MICROFLUIDIC APPROACH TO PREPARING POLYMER MICROSPHERES FOR ENHANCED ORAL PROTEIN DRUG DELIVERY

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Tiivistelmä – Referat – Abstract

Polymer microspheres hold great potential as oral drug delivery system for therapeutic proteins. Microspheres prepared with biocompatible and biodegredable polymers have been extensively studied, since the oral delivery of therapeutic proteins is challenging due to the conditions in the GI-tract. The aims of this research were to apply microfluidics on polymeric microsphere preparation process, to determine what kind of formulations are suitable for this technology, to establish a controlled preparation process that produces advanced particles and to create a template for oral protein drug delivery.

With microfluidic fabrication it is possible to gain control over the process and content of each droplet. However, finding suitable formulations for microfluidics is demanding. In this study, biphasic flow was employed to successfully produce double (W/O/W) emulsion droplets with ultra thin shells. Once the process and formulation variables were optimized constant droplet production was achieved. Flow rates used were 500 µl/h in the inner and in the middle phase and 2500 µl/h in the outer phase, respectively.

Two formulations were selected for further characterization: 5 % poly(vinyl alcohol) in water in the outer phase, 3 % polycaprolactone in ethyl acetate in the middle phase and either 10 % or 20 % poly(vinyl alcohol) and polyethylenglycol (1:4) in water in the inner phase. All the particles were found to be intact and contain the inner phase, as verified by confocal microscopy. Further, the particles were monodisperse and non-porous, as observed by scanning electron microscopy. Particle size was found to be around 20-40 µm, variation in the particle size within one batch was small and the particles were stable up to 4 weeks. The encapsulation efficiency of the particles was remarkable; as high as 85 % loading of the model compound, bovine serum albumin. Particles released 30 % of their content within 48 hours. In conlusion, developing functional formulations for micfoluidic technology was possible, the microparticles encapsulated the model protein extremely well and all in all microfluidic technology had a lot of potential for droplet manufacturing for pharmaceutical applications.

Avainsanat – Nyckelord – Keywords
Microparticles, polymers, microfluidics, therapeutic proteins, drug delivery

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Tiivistelmä – Referat – Abstract

Polymeerimikropartikkeleilla on suuri potentiaali terapeuttisten proteiinien oraalisessa lääkkeenannossa. Bioyhteensopivista ja biohajoavista polymeereistä valmistettuja mikropartikkeleita on tutkittu laajasti, sillä oraalinen proteiinilääkkeenanto on haastavaa ruoansulatuskanavan olosuhteiden vuoksi. Tämän tutkimuksen tavoitteena oli käyttää mikrofluidistiikkaa mikropartikkelien tuottamiseen, etsiä mikrofluidistiikkaan sopivia formulaatioita ja saada aikaan hallittu valmistusprosessi, joka tuottaa edistyksellisiä mikropartikkeleita sekä luoda pohjaa proteiinien oraaliseen lääkkeenantoon.

Mikrofluidistisen valmistuksen avulla on mahdollista hallita valmistusprosessia ja muokata jokaisen partikkelin sisältöä. Mikrofluidistiseen teknologiaan sopivia formulaatioita on kuitenkin vaikea kehittää. Tässä tutkimuksessa käytettiin kaksivaiheista virtausta tuottamaan kaksinkertaisia emulsiopisaroita (v/o/v) erittäin ohuilla kuorilla. Kun prosessi- ja formulaatiomuuttujat oli optimoitu, vakaa emulsiotuotanto saavutettiin. Käytetyt virtausnopeudet olivat noin 500 µl/h keski- ja sisäfaasissa ja 2500 µl/h ulkofaasissa.

Kaksi formulaatiota valittiin tarkempaan karakterisointiin: 5 % polyvinyylialkoholia vedessä ulkofaasina, 3 % polykaprolaktonia etyyliasetaatissa keskifaasina ja joko 10 % tai 20 % polyvinyylialkoholia ja polyetyleeniglykolia (1:4) vedessä sisäfaasina. Kaikki partikkelit olivat ehjiä ja niissä oli sisäfaasi, kuten todennettiin konfokaalimikroskopialla. Elektronipyyhkäisymikroskopialla havainnoitiin partikkelien olevan monodispersejä ja houkosettomia. Partikkelikoko oli noin 20-40 µm ja erien sisäinen vaihtelu pientä. Partikkelit pysyivät stabiileina 4 viikkoa. Tämän jälkeen partikkelit luhistuivat 15 %:n viikkonopeudella. Partikkelien kapselointitehokkuus oli huomattava, jopa 85 %:a malliproteiinina käytetystä naudan seerumin albumiinista kapseloitiin. Partikkelit vapauttivat noin 30 %:a malliproteiinista dissoluutiokokeissa. Johtopäätöksenä todetaan, että mikrofluidistisella teknologialla valmistettavia formulaatioita oli mahdollista kehittää, partikkelit kapseloivat malliproteiinia erittäin hyvin ja kaiken kaikkiaan mikrofluidistiikalla on erityisen paljon potentiaalia partikkelituotannossa farmaseuttisia sovelluksia varten.

Avainsanat – Nyckelord – Keywords

Mikropartikkelit, polymeerit, mikrofluidistiikka, lääkkeen kuljetus, terapeuttiset proteiinit

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DCM	Dichloromethane
EtOAc	Ethyl acetate
HPLC	High performance liquid chromatography
Mw	Molecular weight
MWCO	Molecular weight cut off
PCL	Polycaprolactone
PDMS	Poly(dimethylsiloxane)
PEG	Polyethylene glycol
PLA	Poly(lactic acid)
PLGA	Poly(lactic glycolic acid)
PVA	Poly(vinyl alcohol)
SEM	Scanning electron microscopy

1. INTRODUCTION

Novel aspects for oral administration of therapeutic proteins and peptides are needed. Increasing the bioavailability of orally administered peptides and proteins is the main challenge, since the gastro-intestinal tract has various barriers for the delivery, such as proteolytic degradation resulting in degradation of the compound prior to absorption and the inability of the macromolecules to penetrate the intestinal cell wall (Zhou 1994). Thus the drug carrier system in oral delivery of therapeutic proteins has an important role (Langer 1998). The drug carrier protects the protein structure that is essential for preserving the bioactivity of the protein. Additionally, with controlled release prolonged delivery and maintaining concentration within therapeutic limits is possible and thus toxicity and systemic side effects can be reduced. Even though, protein and peptide drugs have typically been administered by injection, the oral route provides less invasive administration route and improves patient compliance.

Polymer microspheres hold great potential as delivery systems for oral protein drug delivery (Freiberg 2004). Polymer microspheres can be used widely applied to many situations where continuous and controlled drug administration is essential and the use of microspheres for drug delivery is not limited to any specific illness. There are various microsphere preparation methods, however, the conventional bulk methods often result in polydisperse microspheres with poor encapsulation efficiencies.

Microfluidic technology has various advantages as polymer microsphere preparation method (Utada et al. 2005). Precise manufacturing and gaining control over the process is possible. Exploring the possibilities of using microfluidic technology in polymer microsphere preparation is an interesting research topic for pharmaceutical sciences.

I LITERATURE REVIEW

2. ORAL PROTEIN DRUG DELIVERY

Oral delivery systems for proteins and peptides have been widely studied for the past few decades and yet challenges remain (Zhou 1994). For example, the production of therapeutic proteins is possible in large scales, but the bioavailability of the proteins is low when administered via the oral route. Proteins and peptides have large molecular size, and are very sensitive to enzymatic degradation, aggregation, adsorption, and denaturation, with short plasma half-life, ion permeability and immunogenicity (Saffran et al. 1986; Fix 1996). When developing carrier systems for therapeutic proteins and peptides, providing protection against proteases and digestive enzymes in the gastrointestinal (GI) tract, and also enhancing the permeation and bioavailability of the encapsulated therapeutics, should be taken into account (Morishita and Peppas 2006).

2.1 Challenges in oral delivery of proteins

Various GI barriers inhibit the oral administration of proteins (Rekha and Sharma 2011). The acidic conditions in the stomach cause degradation of the proteins. In the small intestine, where drugs are mainly absorbed, the enzymatic activity of proteases is also higher than in any other part of the GI tract. Next, the enzymes in the intestine destroy the structure of the protein, such as aminopeptidase, trypsin, chymotrypsin, elastase, pepsin, and carboxypeptidase A and B.

Poor absorption of macromolecules such as proteins and peptides limits the oral bioavailability of the therapeutics. Crossing the epithelial intestinal cell layer is possible via diffusion through the hydrophobic tight junctions by passive transport, via facilitated transcellular diffusion through the lipophilic absorptive cells, or via active carrier mediated transport systems or transcytosis (Ingemann et al. 2000). Proteins and peptides mostly absorb through the enterocytes or tight junctions of the epithelial intestinal cells (Rekha and Sharma 2011) (**Figure 1**).

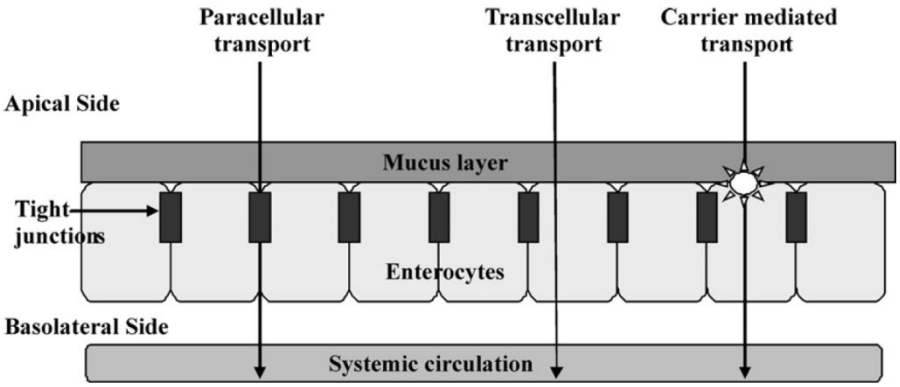

Figure 1. The transport pathways for peptides and proteins across the intestinal cell epithelium (Rekha and Sharma 2011).

Additionally, absorbed peptides and protein may undergo efflux from the cells due to their affinity to P-glycoprotein that decreases absorption rates and lowers the bioavailability (Li 2001). The P-glycoprotein substrates also often undergo metabolism via the CYP3A4 that again lowers the bioavailability. Thus, in general, the oral bioavailability of most peptides and proteins is less than 1% (Mahato et al. 2003).

Other physical barriers for proteins and peptides absorption are the size, charge and solubility constraints (Cox et al. 2002). Paracellular protein transport across the aqueous channels and tight junctions between the epithelial cells is limited due to the physical properties of the molecules. Size-dependent transport with constant size and charge indicates that various physical properties act in unison affecting the permeability of proteins and peptides. The positively charged peptides permeate better through the epithelial cells, indicating an interactive environment wherein the penetrating peptide and protein interacts with lipids and proteins lining the aqueous pores. Also this macromolecule permeability barrier is developed as the human being grows older (Udall et al. 1981). During a short period after birth, the GI tract is more permeable to macromolecules, and thus, the permeability of peptides and proteins across the GI tract of neonates is higher than that of the adult.

2.2 Applications for oral delivery of proteins

The charge and solubility parameters of therapeutics can be changed by formulation and chemistry adjustments (Mahato et al. 2003). The solubility can be affected by using of a salt form or by covalent attachment of hydrophilic polymers such as PEG or hydrophobic lipids. Chemical modifications of peptides and proteins have been shown to improve also the stability and membrane penetration. These modifications can be done either by direct modification of exposed to the side-chain amino acid groups of the proteins (Murphy and óFágáin 1996) or through the carbohydrate part of the glycoproteins and glycoenzymes (Barbaric et al. 1988).There are formulation vehicles that are used to overcome different biological barriers within the GI tract, including hydrogels, emulsions, microemulsions, microparticles, nanoparticles, coated liposomes and mucoadhesive polymers. Also, protease inhibitors such as FK-448, which inhibits chymotrypsin (Fujii et al. 1985; Shinomiya et al. 1985), and absorption enhancers (Leone-Bay et al. 2001; Stoll et al. 2000) have been studied in order to enhance the oral drug delivery of proteins.

As an example, cyclosporine A has been successfully formulated into oral protein drug dosage form (Sandimmun Neoral, Novartis). Cyclosporine is an atypical cyclic peptide which consists of 11 aminoacids, is highly hydrophobic, possesses significant oral activity as an immunosuppressant, and is also resistant to proteolytic degradation (White 1982). Cyclosporine A absorption takes place via transcellular diffusive route through the lipid membrane and it is substrate for P-glycoprotein (Saeki et al. 1993). Cyclosporine A has been formulated with peglicol-5-oleate, olive oil and ethanol at a ratio of 30:60:10 followed by aqueous dilution for emulsification in order to avoid poor aqueous solubility, slow and incomplete absorption, and overall low oral bioavailability (Grevel 1986). The mean cyclosporine bioavailability is 30% in normal subjects and the absolute oral bioavailability varies from 5 to 90% in adult kidney transplant patients (Ptachcinski et al. 1985). Cyclosporine A proves that production of commercially successful oral medicines of proteins and peptides drugs possible. Another remarkable example is the oral delivery of insulin, that is now also possible via oral route (Stanton 2013). The development process for oral insulin preparation has reached patenting stage at NovoNordisk.

3. POLYMER MICROSPHERES

Polymeric microcapsules hold great potential as oral drug delivery systems for therapeutic proteins (Freiberg and Zhu 2004). The preparation of the first polymer microspheres took place in the 1960s in order to better control the drug release: the first polymers used were silicone rubber (Folkman and Long 1964) and polyethylene (Desai et al. 1965). About a decade later the microsphere preparation with biodegradable polymers began (Mason et al. 1976). In this literature review the focus is on the polymers used in this research poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PLGA), however, a number of other biocompatible and biodegradable polymers suitable for medical applications also exist.

3.1 Preparation of microspheres

Various techniques for polymer microsphere preparation have been reported. Solvent evaporation technique to obtain oil-in-water (O/W) or water-in-oil-in-water (W/O/W) droplets is the most common method, and with this technique droplets are formed in an emulsion followed by evaporation the organic solvent (Freiberg and Zhu 2004; Jalil and Nixon 1989) (**Figure 2**). These emulsions can also be created by stirring the solutions with high speed homogenizers (Barbato et al. 2001) or sonicators (Tomar et al. 2011). After stirring, the microsphere polymerization takes place as the solvent is allowed to evaporate.

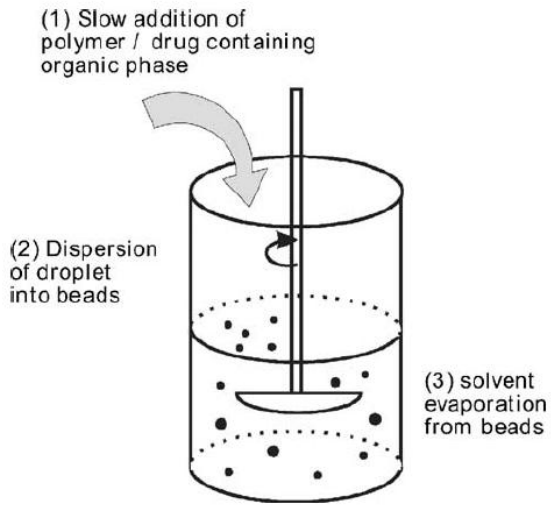

Figure 2. Solvent evaporation technique for preparing single or double emulsions in order to obtain polymer microspheres (Freiberg and Zhu 2004).

Other bulk methods for preparing polymer microspheres are the spray drying technique, the solution-enhanced dispersion method, the hot melt technique and coaservation. The spray drying method has been used widely for producing dry powders, granules or agglomerates, and can also be employed in the microsphere preparation processes (Bodmeier and Chen 1988). The solution-enhanced dispersion method enables the creation of microspheres without using organic solvents, forming stable microspheres with high encapsulation efficiencies by preparing microspheres using supercritical fluid (Bodmeier et al. 1995). With the hot melt technique the polymer used is melted, dispersed in a suitable dispersion medium and slowly cooled, and thus, forming microspheres (Mathiowitz and Langer 1987). This method is suitable for polymers with low melting points and for microspheres, which are susceptible to hydrolysis. Coaservation or phase separation consists of decreasing the solubility of the encapsulating polymer by addition of a third component to the polymer solution in an organic solution (Lewis 1990; Jalil and Nixon 1990). In this process, the drug is dispersed in the polymer solution and coated by the coacervate. The process consists of phase separation of the polymer solution, adsorption of the coacervate around the particle containing the drug and solidication of the microspheres (Edelman et al. 1993).

The methods using shear in the emulsification process often result in polydisperse emulsions and to obtain small-size dispersions often requires shear forces that may degrade the peptides and proteins. Methods that produce more monodisperse particles are, for example, microporous membranes, where monodisperse emulsions are produced by extruding a coarse emulsion through porous glass membranes (Vladisavljević et al. 2006), droplets formation in microchannels (Sugiura et al. 2004), or microfluidics (Kim et al. 2011; Kim et al. 2013; Datta et al. 2012; Duncanson et al. 2012a; Duncanson et al. 2012b) as discussed in this master's thesis research work.

The outcome of the polymer microsphere preparation process does not only depend on the production method, but also on the substances used in the process. Especially, the polymer molecular weight affects the qualities of the microspheres prepared (Park 1994). Shape, size and the degradation rate are connected to the molecular weight of the polymer. With microspheres that contain polymer chains of lower molecular weight, the quantity of the degradation products increases. The differences in the degradation profiles occur due to the differences in glass transition temperatures (*T*g) and crystallinity associated with polymers of different molecular weights.

The microspheres produced from double emulsions usually contain additional polymer with the water soluble drug in the inner phase, and thus, in the core of the microsphere (Freiberg and Zhu 2004) (**Figure 3**). The outer shell consists of polymer in the organic solvent that is later evaporated as the shell is polymerized.

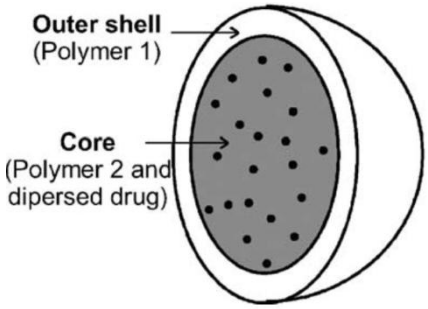

Figure 3. Cross-section of the structure of a polymeric microsphere prepared by the solvent evaporation method from double emulsions (W/O/W) (Freiberg and Zhu 2004).

3.2 Physicochemical properties of polymers used in this research work

Aliphatic polyesters, such as poly(lactide), poly(glycolide), PCL and additionally their copolymers have been widely studied as biodegradable polymers for controlled drug delivery applications (Thombre and Cardinal 1990; Albertsson et al. 1992). The focus in this research was set on the following polymers due to their wide used in pharmaceutical industry.

3.2.1 Poly(lactic acid), poly(glycolic acid) and poly(lactic-co-glycolic acid)

Poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) are thermoplastic aliphatic polyesters that are biodegradable and biocompatible (Wu 1995; Heller 1980; Kitchell and Wise 1985). Poly(lactic-co-glycolic acid) or poly(lactide-co-gycolide) (PLGA), is the copolymer of PLA and PGA (**Figure 4**). There are two forms of PLA: (1) an optically active stereoregular form ($_{L}$-PLA) that has high regularity in its polymer chain, and (2) an optically inactive racemic form ($_{D,L}$-PLA) that is an amorphous polymer because of the irregularities in the polymer chain structure (Tice and Cowsar 1984). PLGA prepared from $_{L}$-PLA and PGA is crystalline while PLGA prepared from $_{D,L}$-PLA and PGA is amorphous (Lewis 1990; Wu 1995). Lactic acid is more hydrophobic than the crystalline glycolic acid, and thus, PLGA copolymers that contain more lactide are less hydrophilic, absorb less water and degrade more slowly than PLGA copolymers which contain less lactide (Wu 1995). The *T*g of PLGA varies between 40–60 °C depending on the ratio of lactide and

glucolide. The solubility of PLA, PGA and PLGA also varies according to the structure of the polymer. Generally, PLA, PGA and PLGA are soluble, for example, in dichloromethane, toluene and dimethyl sulfoxide.

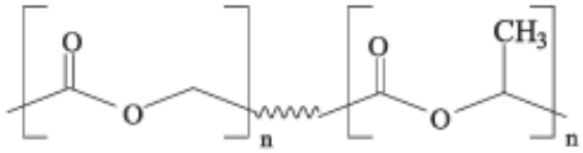

Figure 4. Structure of PLGA with a glycolic acid part (left) and lactic acid part (right) for the copolymer and the portions of these defines the properties of the copolymer (Langer and Vacanti 1993).

The synthesis of PLA, PGA and PLGA can be conducted with direct polycondensation reaction of lactic or glycolic acid resulting in low molecular weight products (Fukuzaki et al. 1988). Other possible synthesis method is the ring-opening polymerization of cyclic dimers (Deasy et al. 1989). Thus, high molecular weight polymers are synthesized using metal catalysts. The intrinsic viscosity is directly related to the molecular weights of PLA, PGA and PLGA (Wu 1995).

PLA, PGA and PLGA biodegrade correspondingly into lactic and glycolic acids (Wu 1995). The biodegradation process presumably takes place purely through hydrolysis, yet differences have been found between *in vitro* and *in vivo* degradation rates, possibly due to some enzymatic activity (Lewis 1990; Wu 1995). The number of carboxylic end groups present in the PLGA chains increases during the biodegradation process, and thus, catalyze the biodegradation process. PLGA (50:50) hydrolyzes much faster than those containing higher proportion of either of the two monomers (Lewis 1990). The biodegradation process can create acidic microenvironment that can cause issues with biocompatibility and protein degradation (Fu et al. 2000). Since PLGA is one of the first FDA approved polymers, it has been widely used in various studies where polymer microspheres have been successfully manufactured with vancomycine (Atkins et al. 1998), polypeptide (Li et al. 1995) and with bovine insulin (Uchida et al. 1997).

PLGA microspheres have also been used in the development of oral vaccines. Ovalbumin as a model antigen has been successfully administered orally from PLGA particles (Challacombe et al. 1997; Uchida et al. 1994). In addition, also microspheres in oral administration of tuberculosis vaccinations have been reported (Vordermeier et al. 1995).

3.2.2 Polycaprolactone

Polycaprolactone, poly (ε-caprolactone) or poly (epsilon-caprolactone) (PCL), is a semi-crystalline and hydrophobic polymer (Chandra and Rustgi 1998). The melting point of PCL is 60 ºC and is soluble in dichloromethane, chloroform, carbon tetrachloride, benzene, toluene, cyclohexanone, acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile (Coulembier et al. 2006). The *T*g of PCL is –60 °C and the melting point is between 59 and 64 °C (Hayashi 1994). PCL is biocompatible and biodegradable (Pitt 1990; Chen et al. 2000). Degradation of PCL is an autocatalyzed reaction, where the liberated carboxylic acid end groups catalyze the hydrolysis of additional ester groups (Pitt 1990).

There are two methods used to prepare PCL: (1) though a free radical ring-opening polymerisation from 2-methylene-1-3-dioxepane, and (2) more commonly by using a ring-opening polymerisation from ε-caprolactone using a variety of anionic, cationic and coordination catalysts (Pitt 1990). The ring-opening polymerization is catalyzed with stannous actuate and the molecular weight can be controlled with low molecular weight alcohols (Storey and Taylor 1998) (**Figure 5**). Various molecular sizes of PCL are available and the bigger the molecular weight is the less crystalline is its structure (Chandra and Rustgi 1998). The molecular weight can vary from 3,000 to 80,000 g/mol (Hayashi 1994).

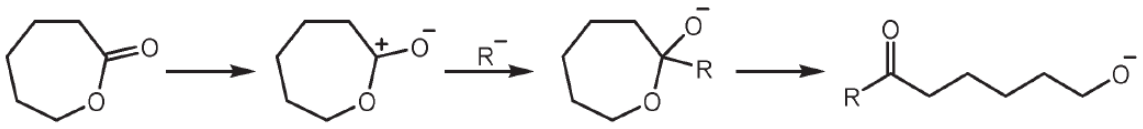

Figure 5. Example of the ring-opening polymerization of PCL with anionic catalyst (R^-) (Labet and Thielemans 2009). After this initial reaction the anionic catalyst is removed and the PCL chain is complete.

PCL has been widely used in the preparation of microparticles for drug delivery (Tomar et al. 2011; Somavarapu et al. 2005; Scala-Bertola et al. 2012, Natarajan et al. 2011) (**Figure 6**). PCL microspheres have been mainly manufactured with the traditional emulsion methods containing, for example, BSA (Bolzinger et al. 2007; Coccoli et al. 2008), taxol (Dordunoo et al. 1995), cyclosporine (Aberturas et al. 2002), ketoprofen (Guzman et al. 1996), and insulin (Shenoy et al. 2003). Additionally, microfluidic preparation of PCL microspheres has been studied without loading the particles (Liu et al. 2009).

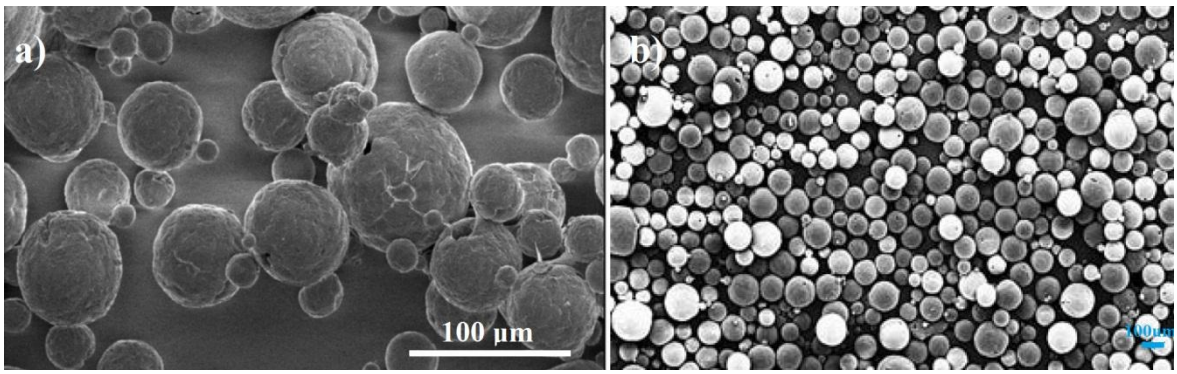

Figure 6. PCL particles prepared from PCL with bulk methods (a) PCL particles prepared with vigorous magnetic stirring and 2.5 % PCL in DCM as the middle phase (Scala-Bertola et al. 2012) and (b) PCL particles prepared using ultrasonic bath and 1.7 or 3 % of PCL in DCM as the middle phase that forms the shell (Coccoli et al. 2008).

PCL also has good blend-compatibility and various co-polymers have been prepared from PCL (Chandra Rustgi 1998; Chang et al. 1986). PCL has also been combined, for example, with PLA, PLGA, cellulose propionate and cellulose acetate butyrate. The modifications affect the release kinetics of the microparticles.

3.3 Characterization methods for microspheres

In vitro characterization of the microspheres is essential for the early stages of drug product development (Gibson 2001). Resources in the drug development process can be significantly saved when the basic characterization of the product is done thoroughly in the beginning. The *in vitro* characterization in the early stages consists of evaluating the particle size, stability, encapsulation efficiency, surface properties and drug release. The particle size can be observed with optical microscopy or more advanced techniques, such as Coulter counter (Gee and Bauder 1986) or light blocking methods (Gibbs 1982). The drug release profiles are often affected by the size distribution and in many cases the rate of drug release has been found to decrease with increasing sphere size (Narayani and Rao 1994; Akhtar and Lewis 1997).

With fluorescent samples, characterization in terms of the content of the particles, by confocal microscopy is useful. This form of microscopy is an optical imaging technique that uses point illumination and a spatial pinhole to eliminate out-of-focus light in the sample that are thicker than the focal plane (Pawley 2006). With confocal microscope it is possible to observe the sample on a depth level at a time and when using fluorescent agents in the samples, lasers can be employed to produce emission-excitation spectra from the sample. Stability studies are conducted for the products in stressed conditions in order to evaluate the performance of the product over the preservation periods (Gibson 2001).

Scanning electron microscopy (SEM) is also used to observe the morphology of an object and can be used to evaluate the surface properties and the particle size of the microspheres (Hamley 2007). When using SEM, an electron beam is scanned across an object, knocking secondary electrons out of its surface atoms. The secondary electrons are then detected with appropriate detector. The actual image is produced using this data.

Another important characterization parameter is the encapsulation efficiency. Increasing or controlling the encapsulation efficiencies is desirable, because it can prevent the loss of expensive medicines and it can help to extend the duration and dosage of treatment (Gupta and Kumar 2001). The drug content of the encapsulated microspheres can be described by

two quantities: the total amount of drug employed minus the amount of unloaded drug. From the results of the encapsulation efficiency studies the preparation method can be evaluated in terms of the ability to incorporate the model drug into the microspheres (Judefeind and De Villiers 2009). The encapsulation efficiency is used to evaluate the performance of the drug delivery system and to compare the quality of different formulations.

Dissolution testing is also an important part of drug product development (Lee et al. 2008). Dissolution testing provides data regarding the rate and extent of drug absorption in the body and it can assess the formulation principles on the release properties of a drug product. For conventional dosage forms there are a set of procedures for the drug release tests (Siewert et al. 2003). For novel dosage forms, in which the formulation design and the physicochemical properties vary, the development of the dissolution test system is more demanding. However, the general principles of dissolution tests for solid oral dosage forms should also be applicable to *in vitro* drug release tests for novel dosage forms, such as microparticles. The goal of the dissolution tests is to use the test for the biopharmaceutical characterization of the drug product, and for ensuring consistent product quality within a defined set of specification criteria.

Polymeric microspheres have also been widely characterized *in vivo* and *in vitro* in order to establish an *in vitro–in vivo* correlation. For example, the *in vivo* results using PGLA microsphere systems have been obtained from rats for the release of nifedipine (Sandstrap et al. 1999; Tuncay et al. 2000).

4. DROPLET-BASED MICROFLUIDICS

With microfluidic devices it is possible to mix immiscible liquids with precise control (Squires and Quake 2005). The microfluidic technology has various advantages, particularly the ability to create actually three-dimensional flows (Utada et al. 2005). This makes the precise manufacturing process possible and enables gaining the control over the immiscible fluids.

4.1 Physics of microfluidics

The droplet formation in microfluidic devices is based on jetting to dripping transition and by taking advantage of the hydrodynamic instability (Powers et al. 1998). With the jetting to dripping transition the drop formation involves a balance between the viscous drag of the coaxial fluid that pulls on the drop and the surface tension forces (Umbanhowar et al. 2000). The surface energy is decreased as the jet breaks into drops, and thus, the drop formation can be understood via the Rayleigh-Plateau instability (Squires and Quake 2005; Utada et al. 2007). The stream breaks into drops as the Laplace pressure increases within the thinner parts of the stream. The Laplace pressure is the internal pressure of water caused by the curvature of the interface. This high pressure pushes the fluid within the jet to either side causing the thin region to become thinner and form the drops. Additionally, the jetting to dripping transition is affected by the capillary number that is the balance between the force caused by the viscous drag and the force caused by surface tension. When there is little viscous drag and the capillary number is low, the Weber number is necessary to describe the balance between the inertial and the surface tension forces. Then, the inertial force of the fluid must overcome the surface tension forces, and thus, leading to pinch-off and to create the drops.

The modelling of the behaviour of fluids in microscale can also be described with additional dimensionless numbers, as described representatively in the review article by Squires (Squires and Quake 2005) (**Table 1**).

Table 1. The dimensionless numbers that are used to describe the physics of microfluidics (Squires and Quake 2005): ρ is the density, L_0 is the length scale, U_0 the is the flow velocity, η the is shear viscosity, D is the diffusivity, γ the is surface tension, τ_p is the polymer relaxation time, τ_{flow} is the oscillation time, h is the shortest dimension setting the shear rate, U_b is the buoyant velocity scale, and β is a slip length of order.

