encapsular materiales hidrofílicos e hidrofóbicos. Dado que el tamaño de las microcápsulas queda definido por la gota usada como plantilla, y dados los avances en tecnología de emulsificación, la construcción de microcápsulas casi perfectamente monodispersas se encuentra al alcance. La elección del tamaño, geometría y origen de las partículas coloidales que pueden ser usadas para ensamblar el revestimiento, y los métodos para inmovilizarlas, otorgan control sobre la focalización y el mecanismo de liberación de los coloidosomas. El requerimiento sine qua non es que las partículas deben tener afinidad tanto por el agua como por el aceite en la interfase, imponiendo una restricción en la elección de los materiales a ser usados. Además, los métodos aplicados usualmente para inmovilizar las partículas al formar el revestimiento (por ejemplo, calentar las microcápsulas para fundir las partículas entre sí) presentan una desventaja para la encapsulación de materiales alimenticios delicados, de los cuales muchos de sus componentes activos son termolábiles o volátiles. No obstante, sin un fundido suficiente

de las partículas la cápsula no otorgará sus propiedades protectivas. Los capítulos siguientes están, por lo tanto, orientados a nuevas técnicas de microencapsulación que complementen las rutas de coloidosomas y adsorción capa-a-capa.

En el Capítulo 3 se presentan los primeros resultados de microcápsulas preparadas por adsorción electrostática secuencial de fibrillas protéicas (de dimensiones aproximadas de 1 μm de largo y 4 nm de espesor) y pectinas fuertemente metiladas. El procedimiento de encapsulación se efectuó a pH bajo (3.5), lo cual permitió usar como plantilla gotas de aceite estabilizadas por proteínas del suero, y por lo tanto cargadas positivamente. Observaciones a través de microscopía confocal láser de barrido demostró que las fibrillas se adsorben, en cantidades mucho mayores que una monocapa, como una estructura difusa sobre una capa de pectina. Esto fue confirmado con la determinación del espesor del revestimiento a partir de imágenes obtenidas con el microscopio electrónico de barrido. Las fibrillas permanecieron atrapadas en la superficie tras la adsorción de otra capa de pectina. Pudo ser demostrado indirectamente que la inclusión de fibrillas en el revestimiento tiene un tremendo impacto en la resistencia mecánica, en comparación a microcápsulas hechas solamente con capas de proteínas y pectinas. Dado que las microcápsulas son ensambladas a bajo pH, dependiendo de interacciones electrostáticas subordinadas al pH y a la fuerza iónica, se consideró que estas capsulas podrían sobrevivir a bajo pH y aumentar su permeabilidad, o ser totalmente desorganizadas cuando son expuestas a pH neutro. Esto se analiza más profundamente en el siguiente capítulo.

En el Capítulo 4 se presenta la caracterización exhaustiva de microcápsulas reforzadas con fibrillas. Cada capa (de fibrillas o pectina) aumenta aproximadamente en 30 nm el espesor total, siendo esto considerablemente mayor que la cobertura que otorgaría una monocapa, tal como pudo confirmarse a través de reflectometría. Las microcápsulas mantienen su integridad cuando son expuestas a pH bajo 5.2 (mostrando una disolución lenta a pH 2), pero se desintegran a pH 7 o mayor. Esta respuesta no es lineal, siendo también éste el caso de la resistencia mecánica. Mientras las microcápsulas con 7 o más capas tuvieron un módulo elástico similar, las microcápsulas con 8 o más capas

presentaron el doble de la resistencia, alrededor de 0.6 GPa. Este último valor es comparable con la resistencia de microcápsulas poliméricas que han sido modificadas por entrecruzamiento. Esto se suma al efecto de los defectos presentes en el revestimiento, tal como se observó con el microscopio electrónico de barrido: las cápsulas con 8 o más capas presentan revestimientos lisos y libres de defectos, lo que resulta en una alta estabilidad frente a cambios de pH.

