Polyelectrolyte Microcapsules for Pharmaceutical Applications

Ir. Bruno De Geest
POLYECTROLYTE MICROCAPSULES FOR PHARMACEUTICAL APPLICATIONS

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Voor Elsje
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## LIST OF ABBREVIATIONS AND SYMBOLS

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<tr>
<td>H-NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>AAPBA</td>
<td>acryl aminophenylboronic acid</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>APBA</td>
<td>aminophenylboronic acid</td>
</tr>
<tr>
<td>AuNP</td>
<td>gold nanoparticle</td>
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<tr>
<td>CDI</td>
<td>1,1'-carbonyldiimidazole</td>
</tr>
<tr>
<td>CHIT</td>
<td>chitosan</td>
</tr>
<tr>
<td>CHON</td>
<td>chondroitin sulfate sodium salt</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
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<tr>
<td>dex</td>
<td>dextran</td>
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<tr>
<td>DEXS</td>
<td>dextran sulfate sodium salt</td>
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<tr>
<td>DMAE</td>
<td>dimethyl aminoethyl</td>
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<tr>
<td>DMAEA</td>
<td>dimethyl aminoethyl acrylate</td>
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<td>DMAEMA</td>
<td>dimethyl aminoethyl methacrylate</td>
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<tr>
<td>DMAP</td>
<td>4-N,N-dimethylaminopyridine</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DS</td>
<td>degree of substitution</td>
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<tr>
<td>DOPA</td>
<td>dioleoyl phosphatic acid</td>
</tr>
<tr>
<td>DOPE</td>
<td>dioleoyl phosphatidylethanolamine</td>
</tr>
<tr>
<td>DOTAP</td>
<td>dioleoyl trimethylammoniumpropane</td>
</tr>
<tr>
<td>GMA</td>
<td>glycidyl methacrylate</td>
</tr>
<tr>
<td>HEMA</td>
<td>hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HPMA</td>
<td>hydroxypropyl methacrylamide</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>KPS</td>
<td>potassium peroxodisulfate</td>
</tr>
<tr>
<td>IR</td>
<td>infra red</td>
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<tr>
<td>LbL</td>
<td>Layer-by-Layer</td>
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<tr>
<td>MAA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>MA</td>
<td>methacrylate</td>
</tr>
<tr>
<td>PAH</td>
<td>poly(allylamine hydrochloride)</td>
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<td>PARG</td>
<td>poly(L-arginine hydrochloride)</td>
</tr>
<tr>
<td>PASP</td>
<td>poly(L-aspartic acid sodium salt)</td>
</tr>
<tr>
<td>PEI</td>
<td>poly(ethylene imine)</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PGLU</td>
<td>poly(L-glutamic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PSS</td>
<td>poly(sodium styrene sulfonate)</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
</tr>
<tr>
<td>RITC</td>
<td>rhodamine B isothiocyanate</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SOPC</td>
<td>stearoyl oleoyl phosphatidylcholine</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethyl thodamine isothiocyanate</td>
</tr>
<tr>
<td>XRR</td>
<td>X-ray reflectometry</td>
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<td>ζ</td>
<td>zeta</td>
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INTRODUCTION TO PULSED DRUG RELEASE

Parts of this chapter were published in:


1 Laboratory of General Biochemistry and Physical Pharmacy, Department of Pharmaceutics, Ghent University, Ghent, Belgium.
2 Pharmaceutical Care Unit Ghent, Department of Pharmaceutics, Ghent University, Ghent, Belgium
3 Drug & Patient Information, Faculty of Pharmacy, Katholieke Universiteit Leuven, Leuven, Belgium.
INTRODUCTION TO PULSED DRUG RELEASE

During the past decades much effort has been made on the development of dosage forms for sustained drug release, providing a constant release rate and concentration in the bloodstream. Pulsed drug release can be defined as the rapid and transient release of a drug after a lag-phase with no or little drug being released. ¹ Pulsed drug release could be of interest e.g. to prevent drug tolerance (as in the case of nitrate therapy where a sustained therapy leads to the rapid development of tolerance), to satisfy circadian requirements (which can modulate the pharmacokinetic and pharmacodynamic responses, as in the case of attaining night time theophylline levels in anti-asthmatic therapy) and to achieve ‘on demand’ responses (as in insulin therapy).

Drug delivery scientists have worked on several concepts holding promise to establish pulsed drug release profiles. One way to classify the pulsed drug delivery systems under development is based on the physicochemical and biological principles that trigger their release. In ‘programmed’ delivery systems the release is completely governed by an inner mechanism of the device, i.e. the lag time prior to the drug release is controlled primarily by the delivery system. In ‘triggered’ delivery systems the release is governed by changes in the physiological environment of the device (‘biologically triggered systems’) or by external stimuli (like the application of e.g. an electromagnetic field, ultrasound or laser light).

IMPLANTABLE MICROCHIPS FOR PULSED DRUG RELEASE

Several approaches to achieve pulsed drug release have already been presented. E.g. Santini et al. reported on the fabrication of implantable microchips for controlled release of therapeutics. ² The microchips consist of several micro-reservoirs, containing the therapeutics, embedded in a matrix. The drug release from any desired micro-reservoir can be triggered by applying an electric current to the seal of the corresponding micro-reservoir, inducing the dissolution of the seal leading to release of drug molecules. In another approach
biodegradable polymers, such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), were used to fabricate the seals of the micro-reservoirs. By tailoring the degradation rate of the seal material it was possible to tailor the exact moment at which the drug molecules were released from a specific micro reservoir, creating a steep release profile.

EXPLODING MICROCAPSULES FOR PULSED DRUG RELEASE

Microcapsules, releasing their content in response to an internal or external trigger, are of great interest in the field of pulsed drug delivery. Due to their micrometer dimensions they can be administered by injection, offering considerable advantages towards patient compliance as they are less invasive than implants. Recently, the Sukhorukov and Caruso research groups reported on ‘exploding’ microcapsules. Polyelectrolyte microcapsules containing gold nanoparticles were fabricated using the layer-by-layer (LbL) technique. Therefore a sacrificial colloidal template was coated with several polyelectrolyte layers of opposite charge and also one layer of gold nanoparticles, followed by the dissolution of the template resulting in the formation of hollow capsules. Afterwards the capsules could be filled with macromolecules by reversibly changing the permeability of the LbL membrane. Irradiation of the microcapsules with IR-light induced a local heating of the gold nanoparticles causing a disruption of the LbL membrane and thus release of encapsulated molecules. As IR-light is able to penetrate the skin over several millimetres, such microcapsules, once injected subcutaneously, could be interesting for pulsed drug delivery.

INTRACELLULAR PULSED DRUG RELEASE

On the other hand, instead of pre-programmed pulsed drug delivery, one could also aim to develop drug delivery systems able to release their payload after a specific physicochemical trigger. Especially triggers which can be evoked by the human body itself are of interest. E.g. to deliver therapeutic molecules, such as peptides and nucleic acids, to an intracellular target it could be interesting to make use of physicochemical differences between the extra- and intracellular medium. It is well known that the pH in the endo/lysosomes is up to two units lower than the extracellular pH (i.e. pH 7.4). Recently Lynn et al. reported on a novel family of pH-sensitive degradable polycations (so called poly-β-aminoesters). These polymers exhibit some unique features as they appear to be only
soluble at a pH below 6.5. Poly-β-aminoester based micro- or nanoparticles rapidly dissolve when they are transferred from a pH 7.4 to a slightly acidic pH. These characteristics are highly interesting as it would allow such microparticles to release their drug content upon cellular internalisation. DNA and anti-tumour agents have been encapsulated in such microparticles and successfully used for cellular delivery in vitro.

**PULSED DRUG RELEASE – HOPE OR HYPE?**

“Precisely timed drug delivery may maximize therapeutic efficacy, may minimize dose frequency and may reduce toxicity by avoiding side effects and drug tolerance”, an often cited slogan. Will it remain hype or will it become reality?

It is outspoken claimed by the World Health Organization and research funding foundations (like e.g. the Bill and Melinda Gates Foundation) that ‘single-shot’ vaccines, where the initial and booster doses are contained in one delivery system, could improve vaccination coverage by reducing the number of vaccination sessions required to generate immunity. Single-shot vaccination would be a major step forward not only in delivering established vaccines but also future vaccines. As an example, it is expected that women will need three to four antigen doses, spread over a number of months, to become well protected against human pappiloma virus induced cervical-cancer. Thus both the developing countries as well as the western world could benefit from single-shot vaccines. To develop single-shot vaccines many pre-clinical studies, (often based on poly-lactic acid /poly-glycolic acid which release the antigen) undertaken in recent years, have proven that biodegradable microspheres in a sustained (i.e. not pulsed) way are promising in generating immunity. Clinical testing would be a next step, however, the financial resources available today seem to be a major bottleneck for clinical research on single-shot vaccination. Also the major question whether ‘pulsing single-shot vaccines’ should be preferred above ‘slowly releasing single-shot vaccines’ remains currently unanswered. Considering the impact single-shot vaccines could have on human health we strongly encourage pharmaceutical and clinical researchers to focus on pulsed delivery of antigens. Both the implantable microchips as well as the exploding microcapsules described above should be evaluated for this purpose.

In the last decade material scientists already have shown to be able to fabricate new materials with unique properties that respond to biological triggers, at least in vitro and in vivo. Biocompatible glucose sensors can open opportunities as triggered devices to guide insulin release from implanted subcutaneous supplies. It is important to notice that the major part of the pulsed drug delivery devices reported are prototypes performing well in
vitro, however no data are available on their performance in vivo. Therefore it remains a huge challenge to cope with the influences of a biological environment on the integrity of the drug delivery devices and on their drug release profile. Also, are fluctuations in temperature, pH… under physiological or pathophysiological conditions large enough to meaningfully influence the drug release?

The rather limited progress in in vivo pulsed delivery of therapeutic agents is also partially due to limited knowledge on which drugs and indications would benefit from pulsatile or temporally modulated release. Quite a lot of challenges remain with this regard. Many diseases display symptoms and onset characteristics that are randomly distributed within 24 hours: e.g. coronary infarction and angina pectoris (activity related), asthmatic attacks (early morning) and peptic ulcer pain (night). Data, primarily concerned with the chronopharmacokinetics of propranolol, organic nitrates, nifedipine, antiasthmatics and histamine H2-blockers can be taken as examples. \(^{12, 13}\) Pulsed drug delivery should make glucocorticoid replacement therapy as physiological as possible, the normal pattern of secretion including a diurnal rhythm and a pulsatile ultradiurnal rhythm. It may enable splitting the daily delivered dose and temper the long term side effects like adrenal suppression and lowered bone mineral density. \(^{14}\)

Even with relatively small and well known molecules indirect approaches can be hypothesised to pathological conditions. It has been clearly demonstrated hat benzodiazepine administration affects the activity of the hypothalamus-pituitary-adrenal axis (HPA) with a variety of effects at both the central and peripheral level. An inhibitory effect of diazepam on the adrenocorticotropic hormones (ACTH) has been reported. Alprazolam possesses the most remarkable inhibitory effect on the HPA axis, being 40 times more potent than diazepam. This finding could enable programmed release in case of panic provoked by central HPA axis hyperactivation. \(^{15}\)

As benzodiazepines are concerned, pulsed drug delivery of diazepam can render service in case of addiction by releasing appropriate doses diminishing in time. It seems that the consequences of circumstantial change of sleep and wakefulness by shift work are largely underestimated. Disrupted nocturnal production of melatonin and reproductive hormones are of relevance for breast cancer ethiology. Studies completed to date have found an increased risk of breast cancer associated with indicators of exposure to light-at-night and night shift work. \(^{16}\) More insight in the neurophysiology of sleep and wakefulness may enable to install a ‘programmed’ sleep pattern by releasing agents proper to different sleep phases. \(^{17}\)

It should be encouraged to challenge more the pharmacokinetic paradigm that ‘flatter is better’: what drugs and/or therapeutic indications will benefit indeed from temperature, pH, ionic strength… induced release? Further pharmaceutical research in pulsed drug delivery...
should be also supported by biologists who should try to obtain better insights into circadian rhythms. On the other hand, assuming that we would know how the optimal 'delivery equation' looks like for a certain drug, as material scientists we wonder whether we will ever be able to design delivery systems, based on biocompatible building blocks as this is a primary requirement to reach clinical phase testing, which truly meet the (highly likely complicated) delivery characteristics. Considering these criticisms, we believe that in the coming years 'pulsed drug delivery scientists' should define (in a trans-disciplinary approach) which type of drugs/diseases will be the focus in pulsed drug delivery research with the hope that within the next decade injectable pulsed drug delivery system will become available to (hopefully a large group of) patients.

REFERENCES

OUTLINE AND AIMS OF THIS THESIS
OUTLINE AND AIMS
OF THIS THESIS

Upon administration into the body many (biotechnological) therapeutics, such as e.g. proteins and peptides, face a threatening environment and become readily degraded when not encapsulated in a suitable pharmaceutical carrier. Besides protecting the drug from degradation, the carrier should deliver it at the desired target site and/or at the desired moment. Generally speaking, this thesis deals with the design of vehicles for pharmaceutical purposes. The vehicles reported in this thesis are prepared by the ‘layer-by-layer’ (LbL) technique. The LbL technique is based on the sequential adsorption of charged polyelectrolytes onto an oppositely charged surface. When this technique is applied on a colloidal template one can encapsulate this template. It is even possible to fabricate ‘hollow capsules’ after dissolution of the template.

Several types of so called ‘polyelectrolyte microcapsules’, each able to release their content after a specific internal or external stimulus, are presented in this thesis. Two categories of polyelectrolyte microcapsules were investigated. The first category of polyelectrolyte microcapsules releases their content upon an internal trigger inside the microcapsules. This type of capsules was denoted as ‘self-exploding microcapsules’. The second category of microcapsules has specific properties in the LbL membrane, making them sensitive to specific physicochemical stimuli. This stimuli responsive behaviour is achieved by incorporation of specific moieties, such as chemical groups, degradable polymers, nanoparticles, etc … within the LbL membrane.

CHAPTER 1 overviews the layer-by-layer technique and describes how polyelectrolyte microcapsules are fabricated by LbL coating of a sacrificial template. We particularly review on stimuli responsive polyelectrolyte microcapsules which decompose and release their content after a specific physicochemical trigger. CHAPTER 1 also describes how these capsules may be used for biomedical/pharmaceutical applications and compares the potential of these microcapsules to other drug delivery systems under investigation.

CHAPTER 2 – 5 report on self-exploding microcapsules. CHAPTER 2 describes that it is possible to obtain self-exploding microcapsules by surrounding a biodegradable microgel core with a semi-permeable membrane based on synthetic polyelectrolytes. CHAPTER 3 reports in detail on the physicochemical properties of the self-exploding microcapsules.
In **CHAPTER 4** we use bio-polyelectrolytes, instead of synthetic polymers, to coat the microgels and show that the ‘exploding behaviour’ of the microcapsules strongly depends on the type of bio-polyelectrolytes the membrane consists of. It is shown that choosing suitable bio-polyelectrolyte microcapsules allows making self-exploding microcapsules which explode upon incubation under physiological conditions. In **CHAPTER 5** an alternative technique for the coating of the microgels is reported. By adsorbing charged lipid vesicles onto the surface of oppositely charged microgels it is possible to surround the microgels with a continuous lipid coating, also leading to self-exploding microcapsules.

**CHAPTER 6** describes the fabrication of monodisperse microgels using a microfluidic emulsification device. The use of monodisperse microgels, compared to polydisperse microgels fabricated by ordinary emulsification, should contribute to a more uniform behaviour of a large population of self-exploding microcapsules.

**CHAPTER 7 – 10** report on hollow polyelectrolyte microcapsules able to release their content due to an external trigger. **CHAPTER 7** describes the fabrication of polyelectrolyte microcapsules responsive to glucose using a new phenylboronic acid containing polycation whose charge changes upon binding with glucose. Polyelectrolyte microcapsules containing this polycation decompose when glucose is present in the surrounding medium. **CHAPTER 8** reports on the use of degradable polyelectrolytes to fabricate polyelectrolyte microcapsules and show that after intracellular uptake, such microcapsules become digested by the cells.

**CHAPTER 9 & 10** deal with the fabrication of hybrid gold nanoparticle/polyelectrolyte microcapsules. It is proven that such capsules can be decomposed by a variety of physicochemical triggers such as a change in pH, IR-laser light and ultrasound energy.
Chapter 1

Release Mechanisms for Polyelectrolyte Microcapsules

Parts of this chapter are in press:

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Chemical Society Reviews

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2 Centre for Material Research, Queen Mary University of London, London, United Kingdom.
Polyelectrolyte microcapsules have recently been introduced as new microscopic vehicles which could have high potential in the biomedical field. In this chapter we give an introduction on the layer-by-layer (LbL) technique which is used to fabricate these polyelectrolyte microcapsules as well as on the different triggers that have been exploited to obtain drug release from these microcapsules. Furthermore, other delivery systems that use the same trigger for drug delivery are compared and critically discussed with regard to their biomedical relevance.
Chapter 1

Release Mechanisms for Polyelectrolyte Microcapsules

INTRODUCTION

The field of drug delivery focuses on the development of suitable carriers for therapeutic molecules.\(^1\) The recent availability of many biotechnological therapeutics, such as peptides, proteins and oligonucleotides has challenged and thus stimulated the advanced drug delivery research field as such therapeutics depend on suitable carriers to protect them from extracellular enzymes and to deliver them to the target cells. During the past decades a large variety of micro- and nanocarriers have been developed to serve this purpose. Liposomes were amongst the first nanocarriers studied for the delivery of a large variety of both low and high molecular weight therapeutics.\(^2\)

Since the beginning of the nineties controlled radical polymerisation techniques\(^3, 4\) such as atom transfer radical polymerisation (ATRP) and nitroxide mediated polymerisation (NMP) have offered new tools to polymer chemists for the design of well defined polymeric architectures\(^3\) such as block copolymer micelles and polymersomes.\(^5\) The latter supramolecular structures can be seen as the synthetic analogue of liposomes and have, to a certain extent, also been explored for the encapsulation of therapeutics. The major advantage of these synthetic structures is that their properties can be tailored by varying their chemical composition. This allows the design of vesicles which respond to specific stimuli (such as pH, redox-potential, magnetic field etc.) which may trigger them to release their content at the desired site and time. However, there are several drawbacks to these vesicles. They are not yet commercially available and their fabrication requires complex chemical syntheses and purification making them expensive to fabricate and hardly accessible to a broad public in the biomedical field.

A few years ago, a novel type of vesicles, called polyelectrolyte microcapsules, were introduced.\(^6, 7\) They are fabricated using the Layer-by-Layer (LbL) technique, i.e. the self assembly of charged species onto an oppositely charged sacrificial colloidal substrate.
followed by the dissolution of this substrate. Possible advantages of polyelectrolyte microcapsules are the absence of hazardous procedures and the use of simple building blocks along with the possibility to introduce a high degree of multifunctionality within their shell. The promising expectations of polyelectrolyte microcapsules for biomedical applications have evoked a synergetic effect between scientists from different fields such as chemists, material scientists, pharmacists, biologists and even theoretical and experimental physicists.

While other reviews have reported on the physicochemical properties,8, 9 permeability,10 use as microreactor11 or biofunctionalisation 12 of polyelectrolyte microcapsules, this review aims to outline the efforts made towards the development of polyelectrolyte microcapsules which are ‘intelligent’ in the sense that they release their content as a consequence of external or internal stimuli. We especially tried to find out the strengths and weaknesses of intelligent polyelectrolyte microcapsules versus other types of stimuli-sensitive delivery systems and try to determine what specific opportunities there are for polyelectrolyte microcapsules in the field of drug delivery. The discussion brought in this chapter should stimulate scientist from multidisciplinary fields to focus on the development of polyelectrolyte microcapsules which could offer a solution to persisting needs in drug delivery.

THE LAYER-BY-LAYER TECHNIQUE

The Layer-by-Layer (LbL) technique was introduced in the beginning of the nineties by Gero Decher.13 Originally this technique was based on the sequential adsorption of oppositely charged polymers (i.e. polyelectrolytes) on a charged planar substrate. Upon adsorption of a polyelectrolyte layer, charge overcompensation takes place, leading to a reversal of the surface charge, promoting the adsorption of a next, oppositely charged, polyelectrolyte. Figure 1 is a schematic illustration of the LbL process. A charged substrate, e.g. a silicon wafer, is immersed into an aqueous solution of an oppositely charged polyelectrolyte. After a certain adsorption time the substrate is removed and washed with water in order to remove excess polyelectrolyte. In the next step the substrate is immersed into the second polyelectrolyte solution which has a charge opposite to the first polyelectrolyte. This second polyelectrolyte adsorbs onto the layer of the first polyelectrolyte which reverses the surface charge. Again a washing step is performed and the whole procedure can be repeated as many times as one desires. In this way one can easily
prepare multilayered films with tuneable physico-chemical properties as both the number of layers as well as their composition can easily be varied.

Figure 1. (A) Schematic representation of the deposition of a polyelectrolyte film on a slide. Steps 1 and 3 represent the adsorption of respectively a polyanion and polycation, steps 2 and 4 are washing steps. (B) Simplified molecular picture of the first two adsorption steps, depicting film deposition starting with a positively charged substrate. Counterions are omitted for clarity. The polyion conformation and layer interpenetration are an idealization of the surface charge reversal with each adsorption step. (C) Chemical structure of poly(styrene sulfonate) (PSS) (left) and poly(allylamine hydrochloride) (PAH) (right), often used polylions to build LbL films.

Several techniques have been used to characterize the multilayer build-up on planar surfaces. Amongst them UV-VIS adsorption, quartz crystal microbalance (QCM) and ellipsometry are the most widespread and allow one to determine both the mass and thickness increment upon adsorption of a single polyelectrolyte layer. The internal structure of the polyelectrolyte films (whether it is ordered or rather ‘fuzzy’) can be investigated by X-ray techniques while their surface morphology can be adequately analyzed by atomic force microscopy (AFM).

Currently, a large number of components, other than charged polymers, have been used to build multilayered films. DNA, proteins, nanoparticles, lipids, viruses etc. have been included in the multilayers yielding to thin films with tailor made properties. Besides electrostatic, other interactions such as H-bonds, covalent bonds, biospecific interactions, stereocomplex formation etc... have also been used in
order to accomplish a layer-by-layer build-up. Additionally, pH \(^ {34, 35}\), temperature, \(^ {36, 37}\) glucose \(^ {38}\) and biotin \(^ {39}\) responsive LbL films have been made by varying the chemical nature of the polyelectrolyte film.

Most of the applications cited above involve the design of planar films. One of the most eye-catching applications of the LbL technology involves polyelectrolyte microcapsules fabricated by LbL coating of colloidal templates. \(^7\) The potential use of LbL microcapsules as drug carriers has extended LbL research in the field of drug delivery \(^{11,40}\). The following paragraphs give additional information on these microcapsules.

**POLYELECTROLYTE MICROCAPSULES**

**Fabrication of polyelectrolyte microcapsules**

Polyelectrolyte microcapsules, introduced in 1998, are obtained by LbL coating of a colloidal substrate followed by the dissolution of this template as schematically presented in Figure 2. \(^6, 7, 41\) For the fabrication of hollow polyelectrolyte microcapsules poly(styrene sulfonate) / poly(allylamine hydrochloride) (PSS/PAH) is a very popular polyelectrolyte pair as PSS/PAH multilayer films deposited on planar substrates have been well studied. Also, the preparation of PSS/PAH microcapsules is reproducible and does not suffer from capsule aggregation or capsule decomposition upon removal of the core template, as often observed in the case of microcapsules fabricated from biopolymers, having a lower charge density. The physicochemical \(^{10}\) and mechanical \(^{42, 43}\) properties of PSS/PAH microcapsules have been investigated by several groups. Initially hollow polyelectrolyte microcapsules were made using organic templates (like polystyrene (PS) or cross-linked melamine formaldehyde (MF) microparticles) which were dissolved after deposition of the LbL coating using both organic solvents and acidic solutions (0.1 M HCl). However, as discussed below, a main issue during the core removal is the integrity of the capsule wall. \(^{44-46}\) It is known that organic solvents create pores in polyelectrolyte multilayers allowing the polystyrene to diffuse outwardly from the microcapsules. \(^{47}\) However, removal of MF-templates is more difficult as it has been reported that MF oligomers stay complexed to the capsule wall and/or in the capsule interior, even after prolonged incubation in an acidic environment. \(^{44, 45, 48, 49}\) Therefore MF microparticles became less frequently used as templates, because MF oligomers remaining in the capsule wall lead to a rather undefined structure of the polyelectrolyte microcapsules and may also be toxic. Although the removal of a PS-core is easier, the fast dissolution of PS in organic solvents creates an osmotic pressure which may
destroy the polyelectrolyte shell. 47 To overcome this inconvenience inorganic carbonates, such as calcium carbonate (CaCO$_3$), 50-54 manganese carbonate (MnCO$_3$) and cadmium carbonate (CdCO$_3$) 50, 55 have recently been introduced as a template for the fabrication of hollow polyelectrolyte microcapsules. These microparticles are easily made by mixing calcium chloride and sodium carbonate and become easily dissolved by EDTA (when CaCO$_3$ is used) or by a low pH (in the case of MnCO$_3$ and CdCO$_3$). The major advantage of inorganic templates is the low molecular weight of the ions. Polyelectrolyte shells are, generally speaking, known to be permeable to molecules with a molecular weight below 5 kDa 56 and should therefore not undergo an osmotic stress during the dissolution of the inorganic templates.

![Figure 2](image)

**Figure 2.** Schematic illustration showing the preparation of ‘hollow’ polyelectrolyte microcapsules. The initial steps (a through d) involve stepwise film formation by repeated exposure of the colloids to polyelectrolytes of alternating charge. Between each step the excess polyelectrolytes are removed before the next layer is deposited. When the desired number of polyelectrolyte layers is obtained the core is decomposed (e) resulting in a suspension of ‘hollow’ polyelectrolyte microcapsules (f).

**Loading of polyelectrolyte microcapsules**

CaCO$_3$ microparticles (Figure 3) have proved to be excellent sacrificial templates not only for the fabrication of hollow polyelectrolyte microcapsules but also for making ‘filled’ polyelectrolyte microcapsules since CaCO$_3$ microparticles can be easily loaded with macromolecules (e.g. proteins) during 51 or after their preparation. On top, the mild
dissolution conditions do not destroy the encapsulated macromolecules. Also silica has been used as core for the fabrication of hollow polyelectrolyte microcapsules.  

This approach has advantages as monodisperse non-porous silica particles are commercially available allowing the preparation of monodisperse polyelectrolyte microcapsules. However, the hydrofluoric acid solution, required to dissolve the silica core, requires extreme caution when handled. This possibility of encapsulating macromolecules is a great advantage over microcapsules made using PS or MF-templates. Indeed, the latter have to be loaded afterwards by varying the solvent polarity, salt concentration or pH of the medium in order to reversibly create pores to allow the inward diffusion of macromolecules. Subsequently the pores are closed by dispersing the microcapsules in their original medium as shown in Figure 4. A variation on this route is filling the microcapsules with macromolecules followed by a cross-linking of the shell leading to the entrapment of the macromolecules. It is very likely that the conditions used to ‘post-load’ polyelectrolyte microcapsules will affect the integrity of many therapeutic macromolecules like peptides and proteins. The Caruso group recently reported on the fabrication of mesoporous silica particles which could easily be filled with proteins in their pores and subsequently used as template for the fabrication of protein filled microcapsules. These pre-loading procedures should offer the same advantages as in case of coprecipitated CaCO₃.

Figure 3. SEM images of CaCO₃ microparticles: (A) in overview, (B) single particle, and (C) broken particle.
Macromolecular drugs or nanoparticles, 6 lipids, 63 dendrimers, 64-66 enzymes, 67 DNA, 68 and empty viruses 69 can also be incorporated in the polyelectrolyte multilayer wall. Finally, polyelectrolyte microcapsules may be loaded with charged molecules by electrostatic interactions with an oppositely charged matrix present inside the polyelectrolyte microcapsules. 64, 70, 71 Clearly, for this purpose the charged molecules should be able to diffuse through the LbL shell. This type of ‘post-loading’ has been used to encapsulate negatively charged species in polyelectrolyte microcapsules derived from MF templates, through interaction with MF remnants. 48, 49 Positively charged species have been encapsulated by using an alginate 71 or PSS 70 matrix inside the microcapsules.

![Figure 4. Loading of (green) fluorescently labelled urease in ‘hollow’ polyelectrolyte microcapsules.](image)

**Drug delivery applications of polyelectrolyte microcapsules**

Polyelectrolyte microcapsules may find applications in very distinct fields. They may be used as microreactors for the synthesis 11 or separation 72 of materials or they may act as sensors. 73-75 For a couple of years there has been serious interest in exploring their potential as drug delivery vehicles. 40 In the field of drug delivery there is an urgent need for time and space controlled drug delivery systems. Therefore, the LbL technique has recently been applied to prepare stimuli responsive polyelectrolyte microcapsules for controlled drug delivery. The following paragraphs review stimuli that have been exploited to induce the release from polyelectrolyte microcapsules.
**pH-responsive polyelectrolyte microcapsules**

Generally speaking, polyelectrolyte microcapsules composed of weak polyelectrolytes are responsive to the pH of the environment. PAH, one of the most popular polyelectrolytes for the fabrication of hollow polyelectrolyte microcapsules is a weak polyelectrolyte (pKₐ of 8.7 in salt free solution). When complexed to PSS the apparent pKₐ changes to 10.7 as described by Petrov et al. 76 When the environmental pH becomes higher (in the case of a polybase) or lower (in the case of a polyacid) than the pKₐ, the polyelectrolytes become uncharged, which in turn, disassembles the microcapsules.

![Figure 5. AFM images of PSS/PAH based polyelectrolyte microcapsules treated with pH 3.5 (left) and pH 12 (right) buffers before drying. The microcapsules were prepared on MF particles. The porous structure of the polyelectrolyte shell treated with the acidic solution is clearly visible.](image)

Rubner et al. described the influence of pH on the charge density and morphology of PAH based planar multilayers. 77 Using AFM they observed that pore formation occurs when PAH containing multilayers are placed in an acidic environment, whereas the multilayers exhibit a smooth surface at a higher pH. Also, pore formation seemed to be a reversible process as the pores disappeared when the pH was increased. Antipov et al. made similar observations on PSS/PAH-based polyelectrolyte microcapsules. 78 In Figure 5 one can clearly see pores in the polyelectrolyte microcapsules treated with an acidic solution (left image), whereas the shell of microcapsules treated with an alkaline solution is intact (right image). The pH dependent integrity of the shell makes the permeability of the microcapsules to high molecular weight substances also pH dependent. As shown in Figure 6, the microcapsules are permeable in the acidic region whereas they are impermeable in the
alkaline region. Using this reversible pore formation Sukhorukov et al. filled PSS/PAH polyelectrolyte microcapsules with FITC-dextrans: dextrans were allowed to diffuse in the microcapsules at low pH while they became entrapped in the microcapsules by increasing the pH.  

The pH dependent behaviour of polyelectrolyte microcapsules containing one or two weak polyelectrolytes has been also described by Déjugnat et al. 47 for PSS/PAH microcapsules and by Mauser et al. 79 for PMA/PAH (PMA: poly(methacrylic acid)) microcapsules. Both authors reported swelling of the microcapsules when the pH was shifted towards the pK_a of one of the polyelectrolytes. PSS/PAH microcapsules started to swell when the pH was above 11 and disassembled when the pH was above 12. Concerning PMA/PAH microcapsules, swelling, followed by dissolution of the microcapsules, could be observed both at low and high pH, thus resulting in a dual pH-responsive behaviour of PMA/PAH microcapsules. The permeability of such dual responsive microcapsules was studied by Shutava et al. 80 for microcapsules based on tannic acid and PAH: the microcapsules seemed impermeable to FITC-dextrans at neutral pH but became permeable at both low and high pH.

The reversible pH dependent swelling of PSS/PAH microcapsules has been used by Déjugnat et al. 81 for the encapsulation of high molecular weight compounds such as rhodamine-labelled PSS and FITC-dextrans. By shortly exposing the microcapsules to a pH above 11 they swell dramatically and become largely permeable to high molecular weight compounds. A rapid lowering of the pH below 11 shrinks the microcapsules, returns them to their closed state, which entraps the molecules. Similarly, release of high molecular weight compounds can be obtained from the filled microcapsules by transferring them from neutral pH to a pH above 11.