Re	Reynolds	$\frac{\rho U_0 L_0}{\eta}$	inertial/viscous	Eq. (1)
Pe	Péclet	$\frac{U_0 L_0}{D}$	convection/diffusion	Eq. (2)
Ca	capillary	$\frac{\eta U_0}{\gamma}$	viscous/interfacial	Eq. (3)
Wi	Weissenberg	$\tau_p \dot{\gamma}$	polymer relaxation time/shear rate time	Eq. (4)
De	Deborah	$\frac{\tau_p}{\tau_{\text{flow}}}$	polymer relaxation time/flow time	Eq. (5)
El	elasticity	$\frac{\tau_p \eta}{\rho h^2}$	elastic effects/inertial effects	Eq. (6)
Gr	Grashof	$\frac{\rho U_b L_0}{\eta}$	Re for buoyant flow	Eq. (7)
Ra	Rayleigh	$\frac{U_b L_0}{D}$	Pe for buoyant flow	Eq. (8)
Kn	Knudsen	$\frac{\beta}{L_0}$	slip length/macroscopic length	Eq. (9)

The Reynolds number (Eq. 1), relates the inertial forces to the viscous forces; the Péclet number (Eq. 2) relates the convection to diffusion; the capillary number (Eq. 3) relates the viscous forces to the surface tension; the Deborah (Eq. 4), Weissenberg (Eq. 5), and the elasticity numbers (Eq. 6) express the elastic effects; the Grashof (Eq. 7) and the Rayleigh (Eq. 8) numbers relate the transport mechanisms in the buoyancy-driven flows; and the Knudsen number (Eq. 9) relates the microscopic to the macroscopic length scales (Squires and Quake 2005).

Using the Reynolds number the magnitude of the inertial and the viscous force densities are compared (Squires and Quake 2005). The viscous force densities result from gradients in viscous stress. When it comes to the scale used in microfluidics, the Reynolds number is often small enough for the inertial effects to be irrelevant. Thus, the viscosity has a greater

effect when it comes to fluid behaviour in microfluidics. The viscous forces typically overwhelm the inertial forces and this is why the flow is linear. The Péclet number referrers to the relative importance of convection to diffusion and describes how far down the channel must the fluids flow before the channel is homogenized. This diffusive mixing can be desirable depending on the application. The Péclet number is more relevant when the fluids used in the microfluidic system are miscible.

The capillary number is important when the fluids in the microfluidic system are immiscible (Squires and Quake 2005). Between immiscible fluids the surface tension affects the dynamics of the free surface, and due to the Rayleigh-Plateau instability, the stream of the fluid breaks into drops (De Gennes et al. 2004). Thus, microfluidic devices can be used to create controllable droplet emulsions in immiscible fluids (Thorsen et al. 2001). Competing stresses drive the interface. The surface tension works to reduce the interfacial area and the viscous stress works to extend and drag the interface downstream. The droplets form as the interphase is destabilized. The capillary forces can also be used to manipulate and transport fluids with free surfaces, and usually modifying the interfacial forces disrupts the balance and causes motion (Squires and Quake 2005).

The Weissenberg and Deborah numbers help to evaluate the elastic component to the fluid caused by the dissolved polymers (Squires and Quake 2005). Adding polymers enriches the flow behaviour. Weissenberg number describes the spring forces balancing the Brownian forces to give a characteristic polymer size and Deborah number describes the time scale characteristic of the flow geometry. The flow time scale can be long or short compared with the polymer relaxation time resulting in a dimensionless ratio. The elasticity number evaluates the change of the elastic effects as the flow velocity increases. With the increase of the elasticity number also the Weissenberg and Deborah numbers increase. In addition, Reynolds number increases in the same way and the inertial effects become more important. The Grashof and Rayleigh numbers help understand the effects that density differences have on the fluid behaviour. The Grashof and Rayleigh numbers compare the same fundamental effects as the Reynolds and Péclet numbers. The Knudsen number matters when the fluid cannot be treated as a continuum. Non-continuum effects have an increasingly important role as the Knudsen number increases.

4.2 Microfluidic devices

Microfluidic devices at Prof. Weitz's laboratory are hand-made glass capillary devices (Duncanson et al. 2012a; Chu et al. 2007; Shum et al. 2011; Kim and Weitz 2011), similarly to the microfluidic devices that have been manufactured in other research works (Liu et al. 2009), or poly(dimethylsiloxane) (PDMS) devices (Abate and Weitz 2009; Thiele et al. 2010) which are manufactured with soft lithography (Whitesides and Stroock 2001). Soft lithography at Harvard University takes places in the Center for Nanoscale Systems and the manufacturing is based on automated manufacturing systems employing matrixes made for each device design.

Microfluidic devices can employ whether coaxial flow, hydrodynamic flow-focusing or combination of these two. With co-flow (**Figure 7**) one fluid flows on the outside of the circular capillary through the square capillary and the other flows through the inner circular capillary (Umbanhowar et al. 2000). The result is a coaxial flow of the two fluids that easily form drops. The alternative for co-flow is flow-focusing of the inner fluid by the outer fluid (Gañán-Calvo and Gordillo 2001) (**Figure 7**). The outer fluid is introduced into the device as in the co-flow device, yet the inner fluid is being introduced from the opposite side and both fluids are collected, and exit through the cylindrical capillary. The process is basically the same as with the co-flow device design, but one fluid is flowing in the opposite direction and is hydrodynamically focused through the narrow orifice by the outer fluid. The advantage of this method is the production of a stream that is narrower compared to the orifice size.

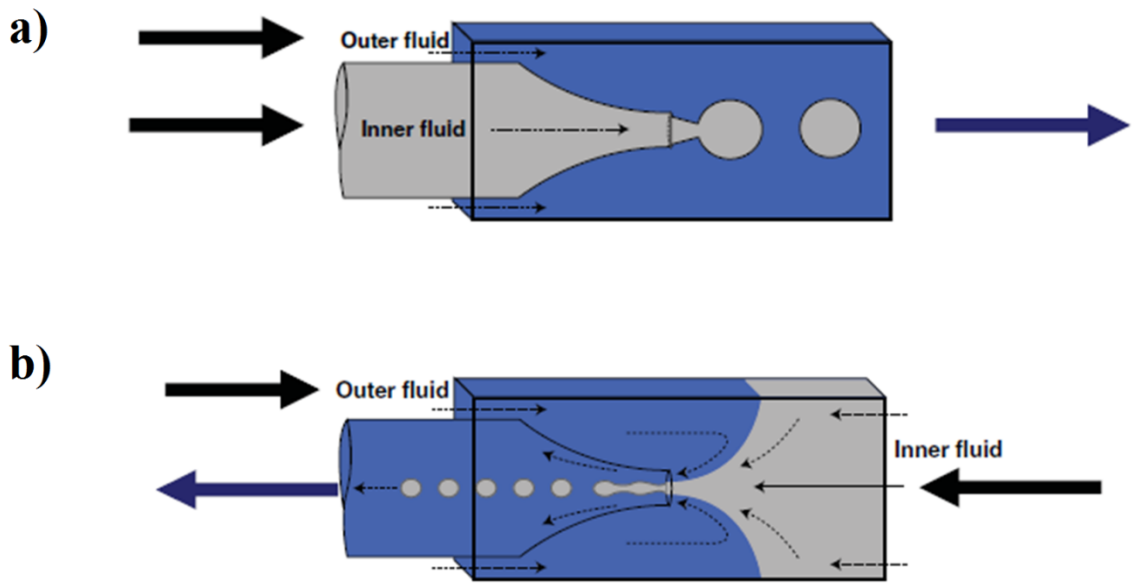

Figure 7. (a) A co-flow microcapillary device for producing single emulsion droplets () and (b) a flow-focusing microcapillary device for making single emulsion droplets (Utada et al. 2007).

By combining co-flow and flow-focusing, the preparation of more complex materials is possible (Utada et al. 2005). The designs of these devices are more complicated and perfect alignment of the tapered capillaries is required (**Figure 8**). Drops are thus created at the orifice from a coaxial flow of two fluids.

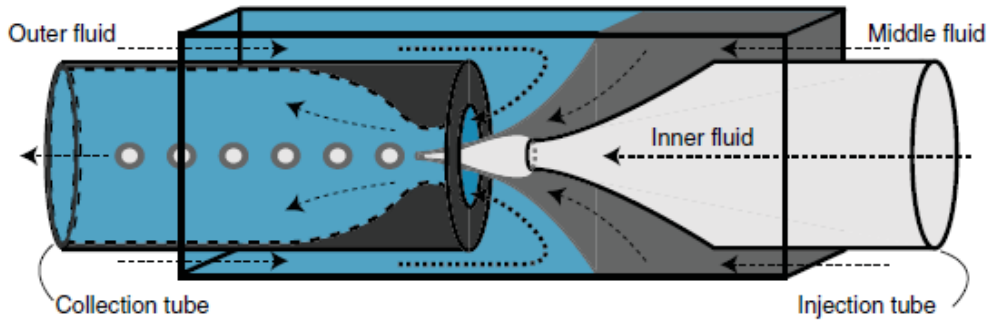

Figure 8. Microfluidic device that combines co-flow and flow-focusing thus creating double emulsion droplets (Utada et al. 2007).

The process for double emulsions is completely scalable (Chu et al. 2007; Wang et al. 2011). The desired number of layers can be added to the procedure by repeating the flow-focusing and co-flow parts to the devices used (**Figure 9**). However, the more complex the emulsion structure is, the more control is needed for the process to succeed (Utada et al. 2007). The process can be adjusted by controlling the size and number of drops. Also, a large number of droplets can be encapsulated and stabilized within another droplet (Adams et al. 2012). Preparing more complex structures is generally more time consuming than preparing single emulsions.

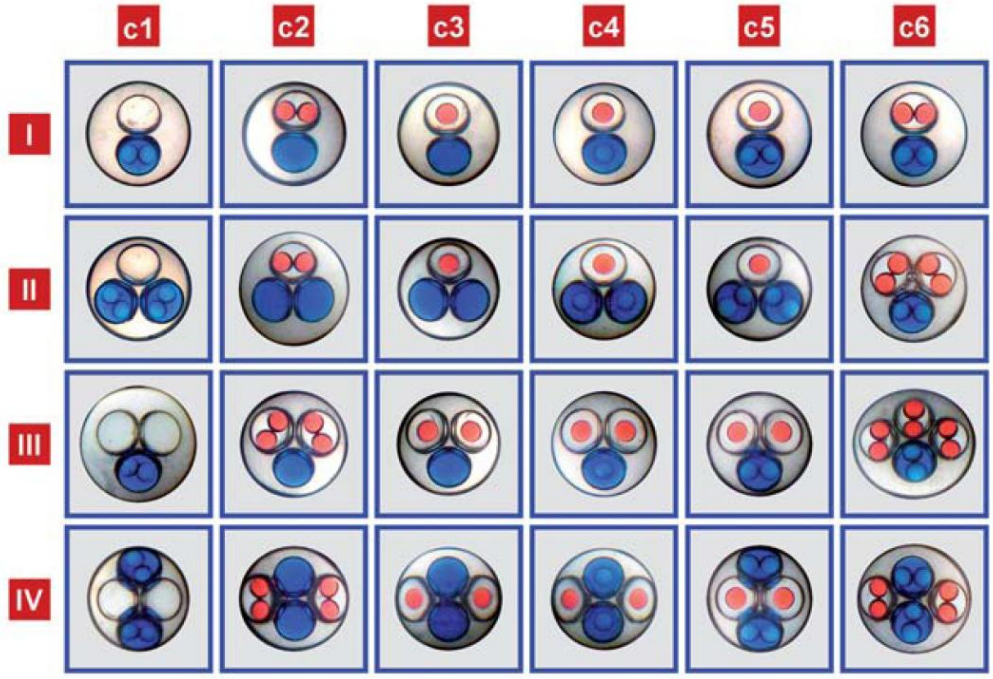

Figure 9. Multiple emulsion droplets with varying structures (Wang et al. 2011).

Besides repeating the structures of the device to obtain multiple emulsions, it is also possible to fabricate one-step emulsification of multiple concentric shells capillary microfluidic devices (Kim and Weitz 2011) (**Figure 10**). The formation of a stable coaxial interfaces and subsequent breakup provides a facile way to produce monodisperse multiple emulsion drops of high order and has potential as advanced microcapsules.

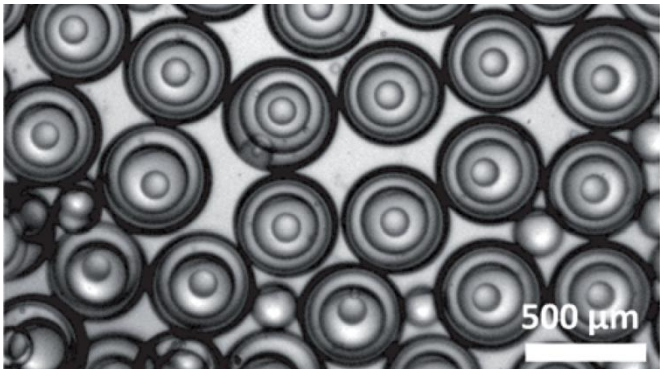

Figure 10. Advanced microcapsules manufactured by a single-step emulsification process (Kim and Weitz 2011).

Similar functions are achieved with different designs of PDMS devices (Abate and Weitz 2009) (**Figure 11**). The channels contain certain number of T-junctions. Engineering the channels carefully optimizes the drop formation and enables controlling the droplet size and production of the monodisperse droplets. Also, the droplet production process with PDMS devices is scalable and it is possible to specify the multiple emulsion order. As the devices are prepared lithographically, the wettability of the channels in the device can be alternated to optimal for the production of the multiple emulsions using hydrophobic or hydrophilic coating.

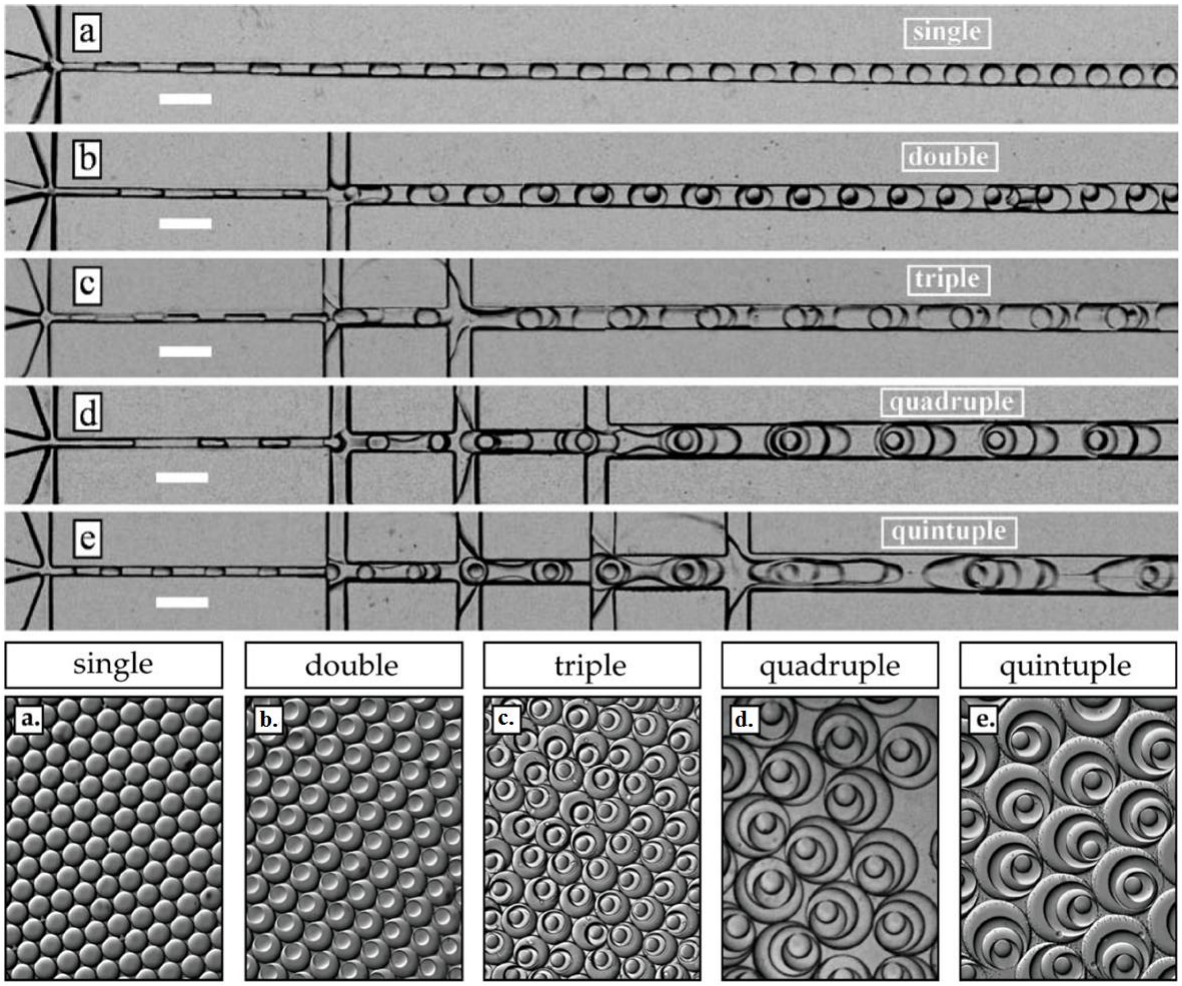

Figure 11. Channels in PDMS devices. By adding T-junctions multiple emulsions can be prepared, modified from (Abate and Weitz 2009). Photomicrographs of (a) single, (b) double, (c) triple, (d) quadruple and (e) quintuple emulsion drop maker arrays. The scale bars indicate 100 µm.

4.3 Applications of microfluidics

With microfluidic technology it is possible to independently choose the chemical compositions and structures of the prepared particles (Duncanson et al. 2012b). The modification of the particle properties is done with the selection of fluids to certain device design. With microfluidics synthesizing a range of microparticles with distinct composition and structure is possible. Next, the applications of microfluidics done at Weitz's laboratory at School of Engineering and Applied Sciences at Harvard University are described.

4.3.1 Microfluidics used to produce microparticles

Various polymeric microparticles as single emulsion droplets have been prepared with microfluidics, for example, using PLA in a flow-focusing device (Vladisavljević et al. 2012; Duncanson et al. 2012c). With these processes the monodispersity and the size of the particles are mainly adjusted with the phase flow rates. With PDMS devices the preparation of highly monodisperse, sub-micrometre conjugated polymer particles has also been reported (Kuehne and Weitz 2011). In addition to the flow rates, the particles size can be controlled by the polymer concentration with as small particles as 150 nm to 2 µm. As additional layer, usually W/O/W is added to the emulsification, and the process creates hollow polymer microspheres (Duncanson et al. 2012b; Liu et al. 2009). Double emulsion for preparing microspheres with ultra-thin shells can be created using biphasic flow in the inner capillary of the glass capillary devices combining co-flow and flow-focusing (Kim et al. 2011; Kim et al. 2013a)

Additional elements such as size-tunable pores can be added to the microparticles prepared as emulsion droplets by microfluidics (Duncanson et al. 2012c). Tunable active release mechanisms can also be added to the microparticles (Abbaspourrad et al. 2013). These particles are triggered by a plasticizing stimulus that indicates a phase change transition of the polymeric membrane from a solid form to a fluidized form. The absorption of the liquid plasticizing stimulus, a solid-to-liquid phase change is initiated within the capsule membrane. This enables controllable release kinetics as the cargo is actively driven out of the microcapsule through a defect at the particle shell. Tuning of the fluidity of the membrane is possible by altering the amount of plasticizing stimulus. Stimuli-responsive microcapsules that selectively release their contents through head-to-tail depolymerization of poly(phthalaldehyde) have been prepared with flow-focusing microfluidic technology (DiLauro et al. 2013). Poly(phthalaldehydes) depolymerize completely from head-to-tail in response to fluoride, which provides an amplified response to the applied chemical signal and the rate of the response can be tuned both by varying the length of the polymer and the thickness of the shell wall.

4.3.2 Thermosensitive products used in microfluidics

Adding another fluid input downstream by introducing an activator or accelerator for the gelation reaction can be used to produce different kinds of gels (Utada et al. 2007). Taking advantage of the fine control of the mixing fluids, creating a gel that shrinks in response to heat is also possible.

Droplet-based microfluidics can be used to produce thermosensitive poly(*N*-isopropylacrylamide) (pNIPAm) gel particles (Shah et al. 2008). In addition, with the microfluidic preparation technique controlling both outer dimensions and inner morphology of the particles is possible. These techniques are applicable for the synthesis of particles of a variety of chemical compositions and for the generation of higher order supraparticles using directed assembly of colloidal particles in droplets. Also, pNIPAm microparticles have been produced with microfluidics and these thermosensitive structures enable more accurate drug release properties for advanced drug delivery applications (Duncanson et al. 2012b).

4.3.3 Polymersomes used in microfluidics

A variety of polymersomes, vesicles with a membrane composed of a bilayer of amphiphilic block-co-polymers (Discher et al. 1999), have been prepared with the glass capillary devices. Multi-compartment polymersomes can be used for storing multiple drugs in a single carrier and for enabling simultaneous release of two active agents (Zhao et al. 2011). Polymersomes can be stabilized with hydrogel cores and induced UV-polymerization (Kim et al. 2013b). Polymersomes for triggered release can be produced using photo- and thermo-sensitive polymers (Amstad et al. 2012). Polymersomes with potential for extremely accurate content release can be created using a capillary microfluidic device using W/O/W double emulsion drops with the middle oil phase containing a mixture of thermoinsensitive amphiphiles, thermosensitive amphiphiles, and photothermal gold nanoparticles. Additionally, polymersomes can also be used as artificial cells in biomimetic studies to model protein expression and aggregation more effectively than it is possible to model with artificial cells produced with other methods (Martino et al.

2012). These artificial cell structures can be specifically modified with microfluidics to obtain optimized modelling properties. For example, in the study of Martino et.al in additional homopolymer layer was included in the shell to enhance stability and prevent protein aggregation into the shell structures.

4.3.4 Applications for cells using microfluidics

The encapsulation of single cells is possible with droplet based microfluidics (Köster et al. 2008). Thus, it is possible to encapsulate, incubate, and manipulate individual cells in picoliter aqueous drops in a carrier fluid and using drop-based microfluidics to create preconditions for single cell experiments (e.g., as screening for monoclonal antibodies). Microgel gelation for micrometer-sized hydrogel particles that contain living cells without using reaction involving free radicals can be produced with microfluidics (Rossow et al. 2012). Thus, the viability of the cells is ensured and the microfluidic technology offers additional advantages for cell cultural systems.

High-throughput analysis and sorting of single cells is also possible with microfluidics (Mazutis et al. 2013). Compartmentalization of single cells in droplets enables the analysis of proteins released from or secreted by cells, and thus, overcoming the limitations of traditional flow cytometry and fluorescence-activated cell sorting. The microfluidic systems are easily adapted for screening other intracellular, cell-surface or secreted proteins and for quantifying catalytic or regulatory activities.

4.3.5 Other applications of microfluidics

Fabrication of liquid crystals by making the middle fluid a liquid crystal mixed with chloroform, lowers its viscosity and makes it isotropic (Nelson 2002). After the shell is formed the chloroform evaporates and a shell of liquid crystal is produced. Predicting a variety of different defect structures results in making a shell of liquid crystal. Also, gas filled particles, bubbles and anti-bubbles can be manufactured with droplet-based microfluidic production (Duncanson et al. 2012a). The generation of water-in-water (w/w)

jets and emulsions by combining droplet microfluidics and aqueous two-phase systems is also possible (Shum et al. 2012).

Microfluidics can also be used for observing various phenomena, for example, the breaking behaviour of droplets (Chen et al. 2011) or buckling of colloidal capsules in order to create advanced capsule shells (Datta et al. 2012). Another application for microfluidic drop technologies is the use of the drops as isolated microreactors for chemical reactions (Utada et al. 2007). Water-based assays are most commonly used and suitable also for bio-assays. For example, synthesizing mesoporous hydroxyapatite is possible using double emulsion droplets as microreactors (Shum et al. 2009). Double emulsion droplets are highly versatile microreactors, because offer the combined advantages of both shielding the reactants and on-demand addition of reactants, and also enable simple visualization of the hydroxyapatite formation process as well as control over the porosity in the hydroxyapatite being synthesized. Stabilizing the drops against coalescence while preventing any of the contents of the drops from dissolving in the continuous phase is a challenge when preparing these microreactors.

Scaling up of the materials produced by microfluidics is possible up to the range of a few kilograms per day (Utada et al. 2007). The scaling up possibility is based on the use of a large number of the aligned capillaries in PDMS devices, operating in parallel and the primary applications for such encapsulation materials would likely be for high-value-added materials.

5. AIMS OF THE STUDY

The research conducted in this master's thesis had four main goals:

(1) To apply droplet based microfluidics on polymeric microsphere preparation process, and thus, to employ the advantages that microfluidic technology offers in terms of producing stable, monodisperse double emulsion (W/O/W) droplets with high encapsulation efficiency of therapeutic molecules.

(2) To determine suitable formulations for microfluidics and to study the general limitations concerning the formulations, as well as to adjust them to the use with biocompatible and biodegradable materials.

(3) To control the preparation process of the formulations and to establish a stable and precise preparation system, and thus, to create a new paradigm for microsphere production and to produce sophisticated droplets that are superior to those manufactured with conventional bulk methods.

(4) To characterize the droplets, to prove their quality and to load the droplets with therapeutic proteins in order to create templates for enhanced oral protein drug delivery using the microfluidic process developed as mild processing option suitable for protein drug product preparation.

II EXPERIMENTAL PART

6. MATERIALS AND METHODS

The following reagents or solvents were used in the experimental part of this work (**Table 2**). The reagents or solvents are listed with the essential information, purity (if provided) and manufacturer.

Table 2. List of reagents and solvents used in this work.

Reagent or solvent	Purity	Manufacturer
Trimethoxy(octadecyl)silane	90 %	Sigma-Aldrich, U.S.
2-[methoxy(polymethyleneoxy)propyl] 9-12 trimethoxysilane	90 %	Gelest Inc., Netherlands
Poly(vinyl alcohol) (PVA) 87-89 % hydrolyzed, Mw 13,000–23,000		Sigma-Aldrich, U.S.
Poly(D,L-lactide-co-glycolide) (PLGA) 85:15, Mw 50,000–70,000		Sigma-Aldrich, U.S.
Poly(D,L-lactide-co-glycolide) (PLGA) 50:50, i.v. 0.5–0.65		Polysciences Inc., U.S.
Poly(L,D-lactic acid) (PLA), i.v. 0.2		Polysciences Inc., U.S.
Polyethylene glycol (PEG) 6000, Mw 5,000–7,000		Fluka Analytical, Germany
Polycaprolactone (PCL), Mw 70,000–90,0000		Sigma-Aldrich
Tween® 20		Sigma-Aldrich, U.S.
Dichloromethane (DCM)	≥ 99.8 %	Sigma-Aldrich
Ethyl acetate (EtOAc)	≥ 99.5 %	Sigma-Aldrich, U.K.
Sodiumchloride (NaCl)	>99 %	BHD, U.S.
	≥ 99.5 %	Fluka Analytical, Germany
Nile red		Sigma-Aldrich, Germany

FITC-dextran, Mw 10,000		Molecular Probes, U.S.
3,4,9,10-perylene-tetracarboxylic dianhydride	97 %	Sigma-Aldrich, Germany
β-galactosidase (from *Aspergillus oryzae)* 10.3 units/mg		Sigma-Aldrich, Japan
Salbutamol sulphate		Alfa Aesar, U.S.
Bovine serum albumin (BSA)	≥ 96 %	Sigma-Aldrich, Germany
2-nitrophenyl β-D-galactopyranoside (ONPG)	>98 %	Sigma-Aldrich, Switzerland
2-mercaptoethanol	≥ 99 %	Sigma-Aldrich, Germany
Magnesium chloride hexahydrate ($MgCl_2$)	≥ 99 %	Sigma-Aldrich, Japan
Sodium carbonate ($NaCO_3$)	≥ 99.5 %	Sigma-Aldrich, U.S.
Methanol (MeOH) HPLC gradient grade	≥ 99.9 %	BDH, EC
Acetonitrile (ACN) HPLC gradient grade	≥ 99.9 %	BDH, EC
Trifluoroacetic acid (TFA)	99 %	Sigma-Aldrich, Germany
Potassiumdihydrophosphate (KH_2PO_4)	99.4 %	Mallinckrodt, U.S.
	> 99.5 %	Riegel-de Haën, Germany
Potassium chloride (KCl)	≥99 %	Sigma-Aldrich, Germany
Disodium phosphate monobasic (Na_2HPO_4)	>98 %	Sigma-Aldrich, Germany
Sodiumhydroxide (NaOH)	≥ 98 %	Sweden
Hydrochloric acid (HCl) 1M		BDH, France

The following solutions were prepared for the experiments. The collection media for the emulsion droplets was made with 0.2922 g of NaCl in 100 mL of MQ-water (50 mM) or with 0.911 g of NaCl in 100 mL of water (156 mM).

The activity assay solution was prepared as a mixture containing 0.05 mL of 68 mM ONPG solution, 0.05 mL of 30 mM $MgCl_2$ solution, 0.05 mL of 3.36 M 2-mercaptoethanol solution and 1.3 mL of 100 mM saline phosphate buffer solution (PBS). PBS buffer consisted of 0.8 g of NaCl, 0.02 g of KCl, 0.144 g of Na_2HPO_4 and 0.024 g of KH_2PO_4 in 100 mL of MQ-water. 1 M HCl was used to adjust the pH of the solution to 7.4.

The mobile phases used in the HPLC experiments were as follows:

0.1 % TFA in MQ-water was prepared by mixing 1 mL of TFA in MQ-water and filtering before use. PBS (25 mM) was prepared by adding 3.9 g of KH_2PO_4 to 1000 mL of water, adjusting the pH to 3.0 and filtering the solution before use. Buffers for dissolution tests were prepared according to the European Pharmacopoeia, 7^{th} edition. PBS (100 mM) was prepared by mixing 0.68 g of KH_2PO_4 and 0.14 g of NaOH to 100 mL of water and adjusting the pH to 7.2. HCl buffer (100 mM) was prepared by mixing 8.5 mL of 1 M HCl and 0.27 g of NaCl in 100 ml of MQ-water and adjusting the pH to 1.2.

6.1 Microfluidic devices and production of droplets

In the following experiments droplets were produced with three different designs of microfluidic glass capillary devices. Each design was optimized to prepare the desired type of droplets with certain flow properties. First, double emulsion droplets were produced with two tip glass capillary device designed for double emulsions. Then, a single emulsion was produced with one tip glass capillary device. Finally, double emulsion droplets were produced with glass capillary device designed for biphasic flow.

Devices were assembled on glass slides and they consisted of square capillaries (outer diameter = 1.5 mm; inner diameter = 1.05 mm; Harvard borosilicate square tubing; Atlantic International Technology, U.S.) and cylindrical capillaries (outer diameter 1.0 mm; inner diameter 580 µm; borosilicate glass tubing; World Precision Instruments Inc., U.S.). Cylindrical capillaries were placed inside the square capillaries and aligned with them. Capillaries were glued on the glass slide with epoxy glue and the needles (Type 304 SS Dispensing Needle 20 Gauge, U.S.) were set at the ends of the capillaries as inlets and also glued on the glass slides (**Figure 12**).