La disponibilidad de materiales de grado alimentario permitieron definir un segundo sistema (para ser ensamblado también a bajo pH), presentado en el Capítulo 5. Una sola capa de partículas de sílica cargada se adsorbió en capas de proteína y pectina adsorbidas secuencialmente, reduciendo el número de ciclos de adsorción. Las capas pre-adsorbidas estabilizan las partículas hidrofílicas de sílica que, de otra forma, producirían una pobre estructura para un coloidosoma. De esta manera se obtuvo una estructura altamente porosa pero resistente, que podría ser fácilmente rellenada. Después de llena, los poros pueden cerrarse con la adsorción de capas adicionales de proteína y pectina.

La desventaja de los dos sistemas de microencapsulación descritos previamente es que los polielectrolitos necesitan ser usados en exceso, haciendo esencial contar con pasos intermedios de lavado para eliminar el material no adsorbido. Para superar esto presentamos en el Capítulo 6 un artefacto microfluídico para efectuar la adsorción capa-a-capas de hasta 10 capas de fibrillas proteicas y pectina de modo continuo. El diseño del chip es simple y no requiere una infraestructura compleja a su alrededor, dado que el control de la dosificación de los materiales para el ensamblaje de las microcápsulas se basa solo en el adecuado balance de las resistencias hidráulicas en diferentes secciones del circuito microfluídico. A pesar de que asuntos como la modificación de la superficie de los microcanales para su operación continuada, y el escalamiento del proceso a volúmenes de interés industrial son aun un desafío, sentimos que es una contribución importante hacia la producción controlada de microcápsulas.

La integración de conocimiento sobre auto-ensamblado, del cual algunos ejemplos pueden encontrarse en esta tesis; la búsqueda de nuevos materiales de grado alimentario, que pueden actuar sinérgicamente para ensamblar un revestimiento multifuncional más inteligente; y un mejor diseño de artefactos microfluídicos, para un control más estrecho de este proceso, son fundamentales para que la producción de microcápsulas alcance la madurez que le transforme en una herramienta real para la industria de alimentos. El Capítulo 7 discute los requerimientos para la producción de microcápsulas reforzadas producidas capa-a-capla utilizando aparatos microfluídicos, y concluye esta tesis con una discusión general de los resultados a la luz de posibles desarrollos futuros en el área de la microencapsulación.

Acknowledgements

At a farewell dinner in Chile some friends asked me if I was afraid to go abroad to work on a PhD project. I answered '*what could be so difficult? If it's about studying then I'll study; if it's about working then I'll work*'. I could not have been more mistaken in my enthusiastic oversimplification: coming to The Netherlands to work towards a PhD degree was a life-changing experience, far beyond work and study. And that is due to people for whom I am truly thankful.

First things first: the beginning of this adventure would not have been possible without the support and guidance of Beatriz Cancino, who contacted me with the Food Process Engineering group and supported my application for the Alþan scholarship. At just about the same time, Valentina, another of her pupils, came to France for her PhD degree, which allowed us to support each other during the application process and later, in sharing our experiences in starting up our respective projects. *Gracias Vale!*

On my arrival to Schiphol Airport, in the midst of terrible jet-lag after the 30 hour flight - my first ever journey by airplane - I met my supervisor to-be, Karin Schroën. We had met by email long before, during the (extremely) tedious and prolonged process of getting my visa ready. At that time, I was Mr. Rossier to her and she was Dr. Schroën to me. I'm very happy that after many Friday meetings and hours of scientific discussions, the efforts to tame my Chilean *Spanglish*, and the trips together to conferences, that I can simply be Francisco and she, Karin. And that we ended up also sharing non-scientific interests, like photography. Thanks a lot for all the knowledge and experience transmitted, and the time you shared with the many special guests who visited me over the past four years.