Pharmaceutical applications of pH-responsive polyelectrolyte microcapsules may be the delivery of entrapped drugs at locations with a pH lower than that of serum (i.e. 7.4). A lower pH is found for example in the stomach, 82 vagina, 83 extracellular matrix of tumours, 84, 85 inflammatory and ischemic regions, intracellular vesicles like endosomes, lysosomes and secretory granules. Importantly, except in the stomach, the pH in these tissues and cellular organelles is only slightly lower than 7.4. It will therefore be a challenge to design polyelectrolyte microcapsules that are stable in the blood but release their payload at a pH of e.g. ~6.8. None of the present pH-responsive polyelectrolyte microcapsules fulfil this requirement. This is in contrast with the well studied pH-responsive microgels, 86-92 fabricated from weakly acidic or basic polymers, which swell at lower pH and release encapsulated molecules. Other hydrogels, like the commercially available pH-sensitive SQZ GelTM (Macromed Inc, USA), shrink upon lowering the pH and squeeze out the drug molecules. 93 The pH at which these hydrogels start to swell or shrink has been fine-tuned to
physiologically relevant pH's by playing around with the hydrophobicity of the hydrogel. 94, 95 Apart from microgels that show a pH dependent swelling, also microgels 96-99 and liposomes 100, 101 which start to degrade or to dissolve at a pH lower than 7.4 have been proposed as a pH sensitive delivery system. Most of the pH-responsive hydrogels are prepared by radical crosslinking of derivatives of polyacrylamide, vinylpyridine, 102 vinyl imidazole 103 and amino acids. 93 This is a major drawback as the radical crosslinking may affect the integrity of the encapsulated drugs like proteins or DNA. Therefore, physically cross-linked pH sensitive hydrogels have been recently described. 104, 105 Besides pH sensitive microgels and liposomes, pH responsive polymeric micelles have also gained attention as an extra- or intracellular drug delivery system, especially in the treatment of cancer. 106-108 Excellent reviews on pH-sensitive micelles have been recently published. 109, 110 Important to note is that intracellular drug delivery by pH sensitive polyelectrolyte microcapsules will probably be limited to phagocytes and cancerous cells as mostly those cells are able to internalize micrometer-sized particles, being the size range of most polyelectrolyte microcapsules studied today.

Figure 6. Open (a, c) at pH 3.5 and closed (b, d) at pH 10 states of polyelectrolyte shells prepared on MF particles (a, b) and CdCO₃ crystals (c, d).
CHAPTER 1 – RELEASE MECHANISMS FOR POLYELECTROLYTE MICROCAPSULES

Figure 7. Confocal microscopy images showing microcapsules consisting of 8 layers PSS/PAH (a) being impermeable to fluorescently labelled PAH (Mw~70kDa) in the absence of salt, (b) being impermeable to fluorescently labelled PAH in the presence of $10^2$ M NaCl, (c) coloured with rhodamine 6G and (d) filled with fluorescently labelled PAH after removal of the excess PAH following a washing/centrifugation step of the microcapsules in image (b).

Salt responsive polyelectrolyte microcapsules

The first report on polyelectrolyte microcapsules decomposable by salts was made by Caruso et al. $^{68}$ They fabricated polyelectrolyte microcapsules using DNA/spermidine. It is known that DNA-spermidine interactions are reduced at higher ionic strength. When the DNA/spermidine microcapsules were immersed in a solution containing 5 M of salt, the multilayers completely dissolved leading to the destruction of the microcapsules. Ibarz et al. reported that the permeability of hollow PSS/PAH polyelectrolyte microcapsules, templated on MF cores, for high molecular weight compounds, sharply improved when the salt concentration exceeded $10^2$ M. The higher permeability was not due to the formation of pores, $^{111}$ this is in contrast to the pH induced pore formation in PSS/PAH microcapsules as
reported above. Föster resonance energy transfer between rhodamine and fluorescein labelled PAH revealed structural changes within the polyelectrolyte multilayers. A sharp decrease in FRET (indicating a longer distance between the polyelectrolyte layers) was observed around a $10^{-2}$ M salt concentration, which does correspond with the higher permeability of the capsule wall. The salt induced permeability of the microcapsules was explained by a shielding of the charges on the polyelectrolytes that lowers the interactions between the oppositely charged polyelectrolytes and facilitates the diffusion of macromolecular substances through the multilayers.

The reversible switching of the permeability of polyelectrolyte microcapsules through variation of the salt concentration has also been used to encapsulate high molecular weight compounds. 111 (Figure 7) At higher salt concentration the PSS/PAH capsule are open to macromolecules with a molecular weight of up to 70 kDa while at lower salt concentration the wall closes. Antipov et al. reported similar findings on PSS/PAH microcapsules fabricated on inorganic CdCO₃ crystals as sacrificial template. 78

Although some salt responsive drug delivery systems have been proposed, 112-115 salt responsive polyelectrolyte microcapsules will probably have no application in drug delivery as ionic strength variations in the human body do not exist. However, the intra- versus extracellular concentration of a number of ions differs significantly. For example, the concentration of sodium, calcium and potassium inside cells (respectively ~11 mM, ~230 nM, ~115 mM) is significantly different from the concentration in serum (respectively ~140 mM, ~2 mM, ~4.5 mM). 116-118 Additionally, the intracellular concentration of calcium significantly varies from cell organelle to cell organelle, with the highest calcium concentrations in the mitochondria and sarcoplasmic reticulum. Micogels that swell/shrink upon sensing a specific ion may be suitable to deliver drugs in the cytosol or in certain cell organelles. As an example, polyacrylate hydrogels were described by Horkay et al. 119, 120 which significantly swell when the calcium concentration in the environment becomes lower than 1 mM (note that the intracellular concentration is ~230 nM). Also, diseases exist which are characterized by a non-physiological plasma concentration of one or more ions. For example, a low calcium concentration in serum has been exploited as a trigger for the delivery of oestradiol in the treatment of osteoporosis. 121 However, the relevance of this system is limited as it is well-known that osteoporosis is not always characterized by low calcium levels in the serum.

Light-responsive microcapsules

The first report on optically sensitive polyelectrolyte microcapsules was made by Tao et al. 122 They reported on the use of the azo dye Congo red as a constituent of polyelectrolyte multilayers. Irradiation of such polyelectrolyte microcapsules with visible light
(for 120 min) slightly distorted the polyelectrolyte multilayers, enhancing their permeability for fluorescently labelled dextrans with a molecular weight of up to 464 kDa. Both the long irradiation time and the use of visible light (which does not sufficiently penetrate the skin) limit, however, the in vivo use of light responsive polyelectrolyte microcapsules for drug delivery.

Figure 8. Confocal microscope images demonstrating remote release of encapsulated rhodamine-labelled PSS polymers from a polyelectrolyte multilayer capsule containing gold sulphide core/gold shell nanoparticles in its walls. Fluorescence intensity profiles along the line through the capsule show that it is filled with fluorescent polymers before (a) and empty after (b) laser illumination. After the release of encapsulated polymers, the leftover fluorescent intensity is observed only in the walls of the capsule, (b). Insets show black and white transmission microscope images of the same capsule. Incident intensity of laser diode operating at 830 nm was set at 50 mW.

Near infrared (IR) laser light is interesting for drug delivery applications as most tissues show negligible adsorption in the 800-1200 nm region, making IR-laser light attractive for inducing structural changes in drug containing microcapsules injected in tissues located at the surface of the body. IR-light sensitive polyelectrolyte microcapsules have been fabricated by incorporating gold nanoparticles in their PSS/PAH polyelectrolyte shell. Upon irradiation with IR-light (short pulses of < 10 ns) the gold nanoparticles absorb the energy and transform it into heat, which locally disturbs the integrity of the polyelectrolyte microcapsules. The Caruso group was the first to demonstrate the release of encapsulated biomacromolecules upon IR irradiation of polyelectrolyte microcapsules functionalised with gold nanoparticles. Also Skirtach et al. reported recently on near IR sensitive polyelectrolyte microcapsules. Figure 8a shows confocal and transmission images of a PSS/PAH capsule, doped with gold nanoparticles, which is filled with rhodamine-labelled PSS. Upon irradiation with IR-laser light (Figure 8b) the capsule breaks open, as can be seen from the strongly deformed structure, leading to the release of the encapsulated
material. Figure 9 shows SEM images of microcapsules (a) before irradiation, (b) after irradiation at moderate intensity and (c) after irradiation at high intensity. They clearly demonstrate the drastic impact of IR-light on the morphology of the microcapsules.

![SEM images of microcapsules](image)

*Figure 9.* Irradiation of the microcapsules with multiple laser pulses. SEM images of the microcapsules: a) before irradiation; b) after moderate radiant exposure (30 mJ/cm²); and c) after radiant exposure of 50 mJ/cm² and higher. The insets are the corresponding TEM images.

‘Photo-controlled’ release of drugs remains an attractive approach as it should allow controlling the delivery in time and space. Therefore, IR-sensitive polyelectrolyte microcapsules may be useful for controlled drug release, because after subcutaneous injection the release of encapsulated material may be triggered by local irradiation of the skin with IR-light. Besides IR-sensitive polyelectrolyte microcapsules, also photo-sensitive liposomes and photo-sensitive polymer micelles are under development. 

Light-triggered release of drugs from liposomes is due to a light induced destabilization of the lipid bilayer that can occur via (1) light sensitized production of reactive species (such as singlet oxygen) that cause fragmentation of the lipids in the liposomes, (2) photo-polymerization or (3) photo-isomerization of the lipids. Additionally, local heating upon
irradiation of gold nanoparticles incorporated in the liposomes may also induce drug release. In case of polymer micelles the release is due to a photo-chemical reaction that alters the hydrophobicity of the micelle-forming polymers, leading to the release of encapsulated material. However, most of the photo-sensitive liposomes and micelles are responsive to the shorter wavelengths which limits their application in vivo. Indeed, UV-light may damage cells and shows a limited penetration depth (<0.5 mm) in tissues. Therefore, we believe that research on light-sensitive delivery systems should focus on IR-light sensitive systems because IR-light is less harmful and has a much deeper penetration depth in tissues (e.g. 8 mm in the liver at a wavelength of 1070 nm).

Magnetic field responsive microcapsules

Lu et al. reported on hollow PSS/PAH-based microcapsules (using MnCO₃ as sacrificial template) which are addressable by a magnetic field due to the incorporation of one layer of positively charged gold coated cobalt nanoparticles (Co@Au) into the polyelectrolyte shell of the microcapsules. Microcapsules with a rather thick wall (approximately 250 nm) were observed by AFM. The thick walls are probably due to the aggregation of Co@Au nanoparticles. Upon applying an oscillating magnetic field, the ferromagnetic Co@Au nanoparticles twist, which disturbs the structure of the polyelectrolyte multilayers and, consequently, allows the diffusion of macromolecules through the capsule wall (Figure 10). Furthermore it was shown that only microcapsules having one layer of Co@Au nanoparticles could switch their permeability upon application of a magnetic field, whereas microcapsules having multiple Co@Au nanoparticles layers remained impermeable upon applying a magnetic field. The magnetically induced permeability could be of interest for drug loading and release from polyelectrolyte microcapsules. However, one should note that the long exposure time (30 min) and strong magnetic field (1200 Oe, 150 Hz) required to permeabilize the Co@Au microcapsules described above led to a 30°C increase in temperature of the capsule suspension which is, highly likely, problematic for the loading of thermosensitive drugs (like e.g. proteins) in the microcapsules. Clearly, magnetically induced drug release from microcapsules injected in the body would be also problematic.

Some other magnetic-responsive delivery systems have been reported as well. The group of Langer et al. embedded magnetic beads together with insulin in a ethylenevinylacetate copolymer matrix and demonstrated, after implantation of the matrix in diabetic rats, that the blood glucose levels drastically decreased each time an external oscillating magnetic field was applied. Couvreur et al. made alginate spheres containing insulin and ferrite microparticles and showed a 50-fold increase in insulin release in the presence of an oscillating magnetic field. It was believed that the vibrating magnetic
particles induced openings in the polymer matrix facilitating the release of insulin. Currently there is a renewed interest in magnetically controlled drug delivery systems. Babincova et al. developed doxorubicin loaded magnetoliposomes which are first targeted into tumours by a static magnetic field and, consequently, massively release the doxorubicin upon application of an oscillating magnetic field. The release occurs due to a local increase in temperature (up to 42°C) which ‘melts’ the liposomes.

Figure 10. Assembly and magnetically permeabilization of the polyelectrolyte microcapsules by oscillating magnetic field (1200 Oe, 150 Hz) for 30 min. The molecular weight of the FITC-dextran was 2000 kDa.

Glucose-responsive polyelectrolyte microcapsules

Glucose responsive microcapsules, encapsulating insulin, could be promising for the treatment of diabetes mellitus patients as the insulin would only be released when the glucose concentration in the blood exceeds a physiological value.

Mc Shane et al. fabricated polyelectrolyte microcapsules containing glucose oxidase within the multilayers. They expected the microcapsules to be glucose sensitive because the glucuronic acid, occurring from the oxidation of glucose by the immobilized glucose oxidase, would drop the pH at the surface of the microcapsules and hence modify its permeability. However, glucose did not disassemble the microcapsules, nor did the authors report on any possible change in permeability of the microcapsules. Other glucose-sensitive delivery systems for insulin have been described. Indeed, pH-sensitive hydrogels loaded with insulin, glucose oxidase and catalase have been well-studied. In these hydrogels glucose oxidase also generates glucuronic acid when glucose enters the hydrogels. This lowers the pH which swells or shrinks the hydrogel and which leads to the release of insulin. The
function of catalase in these hydrogels is to convert the aggressive hydrogen peroxide, occurring from the enzymatic conversion of glucose, to oxygen and water. Unfortunately these systems have important shortcomings. Firstly, they lack a reproducible and rapid response on a long-term basis. Secondly, these hydrogels are often neither biocompatible nor biodegradable. Also, many of the reported systems are only glucose-responsive at very high (4 to 36 mg/ml) (clinically irrelevant) glucose concentrations. Indeed, in a healthy person the blood glucose concentration is around 1 mg/ml, whereas glucose levels above 2 mg/ml are already common in diabetic patients. Therefore, glucose-responsive delivery systems should ideally start to release insulin as soon as the glucose level reaches about 2 mg/ml.

*Degradable polyelectrolyte microcapsules*

For many biomedical applications, especially for drug delivery, biodegradable polyelectrolyte microcapsules would be preferred to non-degradable ones. Several reports on degradable LbL films deposited on planar substrates have been published. Lynn *et al.* reported on degradable polycations (poly-β-aminoesters) to make LbL films for the controlled release of small drug molecules and DNA. Serizawa *et al.* and Picart *et al.* reported on enzymatically degradable polysaccharide-based polyelectrolyte films, which seemed to be degradable in the mouths of rats. Biodegradable polyelectrolyte films could be of interest for the surface modification of implants by rendering them more biocompatible or by making them drug eluting by the incorporation of drug molecules within the multilayers.

The first paper on degradable polyelectrolyte microcapsules was published by Mohwald *et al.* Polyelectrolyte microcapsules were covered with a lipid bilayer rendering them impermeable to low molecular weight compounds such as carboxyfluorescein. When phospholipase A2 was added, the lipid bilayer degraded resulting in the formation of pores rendering the microcapsules permeable to carboxyfluorescein. Several papers reported on the use of biopolymers such as polypeptides, polysaccharides and DNA. Although the authors did not report on the degradation of these microcapsules, highly likely they will degrade in vivo.

*Redox responsive microcapsules*

It is well-known that the colon and the cytosol of cells have a more reducing environment compared to other locations in the body. This has been exploited for colon
specific delivery of drugs using hydrogels that contain azo-bounds which are reductive-sensitive.\textsuperscript{154} Also redox-sensitive polymers containing a disulfide linkage in their backbone have been synthesized.\textsuperscript{155} The high redox-potential inside cells has recently been used as a trigger to enhance the intracellular release of plasmid DNA, antisense oligonucleotides, or small interfering (si) RNAs from pharmaceutical carriers containing disulfide linkages.\textsuperscript{156}

Polyelectrolyte microcapsules that are redox-sensitive have recently been reported by Haynie \textit{et al.} They were fabricated from anionic and cationic polypeptides containing cysteine groups.\textsuperscript{151} Upon cross-linking of the cysteine’s thiol groups (leading to disulfide bonds) microcapsules were found to be stable at both neutral and acidic pH. However, after reducing the disulfide bonds, the microcapsules disassembled at a pH lower than the pK\textsubscript{a} of the anionic polypeptides as the multilayers were then no longer stabilized by electrostatic or covalent bonds. Although a redox triggered deconstruction of polyelectrolyte microcapsules is highly suitable for \textit{in vivo} applications, the system reported by the Haynie groups requires an acidic pH to disassemble the microcapsules. At pH 7.4 the microcapsules will remain stabilized through the electrostatic interactions between the cationic and anionic polypeptides as both are charged.

The Caruso group recently fabricated microcapsules with a shell composed of multiple layers of thiol-modified polymers, bound to each other through hydrogen bonds and which can be cross-linked via disulfide bridges.\textsuperscript{157} As described in the introduction of this chapter it is possible to make multilayers based on interactions other than electrostatic interactions. Hydrogen-bound multilayers deposited on flat substrates have been well-studied. However, polyelectrolyte microcapsules fabricated from hydrogen-bound multilayers are less investigated. The attractiveness of hydrogen-bound multilayers lies in the fact that some of them can be deconstructed under physiological conditions. For example hydrogen-bound multilayers containing poly(methacrylic acid) (PMA) as a proton donor are fabricated at low pH in order to have the PMA in its uncharged form, whereas at pH 7.4 the PMA becomes charged and repulsion between the PMA chains will occur, disassembling the multilayer film. Multilayered microcapsules based on hydrogen bond interactions between PMA and poly(vinylpyrrolidone) (PVPON) were fabricated on protein filled mesoporous silica (note that silica can be dissolved in a hydrogen fluoride (HF) solution).\textsuperscript{157} Cross-linking through disulfide bonding was accomplished using PMA functionalized with cysteinamine moieties. Microcapsules cross-linked through disulfide bonds were found to be stable under physiological conditions. However, when the disulfide bonds were reduced, the microcapsules disassembled readily and released their content.
CONCLUSIONS AND OUTLOOK

Drug delivery systems that can release their payload in response to internal or external triggers may offer great advantages. The potential of the LbL technique to make (bio)responsive drug delivery systems has recently been discovered. We have reviewed such (bio)responsive polyelectrolyte microcapsules and compared them with other types of (bio) responsive drug delivery systems which are under development.

In our opinion, currently only the IR-sensitive and the biodegradable polyelectrolyte microcapsules seem realistic for in vivo drug delivery. Unfortunately, detailed in vivo data on such microcapsules are lacking. Indeed, most of the so far studied polyelectrolyte microcapsules only respond to extreme stimuli that do not occur or cannot be applied in vivo. One should realize that the physiological processes during which release of drugs from the polyelectrolyte microcapsules should occur create only subtle physicochemical changes in the human body. Consequently, polyelectrolyte microcapsules which are sensitive to such subtle changes are required. A clear challenge is to synthesize polyelectrolytes that allow the design of polyelectrolyte microcapsules sensitive to small (and physiologically) relevant changes of pH, salt concentration, glucose concentration and redox potential. For example, it would be of interest to build polyelectrolyte microcapsules which could escape from endosomes at the time acidification starts in the endosomes. Subsequently they should release their payload in the cytosol or nucleus in response to the high reductive environment of the cytosol or in response to nucleus specific enzymes.

Another important issue in capsule science is promoting cellular uptake, specific targeting or just avoiding uptake or adsorption on the microcapsules. It is well known that aspecific adsorption to particles for intravenous injection should be avoided in order to avoid the formation of large aggregates or to avoid uptake by fagocyting cells from the immune system. The issue of biofunctionalisation has been addressed by several groups. Shielding of polyelectrolyte microcapsules from non-specific adsorption has been performed by pegylation of the capsule surface using a polyelectrolyte grafted with polyethylene glycol (PEG) side chains. Targeting of microcapsules using an external magnetic field to concentrate the microcapsules at a certain location has been demonstrated by Zebli et al. using microcapsules functionalised with magnetic particles. The Donath group has provided microcapsules with virus functionalities by incorporating rubella like particles in the polyelectrolyte shell. Such microcapsules could hold promise to enhance endosomal uptake/escape or promote the delivery of encapsulated material to the cell nucleus. Lipid coating of microcapsules was first demonstrated by Moya et al. Recently the Caruso group coupled antibodies to microcapsules covered with lipid bilayers and showed that those
antibodies could bind the secondary antibodies. This approach is promising towards the selective uptake of microcapsules by specific cell types.

Until now the pharmaceutical technological aspects of polyelectrolyte microcapsules have not thoroughly been studied. Capsule formulations, stable for extended periods, should be developed using freeze-drying or other conservation techniques. With respect to clinical applications, sterilisation and up scaling will become important issues. Finally, the toxicological and immunological aspects of the microcapsules should be explored into detail. This issue has briefly been addressed in one paper indicating no significant effect on cell viability at low capsule concentration. It remains however important to further concentrate on this topic and to evaluate the toxicity of the microcapsules in vivo.

Clearly, polyelectrolyte microcapsules are an intriguing new type of vesicle which may offer potential for biomedical applications. Also, the science of layer-by-layer assembly is gaining interest from scientists in a broad field which is a very interesting evolution. This multidisciplinary approach is the prerequisite to find effective applications for polyelectrolyte microcapsules. It is very important that people with different backgrounds combine their efforts in order to come up with new applications for these microcapsules in medicine. Finally, keeping in mind that other concepts and devices do exist, the major challenge for polyelectrolyte microcapsules research is also to determine precisely for which applications they offer distinct advantages, compared to other vesicles.

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CHAPTER 2 SELF-EXPLODING MICROCAPSULES

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ABSTRACT

In this chapter we describe the design of self-exploding microcapsules by layer-by-layer (LbL) coating of degradable dextran-hydroxyethyl methacrylate (dex-HEMA) microgels with the polyelectrolytes poly(allylamine hydrochloride) (PAH) and sodium polystyrenesulfonate (PSS). When the microgel core degrades, the swelling pressure increases and at a critical value of the swelling pressure the surrounding membrane will suddenly rupture. This type of microcapsules could be very promising for pulsed drug delivery.
INTRODUCTION

Pharmaceutical research strives to design systems that deliver drugs according to therapeutic needs. For example, to avoid multiple dosing, scientists have designed controlled drug delivery tablets, pumps, implants, and patches that provide a continuous drug release over a prolonged period of time. However, there are many applications in medicine where a non-uniform release profile could be more beneficial.\textsuperscript{1-4} For example, for bioactive agents such as hormones, many have suggested that pulsed release may offer advantages over continuous release.\textsuperscript{5-7} A pulsed release pattern could also be advantageous for drugs that develop biological tolerance when they are constantly present at their target site. Additionally, a device that could release an initial dose and a booster dose of a vaccine at different times after a single injection (‘single-shot vaccination’) would be promising as it would reduce the number of injections required to generate immunity.\textsuperscript{8}

To achieve pulsed delivery we try to design ‘self-exploding microcapsules’. As Figure 1 shows, we envision (bio)degradable microgels surrounded by a membrane that is permeable to water but impermeable to both the microgels’ degradation products and the entrapped drugs. As the microgels degrade their swelling pressure will increase which should rupture the membrane. Rupture is followed by a sudden release of the entrapped drug. The release time of the drug will therefore be governed by the degradation kinetics of the microgels, as this will determine the increase in swelling pressure and thus the time of explosion of the microcapsules. This chapter aims to investigate whether we can experimentally achieve such self-exploding microcapsules. Microcapsules which rupture upon applying an external trigger such as an electric field,\textsuperscript{9,10} IR light,\textsuperscript{11-15} pH\textsuperscript{16,17} etc, have been reported before. However, the requirement of an external trigger greatly limits the \textit{in vivo} applicability. To the best of our knowledge self-exploding microcapsules, in which an internal mechanism governs the explosion, have never been reported.
CHAPTER 2 – SELF-EXPLODING MICROCAPSULES

Figure 1. Schematic representation of self-exploding microcapsules. (I) Before degradation the polymer chains in the microgels are connected in a three-dimensional network by chemical cross-links ( ). The yellow discs represent the encapsulated drugs. The microgels are surrounded by a polyelectrolyte membrane. (II) The microgels degrade by hydrolysis of the cross-linkers. As degradation proceeds, the cross-link density decreases and free polymer chains occur. (III) At the end of the degradation process, the core of the microcapsules has become a polymer solution and the corresponding swelling pressure causes the membrane to rupture.

MATERIALS AND METHODS

Materials.

1,1’-carbonyldiimidazole (CDI), 2-hydroxyethyl methacrylate (HEMA), N,N,N’,N’-tetramethylenediamine (TEMED), 4-N,N-dimethylaminopyridyne (DMAP), dimethyl sulfoxide (DMSO), magnesium sulfate (MgSO₄), hydroquinone monomethyl ether, dimethyl aminoethyl methacrylate (DMAEMA), tetramethyl rhodamine B isothiocyanate (TRITC), PSS (Mw~70 kDa), PAH (Mw~70 kDa), dextran (Mw~19 kDa) and FD (Mw~4kDa, 20 kDa and 150 kDa) were purchased from Sigma-Aldrich-Fluka. Dichloromethane (CH₂Cl₂), Potassium peroxodisulfate (KPS) and polyethylene glycol (PEG; Mw~20 kDa) were purchased from Merck. Dextran (Mw~19 kDa) was obtained from Fluka. TRITC labeled PAH was prepared as reported in literature. ¹⁸

Synthesis of dex-HEMA

Dex-HEMA with a degree of substitution (DS; the number of HEMA groups per 100 glucopyranose units in dextran) of 2.5 was synthesized in a two step reaction according to literature. ¹⁹ ²⁰ The reaction scheme is shown in Figure 2. Flasks, stoppers, stirring bars and dextran were dried overnight under vacuum at 40 °C. In a the first step, CDI (2.43 g; 15 mmol) was dissolved in 25 ml CH₂Cl₂ and stirred under a nitrogen atmosphere. HEMA
(1.3 g; 10 mmol) was added drop wise and stirred for 1 h under a nitrogen atmosphere. The CH₂Cl₂ phase is extracted twice with water and MgSO₄ is added to dry the CH₂Cl₂ phase. The MgSO₄ is filtered and a spatula tip hydroquinone monomethyl ether is added to prevent spontaneous polymerisation. The CH₂Cl₂ is evaporated yielding carbonylimidazole activated HEMA (HEMA-CI). The purity of the HEMA-CI is investigated by ¹H-NMR in CDCl₃.

In the second step, dextran (50 g) and DMAP (10 g) are dissolved in 500 ml DMSO and stirred until completely dissolved. HEMA-CI was added to the reaction mixture taking into account the purity of the HEMA-CI and conversion efficiency of the reaction as previously reported.¹⁹,²⁰ The reaction mixture is stirred for 4 days at room temperature under a nitrogen atmosphere and subsequently put in dialysis bags with an Mw cut-off of 14 kDa. After 5 days of dialysis against pure water the solution is freeze dried and dex-HEMA is obtained as a white fluffy product. The DS of the dex-HEMA is determined by ¹H-NMR in D₂O.

![Reaction scheme for the synthesis of dex-HEMA.](image)

**Preparation of dex-HEMA-DMAEMA microgels.**

Dex-HEMA-DMAEMA microgels with initial water content of 70% (w/w) were prepared according to Franssen et al.²¹ In detail, 71 mg dex-HEMA, 20 µl FITC-dextran solution (50 mg/ml) and 35 µl DMAEMA were dissolved in 1.577 ml water and subsequently emulsified with a 3.35 ml 24 % (v/v) aqueous PEG solution. Radical polymerisation of the aqueous dex-HEMA emulsion droplets was initiated by adding TEMED (100 µl pH neutralized with 4 N HCl) and KPS (9 mg). The reaction was carried out at room temperature for 1h. Afterwards the obtained microgels were washed three times with pure water to remove PEG, KPS and TEMED. Finally the microgels were suspended in 5 ml and stored at -20 °C.
**LbL coating of the dex-HEMA microgels.**

500 µl (15 mg/ml) microgels were dispersed in 1 ml polyelectrolyte solution (2 mg/ml in 0.5 M NaCl) and shaken for 15 min. The excess polyelectrolyte was removed by 2 centrifugation steps. This procedure was repeated until 3 polyelectrolyte bilayers were obtained.

**Confocal laser scanning microscopy (CLSM).**

CLSM experiments were performed using a MRC1024 Bio-Rad scanning system equipped with a 60x water immersion objective. Permeability experiments were performed in 96 well plates by mixing a 50 µl FD (1 mg/ml) solution with 50 µl microgel dispersion, both in 0.1 M carbonate buffer at pH 9 or in a 0.1 M phosphate buffer at pH 7. FRAP experiments were performed by bleaching a circular region inside the microcapsules. The fluorescence recovery was quantified by calculating the ratio of the fluorescence intensity of the recovering region to the fluorescence intensity of a non-bleached region in the same capsule.

![Figure 3. Reaction mechanisms for the hydrolysis and polymerisation of dex-HEMA.](image-url)
CHAPTER 2 – SELF-EXPLODING MICROCAPSULES

RESULTS AND DISCUSSION

Dex-HEMA was synthesized as reported in literature by a two step reaction. In a first step HEMA was activated by reaction with carbonyldiimidazole. Secondly the activated HEMA was conjugated to dextran yielding dex-HEMA, bearing methacrylate groups connected to the dextran backbone by a carbonate ester bond. Biodegradable dextran based microgels, with an average diameter of 7 µm, were prepared from dextran-hydroxyethyl methacrylate (dex-HEMA) as described by the Hennink group. Radical polymerisation was performed at ambient conditions using the KPS/TEMED initiator/catalyst system. Figure 3 shows the reaction mechanism of the dex-HEMA polymerisation. The obtained three dimensional structure is schematically represented in Figure 4. Because the methacrylate groups are connected to the dextran backbone by a carbonate ester, the degradation of the microgels occurs by hydrolysis of the cross-links, yielding both the original dextran chains and HEMA oligomers as degradation products. The reaction mechanism of the hydrolysis of the carbonate ester in dex-HEMA is shown in Figure 3. The degradation of dex-HEMA gels can be tailored from days to months by changing the cross-link density. To obtain positively charged microgels we copolymerized dex-HEMA with dimethyl aminoethyl methacrylate (DMAEMA; pKa 8.3). These microgels are hereafter referred to as dex-HEMA-DMAEMA microgels.

Figure 4. Schematic representation of the polymerization of dex-HEMA (step A), leading to the formation of intra- and intermolecular cross-links which form the three dimensional hydrogel network, and (step B) the hydrolysis of the dex-HEMA hydrogels leading to the formation of dextran chains and oligomethacrylates as degradation products.

The dex-HEMA-DMAEMA microgels were coated with polyelectrolytes using the Layer-by-Layer (LbL) technology. Briefly, this technique is based on the sequential adsorption of oppositely charged polyelectrolytes on a charged substrate. The major
advantage of this technique is that the properties of the obtained membrane can be created to within nanometers. To the best of our knowledge LbL coating of micron sized hydrogel particles has not been reported yet. Sodium poly styrenesulfonate (PSS) (Figure 5C) served as polyanion while polyallylamine hydrochloride (PAH) (Figure 5B) served as polycation. This polyelectrolyte pair has been thoroughly studied and has proved to be ideally suited to coat colloids. 26-28 The adsorption procedure was monitored by measuring the electrophoretic mobility after each adsorption step. The initial $\zeta$-potential of the dex-HEMA-DMAEMA microgels was +29 mV and oscillated steadily between -50 mV and +50 mV upon alternating adsorption of PSS and PAH, well in agreement with published data on PSS/PAH coating of other types of particles. 28 To visualize the coating, rhodamine-labeled PAH (PAH<sub>TRITC</sub>) was used. Figure 5A shows a confocal laser scanning microscopy (CLSM) image of (PSS/PAH)<sub>3</sub>-coated dex-HEMA-DMAEMA microgels; a clear fluorescent ring can be observed, which indicates the formation of a polyelectrolyte membrane surrounding the microgels.

Figure 5. (A) CLSM image of (PSS/PAH)<sub>3</sub>-coated dex-HEMA-DMAEMA-microgels. PAH was fluorescently labeled with TRITC. Molecular structure of poly(allylamine hydrochloride) (B) and poly(styrene sulfonate) (C), both in their charged form.

As outlined above, to obtain self-explooding microcapsules the membrane should be impermeable to the degradation products of the microgels. As the microgels degrade by hydrolysis of the HEMA-crosslinks, the major degradation product is dextran (being the chains used to synthesize the dex-HEMA which had an average molecular weight of 19 kDa). By CSLM we investigated the permeability of the (PSS/PAH)<sub>3</sub> coating to FITC-dextrans (FD) of various molecular weights (4 kDa and 20 kDa) at respectively pH 7 and 9. Uncoated and (PSS/PAH)<sub>3</sub>-coated dex-HEMA-DMAEMA microgels were immersed in FD-solutions.
Chart 1 shows that the uncoated dex-HEMA-DMAEMA microgels were permeable to both FD 4 kDa and 20 kDa, independent of the pH. The permeability of the (PSS/PAH)$_3$-coated dex-HEMA-DMAEMA microgels was however pH dependent: at pH 7 the coated microgels were permeable to both 4 kDa and 20 kDa FD’s while at pH 9 they were impermeable. Similar findings on the pH dependent permeability of (PSS/PAH)$_3$ coatings were reported by Antipov et al. These permeability observations allowed us to suggest that at pH 9 the polyelectrolyte membrane should be impermeable to the degradation products of the microgels, being dextrans chains of 19 kDa. This should allow a build-up of the osmotic pressure upon degradation of the gel in the microcapsules. To the contrary, at pH 7 the (PSS/PAH)$_3$ coating is expected to be permeable to the degradation products and, consequently, a rise in osmotic pressure is not expected.

Chart 1. Permeability of uncoated and (PSS/PAH)$_3$-coated dex-HEMA-DMAEMA microgels to FD’s. Images A and B illustrate the (PSS/PAH)$_3$-coated microgels immersed in a 20kDa FD solution at pH 7 (A) and pH 9 (B).