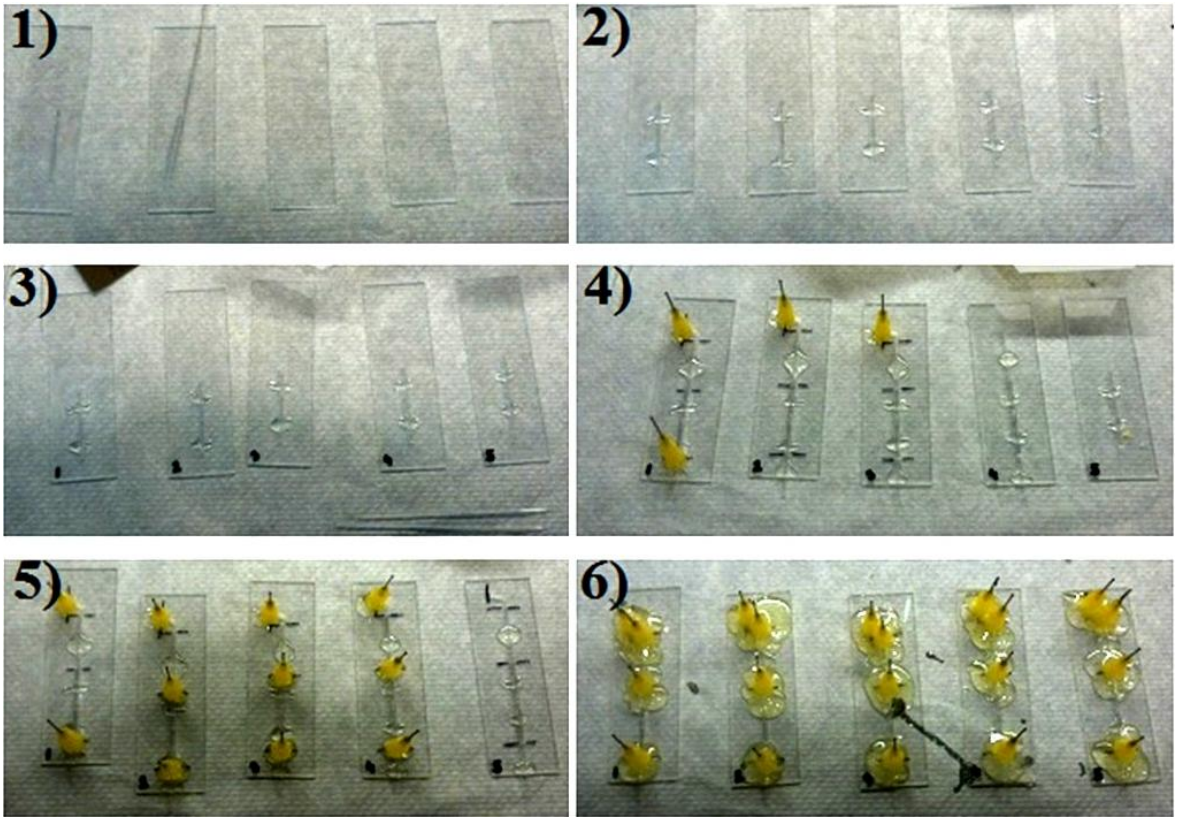

Figure 12. Assembling process of microfluidic glass capillary device for biphasic flow; 1) square capillaries are cut; 2) square capillaries are glued on the glass slide; 3) cylindrical capillaries are pulled and placed in the square capillaries and after that the alignment is glued on the glass slide; 4) stretched cylindrical capillary for inner phase is added; 5) needles as inlets are added; and 6) glued carefully on the glass slide.

Cylindrical capillaries were pulled with a Flaming/Brown micropipette puller (Model P-97, Sutter Instrument Co., U.S.) to obtain tapered tips and to form tips of desired diameter. Tips were formed under the following parameters of the micropipette puller: heat = 260 C°; pull = 3 N/m; velocity = 3 m/s; and time = 150 s. The largest diameter possible to obtain with the micropipette puller was 40 μm. When larger diameters were needed the tips were carefully sanded with fine sand paper (P2000).

Cylindrical capillaries were coated with hydrophilic or hydrophobic coating, corresponding to whether they contained the water or the oil phase. Tips were dipped in either the hydrophobic trimethoxy(octadecyl)silane (Sigma-Aldrich, U.S.) or the hydrophilic 2-[methoxy(polymethyleneoxy)propyl] 9-12 trimethoxysilane (Gelest Inc., Netherlands) and were dried with pressurized air after 30 minutes of the coating process.

Emulsion phases (either W/O or W/O/W) were pumped into the glass capillary devices with syringes and Harvard pumps (Harvard Apparatus Hollston, U.S.). Syringes were attached to the inlets with plastic tubing (PE5 0.86 mm x 1.32 mm, Scientific Commodities Inc., U.S.). Flow rates were controlled with Harvard pumps starting with higher flow rates in order to form the interphase in the correct location and then gradually reduced to start the droplet formation. Flow rates were optimized for each formulation and each device.

The drop formation was observed with optical microscopes (Leica, Germany and Edmund Scientific, Germany) and high speed cameras (Phantom High Speed Cameras: V7, V7.3, V9; Vision Research Inc., U.S.) (**Figure 13**). Recordings with the cameras were done with 250 time deceleration, the videos showing the droplet formation phenomenon 250 times slower than in actuality.

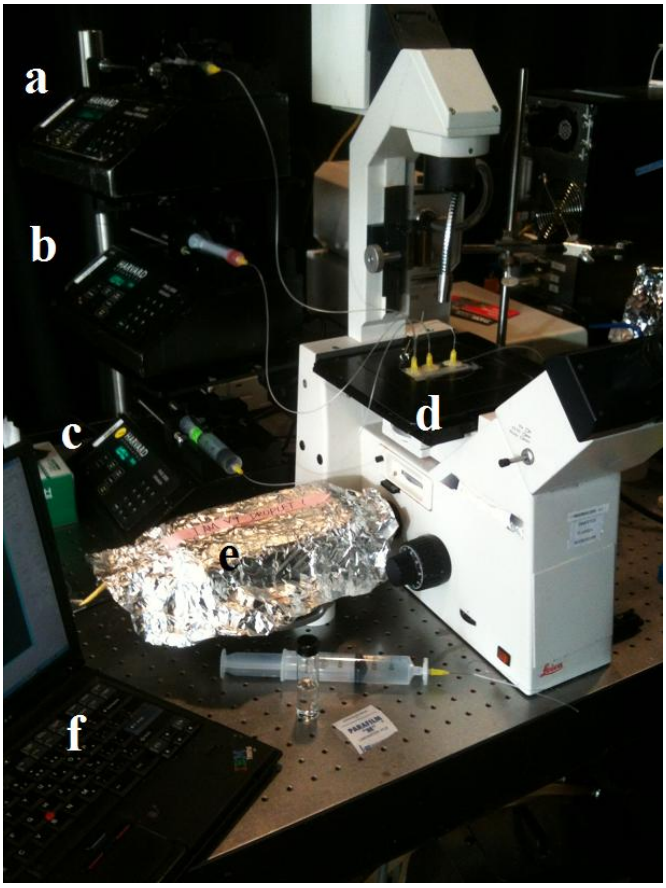

Figure 13. Microfluidic droplet production: (a)–(c) Harvard pumps with syringes containing inner, middle and outer phases; (d) microfluidic device and optical microscope; (e) high speed camera (covered with tin foil); and (f) computer for monitoring the process.

The phases were filtered (Acrodisc Syringe Filter, 0.45 µm Super Membrane, Life Sciences, U.S.) before the preparation process. Glass capillary devices were washed with MQ-water before the preparation process to remove all the air from the capillaries. Droplet formation was monitored throughout the process and possible problems were recorded.

6.2 Formulation screening study

6.2.1 Double emulsion with two tip glass capillary devices

The two tip glass capillary device consisted of three inlets and two tips in the middle of the device (**Figure 14**). This device combined co-flow and flow-focusing and it was ideal for producing monodisperse double emulsion droplets with rather thick shell as a continuous process. The inner phase flows through the cylindrical capillary with smaller tip, and the middle and outer phases flow to opposite directions in the square capillary. Droplets were collected in a vial containing water or collection media with osmolarity corresponding to the osmolarity of the inner phase. Osmolarity was adjusted by measuring the osmolarity of the inner phase with an osmometer (The Advanced Micro Osmometer, Model 3300, Advanced Instruments Inc., U.S.).

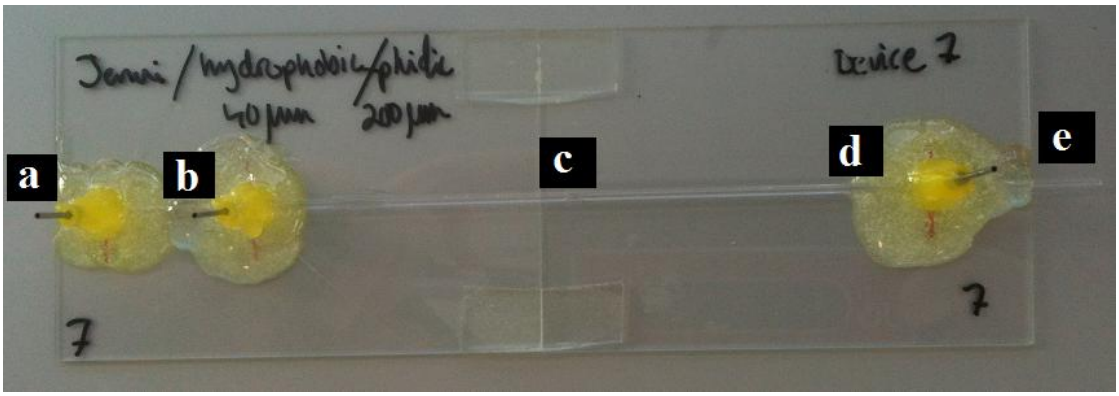

Figure 14. Two tip glass capillary devices for preparing double emulsion droplets: (a) inlet for the inner phase; (b) inlet for the middle phase; (c) hydrophobic and hydrophilic tips 40 and 200 µm, respectively, with the latter being the collection tube; (d) inlet for the outer phase; and (e) outlet for double emulsion droplets.

Within the square glass capillary, the tips of the cylindrical capillaries were at 80 µm distance from each other (**Figure 15**). Flow rates were adjusted so that the interphase was formed at the tip of the cylindrical capillary of the inner phase. The flow rates were varied according to the formulation used as follows: 500–10,000 µL/h in the outer phase, 75–8,000 µL/h in the middle phase, and 50–5000 µL/h in the inner phase.

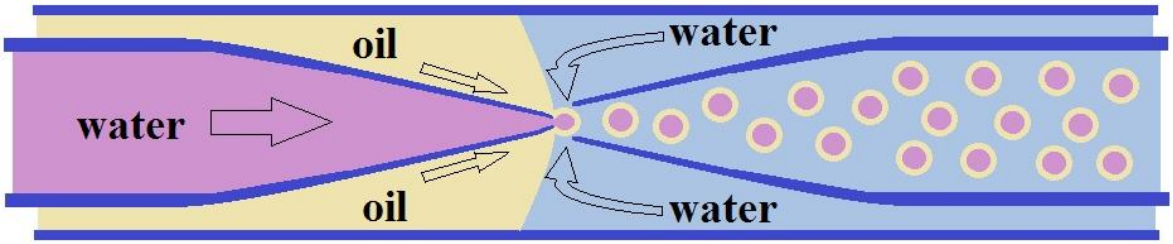

Figure 15. Flows of the phases in the two tip glass capillary devices for double emulsion formation (W/O/W). The inner phase flows through the cylindrical capillary, and the middle and outer phase in the square capillary where they form an interphase at the tip of the cylindrical capillary of the inner phase. Double emulsion droplets are formed at this interphase.

The droplet preparation process started with the higher flow rates and the rates were gradually decreased to very slow rates to allow the jetting to dripping transition. In the beginning of the process, higher flow rates were used to form a jet where the inner phase was jetting inside the middle phase.

The formulation screening process was started with PLGA (85:15, M_w = 50,000–70,000) in the middle phase and PVA (M_w =13,000-23,000, 87–89 % hydrolyzed) in the inner and in the outer phase (**Table 3**). The viscosities of the phases were attempted to adjust to correspond with each other by gradually reducing the quantity of PLGA in the middle phase.

Table 3. Formulations 1–4 of PLGA and PVA.

#	Outer phase	Middle phase	Inner phase
Formulation 1	5 % PVA in water	0.1 % PLGA in DCM	5 % PVA in water
Formulation 2	5 % PVA in water	0.02 % PLGA in DCM	5% PVA in water
Formulation 3	5 % PVA in water	0.01 % PLGA in DCM	5% PVA in water
Formulation 4	5 % PVA in water	0.005 % PLGA in DCM	5% PVA in water

The PLGA used above was then changed to another PLGA (50:50; M_w = 50,000–65,000, Polyscience Inc., U.S.) (**Table 4**). The concentration similar to PLGA of 85:15 was tested and the viscosity of the outer and inner phases, first were increased, then the viscosity of the inner phase decreased, and finally, attempted to stabilize with PEG 6000 (M_w = 5,000–7,000, Sigma-Aldrich, Germany).

Table 4. Formulations #5–13 of PLGA and PVA.

#	**Outer phase**	**Middle phase**	**Inner phase**
Formulation 5	5 % PVA in water	0.06 % PLGA in DCM	5 % PVA in water
Formulation 6	5 % PVA in water	0.03 % PLGA in DCM	5 % PVA in water
Formulation 7	10 % PVA in water	0.06 % PLGA in DCM	10 % PVA in water
Formulation 8	10 % PVA in water	0.03 % PLGA in DCM	10 % PVA in water
Formulation 9	10 % PVA in water	0.03 % PLGA in DCM	2 % PVA in water
Formulation 10	10 % PVA in water	0.03 % PLGA in DCM	0.5 % PVA in water
Formulation 11	10 % PVA in water	0.03 % PLGA in DCM	only water
Formulation 12	10 % PVA in water	0.014 % PLGA in DCM	only water
Formulation 13	10 % PVA in water	0.014 % PLGA in DCM	10 % PVA and PEG (1:4) in water

Alternative polymers were tested for the outer and the inner phases (**Table 5**). PLGA concentrations in the inner phase were kept minimal and the effect of Tween® 20 (Sigma-Aldrich, U.S) in the outer phase was also tested. PCL (M_w = 70,000–90,000, Sigma-Aldrich, U.S.), which is soluble in nontoxic ethyl acetate (Sigma-Aldrich, U.S.), was tested as an alternative polymer in the middle phase. The viscosity of the inner phase was kept minimal by not adding polymers.

Table 5. Formulations #14–17 containing Tween and PCL as alternative polymers.

#	Outer phase	Middle phase	Inner phase
Formulation 14	0.1 % Tween 20 in water	0.014 % PLGA in DCM	10 % PVA and PEG (1:4) in water
Formulation 15	0.1 % Tween 20 in water	0.014 % PLGA in DCM	only water
Formulation 16	10 % PVA in water	2 % PCL in DCM	only water
Formulation 17	10 % PVA in water	2 % PCL in ethyl acetate	only water

Finally, PLA (Polysciences Ins., U.S.) was chosen for the polymer of the middle phase (**Table 6**). A slight increase of the viscosity for the inner phase was also tested.

Table 6. Formulations #18–20 containing PLA.

#	Outer phase	Middle phase	Inner phase
Formulation 18	10 % PVA in water	5 % PLA in DCM	only water
Formulation 19	10 % PVA in water	5 % PLA in DCM	1 % PVA in water
Formulation 20	10 % PVA in water	5 % PLA in DCM	0,5 % PVA in water

6.2.2 Double emulsion with combining microfluidics and bulk method

The most simple microfluidic production of droplets is the one with tip glass capillary device producing single emulsion droplets using flow-focusing (**Figure 16**). Droplet production was easy to achieve and it was constant despite the slight changes in the flows. To obtain double emulsion droplets this system was connected to the outer phase outside the microfluidic system. The single emulsion droplets were collected to a vial containing

the outer phase and mixed there with a magnetic stirring in order to form double emulsion droplets.

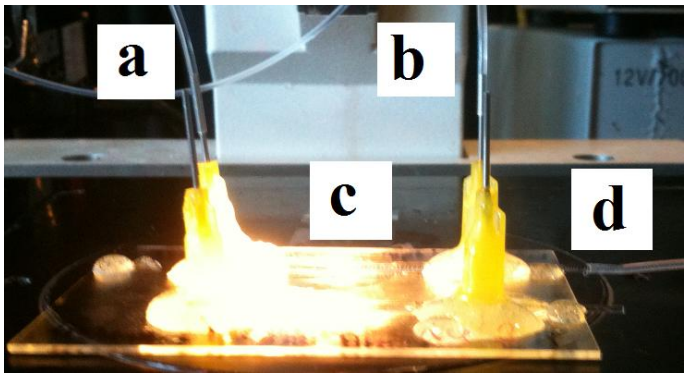

Figure 16. One tip glass capillary device for formation of single emulsion (W/O). The device consists of: two inlets for the inner phase (a) and two for the outer phase (b); the hydrophobic tip (c); and the outlet for the droplets (d).

Single emulsion droplets were rather easily formed with a wider range of flow rates (**Figure 17**). The flow rates in the oil phase were 3,000–20,000 µL/h and 100–2,000 µL/h in the water phase. The process started with higher flow rates and they were adjusted so that the interphase was formed at the tip of the collection capillary.

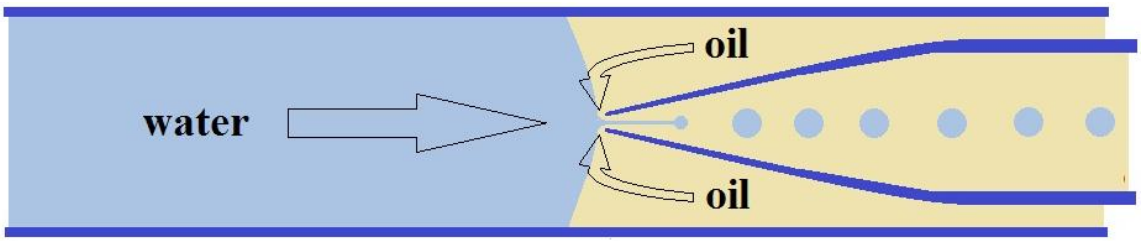

Figure 17. Flows of the phases in one of the tip glass capillary device for single emulsion (W/O). Phases flow in the square capillary and form an interphase at the tip of the collection tube. Single emulsion droplets form from the innerphase as they move through jetting to dripping transition at the beginning of the collection capillary.

Formulation screening for this method was started with lower concentrations, more similar to the bulk formulations found in the literature (Tomar et al. 2011; Coccoli et al. 2008) (**Table 7**). PCL was used in the inner phase and the concentrations varied in order to form stable W/O emulsions. Before mixing with the outer phase the stability of the W/O droplets was followed. Also, PLGA and PLA (W/O) single emulsions were tested, but the droplets were not stable due to the fast evaporation of DCM. Out of three polymers, PCL was the only one that dissolved in ethyl acetate. Dissolution of PCL was accelerated by heating ethyl acetate to 40 °C and stirred for 1 hour.

Table 7. Formulations #21–23 of PCL with low concentrations of PVA.

#	Outer phase	Middle phase	Inner phase
Formulation 21	0.5 % PVA in water	3 % PCL in ethyl acetate	0.5 % PVA in water
Formulation 22	0.5 % PVA in water	1.7 % PCL in ethyl acetate	0.5 % PVA in water
Formulation 23	1 % PVA in water	3 % PCL in ethyl acetate	0.5 % PVA in water

The quantities of PVA in the outer and in the inner phase were increased to stabilize the droplets (**Table 8**). The PVA concentrations of the outer phase were adjusted so that the mixing rate, and thus, the droplet formation, was ideal. The inner and middle phases were adjusted optimally for stability and flow in the microfluidic device. Finally, the fluorescent agent FITC-dextran (M_w = 10,000, Molecular Probes, U.S.) was added to the inner phase for the confocal studies for the particles. Particles prepared with the combination method were also collected, washed and dried.

Table 8. Formulations #24–28 of PVA and PCL.

#	Outer phase	Middle phase	Inner phase
Formulation 24	1 % PVA in water	3 % PCL in ethyl acetate	5 % PVA in water
Formulation 25	10 % PVA in water	3 % PCL in ethyl acetate	1 % PVA in water
Formulation 26	5 % PVA in water	3 % PCL in ethyl acetate	1 % PVA in water
Formulation 27	1 % PVA in water	3 % PCL in ethyl acetate	1 % PVA in water
Formulation 28	1 % PVA in water	3 % PCL in ethyl acetate	1 % PVA in water and FITC dextran

Alternatively, also Tween 20 was tested and the effect on the particle size was evaluated (**Table 9**). Two different concentrations were tested.

Table 9. Formulations #29 and 30 of Tween, PCL and PVA.

#	Outer phase	Middle phase	Inner phase
Formulation 29	1 % Tween 20 in water	3 % PCL in ethyl acetate	1 % PVA in water
Formulation 30	10 % Tween 20 in water	3 % PCL in ethyl acetate	1 % PVA in water

6.2.3 Double emulsion with biphasic flow

In the glass capillary devices with biphasic flow, the inner and middle phase flow is the same cylindrical capillary (**Figure 18**). An extra cylindrical capillary is placed in the cylindrical capillary for the middle phase. This capillary is stretched using a flame. The inner phase flowed through this capillary into the middle phase forming water drops in the middle phase.

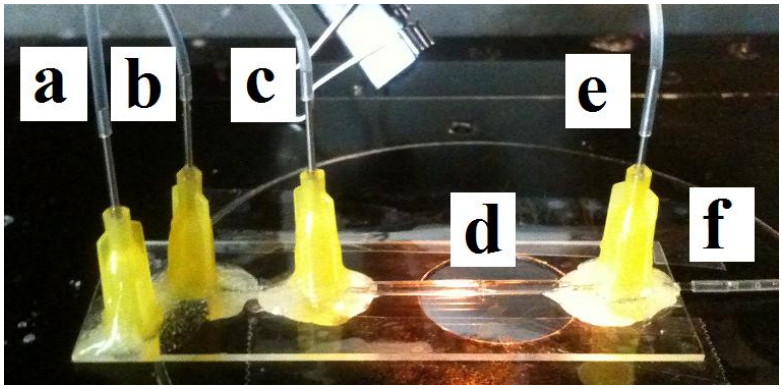

Figure 18. Glass capillary device for biphasic flow: (a) inlet for the inner phase; (b) inlet for the middle phase; (c) inlet for the outer phase; (d) hydrophobic and hydrophilic tips of 100 and 150 µm, respectively, with the latter being the collection tube; (e) closed inlet; and (f) outlet for the droplets.

In this device it was essential that the cylindrical glass capillary for the inner and the middle phase was well coated hydrophobic in order to prevent the inner and the middle phase from reversing and forming oil drops in water, instead of forming water drops in oil as desired. As the water drops surrounded with oil reached the tip, double emulsion droplets with ultrathin shells were formed (**Figure 19**). With the biphasic flow range of flow rates used was smaller. The inner and middle phases flowed always at the same rate of 1500, 1000 or 500 µL/h. The flow rate of the outer phase was either 3000 or 2500 µL/h.

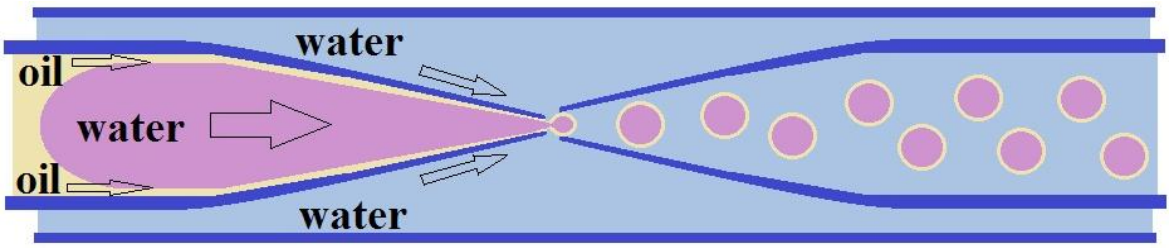

Figure 19. Flows of the phases in the glass capillary device for biphasic flow for double emulsion droplets (W/O/W). The inner and middle phases' flow in the first cylindrical capillary. Stretched capillary is inserted into the cylindrical capillary and the inner phase flows through it, forming water phase droplets into the oil phase. This forms droplets with ultrathin shells as they move to the collection capillary and interact with the outer phase flowing from the square capillary.

In between the water droplets, the leftover oil formed O/W single emulsion droplets. Double emulsion droplets were separated from the single emulsion droplets as they were collected into the vial with collection media (NaCl in water). Double emulsion droplets sink into the bottom of the vial and single emulsion droplets, with only ethyl acetate inside, float to the surface of the collection media due to their lower density. The osmolarity of the collection media corresponded to the osmolarity of the inner phase. The osmolarity of the inner phase was studied as described earlier.

Formulation screening for this device design started with PLA in DCM and was continued with PCL in the middle phase (**Table 10**). DCM was not suitable for the glass capillary device with biphasic flow, and thus, PCL was the only ethyl acetate soluble polymer used in these formulations. The viscosity of the inner phase was kept high in order to co-flow with the viscose middle phase.

Table 10. Formulations #31–33 of PVA, PLA and PCL.

#	**Outer phase**	**Middle phase**	**Inner phase**
Formulation 31	10 % PVA in water	5 % PLA in DCM	10 % PVA and PEG 6000 (1:4) in water
Formulation 32	10 % PVA in water	5 % PCL in ethyl acetate	10 % PVA and PEG 6000 (1:4) in water
Formulation 33	5 % PVA in water	2.5 % PCL in ethyl acetate	10 % PVA and PEG 6000 (1:4) in water

Concentrations of polymers were further adjusted and optimized for stable particle production. Two fluorescent agents were used: hydrophilic FITC-dextran (M_w = 10,000, Molecular Probes, U.S.) for the inner phase and hydrophobic 3,4,9,10-perylene-tetracarboxylic dianhydride (Sigma-Aldrich, China) for the middle phase (**Table 11**). The fluorescent agents were chosen so that the excitation-emission spectra did not overlap.

Table 11. Formulations #34–38 of PVA, PLA and PCL. Final optimization of the concentrations and formulations with fluorescent agents.

#	Outer phase	Middle phase	Inner phase
Formulation 34	5 % PVA in water	3 % PCL in ethyl acetate	10 % PVA and PEG (1:4) in water
Formulation 35	5 % PVA in water	5 % PCL in ethyl acetate	10 % PVA and PEG (1:4) in water
Formulation 36	10 % PVA in water	3 % PCL in ethyl acetate	10 % PVA and PEG (1:4) in water
Formulation 37	5 % PVA in water	3 % PCL in ethyl acetate	10 % PVA and PEG (1:4) and FITC-dextran in water
Formulation 38	5 % PVA in water	3 % PCL in ethyl acetate and perylene	10 % PVA and PEG (1:4) and FITC-dextran in water

Particle loading was done first with a model protein β-galactosidase (from *Aspergillus oryzae*, Sigma-Aldrich, Japan) (**Table 12**). Due to the dextrin used to stabilize the protein, the water solubility was low, and thus, changes in the inner phase were attempted.

Table 12. Formulations #39–42 of PVA and PCL loaded with β-galactosidase.

#	Outer phase	Middle phase	Inner phase
Formulation 39	5 % PVA in water	3 % PCL in ethyl acetate	0.5 % PVA and 5 % β-galactosidase in PBS
Formulation 40	5 % PVA in water	3 % PCL in ethyl acetate	1.5 % PVA and PEG (1:2) and 5 % β-gal in PBS
Formulation 41	5 % PVA in water	3 % PCL in ethyl acetate	10 % PVA and PEG (1:4) and 0.4 % β-gal in water
Formulation 42	5 % PVA in water	3 % PCL in ethyl acetate	10 % PVA and PEG (1:4), 0.4 % β-gal in water

The properties of the inner phase were further determined with rheology tests. Rheological measurements were performed for the inner phases of formulations #37, 38 and 39. The instrument used for the rheology measurements was Ares-G2 Rheometer (TA Instruments Inc., U.S.). Ares G2 was chosen for this experiment, since it had separate motor and

transducer, and thus, enables measuring stress independently of the applied shear deformation. Two parallel measurements of 3 min for each sample were conducted. Optimization of the inner phase was continued and the phase further developed by increasing the quantity of PVA and PEG (**Table 13**).

Table 13. Formulation #43 and 44 for increasing the viscosity of the inner phase.

#	Outer phase	Middle phase	Inner phase
Formulation 43	5 % PVA in water	3 % PCL in ethyl acetate	20 % PVA and PEG (1:4) in water
Formulation 44	5 % PVA in water	3 % PCL in ethyl acetate	20 % PVA and PEG (1:4) and FITC-dextran in water

Finally, the inner phase with 20 % PVA and PEG was loaded with 2.5 % of salbutamol sulphate (Alfa Aesar, U.S.) and 1 % of bovine serum albumin (Sigma-Aldrich, Germany) (**Table 14**).

Table 14. Formulations #45 and 46 loaded with salbutamol sulphate and bovine serum albumin.

#	Outer phase	Middle phase	Inner phase
Formulation 45	5 % PVA in water	3 % PCL in ethyl acetate	20 % PVA and PEG (1:4) 2.5 % salbutamol in water
Formulation 46	5 % PVA in water	3 % PCL in ethyl acetate	20 % PVA and PEG (1:4) 1 % BSA in water

Drying of the particles was also attempted. However, a suitable drying method was not found and only very small quantities of the particles were dried.

Double emulsion (W/O/W) with only bulk method was prepared as a reference. The emulsion consisted of 10 % PVA and PEG (1:4) and 0.4 % (w/w) β-galactosidase in the inner phase, 3 % PCL in ethyl acetate in the inner phase and 5 % PVA in the outer phase.

The emulsion was produced with sonication (Ultrasonic processor, Ace Glass, U.S.). The quantities of the phases were in the ratio 1:10:100.

6.3 Characterization of the microspheres

6.3.1 Particle size

The particle size was determined by optical microscopy and diameter measurements. Diameter measurements were done with Image J software for scientific image analysis (National Institutes of Health, U.S.) and measured according to 1 mm scale for the optical microscope. The average diameter and standard deviation were also calculated. The Student's *t*-test was conducted to compare the batches, and thus, possible significant statistical differences evaluated.

The particle size was measured from the collected particles of 5 batches of formulations containing 5 % PVA in the outer phase, 3 % PCL in the middle phase and either 10 or 20 % of PVA and PEG (1:4) in the inner phase (formulations #34, 37, 42 and 43; n = 100). As a reference, also the particle size of the droplets prepared with bulk method was determined (n = 100).

In addition, the particle size during the microfluidic preparation process was determined (formulations #37 and 42; n = 100) and compared with the collected particles from corresponding batches. Also, the particles sizes of a small batch of dried particles were measured.

The shell thickness of two batches of the particles during the preparation process (n = 100) and after collection (n = 100) was measured from the formulation #38 as described above. The shell thickness was determined with optical microscope (Leica, Germany) during the preparation process and with confocal microscope (Leica Microsystems CMS GmbH, Germany) from the collected particles. The ratio of the shell thickness and diameter of the whole particle was determined and evaluated.

6.3.2 Number of successful double emulsion droplets

One of the parameters that determined whether an emulsification process was working properly is the number of successful double emulsion droplets. The number of droplets containing the inner phase was determined with confocal microscope (Leica Microsystems CMS GmbH, Germany). The content of the droplets was examined from 3 batches of formulation #37, where the inner phase was made fluorescent with FITC-dextran (M_w = 10,000, Molecular Probes, U.S.; n = 200). The percentage of droplets containing the inner phase was calculated. As a reference, double emulsion with bulk method was prepared with FITC-dextran in the inner phase and the number of successful double emulsion droplets was calculated (n = 200).

6.3.3 Short time stability

Short time stability studies were conducted observing variation in the particle sizes with optical microscopy and Image J software as described above (6.3.1). Particles were measured (n = 100) and the Student's t-test used to evaluate whether the variation was statistically significant. Batches for the short time stability tests were stored in a refrigerator at 8 °C.

Three batches (formulations #34 and 41) were monitored for 4 weeks and the samples were taken when the time elapsed was 0, 1, 3, 7, 14, 21 and 28 days (with exception to earlier the third small batch n = 50, other two n = 100). Two batches (formulations #34 and 37) were monitored for 6 weeks and the samples were taken when the time elapsed was 0, 28, 35 and 42 days. The batch size was the main limiting factor for monitoring the stability at longer times or more frequently. For reference, the stability of bulk emulsion droplet was monitored and the samples were taken when the time elapsed was 0, 1 and 3 days. In addition, the stability of the dried particles was also monitored from a small batch for 28 days.

6.3.4 Surface properties of the particles

Topographical properties of the particles were studied with SEM microscope (Carl Zeiss AG, EVO 55 Environmental SEM, Germany), as shown in **Figure 20**. EVO series was chosen because it has a wet stage system that allowed taking pictures of the sample in liquid without the need of coating, freeze-drying or preparing the sample in any method. With polymer microspheres the coating easily damages the particle surface and influences the quality of the results. The wet stage method was the most suitable for small particle batches and the original state of the outer shell was preserved. With this method the chamber is cooled down using liquid nitrogen as also in other SEM methods. The main difference was that the conditions in the chamber were set to maintain water vapour in the chamber. The vapour present in the chamber made obtaining the images possible.