I met Remko soon after and could not have fallen into better hands. I had never before met someone with your vision for the future, broad knowledge of so many fields and with such kindness. I was really surprised when I discovered that your talents didn't stop there. In Japan you drew a great cartoon for a set-up in two minutes flat, started improvising on the piano at Nara Women's University, and finished the day decorating a

tile for the Todai-ji temple. I received from you invaluable scientific guidance during this project, and you and Karin provided tremendous personal support when the twists and turns of life caused me to suddenly return to my country in July 2007 to comfort my family. I hope the future grants us many occasions for collaboration and visits to Chile, where I can show you new places to enjoy with Astrid.

I would also like to thank Leonard Sagis for his generous input at the beginning of the project, and Adriaan van Aelst, Henk Kieft, Mieke Kleijn and Remco Fokkink for helping me adapt characterization techniques to the (not always very cooperative) microcapsules. The good humor and willingness of Jos Sewalt made setting up experiments at lab 627 and to learning how to use new equipment, very pleasant. Martin de Wit was always there to walk me through the brave new world of paperwork and timesheets. Joyce, thanks for all your help with those little, but nonetheless crucial aspects of everyday life on the 6th floor of Biotechnion and for always trying to squeeze some Dutch out of me.

I was happy to share many technical issues, courses (Han-sur-Lesse!) and scientific discussions about our mutual results at our workpackage of MicroNed with my colleagues at the 3rd floor, Yul and Ardy. I was also very fortunate to work together with Rielle, Jolet, Yi, Shuyan, Xiaoli, and Miguel with whom I shared both a considerable amount of experimental work and the joy of new results. And, of course, thanks for giving me the opportunity to develop the art of supervision with you.

I happen to have a very big family and good friends back home with whom I am very close. They sent me their love whenever they could, especially Cecilia, Julio, Renzo, Gustavo, Nacho, Sofi, Pablo, Pili, and Felipe. Life far away from Valparaíso without a proper substitute family would have been impossible, but a couple of weeks after my arrival to Wageningen I was adopted by the few Chileans studying at WUR: Alejandra, Gustavo and Christian. Their experience as to how a Chilean can 'survive' in The Netherlands was highly appreciated, most of all because their advice often came wrapped in barbecued lamb – Chilean style! Through these three, I was introduced to a

big friendly bunch of Latin Americans and Latin-Americanized Wageningeners. Marcos and Roxina quickly became (and remain) my ‘fairy godparents’ and I was happy to be a witness at their wedding, and meet little Luca when he arrived soon after. Rhiannon polished my wild English through talking about dreams, popular expressions, and Chilean wine, and even tried to get me into Yoga (a fruitless effort given my strong tendency to snore during meditation). Many others brought their Latin American warmth to barbecues, dinners and guitar-filled evenings, and I am thankful to them for making real, far from home, that old Bolivarian dream. New Chileans arrived to Wageningen and the stories with Gabi, Lucero, Paula, Marianne, Tomás y Pia, Roberto y Marcela, Carlos y Alicia, Nico y Rosita could easily fill another thesis, including episodes like meeting our national President, Michelle Bachelet, Queen Beatrix and Princess Máxima, or the support activities we organized to help our country after the earthquake in 2010. And how can I ever forget discussions and reconciliations with Tati debating my origins: whether I am technically from Valparaíso or from Viña del Mar. My Latin American(-ized) friends, I hope you know that you have been true brothers and sisters to me in The Netherlands. A family always has two branches; the other one came from Marzia who included me (or maybe just my guitar, but I joined her anyway) in barbecues in Arnhem, where Jorge, Gerardo, Aldana, Gus, Clara, Sabina, and Ishai became part of my story. One great chapter took us walking, in 2009, for more than 100 km, 4000 meters above sea level, in the Huayhuash mountain range in Perú. *Gracias chiquillos!*

Music is an intrinsic part of being; without it I would have been but half alive while in Wageningen. Luckily I discovered, and quickly joined the Wagenings Studenten Koor en Orkest Vereniging and enjoyed performing many concerts with them over seven seasons. Thanks to Ger Vos, for all his teachings and Annette, Jeroen, Irene, Lisette, Martin, Maurits, Joel, amongst many others, for all the fun and music we have shared together. And though they may not know it, Ali Hendriksen and Ella Hoeneveld also played an important role keeping science and music in balance over this past year.