<table>
<thead>
<tr>
<th>Uncoated microgels</th>
<th>Coated microgels</th>
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<tr>
<td>FD 4kDa</td>
<td>FD 20kDa</td>
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<tr>
<td>Permeable</td>
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<td>pH 7</td>
<td>Permeable</td>
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<tr>
<td>pH 9</td>
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To verify this we studied the behavior of uncoated and (PSS/PAH)$_3$-coated dex-HEMA-DMAEMA microgels during degradation. Both microgel types were incubated at 37 °C at pH 7 as well as pH 9. Microscopy experiments on uncoated microgels revealed that at pH 7 the microgels were completely degraded after 5 days while at pH 9, they were completely...
Figure 6. (A) CLSM image of (PSS/PAH)$_2$-coated dex-HEMA-DMAEMA microgels 5 days after degradation at pH 7. 150 kDa FD were encapsulated in the microgels. (B) Fluorescence recovery curves after bleaching a spot inside the microcapsules. The red curve corresponds with non-degraded microcapsules (lower images) while the black curve corresponds with microcapsules degraded at pH 7 (upper images). (C) CLSM image of (PSS/PAH)$_2$-coated dex-HEMA-DMAEMA microgels 1 day after degradation at pH 9. Only remnants of broken microcapsules are present.

degraded in one day, due to accelerated hydrolysis at an alkaline pH. Figure 6A shows a CLSM image of (PSS/PAH)$_2$-coated dex-HEMA-DMAEMA microgels 5 days after degradation at pH 7. To prove that at pH 7 degradation of the microgels had indeed occurred we studied the mobility of encapsulated 150 kDa FD by fluorescence recovery after photobleaching (FRAP) before and after incubating the (PSS/PAH)$_2$-coated dex-HEMA-
DMAEMA microgels for 10 days in buffer at pH 7. Figure 6B clearly shows that in non-
degraded microgels the fluorescence does not really recover after the bleaching step
indicating that the FD chains are (sterically) immobilized in the microgel network. In
(PSS/PAH)$_3$-coated dex-HEMA-DMAEMA microgels incubated for 10 days at pH 7 the
fluorescence significantly recovers, indicating that the FD chains became mobile due to
degradation of the network in the microgels. Nevertheless the microgels were degraded, and
clearly explosion did not occur.

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**Figure 7.** Snapshots of (A) (PSS/PAH)$_3$-coated dex-HEMA-DMAEMA microgels during the rupturing of the
membrane and (B) uncoated dex-HEMA-DMAEMA microgels. The time interval between the snapshots is 15 min.

Consequently we studied the behavior of the (PSS/PAH)$_3$-coated dex-HEMA-
DMAEMA microgels at pH 9. As shown in Figure 6C, only remnants of broken polyelectrolyte
shells were visible after degradation of the dex-HEMA-DMAEMA microgels. To prove the
self-explosion of the particles we tried to witness the rupturing of the membrane. In this
experiment (PSS/PAH)$_3$-coated dex-HEMA-DMAEMA microgels with encapsulated 150 kDa
FD were placed at 80°C for 4 minutes (to accelerate the degradation) in buffer at pH 9. The
particles were subsequently put under the CLSM (at 40 °C) and followed over time.
Figure 4A shows four snapshots of the particles taken after respectively 60, 75, 90 and 105
min: rupturing of the coating occurs and the encapsulated 150 kDa FD is suddenly released
from the particles (Figure 7A).$^{30}$ Due to Laplace’s law larger microcapsules explode earlier
than smaller microcapsules. However, the time-interval during which all the microcapsules
ruptured is less than 10% of the total time required for the degradation of the microgel core.
As a negative control we also followed the behavior of non-coated dex-HEMA-DMAEMA
microgels. As shown in Figure 7B the microgels dissolve gradually in the alkaline solution.$^{30}$
As outlined above, when completely degraded a dex-HEMA gel turns into a solution of dextran and HEMA oligomers. In case the LbL membrane is permeable to the HEMA oligomers one can assume that, when the dex-HEMA-DMAEMA microgel is totally degraded, the swelling pressure of the microcapsule becomes the osmotic pressure of the corresponding dextran solution which is estimated to be 150 kPa for the dex-HEMA-DMAEMA microgels used in this study. 31 Therefore, assuming a particle mean diameter of 7 µm and using Laplace’s law ($\tau = \Delta \pi / 2r$; where $\tau$ is the the tensile strength [N/m], $\Delta \pi$ the osmotic pressure gradient [N/m²] and $r$ the radius [m]), an upper limit of the tensile strength of the (PSS/PAH)$_3$ membrane is estimated to be 0.26 N/m. The osmotic pressure of 150 kPa which is able to rupture the microcapsules is in accordance with the reported data on the osmotic pressure required to induce severe deformations in hollow polyelectrolyte microcapsules. 32, 33

CONCLUSIONS

In conclusion, this study shows that charged dex-HEMA microgels can be coated with polyelectrolytes using electrostatic interactions: CSLM and zeta-potential measurements proved that sequential adsorption of PSS and PAH at the surface of the dex-HEMA-DMAEMA microgels could be achieved. We observed that the permeability of the (PSS/PAH)$_3$ coating was pH dependent: while it was permeable to 20 kDa FITC-dextran at pH 7 it was impermeable at pH 9. Consequently, as the major degradation product of dex-HEMA-DMAEMA microgels is dextran of 19 kDa we expected that at pH 9 the (PSS/PAH)$_3$ coating should be impermeable to the degradation products. This should increase the (inner) swelling pressure of the microcapsules upon degradation of the entrapped gel, which could lead to a rupturing of the surrounding membrane. Indeed, a sudden rupturing of the coating due to the degrading gel could be experimentally confirmed. In this way self-exploding microcapsules are obtained as they explode without the need of an external trigger: the time of explosion being completely governed by the degradation kinetics of the entrapped gel which governs how the swelling pressure of the microcapsules increases as a function of time.

The dextran-based microgels described above are promising for biomedical applications based upon reports that dex-HEMA is biocompatible 34, 35 and because proteins can be easily incorporated inside dex-HEMA microgels with encapsulation efficiencies up to 90 %. 36 Our on-going research is focusing on the development of monodisperse microgels.
which should significantly enhance the simultaneous explosion of the microcapsules and we will further evaluate the potential of the self-exploding microcapsules for pulsed drug delivery.

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REFERENCES


CHAPTER 3

LAYER-BY-LAYER COATING OF DEGRADABLE MICROGELS FOR PULSED DRUG DELIVERY

Parts of this chapter are in press:


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CHAPTER 3 - LAYER-BY-LAYER COATING OF DEGRADABLE MICROGELS FOR PULSED DRUG DELIVERY

ABSTRACT

In the previous chapter we reported on ‘self-rupturing’ microcapsules which consist of a biodegradable dextran-based microgel surrounded by a polyelectrolyte membrane. Degradation of the microgel increases the swelling pressure in the microcapsules which, when sufficiently high, ruptures the surrounding polyelectrolyte membrane, applied by layer-by-layer (LbL) technology on the microgels’ surface. In this chapter we characterize in detail the morphology and permeability of the LbL coatings surrounding the dextran microgels and investigate how they behave during degradation of the microcapsules. We show that by fine-tuning the properties of both the microgel core and the LbL membrane a ‘pulsed release’ from the self-rupturing microcapsules can be obtained. Compared to ‘sustained’ release, ‘pulsed’ delivery could be an advantage for the delivery of certain drugs.
CHAPTER 3

LAYER-BY-LAYER COATING OF DEGRADABLE MICROGELS FOR PULSED DRUG DELIVERY

INTRODUCTION

Due to the ever increasing amount of biopharmaceuticals there is a growing need for advanced drug delivery systems. Instead of sustained drug release, pulsed drug delivery could be more attractive to deliver certain therapeutics. For example, a pulsed release pattern could be advantageous for drugs that develop biological tolerance when they are constantly present at their target site or for drugs that require dosing at night. Also, microparticles that could suddenly release antigens at well defined times after injection could show potential for ‘single-shot vaccination’. For this purpose the injectable device should consist of different types of micro particles, each type of particle suddenly releasing its encapsulated antigen at a well defined time after injection. Devices which show pulsed release upon applying an external trigger such as pH, electric field, IR-light, etc. have been described, however, the reported triggers might not always be applicable in vivo. Also implants for pulsed drug delivery have been reported but, for obvious reasons, injectable microparticles offer benefits.

In the previous chapter we introduced a new type of microparticles which we termed ‘self-exploding microcapsules’. A self-exploding microcapsule consists of a biodegradable microgel core surrounded by a suitable membrane (Figure 1). It was shown that upon degradation of the microgels the swelling pressure of the degrading microgels was able to suddenly rupture the membrane. In this system the membrane surrounding the microgels plays a major role as it has to be (i) permeable to water, (ii) impermeable to the degradation products of the microgels (upon degradation the gels turn into a polymer solution) and (iii) rupture when the swelling pressure reaches a critical value.
We applied a membrane at the surface of the microgels by layer-by-layer electrostatic self-assembly (LbL-ESA) which is a very promising technology for the coating of microparticles. First developed by Decher et al. on planar substrates, this technique has been extended to the coating of colloidal particles by Sukhorukov et al. Briefly explained, as illustrated in Figure 1, this approach is based on the alternate adsorption of oppositely charged polyelectrolytes (PE’s) on a charged surface, driven by the electrostatic interaction at each step of adsorption. It has been shown that a wide variety of colloidal particles can be coated by polyelectrolyte multilayer deposition: organic latex particles, inorganic particles, dye and drug nanocrystals, protein aggregates and biological cells. A major advantage of the LbL technique is the possibility to tune the layer thickness on the nanometer scale and thus control the mechanical properties and the permeability of the polyelectrolyte shell.

As the LbL membrane of the self-rupturing microcapsules has a major impact on the release features of this new type of microparticles, this chapter especially aims to characterize in detail the LbL films deposited on the surface of the microgels. We also aimed to show that choosing an optimal LbL-coating significantly reduces burst release and makes release to occur at the time the microcapsules rupture.
CHAPTER 3 - LAYER-BY-LAYER COATING OF DEGRADABLE MICROGELS FOR PULSED DRUG DELIVERY

MATERIALS AND METHODS

Materials.

Dextran (Mw~19 kDa), fluorescein isothiocyanate-dextrans (FITC-dextran, Mw~respectively 20 and 150 kDa), tetramethyl rhodamine B isothiocyanate-dextrans (TRITC-dextran, Mw~158 kDa), N,N,N',N'-tetramethylethylenediamine (TEMED), methacrylic acid (MAA) and dimethyl aminooethyl methacrylate (DMAEMA), rhodamine B isothiocyanate (RBITC), sodium poly(styrene sulfonate) (PSS, 70 kDa) and poly(allylamine hydrochloride) (PAH, 70 kDa) were purchased from Sigma-Aldrich-Fluka. Potassium peroxodisulphate (KPS) and poly(ethyleneglycol) (PEG; 20 kDa) were purchased from Merck. RBITC labelled PAH was synthesized as reported in literature. 31

Synthesis of dex-HEMA.

Dextran-hydroxyethyl methacrylate (dex-HEMA) was prepared and characterized according to a method described elsewhere. 32 Dextran with a number average molecular weight of 19 kDa was used. The degree of substitution (DS, the number of HEMA groups per 100 glucopyranose residues of dextran) was determined by proton nuclear resonance spectroscopy (1H-NMR) in D2O with a Gemini 300 spectrometer (Varian). 33 The DS of the dex-HEMA used in this study was 2.5.

Preparation of dex-HEMA microgels.

Dex-HEMA microgels, with an initial water content of 70 % (w/w), were prepared according to Franssen et al. 34 In detail, 71 mg dex-HEMA was dissolved in 1.577 ml water and subsequently emulsified by vortexing with a 3.35 ml 24 % (v/v) aqueous PEG solution. Radical polymerisation of the aqueous dex-HEMA emulsion droplets was initiated by adding TEMED (100 µl pH neutralized with 4 N HCl) and KPS (9 mg). The reaction was carried out at room temperature for 1h. Afterwards the obtained microgels were washed three times with pure water to remove PEG, KPS and TEMED. Finally the microgels were suspended in 5 ml pure water and stored at -20 °C. To prepare negatively and positively charged dex-HEMA microgels, 35 respectively methacrylic acid (MAA; 25µl) or dimethyl aminooethyl methacrylate (DMAEMA; 35µl) was added to the dex-HEMA/PEG mixture just before vortexing it. In this chapter 'dex-HEMA-MAA microgels' and 'dex-HEMA-DMAEMA microgels' refer to negatively charged and positively charged microgels, respectively. FITC/TRITC-dextrans were
incorporated in the microgels by adding 100µl of a FITC/TRITC-dextran solution (50 mg/ml) to the dex-HEMA/PEG mixture just before the vortexing step.

**LbL coating of the dex-HEMA microgels.**

Dex-HEMA microgels were coated by the consecutive adsorption of oppositely charged polyelectrolytes using the centrifugation technique. The microgels (500 µl from the original suspension) were dispersed in 1 ml of polyelectrolyte solution (2 mg/ml in 0.5M NaCl). The polyelectrolytes were allowed to adsorb for 15 min, under continuous shaking. The dispersion was then centrifuged at a speed of 300 g for 3 min. Subsequently the supernatant was removed and the microgels were redispersed in Milli-Q water to remove the non-adsorbed polyelectrolytes. This washing was repeated twice before the second polyelectrolyte solution was added. The process was repeated until the desired LbL coating was reached.

**Release experiments.**

TRITC-dextran containing dex-HEMA-DMAEMA microgels were prepared and LbL coated as described in the paragraphs above. However, the scale on which the LbL-coating was performed was 10 ml instead of 1 ml. After preparation, the uncoated and (PSS/PAH)3-coated dex-HEMA-DMAEMA microgels were centrifuged, the supernatant was removed and the microgels/microcapsules were redispersed in centrifugation tubes containing 50 ml 0.1 M carbonate buffer (pH 9). The centrifugation tubes were thermostatised at 37 ± 0.5 °C and the microgels/microcapsules were kept in suspension by mechanical agitation. 1 ml samples were withdrawn at 2.5 h time intervals (by a VenKel Industries 800 automatic sampling station) and filtered to remove the microcapsules. The fluorescence intensity of the samples was measured with a Wallac Victor 2 (Perkin Elmer) plate reader. The measured fluorescence values were normalized against the fluorescence values measured at the end of the release experiments. It was verified that the measured fluorescence values belonged to the range in which a linear relation exists between the concentration of the TRITC-dextran solutions and their fluorescence.

**Confocal laser scanning microscopy.**

Confocal micrographs of the LbL coated microgels were taken with a MRC1024 Bio-Rad confocal laser scanning microscope (CLSM) equipped with a krypton-argon laser. An
inverted microscope (Eclipse TE300D, Nikon) was used which was equipped with a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon).

**Electrophoretic mobility.**

The electrophoretic mobility of the LbL coated microgels was measured using a Malvern Zetasizer 2000 (Malvern Instruments). The \( \zeta \)-potential was calculated from the electrophoretic mobility (\( \mu \)) using the Smoluchowski relation: \( \zeta = \mu \eta / \varepsilon \) where \( \eta \) and \( \varepsilon \) are the viscosity and permittivity of the solvent, respectively. Since the largest microgels did not allow \( \zeta \)-potential measurements (they sedimented too quickly in the cuvette of the instrument), we measured the \( \zeta \)-potential of the smallest microgels (approximately 1 \( \mu m \)). Therefore the dex-HEMA microgel dispersions were centrifuged (1 min at low speed (100 g)) and the \( \zeta \)-potential measurements were done on dex-HEMA microgels which remained in the supernatant.

**Scanning electron microscopy (SEM).**

Scanning electron microscopy measurements on (LbL coated) microgels were carried out using a Zeiss DSM 40 instrument operating at an accelerating voltage of 3 kV.

**Atomic force microscopy (AFM).**

Experiments were performed on air-dried (LbL coated) microgels deposited onto microscope glass slides. Images were obtained in tapping mode under ambient conditions with an AutoProbe CP system (Park Scientific Instruments) using a 100 \( \mu m \) scanner. Si\(_3\)N\(_4\) cantilevers (spring constant about 0.1 Nm\(^{-1}\)) with integrated pyramidal tips were used. The AFM-tip was positioned on top of rather large microgels (diameter of 15 \( \mu m \)) in order to avoid the effect of the curvature on the image.

**RESULTS AND DISCUSSION**

To synthesize biodegradable dextran hydrogels, \(^{36}\) methacrylate moieties were linked to the dextran backbone by hydrolysable carbonate esters (Figure 2A). Radical polymerization of the methacrylate moieties cross-links the dextran chains. The fabrication of
dex-HEMA microgels using the water-in-water emulsion technique is based on the immiscibility of the PEG and dex-HEMA solutions. Microgels with an average diameter of 7 µm were obtained. It could be expected that charged dex-HEMA microgels would be more suitable for LbL coating than neutral ones. Van Tomme et al. recently reported that charged dex-HEMA microgels can be prepared by copolymerization of dex-HEMA with MAA (pKₐ 4.5) (Figure 2B) or DMAEMA (pKₐ 8.4) (Figure 2C). The incorporation of these charged groups into the microgels was verified by measuring the ζ-potential of the dex-HEMA microgels. Indeed, a negative ζ-potential was measured in case MAA was used, while a positive ζ-potential was observed in case DMAEMA was used, indicating the successful charge loading of the dex-HEMA microgels.

We observed that the charge of the microgels strongly influences their degradation rate. While the positively charged dex-HEMA-DMAEMA (70 %; DS 2.5) microgels were completely degraded within 5 days (at 37 °C and pH 7.4), it took up to 30 days to completely degrade the negatively charged dex-HEMA-MAA (70 %; DS 2.5) microgels. It has been reported by van de Wetering et al. that the degradation rate of alkaline ester hydrolysis can be influenced by neighbouring ternary amine groups. After nucleophilic attack of the hydroxyl ion on the ester group, the intermediate is stabilized by resonance stabilisation which promotes the alkaline hydrolysis of the ester group thus increasing the degradation rate of the dex-HEMA-DMAEMA microgels. On the other hand, in case of dex-HEMA-MAA microgels the presence of the carboxyl group of the MAA will destabilize the intermediate and thus defavourize the alkaline hydrolysis of the ester group, resulting in a slower degradation of the dex-HEMA-MAA microgels, which was indeed observed.

To perform the LbL-coating of the microgels we used poly(allylamine hydrochloride) (PAH) as polycation and sodium poly(styrene sulfonate) (PSS) as polyanion. This polyelectrolyte pair is well studied for the coating of both flat as well as colloidal templates. Initially we deposited 3 PSS/PAH bilayers onto the microgels.
Figures 3A-B show the results of \( \zeta \)-potential measurements on uncoated and LbL coated dex-HEMA microgels. Before coating, the \( \zeta \)-potential of neutral, dex-HEMA-MAA and dex-HEMA-DMAEMA microgels were respectively 0 mV, -30 mV and 28 mV; The \( \zeta \)-potential values proved the successful incorporation of respectively MAA and DMAEMA. Figures 3A and B clearly show that the charge of the microgels changes upon submerging them in PSS and PAH solutions, indicating that multilayer build-up takes place. The \( \zeta \)-potential profile observed upon exposure of the microgels to PSS/PAH solutions agrees well with literature.
data on PSS/PAH coating of other types of particles. Figure 3B shows that LbL coating of the neutral dex-HEMA microgels is also possible. The obtained $\zeta$-potential profile is however quite irregular. For charged microgels (Figure 3A), electrostatic interactions between the microgels and the polyelectrolytes are the main driving force for polyelectrolyte adsorption. However, for neutral microgels other interactions probably play a role. These interactions seem to be strong enough because otherwise the adsorbed layers would be removed upon adsorption of the next polyelectrolyte layer. Physical entanglements between the polyelectrolyte chains and the hydrogel matrix may considerably promote the adsorption of polyelectrolytes to the neutral gel surface.

![Figure 3](image)

*Figure 3.* CLSM images of dex-HEMA-DMAEMA microgels coated with (PSS/PAH)$_3$ (A), dex-HEMA-MAA microgels coated with (PAH/PSS)$_3$ (B) and dex-HEMA microgels coated with (PSS/PAH)$_3$ (C). The PAH was fluorescently labelled with RITC.

The obtained microcapsules were visualized by confocal laser scanning microscopy (Figure 4 A-C). When fluorescently labelled PAH (PAH-RITC) was used in the LbL assembly on the dex-HEMA-DMAEMA microgels (Figure 4A) a sharp contour could be observed. When dex-HEMA-MAA microgels were coated using PAH-RITC (Figure 4B), inwards diffusion of PAH-RITC was observed. This indicates that besides a shell at the surface, also a polyelectrolyte matrix is formed inside the microcapsules. Figure 4C shows a confocal microscopy image of LbL coated neutral dex-HEMA microgels. In this case it becomes even impossible to distinguish a core-shell structure. Probably the absence of charges on the surface of the microgels is fatal to an adequate multilayer build-up and one can thus question the quality of the coating.
Figure 5. SEM images of (A) uncoated and (B) coated microgels.
Figure 6. AFM images of uncoated (left) and PSS/PAH coated microgels (right) recorded in tapping mode. (A1) and (B1) show the topology of the surface. (A2) and (B2) show the roughness of the surface along the line marked on (A1) and (B1). (A3) and (B3) are the 3D images of the surface.

Since confocal microscopy does not give any information about the morphology of the LbL-coated microgels, SEM and AFM were used. Figure 5 depicts SEM images of both uncoated and coated microgels. It reveals that the surface of the uncoated particles is rather smooth compared to the coated ones, which show a more granular structure. Especially when PAH was the outermost polyelectrolyte layer a remarkable ‘brain-like’ structure appeared. A similar morphology was observed using AFM by McAloney et al. 44, 45 when studying the deposition of PSS/PAH multilayers on planar substrates at high salt concentrations. The difference between uncoated and PSS/PAH coated dex-HEMA-DMAEMA microgels was confirmed by AFM measurements. Uncoated microgels (Figure 6A) show irregularities ranging from 2 to 8 nm while PSS/PAH coated microgels (Figure 6B)
show irregularities ranging from 20 to 50 nm. The irregularities in the morphology of the LbL coating are highly likely due to the differential drying between the gel core and the LbL coating.

As outlined in Figure 1, the rupturing of the microcapsules is triggered by the swelling pressure of the degrading microgels. To exert a sufficient pressure it is important that the degradation products of the microgels, i.e. 19 kDa dextran chains, do not diffuse through the coating during the degradation of the microgels. In the previous chapter we reported that the permeability of the (PSS/PAH)$_3$ membrane seems to be pH dependent as self-rupturing microcapsules could only be obtained upon incubation of the microcapsules at pH 9 (thus suggesting that at pH 9 the coating is impermeable for 19 kDa dextran chains) while upon incubation at pH 7 the microcapsules’ membrane remained intact (thus suggesting that at pH 7 the coating is permeable for 19 kDa dextran chains). To further investigate the permeability of the (PSS/PAH)$_3$ coating we used 20 kDa FITC-dextran as its molecular weight corresponds well to the molecular weight of the dextran chains the microgels are composed of.

**Figure 7.** Permeability of the LbL coating surrounding the dex-HEMA-DMAEMA microgels to 20 kDa FITC-dextran. The LbL coated microgels are immersed into a buffered solution containing 1 mg/ml FITC-dextran and are visualized by confocal microscopy.
The (PSS/PAH)$_3$ coated dex-HEMA-DMAEMA microgels were incubated in a 1 mg/ml FITC-dextran solution. The experiment was performed at pH 7 and pH 9. From the confocal images in Figure 7 it is clear that at pH 7 the microcapsules’ wall is permeable to the 20 kDa FITC-dextrans while at pH 9 it is impermeable. When the number of polyelectrolyte bilayers is increased from 3 to 6 we observed that also at pH 7 the coating becomes impermeable. As a control, when non-coated dex-HEMA-DMAEMA microgels were incubated in a FITC-dextran solution they appeared to be permeable to the FITC-dextrans (data not shown). As PAH is a weak polyelectrolyte (pK$_a$ 8.5 46) it is not surprising that the properties of PSS/PAH polyelectrolyte multilayers are pH-dependent. The pH dependent stability 47 and permeability 38 of PSS/PAH based polyelectrolyte microcapsules has indeed been shown. Antipov et al. reported that PSS/PAH based microcapsules become impermeable to high molecular weight species upon increasing the pH from 7 to above 8. 38 Apparently an analogue phenomenon is observed in our case.

To evaluate the effect of the degradation of the microgel core on the integrity of the polyelectrolyte membrane, the microcapsules were incubated at 37 °C at pH 7 (for 10 days) and at pH 9 (overnight). Optical microscopy on non-coated dex-HEMA-DMAEMA microgels showed that these degradation times ensure complete degradation of the microgel core. Two different types of microcapsules were used: dex-HEMA-DMAEMA microgels coated with (PSS/PAH)$_3$ and dex-HEMA-DMAEMA microgels coated with (PSS/PAH)$_5$.

When the microcapsules were put under the confocal microscope after the degradation of the microgel core it was observed that only the (PSS/PAH)$_3$-based microcapsules incubated at pH 9 were ruptured (data not shown), in agreement with our previous observations. 18 In all other cases the polyelectrolyte membrane remained intact (Figure 8A). To prove that the membranes remained indeed intact the experiments were repeated with microgels which were initially loaded with 150 kDa FITC-dextrans. Upon degradation of the microgel core we observed that polyelectrolyte microcapsules filled with FITC-dextrans remained (Figure 8B). Figures 8C and 8D are SEM images corresponding to the microcapsules shown in respectively Figure 8A and 8B. In case 150 kDa FITC-dextrans were encapsulated the microcapsules showed a more granular structure due to the precipitation of the FITC-dextrans upon drying of the sample. A similar morphology of filled microcapsules after drying has been observed by Sukhorukov et al. 48

To visualize the rupturing of the (PSS/PAH)$_3$-coated dex-HEMA-DMAEMA microgels it was investigated up to which pH the (PSS/PAH)$_3$ coating was stable. Déjugnat et al. have reported on the pH responsive properties of hollow PSS/PAH-based microcapsules and from these results it can be concluded that the PSS/PAH coating remains intact until a pH of 11 whereas at a pH above 12 the multilayer membrane become irreversibly destroyed. 47
Therefore the (PSS/PAH)\textsubscript{3} coated dex-HEMA-DMAEMA microgels were incubated in a solution buffered at pH 11 and the degradation process was monitored by confocal microscopy. Figure 9 shows snapshots of the microcapsules during the degradation. Initially they start to swell and at a certain moment they rupture leading to the release of the encapsulated material, which was 150 kDa FITC-dextran.

In a next step the effect of the (PSS/PAH)\textsubscript{3} coating on the release of 158 kDa TRITC-dextrans from dex-HEMA-DMAEMA microgels, degrading at pH 9, was investigated. We expected to observe a pulsed release for the following reasons. First, at pH 9 the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure8.png}
\caption{Confocal images of (PSS/PAH)\textsubscript{3} coated empty dex-HEMA-DMAEMA microgels (A) and dex-HEMA-DMAEMA microgels loaded with 150 kDa FITC-dextrans (B), after degradation at pH 7. The PAH was fluorescently labelled with RITC. Figures C and D show the SEM images corresponding to (A), respectively (B).}
\end{figure}
(PSS/PAH)$_3$ coating surrounding dex-HEMA-DMAEMA microgels should not be permeable to 158 kDa TRITC-dextrans as it is even impermeable to 20 kDa dextrans (Figure 7). In other words, the 158 kDa TRITC-dextrans should not leak through the membrane during degradation of the microgels. Second, at pH 9 the (PSS/PAH)$_3$ microcapsules are self-rupturing i.e. the membrane breaks due to the increase in swelling pressure of the microgels.

Figure 9. CLSM images of (PSS/PAH)$_3$ coated microgels during the degradation at pH 11.

Figure 10 shows that the release of 158 kDa TRITC-dextrans from uncoated and (PSS/PAH)$_3$ coated dex-HEMA-DMAEMA microgels differs significantly. Uncoated microgels show a substantial burst release followed by a continuous release until the microgels are completely degraded after 20 h. A burst release does not occur in case of (PSS/PAH)$_3$ coated dex-HEMA-DMAEMA microgels, the 158 kDa TRITC-dextran release remains even low during the first 10 hours of degradation. After this initial phase the majority of the encapsulated 158 kDa TRITC-dextran molecules are released in a couple of hours allowing to conclude that (PSS/PAH)$_3$ coating of the dex-HEMA-DMAEMA microgels causes the release of the 158 kDa TRITC-dextrans to be much more pulsatile.
Figure 10. Cumulative release curves of 158 kDa TRITC-dextran from uncoated dex-HEMA-DMAEMA (open symbols) and (PSS/PAH)_3-coated dex-HEMA-DMAEMA microgel (closed symbols). The data points are interconnected with a cubic B-spline. The experiments were run in duplicate.

One could, however, wonder why the observed release pulse is less steep than one would expect when all the microcapsules would simultaneously rupture. This may be partially explained by Laplace’s law:

\[ p = \frac{2\gamma}{r} \]

With \( p \) the pressure, \( \gamma \) the membrane tension and \( r \) the radius. In other words, the internal pressure needed to destroy a given LbL membrane depends on the radius of the microgel; one needs a higher pressure for rupturing the membrane surrounding smaller microgels than for destroying the same membrane surrounding larger microgels. As the microgels reported in this chapter are polydisperse in size, one could indeed expect that not all the microcapsules will self-rupture at the same time but the time of rupturing will show a distribution. Recently we reported on the fabrication of highly monodisperse dex-HEMA microgels using a microfluidic emulsification device. Future work will focus on the use of these monodisperse microgels to produce a highly uniform population of microcapsules. Another aspect which should be addressed in future work is how to modify the properties of the LbL coating surrounding the microgels in order to render this coating sufficiently impermeable under physiological conditions without increasing the mechanical strength of this coating to a point where it can no longer be ruptured by the swelling pressure of the degrading microgel.
CONCLUSIONS

Dextran based microgels, with different surface charges, were used as template for the LbL assembly of the polyelectrolytes PSS and PAH. ζ-potential measurements and CLSM proved that polyelectrolytes can be sequentially adsorbed onto the surface of neutral, positively as well as negatively charged dextran microgels leading to microcapsules. It was observed that the positively charged dex-HEMA-DMAEMA microgels were the most promising as template for LbL assembly. The permeability of PSS/PAH based LbL coatings surrounding dex-HEMA-DMAEMA microgels was investigated. It was found that 3 bilayers of PSS/PAH rendered the microcapsules impermeable to 20 kDa FITC-dextran at pH 9 while they were still permeable at pH 7. Increasing the number of polyelectrolyte bilayers to 6 rendered the microcapsules impermeable also at pH 7.

We showed that upon degradation at pH 9 the dextran microgels were able to rupture their surrounding (PSS/PAH)$_3$ coating, resulting in ‘self-rupturing microcapsules’, while when degraded at pH 7 the (PSS/PAH)$_3$ coating did not rupture, leading to hollow (PSS/PAH)$_3$ microcapsules. This was explained by the pH dependent permeability of the (PSS/PAH)$_3$ coating to the degradation products of the microgels. When degraded at pH 9 the release of high molecular weight TRITC-dextran, encapsulated in (PSS/PAH)$_3$ coated microcapsules, was significantly more pulsatile compared to the TRITC-dextran release from uncoated dextran microgels.

The concept presented in this chapter may be promising towards biomedical applications, especially in the field of pulsed drug delivery, as the time of rupturing is determined by the degradation rate of the microgels, which can be tailored from days to several weeks by varying the cross-link density of the microgels. Our further research will focus on LbL coating of degradable microgels making use of biocompatible polyelectrolytes with the aim to obtain microcapsules which are under physiological conditions impermeable to both the encapsulated drugs as well as the degradation products of the dextran gels, two major requirements to obtain pulsed delivery from this type of micromaterials.

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CHAPTER 4

SELF-RUPTURING AND HOLLOW MICROCAPSULES PREPARED FROM BIO-POLYELECTROLYTE COATED MICROGELS

Parts of this chapter are in press:

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ABSTRACT

This chapter reports on microcapsules obtained by layer-by-layer deposition of bio-polyelectrolyte multilayers at the surface of biodegradable dextran microgels. It shows that the behaviour of the layer-by-layer coating upon degradation of the microgel core strongly depends on the bio-polyelectrolytes used. Two types of microcapsules, ‘self-rupturing’ microcapsules and ‘hollow’ microcapsules are presented. Self-rupturing microcapsules were obtained when the swelling pressure of the degrading microgel core was sufficiently strong enough to rupture the surrounding bio-polyelectrolyte membrane. Self-rupturing microcapsules could be of interest as a pulsed drug delivery system. Hollow microcapsules were obtained after applying multiple layers of bio-polyelectrolyte that could withstand the swelling pressure of the degrading microgel core. It was observed that biomacromolecules (like albumin and dextran) spontaneously accumulate in the hollow microcapsules prepared from dex-HEMA microgels, which could be of interest for drug encapsulation purposes.
Chapter 4

Exploding and Hollow Microcapsules from Bio-Polyelectrolyte Coated Microgels

Introduction

The advent of new biotechnological therapeutics has evoked novel challenges in drug delivery. For several therapeutics such as vaccines and hormones, a pulsatile release pattern could be beneficial. For example, a vaccine delivery system able to release the antigen in multiple pulses after a single injection could replace the multiple injections which are currently required to generate sufficient immunity. Also, for drugs that develop biological tolerance when they are constantly present in the bloodstream, pulsed drug release instead of sustained drug release could be attractive. A way to achieve pulsed drug delivery could be the encapsulation of the drug in micro- or nanoparticles which release their content at pre-programmed times after injection.