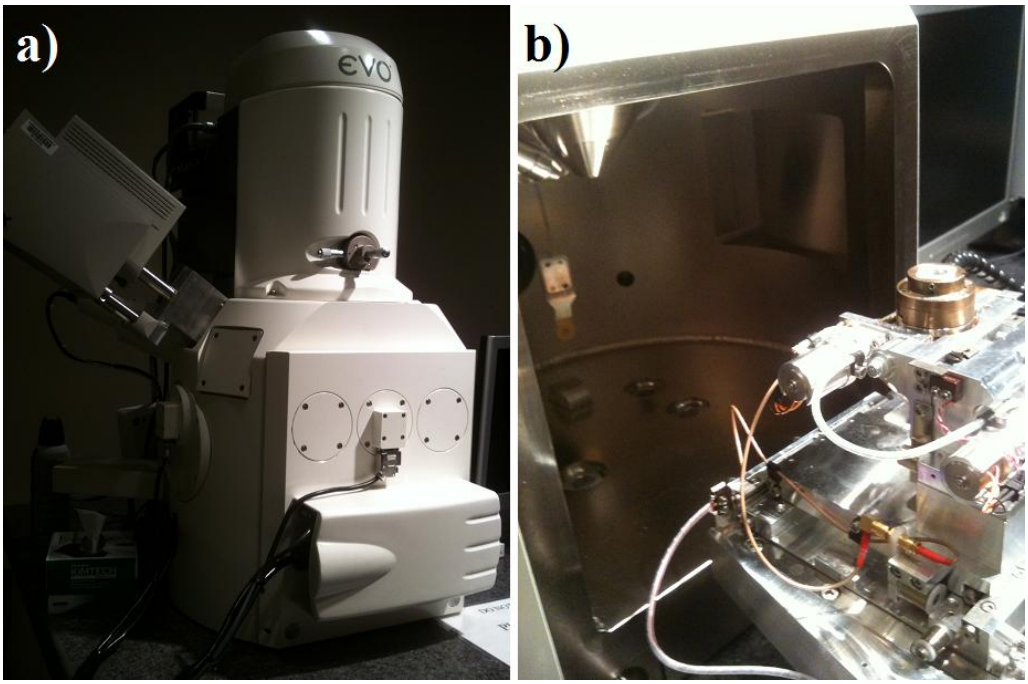

Figure 20. (a) EVO 55 Environmental SEM microscope, and (b) chamber and the wet stage.

The sample used was the particles with 0.4 % loading of β-galactosidase. The sample was placed on a paper platform in the collection media with a pipette. The chamber was cooled

with liquid nitrogen to water vapor state and the electric beam was run through upper and lower aperture of 100 and 500 µm.

6.3.5 Encapsulation efficiency

The encapsulation efficiency was determined for PCL particles loaded with 0.4 % β-galactosidase, 2.5 % salbutamol and 1 % BSA (formulations #42, 45 and 46). Samples were taken from the supernatant of 3 different batches of each formulation. Samples of 1 mL were taken immediately after the droplet preparation process was completed. Batches of formulation #44 containing 2.5 % of salbutamol were additionally sonicated in an ultrasound bath (Fritsch, Ultrasonic-cleaner, Laborette 17, Germany) for 3 h to determine the total quantity of salbutamol released. Samples were taken immediately after the sonication.

Samples of β-galactosidase were analyzed with modified activity assay (altered Sigma-Aldrich protocol). 0.1 mL of the sample was added to the ONPG solution and the mixed solution was allowed to react at 37 °C for 10 min. After that the reaction was stopped with 0.3 mL of 5 M $NaCO_3$ solution. The protein activity was determined by absorbance of the reaction product of ONPG at 420 nm (showing a yellow color), with an accepted absorbance being between 0.2 and 0.5. The instrument used for the absorbance determination was Nanodrop spectrophotometer (ND-1000, U.S.). A standard curve for the activity assays of β-galactosidase solutions in water with concentrations from 10 to 500 µg/mL was made (R^2 = 0.981).

Samples of salbutamol and BSA were analyzed with high performance liquid chromatography (HPLC Thermo System Products, Agilent 1200 Infinity Series, Agilent Technologies, Germany). The HPLC salbutamol method was developed using a Discovery® C18 column (Supelco Analytical, U.S.), flow rate of 1 mL/min with a mobile phase consisting of methanol and PBS (25 mM; pH 3) at a ratio of 25:75 (v/v) operating at 25 °C. The running time was 4 min and the UV detection of salbutamol was set at 270 nm with a retention time of 2.6 min. A standard curve for the salbutamol quantification at concentrations from 0.5 to 25 µg/mL was made (R^2 = 0.99988). The BSA method was

developed using a Vydac 214MS C4 column (Grace Davison Discovery Science, U.S.), suitable for protein analysis, flow rate of 1 mL/min with a mobile phase consisting of ACN and 0.1 % TFA operating at 40 °C. The protein analysis required a gradient of TFA and ACN of ratios of 80:20 (v/v) to 35:65 (v/v) within 12 min and reversing back to 80:20 (v/v) within 8 minutes, with a total run time of 20 min. The UV detection of BSA was set at 210 nm with a retention time of 8.5 min. A standard curve for BSA quantification from concentrations of 5 to 500 µg/mL in NaCl corresponding to the collection media was made (R^2 = 0.99991). BSA method was adapted for Agilent 1200 combining three BSA methods found in literature (Umrethia et al. 2010).

The encapsulation efficiencies were calculated from the HPCL results using Equations 10 and 11. The encapsulation efficiency for salbutamol was determined by comparing the total quantity of salbutamol with the quantity of salbutamol in the supernatant. The experimental total quantities of salbutamol were compared with the theoretical total quantities of salbutamol.

$$EE(\%) = \frac{Total\ quantity\ of\ salbutamol - Salbutamol\ quantity\ in\ supernatant}{Total\ quantity\ of\ salbutamol} \times 100 \qquad (10)$$

The encapsulation efficiency of BSA was calculated based on the theoretical total quantities of BSA. These theoretical quantities were determined by calculation in amount of BSA in the inner phase used to prepare droplet for each experiment. The experimental quantities were not obtained, since the sonication process would have led to degradation of the protein structure.

$$EE(\%) = \frac{Total\ quantity\ of\ BSA - BSA\ quantity\ in\ supernatant}{Total\ quantity\ of\ BSA} \times 100 \qquad (11)$$

6.3.6 Drug release

Drug release from the particles was evaluated with confocal microscopy and dissolution tests. With confocal the follow-up of one PCL particle was observed for 2 h. The confocal observation begun as the time elapsed from the beginning of particle preparation process was 0.5 h and the additional images were taken with time points of 1, 1.5 and 2 h.

Drug release from the particles was evaluated with dissolution tests using diffusion cells (Snapwell diffusion chambers, Grown Glass Company Inc., U.S.) and the release tests were made in glass containers. Additionally the release of FITC-dextran was observed with confocal microscope. Both dissolution tests were conducted with two dissolution media: a PBS at pH 7.2 and a HCl buffer at pH 1.2. Samples used were PCL particles (formulation #46) loaded with 1 % of BSA and pure BSA powder was used as reference.

Snapwell diffusion chambers consisted of inner and outer chambers: outer chambers to control the temperature and inner chambers to serve as donor (3 mL) and receiver (1 mL) compartments (**Figure 21**). The outer chambers were connected to a pump and the water heater system with silicone tubes. The water heater and pump system kept the water running through the system with constant temperature of 37 °C during the experiment. Small stirring magnets were placed in the bottom of the donor and receiver compartments to stir the samples. Silicone rings were used in between the chambers to prevent them from leaking or breaking. Between the silicone rings cellulose ester membrane (Spectra/Por Biotech CE Membranes, Spectrum Labs, U.S.) with MWCO of 100,000 or 1,000,000 Da was used.

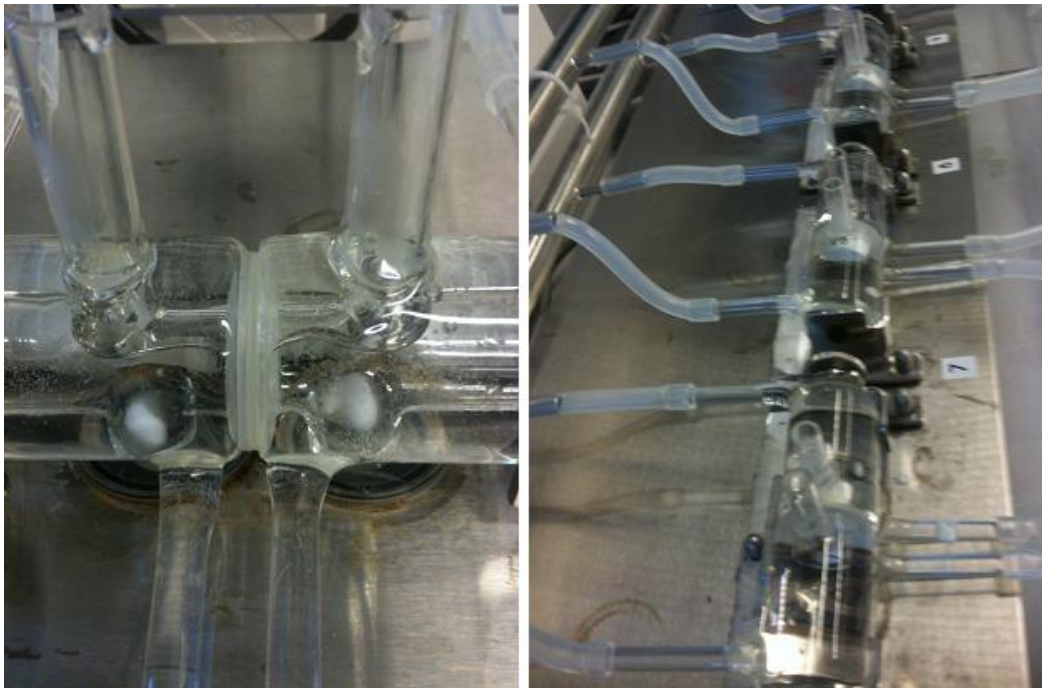

Figure 21. Diffusion cell device and Snapwell diffusion chambers (Grown Glass Company Inc., U.S.).

The particles were placed in the donor compartment and the aliquots were taken from the receiver compartment with a Finnpipette when the elapsed time was 0, 10, and 30 min, and 1, 2, 3, 4, 5, 6, 24 and 48 h. At 48 h an aliquot from the donor compartment was also taken in order to evaluate the diffusion through the membrane during the experiment. Each aliquot taken was 0.5 mL and the same volume of fresh media was replaced back in order to keep the volume of the dissolution constant.

Drug release tests (**Figure 22**) in glass containers were conducted on heating and stirring plates (H+P Labortechnik AG, Multitherm, Germany). The volume of the releasing media was 20 mL. The temperature was monitored during the dissolution tests and kept at 37°C ± 0.5 °C. Each aliquot taken was 1 mL and the same volume of fresh media was replaced back in order to keep the volume of the dissolution constant. Aliquots were taken when the elapsed time was 30 sec, 1, 2, 3, 4, 5, 10, and 30 min, 1, 2, 3, 4, 5, 6, 24, and 48 h, 1 and 2 weeks. More aliquots were taken in the beginning of the dissolution test, since the effect of the membrane was not present in this experiment. After 2 weeks the glass containers with

the dissolution test system were sonicated (Sonics Vibra-cell, Sonics and Materials Inc., U.S.) in order to release the total amount of BSA remaining in the particles.

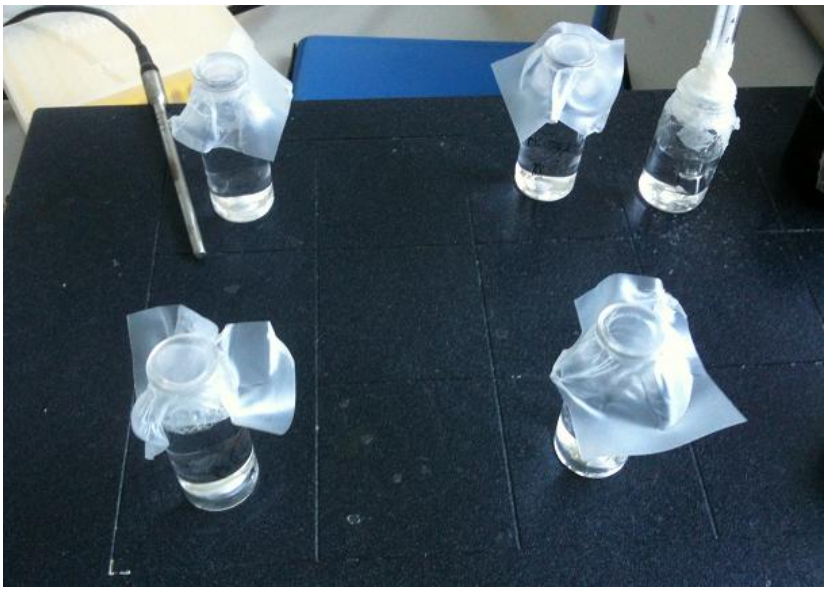

Figure 22. Drug release tests in glass containers on stirrer and heating plates (H+P Labortechnik AG, Multitherm, Germany).

The aliquots taken were analyzed with HPLC for BSA as described in section 6.3.5. Standard curves for the quantification of BSA from concentrations of 5 to 500 µg/mL at pH 7.2 (R^2 = 0.99992) and pH 1.2 (R^2 = 0.99965) were made. The results were processed so that the quantity of the drug in the samples was cumulatively added to the total quantity of released drug, which was then compared to the total quantity of the drug in the aliquot to obtain the quantity of the released drug as a percentage (Equation 12).

$$RD(\%) = \frac{\mathrm{C} \times 20\ \mathrm{mL} + (m_0 + m_1 + \cdots + m_n)}{Total\ input\ of\ BSA - BSA\ in\ supernatant}\ x\ 100 \qquad (12)$$

Where C is the concentration of BSA in the aliquot multiplied with the total quantity of media, added to the cumulative quantities (m_0, m_1, m_n) of previous aliquots divided by the

difference between the total quantity of BSA used in the particle preparation process and the total quantity of BSA in the supernatant.

7. RESULTS AND DISCUSSION

7.1 Microfluidic devices and production of droplets

Successful droplet production was dependent on the device and formulation used. Formulations that could be used to produce double emulsion droplets in bulk, would generally not work with microfluidics. The common ratio of phases in W/O/W emulsion was around 1:10:100 (Jeong et al. 2003) to 1:25:100 (Coccoli et al. 2008). In microfluidics the ratios varied according to the device design used and were determined directly by the flow rates. The production rate was also dependent on the flow rates. Overall, the production using one glass capillary device was slow and for actual industrial scale production scaling-up would be necessary.

Microfluidic devices were hand-made, and thus, the design was not always identical. Additionally, all of the devices could not be considered fit for droplet production. When producing droplets with polymers in organic solvents as the middle phase each device could only be used once. Due to this, variation between the batches was more significant. If PDMS devices would have been used in these experiments, the structure of the devices would have been identical and there would not have been differences caused by the device structure in the batches.

7.2 Formulation screening study

7.2.1 Double emulsion with two tip glass capillary devices

The two tips of glass capillary devices were the most challenging design out of the three device designs used in these experiments. Formulations #1–4 with PLGA (85:15) were not successful. With higher concentrations of PLGA droplet formation could not be achieved. With the extremely low concentration of 0.005 % of PLGA single emulsion droplets were formed. However, even the single emulsion droplets were not stable. The higher portion of lactic acid with the PLGA was, the more viscous middle phases were formed (Wu 1995). Thus, the formulations #5–13 PLGA (85:15) were replaced with PLGA (50:50) where the portion of glycolic acid was smaller.

The formulations #5 and 6 produced single emulsion droplets. With these formulations the inner phase jetting within the middle phase was possible, but jetting to dripping transition was not achieved. The viscosities of the inner and the outer phase were increased in formulations #7 and 8, but this failed to solve the issue. In formulations #9–11 the polymer content of the inner phase was decreased, but the inner phase contained any quantity of protein, double emulsion droplet production was not possible.

With the formulations #11–13 the content of the inner phase was further altered. As the inner phase contained only water, partial double emulsion droplet formation was possible. Partial success rate was approximately from every third to every seventh droplet being double emulsion droplet and the droplets formed in between being single emulsion droplets. Perfect double emulsion droplet production was not achieved. In formulations #14 and 15 the PVA in the outer phase was replaced with Tween 20, but it did not stabilize the droplets nearly as well as PVA. In the formulations #16 and 17, PLGA was replaced with PCL, but either the solvent used was DCM or ethyl acetate, the middle phase turned out to be even more viscose than with PLGA, and thus, the droplet production was impossible.

Neither PLGA (85:15), PLGA (50:50) nor PCL was suitable for droplet production in the two tip glass capillary device due to the excessive viscosity in the middle phase. Thus,

PLGA was replaced with PLA (intrinsic viscosity from 0.5–0.65 to 0.2) that would form less viscous middle phase.

With the formulation #18 successful double emulsion droplet production was accomplished (**Figure 23**). However, the inner phase contained only water, and thus, the particles were not stable and collapsed at the end of the collection tube. For more stable droplets the PVA content of the inner phase was increased (formulations #19 and 20); as the viscosity of the inner phase increased the droplet production was not achieved.

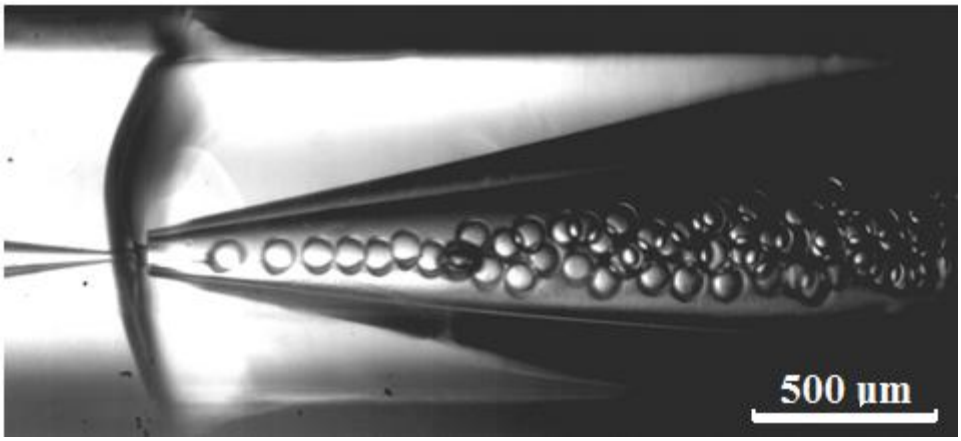

Figure 23. Optical microscope image showing the successful production of double emulsion droplets with the two tip glass capillary device.

The droplet production with the two tip glass capillary device had various problems (**Figure 24**). For example, the production process was often disrupted because of these issues and had to be started from the beginning. Due to these issues with the two tip glass capillary devices no constant droplet production was achieved.

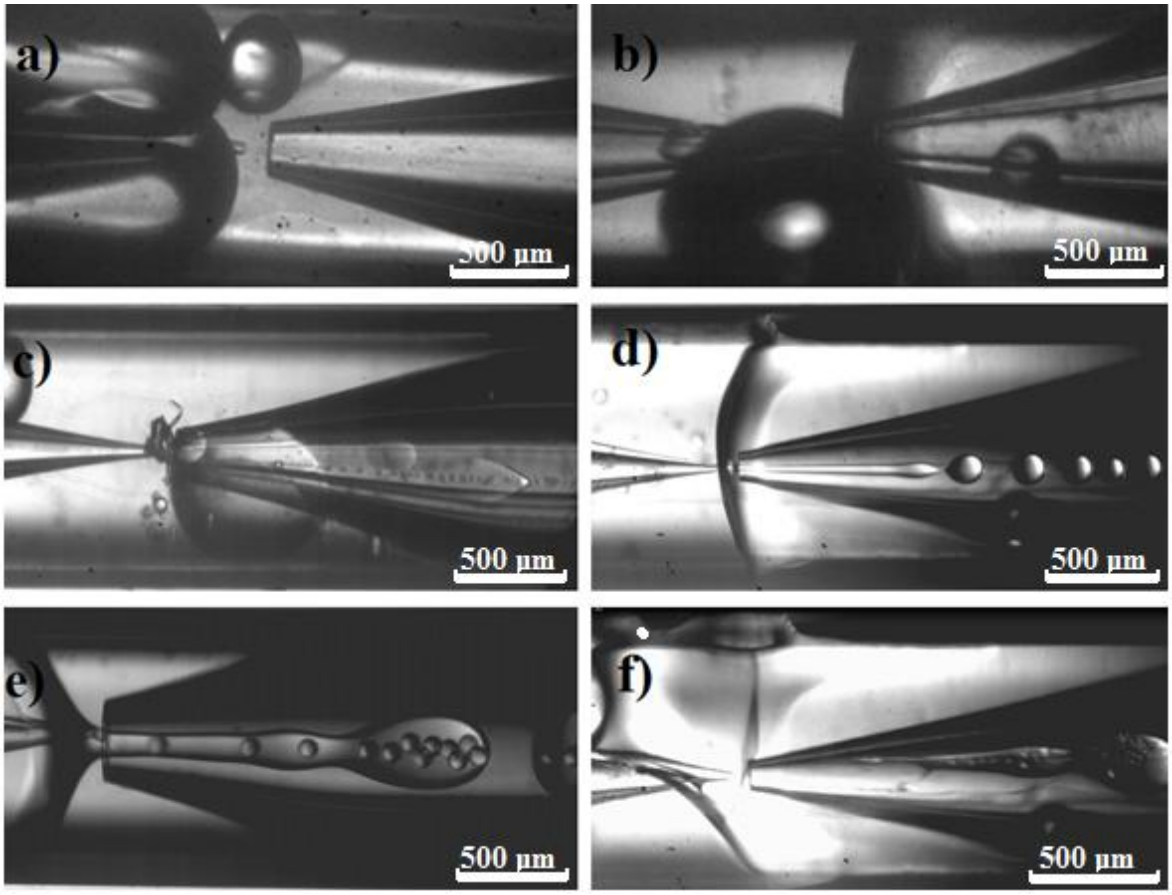

Figure 24. Optical microscope images showing the problems with double emulsion droplet production: (a) viscosity, (b) air bubbles, (c) clogging, (d) formation of single emulsion droplets, (e) formation of droplet with multiple cores, and (f) breaking of the interphase.

The viscosity of the middle phase often led to formation of uneven interphase. The more viscous the middle was the easier it formed along the square capillary, forming two streams instead of one. The impact of viscosity is more significant in microscale due the low Reynold's number (Squires and Quake 2005), and thus, the behavior of the phases could not be controlled with the changes of the flow in order to overcome the effect of viscosity.

The preparations taken before the preparation process, washing the device and filtering the phases in some cases failed to prevent clogging or air bubbles in the device. Clogging was caused mostly by the polymers precipitating in the phases, especially in the middle phase were the polymers used were soluble only in organic solvents and the solubility to even

those was limited. Clogging was more likely to take place as the polymer concentrations in the phases were higher. The statistic probability of clogging could be reduced with heating and stirring of the middle phase during the production process. However, the temperature should be carefully controlled in order to keep the overall temperature of the emulsion under 42 °C to protect the protein from degradation. Changes in the temperature would also affect the viscosity of the middle phase and the flow rates should be adjusted carefully for changing the process variables. The viscosity of the phases was also the main cause for air bubbles in the capillary system. The bubbles were more persistent and tended to form easier in the more viscous phases. With the two tip glass capillary devices air bubbles disturbed the interphase and the droplet production ceased. In most cases the air bubble could be removed from the tip area with high flow rates. However, the procedure required beginning the flow rate adjustment and droplet production process from the starting.

A common phenomenon with the two tip glass capillary devices was single emulsion droplet (O/W) production or double emulsion droplet production (W/O/W) with multiple cores. Single emulsion droplets were formed when the inner phase would not reach the interphase at the tip with correct flow rate. Droplets with multiple cores were produces as the flow rate of the inner phase was higher. The double emulsion droplets with multiple cores were not stable and collapsed in the beginning of the collection tube. In some cases, the interphase would breakdown from the side of the middle phase. This was yet a viscosity issue and required restarting the production process again.

The two tip glass capillary devices themselves worked with different formulations, such as polymersome formulations with glucose in water in the middle phase PEG-b-PLA in chloroform and toluene, and PVA in water in the outer phase (Duncanson et al. 2012b). With this formulation the viscosity of the inner phase is close to the viscosity of water and the middle phase is less viscous, yet not suitable for drug formulations. Also, the droplets have been stabilized with hydrogels to ensure more stable production and droplets (Kim et al. 2013). These droplets contained 15 or 10 % (w/w) of poly(ethylene glycol)diacrylate (PEGDA) in water as the inner phase, 5 % (w/w) of PEG-b-PLA in chloroform and hexane (38:62) and 10% (w/w) of PVA in the outer phase. Adding 0.2% (w/w) of photoinitiator in the inner phase polymerizing the droplets was possible. This made the inner phase more

rigid, and thus, the droplets remained intact. The two tip glass capillary devices have also been used to produce multiple emulsions with phases that have viscosities better suitable for the application (Kim and Weitz 2011).

In this study, for example, triple emulsion droplets (O/W/O/W) were prepared using water as the inner phase, hexadecane and 1% (w/w) Span 80 in the first oil phase, 3% (w/w) PVA and 1% (w/w) F108 in water in the second water phase, and finally hexadecane and 1% (w/w) Span 80 as the outer phase. Another sort of polymersomes were created with 10% (w/w) of PVA in water as the outer phase, PEG-b-PLA and PLA in a mixture of chloroform and hexane (38:62%, v/v) as the middle phase, and as the formulation was designed for protein expression bacterial ribosomal extract (*E. coli*) and MreB DNA plasmid in water as the inner phase (Martino et al. 2012). Also, in this study the chloroform residues in the product would be challenging in medicinal product, yet the co-polymer in these organic solvent enabled the droplet formation.

However the desired formulation for protein drug encapsulation in this study contained only one polymer (PLGA, PLA or PCL) in the middle phase making it highly viscous, and thus, the two tip glass capillary devices could not be considered for producing of these particles. The two tip glass capillary devices required more complicated, and thus, significantly more expensive formulations. Scaling-up of a process with no guarantee of functioning would be highly unprofitable. Thus, the technology for droplet production was changed to more practical alternatives, such as combining microfluidics with bulk method and biphasic flow.

7.2.2 Double emulsion with combining microfluidics and bulk method

Single emulsion droplet production with one tip glass capillary devices proved to be a simpler droplet production method than the method using the two tip glass capillary devices. Droplets with microfluidics were chosen to produce with PCL in ethyl acetate due to the stability of W/O emulsion. This made also the manufacturing process possible outside of a fume hood since ethyl acetate is nontoxic (Ramalakshmi and Raghavan 1999). The formulations #21–23 contained very moderate quantities of polymers, especially in the

inner and in the outer phases. As the viscosities failed to match between the phases, very unstable droplets were produced with microfluidics and the lack of PVA working as a surfactant in the outer phase prevented the formation of stable droplets.

In formulations #24–26 the polymer concentrations were increased significantly. Double emulsion droplet formation was achieved and the formed droplets were even possible to collect and dry. In the formulation #24 the inner phase contained 5% (w/w) PVA in order to stabilize the particles. The increase of PVA quantity failed to do so and instead the size of the W/O droplets increased, and the single emulsion particles became less stable. Thus, 1% (w/w) of PVA in the inner phase proved to be the ideal concentration. With the formulations #25 and 26 where PVA concentrations of 5% (w/w) and 10% (w/w) were tested, the more viscous outer phase slowed down the droplet formation and prevented the ethyl acetate from evaporating, thus creating large and slightly unstable droplet. The ideal formulation for the combination technique was the formulation #27 with 1% (w/w) of PVA in the inner and in the outer phase. The maximum concentration for PCL in ethyl acetate was 3% (w/w) due to solubility and viscosity issues.

Spherical particles were formed when the polymer worked as a surfactant in the outer phase was PVA. In the formulations #29 and 30 when Tween 20 was tested in the outer phase the system failed to produce spherical droplets or encapsulate the first emulsion into double emulsion in the outer phase. The spherical particles from the formulation #27 were polydispersed and part of the droplet population lacked the inner phase. This was caused by the lack of control with the latter emulsification process (**Figure 25**).

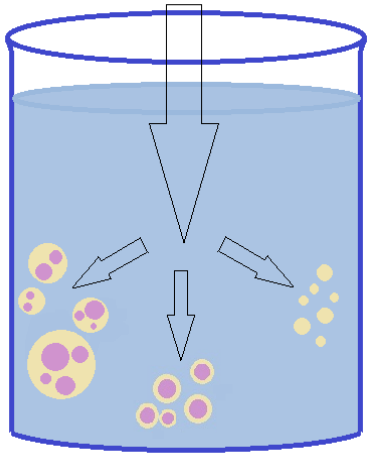

Figure 25. Schematic of the droplet formation with the combination method. As the first emulsion (W/O) is mixed to the outer phase the double emulsion droplets form, and thus, the variation in the particle size and content is significantly heterogeneous.

The particle formation was fast and drying of the particles was possible with filter paper. The dried particles preserved their spherical forms. This simple method was successful in the drying of the combination method particles due to the size and PCL quantities in the particles.

The sole advantage of combining bulk method with microfluidics was the preservation of the protein structure with mild emulsification technique for the first emulsion in the process. However, the double emulsion droplet production was not as sophisticated as in the methods that produces double emulsion droplets directly. All the advantages of microfluidics were not exploited and the double emulsion droplet formation process was not controlled nor could it have been monitored with the fast cameras. Also, similar issues with clogging and air bubbles as described earlier emerged with the one tip glass capillary devices.

With microfluidics various formulations for single emulsion droplet manufacturing have been developed. With these glass capillary devices, O/W single emulsion droplets were produced. Extremely monodisperse droplets were produced with polyfluorene (PFO) in toluene as the inner phase and PVA in water as the outer phase (Kuehne and Weitz 2011).

The particle size was controlled by adjusting the concentrations. Also, PLGA and PLA were possible to formulate as a single emulsion (O/W) (Duncanson et al. 2012c). In this droplet formation process the outer water phase contained 10% (w/w) of PVA and the inner phase varying concentrations of PLA and PLGA. Similar particles with 5% (w/w) of PVA in the outer phase and 5% (w/w) of PLA in the inner phase were prepared (Vladisavljević et al. 2012). The single emulsion droplets were stable and monodisperse.

However, even though these formulations were often meant as templates for drug delivery, loading them with water soluble drugs is self-evidently impossible and would require changing the system into double emulsion droplet production. This again would set new requirements for the formulations. Also, DCM and toluene are toxic and especially toluene is unsuitable for drug formulations.

7.2.3 Double emulsion with biphasic flow

With the biphasic flow droplet production from more viscous phases was possible, since an interphase is not formed in the square capillary, and thus, minor changes in the flows of the phases did not disturb the droplet production on a larger scale. In addition, since the interphases formed within the first cylindrical capillary the flow in the square capillary affected only the dripping to jetting transition, and thus, also the flow rates needed lesser changes to achieve the droplet production process than in the other devices designs used in this study.

When the process was successful all of the water drops formed double emulsion (W/OW) drops (**Figure 26**). The flow rates were adjusted to low speeds (500–1000 µL/h) as the biphasic flow was formed in the first cylindrical capillary. Once started and undisrupted the droplet formation was stable and continuous for several hours.