The PRELBIT (Process Engineering Lunch Break International Table) made its appearance just when I thought that my sense of humor would be lost in translation.

Since then 12.15 has been a refreshing moment during my workday; a moment of inspiration that took the form of the Labuitje 2009, with the contribution of Miranda. Thanks Carsten, Benjamin, Judith, Anna, Nirmal, Leila and, more recently, Thomas, Ivette, Nicolas, Rianne, Marta and Lena. *Dank je wel, dhanyavad, merci, obrigado, gracias und danke schön!*

But working days are not only about the lunch breaks, and I must say that the working atmosphere was more than pleasant at Process Engineering. I will keep warm memories of all my colleagues, especially from Tamara, Koen, Eduard, Hassan, Edwin, David, Helena and Catarina, who included me in their lives beyond just work. Marieke, Frank, Mgeni and I shared a lot of good laughs in room 620 (with a very nicely decorated door and an eternally Christmas-inspired ceiling). And with Elsbeth, Anja, and Maurice I enjoyed work, dinners, more work and the great satisfaction of organizing and accomplishing the PhD trip 2010 that took us all to the USA. I find myself missing our meetings, guys!

From the PRELBIT I am very happy to count on two great neighbors who accepted the responsibility of acting as my paranymphs: María Cuaresma and Petra Vossenbergh. Dear Petra, we shared many work hours at the lab but overall we shared a love for music: it is maybe due to that ability to listen (rather than to just hear) that we need to say very little to understand each other and feel in good company. Dear María, you have been the closest friend, even from the distance existing between Spain and Wageningen. Thanks for all the advice and for making me feel at home when I visited your homeland of Huelva.

Silvia, Silvana, Sofía y Francisco en una forma muy especial: hemos sido solo uno a través de estos cuatro años. Estás páginas son tan tuyas como mías.

A handwritten signature in black ink, appearing to read 'Francisco', with a stylized flourish underneath.

About the author

Francisco José Rossier Miranda was born in Valparaíso, Chile, on April 16th, 1978. His education started at the Seminario San Rafael (Valparaíso) and continued at the Colegio Rubén Castro (Viña del Mar), from where he graduated in 1995 receiving the '*Ruben Castro*' prize and a special distinction from the Music School of the Pontificia Universidad Católica de Valparaíso (PUCV). In 1996 he started his studies at the



School of Food Engineering of the Faculty of Natural Resources of the PUCV, with a scholarship awarded to the best selection scores of that year. During his studies he performed internships working in optimization of the production schemes for salmon fish processing plants and fruit juice concentration plants. In 2004 he completed his training as Food Engineer with a thesis focused on the optimization of the operation conditions for a tray dryer, graduating with the '*Luis Contreras*' distinction. In the following years he worked as research assistant in projects related with the integration of membrane filtration in the treatment of liquid waste, mainly from starch production, designing and constructing a pilot plant. In March 2006 he was awarded with an Alþan Scholarship to start his PhD-project in June at the Food Process Engineering Group of Wageningen University, The Netherlands. The project, under the supervision of Prof. Remko Boom and Dr. Karin Schroën, aimed to the design of microstructures suitable for microencapsulation in the food industry. The result of this research during the last 4 years are presented in this thesis. Since June 2010 Francisco works in a post-doc position in a collaboration project between the Food Process Engineering and the Organic Chemistry Group of Wageningen University.

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List of Publications

Peer reviewed journals

Sagis, L. M. C.; de Ruiter, R.; Rossier-Miranda, F. J.; de Ruiter, J.; Schroën, K.; van Aelst, A. C.; Kieft, H.; Boom, R.; van der Linden, E., Polymer Microcapsules with a Fiber-Reinforced Nanocomposite Shell. *Langmuir* **2008**, 24, 1608-1612.