In the chapters 2 and 3 we introduced microcapsules that are able to explode, and thus release their content, without the use of an external trigger. As Figure 1A schematically represents, the so-called ‘self-exploding’ microcapsules consist of a biodegradable microgel surrounded by a semi-permeable membrane. The microgels are based on dextran-hydroxyethyl methacrylate (dex-HEMA; Figure 1B). Dex-HEMA microgels are biodegradable through hydrolysis of the carbonate esters in the cross-links which connect the dextran chains. Upon cleavage of the cross-links the swelling pressure of the dex-HEMA microgels increases. This swelling pressure may rupture the surrounding membrane, depending on the properties of the membrane and the pH of the environment. In the chapters 2 and 3 we reported that ‘self-exploding’ microcapsules could be obtained when the dex-HEMA microgels were coated with (PSS/PAH), i.e. a membrane consisting of 3...
polyelectrolyte bilayers, each bilayer composed of a sodium poly(styrene sulfonate) layer and a poly(allylamine hydrochloride). The (PSS/PAH)\textsubscript{3} layer was applied by the layer-by-layer (LbL) technique.\textsuperscript{14-17} LbL coating is the sequential adsorption of charged species, such as polyelectrolytes,\textsuperscript{16} nanoparticles,\textsuperscript{18-20} nanotubes,\textsuperscript{21} lipids,\textsuperscript{22} viruses,\textsuperscript{23} etc. on an oppositely charged planar or colloidal substrate. An important observation was that the (PSS/PAH)\textsubscript{3} coated dex-HEMA microgels only exploded when they were incubated at pH 9. Rupturing of the (PSS/PAH)\textsubscript{3} coating did not occur when the microcapsules were dispersed in buffer at a physiological pH (7.4). This was attributed to the pH dependent permeability of the (PSS/PAH)\textsubscript{3} membrane. At pH 7 the (PSS/PAH)\textsubscript{3} membrane was permeable to the degradation products of the dex-HEMA microgels while at pH 9 it was impermeable.\textsuperscript{24} Consequently, at pH 9 the degradation products remained inside the microcapsules during the degradation process of the microgels and thus increased the osmotic/swelling pressure of the core of the capsules, finally rupturing the (PSS/PAH)\textsubscript{3} membrane.

As self-exploding microcapsules may have great potential as a pulsed drug delivery system\textsuperscript{6-8} we aimed to design self-rupturing microcapsules that (a) solely consist of biopolymers instead of synthetic polymers like PSS and PAH and (b) rupture upon incubation at a physiological pH. In this chapter we show that this is possible by carefully choosing the appropriate bio-polyelectrolytes for building the LbL membrane. Furthermore, we demonstrate that depending on the composition of the bio-polyelectrolyte membrane, self-rupturing microcapsules as well as ‘hollow’ microcapsules can be fabricated using the degradable dex-HEMA microgels as sacrificial templates. Hollow bio-polyelectrolyte microcapsules, prepared by LbL coating of poly(lactic acid-co-glycolic acid), melamine formaldehyde and calcium carbonate microparticles as sacrificial template, have been

![Figure 1. (A) Schematic representation of a microcapsule. The inner core of the microcapsule is a microgel composed of dextran chains, which have been cross-linked (black dots). The microgel core has been coated with several polyelectrolyte bilayers of opposite charge (grey curves) using the LbL technique. (B) Molecular structure of dex-HEMA. Dex-HEMA are dextrans that have been derivatized with methacrylate groups connected to the dextran backbone by hydrolysable carbonate esters. The dextran chains can be cross-linked by radical polymerization of the methacrylate groups, forming a three dimensional network. Hydrolysis of the carbonate esters in the cross-links leads to the formation of the original dextran chains and polyHEMA fragments as degradation products.](image-url)
reported before. However, the use of organic solvents to dissolve the core material may be disadvantageous, especially where the encapsulation of drug molecules is concerned.\textsuperscript{25, 26} Also, remnants of toxic melamine formaldehyde oligomers may remain in the capsules' walls after dissolving the core.\textsuperscript{27-29} Severe aggregation of the hollow microcapsules has also been reported.\textsuperscript{30} In this study we show that dex-HEMA microgels can be dissolved under mild conditions yielding non-aggregated hollow microcapsules.

**EXPERIMENTAL**

*MATERIALS.*

Dextran (Mw~19 kDa), N,N,N’,N’-tetramethylmethylenediamine (TEMED), dextran sulfate (DEXS; Mw~10 kDa), poly-L-arginine (PARG; Mw~100-200 kDa), chondroitin sulfate (CHON), poly-L-aspartic acid (PASP; M w~15-50 kDa), poly-L-glutamic acid (PGLU\textsubscript{low}; M w~15-50 kDa and PGLU\textsubscript{high}; Mw~50-100 kDa), FITC-bovine serum albumin (FITC-BSA), FITC-dextran (Mw~20, 40, 70, 150, 500 and 2000 kDa) and rhodamine B isothiocyanate (RITC) were purchased from Sigma-Aldrich-Fluka. Potassium persulfate (KPS), dimethyl aminoethyl methacrylate (DMAEMA), polyethylene glycol (PEG; Mw~20 kDa), citric acid, sodium hydroxide (NaOH) and hydrochloric acid were purchased from Merck. RITC-labelled PARG was synthesized by reacting RITC and poly-L-arginine in 0.1 M borate buffer at pH 8.5 followed by dialysis with pure water for several days.

*Synthesis of dex-HEMA microgels.*

Dex-HEMA was synthesized as previously reported.\textsuperscript{10, 11} The degree of substitution (DS; the number of HEMA groups per 100 glucopyranose units) was 2.5 as determined by \textsuperscript{1}H-NMR spectroscopy. Fluorescent dex-HEMA microgels were fabricated according to Franssen et al.\textsuperscript{31, 32} by dissolving 71 mg dex-HEMA, 20 µl FITC-dextran solution (50 mg/ml) and 35 µl DMAEMA in 1.577 ml water and subsequently emulsifying this solution with a 3.35 ml aqueous PEG solution (24 % (v/v)). Radical polymerization of the aqueous dex-HEMA emulsion droplets was initiated by adding TEMED (100 µl pH neutralized with 4 N HCl) and KPS (180 µl, 41 mM). The reaction was carried out at room temperature for 1h. Afterwards the microgels obtained were washed three times with pure water to remove PEG, KPS and TEMED. Finally the microgels were suspended in 5 ml water and stored at -20 °C. The size distribution of the microgels was determined by laser diffraction (Malvern Mastersizer).
**Polyelectrolyte coating of the microgels.**

Dex-HEMA microgels were coated by the consecutive adsorption of oppositely charged polyelectrolytes. 33 500 µl of dex-HEMA microgel dispersion was mixed with 1 ml of polyelectrolyte solution (1 mg/ml in 0.5 M NaCl). The polyelectrolytes were allowed to adsorb for 10 min, under continuous shaking. The dispersion was then centrifuged at a speed of 300 g for 3 min. Subsequently the supernatant was removed and the microgels were redispersed in Milli-Q water to remove the non-adsorbed polyelectrolytes. This washing was repeated twice before the second polyelectrolyte solution was added. As the microgels were positively charged we started the LbL coating with the polyanion. In total 4 polyelectrolyte bilayers were deposited.

**Preparation of hollow microcapsules using dex-HEMA microgels as template.**

To obtain hollow microcapsules, dex-HEMA microgels coated with 4 bilayers DEXS/PARG were dispersed for 30 min in 0.1 M NaOH (to degrade the dex-HEMA microgel cores), followed by 3 washing steps with pure water to remove the degradation products of the microgels and the NaOH. Finally the hollow microcapsules were dispersed in 1 ml Milli-Q water.

**Preparation of hollow microcapsules using MnCO₃ as template.**

Manganese carbonate (MnCO₃) particles with an average diameter of 5 µm were prepared as reported by Zhu et al. 34 LbL coating of the MnCO₃ particles occurred as described above for the LbL coating of dex-HEMA microgels. The MnCO₃ cores were dissolved by adding 1 ml of 0.2 M citric acid in 0.1 M HCl to the coated MnCO₃ particles, followed by several washing steps with pure water. 35

**Scanning electron microscopy measurements.**

Scanning electron microscopy (SEM) measurements were conducted on a Gemini Leo instrument operated at an acceleration voltage of 3 kV. For a sample preparation, a drop of microcapsules suspension was applied to a glass slide, dried overnight, and sputtered with gold.
**Atomic force microscopy measurements.**

Atomic force microscopy (AFM) images were taken on a Nanoscope IIIa Multimode SFM (Digital Instruments Inc.) in air at room temperature using the tapping mode. Samples were prepared by applying a drop of microcapsules suspension onto a freshly cleaved mica substrate followed by drying under a gentle stream of nitrogen.

![Figure 2](image)

**RESULTS AND DISCUSSION**

Biodegradable dex-HEMA microgels were fabricated according to Franssen et al. by a completely aqueous water-in-water emulsion technique based on the immiscibility of an aqueous polyethylene glycol phase and an aqueous dextran phase. 31, 32 Radical polymerization of the pending HEMA groups leads to the formation of a three-dimensional network. Positively charged microgels (as evidenced from electrophoreric mobility measurements) with an average diameter of 7 µm were obtained by copolymerizing dex-HEMA with dimethyl aminoethyl methacrylate (DMAEMA), which is positively charged at neutral pH. 36 While Figure 2A shows the size distribution of the microgels, as determined by laser diffraction, Figure 2B is a confocal image of dex-HEMA microgels homogenously loaded with FITC-dextran. The FITC-dextrans stay encapsulated inside the dex-HEMA microgels and become slowly released during the degradation of the microgel core, during up to 7 days.
In the next step we tried to coat the dex-HEMA microgels with four bio-polyelectrolyte bilayers using the LbL technique. The bio-polyelectrolyte coated dex-HEMA microgels are termed ‘microcapsules’ in the continuation of this chapter. Initially a large number of bio-polyelectrolytes were attempted to coat the dex-HEMA microgels. Chitosan, poly-L-lysine, poly-L-ornithine, alginic acid and hyaluronic acid appeared to be unsuitable bio-polyelectrolytes because severe aggregation of the microgels took place; mostly the dex-HEMA microgels aggregated instantaneously upon dispersing in the bio-polyelectrolyte solution. The low charge density and relatively high viscosity (especially in case of chitosan, alginic acid and hyaluronic acid) of the bio-polyelectrolytes may explain their failure. The only bio-polycation that allowed coating the dex-HEMA microgels without aggregation seemed to be poly-L-arginine (PARG). Poly-L-arginine is the only polypeptide having side groups that are charged at almost every pH (the $pK_a$ of the side groups is 12.5) and is therefore a strong polyelectrolyte. Using PARG as polycation, chondroitin sulfate (CHON), poly-L-aspartic acid (PASP), low molecular weight poly-L-glutamic acid (PGLU$_{low}$), high molecular weight poly-L-glutamic acid (PGLU$_{high}$) and dextran sulfate (DEXS) seemed to be suitable polyanions for LbL coating of the dex-HEMA microgels. Figure 3 shows confocal microscopy images of dex-HEMA microgels coated with different combinations of bio-polyelectrolytes. A clear ring of red fluorescence (due to the PARG which was fluorescently red labelled) surrounding the microgels can be observed indicating a successful LbL coating of the dex-HEMA microgels.

![Figure 3](image)

**Figure 3.** Confocal microscopy images of the microcapsules obtained after LbL coating of the dex-HEMA microgels with 4 bio-polyelectrolyte bilayers. The microgels were fluorescently labelled with 150 kDa FITC-dextran (green colour) while the PARG in the LbL coating was fluorescently labelled with RITC (red colour).

To investigate how the degradation of the dex-HEMA microgel core influences the bio-polyelectrolyte coating, the microcapsules were incubated in a 0.1 M phosphate buffer (pH 7.4) at 37 °C. After 5 days (being the time it takes to fully degrade the dex-HEMA microgel core at pH 7.4 and 37 °C) the microcapsules were investigated under the confocal microscope (Figure 4). The result was clearly dependent on the composition of the LbL membrane. In Figure 4A all microcapsules were ruptured; ‘self-rupturing’ capsules were thus obtained. In Figure 4B some microcapsules were ruptured while others remained intact and filled with FITC-dextran. In Figure 4C ‘hollow’ microcapsules were obtained i.e. the
microcapsules did not rupture but had released the 150 kDa FITC-dextran as no significant fluorescence could be detected within the microcapsules.

![Image](image.png)

**Figure 4.** Confocal images of dex-HEMA microgels (which were fluorescent labelled with 150 kDa FITC-dextrans; green colour) coated with respectively (A) (CHON/PARG)₄, (B) (PGLUₕₘ/PARG)₄ and (C) (DEXS/PARG)₄ after degradation of the microgel core. In (A) all microcapsules were broken and had released their contents. In (B) both broken as well as intact (still filled with 150 kDa FITC-dextrans; green colour) were observed. The capsules in (C) remained intact but had released their content by diffusion through the bio-polyelectrolyte coating. In all cases the PARG was fluorescently labelled with RITC (red colour).

**Table 1.** Depending on the membrane composition, degradation of the dex-HEMA microgel core results in self-rupturing and/or hollow microcapsules.

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Table 1 shows which LbL membrane composition resulted in either self-rupturing or hollow microcapsules. The behaviour of the LbL membrane during degradation of the microgel core depends most likely on its mechanical strength and its permeability to the degradation products (i.e. 19 kDa dextran chains) of the microgel. (DEXS/PARG)₄ membranes remain intact probably due to the fact that they are permeable not only to the 150 kDa FITC-dextrans but also to the degradation products of the microgels, which hampers the build-up of a significant osmotic pressure. The other bio-polyelectrolyte coatings are apparently less permeable to the degradation products of the microgels allowing the osmotic pressure to build-up and rupture the membrane. It is also interesting to note that an increase in molecular weight of the PGLU clearly influences the (mechanical and/or permeability) behaviour of the microcapsules. Microcapsules fabricated with PGLUₙ all rupture. The ones fabricated with PGLUₕₘ/PARG do not all explode, a percentage of them remains intact keeping the FITC-dextrans encapsulated. This indicates that the increase in
molecular weight of the PGLU changes the interplay between the mechanical properties and the permeability of the bio-polyelectrolyte coating.

Figure 5. Confocal snapshots of (PGLU\textsubscript{low}/PARG)\textsubscript{4}-coated microcapsules during degradation of the microgel core at alkaline pH. The microgels contained 150 kDa FITC-dextran (green colour) and the PARG was fluorescently labelled with RITC (red colour). The time-interval between the successive snapshots is 20 s.

We took a closer look at the behaviour of the (PGLU\textsubscript{low}/PARG)\textsubscript{4} microcapsules during degradation of the microgel core (Figure 5). The degradation was accelerated by increasing the pH of the dispersion (which accelerates the hydrolysis of the esters in dex-HEMA). Since PARG is a strong polyelectrolyte, its cationic groups are positively charged over a wide pH range. Therefore we could expect that the alkaline pH does not influence the properties of the polyelectrolyte membrane. This is crucial, as it has been reported that the properties of polyelectrolyte membranes containing one or more weak polyelectrolytes may change significantly as a function of the pH. \textsuperscript{35, 38-40} Figure 5 shows confocal images taken at 20 s time intervals after the addition of 5 µl of 0.5 M NaOH to 5 µl of the (CHON/PARG)\textsubscript{4} microcapsule suspension. The microcapsules start to swell (Figure 5A-D) and at certain time (Figure 5F) all microcapsules rupture, most likely when the swelling pressure of the degrading microgels exceeds the tensile strength of the bio-polyelectrolyte membrane. After rupturing, only remnants of broken microcapsules are observed (Figure 5F) having completely released the encapsulated FITC-dextran.
Figure 6 shows confocal microscopy images of (DEXS/PARG)$_4$ microcapsules both before (A) and after (B) degradation of the microgel core. After degradation, the interior of the microcapsules is no longer (green) fluorescent which indicates the outward diffusion of the FITC-dextran during the degradation process of the microcapsules. Figure 7 shows SEM images of the microcapsules (A) before, and (B) after degradation of the microgel core. The rough surface in Figure 7A is probably due to the differential drying between the dex-HEMA microgel core and the (DEXS/PARG)$_4$-coating upon sample preparation. In Figure 7B one can see a collapsed microcapsule, which confirms that the microgel core has indeed been degraded, yielding a hollow microcapsule. Using AFM, the thickness of the bio-polyelectrolyte membrane could be estimated (Figure 8A-D) according to the method reported by Leporatti et al. Taking into account that the measured height is twice the thickness of a single microcapsule wall, the thickness is estimated to be 45 ± 4 nm (as obtained from measurements on three different microcapsules). Compared to other LbL membranes, the (DEXS/PARG)$_4$-coating seems to be rather thick. While Déjugnat et al. reported a mean bilayer thickness of 3.5 nm for poly(styrene sulfonate)/poly(allylamine hydrochloride) (PSS/PAH) we measured a mean thickness of 11 nm per DEXS/PARG bilayer. Bio-polyelectrolyte multilayers deposited on flat substrates have been studied by Picart et al. They reported that the thickness of the membranes increases exponentially as a function of the number of adsorbed layers. Also LbL multilayer films from polysaccharides and polypeptides seem to show an exponential growth, contrary to PSS/PAH multilayers which show a linear growth step as a function of layer number. It could also be possible that in Figure 4C not all of the degradation products of the microgel
were expelled from the microcapsules' interiors (see below). These residual dextrans could result in a higher estimation of the shell thickness from AFM measurements.

**Figure 7.** SEM images of (DEXS/PARG)$_4$ coated dex-HEMA microgels before (A) and after (B) degradation of the microgel core.
Figure 8. AFM image of a hollow (DEXS/PARG), microcapsule fabricated from a dex-HEMA microgel as sacrificial template. (A) topology image, (B) error image, (C) Height profile along the line indicated in (A) and (D) 3D image.
Hollow (DEXS/PARG)$_4$ capsules, obtained after degradation of the dex-HEMA microgel cores, were incubated for 30 min in FITC-BSA or 40 kDa FITC-dextran solutions (0.5 mg/ml). Figures 9A and 9B show the FITC-BSA and FITC-dextran strongly accumulated inside the hollow microcapsules. Nearly no fluorescence remained in the solution surrounding the microcapsules. Similar results were obtained FITC-dextrans with a molecular weight ranging from 4 to 2000 kDa (data not shown). On the contrary, hollow (DEXS/PARG)$_4$ capsules fabricated from MnCO$_3$ microparticles as sacrificial template did not become filled with FITC-BSA (Figure 9C), in agreement with earlier findings. $^{44, 45}$

**Figure 9.** Confocal microscopy images of hollow (DEXS/PARG)$_4$ microcapsules, fabricated from dex-HEMA microgels as sacrificial template, dispersed in (A) a FITC-BSA solution and (B) a 40 kDa FITC-dextran solution. (C) Confocal microscopy image of hollow (DEXS/PARG)$_4$ microcapsules, fabricated from MnCO$_3$ microparticles as sacrificial template, dispersed in a FITC-BSA solution.

Currently, it remains speculative why hollow (DEXS/PARG)$_4$ dex-HEMA microgel-templated microcapsules so strongly absorb FITC-BSA while hollow (DEXS/PARG)$_4$ microcapsules templated on MnCO$_3$ exclude FITC-BSA. It is known that the permeability of polyelectrolyte membranes not only greatly depends on external conditions, such as pH and ionic strength, but also on the type of sacrificial template from which they are fabricated. $^{46-49}$ As 150 kDa FITC-dextrans are able to diffuse through the (DEXS/PARG)$_4$ membrane during degradation of the dex-HEMA microgel core (Figure 6), it is very likely that most of the 19 kDa dextran chains, from which the microgels were fabricated, are also able to permeate the (DEXS/PARG)$_4$ membrane. It remains possible, however, that not all the dextran chains left the microcapsules. Indeed, when the hollow (DEXS/PARG)$_4$ microcapsules were viewed with the confocal microscope at maximum laser intensity, traces of green fluorescence (due to remaining 150 kDa FITC-dextran) could still be observed inside the microcapsules (data not shown). It is known that many proteins show affinity for dextrans, $^{50}$ therefore remnants of dextran in the hollow capsules might explain the accumulation of FITC-BSA.
CONCLUSIONS

This study reported on the successful fabrication of microcapsules consisting of a biodegradable dextran microgel core surrounded by a bio-polyelectrolyte multilayer coating applied to the microgels by layer-by-layer technology. Upon degradation of the microgel core, depending on the composition of the bio-polyelectrolyte coating, either ‘self-rupturing’ or ‘hollow’ microcapsules were obtained.

Self-rupturing microcapsules, being microcapsules that suddenly rupture due to the swelling pressure of the degrading microgel core, were obtained by LbL coating of dex-HEMA microgels with 4 bio-polyelectrolyte bilayers of CHON/PARG, PGLU_low/PARG or PASP/PARG. The major difference between the self-rupturing microcapsules described in this chapter and those reported in chapter 2 is that the former explode at a physiological pH. Their exterior coating consists not of synthetic, but biological polyelectrolytes. Both the rupturing at a physiological pH and the use of bio-polyelectrolytes are promising features in the development of self-rupturing microcapsules for drug delivery purposes.

Hollow capsules could be obtained by degrading dex-HEMA microgels after they were coated with 4 bio-polyelectrolyte bilayers of DEXS/PARG. It was shown that FITC-BSA and FITC-dextran spontaneously accumulate in the hollow capsules while they did not accumulate in (DEXS/PARG)_4 hollow microcapsules prepared from MnCO₃ microparticles as sacrificial template. Previously reported procedures to load hollow polyelectrolyte microcapsules with macromolecules often require the inclusion of oppositely charged species or an additional step, such as a change in pH, ionic strength, solvent polarity, cross-linking, to temporarily change the permeability of the microcapsule’s membrane. The spontaneous deposition of biomacromolecules in hollow microcapsules prepared from dex-HEMA microgels as sacrificial template could be of interest for drug encapsulation purposes.

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REFERENCES

CHAPTER 5
SELF-EXPLODING LIPID COATED MICROGELS

Parts of this chapter were published in:


1 Laboratory of General Biochemistry and Physical Pharmacy, Department of Pharmaceutics, Ghent University, Ghent, Belgium.

2 Unité de Physique et de Chimie des hauts Polymères, Université catholique de Louvain, Louvain-la-Neuve, Belgium.
**ABSTRACT**

Self-exploding microparticles show potential for advanced delivery of certain therapeutics. This study evaluates (1) whether degrading biodegradable dextran hydroxyethyl methacrylate (dex-HEMA) microgels can be coated by a lipid membrane and (2) whether the surrounding membrane can be ruptured by the increasing swelling pressure of the degrading microgel. We found that adsorption of charged liposomes to oppositely charged dex-HEMA microgels provides efficient coating of the microgels whereby microparticles with a ‘core-shell’ structure were clearly obtained. Especially, we could confirm experimentally that the swelling pressure increase of degrading dex-HEMA microgels can destroy the lipid membrane surrounding the microgels.
INTRODUCTION

Thanks to recent progress in biotechnology and medicine an increasing amount of macromolecular therapeutics (such as proteins and DNA) has become available. As these drugs have to be protected from threatening environments during administration, a lot of scientific effort has been focused on developing appropriate matrices. Due to their high biocompatibility and tuneable properties, hydrogel matrices are an attractive method to encapsulate such drugs. Our research group is focusing on ‘exploding microgels’ for pulsed drug delivery. ¹ We envision micron-sized (bio)degradable gel particles surrounded by a membrane that is permeable to water but impermeable to both the entrapped drugs and the degradation products of the gel. As the microgel degrades its swelling pressure increases. ²,³ At a critical value of the swelling pressure we foresee that the membrane will rupture and the entrapped drugs will be released.

Clearly, to realize this concept, one requires a (water permeable) coating surrounding the microgels. Technology that allows coating the surface of hydrogels is very attractive for numerous applications in biomedicine and pharmacy, e.g. for tailoring the hydrophilicity or permeability. However, a general method for the coating of hydrogel surfaces does not yet exist. Obviously, coating microgels demands additional requirements when compared to coating planar supports, a main issue being that the microgels need to remain colloidally stable. Some strategies have been developed to coat microgels with lipids. Kiser et al. ⁴,⁵ centrifuged drug loaded microgels into a lipid film deposited on a glass vial. Several groups modified the hydrogel surface by inserting lipid anchors at the microgel surface, which promote the self-assembly of lipid membranes. ⁶-⁹ Although attractive, these methods do show some limitations. For example, when we coated dextran-based microgels by the method of Kiser et al. we observed that only a marginal part of the microgels became lipid
coated. Also, the grafting of fatty acid chains on the microgels’ surface, requiring the use of organic solvents, may be hazardous to many biotechnological drugs.

The aim of this chapter is twofold. In the first part we aim to demonstrate how dextran hydroxyethyl methacrylate (dex-HEMA) microgels can be coated with lipids, using electrostatic interactions as a driving force. In the second part we aim to show that the increase in swelling pressure, due to the degradation of the dex-HEMA microgels, can rupture the surrounding lipid membrane.

Table 1. List of abbreviations of the polymers and lipids used in this study

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<tr>
<th>abbreviation</th>
<th>full term</th>
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<td>dex-MA</td>
<td>dextran-methacrylate</td>
<td>0</td>
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<td>-</td>
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<tr>
<td>DOPE</td>
<td>dioleoyl phosphadylethanolamine</td>
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*under physiological conditions

**MATERIALS AND METHODS**

**Materials.**

Sulfuric acid (98 %), hydrogen peroxide (H₂O₂) (30–%) and potassium persulfate (KPS) were purchased from Merck VWR. Dimethyl sulfoxide (DMSO), 4-N,N-dimethyaminopyridine (DMAP), glycidyl methacrylate (GMA), N,N,N’,N’-tetramethylethlenediamine (TEMED), methacrylic acid (MAA), dimethyl aminoethyl methacrylate (DMAEMA), chitosan (CHIT; low molecular weight), sodium poly(styrenesulfonate) (PSS, Mw~70 kDa), poly(allylamine hydrochloride) (PAH, Mw~70 kDa) and poly(vinyl alcohol) (PVA, Mw~70 kDa) were obtained from Aldrich. Dextran (Mw~19
kDa) and dextran sulfate (DEXS, Mw~500 kDa), were obtained from Fluka. Tetramethyl rhodamine isothiocyanate-dextrans (TRITC-dextran, Mw~158 kDa) and fluorescein isothiocyanate-polyallylamine hydrochloride (FITC-PAH, Mw~70 kDa) were purchased from Sigma. The lipids SOPC (stearoyl oleoyl phosphatidylcholine), DOPE (dioleoyl phosphatidylethanolamine), DOPA (dioleoyl phosphatidic acid) and DOTAP (dioleoyl trimethylammoniumpropane) were obtained from Avanti Polar Lipids. Cholesteryl BODIPY FL C₁₂ was obtained from Molecular Probes. Poly(lactic-co-glycolic) acid (PLGA; 5050 DL 4A) was obtained from Alkermes. One-side polished 475 µm-thick silicon wafers were obtained from ACM (Applications Couches Minces, France) with (100) orientation. Table 1 gives an overview of the abbreviations of the polymers and lipids used in this chapter.

**Synthesis of dex-HEMA and dex-MA.**

Dex-HEMA with a DS of 2.5 and dex-MA with a DS of 5 were prepared and characterized as previously described. The synthesis of dex-MA was as follows (Figure 1 gives the reaction scheme for the synthesis of dex-MA). Dextran (50 g) and DMAP (10 g) were dissolved in 500 ml DMSO under a nitrogen atmosphere. GMA (14.5 mmol; 2 g) was added and the reaction mixture was stirred for 48h under a nitrogen atmosphere. Afterwards the reaction mixture was put in dialysis bags (Mw cut off of 14kDa). After 5 days of dialysis the solution is freeze dried and dex-HEMA us obtained as a white fluffy product. The DS of the dex-MA is determined by H-NMR in D₂O.

![Figure 1. Reaction scheme for the synthesis of dex-MA.](image-url)

**Preparation of dex-HEMA microgels.**

Dex-HEMA microgels were prepared according to Stenekes et al. In detail, deoxygenated aqueous solutions of dex-HEMA (25 % w/w solution) and polyethylene glycol (PEG; 24 % w/w solution; 20 kDa) were prepared. The dex-HEMA and PEG solutions were
vigorously mixed with a vortex for 1 min under a nitrogen atmosphere to obtain a water-in-water emulsion. A PEG/dex-HEMA ratio of 19 (v/v) was used; the total volume amounted to 5 ml. The resulting emulsion was allowed to stabilize for 10-15 min. Subsequently TEMED (100 µl pH neutralized with 4 N HCl) and KPS (180 µl, 41 mM) were added to cross-link the dex-HEMA. After gentle mixing, the emulsion was incubated without stirring for 30 min at 40°C yielding microgels with an estimated water content of approximately 70 % (w/w). Three washing and centrifugation steps with 50 ml Milli-Q water removed the residual KPS and TEMED. The remaining pellet was suspended in 5 ml Milli-Q water. To prepare negatively and positively charged dex-HEMA microgels, respectively methacrylic acid (MAA; 25 µl) or dimethyl aminoethyl methacrylate (DMAEMA; 35 µl) were added to the mixture just before vortexing the dextran and PEG solutions as described in the paragraph above. In this chapter ‘dex-HEMA-MAA microgels’ and ‘dex-HEMA-DMAEMA microgels’ refer to negatively charged and positively charged microgels, respectively. Dex-MA microgels were fabricated in an identical way as dex-HEMA microgels.

**Preparation of PLGA microparticles.**

100 mg PLGA was dissolved in 10 ml dichloromethane. 100 mg PVA was dissolved in 100 ml water. Both solutions were mixed by vortexing for 1 min yielding an emulsion of dichloromethane in water. 50 ml water was added and the emulsion was stirred for 3 hours at 40 °C to evaporate the dichloromethane. The resulting PLGA microparticles were centrifuged and washed twice with Milli-Q water. Afterwards they were stored at 4 °C.

**Lipid coating of dex-HEMA microgels.**

The lipid vesicles were prepared as follows. The lipids were first dissolved in chloroform (2 mg/ml). Subsequently the chloroform was evaporated yielding a lipid film on a glass vial. To obtain lipid vesicles (liposomes), water was added up to a final lipid concentration of 1 mg/ml and sonicated (Branson 32, Branson Ultrasonics, 150 Watt) for 5 min. To reduce the size of the liposomes, the liposomes were extruded through a polycarbonate membrane with a pore size of 100 nm. The (charged) liposomes (500 µl) were mixed with a suspension (200 µl) of (oppositely charged) microgels and shaken for 20 min to allow adsorption of the lipid vesicles to the surface of the microgels. Then the samples were centrifuged for 3 min at 500 g and the supernatant was removed. The centrifugation and resuspension procedure was repeated three times. Finally the microgels were redispersed in 500 µl Milli-Q water and stored at 4 °C.
Lipid coating of PLGA microparticles.

The PLGA microparticles were first ‘pre-coated’ with 3 polyelectrolyte bilayers by alternating immersion in aqueous solutions of dextran sulfate (2 mg/ml) and chitosan (1 mg/ml) in the presence of 0.5 M NaCl. The polyelectrolytes were allowed to adsorb for 15 min, under continuous gentle shaking. The dispersion was then centrifuged and the supernatant was removed. Afterwards, the microparticles were redispersed in Milli-Q water to wash away the non-adsorbed polyelectrolytes. This washing was repeated twice before the second polyelectrolyte solution was added. The process was repeated until 3 bilayers were deposited. In a final step, the pre-coated PLGA particles were lipid coated, following the same procedure as for the lipid coating of microgels.

Degradation experiments.

In order to visualize the degradation behavior of the (lipid-coated) microgels a 10 µl drop of microgel suspension was put at room temperature on a coverslip under the confocal microscope. To accelerate the degradation rate of the (lipid coated) microgels 10 µl of a 1 M NaOH solution was added to the drop of microgel suspension and the behavior of the microgels was followed under the confocal microscope.

Confocal laser scanning microscopy.

Confocal micrographs of the lipid coated microgels and PLGA microparticles were taken with a MRC1024 Bio-Rad confocal laser scanning microscope (CLSM) equipped with a krypton-argon laser (Biorad, Cheshire, UK). An inverted microscope (Eclipse TE300D, Nikon, Japan) was used which was equipped with a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon, Japan). Dex-HEMA microgels were made fluorescent by adding 1 mg TRITC labelled dextrans to the dex-HEMA/PEG mixture before the cross-linking.

Electrophoretic mobility.

The electrophoretic mobility of the layer-by-layer (LbL) coated microgels was measured using a Malvern Zetasizer 2000 (Malvern Instruments). The ζ-potential was calculated from the electrophoretic mobility (μ) using the Smoluchowski relation: \[ ζ = \frac{ημ}{ε} \] where η and ε are the viscosity and permittivity of the solvent, respectively.
**Scanning electron microscopy (SEM).**

Samples for Scanning electron microscopy (SEM) imaging were prepared by drying under vacuum followed by sputtering with chrome. SEM images were recorded using a Gemini Leo 982, operating at an accelerating voltage of 1 kV.