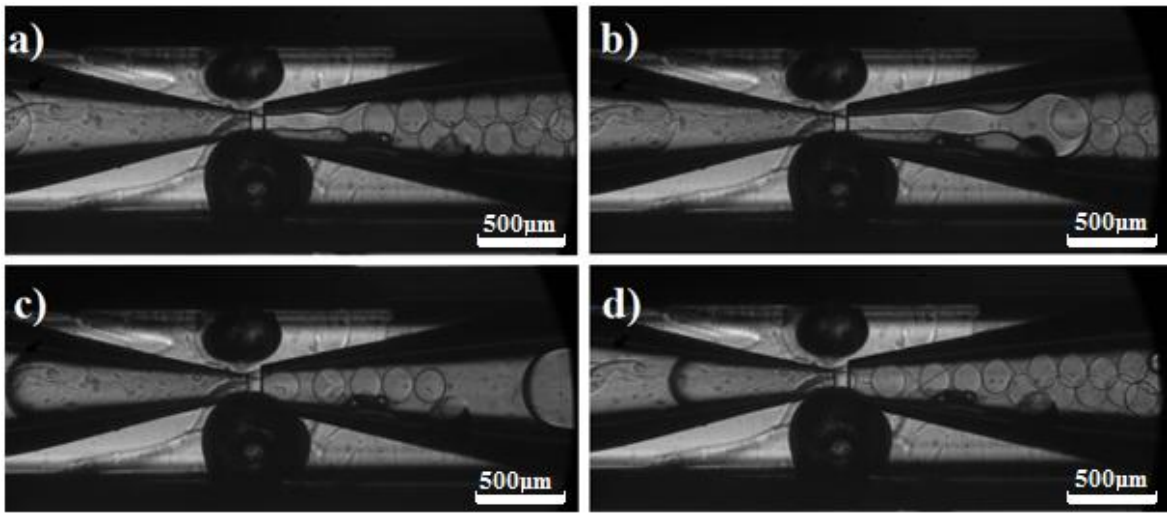

Figure 26. Optical microscope images showing the droplet formation with biphasic flow: (a) oil phase reaching the tip of the first capillary, (b) oil droplets (O/W) forming, (c) beginning of the formation of double emulsion (W/O/W) droplets, and (d) continuing of the droplet formation from the water drop.

Within the process a change in the size of the water drops in the first cylindrical capillary was observed. The water particles started smaller and gained more size during the preparation process. This was caused by the wearing out of the hydrophobic coating in the first cylindrical capillary.

The collection of the droplets was made possible by the density differences with the single emulsion droplets and double emulsion droplets. When the oil phase was stained with Nile red, it was possible to observe the separation and formation of the droplets (**Figure 27**).

Figure 27. Collection and separation of the droplets from the biphasic flow. Bigger oil droplets floated and the smaller double emulsion droplets with less of the pink dye sank in the bottom of the collection vial.

As the molarity of the collection media was set according to the molarities of the inner phases of formulations #34 and 43, 50 mM with 10% (w/w) PVA:PEG 6000 and 156 mM with 20% (w/w) PVA:PEG 6000. The molarities did not change as the small quantities of fluorescent agents or model drugs were added to these inner phases.

The formulation screening for the biphasic flow began with the formulation #31: 5% (w/w) PCL in the middle phase with DCM as the organic solvent. DCM was not suitable solvent for this system, since caused PVA to precipitate in the first cylindrical capillary. Due to this, neither PLA nor PLGA were suitable options for the polymer in the middle phase, as they failed to dissolve in ethyl acetate. The obvious choice within the three polymers used in the study was PCL. Additionally, ethyl acetate was a non-toxic option for the middle phase and since it is used mainly in pharmaceutical industry (Dutia 2004), and also in food industry to decaffeinate tea and coffee (Ramalakshmi and Raghavan 1999).

In the formulation #32 the polymer in the ethyl acetate middle phase was PCL with concentration of 5% (w/w). The 5% concentration for PCL in ethyl acetate was too high in terms of solubility. The middle phase was too viscose and impossible to handle within the droplet preparation process. For the formulation #33, the PCL concentration was reduced to 2.5% (w/w) with decreasing the quantity of PVA in the outer phase to match the viscosities and droplet production was possible.

The contents of the phases were further adjusted in the formulations #34–36. 3% (w/w) proved to be the maximum concentration for PCL. Optimal droplet production was achieved with 5% (w/w) of PVA in the outer phase and 10% (w/w) PVA and PEG 6000 (1:4) in the inner phase. The formulation development was continued with successfully adding fluorescent FITC-dextran to the inner phase and perylene to the middle phase. The results from observing the droplets with confocal microscopes are discussed later in section 7.3.2.

Drug loading to the formulations started with *β*-galactosidase as a model protein. The inner phase was altered in the formulations #39 and 40 due to the poor water-solubility of *β*-galactosidase. Poor water-solubility was caused by dextrin chain used as a preservative in the *β*-galactosidase (Sigma-Aldrich, U.S.). The quantity of *β*-galactosidase was increased to 5% (w/w) as the quantities of the polymers decreased. These formulations failed to produce double emulsion droplets and as a result the polymer content of the inner phase was reversed to the 10% (w/w) of PVA and PEG 6000 (1:4), and the maximum amount of *β*-galactosidase possible to dissolve to that interphase was determined to be 0.4% (w/w). With the therapeutic proteins used as medicines, the protein are usually so potent that for the desired effect already very small quantities are sufficient. The dosage is dependent on the protein drug used. For example, the therapeutic dosage for insulin is 10 ng/mL (Van den Berghe et al. 2003) and for botulinum toxine 10 µg/mL (Borodic et al. 1994).

The effect of the content of the inner phase was further determined by the viscosity observations (see Appendix 1). The results of the rheology measurements conducted for the inner phases of the formulations #39–41 are presented in **Figure 28**. The significant data in **Figure 28** is between 100 and 130 seconds when the viscosity measurement process has settled down. It is possible to determine that the viscosity of the inner phase of the formulation #39 is 4 times the viscosity of the other two inner phases. This explains why the droplet production was only successful with the formulation #39. The dynamic viscosities of the formulations #40 and 41 were almost corresponded to the viscosity of water. This affected the droplet formation process so that it failed to succeed.

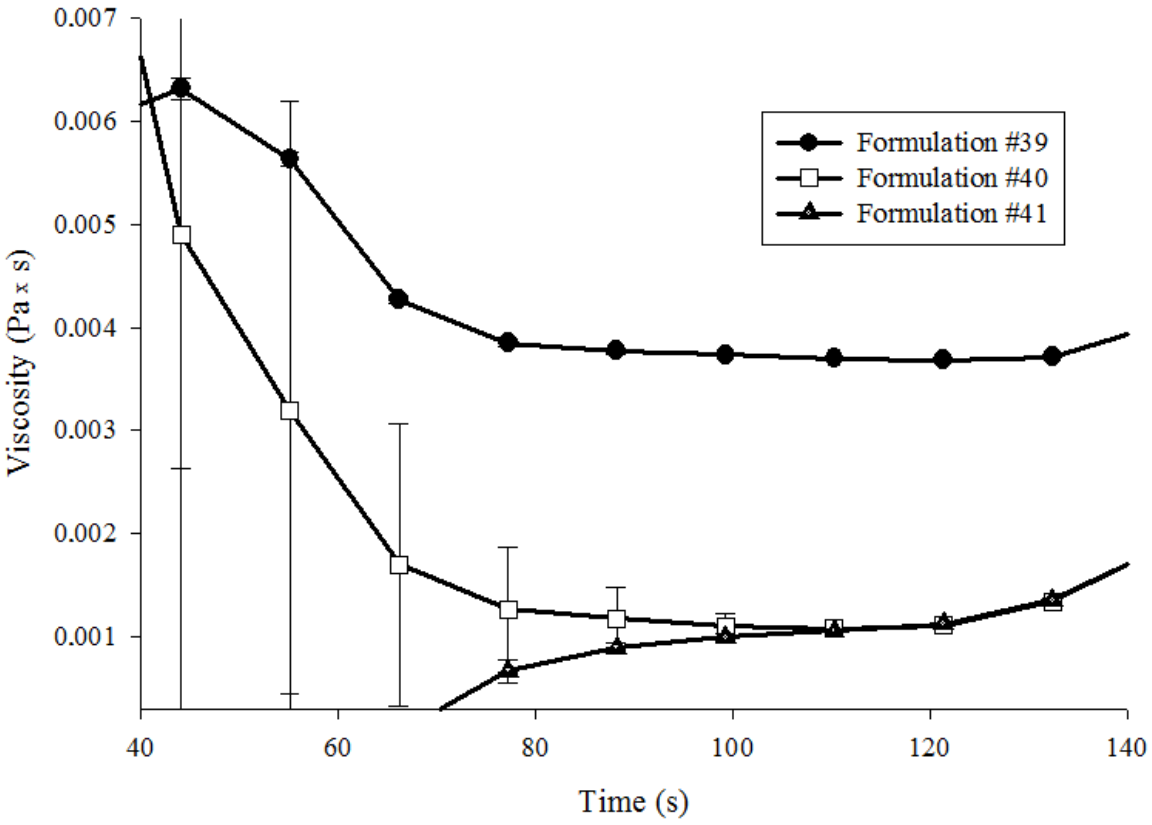

Figure 28. The viscosities of three different inner phases. Two parallel measurements with Ares G2 were conducted. The viscosity is expressed as dynamic viscosity, Pascal seconds (Pa × s).

The results from these experiments were used to adjust the content of the inner phase. The drug loading had to be kept small due to the necessary polymer content in the inner phase. In the *β*-galactosidase preparation the protein was stored with dextrin. These dextrin chains decreased the solubility of the *β*-galactosidase preparation. In case of more pure preparation of the protein higher drug loadings could have been achieved.

The formulation development was continued with adding PVA and PEG to the inner phase to the concentration of 20% (w/w) and increase in the polymer quantity in the inner phase, facilitating slightly the droplet preparation process. The droplet produced with the formulations containing 20% (w/w) (formulations #43–46) were otherwise identical to the droplets containing 10% (w/w) of PVA and PEG (formulations #34–38, 41, and 42). The formulation with 20% (w/w) was chosen for further development and the loading with

FITC-dextran was successful. The model protein was changed to BSA due to the problems observed with β-galactosidase, such as the water-solubility, purity and analysis. BSA loading with 1% (w/w) was possible and constant droplet production was achieved. 1% (w/w) loading for a protein drug was already quite acceptable. Other model drug, salbutamol sulphate was used with 2.5% (w/w) loading. Due to the small size and excellent solubility to water, salbutamol performed excellently as a model drug.

Overall, the double emulsion droplet production with the biphasic flow was successful and the production rates were decent enough to prepare the necessary quantities of the droplets to further characterization. Nonetheless, problems common to the microfluidic droplet production were present also in the devices designed with biphasic flow. Main problems with the devices were alignment mistakes. Problems with the droplet production were similar to the issues discussed earlier. The main problem was the clogging of the capillaries. Additionally, the problems discussed earlier (**Figure 24**) also affected the success rate of droplet formation in biphasic flow.

Specific problems in the biphasic flow were diameter of the additional cylindrical capillary for the inner phase and disruption of the double emulsion droplet production in the middle of the water drop movement to the collection capillary. The additional cylindrical capillary was made with stretching the capillary in a flame. Due to this method variations in the shape were significant and resulted in either too small water droplets that could not be considered as successful biphasic flow, or a jet of the inner phase, which failed to reach droplet production. Disruption with the water drop took place when the oil phase did not distribute evenly along the wall of the first cylindrical capillary, and thus, the double emulsion droplet formation ceased (**Figure 29**). Thus, the inner and outer phases mixed and small single emulsion droplets were formed from the middle phase. This phenomenon is the main reason for the leaking of the model drug from the double emulsion particles decreasing the encapsulation efficiencies achieved.

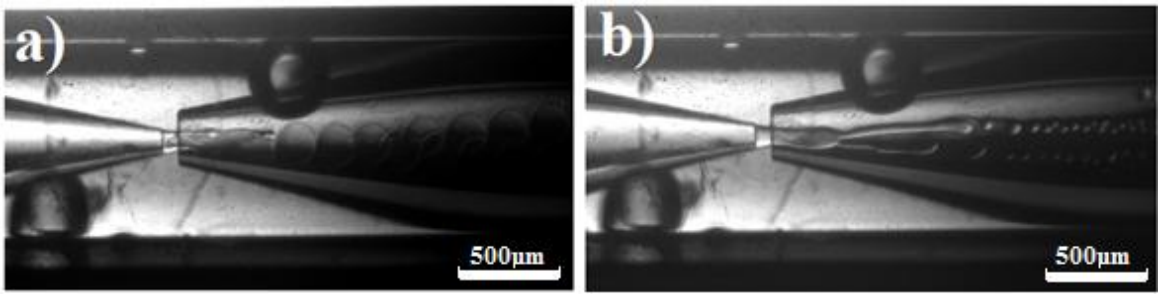

Figure 29. Optical microscope images showing the disruption in droplet formation with biphasic flow: (a) normal production of double emulsion droplets with thin shells, and (b) breaks and droplet forming are single emulsion droplets from the oil phase.

Drying of the particles failed to succeed. Even though various filters were used the process did not provide decent quantities of dried particles. Drying attempts indicated that the particles were adhesive. The dried particles also flocculated as preserved (see section 7.3.3).

Other formulations for the biphasic flow have been developed (Kim et al. 2011). The droplets produced with 25% (w/w) PEG6000 in the inner phase, 1% (w/w) of Span 80 in the middle phase and 10% PVA in the outer phase. The droplets produced in this study were more successful (see section 7.3.2). However, 25% (w/w) of PEG in the inner phase would have inhibited loading the inner phase with any quantity of protein drug. In the same study the double emulsion droplets with biphasic flow were also manufactured from 10% (w/w) PVA in water as the inner phase, 20% (w/w) PLA in toluene as the middle phase and 3% (w/w) PVA in the outer phase. The double emulsion droplet preparation with these compounds was successful, yet toluene as the organic solvent in the middle phase is an immediate backset for employing this formulation to actual medical preparations. Biphasic flow has also been used for manufacturing of liposomes, giant lipid vesicles and Janus liposomes with different lipid in outer and in the inner part of the bilayer (Arriaga, L., personal communication, SEAS, January 2013).

Even though the formulations developed with PCL produced continuously stable double emulsion droplets, the formulation could be further improved with diversifying the components in the phases. The formulation for the biphasic flow of this study could be

further improved, for example, with UV polymerization (Kim et al. 2013) or co-polymers (Duncanson et al. 2012d).

7.3 Characterization of the microspheres

7.3.1 Particle size

Particles from the formulations #34 and 43 as well as the loaded particles with the same phase components were fairly monodisperse and spherical (**Figure 30**). Particle observation and diameter determination using optical microscopy was successful.

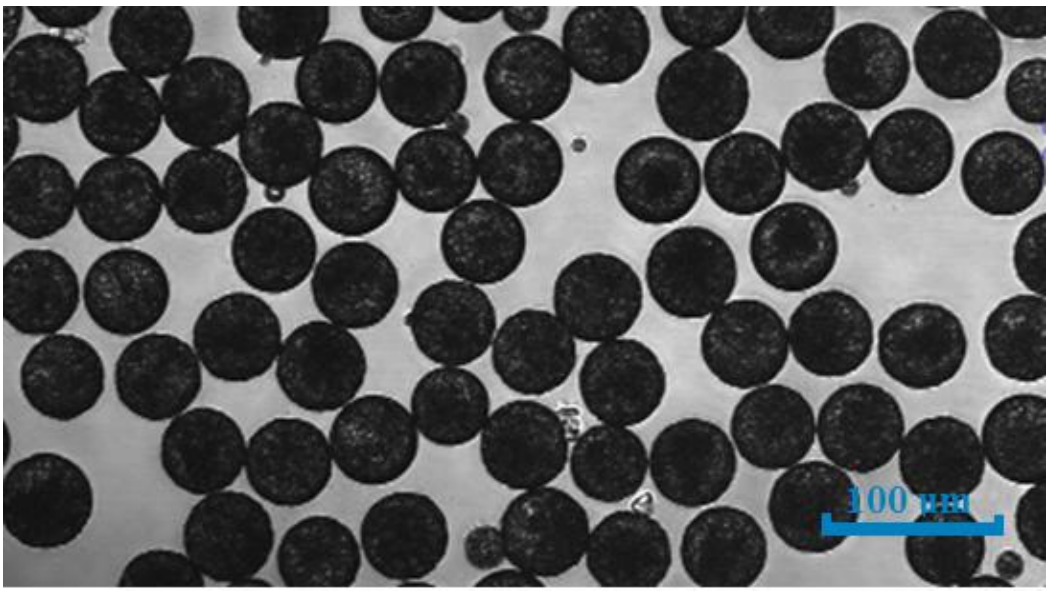

Figure 30. PCL particles observed with an optical microscope.

The particle sizes with standard deviations of the 5 batches observed are presented in **Table 15**. All the batches were found not to be significantly different using the Student's *t*-test analyses. Due to the variance within the batches they could all be considered to be similar.

Table 15. Particle sizes of 5 different batches collected and observed with optical microscope. Batches 1 and 2 are particles of formulation #34, batch 3 is of formulation #37, batch 4 is of formulation #42, and batch 5 is of formulation #43.

	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Average
Particle size (µm)	47	23	35	37	41	36
Standard deviation (±µm)	6	8	6	5	7	6

According to the Student's *t*-tests performed, statistically significant differences could be found in the particle sizes between all the batches observed. The standard deviation was not as significant as with particles prepared with bulk methods, and thus, error bars of the particle populations did not overlap and statistical differences were observed with the Student's *t*-tests.

ImageJ software was also used to determine the particle sizes (see Appendix 2), since the program defines the diameter extremely precisely, and thus, the standard deviating was more significant than it would have been, if the particles sizes were determined in a less accurate method. Overall, optical microscopy was not the most convenient tool for determining the particle sizes. The samples observed were limited to small quantities of particles that did not necessarily represent the size distribution of the whole batch. Alternative methods for determining the particle size could be considered to produce results with better quality, for example, with Coulter counter (Gee and Bauder 1986) or light blocking methods (Gibbs 1982). All the batches were manufactured with different glass capillary devices, and thus, the particles produced were not identical. This issue could be overcome by using PDMS devices and ensuring standard conditions for the droplet production.

Additionally, significant differences in the particle sizes were observed when the particle size during the preparation process was compared with the size of the collected particles. Particles were larger when prepared. The process particle size was 6 times the size of the collected particles as the average diameter of the particles during the process was 218 ± 39

µm and it decreased to 36 ± 6 µm, which was the average diameter of the collected particles.

As the diameter of the particles decreased the shell thickened. The shell of the collected particles was 3.3 times thicker than the shell of the particles during the preparation process. The average shell thickness was 2.1 ± 0.7 µm during the particle preparation process. The shell thickened to 7.0 ± 2.0 µm as the particles were collected. The change in the particle size and in the shell thickness could not be explained by the differences in osmotic pressure (**Figure 31**). The flow according to the osmolarity of the phases should have been from outside into the particles. However, the particles shrank and lost part of their water content.

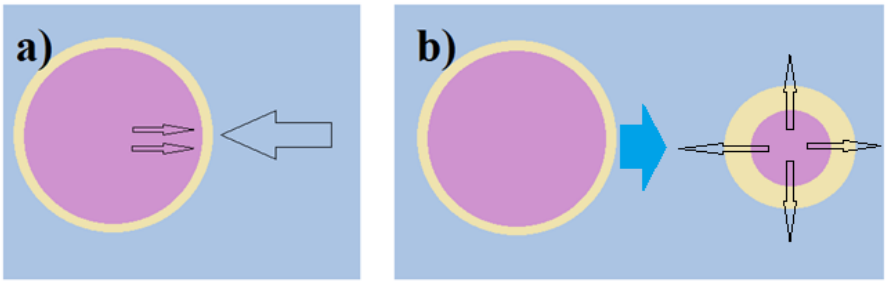

Figure 31. Schematics of the (a) water flow caused by the differences in osmotic pressure and (b) the actual change in the particle size.

The shrinking of the particles was caused by crystallization of the PCL in the middle phase. As the PCL crystallizes, the PCL units were drawn closer together and the water is pushed out of the particle. As a result, as the size of the inner sphere decreases, the shell thickens as observed when comparing the process particles size and the size of the collected particles with optical microscope. The crystallization was driven by van der Waals forces between the PCL units. The existence of these forces was determined by the molecular structure of PCL.

Also, the particle sizes of the combination method and the bulk method were determined successfully. The size of the particles produced with the combination method was 155 ± 86

μm. The standard deviation was more significant than with the microfluidics methods, which directly produced the double emulsion droplets. Also, the particle size was significantly bigger in the case of bulk method. The main reason for abandoning this technique was that the particle size was large and is not suitable for drug administration. Generally, the microparticles for oral drug delivery should preferable have diameters of less than 100 μm (Freiberg, Zhu 2004). Smaller particles could have been created with greater input of energy to the system. However, increasing the stirring rate was not an option, since particles only formed with low stirring rates.

The size of the particles produced with the bulk method was 30 ± 20 μm. With sonication it was possible to produce smaller particles. However, sonication is likely to destroy the protein structure and also the encapsulation efficiency is likely to be poorer. Manufacturing double emulsion droplets with biphasic flow proves thus to be more sophisticated droplet preparation methods than the other methods within this research.

Comparing the results discussed earlier with other studies on PCL microparticles showed that in many studies the microparticles manufactured were smaller in size. Microparticles with diameter of 2 μm have been successfully prepared (Somavarapu et al. 2005). In some studies microparticles with more similar sizes were also achieved. For example, particles with diameter ranging from 21.3 to 40.8 μm with quite similar deviation have been produced with the solvent evaporation method (Jeong et al. 2003). Also, larger PCL particles for drug administration have been developed. Particles with diameters ranging from 70 to 80 μm have been designed for controlled release of warfarin (Scala-Bertola et al. 2012), and from 61 to 190 μm for the delivery of quercetin (Natarajan et al. 2011). Various sizes of PCL particles have been produced and particles regardless their accurate size can be used in different medical applications.

7.3.2 Number of successful double emulsion droplets

Microfluidic droplet preparation proved to be sophisticated enough to produce double emulsion droplets with 100% success rate. All the droplets produced and collected were double emulsion droplets that contained the inner phase. Confocal fluorescence microscopy enabled observing the presence of the inner phase (**Figure 32**).

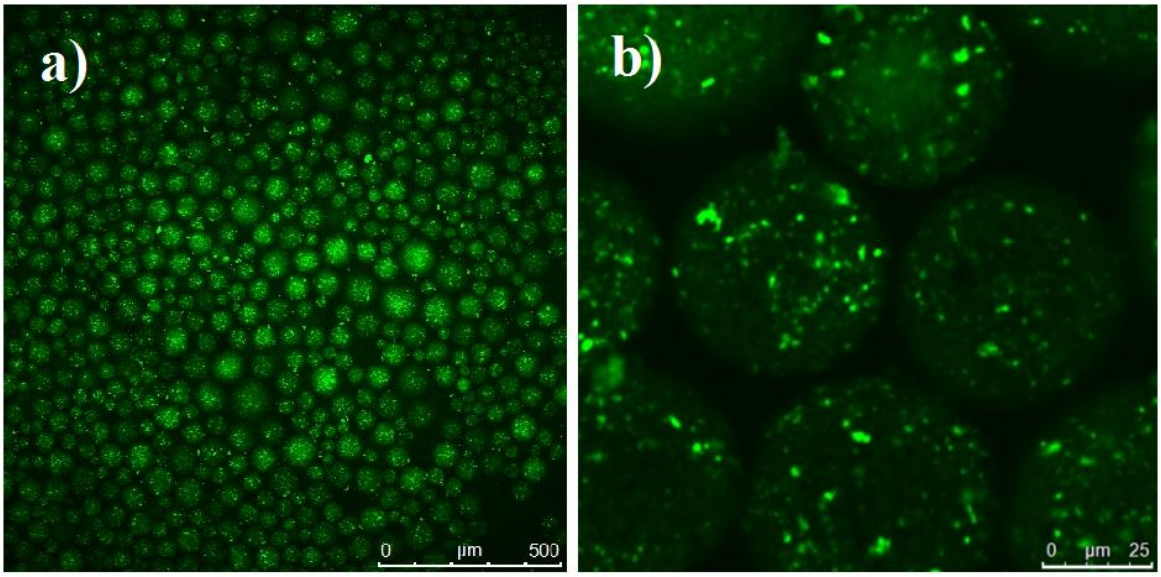

Figure 32. Confocal fluorescence microscopy images of PCL particles with FITC-dextran (green) and *β*-galactosidase in the inner phase.

Moreover, the presence of the middle phase was also observed under confocal fluorescence microscopy (**Figure 33**). With the correct dyes the inner phase and the middle phase were clearly distinguished in the confocal images. Observing the dyed phases separately was also possible, since the excitation/emission spectra did not overlap. All the particles consisted of the inner and the middle phase, the shells were evenly distributed along the inner sphere and particles were fairly monodisperse.

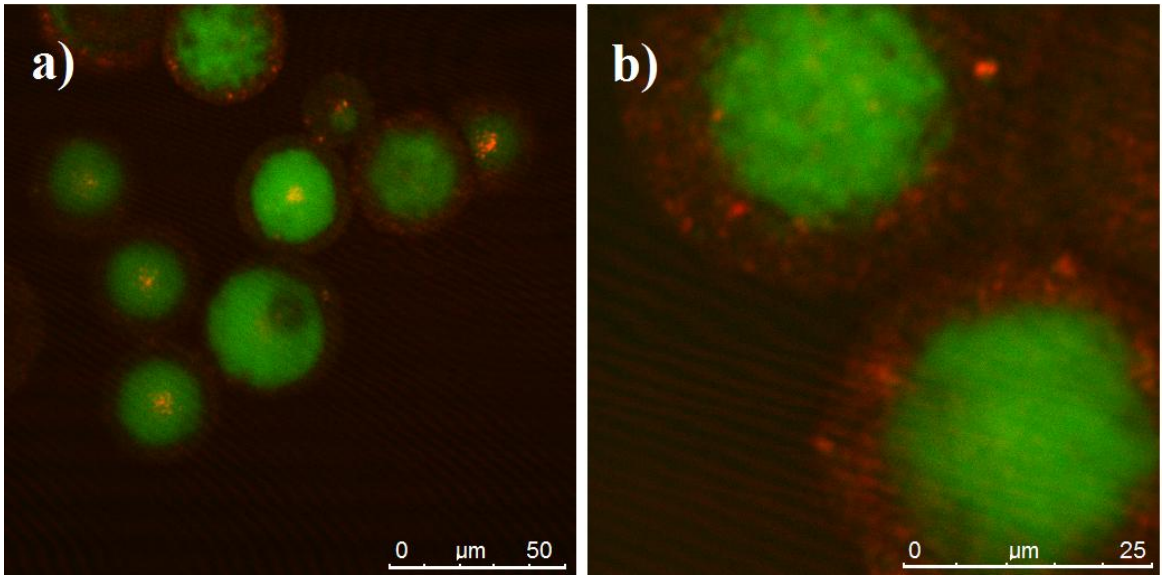

Figure 33. Confocal fluorescence microscopy images of PCL particles with FITC-dextran (green) in the inner phase and perylene (red) in the middle phase.

The particles produced with biphasic flow were compared to the particles produced with the combination technique (**Figure 34**). Apart from being significantly larger, all the combination method particles did not contain the inner phase. Additionally, based on confocal fluorescence microscope observations, the combination method particles seemed to contain varying concentrations of the inner phase. The lack of control in the double emulsion formation process may explain this phenomenon. Also, small quantities of FITC-dextran were observed in the media indicating poor encapsulation efficiency.

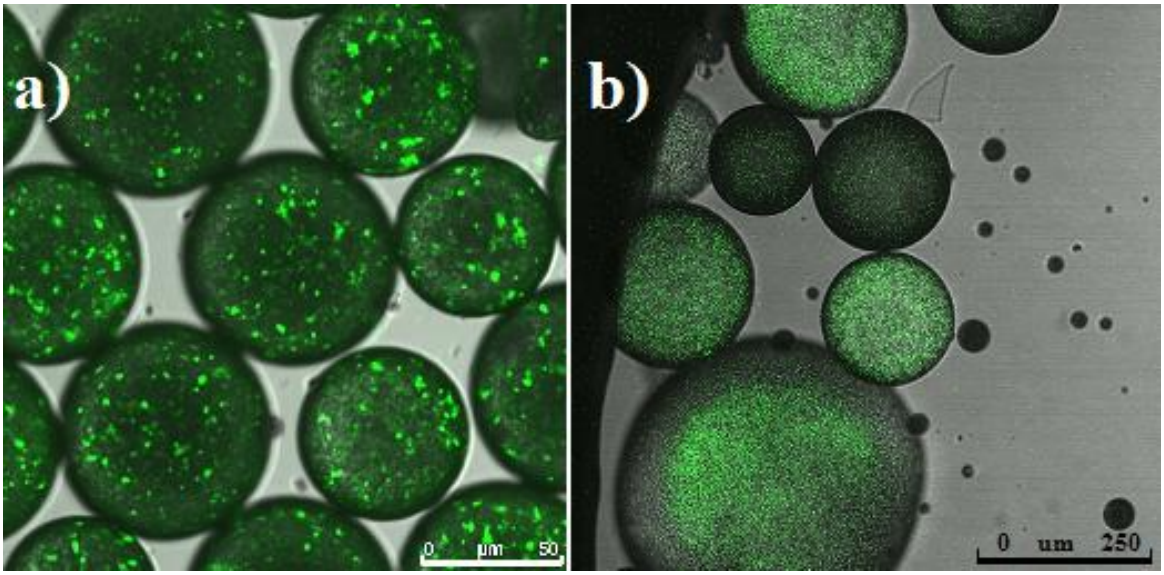

Figure 34. Confocal fluorescence microscopy images of PCL particles with biphasic flow (a), and with combination method (b). The scale bars of the images are significantly different: particles in image (b) are 5 times larger than in image (a).

The observation of the bulk emulsions with confocal fluorescence microscope was challenging (**Figure 35**). Due to the very poor encapsulation efficiency and less clear separation of the particles, the content of single droplet was difficult to observe. However, it was possible to determine that larger quantity of FITC-dextran was not encapsulated within the particles and that the size distribution and variation in the FITC-dextran concentration within the particles was more significant.

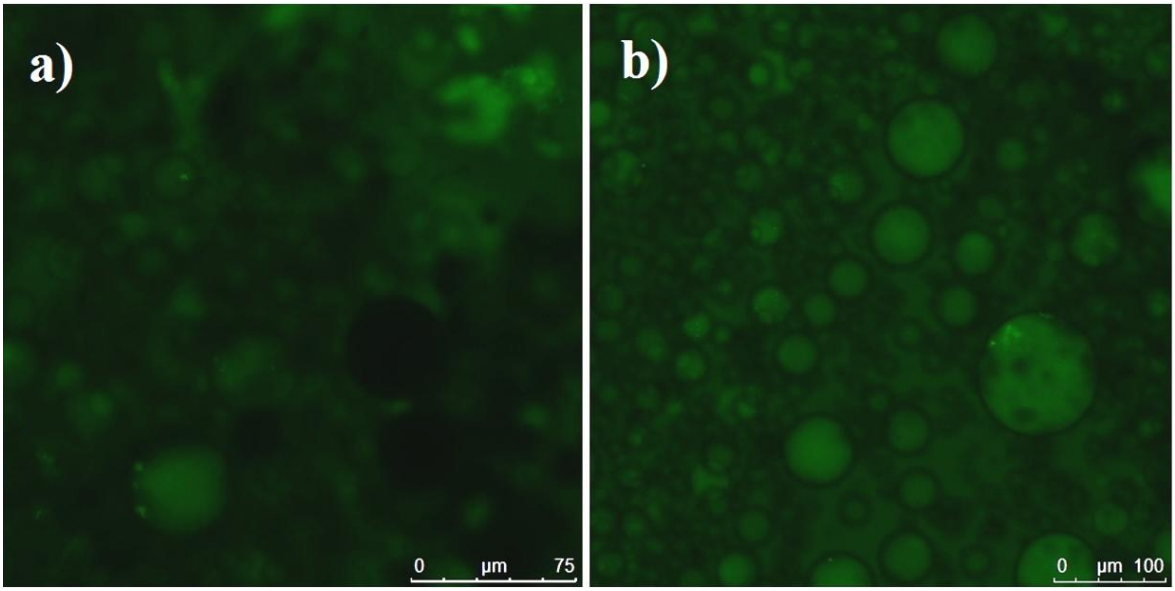

Figure 35. Confocal fluorescence microscopy images of particles of the bulk emulsion batch prepared as a reference with FITC-dextran (red) in the inner phase.

Overall, the particles prepared with microfluidics were more successful as double emulsion droplets. This accuracy achieved in the particles content enabled more precise dosing of microencapsulated drugs. When it comes to, for example, therapeutic proteins the dosages have to be precise and double emulsion droplets prepared with bulk technique are not able to provide such precision. The aspect of polymer particle content has been somewhat overlooked in the literature, since the processes used could not be controlled to affect the success rate of the particles in this level.

7.3.3 Short time stability

Short time stability tests indicated that the particles were stable up to 4 weeks. The particles of all the batches kept their appearance similar during the follow-up (**Figure 36**). The quantity of particles in the images varied according to the sample taken from the batches. Generally, the samples were kept small due to the limited quantities of the batches.