Rossier-Miranda, F. J.; Schroën, C. G. P. H.; Boom, R. M., Colloidosomes: Versatile microcapsules in perspective. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2009**, 343, 43-49.

Rossier-Miranda, F.J., Schroën, K., Boom, R. Mechanical Characterization and pH Response of Fibril-Reinforced Microcapsules Prepared by Layer-by-Layer Adsorption. *Submitted for publication*.

Rossier-Miranda, F.J., Schroën, K., Boom, R., Microcapsule Production by a hybrid colloidosome/layer-by-layer technique. *Submitted for publication*.

This chapter has been submitted as: Rossier-Miranda, F.J., Schroën, K., Boom, R., Integrated microfluidic circuit for electrostatic layer-by-layer microcapsule assembly. *Submitted for publication*.

Oral presentations

Nationale MicroNano Conference '07, **2007**, Wageningen, The Netherlands.

17th International Symposium on Surfactants in Solution, **2008**, Berlin, Germany.

22nd Conference of the European Colloid and Interface Society, **2008**, Krakow, Poland.

Workshop Functionalized Materials and Interfaces COST D43, **2009**, Marcoule, France.

AlBan Conference, **2009**, Porto, Portugal.

European Student Colloid Conference, **2009**, Almería, Spain.

MicroNano Conference '09, **2009**, Delft, The Netherlands.

Poster presentations

Netherlands Process Technology Symposium 7, **2007**, Veldhoven, The Netherlands.

Overview of completed training activities



Discipline specific activities

Courses

Industrial proteins (VLAG, 2006)
A unified approach to mass transfer (OSPT, 2007)
Polysaccharides as food colloids and biomaterials (VLAG, 2007)
Physical Chemistry Han sur Lesse winter school I (TU-Delft, 2008)
Computational Fluid Dynamics of Multiphase Flows (OSPT, 2008)
Physical Chemistry Han sur Lesse winter school II (TU-Delft, 2009)
Sustainable Process, Product and System Design (OSPT, 2009)
Interfacial engineering in nanotechnology (Leibniz-Institut für Polymerforschung, 2009)

Conferences

Netherlands Process Technology Symposium 6 (2006, Veldhoven, The Netherlands)
NanoNed MicroNed Symposium II (2006)
Netherlands Process Technology Symposium 7 (2007, Veldhoven, The Netherlands)
Nationale MicroNano Conference '07 (2007, Wageningen, The Netherlands)
17th International Symposium on Surfactants in Solution (2008, Berlin, Germany)
22nd Conference of the European Colloid and Interface Society (2008, Krakow, Poland)
Workshop Functionalized Materials and Interfaces COST D43 (2009, Marcoule, France)
AlBan Conference (2009, Porto, Portugal)
European Student Colloid Conference (2009, Almería, Spain)
MicroNano Conference '09 (2009, Delft, The Netherlands)

General courses

PhD Competence Assessment (WGS, 2006)
Project and Time Management (WGS, 2006)
VLAG PhD Week (VLAG, 2007)
Teaching and Supervising Thesis students (OWU, 2007)
Techniques for Writing and Presenting a Scientific Paper (WGS, 2007)
Philosophy and Ethics of Food Science & Technology (VLAG, 2008)

Optionals

PhD-trip Process Engineering (Japan, 2008)
PhD-trip Food Process Engineering (USA, 2010)
Organization of PhD-trip to USA (2009/2010)
Brain & Game Day Process Engineering (2008)
Brainstorming Day Food Process Engineering (2009)

The research described in this thesis was financially supported
by the national consortium MicroNed.
Francisco José Rossier-Miranda was supported by Programme Alban,
the European Union Programme of High Level Scholarships for Latin America,
scholarship no. E05D060840CL

Cover: Hybrid LbL-colloidosome and 3D image of broken microcapsules with 9 layers (F.R./L.J.)