**Lipid coating of silicon wafers.**

One-side-polished 475 µm-thick silicon wafers were cut into rectangles (2x3 cm). The wafers were cleaned for 20 min in freshly prepared piranha solutions (H$_2$O$_2$ (30 %): H$_2$SO$_4$ (98 %); 1:1 v/v (Caution: piranha solutions react violently with organic materials and should not be stored in closed containers), then abundantly rinsed with Milli-Q water. Wafers were dried by spinning with a spin coater. Prior to adding the lipid coating to the silicon wafers, polyelectrolyte multilayers were first deposited by alternating dipping of the silicon wafers in aqueous solutions of dextran sulfate (2 mg/ml) and chitosan (1 mg/ml) in the presence of 0.5 M NaCl for 5 min. Three rinsing steps in Milli-Q water and one drying step were applied in-between each adsorption step. This process was repeated 3 times. Thereafter, the SOPC:DOTAP (9:1) lipid layer was deposited by dipping the polyelectrolyte coated silicon wafer in a lipid vesicle dispersion prepared as described above.

**Ellipsometry.**

The thickness of the film deposited on the silicon wafers was determined in air with a null ellipsometer (Multiskop from Optrel, Berlin, Germany) at a fixed incident angle of 70° and a fixed wavelength of 5320 Å. A refractive index of 1.48 was assumed to determine the film thickness.

**X-ray reflectometry (XRR).**

The experimental setup consists of a Siemens D5000 2-circles goniometer of 30 cm radius and 0.002° positioning accuracy. X-rays of 1.5418 Å wavelength (CuK$\alpha$) were obtained from a Siemens rotating anode operated at 40 kV and 300 mA, fitted with a graphite secondary monochromator and a scintillation counter. The beam was defined by a 40 µm-wide slit placed at 17.5 cm from the focal point. Parastatic scattering was decreased by a 200 µm wide slit placed after the 40 µm slit. The sample was placed at the center of the goniometer with an automated procedure, using a vertical stage of 1 µm resolution. The
intensity was scaled to unit incident intensity, and then corrected for spill-over at very low angles of incidence. Data are reported as a function of $k_{z0}$, the vertical component of the wave vector of the incident photons in a vacuum.

XRR data were analyzed in two ways. First a Patterson function was computed from the data as described elsewhere, and the average thickness of the coating was obtained from the position of the subsidiary correlation peak in this function. Second, a model of electron density was fitted to the data. The electron density profile was discretized as a succession of flat slabs of width $\Delta = 0.5$ nm, approximately corresponding to the resolution of the experiments defined by $k_{z0,max}$, the maximum value of $k_{z0}$ for which the signal is not yet dominated by the background ($\Delta = \pi/(2.k_{z0,max})$). The number of slabs required was initially selected from the known thickness of the coating determined by the Patterson function. It was progressively incremented to take into account the width of the interfaces, until the fit was deemed satisfactory as judged from the value of $\chi^2$ obtained. The fit parameters were the height (electron density) of each slab, a general scaling parameter, and a constant background value. The reflectivity was computed using Paratt’s formalism. To ease interpretation, the electron density profiles are represented by dots placed at the centers of each slab; cubic spline interpolation of these profiles was used to draw a smooth line through the dots.

**RESULTS AND DISCUSSION**

Dex-HEMA and dex-MA were synthesized according to van Dijk-Wolthuis et al. by grafting dextran with carbonyl imidazole activated HEMA (HEMA-CI) respectively glycidyl methacrylate (GMA). Microgels were synthesized using an all-aqueous water-in-water emulsion technique based on the immiscibility of a dex-(HE)MA and a polyethylene glycol (PEG) phase. In a first approach we tried to coat the dex-HEMA microgels by centrifuging the microgels into a lipid film at the bottom of the test tube, following the procedure reported by Kiser et al. However, in this way only a low percentage of the dex-HEMA microgels became lipid coated. Looking further for a more efficient way to coat the dex-HEMA microgels with a lipid membrane we thought to make charged microgels and coat them with oppositely charged lipids, using electrostatic attraction as the driving force. Addition of DMAEMA and MAA during synthesis of the dex-HEMA microgels allowed us to obtain positively and negatively charged dex-HEMA microgels, as evidenced from $\zeta$-potential measurements (Figure 2A).
Figure 2. (A) ζ-potential of dex-HEMA-MAA and dex-HEMA-DMAEMA microgels, respectively before and after coating with oppositely charged lipid vesicles. (B) CLSM images of dex-HEMA-DMAEMA microgels coated with SOPC:DOPA, the microgels themselves are fluorescently labelled with TRITC-dextran while the lipids are labelled with BODIPY.

In this study SOPC:DOPA, SOPC:DOTAP and DOPE:DOTAP (all with a molar ratio of 9:1) liposomes were used. SOPC and DOPE are both neutral lipids while DOPA and DOTAP are respectively negatively and positively charged. For the formation of unilamellar liposomes it is important that the temperature at which the experiments are performed is above the phase transition temperature of the lipids. For all the lipids used in this study the phase transition temperature is below or equal to 0 °C, allowing one to perform the experiments at room temperature. The combination of charged lipids with neutral lipids is necessary to avoid too much repulsion within the bilayers and thus allowing stable vesicles to form. CLSM clearly showed that upon exposure to the SOPC:DOPA lipid vesicles all the dex-HEMA-DMAEMA microgels became lipid coated (Figure 2B). Microparticles with a core-shell structure were obtained with lipids only at the surface of the microgels and not in the interior, as schematically represented in Figure 3. We assume that the liposomes electrostatically interact with the charges at the microgel surface. Once the surface of a microgel is covered, the lipid vesicles probably spread open and form a lipid layer. Spreading of liposomes upon adsorption on a solid substrate has been observed by several other groups. Recently Mornet et al. reported on the coating of silica particles with a lipid bilayer. They observed with cryo electron microscopy that the lipid bilayer faithfully follows the contour of the solid support. Also they reported that to achieve spreading and fusing of the liposomes a ratio neutral to charged lipids of at least 3:1 was required. Liposomes with a higher net charge did not tend to spread, possibly due to electrostatic repulsion, this contributes to our choice of using liposomes with a ratio uncharged lipids to charged lipids of 9:1.
Figure 3. Proposed mechanism for lipid coating of microgels using the electrostatic interaction as driving force. The positively charged lipid vesicles adsorb onto the negatively charged microgels' surface (A) and spread open, forming a continuous lipid layer surrounding the microgels (B). When the microgel degrades the number of crosslinks (•) decreases. This decreases the elasticity of the microgels thereby increasing the swelling pressure (C). When the swelling pressure reaches a critical value the lipid bilayer ruptures (D).

Besides CSLM, electrophoretic mobility measurements also proved that the dex-HEMA microgels became lipid coated. Figure 2A shows that the ζ-potential of the microgels indeed inversed upon exposure to oppositely charged lipid vesicles. The morphology of both the lipid coated and uncoated microgels was further characterized by scanning electron microscopy (SEM). Figures 4C and 4D reveal that the surface of the uncoated microgels is smoother compared to the surface of the coated microgels. The observation of a rather rough surface of the lipid coated microgels can most likely be attributed to the differential drying of the microgel core and the lipid coating.

To evaluate to what extent this way of lipid coating is broadly applicable on microparticles we performed experiments on neutral dex-HEMA microgels (Figure 5A and 5B) and PLGA particles (Figures 5C). Both types of particles could be successfully coated with lipids if a polyelectrolyte film was first deposited onto the surface of the particles to introduce charges at the surface. Using the Layer-by-Layer (LbL) technique, as developed on colloidal particles by Sukhorukov et al., we deposited 2 polyelectrolyte bilayers (using poly(styrenesulfonate) (PSS) and poly(allylamine) hydrochloride (PAH)) and one monolayer of PSS (denoted as (PSS/PAH)PSS) on the neutral dex-HEMA microgels. On the PLGA particles a precursor film consisting of dextran sulfate (DEXS) and chitosan (CHIT) (denoted as (DEXS/CHIT)DEXS) was applied. This 'pre-coating step' with polyelectrolytes was
performed by submerging the microgels for 15 min. in a 2 mg/ml polyelectrolyte solution containing 0.5 M NaCl, followed by two rinsing steps with pure water. The pre-coating step with polyelectrolytes was necessary, most likely to create a sufficient surface charge in order to electrostatically attract the liposomes, as a lipid coating could not be applied on PLGA particles or on neutral dex-HEMA microgels without a polyelectrolyte multilayer at their surface. Similarly it was not possible to coat microgels with only neutral charged lipids or with lipids bearing an equal charge as the microgels, indicating that electrostatic interactions play a major role in the lipid coating process we describe in this chapter.

Figure 4. (A) SEM image of uncoated dex-HEMA-DMAEMA microgels. (B) SEM image of SOPC:DOPA coated dex-HEMA-DMAEMA microgels.

Figure 5. (A) Green and (B) red fluorescence image of neutral dex-HEMA microgels first pre-coated with (PSS/PAH)$_2$:PSS and then coated with DOPE:DOTAP; PAH was FITC labelled and DOPE was rhodamine labelled. (C) PLGA microspheres first precoated with (CHIT/DEXS)$_3$ and then coated with DOPE:DOTAP. The lipids were labelled with BODIPY.

Although the deposition of a lipid layer on the dex-HEMA microgels was evidenced we did not know whether one lipid bilayer or multiple lipid bilayers were formed. XRR is
ideally suited to investigate the structure of ordered films. As XRR should be performed in
the dry state and on flat substrates it cannot be applied to microgels. To obtain some more
insight into the deposition of charged lipids onto an oppositely charged surface we exposed
liposomes to a silicon wafer onto which 3 bilayers CHIT/DEXS were deposited. Although this
surface probably differs largely from the microgels’ surface, it can serve as a model surface
and will aid in understanding the lipid coating process. Figure 6 shows the X-ray
reflectograms and the corresponding electron density profiles of (CHIT/DEXS)$_3$ and
(CHIT/DEXS)$_3$/SOPC:DOTAP coated silicon wafers. From the number of Bragg peaks we
estimated that the lipid coating obtained on the silicon wafers consists of approximately 20
lipid bilayers, leading to a total thickness of 120 nm. As verification we measured the film
thickness by ellipsometry and found a thickness of 119 nm which agrees with the XRR data.
The absence of Kiessig fringes points towards a high roughness of the coating, most
probably because the surface consists of steps of discrete height (integral multiples of the
period), giving a cityscape profile to the surface. Clearly, we cannot literally extend the
observations made on the (flat) lipid coated silicon surface to the (spherical) lipid coated
microgel surface. However, these observations allow speculating that the dex-HEMA
microgels are probably covered with multiple lipid bilayers. From the XRR measurements it is
most likely that upon adsorption the liposomes Indeed fuse and spread resulting in a coating
consisting of multiple lipid bilayers. In case the lipid coating would consist of intact non-fused
liposomes it would be probably impossible to detect the 20 Bragg peaks, the width of each
peak corresponding with a bilayer thickness of 6 nm which agrees well with literature data on
the thickness of lipid bilayers.

As outlined in the introduction, the second aim of this work was to evaluate whether
the swelling pressure of the degrading dex-HEMA microgels can rupture the surrounding lipid
membrane. The degradation of dex-HEMA, by hydrolysis of the carbonate esters (indicated
on Figure 1) that connect the methacrylate groups with the dextran backbone, results in high
molecular weight dextran chains (19 kDa) and HEMA oligomers. By hydrolysis of the
carbonate esters, the cross links, which connect the dextran chains in the microgels, cleave.
This lowers the elasticity of the microgels which, in turn, increases the swelling pressure of
the dex-HEMA microgels. In previous work, our group characterized how the swelling
pressure of degrading dex-HEMA gels increases during degradation and showed that, when
the gel becomes totally degraded, the swelling pressure at the end of the degradation equals
the osmotic pressure of the obtained dextran solution. The swelling pressure of the
completely degraded dex-HEMA hydrogels used in this study was previously determined to
be around 150 kPa. As lipids are known to have a limited permeability, we expected that
the degradation products of the dex-HEMA microgels would not diffuse through the lipid
membrane before the rupturing of the membrane. To accelerate the degradation of the lipid
coated dex-HEMA microgels, which ranges at neutral pH from days to weeks depending on the composition of the gel, \(^{17}\) we added a sodium hydroxide solution (NaOH; 1 M) to the dispersion of lipid coated dex-HEMA microgels. Under these alkaline conditions hydrolysis of the dex-HEMA microgels occurs within minutes. Figure 7 shows CLSM snapshots, taken every 15 s, of the process. Initially, during the first 75 s, the lipid membrane inflates and stretches, due to the increasing swelling pressure of the degrading dex-HEMA microgels. Finally, in the time interval between 75 and 90 s, this swelling pressure causes the rupture of the lipid membrane (as supporting information a movie is provided showing the explosion of a larger amount of lipid coated microgels, showing that all particles behaved in a similar way.

**Figure 6.** (A) X-ray reflectivity of silicon wafers coated with respectively (CHIT/DEXS)_3 and (CHIT/DEXS)_3/SOPC:DOTAP. Circles are experimental data. Lines are fits using the electron density profiles shown in part B. For clarity the curves have been displaced vertically. \(k_z\) is the vertical component of the wave vector of the incident photons in vacuum. (B) Corresponding electron density profiles. Circles correspond to computed densities, and continuous lines were obtained by cubic spline interpolation.
during degradation) leading to a collapsed lipid membrane as is clearly shown in the image after 120 s. During the 'stretch-phase' of the lipid membrane we also observed the formation of 'parachutes' on the microgels preceding the complete explosion of the lipid membrane. The formation of these parachutes supports the hypothesis that the lipid film consists of multiple lipid bilayers which elongate, creating additional surface area, when subjected to the swelling pressure of the degrading microgel. As schematically outlined in Figure 8, at certain locations the lipid membrane may be weaker than at other locations due to discontinuities within single lipid bilayers. While the swelling pressure of the degrading microgel inflates the whole lipid membrane, at these weaker places in the membrane the increasing swelling pressure locally inflates the lipid membrane resulting in the formation of parachutes.

**Figure 7.** CLSM snapshots of degrading dex-HEMA-MAA microgels, coated with SOPC:DOTAP. The first image (0 s) is obtained immediately after the addition of sodium hydroxide to the microgels. The subsequent images are taken each 15 s. The lipids are green labeled using BODIPY while the microgels are red labeled using TRITC-dextran. Note that the fade out of the red color is not due to bleaching or leakage of the TRITC-dextran from the microgels but due to the pH dependent fluorescence of TRITC. After explosion of the microgels (120 s) only remnants of the lipids can be observed.
As the microgels immediately began to degrade upon exposure to NaOH it seems that the hydroxyl ions are able to diffuse through the lipid coating. On the other hand, the lipid coating appears to be impermeable to the degradation products of the microgels: they seem to remain in the lipid coated particles as an increase in osmotic pressure occurs which finally results in the rupturing of the lipid membrane. Several groups \cite{18,26,27} have observed that supported lipid bilayers are permeable to small molecules whereas the corresponding liposomes seem impermeable to the same molecules. For example Pennefather et al. reported on lipid membranes supported on hydrogel beads which were impermeable to dextrans with a molecular weight ranging from 1.5 to 3 kDa whereas the membrane appeared to be permeable to calcium ions. \cite{27} This permeability was attributed to the presence of small nanoscopic defects within the lipid bilayers allowing the diffusion of small molecules. Most likely a similar phenomenon occurs in case of lipid coating of dex-HEMA microgels.

**Figure 8.** ‘Parachute’ formation in the top bilayer during degradation of the dex-HEMA microgels. Discontinuities within a single lipid bilayer may allow the swelling pressure to inflate the top layer before complete rupturing of the lipid membrane.

In the degradation experiment presented in Figure 7 the lipid-coated microgels were immersed in an alkaline solution. One could, however, wonder whether the alkaline medium did not damage the lipid coating, leading to desorption from the microgel surface. To elucidate this we prepared lipid coated (SOPC:DOTAP (9:1)) dextran-methacrylate (dex-MA) hydrogels containing MAA. While dex-HEMA hydrogels degrade through hydrolysis, dex-MA gels do not as no carbonate esters are present in the cross-links. \cite{10} When 1 M sodium hydroxide was added to uncoated dex-MA-MAA microgels they remained intact (data not shown). A similar behaviour was observed when lipid coated dex-MA-MAA microgels were brought into contact with sodium hydroxide: the lipid film remained intact (lipid coated dex-MA-MAA microgels were exposed to the NaOH during at least 1 h), parachute formation did not occur and the lipid coating did not desorb (Figure 9), all indicating that addition of NaOH did not influence the lipid bilayer itself within the observation period. This allowed us to
conclude that the rupture of the lipid membrane surrounding the dex-HEMA microgels upon addition of NaOH (Figure 7) is indeed attributed to the increase in swelling pressure of the degrading dex-HEMA core and not to NaOH-induced weakening of the lipid membrane.

In the above sections we have shown that the swelling pressure of the degrading microgels can result in a rupturing of the surrounding lipid membranes. It can be argued that we were not able to deposit only a single lipid bilayer on the surface of the dex-HEMA microgels. However, multiple bilayers surrounding the microgels may provide us with a more robust system being more versatile for real applications. The use of 1 M NaOH to trigger the explosion of the lipid coating is clearly not a stimulus which can be provided by the human body in vivo. However, the only aim of adding NaOH was to accelerate the degradation of the lipid coated microgels in order to visualize their behavior in a reasonable time frame using confocal microscopy. To confirm that the explosion of the lipid coating also occurs at physiological conditions, the lipid coated dex-HEMA-DMAEMA microgels were incubated at 37 °C in phosphate buffered saline. After 7 days lipid coated microgels could not be detected anymore indicating the degradation of the microgels and rupturing of the lipid coating.

**Figure 9.** Lipid (SOPC:DOTAP) coated dex-MA-MAA microgels before and after the addition of sodium hydroxide. The lipid membrane remains intact. Note that the same fading of the red fluorescence occurs as observed in Figure 6, due to the pH dependent fluorescence of TRITC.

**CONCLUSIONS**

In this chapter we have demonstrated a new method for the lipid coating of microgels based on electrostatic interactions between the microgels’ charged surface and oppositely charged lipid vesicles. We have also demonstrated that this way of lipid coating is also applicable on non-charged microgels as well as PLGA particles, given that charges are introduced at their surface by applying a polyelectrolyte multilayer by the LbL approach. The lipid coating thus obtained very likely consists of multiple lipid bilayers and shows certain defects. We showed that the increase in swelling pressure, which occurs in degrading dex-HEMA microgels, did indeed rupture the surrounding lipid membrane.
The prepared microgels may be promising for pulsed drug delivery applications. As the composition (i.e. cross link density) of the dex-HEMA microgels governs their degradation rate, and therefore how their swelling pressure increases, it may allow one to tailor the time of explosion of the lipid coated microgels by varying their composition. Using this concept one may be able to design a drug formulation which delivers the drug in a number of pulses after a single injection: a simultaneous injection of e.g. 3 populations of lipid coated dex-HEMA microgels, each population exploding at a well defined time after injection, may result in 3 drug pulses from a single injection. This clearly differs from other types of degradable microparticles that release the encapsulated drugs in a, more or less, constant rate.

While others have reported on lipid coated microgels in which the lipid membrane is ruptured by an external trigger (e.g. electric field), the lipids surrounding the microgels presented in this chapter are ruptured by an internal trigger, namely at the moment the swelling pressure of the dex-HEMA reaches a critical value. To demonstrate the concept we used NaOH to trigger the rupturing of the coating. However, as the degradation of the microgels also occurs at physiological conditions the lipid-coated microgels presented in this study can be termed as ‘self-exploding microgels’. Our future research will focus on the release of macromolecules from such self-exploding microgels.

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CHAPTER 6

MICROFLUIDIC FABRICATION OF MONODISPERSE BIODEGRADABLE MICROGELS

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ABSTRACT

Microgels are promising materials in drug delivery and biomedicine. Although monodisperse microgels would offer considerable advantages, most microgels investigated and used today are polydisperse in size. We report on the fabrication of 10 µm sized monodisperse microgels by emulsifying an aqueous dextran-hydroxyethylmethacrylate (dex-HEMA) phase within an oil phase at the junction of microfluidic channels. Dex-HEMA microgels are biodegradable and are ideally suited for the controlled delivery of proteins.
CHAPTER 6

MICROFLUIDIC FABRICATION OF MONODISPERSE BIODEGRADABLE MICROGELS

INTRODUCTION

Hydrogels are three dimensional polymer networks, connected chemically or physically, which are able to hold a large amount of water due to their hydrophilic nature. Spherical hydrogel microparticles, or microgels, have attracted large interest in drug delivery due to their biocompatibility and the possibility to package drug molecules within the polymer network. When the microgels are fabricated from (bio)degradable polymers, the degradation of these hydrogels regulates the delivery of the encapsulated drug molecules when introduced into biological systems. 1 Stimuli responsive microgels may also be designed using physico-chemical motifs, which are able to respond to pH, 2 temperature, 3 electric field 4 or glucose 5 changes, and provide an intelligent release of drugs. By tailoring the properties of the microgels, the release-rate and release-profile of the drug molecules can be optimized to suit the specific application. Until now, there is no general method available for the synthesis of monodisperse microgels. The use of monodisperse microgels for use in drug delivery systems or sensing applications should offer considerable advantages compared to polydisperse ones with respect to monitoring, predicting and modelling of their behaviour as they exhibit a constant and predictable response to external stimuli.

‘Microparticles’ can be prepared in various ways. Microparticles have been prepared in bulk emulsions by mechanically shearing monomer into a continuous, immiscible phase, followed by subsequent polymerization of the emulsified droplets. However, the resulting particle size distribution is highly polydisperse. 6-9 Various approaches have been proposed to fabricate monodisperse microparticles. Recently, membrane emulsification techniques, in which the discontinuous phase is forced through a porous membrane into the continuous phase, have been reported to produce monodisperse droplets. 10 Sugiura et al. 11,12
The aim of the present research was to synthesize biodegradable monodisperse microgels by photopolymerisation of monodisperse pre-polymer droplets formed in a microfluidic device. The biodegradable monodisperse microgels were made from dextran-hydroxyethyl methacrylate (dex-HEMA; Figure 1D). The synthesis and characterization of dex-HEMA were previously reported. Unlike previously reported microfluidic devices in which the droplet formation is performed at a T-shaped junction, where water and oil phase meet perpendicular (Figure 1A), an in-line droplet generating channel geometry, were the
aqueous phase is flanked by the oil phase, is utilized (Figure 1B). The performance of the in-line droplet generating geometry is compared to the T-shaped junctions and several distinct advantages of the in-line droplet generating geometry are pointed out.

**MATERIALS AND METHODS**

**Materials.**

Dextran, chlorotrimethylsilane, mineral oil (low viscosity) and FITC-BSA was purchased from Sigma-Aldrich-Fluka. Irgacure 2959 was a gift from Ciba Chemicals. ABIL EM-90 was a gift from Degussa. Photoresist AZ 4620 was purchased from Clariant. Polydimethyl siloxane (PDMS; Sylgard 184) was purchased from Dow Corning. Microscope glass slides (50x24 mm) were purchased from Esco. Air regulators were purchased from Control Air Inc. Digital Pressure gauges were purchased from Dwyer Instruments, Inc. Tygon tubing with an internal diameter of 500 µm was purchased from Cole-Parmer. Steel connector pins were purchased from New England Small Tube Corp.

**Fabrication of silicon wafer moulds.**

The designs of the microfluidic channels was performed with Adobe Illustrator and imprinted on transparencies at a resolution of 5000 DPI. Silicon wafers were pre-treated with Adhesion Promoter (Clariant), followed by spincoating (1 min, 1500 rpm) a 10 µm thick layer of AZ 4620 (Clariant) positive photoresist. The spincoated wafer was put for 5 min at 90 °C. A next 10 µm layer AZ 4620 positive photoresist was spincoated onto the silicon wafer followed by 20 min incubation at 90 °C. The spincoated wafers were UV-irradiated through the transparencies for 1 min applying a 10 s on/off regime. The irradiated parts of the spincoated silicon wafers were washed off with 440 MIF Developer (Clariant) leaving rectangular positive relief mould profiles behind on the surface of the silicon wafers. The positive relief rectangular mould profiles were rounded by reflowing the photo-resist on a hotplate at 150 °C for 5 min.

**Fabrication of PDMS devices.**

The patterned silicon wafers were placed in Petri dishes and pre-treated with 5 drops chlorotrimethylsilane. Sylgard 184 Base Silicon Elastomer was mixed with Sylgard 184 Curing
Agent Silicon Elastomer in a 5:1 ratio and subsequently degassed. An approximately 5 mm thick layer of the mixture was cast onto the patterned silicon wafer in the Petri dish and cured in the oven for 20 min. Sylgard 184 Base Silicon Elastomer was mixed with Sylgard 184 Curing Agent Silicon Elastomer in a 20:1 ratio, degassed and spincoated (1 min, 4000 rpm) on a microscope glass slide followed by curing in the oven for 20 min. The PDMS slabs are peeled off from the silicon wafers and holes for the connection pins are punched with a 20 gauge blunt needle. The PDMS slabs were cleaned with isopropanol, dried with nitrogen and stuck onto the spincoated microscope glass slides. This construct was cured for another 20 min at 80 °C in the oven.

RESULTS AND DISCUSSION

The microfluidic devices were fabricated by soft lithography. In soft lithography elastomeric materials such as poly(dimethyl siloxane) (PDMS) are used as building blocks. PDMS are fluid polymers with a low glass transition temperature that form solid elastomers by cross-linking. Silicon wafer moulds containing the microfluidic channels in positive relief were fabricated by spin-coating a positive photo-resist on the silicon wafers followed by the UV-irradiation of the spin-coated wafer through the transparency printed with the microfluidic channels. After a development step, in order to remove the irradiated material, only the non-irradiated pattern of the microfluidic channels remained on the silicon wafer. By placing the patterned wafer on a hotplate at 150 °C for 5 minutes, the positive relief rectangular mould profiles were rounded by reflowing the photo-resist. While rectangular shaped walls caused the droplets to move along the walls of the channels, round shaped walls kept the droplets centred. The PDMS microfluidic chips were fabricated by casting a 5 mm thick layer of PDMS on the moulds pre-treated with chlorotrimethylsilane. The chips were peeled from the moulds, interconnection ports punched, and sealed to glass coverslips pre-coated with a thin layer of partially cured PDMS. Finally, the microfluidic chips were sealed by overnight curing at 80 °C. The channels were approximately 100 µm wide x 20 µm high, tapering to 10 µm x 20 µm in the region where the oil and aqueous phase merged. Figure 1B shows a microscopy image of the microfluidic channels; Figure 1C shows the PDMS-device connected to the fluid reservoirs, containing respectively the dex-HEMA solution (30 % w/w; kinematic viscosity of 44 mm²/s at 25 °C) and the mineral oil (kinematic viscosity of 28 mm²/s at 25 °C), by 30 cm long Tygon tubing (500 µm internal diameter, ~30 cm long) and steel pins (New England Small Tube Corp.). Compressed air was used to pressurize the reservoirs. Air pressure regulators equipped with digital pressure gauges were
used to individually set the air pressure applied to the input reservoirs. In order to recover the
droplets after manufacturing, a collection tube was connected to the outlet of the device. The
inlet of the oil phases was split into two channels which met in the nozzle. The viscous oil
stream impinged the flow of the aqueous dex-HEMA solution through the central cavity and
created droplets via tip-streaming. Repeated contraction and expansion of the water-tip
sheared a single droplet at each cycle and the stream was focused towards the outlet port for
collection.

As mentioned above, our continuous phase was mineral oil while the emulsified
phase was a 30 % (w/w) aqueous dex-HEMA solution. In the absence of a surfactant, a
continuous aqueous stream was formed co-annular with the oil phase, as shown in
Figure 2A. A non-ionic (cetyl dimethicone copolymer) surfactant, ABIL EM-90 (Degussa),
was added to the oil phase at ~4 % (v/v) to reduce the surface tension between both phases.

**Figure 2.** Optical microscopy images of the production of monodisperse emulsion droplets in the in-line
microfluidic device at (A) low production rate and (B) high production rate. C and D show close-ups of the
corresponding nozzles.
which facilitated emulsion formation and prevented subsequent coalescence prior to curing. In previous reports by our group and the Hennink group, dex-HEMA microgels were fabricated using a water-in-water emulsion technique based on the immiscibility of an aqueous dextran phase and an aqueous polyethylene glycol (PEG) phase. Initially, we have also tried the use of a PEG solution as continuous phase but it was impossible to generate aqueous dex-HEMA droplets, due to the lack of shear between the two phases upon mixing. Figures 2B-D illustrate modes of droplet production in the microfluidic device at various pressure balances between the emulsified and continuous phases. Monodisperse droplets were formed at regular time intervals, at rates of $\sim 10^1-10^2$ Hz. Monodisperse droplets were formed at a low rate, when a low pressure was applied on the reservoirs and when the pressure on the dex-HEMA reservoir was low compared to the pressure on the oil reservoir (Figure 2B). Because the system remained at low Reynolds numbers, the droplets moved in an ordered way along the microfluidic channel without coalescence. By increasing the pressure on the dex-HEMA reservoir the droplets were individually sheared at a higher rate (Figures 2C-D). Figure 3A illustrates a single-file flow of droplets, while Figure 3B illustrates a self-assembled necklace pattern of droplets that results at higher relative pressures of the aqueous phase. Multiple droplets were formed at the nozzle when the instability generated at the droplet tip propagated faster than the liquid tread can retract. At a droplet frequency of $\sim 80$ Hz, $\sim 0.15$ mg of monodisperse microgels was collected per hour. The size of the droplets can be slightly altered by changing the production rate, allowing tight control of final product size by modifying the relative operating pressures of the two inputs.

Figure 3. Optical microscopy images of the emulsion droplets moving in an ordered fashion along the microfluidic channels.
As an alternative to the in-line droplet generating geometry also a T-junction geometry was used. This type of geometry was first reported by Thorsen et al.,\textsuperscript{13} producing water-in-oil emulsions inside microfluidic channels. Furthermore, T-shaped microfluidic junctions have been used by several other groups for the fabrication of monodisperse microparticles.\textsuperscript{11,20} In the T-junction, the aqueous and oil phase meet perpendicular and an aqueous tip is formed from which droplets are sheared off by the oil-phase at regular time intervals. Similar to the in-line droplet generating geometry, the formed droplets move along the microfluidic channels in an ordered way as shown in Figure 4. However, when the droplet generating process is observed more in detail one notices the formation of small droplets, so-called satellites, sheared off simultaneously with the larger droplets. It was not possible to avoid the satellite formation through variation of the relative water/oil pressure as both at low as well as at high production speed satellite formation was observed (right images in Figure 4). When the same experiment was repeated with pure water as aqueous phase instead of an aqueous dex-HEMA phase, no satellite formation was observed (data not shown). Apparently the failure of the T-shaped droplet generating geometry for the formation of monodisperse dex-HEMA droplets is due to the dex-HEMA phase itself. Moreover, also the production speed of this type of droplet generating geometry is limited compared to the in-line droplet generating geometry. Whereas the in-line droplet generator can produce the ‘self-assembled necklace’ structure of droplets moving parallel to each other, the T-shaped droplet generator is only able to produce one row of droplets thus limiting the droplet-formation frequency. Therefore we proceeded further with the in-line droplet generating geometry for the production of monodisperse microgels.

**Figure 4.** Optical microscopy images of the production of monodisperse emulsion droplets in the T-junction microfluidic device. The right images show enlarged images of the nozzle where the emulsion droplets are formed at low (upper right image) and high (lower right image) production speed. The red arrow points out the formation of satellites.
A photoinitiator, Irgacure 2959 (Ciba Chemicals), was added to the aqueous dex-HEMA solution before the emulsification process to allow collected microspheres to be subsequently cured by UV irradiation. When the dex-HEMA droplets containing the photoinitiator left the microfluidic device they were collected in a separate vial and immediately polymerized by UV irradiation while still suspended within oil. The cured dex-HEMA microgels were then separated from the oil by centrifugation followed by several washing steps with deionised water. The removal of the oil from the surface of the microgels was verified by scanning electron microscopy. Figure 5A is an optical microscopy image of the dex-HEMA microgels suspended in water forming a hexagonal closely packed structure which is typical for microparticles exhibiting excellent size uniformity. Figure 5B shows the size distribution of a population of microspheres ($n = 150$), with an average diameter of $9.9 \mu m \pm 0.3 \mu m$. In contrast to the uniform microgels prepared by microfluidic emulsification, Figure 5C illustrates the polydisperse microgels as synthesized by polymerizing droplets obtained by simply vortexing the same dex-HEMA and oil solutions. The main parameter determining the size of the microgels is the width of the channel in which the dex-HEMA phase is focused when leaving the nozzle. While very fine channels may be fabricated using soft lithography, with features on the order of a few microns, the operating pressures required to generate sufficient shear for emulsification becomes prohibitively large due to the increase in hydraulic resistance. When the applied pressure becomes too large, the bond between the PDMS and the coverslip loses integrity, resulting in the destruction of the device. This trade-off limits the size of the microgels produced by our soft microfluidic devices at a given operating point.