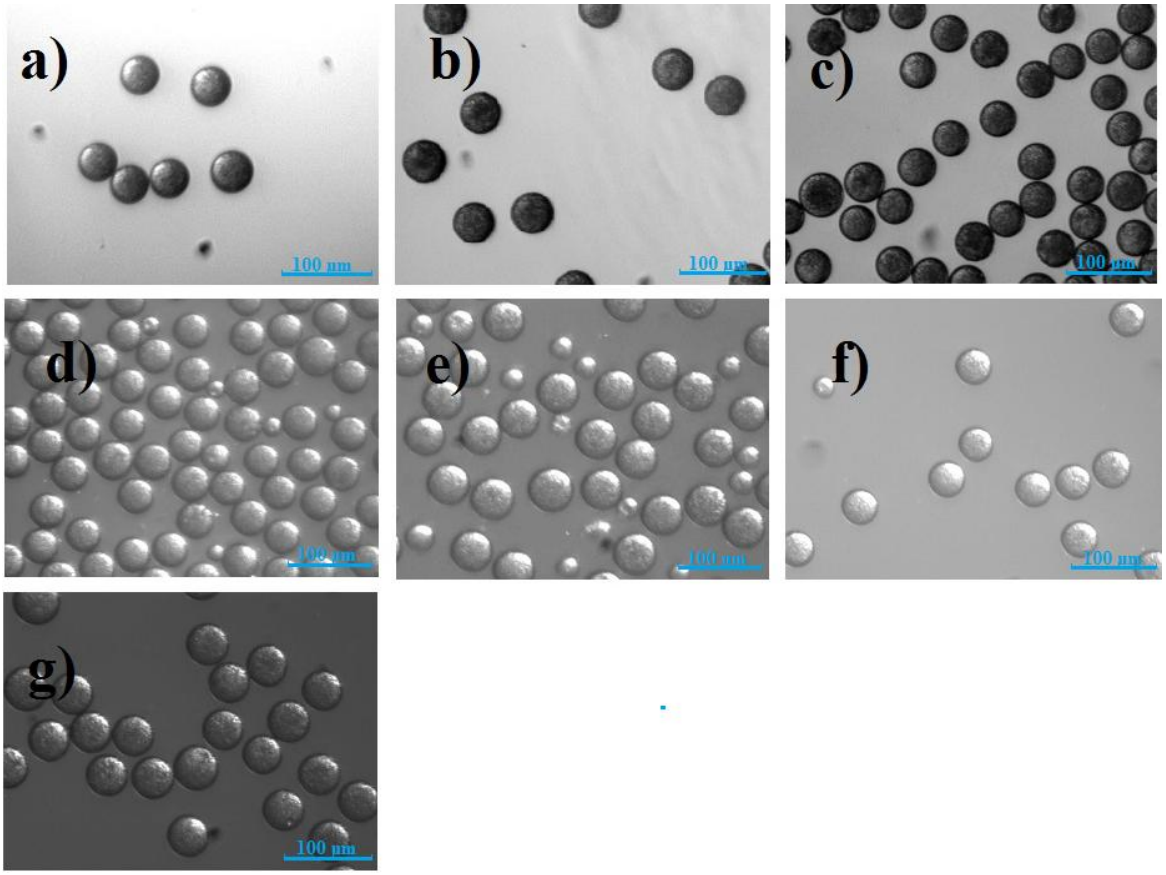

Figure 36. Optical microscope images showing the particles of formulation #34, batch 1 in the short-time stability tests on days (a) 0, (b) 1, (c) 3, (d) 7, (e) 14, (f) 21, and (g) 28. Differences in color are due to the settings of the optical microscope.

Figure 37 elucidates the changes in the particle sizes with the standard deviations. The batches observed were significantly different from each other, and thus, the changes in the particle sizes were compared only within each batch using Student's t-test to determine the statistically significant differences.

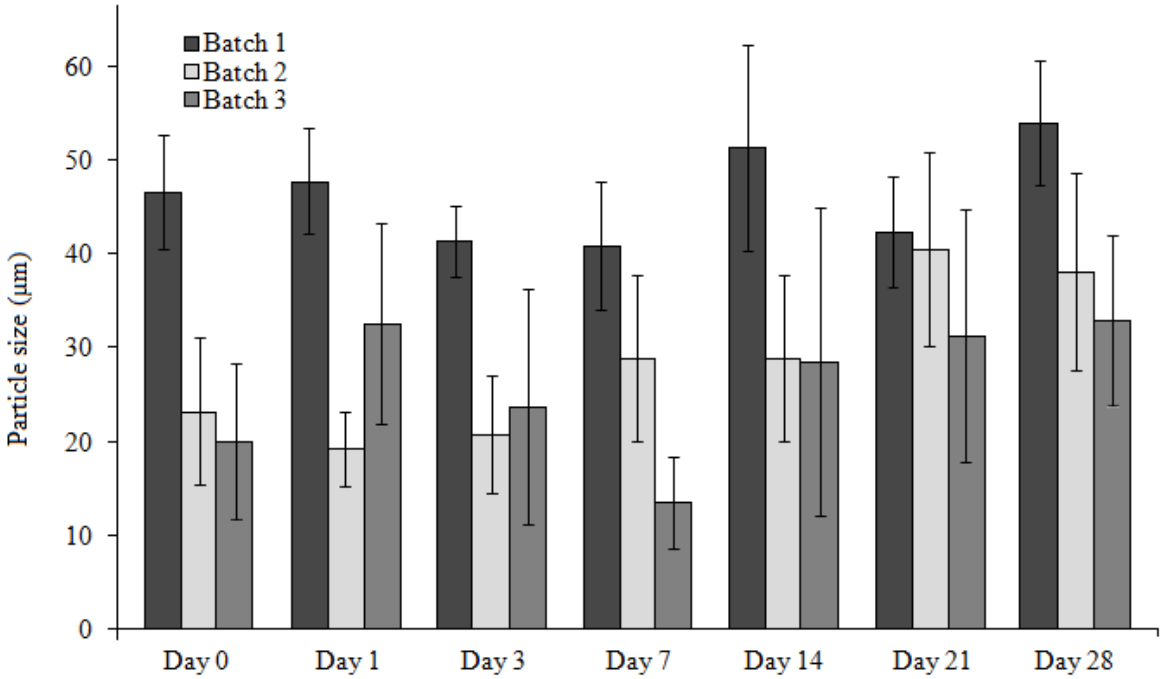

Figure 37 Particle sizes on days 0, 1, 3, 7, 14, 21 and 28. Batches 1 and 2 are particles of formulation #34 and batch 3 contains particles of formulation # 37. Standard deviations are presented as error bars of the 100 particles measured.

Statistical analyses using the Student's *t*-tests conducted to the follow-up batches until day 28 showed the following results. In batch 1, statistically significant differences in the particle sizes occurred between all days except days 0 and 1, as well as between days 3 and 7. With batch 2 the particle size did not change between days 21 and 28. The statistically significant differences in the particle sizes for the third batch were found to be between days 0 and 1, days 1 and 3, days 3 and 7, as well as between days 7 and 14.

The short time stability tests were successfully conducted for 6 weeks for two new batches. In addition to measuring the particle size, the collapsing of the particles was observed in these samples (**Figure 38**).

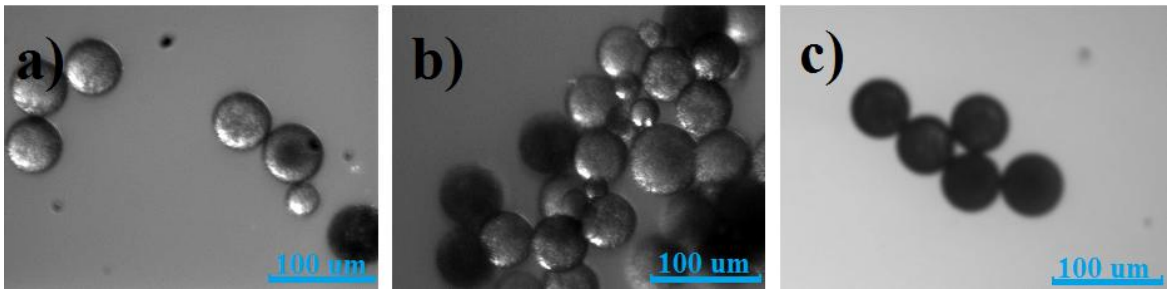

Figure 38. Optical microscope images showing the particles of formulation #34 on days (a) 28, (b) 35 and (c) 42 (Batch 1).

The size of the collapsed particles did not increase and the collapsed particles remained round but flat. Particle sizes of these microspheres are presented in **Figure 39**. The two bathes observed were significantly different in size.

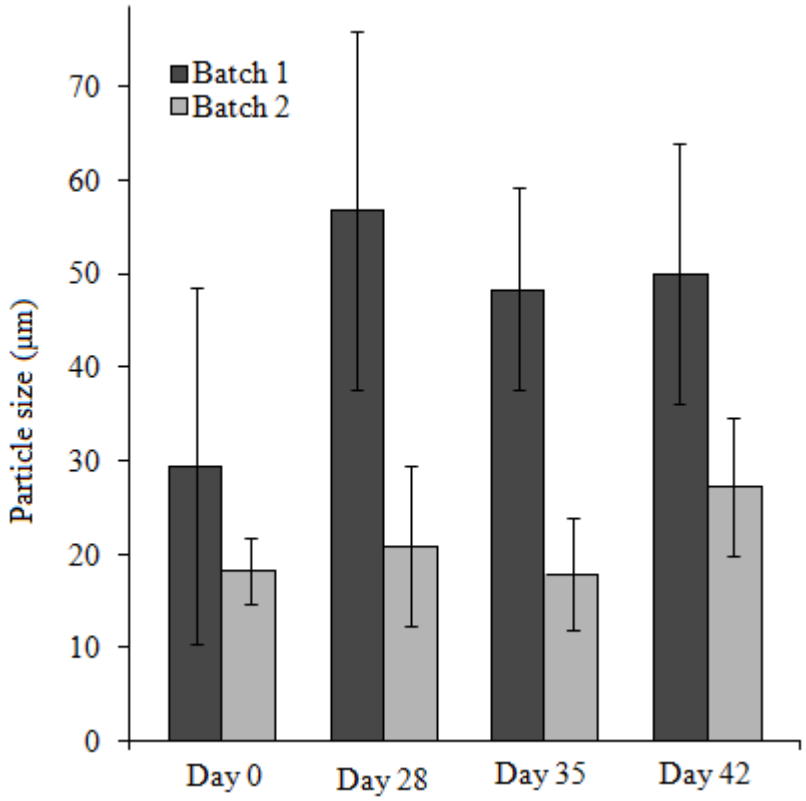

Figure 39 Particle sizes of formulations #34 (Batch 1) and #37 (Batch 2) on days 0, 28, 35 and 42. Standard deviations are presented as error bars of the 100 particles measured.

According to the Student's *t*-test results within batch significant changes in the particle size took place between days 0 and 1, and days 28 and 35. No statistically significant differences were found between days 35 and 42. With batch 2, statistically significant differences were found in between the particle sizes of days 0 and 28, days 28 and 35, as well as between days 35 and 42.

Results for the collapsing of the particles during the short time stability study are presented in **Table 16**. With these batches collapsed particles were detected already on day 28. The collapsing rate between the batches varied.

Table 16. Percentages of collapsed particles on days 28, 35 and 42.

Collapsed particles (%)	**Day 0**	**Day 28**	**Day 35**	**Day 42**
Batch 1	0	5.7	18.3	41.0
Batch 2	0	0.7	15.0	18.9
Average ± standard deviation	0	3.2 ± 3.5	16.6 ± 2.3	30.0 ± 15.6

In many cases all the particles prepared were used in the stability tests. This affected the remaining population and the sample left to be examined the following time. Additionally, other problems with optical microscopy discussed earlier (see section 7.3.1) also affected the results of the stability study.

Particle sizes in the bulk emulsion were observed for 3 days (**Figure 40**). The particles in the bulk emulsion were significantly less stable than the particles prepared with microfluidic technology.

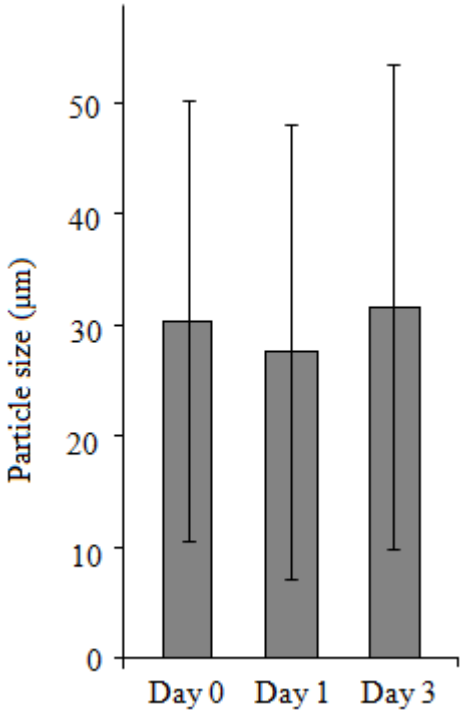

Figure 40. Particle sizes in the bulk emulsion for the days 0, 1 and 3. Standard deviations are presented as error bars of the 100 particles measured.

Deviation in the particle size was significantly greater and the particles were more polydispersed than particles produced with microfluidics. The bulk particles collapsed faster than the particles manufactured with microfluidics. On day 0 ca. 2.9% of the particles were collapsed, on day 1 ca. 24.5% and on day 3 ca. 27.8%. The collapsing rate of 27.8% on third day indicated that the bulk particles were significantly more unstable than the particles prepared with microfluidics. This indicated that the formulation for the bulk droplet production was not ideal and it proved that the particles prepared with microfluidics were significantly more stable.

Particles were stable also when dried (**Figure 41**). Dried particles tended to stick together and form flocculates. The dried particles stayed intact and spherical up to 28 days, and altogether appeared to be more stable than the particles stored in the collection media.

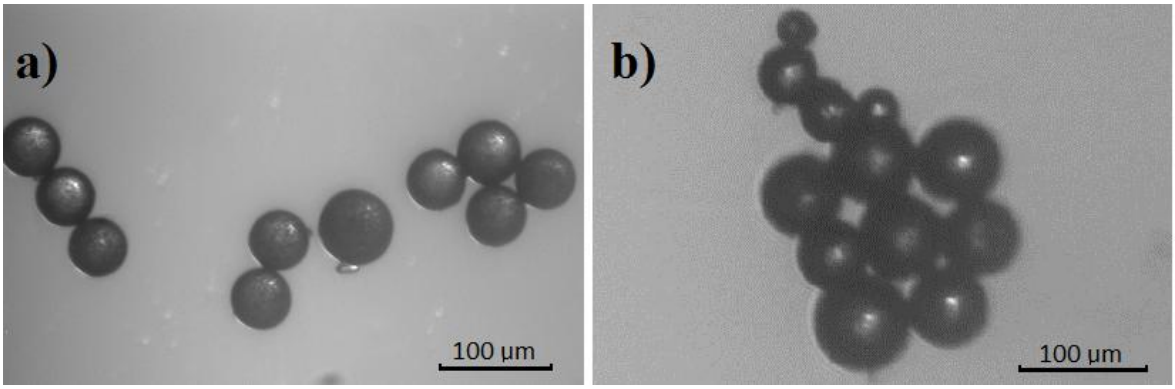

Figure 41. Optical microscope images showing the dried PCL particles: (a) after drying (day 0) and (b) after 4 weeks (day 28).

Stability of the polymer microspheres has been monitored in various methods. Stability studies in stressed conditions and short-time stability studies are conducted as for other drug formulations. Particle size follow-up was not commonly chosen. The collapse of PCL particles at 4 weeks was observed, for example, using SEM, and the percentage of collapsed particles was not determined (Cheng et al. 2010).

7.3.4 Surface properties of the particles

Particles appear to have a non-porous, smooth surface and monodisperse size (**Figure 42**). Evaluation of the surface properties of the particles without coating was made using a wet stage EVO 55 SEM microscope.

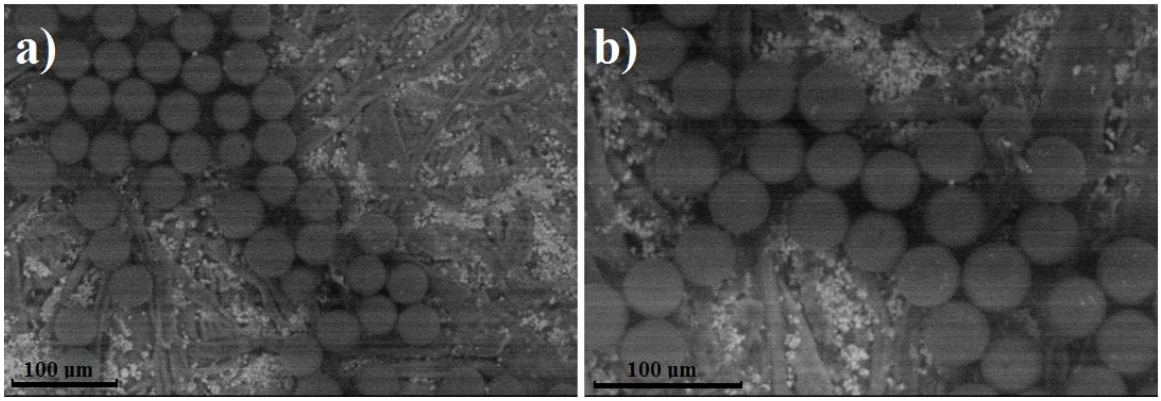

Figure 42. SEM images of PCL particles: (a) is a 506× magnification taken at chamber pressure of 682 Pa with 26 kV, and (b) is a 735× magnification of different particles of the same batch with the same pressure and voltage.

The resolution of the images taken with wet stage was limited and more accurate images were impossible to obtain. Wet stage imaging containing water vapour in the chamber did not provide clearest images. However, the obtained images were clear enough to determine the previously described properties of the particles. The accuracy of the background provided good comparison for the particle surfaces. The paper used as the background was shown highly detailed, and thus, it is possible to conclude that since the particle surface did not have such structure, it was flawless. Particle size determined from the SEM images did not statistically differ from the results of optical microscopy. The batch observed was the first batch used in the 4-week stability test.

PCL particles prepared in other studies also showed non-porous surfaces and altogether similar surface properties from PCL and poly(ε-caprolactone) (Tomar et al. 2011; Somavarapu et al. 2005; Bolzinger et al. 2007; Hnaien et al. 2011; Lee et al. 2000).

7.3.5 Encapsulation efficiency

Encapsulation efficiency determination for *β*-galactosidase failed to succeed. Ethyl acetate residue in the samples effected the degradation of ONPG, thus given erroneous results and causing problems with reproducibility. Loading particles as well as further experiments with *β*-galactosidase were not conducted due the difficulties with analysis.

Encapsulation efficiencies for salbutamol sulphate and BSA were determined successfully and the results were remarkable. The results of the tests were reproducible and the variation between the parallel tests was extremely moderate. Encapsulation efficiencies of salbutamol sulphate are presented in **Table 17**.

Table 17. Encapsulation efficiencies (EEs) of salbutamol.

	1	**2**	**3**	**Average**
EE (%)	86.5	80.5	82.5	83.2

Theoretically, 100% encapsulation efficiency could be achieved with microfluidics, since the particles do not lose their content when stored and the leaking of the inner phase only took place as the double emulsion droplet production was disturbed as described in section 7.2.3 As the PCL particles containing salbutamol were broken with sonication, it was possible to determine the absolute amount of salbutamol encapsulated in the whole system. As these quantities were compared with the theoretical quantities, some differences were found (**Table 18**).

Table 18. Theoretical and experimental determination of the amount of salbutamol encapsulate in the particles.

	1	**2**	**3**
Theoretical amount of salbutamol (µg)	25.0	12.5	25.0
Experimental amount of salbutamol (µg)	20.4	15.7	28.5
Difference between results (µg)	–4.6 (82 %)	3.2 (126 %)	3.5 (114 %)

Differences between the theoretical and the experimental amounts of the encapsulated salbutamol were caused by the variation in the drop size in the first cylindrical capillary during the preparation process. During most processes the drop size grew as the preparation process was continued. Thus, the batches collected in the beginning of the process were most likely to contain less of the inner phase than theoretically assumed. The batches collected towards the end of the process were most likely to contain more of the inner phase than theoretically assumed. This size variation can be explained by the hydrophobic coating slowly wearing off, and thus, the inner diameter of the cylindrical capillary growing and allowing larger drops to form.

The total amounts of BSA were only determined theoretically (**Table 19**). This creates a moderate error source for the encapsulation efficiency of BSA. However, the fact that the BSA batches used in encapsulation efficiency experiments were selected so that the variation of the drop size during the droplet preparation process was as marginal as possible, and should have decreased the effect of this source of error.

Table 19. Encapsulation efficiencies (EEs) of BSA.

	1	**2**	**3**	**Average**
EE (%)	72.2	92.4	87.3	84.7

The encapsulation efficiency measured from the PCL particles either with salbutamol sulphate or BSA produced using microfluidic technology were substantially higher than the encapsulation efficiencies achieved with conventional double emulsion production methods.

Generally, the PCL particles made with microfluidics were significantly better in terms of encapsulation efficiency than the particles prepared by other methods found in the literature. In many studies the encapsulation efficiencies that were achieved were approximately 40–60% (Somavarapu et al. 2005; Coccoli et al. 2008). Other studies indicated poorer encapsulation efficiency of barely 40% (Scala-Bertola et al. 2012,

Bolzinger et al. 2007, Jeong, Lee and Cho 2003, Hnaien et al. 2011). In some other studies, relatively high encapsulation efficiency have been achieved for big particles; these particles had diameters larger than 180 µm, and thus, were not suitable for oral administration (Natarajan et al. 2011). Generally, an encapsulation efficiency of 70% was considered high, with flow-focused jetting cell PCL particles with encapsulation efficiency of 42–79% was achieved (Cheng et al. 2010).

7.3.6 Drug release

Confocal fluorescence microscopy

Figure 43 illustrates the release of FITC-dextran from a single PCL particle. The particle was followed for 2 h during the experimental released of its content.

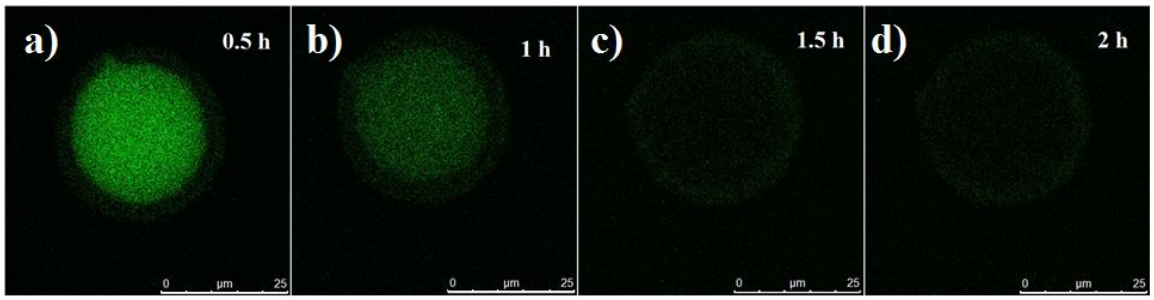

Figure 43. Confocal fluorescence microscope follow-up images of one PCL particle, showing the release of its FITC-dextran (green) content: (a) the shell rupturing from the thinnest spot, (b) particle losing its FITC-content, (c) and (d) particle without FITC-dextran content.

All the particles did not release their content simultaneously. The particle observed with confocal fluorescence microscopy had an uneven shell that wore off quicker that the shells of the other particles and enabled the follow-up when the FITC-dextran was still undoubtedly fluorescent. The degradation of the PCL particles, which was an autocatalyzed reaction (Pitt 1990), occurred in the shell evenly, and thus, the thinnest part of the shell broke first. After the shell broke down, the FITC-dextran content released within 1.5 h. The release kinetics observed this way proved that the droplet did not lose all

its content at once and the dissolution happened gradually and in a somewhat controlled manner. Additionally, the droplet did not collapse as the content of the inner phase was released. The PCL shell remained intact and the particle kept its spherical form.

Nevertheless, the confocal fluorescence microscopy follow-up required approximately 30 min of usage of the laser on the same particle. This could have decreased the excitation from the fluorescent FITC-dextran, and thus, made the particle followed dimmer than it actually was. In addition, this also made the release process appear faster than it is in reality.

Dissolution tests

Dissolution tests performed in the Snapwell diffusion chambers failed to succeed. Even though the membranes chosen had presumably low protein binding and pore size significantly greater than the molecular size of the protein, the membranes hindered the diffusion process so that the results represent rather the quality of the membrane than the actual drug release.

The release tests performed in the glass vials were successful and produced more accurate data on the dissolution process. Physiological BSA dissolved immediately into the dissolution media. BSA was dissolved completely when the time elapsed was 1 min. At 30 s 58% of BSA at pH 7.2 and 19% of BSA at pH 1.2 was dissolved. More acidic conditions retarded slightly the dissolution of BSA. The protein solubility is often dependent on the pH of the dissolution media (Pelegrine and Gasparetto 2005).

The release of BSA from the PCL particles at pH 7.2 is presented in **Figure 44** and **Figure 45**. The results of the tests are presented up to one week, since after that the protein degradation began (see Appendix 3).

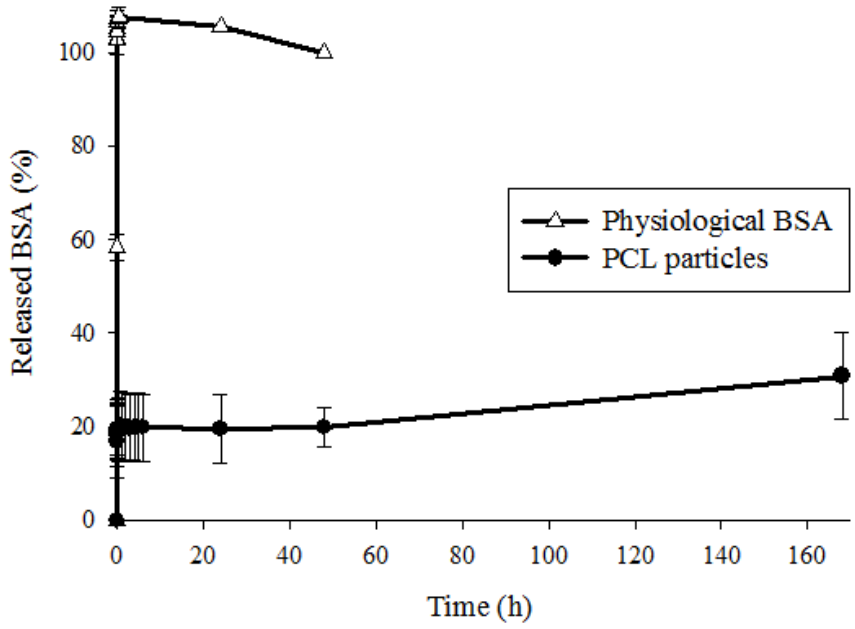

Figure 44. Release profiles of physiological BSA and BSA released from PCL particles at pH 7.2 during one week.

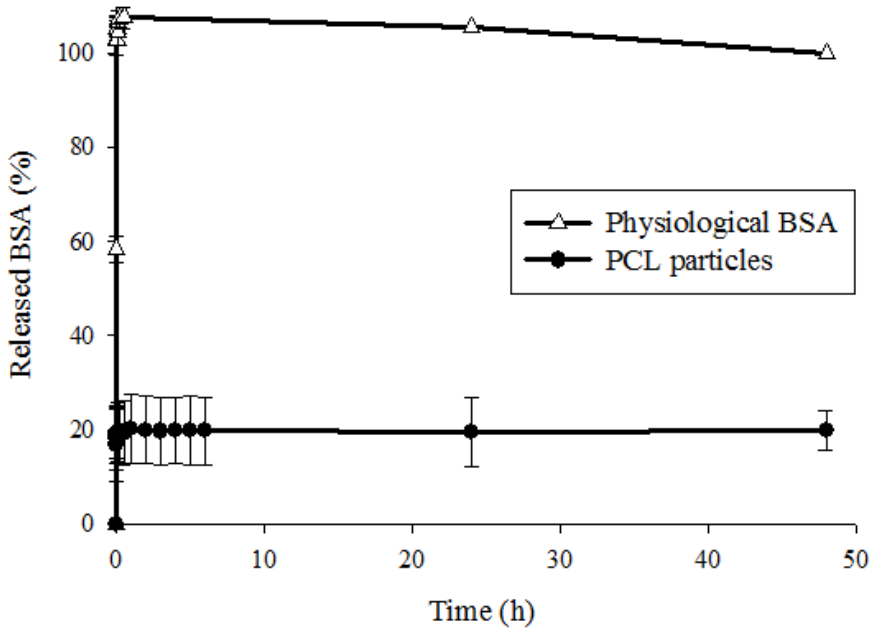

Figure 45. Release profiles of physiological BSA and BSA released from PCL particles at pH 7.2 during the first48 hours of the experiment.

Protein degradation may have begun within hours of starting the experiment. Thus, a larger error in the results is possible. The variation between the results of the two parallel tests was moderate. The shrinking of the particles most likely caused the slow and uncomplete release of BSA. If the particle shell had not thickened, the erosion of shell caused by the autocatalyzed degradation of PCL would have been faster the shell and thus the rate and degree of release would have changed.

The release of BSA from the PCL particles at pH 1.2 is presented in **Figure 46** and **Figure 47**. At lower pH the degradation of BSA began earlier and affected the results more significantly. However, also at lower pH, part of the BSA was released immediately and after that the release rate slowed down. The concentration of BSA started to drop after the elapsed time was 5 h. After this point the results at pH 1.2 are not valid.

The detection limit in the BSA method was 5 µg/mL; the quantities below this concentration were undetectable, and thus, the slight increases in the drug release were possibly undiscovered.

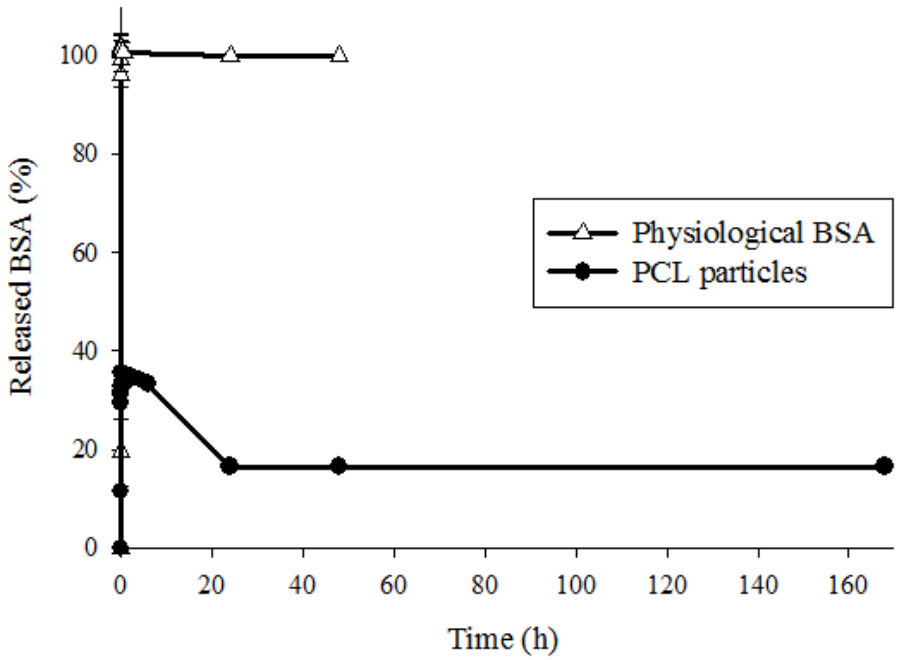

Figure 46. Release profiles of physiological BSA and BSA released from PCL particles at pH 1.2 during one week.

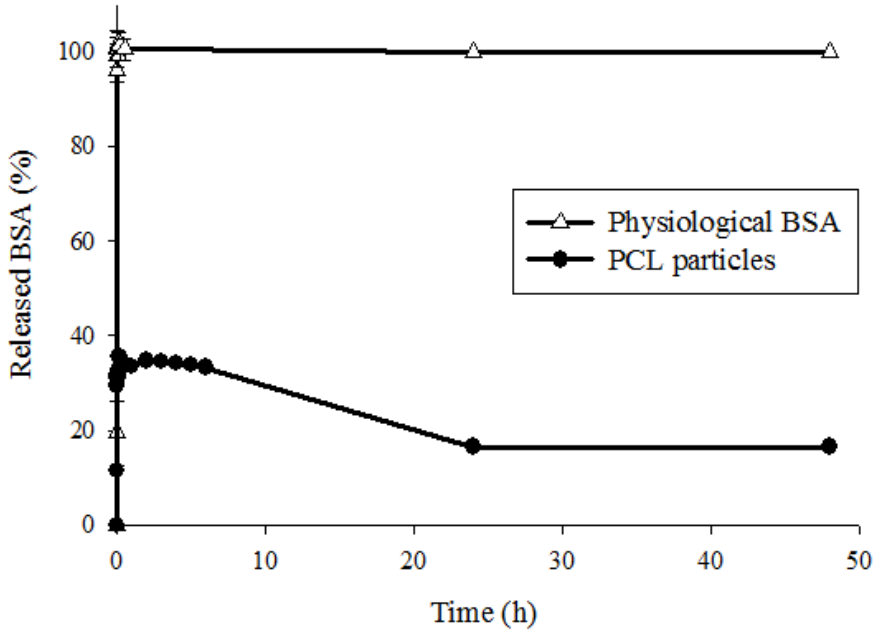

Figure 47. Release profiles of physiological BSA and BSA released from PCL particles at pH 1.2 during the first 48 hours of the experiment.

Sonication in the end of the experiment did not produce valid results. By sonication the protein degradation was faster than the release of the protein from the particles. Thus, HPLC analysis resulted in lower concentrations the longer the sonication was continued. Complete release and analysis of BSA was not possible. Using salbutamol or other hydrophilic small molecule as model drug would have been more suitable for modeling the drug release kinetics from the PCL particles. However, release kinetics and effect of pH with protein and drug with smaller molecular size is not similar and the exact modeling of protein kinetics is not possible. To produce valid results the formulation used and the drug release experiment itself must be further developed.

Problems concerning drug release have also been observed in other studies where the PCL particles have been prepared with various bulk methods. In these studies, the complete release of the drug from the PCL particles has been challenging. The release studies on warfarin showed similar kinetics than those obtained in this study (Scala-Bertola et al. 2012). For example, from the PCL microparticles containing ibuprofen nanoparticles

inside within 24 h approximately 40% of drug was released (Sheikh Hassan et al. 2009), whereas 45–65% of quercetin was released within 700 h (Natarajan et al. 2011).

Protein drug release from various PCL particles has also been widely studied. Differences between the release profiles were caused by the formulation and preparation method used. With BSA approximately 35% was released from the PCL particles within 30 days (Coccoli et al. 2008). In this study, very similar results using sonication and stirring as the production method were obtained. Only 50% of myoglobin was released in 48 h (Hnaien et al. 2011). When studied with recombinant hepatitis B surface antigen PCL particles released 80% of their content in 165 days (Tomar et al. 2011). On the other hand, papaverin was released from the PCL particles up to 80% within 170 h (Jeong et al. 2003). About 90% of lysozyme was possible to release from PCL particles within 30 days (Cheng et al. 2010). The differences in the release profiles were caused by the formulation and preparation method used. However, like in the research found in the literature also in this master's thesis work the release of protein drugs was generally slower than the release of small molecule drugs.

8. CONCLUSIONS

In this study, two functional formulations were developed: 5 % poly(vinyl alcohol) in the outer phase, 3 % of polycaprolactone in the middle phase and either 10 % or 20 % of polyethylene glycol and poly(vinyl alcohol) (1:4) in the inner phase. Overall, in this research work the microfluidic technology was demonstrated to have great potential for droplet manufacturing for pharmaceutical applications and to create templates for protein drug delivery. However, formulation screening process indicated that finding suitable formulations for microfluidics is demanding. Formulations had to contain phases that were correspondent to each other in terms of viscosity and the formulations required prolonged the optimization processes.

Producing double emulsion droplets (W/O/W) with the two tip glass capillary device was impossible using biodegradable polymers in solvents acceptable for medical preparations. Combining microfluidics with bulk method did not provide advantages in the formulation production besides protecting the protein structure in the preparation process. Droplet production was possible with the highly viscous organic solvent using the glass capillary device with biphasic flow. Yet, also this formulation required precise adjustments in order to produce stable droplets. Once the formulation was optimized, constant droplet production was achieved and a stable droplet production system was established.

The double emulsion droplets prepared were significantly better in all aspects examined than the droplets produced with bulk methods. Overall, the microfluidic approach made it possible to develop mild preparation suitable for protein structured drugs, gaining high protein encapsulation efficiency and forming stable, monodisperse, and non-porous particles. The particle size varied according to the device used and even more monodisperse particles could be produced with poly(dimethylsiloxane) (PDMS) devices. Encapsulation efficiency was the most remarkable feature of these particles. Conventional methods are not able to reach encapsulation efficiencies of 85 % for the same size scale of particles.

The only major set-back in the otherwise functional formulations was the unpredicted shrinking of the particles that was caused by the crystallization of PCL and led to imperfect drug release. This phenomenon mostly likely takes place as any sort of PCL particles are prepared, yet only with microfluidics the preparation process is possible to monitor and compare with the actual product. This possibility to monitor particles during formation creates an opportunity to learn more on the polymer behavior in the microparticle preparation processes.

Further research would be required to determine whether the particle size could be more precisely controlled by using poly(dimethylsiloxane) devices and co-polymers, and even try with more complex formulations. To obtain better dissolution profiles the formulation should be further optimized to control the shell thickness of the droplets. Industrial scaling-up production is not possible with the glass capillary devices and it would need a matrix

for poly(dimethylsiloxane) devices develop for biphasic flow and overcoming the issues related to the droplet preparation process. Yet the controlled particle preparation method offers interesting insight for pharmaceutical industry and these carefully optimized dosage forms could be employed for example for developing personalized medications.

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APPENDIX 1

Rheology measurements

Formulation #39

Sweep - 1					
Step time	Temperature	Stress	Shear rate	Viscosity	Normal stress coefficient
s	°C	Pa	1/s	Pa,s	Pa,s²
11.0008	25.778	3.31E-03	0.10002	0.033087	-29.2905
22.04	25.774	2.52E-03	0.215471	0.011717	-12.1372
33.0767	25.766	1.85E-03	0.46421	3.99E-03	-2.27341
44.1118	25.766	6.67E-03	1.00017	6.66E-03	0.544877
55.149	25.764	0.0119723	2.15482	5.56E-03	0.752949
66.1982	25.747	0.0200477	4.64193	4.32E-03	0.288263
77.2335	25.749	0.0387751	10.0014	3.88E-03	0.010961
88.2685	25.744	0.0817472	21.5468	3.79E-03	-1.68E-03
99.3018	25.735	0.17419	46.4214	3.75E-03	-9.50E-04
110.337	25.732	0.370748	100.012	3.71E-03	-3.80E-04
121.37	25.725	0.793482	215.47	3.68E-03	-2.02E-04
132.405	25.717	1.72126	464.214	3.71E-03	-1.52E-04
143.437	25.712	4.04191	1000.12	4.04E-03	-1.39E-04

Sweep - 2					
Step time	Temperature	Stress	Shear rate	Viscosity	Normal stress coefficient
s	°C	Pa	1/s	Pa,s	Pa,s²
11.0008	25.692	2.45E-03	0.100011	0.024459	1.32544
20.04	25.688	2.32E-03	0.215471	0.010771	-5.29873
33.0747	25.682	3.63E-03	0.464249	7.82E-03	-0.918536
44.1118	25.68	5.97E-03	1.00021	5.97E-03	0.627181
55.147	25.675	0.0122962	2.15483	5.71E-03	0.605617
66.1982	25.673	0.0196101	4.64198	4.22E-03	0.197806
77.2355	25.664	0.0382432	10.0012	3.82E-03	-2.12E-03
88.2687	25.664	0.0807071	21.5467	3.75E-03	-3.45E-03
99.3038	25.659	0.172504	46.4215	3.72E-03	-1.36E-03
110.337	25.653	0.369332	100.012	3.69E-03	-4.60E-04
121.372	25.654	0.794264	215.469	3.69E-03	-2.17E-04
132.407	25.646	1.72454	464.215	3.72E-03	-1.53E-04
143.439	25.634	4.03734	1000.12	4.04E-03	-1.38E-04

Formulation #40

Sweep - 1					
Step time	Temperature	Stress	Shear rate	Viscosity	Normal stress coefficient
s	°C	Pa	1/s	Pa,s	Pa,s²
11.0007	26.258	2.01E-04	0.100012	2.01E-03	-31.6295
22.0398	26.251	2.48E-04	0.215471	1.15E-03	-10.9731
33.0745	26.255	2.91E-04	0.46427	6.26E-04	-3.17657
44.1117	26.256	3.90E-04	1.00012	3.90E-04	-0.397196
55.1468	26.253	2.30E-03	2.15484	1.07E-03	8.25E-03
66.1982	26.248	3.41E-03	4.64201	7.35E-04	-8.78E-03
77.2353	26.249	8.36E-03	10.0013	8.36E-04	-2.86E-03
88.2685	26.247	0.0208056	21.5468	9.66E-04	-7.88E-04
99.3037	26.242	0.0466936	46.4216	1.01E-03	-8.18E-04
110.337	26.238	0.104813	100.012	1.05E-03	-3.32E-04
121.372	26.231	0.234577	215.469	1.09E-03	-1.83E-04
132.405	26.231	0.616363	464.215	1.33E-03	-1.47E-04
143.438	26.222	1.85953	1000.12	1.86E-03	-1.37E-04

Sweep - 2					
Step time	Temperature	Stress	Shear rate	Viscosity	Normal stress coefficient
s	°C	Pa	1/s	Pa,s	Pa,s²
11.0008	26.293	8.37E-03	0.100014	0.083715	3.88889
22.04	26.291	8.05E-03	0.215471	0.037344	-3.40971
33.0747	26.291	8.54E-03	0.464246	0.018402	0.554676
44.1118	26.286	9.42E-03	1.00024	9.42E-03	1.21059
55.147	26.287	0.0114446	2.15476	5.31E-03	0.329788
66.1982	26.282	0.0123715	4.64197	2.67E-03	0.0861939
77.2335	26.283	0.016979	10.0013	1.70E-03	7.41E-03
88.2687	26.284	0.0299864	21.5468	1.39E-03	2.00E-03
99.3018	26.274	0.0554195	46.4215	1.19E-03	-1.79E-05
110.337	26.276	0.11078	100.012	1.11E-03	-2.35E-04
121.372	26.277	0.243573	215.469	1.13E-03	-1.56E-04
132.408	26.269	0.630506	464.214	1.36E-03	-1.40E-04
143.439	26.261	1.88094	1000.12	1.88E-03	-1.37E-04

Formulation #41

Sweep - 1					
Step time	Temperature	Stress	Shear rate	Viscosity	Normal stress coefficient
s	°C	Pa	1/s	Pa,s	Pa,s²
11.0008	26.071	9.36E-04	0.100014	9.36E-03	131.662
22.0398	26.071	-2.40E-04	0.215471	-1.12E-03	57.8368
33.0767	26.072	7.98E-04	0.464209	1.72E-03	18.8248
44.1118	26.071	1.64E-03	1.00015	1.64E-03	5.9929
55.149	26.072	4.96E-04	2.15471	2.30E-04	1.46349
66.1982	26.066	1.06E-03	4.64208	2.29E-04	0.245281
77.2335	26.074	7.50E-03	10.0011	7.50E-04	0.0414677
88.2685	26.072	0.019918	21.5469	9.24E-04	8.41E-03
99.3018	26.069	0.0474268	46.4213	1.02E-03	1.35E-03
110.337	26.113	0.106342	100.012	1.06E-03	1.80E-04
121.37	26.068	0.243396	215.469	1.13E-03	-5.99E-05
132.406	26.068	0.633757	464.215	1.37E-03	-1.17E-04
143.437	26.113	1.87632	1000.12	1.88E-03	-1.27E-04

Sweep - 2					
Step time	Temperature	Stress	Shear rate	Viscosity	Normal stress coefficient
s	°C	Pa	1/s	Pa,s	Pa,s²
11.0008	26.036	-5.02E-03	0.100011	-0.050174	5.44401
22.04	26.035	-5.03E-03	0.215471	-0.023352	2.91614
33.0747	26.04	-4.53E-03	0.464252	-9.76E-03	1.78252
44.1118	26.041	-3.18E-03	1.00017	-3.18E-03	0.665913
55.147	26.046	-1.73E-03	2.15469	-8.02E-04	0.262554
66.1982	26.049	-3.64E-04	4.64198	-7.84E-05	0.012364
77.2353	26.056	5.91E-03	10.0013	5.91E-04	7.42E-03
88.2685	26.058	0.0186672	21.5468	8.66E-04	-8.07E-04
99.3038	26.058	0.0448827	46.4217	9.67E-04	-7.22E-04
110.337	26.064	0.105838	100.012	1.06E-03	-3.09E-04
121.372	26.068	0.242489	215.469	1.13E-03	-1.69E-04
132.405	26.072	0.624784	464.214	1.35E-03	-1.40E-04
143.439	26.071	1.84694	1000.12	1.85E-03	-1.30E-04

APPENDIX 2

Particle sizes

Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
43.6752	14.1426	50.327	47.776	34.4088
47.4786	21.5928	30.506	43.572	40.8132
51.777	14.2956	33.588	30.828	35.2008
44.82	28.0062	33.588	36.65	39.6432
50.049	21.8052	34.554	33.127	17.0748
49.3506	33.1272	45.155	31.252	52.272
58.0878	21.87	36.076	36.44	45.5094
44.82	36.8658	36.68	29.777	37.9008
48.3138	23.9076	32.593	37.155	35.8758
44.0208	28.0152	30.506	36.44	37.7118
53.2836	21.492	31.809	32.626	41.6754
45.8784	17.973	29.998	43.065	39.7764
53.8146	35.5284	36.59	41.587	39.4848
56.8512	26.1144	29.998	38.887	36.8784
51.9318	21.5766	32.894	35.617	34.9992
46.971	20.0142	32.727	42.963	34.8966
19.8216	44.1558	32.626	39.364	42.1632
42.4368	42.1308	29.63	35.586	38.7594
47.4786	18.5598	33.292	39.807	42.687
42.1632	19.6434	33.424	37.771	37.9008
39.7764	19.2312	31.497	32.626	34.9992
45.5094	18.0054	31.427	31.427	34.1766
42.9372	21.6252	29.63	33.848	43.0398
53.1324	16.5762	28.304	40.976	36.3924
48.1662	19.1754	33.588	39.89	35.901
49.977	21.2994	27.676	35.556	38.8728
41.868	23.8266	30.828	29.998	38.9412
43.4106	24.2442	35.339	37.303	13.131
50.7726	16.1496	37.067	40.68	31.6926
51.2946	19.5912	26.831	38.774	41.4828
43.9794	17.1972	29.63	49.247	42.9372
50.7726	18.2466	33.424	45.446	43.7364
46.971	21.9294	30.947	37.771	37.9008
54.927	15.0516	29.886	38.774	40.4856
48.9528	24.2442	33.587	31.497	51.4674
52.5276	37.1412	33.155	35.832	34.7688
46.6668	43.9956	37.253	37.944	41.4198
47.4786	19.4832	29.329	38.089	37.7118

47.7036	36.4482	31.213	33.848	37.1898
53.6652	26.9568	39.404	35.679	38.6892
45.6066	27.756	30.769	36.68	34.1766
41.8464	40.5432	31.454	29.219	54.927
39.2148	27.585	40.42	25.316	38.205
53.1	37.3104	31.497	40.301	48.1842
44.7012	25.5096	35.786	37.712	41.868
44.0802	16.7652	36.696	36.44	35.901
43.1838	16.9254	29.117	38.433	38.8278
39.3948	15.2136	30.68	35.121	41.868
50.7726	30.9204	30.947	39.112	37.7118
48.5334	17.559	42.621	37.771	42.1632
43.6752	12.7944	30.023	35.863	43.533
44.82	22.6044	33.832	34.586	28.845
52.7472	29.9214	38.182	31.427	45.333
46.5714	23.229	36.508	33.815	48.0744
42.3522	16.7238	35.651	42.449	42.9984
51.4494	16.4502	27.196	32.356	46.6668
45.5094	19.926	38.972	31.912	48.4794
42.9372	18.0054	31.427	32.491	40.0896
44.3214	23.1246	31.146	39.112	40.5522
50.2614	19.2978	27.196	34.074	41.3982
46.5714	23.9256	60.759	38.774	25.6464
49.6206	28.314	33.522	41.955	47.2536
51.0336	15.516	36.68	45.155	42.6042
50.1912	27.6858	33.815	34.363	42.5412
47.2914	34.3134	37.037	31.427	45.918
46.647	27.5976	31.427	37.067	37.5462
49.6206	30.5568	29.998	34.712	45.6066
41.1822	15.9696	29.518	42.112	40.3092
45.5688	22.9986	34.363	33.783	39.6432
47.5902	20.493	31.146	41.745	52.0686
41.7618	14.841	55.689	33.783	36.27
44.0802	17.991	32.049	40.868	39.2148
45.4122	18.4086	44.568	36.799	44.4024
54.144	17.0136	36.59	34.554	48.5334
25.3332	18.8874	37.973	34.586	42.9372
44.9784	29.8368	38.233	35.121	24.0372
50.6664	16.8012	37.067	41.163	44.3214
48.0006	34.272	41.955	34.106	38.205
43.9992	17.6328	31.146	41.587	39.6432
36.099	14.6286	40.868	40.027	41.1606
36.3924	16.7184	33.783	32.894	51.2244

44.82	18.7344	36.799	40.976	45.5688
43.6752	21.834	38.774	43.192	36.2214
42.9372	15.7554	37.126	40	45.6066
45.9756	14.724	34.074	28.765	46.6866
22.6674	10.0314	25.915	35.679	36.2214
48.663	12.4272	37.944	32.894	50.1192
54.927	30.9402	31.252	41.508	44.8002
53.3502	29.2122	43.268	37.771	44.1414
52.6122	17.7318	39.112	24.117	52.5438
46.6866	13.1238	34.681	32.626	41.526
49.977	41.9292	38.519	33.815	44.4024
53.082	35.9982	40.68	34.586	39.3732
50.7726	21.5154	39.112	44.197	39.5748
46.5714	17.7426	34.106	40.301	42.9372
49.7826	18.3762	30.506	37.155	49.4766
48.9168	34.7004	31.912	38.519	42.1632
47.7954	28.8594	32.626	27.237	45.8208
44.721	22.0572	34.203	43.065	53.748
48.4794	21.2832	29.106	37.712	55.6812

Other particle sizes and measurements

Particle size. combination method (n=100)	Particle size. bulk (n=100)		Process particle size (n=100) Batch 1	Batch 2	Batch 3		Thickness of shells. collected (n=100)	Thickness of shells. process (n=100)
145.526	41.103		257.24	198.78	218.07		4.814	1.084
288.976	43.524		230.01	186.54	191.08		5.589	1.533
169.396	16.756		223.24	189.63	201.42		6.391	2.763
135.248	17.285		305.95	198.78	215.92		4.152	2.423
138.491	26.841		240.04	190.81	210.9		5.058	1.713
370.117	18.946		235.79	177.53	193.44		5.236	2.167
75.123	43.371		261.73	193.53	206.01		4.814	1.713
74.495	53.929		229.36	195.74	209.79		4.785	2.763
79.807	55.962		235.71	198.99	211.1		4.902	1.533
266.601	12.222		307.18	217.47	205.71		5.639	2.423
130.754	11.489		211.81	216.09	196.89		5.035	2.57
145.892	23.193		260.66	213.21	202.78		4.731	3.089
113.862	39.472		233.14	217.15	196.51		6.162	2.763
143.651	52.068		233.32	205.14	198.31		5.748	1.916
92.975	88.003		217.64	205.1	212.09		5.834	1.58
258.719	12.708		236.25	177.61	200.18		7.097	1.626
254.849	17.166		219.78	199.15	204.44		7.283	1.58

278.883	39.628		251.43	212.31	212.09		5.547	1.381
158.24	28.202		214.09	188.64	194.66		6.15	2.234
141.605	16.801		248.93	193.65	184.91		6.131	2.682
99.86	20.253		253.84	183.59	198.42		5.445	2.331
67.912	13.116		258.86	210.19	183.74		6.112	2.234
43.081	25.767		236.09	220.57	209.97		6.614	2.709
261.262	14.207		236.9	468.25	206.19		5.436	1.533
422.823	53.602		254.72	372.02	205.14		5.743	1.533
431.021	60.208		249.98	379	200.35		4.58	2.167
184.017	17.841		266.2	384.62	223.43		3.886	1.533
194.775	40.123		255.49	362.01	188.44		6.266	2.167
148.461	19.504		249.53	350.76	212.97		6.478	1.533
233.581	36.034		224.49	365.78	215.92		5.785	3.427
222.314	63.021		234.12	383.06	207.64		3.452	1.713
182.23	15.494		280.36	387.68	276.59		10.575	1.713
259.392	8.917		223.99	395.44	214.61		9.463	4.468
84.216	10.949		218.18	295.17	209.79		5.436	1.713
67.634	6.498		322.95	212.09	220.95		4.99	1.713
43.457	8.377		306.68	211.16	193.44		6.299	1.713
44.222	31.048		192.05	203.34	216.41		5.634	2.763
25.912	32.115		231.55	222.63	225.93		4.72	3.159
70.072	48.659		252.27	207.77	216.24		7.968	1.533
248.417	55.486		238	194.66	224.91		11.105	2.763
259.327	52.423		244.59	209.03	198.21		9.96	0.766
77.304	22.685		242.83	197.62	212.25		8.287	1.713
212.144	10.17		231.17	199.97	201.23		6.443	1.084
124.43	27.448		239.1	226.07	194.66		10.187	1.713
90.614	14.628		238.53	191.69	201.23		6.653	3.427
131.048	19.216		241.26	212.31	207.16		5.873	2.423
69.52	36.66		220.57	192.76	192.76		8.287	2.57
92.843	7.4772		246.67	215.92	218.93		8.157	2.299
178.72	13.932		262.64	198.21	218.93		5.768	2.57
94.134	20.062		250.88	189.63	181.69		9.112	1.626
88.434	49.71		223.26	222.99	234.18		6.653	1.626
82.882	59.041		235.71	217.21	205.53		9.945	1.626
123.182	64.629		226.59	206.35	215.83		4.942	1.724
85.537	60.766		231.14	211.81	216.24		5.094	2.57
56.163	18.523		234.06	218.84	221.48		6.744	2.874
138.189	6.71		260.39	197.74	196.89		6.467	2.072
213.11	9.072		212.09	232.9	205.6		10.187	1.149
214.819	12.373		248.08	208.31	239.02		10.627	2.57
63.756	20.093		190.91	234.12	200.37		5.094	1.149
6.376	21.241		218.67	210.39	201.19		7.222	1.626

37.066	27.712		254.54	216.52	205.14		6.529	3.448
45.975	9.858		235.79	211.1	206.33		6.065	2.299
266.685	8.48		218.93	192.56	205.51		3.497	1.713
182.874	21.43		261.82	211.03	196.89		4.89	1.084
175.351	27.667		237.37	229.77	218.41		6.529	0.766
129.735	35.683		251.62	226.24	215.4		8.064	0.766
225.916	11.367		250.88	226.3	201.83		5.425	2.423
152.649	5.682		238.02	218.93	183.31		7.222	1.713
84.242	18.253		221.62	207.16	190.41		8.12	1.713
188.316	32.004		231.55	188.91	198.99		5.711	1.149
188.779	44.197		216.24	210.9	218.84		5.95	1.626
387.105	13.386		247.03	212.42	201.21		7.264	1.149
206.487	8.114		222.38	193.65	193.07		8.854	2.299
114.561	64.654		239.72	222.23	215.13		5.237	2.299
261.685	78.881		249.44	217.13	140.71		6.407	2.299
265.21	85.064		228.23	220.95	207.64		2.624	2.57
76.769	57.074		232.19	225.55	194.78		5.249	2.57
153.698	7.979		231.87	229.68	198.09		10.821	2.57
185.872	40.771		255.07	218.07	204.44		10.097	1.533
38.517	20.093		231.63	197.74	194.5		7.423	2.299
164.393	40.131		234.06	213.02	195.81		10.498	2.763
150.234	9.538		235.02	244.13	198.78		7.873	2.423
116.304	48.929		246.02	205.71	206.19		8.216	1.533
168.708	34.425		238.85	235.49	210.45		9.167	2.299
137.712	20.649		224.43	213.98	199.55		10.097	2.423
172.757	6.13		248.78	234.12	205.53		8.939	3.251
142.848	28.688		233.08	197.74	207.28		5.868	1.713
164.819	8.037		231.71	208.71	190.41		8.464	3.251
132.682	43.898		210.9	224.89	192.47		10.028	2.167
100.384	68.023		243.15	220.2	197.36		11.737	1.713
59.962	63.54		238.85	228.54	201.42		9.463	1.713
100.384	12.029		235.24	230.68	201.39		9.39	1.713
308.61	23.07		228.23	220.61	204.98		6.844	2.167
67.431	44.137		216.93	224.56	361.56		5.868	3.159
115.83	35.604		233.54	213.39	235.3		10.028	3.065
173.78	30.405		222.23	212.95	215.66		7.139	1.713
151.242	10.573		218.95	220.2	214.72		11.969	2.763
174.051	47.617		207.97	219.72	217.66		8.939	3.089
139.847	17.771		222.63	212.97	206.19		7.515	2.453
226.695	37.366		246.5	204.44	209.76		10.097	3.089

4-week stability studies

Batch 1 (n=100)						
Day 0	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
43.6752	45.2934	44.3214	34.4088	56.016	39.7764	64.089
47.4786	54.144	37.071	40.8132	58.8024	41.7402	59.4432
51.777	50.4036	37.7352	35.2008	64.0134	40.1112	64.8594
44.82	47.2914	38.205	39.6432	51.777	32.2488	54.6588
50.049	49.6206	41.4198	17.0748	56.3958	39.3948	58.2264
49.3506	46.2258	38.7594	52.272	68.7276	44.1612	55.5264
58.0878	50.6844	39.7764	45.5094	66.6666	37.7118	55.4868
44.82	50.1912	33.993	37.9008	50.9112	37.7586	50.8428
48.3138	45.5094	44.1414	35.8758	34.8966	39.5748	54.2358
44.0208	47.1402	40.3542	37.7118	54.2916	41.1606	54.8856
53.2836	51.9318	38.6658	41.6754	61.4628	39.9564	62.7156
45.8784	44.8002	43.9992	39.7764	62.2548	40.1994	55.6866
53.8146	44.4024	39.9564	39.4848	57.7044	50.1912	56.2194
56.8512	45.117	48.5334	36.8784	30.2292	42.9372	47.3688
51.9318	22.0698	41.3982	34.9992	49.4946	45.5688	56.9862
46.971	49.977	42.6042	34.8966	60.2514	41.868	50.8464
19.8216	46.2078	38.1816	42.1632	56.9592	50.049	53.757
42.4368	49.0626	40.7052	38.7594	61.767	45.117	53.676
47.4786	49.3506	37.143	42.687	37.071	45.117	51.6618
42.1632	49.9248	37.5714	37.9008	58.5756	51.0336	58.437
39.7764	24.8022	39.9996	34.9992	50.2092	41.8464	51.1506
45.5094	48.9168	35.3016	34.1766	53.334	44.4024	55.728
42.9372	45.117	34.6914	43.0398	57.4416	42.9372	49.4946
53.1324	44.82	38.8728	36.3924	34.6662	46.5138	59.1084
48.1662	48.0924	42.3738	35.901	56.016	42.687	54.9558
49.977	44.82	49.4946	38.8728	56.7252	42.5214	56.0628
41.868	48.9168	40.1112	38.9412	52.5942	39.6432	51.0174
43.4106	41.4828	40.7916	13.131	34.1496	35.226	56.5902
50.7726	49.4946	38.6658	31.6926	37.5462	44.3214	53.154
51.2946	43.6752	37.7586	41.4828	64.0278	45.9756	56.8152
43.9794	49.1886	37.0458	42.9372	63.3438	41.868	56.574
50.7726	45.4122	42.75	43.7364	47.1402	40.5522	56.8674
46.971	48.8988	48.663	37.9008	56.2698	44.4024	49.8438
54.927	60.6042	39.2364	40.4856	64.6776	44.82	33.1002
48.9528	46.971	45.3528	51.4674	64.3464	46.5714	52.2792
52.5276	46.6668	42.6672	34.7688	60.0156	49.0266	56.7684
46.6668	45.3528	40.8132	41.4198	54.144	45.9756	51.8058
47.4786	55.071	54.144	37.7118	53.5986	46.0332	60.1362
47.7036	45.8208	39.3948	37.1898	64.0008	51.2244	55.5732

53.6652	47.1402	44.0208	38.6892	50.6844	42.9372	55.5174
45.6066	44.181	42.687	34.1766	48.5334	33.4404	51.6618
41.8464	45.3528	41.4828	54.927	49.4766	24.5862	58.3038
39.2148	41.7402	40.5522	38.205	62.6814	22.1904	44.3088
53.1	45.9756	39.9564	48.1842	52.4772	41.1822	48.5082
44.7012	49.4046	39.9996	41.868	33.7572	51.777	56.7486
44.0802	53.9298	42.687	35.901	37.5462	40.0896	54.2286
43.1838	49.7826	44.0802	38.8278	62.496	28.0314	27.5274
39.3948	49.5486	49.4766	41.868	64.1394	46.8954	55.35
50.7726	27.4878	45.6066	37.7118	54.405	41.355	60.444
48.5334	55.2006	38.8728	42.1632	58.9392	39.2148	60.5898
43.6752	47.7954	41.3982	43.533	52.7472	41.526	56.1186
44.82	48.8988	37.3338	28.845	51.3108	38.7594	57.924
52.7472	48.5334	35.7768	45.333	69.6024	44.3214	58.3038
46.5714	54.2754	50.7366	48.0744	63.3852	43.6752	57.375
42.3522	58.2102	34.1766	42.9984	30.1698	40.6386	54.477
51.4494	46.5138	34.7688	46.6668	41.526	43.3692	56.3598
45.5094	48.0744	40.9014	48.4794	60.3684	51.0516	51.7032
42.9372	44.721	40.023	40.0896	60.2964	44.5626	51.3774
44.3214	60.2964	46.5714	40.5522	53.55	40.8132	51.8472
50.2614	46.8378	42.687	41.3982	49.6206	43.533	55.5732
46.5714	53.1	39.7764	25.6464	51.1038	38.9412	55.1952
49.6206	49.6206	47.3472	47.2536	50.1138	50.9472	52.5258
51.0336	41.355	41.526	42.6042	60.3684	48.8988	56.9106
50.1912	43.9992	48.8988	42.5412	61.29	47.2914	57.2886
47.2914	53.3502	35.8758	45.918	52.7472	45.117	54.648
46.647	45.8208	36.7812	37.5462	54.8622	39.9564	47.6136
49.6206	44.7012	41.6538	45.6066	50.6844	46.8954	57.726
41.1822	44.9784	43.6752	40.3092	56.9592	40.6386	57.15
45.5688	44.3826	44.181	39.6432	50.7726	37.3572	56.5812
47.5902	56.142	42.9984	52.0686	60.2964	37.9476	51.3684
41.7618	49.3326	41.868	36.27	25.6464	44.3214	53.2332
44.0802	57.888	39.9996	39.2148	27.3582	37.9008	51.8544
45.4122	43.6752	43.4304	44.4024	39.9996	39.5982	50.9346
54.144	49.5486	38.6892	48.5334	25.4736	40.8132	53.0586
25.3332	47.7396	41.0742	42.9372	48.8988	40.6386	51.9462
44.9784	49.3326	41.526	24.0372	47.3472	42.9372	56.9016
50.6664	46.647	40.1112	44.3214	33.3342	42.5214	56.3292
48.0006	51.2946	38.4822	38.205	58.6674	43.533	62.0784
43.9992	52.8318	40.617	39.6432	38.7594	37.9008	57.15
36.099	47.178	37.143	41.1606	30.6954	37.7118	56.7486
36.3924	51.3108	47.1402	51.2244	33.993	39.7764	58.7286
44.82	45.6066	39.6432	45.5688	33.993	43.533	56.9106