![Optical microscopy images of microgels](image_url)

**Figure 5.** Optical microscopy images of (A) monodisperse microgels synthesized using the microfluidic device, (B) the size of the monodisperse microgels ($n = 150$) and (C) polydisperse microgels synthesized by polymerization of dex-HEMA droplets obtained by ordinary emulsification of an aqueous dex-HEMA solution in mineral oil.
Dex-HEMA hydrogels are biocompatible and degrade by hydrolysis of the carbonate ester groups which connect the polymerized methacrylate groups and the dextran chains, leading to the formation of both the original dextran chains and low molecular weight oligomethacrylates. This degradation process is schematically represented in step B of Figure 6. The degradation rate of dex-HEMA hydrogels depends on the cross-link density and can be tailored from days to months by varying (i) the number of methacrylate groups per dextran chain and (ii) the initial water content. Dex-HEMA hydrogels are ideally suited for the encapsulation of proteins as the network structure is able to sterically entrap these proteins. Once the hydrogels start to degrade, the size of the pores between the dextran chains increases due to the cleavage of the cross-links. This increase in porosity promotes the diffusion of the entrapped proteins out of the microgel into the bulk environment. To show that the microfluidic based method also allows loading of the microgels with proteins, we added fluorescein-labelled bovine serum albumin (FITC-BSA; Figure 6).

**Figure 6.** Schematic representation of the polymerization of dex-HEMA (step A), leading to the formation of intra- and intermolecular cross-links which form the three dimensional hydrogel network, and (step B) the hydrolysis of the dex-HEMA hydrogels leading to the formation of dextran chains and oligomethacrylates as degradation products.

**Figure 7.** Confocal images of dex-HEMA microgels containing FITC-BSA, respectively before (A), during (B) and after (C) degradation of the microgels.
0.1 mg per mg dex-HEMA) to the dex-HEMA phase. It was verified that the FITC-BSA was insoluble in the oil phase thus assuring total encapsulation efficiency. Moreover, it is known 29 that BSA tends to accumulate in a dextran rich phase. Figure 7A is a confocal microscopy image of a FITC-BSA loaded dex-HEMA microgel produced by microfluidic emulsification. Figures 7A-C show the FITC-BSA containing dex-HEMA microgels respectively during degradation (Figure 7B) and when completely degraded (Figure 7C). In this experiment we used sodium hydroxide to accelerate the degradation of the microgels, which normally takes several days to several weeks, depending on the cross-link density of the dex-HEMA microgels. 28

CONCLUSIONS

In conclusion, we have shown that monodisperse biodegradable dex-HEMA microgels can be prepared by the use of a PDMS microfluidic device. Aqueous dex-HEMA droplets were formed by periodic shearing of an aqueous dex-HEMA stream within a co-flowing immiscible oil stream by an in-line nozzle geometry. The droplets were collected and polymerized by UV-irradiation with the formation of dex-HEMA microgels. A high production rate, by the simultaneous formation of monodisperse droplets, could be obtained by applying a high pressure on the dex-HEMA containing reservoir relative to the pressure on the reservoir containing the mineral oil. We also showed that the dex-HEMA microgels prepared by the microfluidic approach could be readily loaded with proteins. Microfluidic emulsification techniques such as those introduced in this chapter could be useful for rapid prototyping of drug delivery systems based on microgels with narrow size distributions. Further research will focus on the scale-up 30,31 of the process and on the evaluation of the monodisperse biodegradable microgels for specific applications in drug delivery.

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CHAPTER 7

GLUCOSE RESPONSIVE

POLYELECTROLYTE MICROCAPSULES

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¹ Laboratory of General Biochemistry and Physical Pharmacy, Department of Pharmaceutics, Ghent University, Ghent, Belgium.
² Unité de Physique et de Chimie des hauts Polymères, Université catholique de Louvain, Louvain-la-Neuve, Belgium.
ABSTRACT

Phenylboronic acids are known to form covalent complexes with polyol compounds such as glucose. A novel polyelectrolyte, containing phenylboronic acid as glucose-sensitive moiety, has been synthesized and used for the fabrication of glucose-sensitive hollow polyelectrolyte capsules using the layer-by-layer technique. The response to glucose was observed as a rather fast dissolution of the capsules when brought into contact with a glucose-containing medium. These polyelectrolyte capsules are the first polyelectrolyte capsules able to respond to a stimulus that can be provided by the human body (i.e. an increase in glucose concentration). Therefore the concept we present has promising applications in the biomedical field for the controlled delivery of insulin.
CHAPTER 7

GLUCOSE RESPONSIVE POLYELECTROLYTE MICROCAPSULES

INTRODUCTION

Stimuli responsive capsules have gathered major scientific interest in different areas such as drug delivery, diagnostics and sensing. The layer-by-layer (LbL) technique provides an easy, low-cost and versatile method for the fabrication of hollow polyelectrolyte multilayer capsules. Briefly, this technique is based on the alternating adsorption of oppositely charged polyelectrolytes onto a charged substrate. The major advantage of the layer-by-layer technique is its multifunctionality, allowing the incorporation of a broad variety of functional groups, nanoparticles, dyes, enzymes, etc in the multilayers. Hollow polyelectrolyte capsules able to decompose as a consequence of an external stimulus such as pH, salt, IR-laser have been reported. However, the use of e.g. a change in pH as trigger is restricted to few locations in the body (e.g. upon transit from stomach to intestine).

In this chapter we report on glucose-responsive hollow polyelectrolyte multilayer capsules using a novel synthesized polyelectrolyte containing phenylboronic acid as glucose-sensitive moiety. Phenylboronic acids are known to form covalent complexes with polyolcompounds such as glucose. An important property of phenylboronic acid compounds in aqueous medium is that they are in equilibrium between an uncharged and a charged form as shown in Figure 1. Through complexation with glucose one is able to shift the equilibrium in the direction of increasing charged phenylborates because only charged borates can form a stable complex with glucose in aqueous media. Kataoka and co-workers have reported on phenylboronic acid containing poly(N-isopropylacrylamide) hydrogels. The addition of 5 mg/ml glucose, which increases the fraction of charged borate anions rendering the polymer more hydrophilic, shifts the lower critical solution temperature (LCST)
upwards from 22 °C to 35 °C. This results in a swelling and consequent release of the insulin loaded in the gels when the gels are incubated at a temperature between 22 °C and 35 °C. This is a very elegant system. However, its glucose-responsive capacity is limited to a narrow temperature window close to the LCST point. The concept we present in this chapter is not based on a change in LCST caused by glucose, but rather on a glucose-induced change in electrostatic interactions between a polyanion and a phenylboronic acid containing polycation.

![Figure 1. Equilibrium of phenylboronic acid compounds.](Image)

**EXPERIMENTAL**

*Materials.*

4-Sodium polystyrene sulfonate (PSS; Mw~70 kDa), linear polyethylene imine (PEI; Mw~25 kDa), dimethyl aminoethyl acrylate (DMAEA; distilled under reduced pressure prior to use), N,N,N’,N’-tetramethylethlenediamine (TEMED), acrylic acid (AA), [1-ethyl-3-(dimethylamino)propyl]carbo-diimide hydrochloride (EDC), hydrogen peroxide (35 %), FITC-BSA and D-glucose were purchased from Sigma-Aldrich-Fluka. Aminophenylboronic acid (APBA) was purchased from Avocado Research Chemicals. Sodium chloride (NaCl), potassium peroxodisulphate (KPS), diethyl ether, sulfuric acid (98 %) and hydrochloric acid were purchased from Merck. Tetrahydrofuran (THF), methanol and diethyl ether were purchased from Acros Organics. Quartz slides were purchased from Hellma. Monodisperse polystyrene particles with a mean diameter of 10.25 µm were purchased from Microparticles.
GmbH. Dialysis membranes (Mw cut off 25 kDa) were purchased from Spectra Por. Water used in all experiments was of Milli-Q grade. Rhodamine labeled PSS was synthesized as reported in literature.\textsuperscript{21}

**Synthesis of 3-acrylamidophenylboronic acid (AAPBA).**

AAPBA was synthesized according to Kitano et al. In a 250 ml flask, placed in an ice bath, APBA (5.586 g, 30 mmol) was dissolved in distilled water (90 ml) followed by pH adjustment to 4.8 using 1 M NaOH. After dissolution of the APBA, EDC (6.903 g, 36 mmol) was added followed again by pH adjustment to 4.8 using 1 M NaOH. In a second 250 ml flask, AA (2.478 ml, 36 mmol) was dissolved in distilled water (30 ml), followed again by pH adjustment to 4.8 using 1 M NaOH. The acrylic acid solution was drop wise added to the other flask in the ice bath and allowed to stir for 1 h, followed by stirring overnight at room temperature. The reaction mixture was 4 times extracted with diethyl ether followed by the evaporation of the diethyl ether. The resulting product was recrystallized from water and obtained as a white solid. The yield was 33 \% with respect to APBA. \textsuperscript{1}H-NMR in CD\textsubscript{2}OD using a Varian Mercury 300 NMR spectrometer: $\delta$=5.75 (1H, dd, CH\textsubscript{2}=CH-), $\delta$=6.35 (1H, dd, CH\textsubscript{cis}\textsubscript{H}\textsubscript{trans}=CH-) and $\delta$=7.2-7.8 (4H, m, phenyl). The melting point of the AAPBA was 148 °C as determined by DSC (DSC 2920 Differential Scanning Calorimeter (TA Instruments)).

**Synthesis of the AAPBA-DMAEA copolymer (PAD).**

In a 200 ml two-necked flask 200 mg (= 1.047 mmol) AAPBA and 400 µl (= 2.794 mmol) DMAEA were dissolved in 100 ml water. The mixture was deoxygenized by bubbling nitrogen for 30 min. To initiate the polymerization 20 mg KPS and 200 µl TEMED were added and the reaction mixture was stirred for 12 h at room temperature under a nitrogen atmosphere. The mixture was subsequently dialyzed against water (pH adjusted to 3 using HCL) followed by dialysis against distilled water, leading to a partial precipitation of the polymer. After freeze-drying, the polymer was obtained as a white fluffy solid. Because the polymer appeared only soluble at a pH lower than 5 or higher than 11.5, and no organic solvent was suitable to dissolve the polymer, NMR and GPC analysis were not possible. The yield was 67 \% with respect to AAPBA. The composition of the polymer was determined by elemental analysis (Vario EL; Elementar Analysensysteme, GmbH).
**UV-VIS monitoring of the multilayer build-up.**

Quartz slides were cleaned and hydrophylized by treatment with freshly prepared piranha solution (H₂O₂ (35 %)/H₂SO₄ (98 %) 1:1 v/v (caution: piranha solution reacts violently with organic materials and should not be stored in closed containers) then abundantly rinsed with distilled water. The treated quartz slides were precoated with a PEI layer by immersion in a 2 mg/ml PEI solution containing 0.5 M NaCl. Multilayers of PSS/PAD were deposited on the PEI coated quartz slides starting with the negatively charged PSS by immersion in a 2 mg/ml PSS solution (containing 0.5 M NaCl and 0.1 M HCl (pH 1)) during 15 min followed by rinsing with a 0.1 M HCl solution and pure water. Finally the quartz slides were dried under a nitrogen stream. After each deposition of a bilayer the absorbance of the film was measured with a Pharmacia Biochrom 4060 UV-VIS spectrophotometer.

**Fabrication of hollow capsules.**

Monodisperse polystyrene particles with a mean diameter of 10.25 µm served as a sacrificial template. Multilayer build-up was performed by the LbL technique from 1 mg/ml PAD and 2 mg/ml PSS solutions in 0.1 M HCl (pH 1) and 0.5 M NaCl. After each adsorption step, two washing steps with 0.1 M HCl were performed. When the desired amount of layers was deposited, the polystyrene core was dissolved overnight using THF followed by three washing steps with THF and four washing steps with distilled water.

**Electrophoretic mobility.**

The electrophoretic mobility of the LbL coated polystyrene particles was measured in pure water using a Malvern Zetasizer 2000 (Malvern Instruments). The ζ-potential was calculated from the electrophoretic mobility (μ) using the Smoluchowski relation: ζ=μη/ε where η and ε are the viscosity and permittivity of the solvent, respectively.

**Confocal microscopy.**

Confocal images of the hollow capsules were taken with a MRC1024 Bio-Rad confocal laser-scanning microscope (CLSM) equipped with a krypton-argon laser (Biorad). An inverted microscope (Eclipse TE300D, Nikon) was used which was equipped with a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon). To visualize the microcapsules a 5 µL drop of capsule suspension was placed on a cover slip. To asses
the glucose responsive behavior of the microcapsules a second 5 µL drop, containing e.g.
10 mg/ml glucose in a 0.2 M buffer (in order to obtain a total glucose concentration of 5
mg/ml in a 0.1 M buffer), was gently added to the drop of capsule suspension avoiding
capsule movement as much as possible.

**Figure 2.** Polyelectrolytes used for the fabrication of the hollow capsules. PAD (A) serves as polycation and PSS (B) serves as polyanion.

**RESULTS AND DISCUSSION**

An acrylate having phenylboronic acid as a pending group, 3-acrylaminophenylboronic
acid (AAPBA), was obtained by coupling acrylic acid with 3-aminophenylboronic acid
hemisulphate using [1-ethyl-3-(dimethylamino)propyl]carbo-diimide hydrochloride as
condensation reagent. AAPBA was copolymerized by radical polymerization in aqueous
medium at room temperature with dimethyl aminoethyl acrylate (DMAEA) in a 1:2 (w/w) ratio
AAPBA:DMAEA (corresponding with a monomer feed mixture of 40 mol% AAPBA and
60 mol% DMAEA) using potassium peroxodisulphate as radical initiator and N,N,N',N'-'
tetramethyl ethylenediamine as catalyst. After dialysis and freeze-drying, a white powder
was obtained. This product will further on be denoted as PAD (structure A in Figure 2). The
PAD seemed to be only soluble in water at a pH below 5 or above 11. At an intermediate pH,
both the borate and amino groups are charged, which leads to complexation, resulting in the
precipitation of the polymer. The exact composition of the copolymer, as determined from
elemental analysis, was 44 mol% phenylboronic acid groups to 56 %mol amino groups,
which is in agreement with the initial monomer ratio.

UV-VIS spectrophotometry was used to determine whether the synthesized PAD and
polystyrene sulfonate (PSS) could build-up a polyelectrolyte multilayer at the surface of
quartz slides. Quartz slides were first hydrophilyzed by treatment with piranha solution, inducing a negative surface charge. Next, the quartz slides were pre-coated with a polyethylene imine monolayer, which is positively charged, to provide the quartz slides with a surface suitable for layer-by-layer deposition of polyelectrolytes. In total six bilayers PSS/PAD were deposited onto the quartz slides. The coating of the quartz slides with a PSS or PAD layer was performed as described earlier from polyelectrolyte solutions containing 0.5 M NaCl and 0.1 M HCl (pH 1). The high salt concentration is required to keep the polyelectrolytes in a coiled conformation and to allow the deposition of a sufficient amount of polyelectrolyte. The low pH of the polyelectrolyte solutions was used in order to keep the boronic acid moieties uncharged during the layer-by-layer build-up. Figure 3A shows the absorbance spectra of the coated quartz slides recorded after the deposition of each bilayer. The absorbance increases after each deposition cycle, indicating a successful multilayer build-up. Figures 3B and C show the increase in absorbance at 225 nm and 250 nm (attributed to the aromatic groups in PSS and AAPBA) as a function of bilayer number. A nearly ‘linear growth’ of the multilayer can be observed.

Figure 3. UV-VIS spectra of a quartz slide coated with PSS/PAD. 'n’ denotes the number of PSS/PAD bilayers deposited on the surface of the quartz slide. The insets show the increase in absorbance at (B) 250 nm and (C) at 225 nm as a function of the number of PSS/PAD bilayers.
As the PAD copolymer has shown to be suitable for multilayer formation in combination with PSS, the PAD/PSS polyelectrolyte pair was further used for the fabrication of hollow polyelectrolyte capsules. The fabrication of these hollow polyelectrolyte capsules was carried out in two consecutive steps. First the core material (being monodisperse weakly cross-linked polystyrene particles with a diameter of 10.25 µm) was coated with six PAD/PSS bilayers. The deposition conditions were identical to those used for the coating of the quartz slides except that no pre-coating step with PEI was required. In order to separate the polystyrene particles from non-adsorbed polyelectrolytes two washing steps with 0.1 M HCl (using the centrifugation procedure) were carried out followed by the addition of the next polyelectrolyte solution. To monitor the multilayer build-up the \( \zeta \)-potential of the particles was measured after each adsorption step (Figure 4A). The \( \zeta \)-potential of the bare (non-coated) polystyrene particles was \(-21\) mV and changed regularly between \(+45\) and \(-52\) mV upon adsorption of PAD, respectively PSS. The second step in the fabrication of the hollow capsules was the removal of the core material. Therefore THF was added to dissolve the polystyrene. Several washing steps with THF were carried out to remove the polystyrene followed by several washing steps with pure water in order to remove the THF. In this way hollow polyelectrolyte capsules were obtained which were stored in pure water. The hollow capsules could be visualized by confocal microscopy when rhodamine labeled PSS was used during the multilayer build-up (Figure 4B).

When the hollow capsules were placed into a buffered solution at pH 9 (0.1 M carbonate buffer) they remained stable, even after several days (data not shown). Note that upon polymerization and complexation with oppositely charged polyelectrolytes the apparent \( pK_a \) of the amino groups in DMAEA (i.e. 8.4) will increase. This explains why the capsules

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Figure 4. (A) \( \zeta \)-potential as a function of layer number during the multilayer build-up on the polystyrene particles. The measurements were performed in pure water (n=5). (B) Confocal microscopy image of hollow capsules consisting of 6 PAD/PSS bilayers. Note that the PSS was fluorescently labeled with RITC.
Figure 5. Confocal microscopy images (recorded at fixed exposure) of hollow (PAD/PSS)$_6$ capsules gradually disassembling in a 5 mg/ml glucose solution at pH 9. Note that the PSS was fluorescently labeled with RITC. The inset in the upper right corner shows the transmission light image of a capsule.

Figure 6. Confocal microscopy images of the gradual filling and disassembling of hollow (PAD/PSS)$_6$ capsules in a 5 mg/ml glucose solution at pH 9 containing FITC-BSA. Note that the PSS was fluorescently labeled with RITC.
remain intact at pH 9. However, when the capsules were suspended in a solution containing 5 mg/ml glucose at pH 9 they disappeared gradually. As Figure 5 shows, the capsules gradually faded out in time, accompanied by a small degree of swelling, and completely disappeared after 250 s, indicating a rather fast response to the external stimulus. Identical observations were performed on different batches of capsules. Note that the disappearance of the capsules shown in the confocal images was not due to bleaching by the laser. To prove this we added, in the top right corner, the transmission light microscopy image of a disassembling capsule. After 1 min the capsules are barely visible with transmission light microscopy and therefore we opted to visualize the dissolution of the capsules by confocal microscopy. We repeated the same experiment at pH 7 (0.1 M phosphate buffer) and observed that the capsules remained stable in the presence of glucose (data not shown). Also the permeability of the capsules to fluorescently labeled bovine serum albumin (FITC-BSA) was investigated. When the capsules were incubated in a solution containing 0.1 mg/ml FITC-BSA buffered at pH 9 the capsule interior remained dark for at least 30 min (data not shown). When the capsules were incubated in solution of 0.1 mg/ml FITC-BSA and 5 mg/ml glucose, buffered at pH 9, a gradual filling of the capsules with FITC-BSA was observed (Figure 6). Apparently, before complete disassembly of the capsules the wall becomes permeable allowing the inwards diffusion of FITC-BSA. The behavior of the PAD/PSS capsules at glucose concentrations lower than 5 mg/ml was also tested. At a glucose concentration of 2.5 mg/ml (buffered at pH 9) the capsules still decomposed whereas at lower glucose concentrations the capsules remained intact.

Figure 7. Proposed mechanism of the glucose induced decomplexation of a polyelectrolyte bilayer. The red circles represent the sulfonates, the blue circles represent the amino groups. The green circles represent the uncharged phenylboronic acid groups that become anionic (orange circles) after addition of glucose.

It is known that the pH of the environment strongly affects the sensitivity of phenylboronic acid compounds to glucose. The glucose sensitive properties of phenylboronic acid are greatly enhanced when the system is operated near the $pK_a$ of the phenylboronic acid derivatives which is generally situated between 8 and 9. Springsteen et al. have reported that the apparent $pK_a$ of phenylboronic acid/polyol-complexes drops 2 - 4 $pK_a$ units
upon complexation. This drop in pK_a is due to the fact that the polyol complex itself is a stronger acid than the phenylboronic acid derivative. The presence of both the amino groups of the DMAEA and the sulfonate groups of the PSS will influence the apparent pK_a of the phenylboronic acid, thus severely complicating the system. Therefore we will only attempt to give a qualitative interpretation of the experimental observations. When the suspension containing the capsules is buffered at pH 9, which is near the pK_a of phenylboronic acid, the downward shift of the apparent pK_a upon addition of glucose leads to a strong increase in borate anions. From our experimental results this increase in borate anions seems to be sufficient to cause the disassembly of the polyelectrolyte multilayers. When the suspension is buffered at pH 7, the increase in borate anions upon addition of glucose is less pronounced and apparently incapable of causing the disassembly of the polyelectrolyte multilayers. Overall one can state that the addition of glucose at a pH of 9 leads to a situation with an excess of negative charges that are no longer compensated by the positive charges of the DMAEA groups.

The exact mechanism responsible for the disassembly of the capsules is most likely quite complex and not fully understood at this moment. A possible, simplified, explanation is presented in Figure 7. The layer-by-layer build-up is based on the complexation between the anionic sulfonate groups of the PSS and the ternary amino groups of the PAD. Since the multilayer build-up was performed at an acidic pH, the phenylboronic acid moieties are uncharged and did not contribute to the electrostatic complexation during the layer-by-layer build-up. When placed in a buffer at pH 9 already some of the borate groups become charged due to intramolecular complexation, which is likely to occur between amino and boronic acid groups. Upon addition of glucose, an increased amount of phenylboronic acid moieties become negatively charged and start to interfere strongly with the polyelectrolyte multilayers. We expect two phenomena to occur simultaneously. First there is an intermolecular repulsion between the sulfonate groups of the PSS and the glucose/phenylboronic acid complex leading to repulsion between the successive layers. Secondly, there is an intramolecular complexation due the electrostatic interactions between the cationic ternary amino groups and the anionic glucose/phenylboronic acid. Both phenomena contribute to the disassembly of the capsules, indicating that the change in electrostatic interaction between the oppositely charged polyelectrolytes in the multilayer assembly plays a major role in the dissolution of the capsules.
CHAPTER 7 – GLUCOSE RESPONSIVE POLYELECTROLYTE MICROCAPSULES

CONCLUSIONS

In conclusion, a novel glucose-sensitive polyelectrolyte, bearing phenylboronic acid moieties, was synthesized and used for the fabrication of hollow polyelectrolyte capsules using the layer-by-layer technique. When exposed to glucose the capsules disassembled in less than 5 minutes. It is well known that phenylboronic acids do not bind specific to only glucose but form a complex with a wide variety of vicinal diol containing compounds. However, the concentration of non-glucose carbohydrates in the human blood (like galactose) is several orders of magnitude lower than the concentration of glucose and should therefore not be able to cause the disassembly of the capsules (which we indeed observed – data not shown). For healthy human beings the glucose concentration in the bloodstream does not exceed 1.5 mg/ml. As the capsules reported in this chapter only decompose at glucose concentrations above 2.5 mg/ml they will remain intact at normal (i.e. healthy) glucose levels. Capsule decomposition would only occur in those cases where threatening glucose levels are reached. The proposed system can be applied in a broad temperature range and may have significant benefits compared to earlier reported glucose responsive systems which comprise the use of proteins (concanavalin A) or enzymes (glucose oxidase) which may easily denature. Further research aims to design LbL capsules which disassemble at elevated glucose concentrations at more physiological pH (i.e. 7.4) using polymerizable phenylboronic acid derivatives having a pKₐ situated near the physiological pH.

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CHAPTER 8

INTRACELLULARLY DEGRADABLE POLYELECTROLYTE MICROCAPSULES

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ABSTRACT

Polyelectrolyte capsules are very promising for biomedical applications. However, until now there are few techniques available for the release of macromolecules from these capsules. In this communication we are the first to report on the fabrication of polyelectrolyte capsules which can be degraded intracellular after internalization. Both enzymatic as well as hydrolytic degrading capsules are presented. These types of capsules might be promising for the intracellular delivery of peptides and genetic material.
CHAPTER 8

INTRACELLULARLY DEGRADABLE POLYELECTROLYTE MICROCAPSULES

INTRODUCTION

‘Hollow’ polyelectrolyte capsules, 1, 2 fabricated by coating colloidal templates using the layer-by-layer (LbL) technique 3-6 followed by the dissolution of the core material, have recently emerged as attractive materials in the field of controlled drug delivery and catalysis. They can carry high amounts of active compounds in a non-complexed state (i.e. in free form within the cavity of the capsule) 7, 8 and they exhibit the tremendous potential that their physicochemical and mechanical properties can easily be tailored leading to microcapsules with an unmet functionality. The LbL technique is based on the sequential adsorption of oppositely charged substances (polyelectrolytes, 9 nanoparticles, 10 nanotubes, 11 proteins 12 and lipids 13) onto a charged template, being a flat surface 9 or a colloidal core. 14 A wide variety of organic 1 and inorganic 15 materials have been used as colloidal templates for the preparation of hollow polyelectrolyte microcapsules. Major concerns, however, are both the incomplete removal of the core material (which is often toxic) and the hazardous conditions required for removing the core. 16, 17 Recently, calcium carbonate (CaCO₃) based colloidal templates have shown to be promising for the synthesis of hollow polyelectrolyte capsules as CaCO₃ is non-toxic and as the CaCO₃ core can be easily removed by complexation with EDTA (Scheme 1). 18 Moreover, CaCO₃ particles are easy to synthesize and to load up with macromolecules up to a size of about 40 nm due to their high porosity. 19, 20

There are, to the best of our knowledge, no studies reporting on polyelectrolyte capsules which degrade at physiological pH or which can be destroyed by endogenous enzymes. Especially, although cellular uptake of polyelectrolyte capsules has been recently demonstrated, 21-23 intracellularly degradable polyelectrolyte capsules have not been reported. Such microcapsules could be attractive for the delivery of drugs with an intracellular...
target (like e.g. nucleic acids and various proteins). This study aims to design hollow polyelectrolyte capsules which, after cellular entry, degrade intracellularly.

Scheme 1. Schematic representation of the synthesis of polyelectrolyte capsules filled with FITC-dextran using CaCO₃ particles as a template. (A) co-precipitation of FITC-dextran (yellow) in calcium carbonate particles (grey) during the mixing of calcium chloride and sodium carbonate solutions, (B) LbL coating of the calcium carbonate particles (red=polyanion , blue= polycation), and (C) polyelectrolyte capsules filled with FITC-dextran obtained after dissolution of the CaCO₃.

Scheme 2. Chemical structure of the polyelectrolytes used for the fabrication of the polyelectrolyte microcapsules. The arrow indicates the cleavable carbonate ester of the P(HPMA-DMAE) that links the cationic side chain to the backbone of the polymer.
CHAPTER 8 – INTRACELLULARLY DEGRADABLE POLYELECTROLYTE MICROCAPSULES

EXPERIMENTAL

Materials.

Chlorpromazine, filipin III, genistein, FITC-dextran (Mw~2000 kDa), dextran sulphate (DEXS; Mw~10kDa), poly-L-arginine hydrochloride (PARG; Mw>70 kDa), sodium polystyrene sulfonate (PSS; Mw~70 kDa) and polyallylamine hydrochloride (PAH; Mw~70 kDa) were purchased from Sigma-Aldrich-Fluka. Calcium chloride (CaCl₂) and sodium carbonate (Na₂CO₃) were purchased from Merck. LysoTracker® Red was purchased from Molecular Probes. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, heat-inactivated FBS and penicillin/streptomycin were purchased from GibcoBRL. P(HPMA-DMAE) was synthesized according to Funhoff et al. 24

Fabrication of (FITC-dextran filled) CaCO₃ particles.

CaCO₃ particles were fabricated according to Volodkin et al. 18 Briefly, CaCl₂ and Na₂CO₃ solutions (0.33 M) were mixed under vigorous stirring for 30 s leading to the precipitation of CaCO₃ particles. Subsequently 4 centrifugation and washing steps with pure water were performed in order to remove the unreacted species. In a last step the particles were washed with acetone and subsequently air-dried.

FITC-dextrans were incorporated in the CaCO₃ particles by co-precipitation. 20 Therefore 5 mg of FITC-dextran was dissolved in the CaCl₂ solution (3.5 ml) before mixing with the Na₂CO₃ solution.

Fabrication of FITC-dextran filled capsules.

FITC-dextran filled capsules were fabricated in a two step procedure. In a first step the CaCO₃ particles containing the FITC-dextrans were coated using the layer-by-layer (LbL) technique. Therefore 20 mg of FITC-dextran containing CaCO₃ particles were dispersed in a 0.5 M NaCl solution containing the polyanion (1 mg/ml). The dispersion was continuously shaken during 10 min. The excess of polyanion was removed by three centrifugation/washing steps with deionized water. Thereafter, 1 ml of a 0.5 M NaCl solution containing the polycation (1 mg/ml) was added and the dispersion was continuously shaken during 10 min followed again by three centrifugation/washing steps. This procedure was repeated three times for each polyelectrolyte resulting in the deposition of eight polyelectrolyte layers on the FITC-dextran filled CaCO₃ particles. In a second step the
CaCO₃ core was removed by complexation with EDTA. Therefore the coated CaCO₃ particles were shaken during 30 min with 1 ml EDTA-solution (0.2 M, pH 7.5) followed by centrifugation and redispersion in 1 ml fresh EDTA-solution. This procedure was repeated 4 times to assure complete removal of the CaCO₃ core as previously reported by Volodkin et al. Finally the thus obtained hollow microcapsules filled with FITC-dextran were washed 4 times with deionised water.

**Electrophoretic mobility.**

The electrophoretic mobility of the LbL coated microcapsules was measured using a Malvern Zetasizer 2000 (Malvern Instruments). The zeta-potential (ζ-potential) was calculated from the electrophoretic mobility (μ) using the Smoluchowski relation (ζ=μη/ε) where η and ε are the viscosity and permittivity of the solvent, respectively.

**Scanning electron microscopy (SEM).**

A drop of particle or capsule suspension was deposited onto a silicon wafer and dried under a nitrogen stream followed by sputtering with gold. SEM images were recored with a Quanta 200 FEG FEI scanning electron microscope operated at an acceleration voltage of 5 kV.

**Cell culture experiments.**

Vero-1 (African green monkey kidney cells; ATCC number CCL-81) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM glutamine, 10 % heat-inactivated FBS, 100 U/ml penicilline/streptomycine (culture medium) and grown at 37 °C in a humidified atmosphere containing 5 % CO₂. To determine the cellular uptake of the microcapsule we seeded 2.5 x 10⁴ cells per cm² in sterile glass-bottomed culture dishes (MatTek Corporation, MA, USA). Subsequently, 12 h after seeding the cells, they were incubated with 20 µl of the microcapsule dispersions (containing approximately 10⁶ per ml in deionized water; the concentration of capsules was determined by haemocytometry). After 30 min of incubation the culture medium was removed and replaced with fresh culture medium. To visualize the microcapsules in the cells a confocal laser scanning microscope was used (MRC1024 Bio-Rad) equipped with a 60x water immersion objective. To elucidate through which pathway the microcapsules were taken up by the cells we again seeded 2.5 x 10⁴ cells in sterile glass-bottomed culture dishes (MatTek Corporation). Twelve hours
after seeding, the cells were incubated for 1 h at 37 °C in DMEM with respectively chlorpromazine (10 mg/ml), filipin III (1 mg/ml) or genistein (200 µM). Subsequently, the microcapsules were added and the cells were further incubated at 37 °C. Before imaging the cells were washed twice with 0.1 M citrate buffer (pH 4) and twice with DMEM to remove all membrane-associated particles. For lysosome staining the cells were incubated during 30 min at 37 °C with 50 pM of the lysosomal marker LysoTracker® Red before visualization under the confocal microscope.

**Figure 1.** Transmission (A) and confocal (B image) of FITC-dextran (green fluorescence) filled PSS/P(HPMA-DMAE) capsules.

**RESULTS AND DISCUSSION**

Two types of degradable capsules were fabricated using CaCO₃ microparticles as template. The first type of capsules should be susceptible to enzymatic degradation and were made of poly-L-arginine (PARG; Scheme 2) as polycation and dextran sulfate (DEXS; Scheme 2) as polyanion. In the second type of capsules poly(hydroxypropyl methacrylamide - dimethyl aminoethyl) (P(HPMA-DMAE); Scheme 2) and polystyrene sulfonate (PSS; Scheme 2) were used respectively as polycation and polyanion. As Scheme 2 shows, P(HPMA-DMAE) is a polymethacrylamide bearing cationic side chains which can be cleaved by hydrolysis of the carbonate ester. Besides degradable capsules we also prepared non-degradable ‘control capsules’ by coating CaCO₃ particles with PSS and polyallylamine hydrochloride (PAH; Scheme 2).
CaCO$_3$ particles were prepared by a precipitation reaction between calcium chloride and sodium carbonate under vigorous stirring as reported previously.\textsuperscript{18} The average diameter of the CaCO$_3$ particles was 3 µm (as measured by optical microscopy and image analysis software). CaCO$_3$ particles, containing FITC-dextran (Mw=2000 kDa), were obtained via co-precipitation (step A in Scheme 1) and washing away the unincorporated FITC-dextran.\textsuperscript{20} The thus obtained FITC-dextran loaded CaCO$_3$ particles were coated by sequential incubation of the particles in the polyanion and polycation solutions (1 mg/ml; starting with the polyanion) containing 0.5 M of NaCl. After each adsorption step, two washing steps were performed in order to remove the non-adsorbed polyelectrolytes. In total four polyelectrolyte bilayers were deposited (step B in Scheme 1). We preferred to have a polycation as outermost layer as a positive surface charge should enhance the cellular uptake of the capsules considering that most cell types exhibit a negative surface charge. The multilayer build-up was followed by measuring the $\zeta$-potential of the particles after each adsorption step. The $\zeta$-potential of the DEXS/ARG-, PSS/(HPMA-DMAE)- and PSS/PAH
microcapsules changed regularly between -40 and +30 mV upon adsorption of respectively a polyanion and a polycation (data not shown). The CaCO₃ templates were dissolved by adding a 0.2 M EDTA solution (buffered at pH 7.5) to the coated CaCO₃ particles (step C in Scheme 1) leading to the complexation of the Ca²⁺ ions which easily diffuse through the polyelectrolyte coating. Figure 1 shows FITC-dextran filled P(HPMA-DMAE)/PSS polyelectrolyte capsules obtained in this way. SEM images of (PSS/PAH)₄ and (DEXS/PAH)₄ polyelectrolyte capsules are shown in Figure 2. Upon drying of the sample collapsed capsules can be observed. Apparently the (PSS/PAH)₄ capsules exhibit a higher thickness compared to the (DEXS/PAH)₄ capsules which exhibit a flatter morphology.

First we studied whether the DEXS/PARG microcapsules degrade when incubated at 37 °C in a Tris buffer (pH 7.4) containing 1 mg/ml pronase (which is a mixture of proteases). Optical microscopy revealed that after 2 hours of incubation all the microcapsules were disintegrated (data not shown), remnants of capsules could even not be found, indicating that the DEXS/PARG microcapsules are susceptible to degradation by proteases. Consequently we studied whether the PSS/P(HPMA-DMAE) microcapsules degrade when incubated in a phosphate buffer (pH 7.4) at 37 °C (without pronase). After approximately 48 h all the microcapsules had disappeared (data not shown), indicating the disassembling of the multilayers due to the hydrolysis of the cationic side-groups on P(HPMA-DMAE). The degradation period of 48 h is consistent with the report of Funhoff et al. who studied the degradation kinetics of HPMA-DMAE solutions. As expected, the PSS/PAH coated
(control) microcapsules did not degrade nor in the pronase solution neither in the phosphate buffer, even after being incubated for several days.

In a next step the DEXS/PARG-, PSS/P(HPMA-DMAE)- and PSS/PAH-microcapsules, loaded with FITC-dextran, were brought into contact with African green monkey kidney cells (VERO-1 cells). Initially, the microcapsules adhered on the surface of the cells and during the first hour of incubation no uptake of capsules by the cells could be observed (data not shown). However, 20 h later microcapsules were clearly present in most

Figure 4. Transmission (A1,B1,C1) and confocal (green fluorescence (A2,B2,C2) and fluorescence overlay (A3,B3,C3)) images of VERO-1 cells 60h after incubation with FITC-dextran filled (A) PSS/PAH, (B) PSS/P(HPMA-DMAE) and (C) DEXS/PARG microcapsules. LysoTracker® Red was used to stain the lysosomes. The red arrows in A1 stress the presence of intact capsules.
of the cells, as illustrated for DEXS/PARG microcapsules in Figure 3. At this time point the capsules seemed to be intact. Figure 4 (A1, A2 and A3) shows that after 60 h of incubation the (control) PSS/PAH microcapsules were still present in the cells; they remained clearly filled with FITC-dextran and were thus still intact. However, DEXS/PARG and PSS/pHPMA-DMAE microcapsules could no longer be detected in the cells by transmission imaging (Figure 4 B1 and C1). Also, intact FITC-dextran filled capsules could not be detected anymore in the fluorescence images (Figure 4 B2, B3, C2 and C3) all indicating that the DEXS/PARG and PSS/pHPMA-DMAE capsules degraded intracellularly. As Figure 4 B2, B3,

![Figure 5](image)

**Figure 5.** Transmission and confocal images of VERO-1 cells 20h after incubation with FITC-dextran filled DEXS/PARG capsules. A and B are images of VERO-1 cells which have been incubated with microcapsules plus chlorpromazine; C and D are images of VERO-1 cells which have been incubated with microcapsules plus filipin III. The red fluorescence is from LysoTracker® Red.
C2 and C3 show, after intracellular degradation of the capsules green fluorescent spots (much smaller than the original intact capsules) could be observed which are most likely endosomal/lysosomal vesicles containing FITC-dextran. Note that FITC-dextran was not observed in the nucleus, as expected since it is, highly likely, too large to cross the nuclear pores.  

To determine in more detail the mechanism by which the microcapsules were taken up by the cells we exposed the microcapsules to the cells for 20 h in the presence of selective uptake inhibitors. Chlorpromazine was used to inhibit clathrin mediated uptake while filipin III and genistein were used to inhibit lipid raft mediated uptake. Before imaging, the cells were washed with fresh culture medium to remove non-adsorbed capsules. Figure 5 shows transmission and confocal images of cells which were incubated during 20 h with microcapsules in the presence of respectively chlorpromazine (Figure 5 A and B) and filipin III (Figure 5 C and D). Clearly, only the cells co-incubated with chlorpromazine were able to internalize microcapsules while the cells co-incubated with filipin III failed. Co-incubation with genistein yielded identical results as co-incubation with filipin III (data not shown). Therefore one can assume that lipid raft mediated uptake is most probably the mechanism through which the microcapsules enter the cells. Our observations are in accordance to the data reported by Hoekstra et al. who studied the cellular uptake mechanism of polystyrene beads as a function of their size and found that the cellular uptake of micron sized particles (> 1 µm) is mostly caveolae mediated (a subtype of raft lipid mediated uptake) and not clathrin mediated.

CONCLUSIONS

In conclusion, we have shown that polyelectrolyte capsules which contain an enzymatically or hydrolytically degradable polycation, after lipid raft mediated uptake, spontaneously degrade in Vero-1 cells. However stimuli responsive capsules have already been reported, the capsules presented in this chapter have the distinct advantage that no external trigger is required for their decomposition. The in vivo conditions appear to be able to induce this decomposition. These capsules may have high potential for the intracellular delivery of therapeutic nucleic acids (DNA, siRNA) and proteins.
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CHAPTER 9

STIMULI RESPONSIVE MULTILAYERED HYBRID NANOPARTICLE/ POLYELECTROLYTE MICROCAPSULES

Parts of this chapter are in press:

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ABSTRACT

Gold nanoparticles with carboxyl groups on their surface were used in combination with poly(allylamine hydrochloride) for the layer-by-layer coating of calcium carbonate microparticles, followed by the dissolution of the calcium carbonate core. Scanning electron microscopy, transmission electron microscopy and confocal microscopy were used to characterize the hybrid nanoparticle/polyelectrolyte microcapsules. As the gold nanoparticles have carboxyl groups on their surface their charge density is pH dependent, therefore the microcapsules exhibit a pH dependent swelling and can be deconstructed both at low and high pH. Covalent cross-linking between the carboxyl groups of the gold nanoparticles and the amino groups of the poly(allylamine hydrochloride) made it possible to suppress the pH-responsive behavior. The gold nanoparticles incorporated in the microcapsules’ wall rendered the microcapsules responsive to IR-light, which can be used at high intensity to destroy the microcapsules and at low intensity to perform imaging by raman microspectroscopy.
CHAPTER 9
STIMULI RESPONSIVE
MULTILAYERED HYBRID
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MICROCAPSULES

INTRODUCTION

Nanoparticles have become the subject of intensive research during the past decade. Their potential to be used in different fields such as optoelectronics, catalysis, diagnostics, drug delivery, … has stimulated scientists in different fields such as physics, chemistry, biotechnology to become involved with nanoparticle research. The incorporation of nanoparticles (like e.g. gold nanoparticles) into supramolecular structures is of great interest to a broad field as it allows adding extra functionalities to supramolecular structures. The layer-by-layer (LbL) technique is a highly versatile method to fabricate multifunctional supramolecular entities with tunable properties. The LbL technique is based on the sequential adsorption of charged species (like e.g. polyelectrolytes) onto an oppositely charged surface. By consecutive adsorption steps one is able to build-up multilayers which are stabilized through electrostatic interactions. Rather recently, so called polyelectrolyte microcapsules have been designed by LbL coating of a sacrificial template followed by the dissolution of this template. Due to the multifunctionality of the LbL technique it is possible to fabricate polyelectrolyte microcapsules with functionalized or stimuli responsive walls which could be promising for e.g. drug delivery. Polyelectrolyte microcapsules containing nanoparticles within the capsule wall have already been reported, although in all cases only a single layer of nanoparticle was applied. In one study the nanoparticles were introduced in the capsule wall after the fabrication of the microcapsules. Up to our
knowledge multilayered microcapsules consisting of alternating nanoparticle/polyelectrolyte layers have not been reported before.

In this paper we report on the fabrication of pH sensitive gold nanoparticles, 1 to 5 nm in size, and their use for the fabrication of ‘hybrid’ microcapsules from alternating gold nanoparticle/poly(allylamine hydrochloride) layers. As both the gold nanoparticles and the poly(allylamine hydrochloride) have a pH dependent charge density the obtained microcapsules are pH responsive. Further, as gold nanoparticles absorb light, irradiation of the microcapsules destroys the microcapsules and results in the release of the encapsulated material. Clearly, IR-laser sensitive microcapsules could have potential as drug delivery system, e.g. for remote release after subcutaneous injection of microcapsules.

**MATERIALS AND METHODS**

*Materials.*

Poly(allylamine hydrochloride) (PAH; Mw~70 kDa), FITC-dextran (Mw~2000 kDa), hydrogen tetrachloroaureate trihydrate (HAuCl₄⋅3H₂O), mercaptosuccinic acid (MSA) and sodium borohydride (NaBH₄) were purchased from Sigma-Aldrich-Fluka. Methanol, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), calcium chloride (CaCl₂) and sodium carbonate (Na₂CO₃) were purchased from Merck. Quartz slides were purchased from Hellma Optics. All water used in the experiments was of Milli-Q grade.

*Synthesis of gold nanoparticles (Au<sub>NP</sub>).*

425 mg (1.25 $10^{-3}$ mol) HAuCl₄⋅3H₂O was dissolved in 5 ml water. 188 mg (1.25 $10^{-3}$ mol) MSA was dissolved in 245 ml methanol. Both solutions were mixed and stirred during 30 min at room temperature. 473 mg (12.5 $10^{-3}$ mol) NaBH₄ was dissolved in 25 ml water and drop wise added to the HAuCl₄⋅3H₂O/MSA mixture under vigorous stirring. During the addition of the NaBH₄ the solutions gradually turned dark and the Au<sub>NP</sub>’s were formed as a dark precipitate. The reaction mixture was further stirred during 1 h at room temperature. Afterwards the reaction mixture was centrifuged at 4000 rpm during 5 min and the supernatant was discarded. Three washing/centrifugation steps with methanol were performed and finally the Au<sub>NP</sub>’s were dried under vacuum.
**UV-VIS monitoring of the multilayer build-up.**

Quartz slides were cleaned and hydrophylized by treatment with freshly prepared piranha solution ($H_2O_2$ (35 %)/$H_2SO_4$ (98 %) 1:1 v/v (caution: piranha solution reacts violently with organic materials and should not be stored in closed containers) then abundantly rinsed with distilled water. The treated quartz slides were precoated with a PEI layer by immersion in a 2 mg/ml PEI solution containing 0.5 M NaCl. Multilayers of $Au_{NP}$/PAH were deposited on the PEI coated quartz slides starting with the negatively charged $Au_{NP}$’s by immersion in a 1 mg/ml $Au_{NP}$ solution (containing 0.5 M NaCl) during 10 min followed by rinsing with pure water. Finally the quartz slides were dried under a nitrogen stream. Then the PAH layer was deposited from a 2 mg/ml PAH solution (containing 0.5 M NaCl). This was repeated until the desired amount of bilayers was deposited. After each deposition of a bilayer the absorbance of the film was measured with a Pharmacia Biochrom 4060 UV-VIS spectrophotometer.

**Fabrication of calcium carbonate ($CaCO_3$) microparticles.**

$CaCO_3$ particles were fabricated according to Volodkin et al. Briefly, $CaCl_2$ and $Na_2CO_3$ solutions (0.33 M) were mixed under vigorous stirring for 30 s leading to the precipitation of $CaCO_3$ particles. Subsequently four centrifugation and washing steps with pure water were performed in order to remove the unreacted species. In a last step the particles were washed with acetone and subsequently air-dried. FITC-dextrans were incorporated in the $CaCO_3$ particles by co-precipitation. Therefore, 5 mg of FITC-dextran was dissolved in the $CaCl_2$ solution (3.5 ml) before mixing with the $Na_2CO_3$ solution.

**Fabrication of multilayered microcapsules.**

Capsules were fabricated in a two step procedure. In a first step the $CaCO_3$ microparticles were coated using the layer-by-layer (LbL) technique. Therefore, 20 mg of FITC-dextran containing $CaCO_3$ microparticles were dispersed in a 0.5 M NaCl solution containing the $Au_{NP}$’s (1 mg/ml). The dispersion was continuously shaken during 10 min. The excess of $Au_{NP}$’s was removed by three centrifugation/washing steps with deionised water. Thereafter, 1 ml of a 0.5 M NaCl solution containing PAH (2 mg/ml) was added and the dispersion was continuously shaken during 10 min followed again by three centrifugation/washing steps. This procedure was repeated four times until four $Au_{NP}$/PAH bilayers were deposited on the surface of the $CaCO_3$ microparticles. In a second step the $CaCO_3$ core was removed by complexation with EDTA. Therefore the coated $CaCO_3$ particles
were shaken during 30 min with 1 ml EDTA-solution (0.2 M, pH 7.5) followed by centrifugation and redispersion in 1 ml fresh EDTA-solution. This procedure was repeated 4 times to assure complete removal of the CaCO$_3$ core as previously reported by Volodkin et al. $^{17, 18}$ Finally the thus obtained hollow microcapsules filled with FITC-dextran were washed four times with water.

**Electrophoretic mobility.**

The electrophoretic mobility of the Au$_{NP}$'s was measured using a Malvern Zetasizer 2000 (Malvern Instruments). The $\zeta$-potential was calculated from the electrophoretic mobility ($\mu$) using the Smoluchowski relation: $\zeta = \mu \eta / \varepsilon$ where $\eta$ and $\varepsilon$ are the viscosity and permittivity of the solvent, respectively.

**Scanning electron microscopy (SEM).**

A drop of Au$_{NP}$ or capsule suspension was deposited onto a silicon wafer and dried under a nitrogen stream followed by sputtering with gold. SEM images were recorded with a Quanta 200 FEG FEI scanning electron microscope operated at an acceleration voltage of 5 kV.

**Transmission electron microscopy (TEM).**

A drop of Au$_{NP}$ or capsule suspension was deposited and dried onto a Cu-grid modified with amorphous carbon. TEM images were recorded with a CM-200 FEG Philips transmission electron microscope operated at an acceleration voltage of 120 kV.

**Confocal Microscopy.**

Confocal microscopy and transmission light microscopy images were recorded with a Biorad MRC 1024 confocal system. An inverted microscope (Eclipse TE300D, Nikon) equipped with a 60x water immersion objective lens was used.

**Confocal Raman Microscopy.**

A RamanRxn1 spectrometer (Kaiser Optical Systems), equipped with an air cooled CCD Detector (back-illuminated deep depletion design) was used in combination with a
Leica DMLP Raman microprobe equipped with a 100x objective. The laser wavelength during the experiments was the 785 nm line from a 785 nm Invictus NIR diode laser. All spectra were recorded at a laser power of 400 mW.

**IR-laser irradiation of microcapsules**

A drop of microcapsule suspension was deposited onto a microscope slide and positioned under a 100x microscope objective. The collimated IR laser beam of a CW laser diode at 830 nm, with optical power up to 80 mW, was focused on the sample through the objective. A more detailed description of the set-up can be found elsewhere. Visualisation of the microcapsules was performed by a 150 W white light source and a fluorescence lamp. Images were recorded by a charge coupled device (CCD) camera connected to a computer.

**RESULTS AND DISCUSSION**

Gold nanoparticles were synthesized, following the method reported by Kimura et al., by reduction of hydrogen tetrachloroaureate (HAuCl₄·3H₂O) with sodium borohydride (NaBH₄) in methanol. Mercaptosuccinic acid (MSA) was used to cap the formed gold nanoparticles with a monolayer of carboxyl groups. The ζ-potential of the AuNP’s equaled 32 ± 8 mV, indicating that the carboxyl groups of the MSA indeed provide the AuNP’s with a negative surface charge. Figure 1 represents schematically the structure of the obtained AuNP’s in their ionized form. Transmission electron microscopy (TEM) (Figure 2A)
revealed that the AuNP’s are in a non-aggregated state and have a size distribution ranging from approximately 1 to 5 nm. The UV-VIS spectrum of the AuNP’s in concentrated dispersion is shown in Figure 1B, exhibiting the characteristic surface plasmon resonance absorption peak around 560 nm. 22

![Figure 2](image)

**Figure 2.** Schematic representation of the molecular structure of the AuNP’s and molecular structure of PAH.

As the AuNP’s exhibit a negative surface charge they should be suitable to build multilayered films, using the LbL technique, in combination with poly(allylamine hydrochloride) (Figure 1B), which is a positively charged polyelectrolyte (at neutral pH). As AuNP’s absorb, UV-VIS spectrophotometry can be used to monitor the build-up of AuNP/PAH multilayers onto a quartz slide. As shown in Figure 3A, the absorbance of the film increases after every deposited AuNP/PAH bilayer, indicating that multilayer build-up takes place. The absorbance spectrum of the deposited film does not show the characteristic plasmon peak at 560 nm due to the fact that the optical absorbance of extremely thin films is hardly detectable with conventional UV-VIS measurements. 22 Figure 3B shows that the absorbance at 450 nm of the films increases linearly as a function of the number of AuNP/PAH bilayers.

![Figure 3](image)

**Figure 3.** (A) UV-VIS spectra of a quartz slide coated with an increasing number of AuNP/PAH bilayers. (B) Absorbance at 450 nm as a function of the number of AuNP/PAH bilayers.
CHAPTER 9 – STIMULI RESPONSIVE MULTILAYERED HYBRID NANOPARTICLE/ POLYELECTROLYTE MICROCAPSULES

Figure 4. SEM images of (A) CaCO$_3$ microparticles containing FITC-dextrans before LbL-coating and (B) after coating with 4 bilayers Au$_{NP}$/PAH. (C) SEM image of hollow (Au$_{NP}$/PAH)$_4$ microcapsules obtained after dissolution of the CaCO$_3$ core. (D) Confocal microscopy image of FITC-dextran filled (Au$_{NP}$/PAH)$_4$ microcapsules.

Subsequently Au$_{NP}$/PAH microcapsules were fabricated using CaCO$_3$ microparticles as sacrificial templates. 17, 18, 23 FITC-dextran filled CaCO$_3$ particles were synthesized by reacting calcium chloride and sodium carbonate solutions in the presence of FITC-dextran (2000 kDa). 23 Immediately after mixing of the components the precipitation of 4 µm sized CaCO$_3$ microparticles, filled with FITC-dextrans in their pores, took place. The FITC-dextran/CaCO$_3$ microparticles were subsequently coated with Au$_{NP}$’s and PAH. In total 4 Au$_{NP}$/PAH bilayers were deposited onto the surface of the CaCO$_3$ microparticles. After the deposition of the (Au$_{NP}$/PAH)$_4$ coating, the CaCO$_3$ microparticles were dissolved in EDTA solutions. After several washing steps with water, removing the dissolved ions, hollow microcapsules filled with a FITC-dextran solution were obtained. Figure 4 shows SEM images of FITC-dextran filled CaCO$_3$ microparticles (A) before and (B) after coating with 4
AuNP/PAH bilayers. Clearly, the uncoated CaCO$_3$ microparticles exhibit a smooth surface, whereas after coating the CaCO$_3$ microparticles exhibit a rather rough surface. Figure 4C shows SEM images of the microcapsules obtained after dissolution of the CaCO$_3$ core. Collapsed microcapsules, with a rough surface are observed. The collapsed state, which is due to the drying upon preparation of the sample prior to SEM imaging, indicates that hollow microcapsules were present before drying. Figure 4D shows a confocal microscopy image of the microcapsules depicting homogeneous filling of the microcapsules with FITC-dextran.

Figure 5 shows TEM images of a (AuNP/PAH)$_4$ capsule at low (Figure 5A) and high (Figure 5B) magnification. A very dense network of AuNP’s which are ‘glued’ together by electrostatic interactions with the PAH can be observed. In Figure 5B one can clearly distinguish the individual AuNP’s which are closely packed to each other. The TEM images of the (AuNP/PAH)$_4$ microcapsules are substantially different from those reported by Skirtach et al. $^{15}$ where only one layer of AuNP’s was intercalated within the capsule wall. TEM images from such microcapsules show AuNP’s which are sparsely spread within the capsule wall, whereas the AuNP’s in the (AuNP/PAH)$_4$ microcapsules reported in this paper form a tight network.

PAH is a weak polyelectrolyte, meaning that its charge density is dependent on the pH of the environment. $^{24}$ Whereas the amino groups of the PAH are protonated, and thus positively charged, at a pH below 10.7 (the pK$_a$ of PAH when it is complexed to a polyanion was estimated to be 10.7 by Petrov et al.), $^{23}$ they become uncharged when the pH is above 10.7. Several papers have been published describing the swelling and dissolution behavior.
of polyelectrolyte multilayers made from weak polyelectrolytes. As the (AuNP/PAH)$_4$ microcapsules were fabricated by electrostatic build-up between a weak polyacid, as the AuNP’s have carboxyl acid groups on their surface, and a weak polybase (i.e. PAH), we were interested to know how the (AuNP/PAH)$_4$ microcapsules behave both at low and high pH. Figure 6 shows transmission light microscopy images of the (AuNP/PAH)$_4$ microcapsules before and after addition of respectively 0.5 M HCl (Figure 6A) and 0.5 M NaOH (Figure 6B). The microcapsules swell approximately twice in diameter and then gradually dissolve. Clearly, at high pH the PAH amino groups become uncharged and no longer complex with the carboxyl groups of the AuNP, leading to a loosening of the electrostatic interactions within the multilayers. As the negatively charged carboxyl groups are no longer neutralized by the amino groups repulsion between the carboxyl groups occurs, the microcapsules start to swell, and finally dissolve. At low pH (Figure 6B) the inverse phenomenon occurs as the carboxyl groups of the AuNP’s become uncharged and no longer repel the positively charged amino groups of the PAH, again leading to a swelling followed by the dissolution of the microcapsules.

Figure 6. Transmission light microscopy images of (AuNP/PAH)$_4$ microcapsules suspended in pure water (A1 & B1), immediately after addition of 0.5 M NaOH (A2) respectively 0.5 M HCl (B2) and 1 min after the addition of 0.5 M NaOH (A3) respectively 0.5 M HCl (B3).
We were further interested to examine whether it was possible to covalently cross-link the multilayer membrane in order to make microcapsules which remain to exist in a broad pH range. Following the work of Mauser et al. we tried to introduce amide bonds between the carboxyl groups on the AuNP’s and the amino groups on the PAH using carbodiimide chemistry. When such cross-linked (FITC-dextran filled) (AuNP/PAH)$_4$ microcapsules were dispersed in respectively 0.5 M HCl and 0.5 M NaOH they did not swell and remained intact (data not shown). Moreover, the core of the microcapsules remained fluorescent (both in acid and alkaline media) indicating that the cross-linking also prevented the escape of the encapsulated FITC-dextrans. Apparently, the cross-linked (AuNP/PAH)$_4$ microcapsules were able to encapsulate the FITC-dextrans even when the electrostatic interactions were no longer contributing to the integrity of the microcapsules.

![Figure 7](image.jpg)

**Figure 7.** Fluorescence microscopy images of a cluster of FITC-dextran filled (AuNP/PAH)$_4$ microcapsules upon irradiation with IR-laser light. The time interval between the successive images is 2 s.

It has been reported that polyelectrolyte microcapsules containing gold nanoparticles in their shell can be destroyed when irradiated with an IR-laser. The AuNP’s absorb IR-light which locally heats the nanoparticles. As IR-light is not harmful to living tissue, IR-light could be an attractive method to open microcapsules which are injected subcutaneously. The IR-responsive properties of the (AuNP/PAH)$_4$ microcapsules were assessed by irradiating them with a focused laser beam of 30 mW. Figure 7 shows the impact of the irradiation on a cluster of (AuNP/PAH)$_4$ microcapsules. Clearly, shortly after the exposure the microcapsules explode and only debris of broken microcapsules remains. Also the green fluorescence of the encapsulated FITC-dextrans has disappeared. The advantages of the (AuNP/PAH)$_4$ microcapsules described in this paper, which are fabricated from CaCO$_3$ microparticles as sacrificial template, compared to previously reported IR-sensitive microcapsules, which were made using melamine formaldehyde (MF) or polystyrene (PS) microparticles as sacrificial template, are threefold. (1) Whereas the dissolution of MF and PS microparticles occurs under harsh conditions (such as extreme low pH and the use of organic solvents) CaCO$_3$ templates can be easily dissolved at
physiological pH using EDTA. \(^{18}\) (2) When MF or PS microparticles are used, MF or PS oligomers/polymers main remain inside the capsule wall, \(^{29, 30}\) making the microcapsules unsuited as drug delivery system. In contrast, the Ca\(^{2+}\) and CO\(_3^{3-}\) ions can easily permeate through the multilayer wall, moreover they are non-toxic. (3) Polyelectrolyte microcapsules prepared from MF or PS templates need to be post-loaded after the dissolution of the sacrificial template. This requires that the microcapsules' wall can be temporarily reversibly opened, allowing filling of the microcapsules. \(^{31, 32}\) CaCO\(_3\) microparticles can easily be filled with macromolecules by coprecipitation of the macromolecules during the fabrication of the CaCO\(_3\). \(^{19}\) After LbL coating and dissolution of the CaCO\(_3\) one obtains already filled microcapsules.

We wondered whether the (Au\(_{np}/PAH\))\(_4\) microcapsules could be detected by raman microspectroscopy. Raman spectroscopy is based on the shift in wave number of monochromatic IR-light upon inelastic scattering by a substance. \(^{33}\) Raman microspectroscopy allows one to collect data from a well defined microscopic area. The (Au\(_{np}/PAH\))\(_4\) microcapsules were dried onto a quartz slide and put under the confocal raman microscope. Figure 8A shows a raman mapping image of a (Au\(_{np}/PAH\))\(_4\) capsule while

![Figure 8](image)

**Figure 8.** (A) Confocal raman microscopy image of a (Au\(_{np}/PAH\))\(_4\) capsule, (B) the interpolated image and (C) raman spectra taken inside a capsule (gray line) and outside a microcapsules (red line).
Figure 8B shows the interpolated image. The raman spectra inside and outside the capsule are shown in Figure 8C. The raman spectrum in Figure 8C was found to be similar to the one observed in an AuNP’s suspension (in water), proving that the measured raman shifts are due to the AuNP’s in the multilayer of the microcapsules. Therefore, the response of the (AuNP/PAH)$_4$ microcapsules to IR-light is interesting for both release purposes (upon destruction of the microcapsules by highly intense IR light) as well as applications in the diagnostic field.

**CONCLUSIONS**

We reported on the fabrication of hybrid nanoparticle/polyelectrolyte microcapsules by multilayer formation of carboxylic acid functionalized gold nanoparticles and poly(allylamine hydrochloride). Confocal microscopy showed a homogenous filling of the microcapsules with FITC-dextran while electron microscopy clearly revealed the presence of the AuNP’s within the capsule wall. As the gold nanoparticles and the poly(allylamine hydrochloride) contain respectively weak carboxyl and weak amino groups it was possible to deconstruct the microcapsules at both low and high pH. Further it was shown that covalent cross-linking of the capsule wall was an effective method to render the microcapsules invariant to changes in pH. The gold nanoparticles incorporated in the microcapsules’ wall rendered the microcapsules responsive to IR-light, which can be used at high intensity to destroy the microcapsules and at low intensity to perform imaging. The use of CaCO$_3$ as sacrificial template for capsule fabrication makes the microcapsules ideally suited for applications in the drug delivery field. In our ongoing research the microcapsules are currently evaluated for IR induced transdermal drug release.

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CHAPTER 10

ULTRASOUND TRIGGERED
RELEASE FROM
MULTILAYERED MICROCAPSULES

Parts of this chapter are in press:

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ABSTRACT

Multilayered microcapsules fabricated using the Layer-by-Layer (LbL) technology are promising vehicles for the delivery of drug molecules. In this chapter we report on the use of ultrasound for release of encapsulated species from such multilayered microcapsules. It was found that ultrasound has a dramatic effect on the integrity of the capsule membrane leading to destruction of the entire capsule and release of encapsulated material.
CHAPTER 10

ULTRASOUND TRIGGERED
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INTRODUCTION

The growing needs for drug delivery systems releasing their content in a desired fashion have intensified research for 'smart carriers' with unique properties. Among other pharmaceutical carriers, such as polymeric micelles, liposomes, and small colloidal particles, polymeric polyelectrolyte multilayered microcapsules are novel materials holding promise due to their loading capacity and the possibility to precisely tailor their properties by choosing the microcapsules' components. Recently these microcapsules have gathered attention as drug delivery system. Once their target site is reached it is of utmost importance to have a mechanism causing release of encapsulated species from these microcapsules. Externally triggered release has recently been shown to be possible by laser light illumination. The principle is based on heating of metal nanoparticles causing changes in permeability of the outer shell and even total disruption of the shell, finally resulting in the release of encapsulated materials. These laser light sensitive microcapsules could e.g. be activated after cellular uptake or could be activated after subcutaneous injection.

In this chapter we report on the use of ultrasound to trigger release from polyelectrolyte microcapsules. Ultrasound has widely been investigated for biomedical applications, improving drug uptake, anti-inflammatory treatment and imaging. Upon propagation, an ultrasound wave undergoes both viscous and thermal absorption as well as scattering. At low frequency the temperature difference between the particle and the medium will be in equilibrium, whereas at high frequency only a small portion of the surface will be affected by thermal wave. Similar frequency dependence is applicable to viscous
losses, wherein extensive particle motion occurs at low frequency while little movement takes place at high frequencies.

Figure 1 shows schematically which effect ultrasound may have on a polyelectrolyte capsule. When the microcapsules are subjected to ultrasound a morphological change of the capsule wall may occur due to the creation of shear forces between the successive fluid layers, resulting in the disruption of the capsule membrane and release of encapsulated species.

**MATERIALS AND METHODS**

**Materials.**

Poly(allylamine hydrochloride) (PAH; Mw~70 kDa), sodium poly(styrene sulfonate) (PSS, Mw~70 kDa) and FITC-dextran (Mw~2000 kDa) were purchased from Sigma-Aldrich-Fluka. Sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), calcium chloride (CaCl₂) and sodium carbonate (Na₂CO₃) were purchased from Merck. Gold nanoparticles (AuNP) were synthesized according to Kimura et al. All water used in the experiments was of Milli-Q grade.

**Fabrication of calcium carbonate (CaCO₃) microparticles.**

CaCO₃ particles were fabricated according to Volodkin et al. Briefly, CaCl₂ and Na₂CO₃ solutions (0.33 M) were mixed under vigorous stirring for 30 s leading to the precipitation of CaCO₃ particles. Subsequently four centrifugation and washing steps with
pure water were performed in order to remove the unreacted species. In a last step the particles were washed with acetone and subsequently air-dried. FITC-dextrans were incorporated in the CaCO$_3$ particles by co-precipitation. Therefore, 5 mg of FITC-dextran was dissolved in the CaCl$_2$ solution (3.5 ml) before mixing with the Na$_2$CO$_3$ solution.

**Fabrication of multilayered microcapsules.**

Capsules were fabricated in a two step procedure. In a first step the CaCO$_3$ microparticles were coated using the layer-by-layer (LbL) technique. Therefore, 20 mg of FITC-dextran containing CaCO$_3$ microparticles were dispersed in a 0.5 M NaCl solution containing PSS (2 mg/ml) or Au NP’s (1 mg/ml). The dispersion was continuously shaken during 10 min. The excess PSS or Au NP’s was removed by three centrifugation/washing steps with deionised water. Thereafter, 1 ml of a 0.5 M NaCl solution containing PAH (2 mg/ml) was added and the dispersion was continuously shaken during 10 min followed again by three centrifugation/washing steps. This procedure was repeated four times until four bilayers were deposited on the surface of the CaCO$_3$ microparticles. In a second step the CaCO$_3$ core was removed by complexation with EDTA. Therefore the coated CaCO$_3$ particles were shaken during 30 min with 1 ml EDTA-solution (0.2 M, pH 5) followed by centrifugation and redispersion in 1 ml fresh EDTA-solution. This procedure was repeated 4 times to assure complete removal of the CaCO$_3$ core as previously reported by Volodkin *et al.*

Finally the thus obtained hollow microcapsules filled with FITC-dextran were washed four times with water.

**Ultrasonic treatment.**

The experiments were performed with a Branson Sonifier 250 equipped with a titanium microtip, operating at a constant output frequency of 20 kHz. The microtip was positioned in 250 µl of microcapsule suspension in a 500 µl Eppendorf microtube. Subsequently ultrasound was applied at different output power and for different times.

**Scanning electron microscopy (SEM).**

A drop of particle or microcapsule suspension was deposited onto a silicon wafer and dried under a nitrogen stream followed by sputtering with gold. SEM images were recorded with a Quanta 200 FEG FEI scanning electron microscope operated at an acceleration voltage of 5 kV.
Confocal Microscopy.

Confocal microscopy and transmission light microscopy images were recorded with a Biorad MRC 1024 confocal system. An inverted microscope (Eclipse TE300D, Nikon) equipped with a 60x water immersion objective lens was used.

Atomic Force Microscopy (AFM).

AFM images were recorded on a Nanoscope IIIa Multimode SFM (Digital Instruments Inc.) in air at room temperature using taping mode. Samples were prepared by applying a drop of microcapsule suspension on a freshly cleaved mica substrate followed by drying under a gentle stream of nitrogen.

RESULTS AND DISCUSSION

Multilayered microcapsules were fabricated using the LbL technique by successive coating of CaCO₃ microparticles with different layers of polyelectrolytes and gold nanoparticles. During fabrication, the CaCO₃ microparticles were filled with 2000 kDa FITC dextran by co-precipitation of the CaCO₃ with the FITC-dextran. This is an elegant method, allowing a high degree of loading and avoiding a post-filling step of the microcapsules. Two different types of microcapsules were fabricated; type 1 consisted solely of polyelectrolytes while type 2 were 'hybrid' microcapsules consisting of polyelectrolytes and gold nanoparticles (AuNP). Sodium poly(styrene sulfonate) (PSS) was used as polyanion while poly(allylamine hydrochloride) (PAH) was used as polycation. AuNP’s were fabricated according to the method reported by Kimura et al., resulting in AuNP’s with a diameter ranging from 1 to 5 nm (as verified by transmission electron microscopy (data not shown)) and a negative surface charge (as verified by measuring the electrophoretic mobility) due to the presence of carboxyl groups on the surface of the AuNP’s. Multilayer build-up between the PSS/PAH (in case of type 1 microcapsules) and AuNP/PAH (in case of type 2 microcapsules) was driven by the electrostatic interactions between the cationic amino groups of the PAH and the anionic sulfonate, respectively carboxyl groups of the PSS, respectively AuNP’s. For each type of microcapsules in total 4 bilayers were deposited onto the surface of the CaCO₃ microparticles followed by the dissolution of the CaCO₃ by EDTA treatment. EDTA
complexes the calcium and permeates easily through the multilayer membrane, resulting in hollow microcapsules with an average diameter of 4 µm. The FITC-dextrans, initially entrapped inside the pores of the CaCO₃ microparticles, are too large to permeate through the multilayered membrane and stay encapsulated inside the hollow microcapsules. Figure 2 shows optical transmission, confocal and scanning electron microscopy images of the (PSS/PAH)₄ microcapsules while Figure 3 shows the corresponding microscopy images of the (Au NP/PAH)₄ microcapsules. On the transmission image both types of microcapsules appear transparent. However, whereas the (PSS/PAH)₄ microcapsules are hardly visible on the transmission image, the (Au NP/PAH)₄ microcapsules appear significantly darker. These observations are consistent with the appearance of the microcapsules in suspension, being a slightly turbid but clear suspension in case of (PSS/PAH)₄
Chapter 10 – Ultrasound Triggered Release from Multilayered Microcapsules

Microcapsules and a dark brown suspension in case of \((\text{AuNP/PAH})_4\) microcapsules. The dark appearance of the \((\text{AuNP/PAH})_4\) microcapsules is due to the absorption of light by the Au NP’s. On the confocal images in Figures 2B and 3B a homogeneous filling of the microcapsules with green fluorescence is observed, indicating no preferential accumulation of fluorescence in particular parts of the microcapsules. The scanning electron microscopy images in Figures 2C&D and 3C&D show in detail the morphology of the microcapsules. Apparently the \((\text{PSS/PAH})_4\) microcapsules have a much smoother morphology compared to the \((\text{AuNP/PAH})_4\) microcapsules. This is most likely due to the presence of the AuNP’s within the multilayer membrane, offering it a more rigid structure which appears rather rough upon drying.

Figure 3. Optical transmission (A), confocal (B) and scanning electron (C & D – at different magnifications) microscopy images of \((\text{AuNP/PAH})_4\) microcapsules. The green colour in image B is due to the FITC-dextran encapsulated inside the microcapsules.
Chapter 10 – Ultrasound Triggered Release from Multilayered Microcapsules

To evaluate the effect of ultrasound on the integrity of the microcapsules’ wall, the microcapsules, in suspension, were subjected to ultrasonic treatment with an ultrasonic probe operating at a frequency of 20 kHz and a power output of respectively 20, 40 and 100 W during respectively 1, 5 and 10 seconds. Initially both the (PSS/PAH)$_4$ as well as the (Au$_{30}$/PAH)$_4$ microcapsules are nearly monodisperse spherically shaped and homogenously filled with FITC-dextrans, as shown in Figures 2A&B and 3A&B. However, after 1 s sonication at 20 W a large amount of microcapsules are broken and much debris of broken microcapsules can be detected. When the microcapsules are subjected to ultrasound at an output power of 100 W almost no intact microcapsules can be observed and nothing is left but unidentifiably debris of broken microcapsules. The ultrasonic shockwaves originating

**Figure 4.** Optical transmission (A), confocal (B) and scanning electron (C & D – at different magnifications) microscopy images of (PSS/PAH)$_4$ microcapsules after ultrasonic treatment (10 s – 20 W). The green colour in image B is due to residual FITC-dextrans sticking on the wall of broken microcapsules.
from the ultrasound probe propagate through the liquid and probably cause high shear forces between the successive liquid layers. When such shear forces cleave through the membrane of the microcapsules, the membrane is torn apart and the microcapsules are destroyed.

Figures 4 and 5 show optical transmission, confocal and scanning microscopy images of the microcapsules after ultrasonic treatment. The microcapsules are clearly destroyed and empty, the only fluorescence being detected is on the walls of broken microcapsules due to electrostatic or hydrophobic interactions between the fluorescent dye and the remaining polyelectrolytes. Whereas before ultrasonic treatment the microcapsules had a round shape on the electron microscopy images, the microcapsules are extremely deformed after ultrasonic treatment and individual microcapsules can hardly be detected. Figures 4D and 5D
show scanning electron microscopy images at higher magnification of most likely a destroyed capsule. From the irregularities of the surface one can clearly see the impact of the ultrasound on the integrity of the capsule wall as large holes and cracks are present. Figure 6A1 shows an AFM image of a (AuNP/PAH)$_4$ capsule before ultrasonic treatment. The capsule exhibits a round shape and a double wall thickness of 447 nm as observed from the height profile (Figure 6A2). When an AFM image is recorded of the debris after ultrasonic treatment (Figure 6B1) a height of 261 nm (Figure 6B2) is measured. Most likely this corresponds to the thickness of a piece of a single capsule wall which was torn of the capsule due to the sonication.

![AFM images](image)

**Figure 6.** AFM images of a (AuNP/PAH)$_4$ microcapsules before (A) and after (B) ultrasonic treatment. A2 and B2 show the corresponding height profiles along the green line indicated on, A1 and B1. Images A3 and B3 show the corresponding 3D images.
To quantify the effect of ultrasonic treatment on the microcapsules’ integrity the amount of intact and destroyed microcapsules after ultrasonic treatment was determined for the different parameters, being treatment time and power output. Logically, before ultrasonic treatment, no broken microcapsules are observed. After 1 s ultrasonic treatment already a considerable amount of microcapsules are broken and increasing the treatment time results in an increasing amount of broken microcapsules. Also an increase in power input results in an increase of the amount of broken microcapsules. These trends are graphically presented in Figure 7. Remarkably also an influence of the presence of gold nanoparticles can be observed. In case ultrasonic treatment is performed during 10 s, virtually all microcapsules

**Figure 7.** Percentage of broken (A) (PSS/PAH)$_4$ and (B) (Au$_{25}$/PAH)$_4$ microcapsules after ultrasound irradiation as function of irradiation time and output power. For each bar 50 microcapsules were counted.
are destroyed, irrespective of the presence of nanoparticles in the microcapsules’ shell. However, for shorter treatment times, the microcapsules appear mechanically more stable when nanoparticles are included in the shell. When comparing Figure 7A and 7B one observes that after 1 and 5 s of ultrasonic treatment almost the double amount of \((\text{PSS/PAH})_4\) microcapsules are broken compared to the amount of broken \((\text{AuNP/PAH})_4\) microcapsules. The high density of carboxyl groups on the surface of the AuNP’s offers good binding sites with the PAH, making the multilayer structure more rigid than in case of PSS/PAH multilayers. Therefore such hybrid multilayers, consisting of nanoparticles and polyelectrolytes, are most likely more stable towards ultrasound than multilayers consisting solely of polyelectrolytes.

**CONCLUSIONS**

In conclusion, remote release of encapsulated materials from multilayered microcapsules has been performed by ultrasound irradiation. It was shown that ultrasonic irradiation has a dramatic effect on the integrity of multilayered microcapsules leading to their destruction and the release of encapsulated material. Ultrasound offers an easy and fast way of inducing release from multilayered microcapsules and may be of interest to the biomedical field, e.g. for topological application of ultrasound after subcutaneous injection of microcapsules.

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SUMMARY AND GENERAL CONCLUSIONS
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Novel biotechnological macromolecular drugs, such as certain peptides and proteins, require special formulations preventing them from degradation before reaching their target. Moreover, besides protection against enzymatic degradation, also the release pattern plays an important role. For example, in some cases, like the administration of antigens, it could be of interest to release the drug molecules in a pulsatile manner i.e. at different pre-determined time intervals. In other cases the therapeutics should only be released at a specific target site, as a response to a specific local or external trigger. As an example, an appropriate insulin dose should only be delivered to diabetic patients when the glucose concentration in the blood exceeds a critical value.

In this thesis several types of microcapsules are engineered which disassemble, and thus release their content, (1) after a certain incubation time and (2) in response to a specific physicochemical trigger. For the fabrication of these microcapsules we used the Layer-by-Layer (LbL) technique. This technique, based on the sequential adsorption of charged species onto an oppositely charged surface, is highly attractive due to its multifunctionality and ease of use.

In CHAPTER 1 the LbL technique was introduced. The most widespread techniques to characterize LbL films were described. Especially the use of the LbL technique for the fabrication of polyelectrolyte microcapsules has been reviewed in detail. We described in detail the approaches so far reported in literature to induce release from polyelectrolyte microcapsules. On top, the strengths and weaknesses of polyelectrolyte microcapsules towards drug delivery were discussed and compared with the pro’s and con’s of other drug delivery systems under investigation.

In the CHAPTERS 2-5 ‘self-exploding’ microcapsules were presented. These microcapsules can ‘explode’ without the use of an external trigger. The microcapsules consist of a biodegradable dextran-based (dex-HEMA) microgel core surrounded by a semi permeable membrane which is permeable to water but impermeable to the encapsulated drugs and the degradation products of the microgels. When the microgel core degrades, the swelling pressure increases and at a certain moment, when the swelling pressure exceeds the tensile strength of the membrane, the membrane ruptures, releasing of encapsulated material.
In **CHAPTER 2** the ‘proof of principle’ of self-explooding microcapsules, i.e. the fact that the swelling pressure of a degrading microscopic gel particle can rupture a surrounding membrane, was reported. For this purpose positively charged dex-HEMA microgels were coated with 3 bilayers of the synthetic polyelectrolytes poly(sodium styrene sulfonate) and poly(allylamine hydrochloride) (PSS/PAH). We showed that the resulting microcapsules indeed exploded when incubated (during a certain time) at pH 9. However, at physiological pH (i.e. pH 7.4) the (PSS/PAH)$_3$ membrane seemed permeable to the degradation products of the microgels, thus preventing the osmotic pressure to build-up. Consequently, the polyelectrolyte microcapsules were not self-explooding at pH 7.4. In **CHAPTER 3** we investigated the (PSS/PAH)$_3$ microcapsules physicochemically in more detail by different methods.

In **CHAPTER 4** bio-polyelectrolytes, in stead of synthetic polymers, were used for the LbL coating of the dex-HEMA microgels. The main advantage of the bio-polyelectrolytes is their biocompatibility. We showed that the behaviour of the bio-polyelectrolyte microcapsules upon degradation of the microgel core strongly depended on the choice of the bio-polyelectrolyte pair used for the LbL coating. Microcapsules consisting of 4 bilayers of poly-L-glutamic acid / poly-L-arginine exploded and released their content upon incubation under physiological conditions. Microcapsules consisting of 4 bilayers of dextran sulfate/poly-L-arginine did not explode upon incubation under physiological conditions, nor did they explode at non-physiological conditions. ‘Hollow’ microcapsules were obtained. The hollow nature of the resulting microcapsules was proven by scanning electron microscopy and atomic force microscopy.

In **CHAPTER 5** self-explooding lipid (in stead of polyelectrolytes) coated microgels were introduced. We took advantage of the unique structure of lipids, having a polar hydrophilic head group and a hydrophobic hydrocarbon tail. When dispersed in aqueous medium these lipids self-assemble spontaneously and form spherical structures, so called liposomes. Some types of lipids bear a net charge and can be attracted to an oppositely charged surface by electrostatic interactions. Once adsorbed on that surface, the liposomes spread and form a continuous bilayer. This concept was used in **CHAPTER 5** to coat the microgels with lipid bilayers. The deposition of a lipid layer on the surface of dex-HEMA microgels was proven by $\zeta$-potential measurements, confocal microscopy and scanning electron microscopy. When the lipid coated microgels were immersed in a solution at high pH, the lipid layer initially inflated and finally ruptured due to the swelling pressure of the degrading microgel. During the inflation of the lipid membrane so-called ‘parachutes’ were observed on the lipid surface, indicating that the lipid consists of multiple bilayers rather than a single bilayer. This hypothesis was confirmed by X-ray reflectivity studies.
The microgels prepared and investigated in the **CHAPTERS 2 – 5** showed a broad size distribution. According to Laplace’s law, larger microcapsules (as obtained by LbL or lipid coating of larger dex-HEMA microgels) will explode easier than smaller microcapsules as less pressure is required for the elongation of the membrane. Therefore, coating of ‘monodisperse’ microgels would be beneficial in order to narrow the timeframe during which capsule explosion occurs. In **CHAPTER 6** we described the use of a microfluidic device for the fabrication of such monodisperse microgels. By impingement of an aqueous dex-HEMA stream, by a flanking oil stream in a microfluidic nozzle, monodisperse dex-HEMA droplets (in the oil phase) were formed in a continuous way. UV irradiation of the monodisperse dex-HEMA droplets resulted in monodisperse microgels.

The **CHAPTERS 7** to **10** dealt with the fabrication of polyelectrolyte microcapsules which decompose under influence of a natural or external trigger. In **CHAPTER 7** polyelectrolyte microcapsules which dissolve in the presence of glucose were reported. Phenylboronic acid forms a covalent anionic complex with glucose at alkaline pH, leading to a decrease in the apparent pKₐ. A phenylboronic acid containing acrylate was synthesized by coupling aminophenylboronic acid to acrylic acid. This acrylate was copolymerised with dimethyl aminoethyl acrylate, yielding a copolymer containing both amino and phenylboronic acid groups. At low pH this copolymer was used as polycation for multilayer build-up in conjunction with the polyanion poly(styrene sulfonate) (PSS), as evidenced by UV-VIS spectroscopy. Hollow microcapsules were fabricated by LbL coating of polystyrene particles with 6 copolymer/PSS bilayers followed by the decomposition of the polystyrene core in tetrahydrofuran. Upon addition of glucose under buffered alkaline (i.e. pH 9) conditions, the phenylboronic acid moieties change from their uncharged form into their anionic form as their pKₐ shifts, causing repulsion with the sulfonate groups of the poly(sodium styrene sulfonate) and attraction with the amino groups of the dimethyl aminoethyl acrylate. These phenomena of intermolecular repulsion and intramolecular attraction destabilised the multilayer wall of the microcapsules, leading to their dissolution, as shown by confocal microscopy.

In **CHAPTER 8** bio-degradable polyelectrolyte microcapsules were fabricated and investigated in the context of intracellular delivery of molecules. Two types of degradable microcapsules were synthesized. The first type contained a polycation bearing hydrolysable cationic side chains. The second type was fabricated using the bio-polyelectrolytes dextran sulfate and poly-L-arginine. Calcium carbonate (CaCO₃) microparticles were used as sacrificial template for the LbL coating. After LbL coating the CaCO₃ templates were dissolved in an EDTA solution. When the thus obtained polyelectrolyte microcapsules were incubated in a cell culture, the microcapsules were taken up by the cells and degraded intracellularly. Contrary, microcapsules fabricated from the synthetic, non-degradable, polyelectrolytes PSS/PAH did not degrade but stayed intact in the cells. The bio-degradable
Polyelectrolyte microcapsules might be promising carriers for the delivery of antigens and genetic material (e.g. DNA, siRNA...) which have an intracellular target.

In Chapters 9 and 10 we reported on hybrid microcapsules fabricated by consecutive adsorption of negatively charged gold nanoparticles and the polycation PAH onto CaCO₃ microparticles as sacrificial template. Confocal microscopy and electron microscopy were used to reveal the structure of the microcapsules. As the gold nanoparticle had weak acid groups, i.e. carboxyl groups, on their surface and PAH has weak basic groups, i.e. primary amino groups, the microcapsules dissolved respectively at low and high pH as the carboxyl, respectively amino groups, were no longer charged. Covalent cross-linking of the carboxyl and amino groups stabilized the microcapsules. They remained stable in the whole pH range. Further we showed that the microcapsules were remotely activated by IR-laser irradiation by conversion of light energy into thermal energy, heating the gold nanoparticles resulting in the destruction of the microcapsules. In Chapter 10 we evaluated the effect of ultrasound on the integrity of polyelectrolyte and hybrid polyelectrolyte/nanoparticle microcapsules. The shear forces, resulting from the propagating ultrasonic shockwaves in the fluid medium of the microcapsules, completely destroyed the microcapsules. Confocal and scanning electron microscopy could only reveal debris of broken microcapsules after ultrasonic treatment. Both types of microcapsules completely released their content after exposure to ultrasound.

Polyelectrolyte microcapsules are, since their advent in 1998, of interest to both fundamental as well as application oriented researchers. The concepts developed in this thesis may be promising towards biomedical applications of polyelectrolyte microcapsules. However, polyelectrolyte microcapsules are only in their earliest stage and further research is required both to fully understand them as well as to optimise them towards real applications. Indeed, it remains a huge challenge to further develop such microcapsules and to show that, compared to other more established delivery systems such as liposomes and ordinary polymeric particles, they have important benefits.
SAMENVATTING EN ALGEMEEN BESLUIT

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Recente biotechnologische macromoleculaire geneesmiddelen, zoals bepaalde peptiden en eiwitten, vereisen een speciale formulatie om hen te beschermen tegen degradatie alvorens ze hun doel bereiken. Naast bescherming tegen enzymatische afbraak is echter ook het vrijstellingssprofiel van belang. Zo zou het voor het vrijstellen van antigenen interessant zijn een gepulseerd vrijstellingspatroon te bekomen, waarbij de antigenen vrijgesteld worden na vooraf bepaalde tijdsintervallen. In andere gevallen daarentegen moet het geneesmiddel enkel vrijgegeven worden op een specifieke plaats, volgend op een specifieke lokale of externe stimulus. Bijvoorbeeld, een geschikte dosis insuline zou enkel moeten vrijgegeven worden wanneer de glucoseconcentratie in het bloed een kritische waarde overschrijdt.

In deze doctoraatsthesis werden verschillende types microcapsules ontwikkeld die hun inhoud kunnen vrijstellen na (1) een bepaalde incubatietijd en (2) ten gevolge van een specifieke fysicochemische stimulus. Deze microcapsules werden aangemaakt via de Layer-by-Layer (LbL)-techniek. Deze techniek is gebaseerd op de alternerende adsorptie van geladen species op een tegengesteld geladen oppervlak. Grote voordelen van deze techniek zijn de multifunctionaliteit en de eenvoud van gebruik.

In HOOFDSTUK 1 werd de LbL-techniek geïntroduceerd en werden de meest aangewende karakterisatietechnieken besproken. Er werd bijzondere aandacht besteed aan het gebruik van de LbL-techniek voor de aanmaak van polyelektroliet-microcapsules. Daarnaast werden ook de benaderingen gerapporteerd in de literatuur om vrijstelling uit dergelijke microcapsules te bewerkstelligen gedetailleerd beschreven. Tevens werden de sterktes en zwaktes van polyelektroliet-microcapsules met betrekking tot geneesmiddelafgifte besproken en vergeleken met andere geneesmiddelafgiftesystemen.

In de HOOFSTUKKEN 2 – 5 werden ‘zelf-exploderende’ microcapsulesontwikkeld. Deze microcapsules kunnen ‘exploderen’ zonder dat ze een externe stimulus nodig hebben. De microcapsules zijn opgebouwd uit een biodegradeerbare, dextraan gebaseerde (dex-HEMA) microgelkern omgeven door een semi-permeabel membraan dat permeabel is voor water maar impermeabel voor het geëncapsuleerde geneesmiddel en de degradatieproducten van de microgels. Wanneer de microgelkern degradeert, neemt de zwelldruck toe en op een
bepaald ogenblik, wanneer de zweldruk de treksterkte van het membraan overschrijdt, scheurt het membraan en wordt het geëncapsuleerde materiaal vrijgesteld.

In **Hoofdstuk 2** werd de ‘proof of principle’ aangetoond, namelijk dat de zweldruk van een degraderende microgel in staat is een omgevend membraan te doen scheuren. Hiertoe werden positief geladen dex-HEMA microgels gecoat met 3 dubbellagen van de synthetische polyelektrolyten poly(styreen sulfonaat) en poly(allylamine hydrochloride) (PSS/PAH). We toonden aan de verkregen microcapsules exploederen wanneer ze gedurende een bepaalde tijd geïncubeerd werden bij pH 9. Onder fysiologische omstandigheden (pH 7.4) echter bleek het (PSS/PAH)_3-membraan permeabel voor de degradatieproducten van de microgelkern, waardoor er onvoldoende osmotische druk kon opgebouwd worden en de microcapsules niet zelf-exploderend waren bij pH 7.4. In **Hoofdstuk 3** werden de eigenschappen van de (PSS/PAH)_3 coating in detail onderzocht met behulp van verschillende fysicochemische technieken.

In **Hoofdstuk 4** werden voor de LbL-coating van dex-HEMA microgels biopolyelektrolyten gebruikt in plaats van synthetische polymeren. Het voornaamste voordeel van deze biopolyelektrolyten is dat ze biocompatibel zijn. We toonden aan dat het gedrag van de bio-polyelektrolyet microcapsules tijdens degradatie van de microgelkern sterk afhankelijk is van het gekozen bio-polyelektrolyetpaar voor LbL-coating. Microcapsules bestaande uit 4 dubbellagen poly-L-glutaminezuur/poly-L-arginine exploederen en gaven hun inhoud vrij bij incubatie onder fysiologische condities. Microcapsules bestaande uit 4 dubbellagen dextraansulfaat / poly-L-arginine exploederen noch onder fysiologische condities, noch onder niet-fysiologische condities, resulterend in holle microcapsules. Het hol zijn van de microcapsules werd aangetoond met elektronenmicroscopie en atoomkracht-microscopie.

In **Hoofdstuk 5** werden zelf-exploderende met lipiden (in plaats van met polyelektrolyten) gecoate microgels voorgesteld. We maakten gebruik van de unieke structuur van lipiden, die een polaire hydrofiele kop en een hydrofobe koolwaterstofstaart hebben. Gedispergeerd in watertij midden gaan lipiden zich spontaan ordenen en sferische structuren, zogenaamde liposomen, vormen. Bepaalde types lipiden hebben een netto lading en kunnen door middel van elektrostatische interactie aangetrokken worden tot een tegengesteld geladen oppervlak. Eénmaal geadsorbeerd op het oppervlak spreiden de liposomen zich en vormen ze een continue dubellaag. In **Hoofdstuk 5** werd dit concept gebruikt om de microgels met lipiden te coaten. De adsorptie van een lipidenlaag op het oppervlak van de microgels werd aangetoond met ζ-potentiaal metingen, confocale microscopie en elektronenmicroscopie. Wanneer de lipiden-gecoate microgels in een oplossing met hoge pH gebracht werden, begon de lipidenlaag op te blazen ten gevolge van de zweldruk van de degraderende microgels en op een bepaald ogenblik scheurde het
volledige membraan. Gedurende het opblazen van de lipidenlaag observeerden we de vorming van zogenaamde 'parachutes' op het lipidenopervlak. Dit was een aanwijzing dat het lipiden-membraan eerder bestond uit meerdere dubbellagen in plaats van één enkele dubbellaag. Deze hypothese werd ondersteund door X-straal reflectiviteitsmetingen.

De microgels aangemaakt en bestudeerd in de HOOFDSTUKKEN 2 – 5 vertoonden een brede deeltjesgrootte-distributie. Volgens de wet van Laplace zullen grotere microcapsules (verkregen door LbL- of lipiden-coating van grotere dex-HEMA microgels) makkelijker exploderen dan kleinere microcapsules aangezien er minder druk vereist is om het membraan te doen uittrekken. Bijgevolg zou het coaten van ‘monodisperse’ microgels voordelen bieden door het tijdsinterval waarin explosie optreedt te vernauwen. In HOOFDSTUK 6 beschreven we het gebruik van een microfluidic apparaat voor de aanmaak van monodisperse microgels.

De HOOFDSTUKKEN 7 – 10 handelden over de aanmaak van polyelektroliet-microcapsules die konden ontbinden onder invloed van een natuurlijke of externe stimulus. In HOOFDSTUK 7 werden capsules beschreven die konden oplossen in de aanwezigheid van glucose. Fenylboronzuur vormt een covalent anionisch complex met glucose bij alkalische pH, waarbij een daling van de apparente pKₐ optreedt. Een fenylboronzuur bevattend acrylaat werd gesynthetiseerd door aminofenylboronzuur te koppelen aan acrylzuur. Dit acrylaat werd gecopolymeriseerd met dimethyl-aminoethyl-acrylaat, resulterend in een copolymeer dat zowel amine- als fenylboronzuurgroepen bevat. Dit copolymeer werd bij lage pH gebruikt als polykation voor LbL-opbouw in combinatie met PSS als polyanion. UV-VIS spectroscopie werd gebruikt om de LbL-opbouw aan te tonen. Holle microcapsules werden aangemaakt door LbL-coating van polystyreenbolletjes met 6 dubbellagen copolymeer/PSS, waarna de polystyreenbolletjes opgelost werden in tetrahydrofuraan. Wanneer glucose toegevoegd werd onder gebufferde alkalische (pH 9) omstandigheden, veranderden de fenylboronzuurgroepen van een neutrale naar een anionische vorm, ten gevolge van de verschuiving in pKₐ. Hierdoor treedt er afstoting op tussen de fenylboronzuurgroepen en de sulfonaatgroepen van het PSS en treedt er tevens aantrekking op tussen de fenylboronzuurgroepen en de aminegroepen op hetzelfde copolymeer. Door deze intermoleculaire afstoting en aantrekking destabiliseerde het LbL-membraan van de microcapsules en losten deze op, zoals aangetoond met confocale microscopie.

In HOOFDSTUK 8 werden degradeerbare polyelektroliet-microcapsules beschreven en onderzocht met het oog op intracellulaire afgifte van moleculen. Er werden twee types degradeerbare microcapsules. Het eerste type bevatte een polyelektroliet met hydrolyseerbare kationische zijketens. Het tweede type werd aangemaakt gebruik makend van de biopolymeren dextraansulfaat en poly-L-arginine. Calcium-carbonaat (CaCO₃) micropartikels werden gebruikt als sacrificiële kern voor LbL-coating. Na LbL-coating werden
de CaCO₃ micropartikels opgelost met behulp van een EDTA-oplossing. Wanneer de verkregen polyelektroliet-microcapsules toegevoegd werden aan een celcultuur werden de microcapsules opgenomen door de cellen en intracellulair afgebroken. Dit in tegenstelling tot microcapsules bestaande uit de synthetische polyelektrolieten PSS/PAH die niet degradeerden en intact in de cellen bleven. De bio-degradeerbare polyelektroliet microcapsules lijken beloftevol voor de afgifte van antigenen en genetisch materiaal (bijvoorbeeld DNA, siRNA ...), die een intracellulair doel hebben.

**HOOFDSTUK 9** en **10** beschreven de aanmaak van hybride microcapsules door alternerende adsorptie van negatief geladen goud-nanopartikels en PAH op CaCO₃ micropartikels als sacrificiële kern. Confocale en elektronenmicroscopie werden gebruikt om de structuur van de microcapsules te onderzoeken. Aangezien de goud-nanopartikels zwak zure groepen (carboxyl) op hun oppervlak hebben en PAH zwak basische groepen (primair amine) heeft, konden de microcapsules zowel bij lage als bij hoge pH opgelost worden, aangezien de carboxyl-, respectievelijk amine groepen niet langer geladen waren. Door covalente vernetting tussen de carboxyl- en amine groepen konden de microcapsules gestabiliseerd worden waardoor ze stabiel bleven over de hele pH-schaal. Verder toonden we aan dat de microcapsules konden geactiveerd worden door IR-laser-bestraling waarbij lichtenergie omgezet wordt in thermische energie en de microcapsules uiteindelijk scheurden en hun inhoud vrijgaven. In **HOOFDSTUK 10** onderzochten we het effect van ultrasone golven op de integriteit van polyelektroliet- en hybride nanopartikel/polyelektroliet microcapsules. De afschuifkrachten, veroorzaakt door de propagerende ultrasone schokgolven, in de vloeistof rond de microcapsules vernielden de microcapsules volledig. Met behulp van confocale en elektronenmicroscopie konden na ultrasone behandeling enkel nog restanten van gebroken microcapsules gedetecteerd worden. Beide types microcapsules stelden hun volledige inhoud vrij na ultrasone behandeling.

Polyelektroliet-microcapsules wekken, sinds hun uitvinding in 1998, de interesse van zowel fundamentele als toepassingsgerichte wetenschappers. De concepten ontwikkeld in deze doctoraatsthesis zijn een beloftevolle stap in de richting van farmaceutische toepassingen van polyelektroliet-microcapsules. Echter, deze microcapsules zijn nog in het vroegste stadium van ontwikkeling en verder onderzoek is noodzakelijk om volledig inzicht te verwerven en hen te optimaliseren tot echte toepassingen. Inderdaad, het blijft een enorme uitdaging om deze microcapsules verder te ontwikkelen en hun voordeel aan te tonen tegenover meer gevestigde afgiftesystemen zoals liposomen en klassieke polymeerpartikels.
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PERSONAL INFORMATION
Date of birth: November, 26th 1980
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EDUCATION
Ghent University
PhD student, Department of Pharmaceutics,
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Ghent University
MSE Chemical Engineering, graduated June 2003
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Don Bosco College Zwijnaarde
Mathematics (8h) – Science, graduated June 1998

RESEARCH EXPERIENCE
PhD Research
Development of polyelectrolyte microcapsules for
pharmaceutical applications: pulsed drug delivery,
stimuli responsive drug delivery and intracellular
delivery.

Department of Pharmaceutics, Ghent University
Principal investigators: Prof. Dr. Stefaan De Smedt
and Prof. Dr. Joseph Demeester.

M.S. Research
Synthesis and characterization of functional block-
copolymers from dual initiators.

Department of Organic Chemistry, Ghent University
Principal investigator: Prof. Dr. Filip Du Prez
PUBLICATIONS

Papers published in peer reviewed journals:


De Geest, B.G.; Sanders, N.N.; Sukhorukov, G.B.; Demeester, J.; De Smedt, S.C. Release Mechanisms for Polyelectrolyte Capsules. *Chemical Society Reviews* in press


**Papers submitted**


Vandenbroucke, R.E.; De Geest, B.G.; Bonné, S.; Van Haeke, T.; Peeters, L.; Wagner, E.; De Smedt, S.C.; Demeester, J.; Sanders, N.N. siRNA complexed to degradable synthetic pH sensitive polycations is a potent therapeutic against liver diseases. Submitted

**CONFERENCE PRESENTATIONS**

**Oral Presentations**


**Poster Presentations**


*presenting author is underlined*

**AWARDS**

**Student Poster Highlight Award.** Self-exploping biopolymer microcapsules. *Annual Meeting of the Controlled Release Society*. June 18-22, 2005. Miami (FL), USA.


**PATENT APPLICATIONS**

Demeester, J. Stefaan De Smedt, S.C.; Stubbe, B.G.; De Geest, B.G. Pulsed bio-agent delivery systems based on degradable polymer solutions or hydrogels.


**RESEARCH VISITS**


Thorsen Microfluidic Modeling and Design Laboratory, Department of Mechanical Engineering, Massachusetts Institute of Technology, Boston (MA), USA. Under the supervision of Prof. Dr. Todd Thorsen. December 6 – 10, 2004.

Biomaterials & Tissue Engineering Group, Department of Engineering Materials, University of Sheffield, Sheffield, United Kingdom. Under the supervision of Prof. Dr. Giuseppe Battaglia. June 19 - 24, 2006.

JOURNAL REVIEWER

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Macromolecular Rapid Communications
Biomacromolecules
Journal of Controlled Release
Acta Biomaterialia