43.6752	46.6866	41.526	36.2214	23.589	47.3472	33.2604
42.9372	48.1662	41.0742	45.6066	36.3924	18.7146	61.3008
45.9756	46.7424	42.9984	46.6866	55.6974	45.918	51.5358
22.6674	47.1402	38.6658	36.2214	43.9794	38.8728	54.6156
48.663	49.9248	40.8132	50.1192	56.9592	57.951	57.0762
54.927	47.1402	42.4368	44.8002	62.6814	48.4614	53.64
53.3502	43.0812	41.7402	44.1414	49.3506	43.9794	54.522
52.6122	54.144	45.6066	52.5438	54.6822	40.4856	47.997
46.6866	55.6974	40.7052	41.526	49.4046	38.9412	57.402
49.977	42.9372	39.2148	44.4024	59.4036	44.82	24.4314
53.082	46.3806	43.4106	39.3732	53.748	50.1912	31.563
50.7726	44.5014	42.9984	39.5748	53.748	30.4038	55.8216
46.5714	49.4766	49.4046	42.9372	56.0628	49.3326	52.137
49.7826	53.8146	40.7052	49.4766	54.7308	42.75	58.1724
48.9168	48.663	43.4106	42.1632	36	44.5014	54.2394
47.7954	45.333	40.023	45.8208	45.6462	42.9984	54.639
44.721	46.2258	44.82	53.748	62.8938	46.818	60.5664
48.4794	48.0744	40.1112	55.6812	51.1038	47.7954	59.2002

Batch 2 (n = 100)						
Day 0	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
14.1426	19.3212	41.337	67.8258	50.445	31.1274	38.7594
21.5928	17.055	20.6532	37.5696	26.586	43.9794	34.6914
14.2956	19.6488	19.7964	40.1274	47.079	44.3214	29.2734
28.0062	11.7702	9.8856	26.1036	26.2872	45.4122	21.7044
21.8052	11.8872	22.5432	31.3704	38.034	46.5714	46.3806
33.1272	15.0516	22.3038	40.743	24.8526	49.6026	45.5094
21.87	16.2792	32.8248	39.1284	57.3786	42.9372	46.5714
36.8658	17.1828	19.0152	41.4972	18.423	51.4674	31.6926
23.9076	15.9588	19.4724	35.577	26.4042	51.5016	23.9994
28.0152	32.643	10.8522	35.7768	54.675	43.0812	22.1904
21.492	18.0882	30.3822	26.3988	32.4612	36.8784	36.4176
17.973	19.0656	23.436	40.8132	51.8544	78.4746	49.8168
35.5284	18.1458	22.3362	53.6814	30.4416	34.1766	38.7594
26.1144	20.9898	17.9046	39.7764	30.9528	37.0458	45.6066
21.5766	20.3562	14.4108	26.6994	28.251	40.617	26.667
20.0142	14.3082	20.9484	28.3464	23.2794	40.1112	44.82
44.1558	17.7966	24.0534	33.4404	24.903	29.2734	42.1632
42.1308	16.848	22.3794	29.2428	26.586	42.687	42.1002
18.5598	17.757	32.8536	36.0252	29.0754	21.4992	36.0252
19.6434	28.5228	18.468	37.6416	23.4324	38.1816	75.8952
19.2312	19.9782	18.2952	36.612	26.667	35.8758	44.181

18.0054	17.9532	25.2522	25.0506	10.9494	36.3924	24.5862
21.6252	30.8556	14.8608	20.0448	34.1892	37.7352	25.4736
16.5762	15.075	16.3188	32.985	25.929	46.971	21.0816
19.1754	23.499	22.3686	26.667	24.3414	37.5714	38.7594
21.2994	17.9766	23.1624	41.355	30.771	48.4614	45.6462
23.8266	23.3946	27.2628	24.5862	56.2302	31.1274	33.3342
24.2442	18.9378	10.1772	26.6994	27.3978	41.4198	34.1496
16.1496	16.5348	22.9302	28.2528	28.4706	24.4764	42.5214
19.5912	19.926	26.5716	34.0722	32.3982	40.5522	42.3738
17.1972	18.6912	27.1908	34.4088	10.2456	41.0742	30.2292
18.2466	15.4638	23.4468	30.7818	29.871	42.5412	34.4088
21.9294	17.6382	22.3794	30.9258	29.9952	52.272	46.5714
15.0516	20.6802	23.1624	26.667	23.6556	34.6662	21.7044
24.2442	17.4186	26.5626	37.3572	32.3226	44.181	54.9756
37.1412	16.1496	24.1038	33.012	27.9864	46.2834	40.5522
43.9956	17.4546	30.0906	27.3582	25.767	51.8634	29.1204
19.4832	17.2638	11.3796	65.1294	27.6282	45.6066	34.1766
36.4482	20.0142	17.5176	26.9982	27.8856	49.7826	49.1706
26.9568	17.6184	12.8898	33.7572	24.678	30.4038	45.5094
27.756	15.3288	11.6334	37.4292	34.4664	38.8728	48.0744
40.5432	17.6328	12.582	24.0372	30.9528	73.7568	37.9476
27.585	18.5508	21.6702	28.845	26.9802	39.033	64.0278
37.3104	18.9	21.0762	24.0372	29.2446	40.0896	49.4766
25.5096	19.2672	13.9806	26.6994	26.1972	41.355	53.1
16.7652	16.128	10.8738	24.5862	31.4658	37.9008	33.3342
16.9254	20.7558	25.8732	41.4828	26.8992	23.7402	24.0372
15.2136	16.3008	8.8974	41.3982	29.9322	40.617	44.0208
30.9204	25.0398	23.7366	28.782	21.8484	39.2148	33.651
17.559	17.7318	20.5596	31.608	22.6854	41.6754	21.4992
12.7944	22.1868	16.893	18.135	23.2794	43.6752	43.0398
22.6044	17.856	33.5826	29.8134	37.6848	37.7118	42.75
29.9214	18.2088	21.5334	26.6994	31.257	49.7826	25.4736
23.229	19.6254	16.7904	18.8568	26.6148	46.5714	27.8406
16.7238	20.0016	14.1372	33.3342	28.9026	29.1204	25.7508
16.4502	22.4496	6.444	20.3958	21.438	50.1912	37.9242
19.926	15.8832	10.485	19.6866	45.225	36.3924	42.75
18.0054	17.5788	27.414	66.3048	29.0754	36.684	34.6914
23.1246	19.3212	18.468	31.4676	24.1434	16.2756	48.9168
19.2978	20.097	21.9384	36.684	28.7694	41.1822	42.9984
23.9256	19.3626	23.4882	23.2848	13.1166	43.7364	24.9084
28.314	16.9722	20.0178	30.7818	28.3986	45.5094	63.2322
15.516	15.4188	19.8954	33.3342	24.6024	33.4404	30.0816
27.6858	19.3626	28.0404	20.3094	28.7694	40.7052	26.6994

34.3134	20.6874	32.1318	32.1102	28.962	78.1344	47.1402
27.5976	19.3896	19.5732	26.9982	46.1052	16.7058	35.0748
30.5568	16.7184	21.2598	28.0314	27.306	39.3948	43.4106
15.9696	18.1458	19.422	30.4632	23.1786	47.2914	32.1102
22.9986	18.432	20.9484	22.6674	26.2152	38.9412	43.7364
20.493	16.56	19.3338	23.9994	30.0096	49.977	38.8728
14.841	23.0184	22.2156	27.4554	39.1554	32.2488	33.993
17.991	21.312	16.0776	43.0398	29.1762	46.2834	24.0372
18.4086	18.3762	12.6018	31.212	28.0026	20.1762	24.0372
17.0136	17.136	29.862	22.7052	28.935	45.117	38.1348
18.8874	16.3062	19.1952	24.0372	29.2176	43.2666	50.1912
29.8368	18.0054	20.1636	25.1568	30.1644	38.4822	39.9564
16.8012	18.8118	23.4468	25.8894	29.367	38.6658	21.4992
34.272	16.0578	34.281	18.8568	24.3738	28.6272	48.9168
17.6328	16.848	20.3202	48.0924	31.77	36.7812	34.6158
14.6286	20.7846	19.4724	32.1102	27.1692	30.6954	31.0986
16.7184	28.449	20.0178	22.7844	27.3582	51.5196	31.1274
18.7344	25.8048	20.9484	30.1698	22.0806	33.993	46.6866
21.834	17.6382	22.293	28.7208	20.7396	21.0816	34.6158
15.7554	17.46	15.6312	30.7818	28.9026	40.9014	20.6982
14.724	18.9234	21.8502	32.985	35.964	43.0398	47.1402
10.0314	33.1866	17.6418	37.6416	28.0026	27.8406	44.5014
12.4272	19.8036	22.9302	31.0122	19.1916	33.3594	42.6672
30.9402	18.5076	12.2076	9.4284	25.641	42.1632	34.2792
29.2122	18.0306	16.6158	26.3988	23.9886	26.5662	35.2008
17.7318	17.3196	27.2628	36.27	26.7948	37.143	48.1662
13.1238	29.5542	20.97	28.0008	13.293	35.577	52.29
41.9292	17.2782	24.2658	29.5146	33.2604	39.6432	45.3528
35.9982	17.8902	27.2628	29.3328	30.159	37.7118	20.8692
21.5154	18.0306	21.9492	24.5862	15.3324	36.612	27.4878
17.7426	15.8832	25.9488	28.1268	18.2196	52.272	50.6844
18.3762	16.4916	17.1378	25.6122	21.6774	32.0562	34.8966
34.7004	20.3814	6.0138	40.1112	22.194	37.9008	41.7402
28.8594	22.1526	18.5076	42.4368	17.0496	57.348	29.3634
22.0572	31.1562	22.0716	40.5522	54.1368	46.647	46.8954
21.2832	18.8586	18.5076	32.2758	28.9998	40.4856	43.9794

Batch 3 (n = 50)						
Day 0	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
14.4216	22.1238	19.3122	30.8394	33.1002	24.1758	29.871
19.1052	35.1432	10.8198	22.8402	70.1478	30.6702	34.0344
16.2792	21.6774	16.1604	25.5816	41.8284	17.9892	35.4258

12.5388	26.586	21.762	12.8952	36.0342	39.33	30.366
12.4128	9.225	21.9888	11.2194	15.9678	70.668	36.7632
5.94	22.7394	24.444	12.4614	23.0184	14.6592	39.5712
6.7392	35.892	25.047	15.012	18.9234	37.899	34.1766
13.7106	30.897	25.713	11.0214	21.762	21.5046	33.4368
47.5074	27.7128	22.8762	10.809	13.932	17.9118	23.4126
22.8042	23.9886	15.939	10.071	14.2074	20.2626	19.5768
26.4204	21.2202	14.5854	12.5064	12.9978	40.14	30.6558
21.0942	20.619	12.222	11.4984	41.1714	43.4826	29.3184
20.592	40.4712	14.6286	10.071	46.5246	34.0668	34.542
19.8486	24.8526	17.8074	11.2932	31.887	58.176	41.3766
19.8486	20.889	40.959	11.9556	64.3284	13.1166	45.3582
21.0942	44.613	36.2232	11.1456	30.1392	26.3574	16.074
18.4392	40.635	30.1392	13.5306	15.7824	18.2538	19.1754
17.8038	21.1842	21.0654	15.012	19.6308	21.3588	35.8956
16.9704	46.5876	20.4066	15.012	11.9268	12.708	36.819
16.1244	42.3558	13.932	14.1156	13.932	46.3878	41.463
16.1244	44.9298	17.7714	12.6918	11.061	29.6676	41.3316
46.6686	34.3746	28.1142	10.9476	10.17	54.4104	23.3316
20.5182	31.3758	16.9488	15.2676	35.4654	20.1384	38.6496
13.4532	57.9906	17.7372	11.3418	44.8326	26.4042	37.5372
14.9994	42.6924	24.0408	10.2996	53.6148	28.0026	31.887
36.0702	26.7552	18.144	12.1032	23.6556	28.2186	39.1284
19.8486	26.6148	43.4106	12.582	23.7618	14.031	18.0828
26.0766	19.9368	22.1238	12.1032	32.0364	28.0638	45.7506
14.9994	44.9226	22.7466	11.9664	32.004	24.678	38.745
18.4392	45.3186	19.8432	15.561	26.2872	44.1126	14.6286
16.155	42.5862	20.889	12.7998	15.3738	46.0116	35.091
18.8676	32.7276	19.3446	13.3668	35.9424	27.1404	40.2246
15.0336	28.9836	19.719	25.7958	30.2058	16.6248	23.6952
22.8042	60.354	13.0104	13.3452	21.294	27.1746	31.842
15.5556	42.21	13.797	11.8638	15.453	55.7964	25.3296
17.7192	32.3658	51.7428	12.9492	12.8286	40.347	27.8532
15.6528	25.731	81.9216	25.0614	26.6382	52.092	30.7368
19.9998	27.981	28.296	12.9798	26.4042	35.496	35.1432
36.0702	28.791	21.8484	8.5338	21.0654	15.1596	35.8776
14.8662	40.0356	19.5048	22.7142	21.9618	24.7338	40.2012
17.6922	22.0806	30.0096	10.809	16.4556	22.194	31.2282
22.203	39.4056	40.185	11.0592	14.2074	36.9864	40.2354
21.9312	37.287	20.2626	9.4518	17.946	25.2018	15.9678
39.825	36.9396	13.707	12.339	18.4572	49.4694	56.0448
16.155	9.0036	13.932	10.179	24.066	16.2288	39.9564
21.0942	39.5712	43.0074	11.2932	31.941	39.0456	36.6498

19.4166	27.7632	21.294	11.4768	31.6134	31.3308	42.0372
18.8676	36.0864	36.4572	10.971	55.1898	34.0992	13.1526
16.9992	26.856	11.3688	5.3532	90.6174	40.8672	26.7948
14.0364	37.89	8.4636	10.0476	28.6884	21.798	41.103

6-week stability studies

Batch 1 (n = 100)					Batch 2 (n = 100)			
Day 0	Day 28	Day 35	Day 42		Day 0	Day 28	Day 35	Day 42
22.9842	91.9332	51.5016	54.5364		15.9804	30.5478	18.549	23.7402
39.1554	75.2832	52.9506	52.092		17.3556	14.7546	20.1312	48.1842
18.1692	45.918	50.9562	69.7752		17.5212	24.8778	35.3898	32.2218
12.6036	42.687	53.8002	37.6848		13.4784	15.732	25.083	26.667
11.781	57.4722	59.094	56.9106		18.9036	15.3018	14.031	17.8884
11.7234	50.7366	70.8282	55.2492		15.588	18.7326	13.977	22.7052
15.2046	37.3572	49.4604	56.8422		15.4188	14.7222	23.9634	24.5862
8.1558	85.8006	51.0174	55.827		11.0214	15.4836	13.1526	35.0748
11.5452	26.9658	51.6096	56.502		15.2406	14.8068	13.932	44.8002
14.067	77.4018	52.8966	54.7524		17.8092	12.8664	15.2208	28.1268
12.429	53.2332	52.5294	53.667		20.2374	22.0806	13.932	19.0908
12.9492	25.4736	51.0174	51.7158		16.3872	19.062	13.932	23.2848
12.627	59.2254	51.0048	25.2324		13.3974	49.7016	16.7274	36.612
36.36	74.0574	57.204	32.148		12.24	22.2066	16.0452	20.0448
37.1016	41.868	78.5934	44.8776		16.0056	20.6874	11.8476	24.3306
24.9732	71.2188	50.4936	57.114		20.8782	21.294	14.5854	26.5662
17.4276	28.0314	30.897	51.3972		15.2406	13.2102	16.1604	45.333
18.8802	88.6446	56.0448	39.0132		19.341	25.047	27.7182	23.2848
27.0648	69.8562	52.4574	62.505		19.3338	16.866	16.0848	34.7688
20.952	34.6662	55.1898	58.779		15.4818	20.4444	20.4606	27.4878
24.0084	18.666	47.9394	60.1866		16.7184	17.8416	24.5268	21.8682
27.5922	73.4418	53.8146	86.2488		15.9444	27.5274	14.2074	17.7894
35.7768	44.721	48.717	56.088		21.5964	21.6774	7.5798	25.7508
21.1338	74.2122	51.7788	39.0618		13.3452	16.074	9.4896	29.2734
15.1218	83.916	55.2186	59.7528		16.3116	25.8516	16.0452	22.6278
15.1218	57.3948	56.1114	59.4198		16.4628	17.2764	13.3866	37.1898
42.8544	42.6672	53.3268	57.537		18.1692	15.4134	14.2074	40.7916
31.1274	49.0626	49.6566	61.8678		16.8822	15.453	20.8656	34.7688
59.7618	74.6316	20.5056	65.5056		18.5958	16.2	23.3316	20.0448
72.8964	60.3684	55.5264	27.6678		16.3782	16.2	14.6592	26.667
122.9274	60.0156	49.6566	57.5316		18.9396	30.9528	12.4614	25.6464
72.0126	58.2102	46.1826	56.7252		19.9224	13.1526	16.7274	25.6122
65.3886	66.6792	49.7574	55.3086		25.0614	17.9712	15.1596	26.5662
49.0266	40.9014	54.1962	52.9884		22.7628	15.0048	13.7628	28.2834

57.2706	71.6778	32.922	56.3508		17.3646	16.3908	13.4208	26.1954
112.0716	76.653	54.6156	39.1554		18.5292	16.9488	15.6132	22.7052
50.6664	55.458	19.4958	52.8084		13.1076	16.4664	12.3732	31.8042
35.865	67.3038	25.3296	54.63		13.5306	13.932	12.4614	28.1268
34.9218	75.2004	14.9742	53.9442		17.7408	16.2468	13.7628	21.9906
42.2064	62.4528	62.505	53.8362		14.8464	15.6042	15.1398	27.4878
34.5618	71.5536	30.6702	49.6008		19.6794	17.3106	11.7162	24.5124
13.671	64.0692	33.048	50.1552		20.6604	16.569	15.057	27.4878
12.5928	21.0816	51.6618	55.719		25.5816	16.2	29.871	23.7402
34.7004	21.0816	51.426	51.1056		27.2466	17.7012	20.3382	25.8894
32.8464	25.0506	53.5212	66.9564		11.5452	17.7804	17.3106	31.383
36.36	38.8728	50.0922	67.707		15.7284	20.0628	26.3394	29.3634
34.974	45.4122	55.53	53.2944		18.9036	14.6286	16.7184	23.589
35.838	22.7052	30.1392	54.0594		18.6696	12.708	16.9488	27.4554
22.0248	25.3332	56.3508	61.5186		15.525	17.3556	15.6438	34.4088
24.1686	74.9628	55.9728	60.7428		19.0836	16.2468	15.0462	23.9994
36.0216	74.6316	49.851	73.2078		18.0396	14.328	21.9888	19.413
33.9966	44.8002	72.0324	63.0684		18.1692	16.9488	15.7032	29.3328
31.8384	73.0908	49.5972	39.8952		21.393	11.4894	23.2794	22.3506
31.1976	51.8634	53.3358	65.817		15.4188	10.8198	13.932	17.8884
35.406	22.7052	31.6512	57.8196		15.2046	16.1604	15.057	18.666
35.9658	65.6046	28.377	61.9812		16.5798	16.7562	9.4734	20.1762
30.9492	26.4654	29.2446	83.2212		21.6162	16.8012	12.4614	23.3244
36.1476	76.5126	41.7654	21.762		24.4386	13.2462	6.687	29.9628
35.7318	77.1606	49.8222	59.7852		20.169	17.2026	13.5702	18.8568
36.6984	77.1606	48.132	59.5692		22.0248	14.994	17.3106	36.3924
38.619	75.2472	50.6016	56.178		22.8582	26.7552	34.1586	19.8216
34.974	77.1606	50.6592	58.779		18.5508	43.0002	22.9302	29.4534
35.712	41.4198	45.7812	57.537		16.686	33.4044	31.0878	20.0448
37.8378	64.2222	49.068	57.2886		21.6738	32.211	17.946	22.8222
33.57	61.767	51.462	65.448		10.071	36.5094	21.438	34.7688
12.1608	88.1622	31.7646	26.7732		19.7838	26.0082	18.7992	17.7894
10.0854	64.0008	52.3314	25.713		25.992	23.643	12.8286	51.777
13.4388	65.3328	49.3848	23.9688		18.1998	14.274	22.7322	28.0314
31.4964	72.1728	41.5422	26.667		28.6632	39.6648	19.0206	36.27
36.792	29.3634	50.3928	15.0048		16.5798	37.9476	12.7314	34.6662
36.6426	52.0686	50.4198	61.224		15.2406	36.7974	20.0934	16.11
35.9154	39.9564	34.4664	25.941		22.5684	15.8598	12.8664	24.5124
10.0854	69.6024	41.9004	29.554		16.8102	15.7716	16.0452	25.8894
37.7514	76.3614	27.6732	51.215		18.2592	14.031	14.994	24.4764
7.8642	70.7796	56.9412	39.871		15.561	14.7222	10.0458	26.2638
12.1608	46.053	48.1878	51.329		19.0836	19.6308	15.8598	27.8406
13.149	68.832	21.7044	58.75		17.4276	13.2462	18.4572	21.375

36.1134	70.8804	34.7166	58.44		15.9804	19.6308	13.707	31.6926
39.4002	69.9966	56.3544	53.967		16.4628	12.3732	15.7626	45.6462
34.0776	60.8094	51.7032	49.741		28.701	15.0876	19.7028	24.0372
34.6374	74.727	52.3062	45.049		19.1988	21.177	26.7786	24.3306
34.7166	58.545	49.851	38.722		19.3986	17.3106	20.6802	24.0372
16.2594	64.512	61.0722	40.516		19.3338	37.4868	16.1604	19.9998
10.0818	58.8024	54.2502	49.464		24.5466	38.322	24.8778	31.2408
14.2578	65.0196	51.9336	56.679		19.4058	28.5462	21.0294	23.3244
12.24	45.5094	51.0174	25.491		16.0488	12.9258	19.8432	31.383
15.012	57.7188	52.4664	28.491		18.5364	38.4876	18.1944	35.3016
6.399	58.6818	49.9068	26.124		17.9334	46.1736	24.885	45.333
19.7838	69.1794	51.2514	46.667		20.6604	26.4042	25.9956	31.1274
11.511	69.3846	51.1758	56.377		20.7936	21.9618	32.8986	31.8042
9.8928	22.0698	53.2332	34.305		18.5958	13.3974	18.144	25.4736
9.6246	20.1762	52.182	58.547		16.3782	18.2538	19.4652	21.8682
10.2996	34.6662	52.7058	48.787		19.818	17.2854	14.994	25.8894
21.5532	32.2488	52.3368	20.713		17.8938	37.3662	20.1852	28.5354
20.2374	39.2148	48.6882	25.841		19.4616	34.3026	9.4734	23.2848
23.2218	36.7812	47.4858	34.551		16.4124	34.425	16.3134	26.6994
24.327	87.0354	53.8002	49.184		14.2614	15.9588	18.423	30.1392
29.5182	73.9242	52.2414	44.193		18.9396	12.573	8.2278	16.7562
28.9494	45.8784	49.2678	35.572		15.561	20.6262	26.7948	21.762
48.3534	47.178	51.5142	45.016		19.2276	19.062	37.3752	10.3662

Bulk particle stability

Bulk batch (n=100)		
Day 0	Day 1	Day 3
41.103	54.68	94.483
43.524	47.313	80.573
16.756	8.488	83.489
17.285	61.678	71.586
26.841	17.094	57.443
18.946	38.64	34.752
43.371	54.793	23.07
53.929	50.934	10.99
55.962	9.921	12.222
12.222	8.65	23.018
11.489	17.285	18.523
23.193	19.504	14.628
39.472	47.368	33.645
52.068	11.433	28.427
88.003	17.058	62.497

12.708	66.673	35.548
17.166	9.14	11.433
39.628	22.546	57.661
28.202	7.979	52.11
16.801	38.143	5.817
20.253	39.025	31.386
13.116	25.689	48.738
25.767	5.923	53.294
14.207	5.682	20.253
53.602	68.779	18.599
60.208	77.257	10.719
17.841	61.453	27.442
40.123	57.8	34.392
19.504	22.46	64.152
36.034	11.284	13.293
63.021	19.936	26.287
15.494	54.561	7.477
8.917	22.847	12.335
10.949	9.14	10.045
6.498	46.841	26.422
8.377	11.06	23.392
31.048	49.8	22.869
32.115	34.466	34.012
48.659	50.82	60.094
55.486	20.062	23.988
52.423	45.7	32.365
22.685	58.181	57.234
10.17	69.586	15.523
27.448	63.304	78.28
14.628	57.443	28.983
19.216	16.54	10.819
36.66	9.732	17.78
7.4772	9.858	34.569
13.932	7.741	27.009
20.062	16.948	7.7616
49.71	61.171	9.489
59.041	18.381	55.686
64.629	6.156	24.683
60.766	3.389	30.664
18.523	32.34	40.822
6.71	60.226	18.558
9.072	34.335	72.568
12.373	23.727	75.24

20.093	14.659	43.41
21.241	34.909	6.982
27.712	30.655	19.719
9.858	41.421	24.519
8.48	20.687	16.502
21.43	13.797	40.921
27.667	5.517	12.259
35.683	6.229	8.227
11.367	18.45	12.731
5.682	1.762	33.496
18.253	13.977	9.275
32.004	4.057	14.839
44.197	8.377	12.817
13.386	14.065	14.097
8.114	60.278	25.75
64.654	31.152	7.853
78.881	27.869	46.123
85.064	6.687	47.763
57.074	18.466	57.321
7.979	11.104	64.153
40.771	22.69	45.471
20.093	17.622	25.232
40.131	68.099	35.404
9.538	5.572	19.191
48.929	14.207	34.144
34.425	29.662	45.678
20.649	17.71	16.275
6.13	10.704	5.599
28.688	15.859	10.719
8.037	30.695	32.779
43.898	71.366	77.533
68.023	5.817	35.395
63.54	19.384	10.17
12.029	15.139	7.048
23.07	9.522	4.728
44.137	6.687	44.056
35.604	11.367	35.877
30.405	21.682	5.137
10.573	33.528	30.159
47.617	31.77	23.806
17.771	6.778	5.014
37.366	11.755	76.903

APPENDIX 3

Dissolution tests

BSA. Physical mixtures. no membrane. pH 7.2 - series 1						
Time (h)	Concentration (µg/ml)	Quantity of BSA taken in the sample (µg)	Cumulative quantity to be added to the total quantity (µg)	Total quantity of BSA (µg)	Corrected concentration (µg/ml)	Released BSA (%)
0	0	0	0	0	0	0
0.008	97.72297	97.72297	0	1954.5	97.723	62.312
0.017	149.3252	149.3252	97.723	3084.2	154.21	98.331
0.033	141.6996	141.6996	247.048	3081	154.05	98.229
0.05	142.0427	142.0427	388.748	3229.6	161.48	102.97
0.067	137.8074	137.8074	530.791	3286.9	164.35	104.79
0.083	123.8877	123.8877	668.598	3146.4	157.32	100.31
0.17	124.4125	124.4125	792.486	3280.7	164.04	104.6
0.5	117.7142	117.7142	916.898	3271.2	163.56	104.29
24	113.7183	113.7183	1034.61	3309	165.45	105.5
48	113.7183	113.7183	862.227	3136.6	156.83	100

BSA. Physical mixtures. no membrane. pH 7.2 - series 2						
Time (h)	Concentration (µg/ml)	Quantity of BSA taken in the sample (µg)	Cumulative quantity to be added to the total quantity (µg)	Total quantity of BSA (µg)	Corrected concentration (µg/ml)	Released BSA (%)
0	0	0	0	0	0	0
0.008	71.53996	71.53996	0	1430.8	71.54	54.528
0.017	137.3221	137.3221	71.54	2818	140.9	107.39
0.033	130.3658	130.3658	208.862	2816.2	140.81	107.33
0.05	124.054	124.054	339.228	2820.3	141.02	107.48
0.067	119.1602	119.1602	463.282	2846.5	142.32	108.48
0.083	113.7008	113.7008	582.442	2856.5	142.82	108.86
0.17	109.2489	109.2489	696.143	2881.1	144.06	109.8
0.5	104.8966	104.8966	805.392	2903.3	145.17	110.65
24	92.69407	92.69407	910.288	2764.2	138.21	105.34
48	92.69407	92.69407	770.087	2624	131.2	100

BSA. Physical mixtures. no membrane. pH 1.2 - series 1						
Time (h)	Concentration (µg/ml)	Quantity of BSA taken in the sample (µg)	Cumulative quantity to be added to the total quantity (µg)	Total quantity of BSA (µg)	Corrected concentration (µg/ml)	Released BSA (%)
0	0	0	0	0	0	0
0.008	28.13627	28.13627	0	562.73	28.136	24.109
0.017	110.1351	110.1351	0	2202.7	110.14	94.37
0.033	108.7848	108.7848	110.135	2285.8	114.29	97.931
0.05	102.6724	102.6724	218.92	2272.4	113.62	97.354

0.067	101.7011	101.7011	321.592	2355.6	117.78	100.92
0.083	92.72462	92.72462	423.294	2277.8	113.89	97.587
0.17	89.76396	89.76396	516.018	2311.3	115.56	99.022
0.5	85.20962	85.20962	605.782	2310	115.5	98.966
24	82.15631	82.15631	690.992	2334.1	116.71	100
48	82.15631	82.15631	690.992	2334.1	116.71	100

BSA. Physical mixtures. no membrane. pH 1.2 - series 2						
Time (h)	Concentration (µg/ml)	Quantity of BSA taken in the sample (µg)	Cumulative quantity to be added to the total quantity (µg)	Total quantity of BSA (µg)	Corrected concentration (µg/ml)	Released BSA (%)
0	0	0	0	0	0	0
0.008	15.49369	15.49369	0	309.87	15.494	14.648
0.017	103.4562	103.4562	0	2069.1	103.46	97.812
0.033	104.132	104.132	103.456	2186.1	109.3	103.34
0.05	96.27274	96.27274	207.588	2133	106.65	100.83
0.067	93.3924	93.3924	303.861	2171.7	108.59	102.66
0.083	86.99328	86.99328	397.253	2137.1	106.86	101.03
0.17	84.9987	84.9987	484.247	2184.2	109.21	103.25
0.5	79.55046	79.55046	569.245	2160.3	108.01	102.12
24	73.33095	73.33095	648.796	2115.4	105.77	100
48	73.33095	73.33095	648.796	2115.4	105.77	100

BSA. PCL particles. no membrane. pH 7.2 - series 1						
Time (h)	Concentration (µg/ml)	Quantity of BSA taken in the sample (µg)	Cumulative quantity to be added to the total quantity (µg)	Total quantity of BSA (µg)	Corrected concentration (µg/ml)	Released BSA (%)
0	0	0	0	0	0	0
0.008	4.16591	4.16591	0	83.318	4.1659	11.482
0.017	4.71935	4.71935	4.16591	98.553	4.9276	13.581
0.033	4.91937	4.91937	8.88526	107.27	5.3636	14.783
0.05	4.61431	4.61431	13.8046	106.09	5.3045	14.62
0.067	4.7245	4.7245	18.4189	112.91	5.6454	15.56
0.083	4.1595	4.1595	23.1434	106.33	5.3167	14.654
0.17	4.108	4.108	27.3029	109.46	5.4731	15.085
0.5	3.72527	3.72527	31.4109	105.92	5.2958	14.596
1	3.73186	3.73186	35.1362	109.77	5.4887	15.128
2	3.4689	3.4689	38.8681	108.25	5.4123	14.917
3	3.24235	3.24235	42.337	107.18	5.3592	14.771
4	3.18639	3.18639	45.5793	109.31	5.4654	15.063
5	2.86315	2.86315	48.7657	106.03	5.3014	14.612
6	2.79732	2.79732	51.6289	107.58	5.3788	14.825
24	2.52668	2.52668	54.4262	104.96	5.248	14.464

48	3.32608	3.32608	56.9529	123.47	6.1737	17.016
168	5.85314	5.85314	60.2789	177.34	8.8671	24.439
336	3.0627	3.0627	66.1321	127.39	6.3693	17.555

BSA. PCL particles. no membrane. pH 7.2 - series 2						
Time (h)	Concentration (µg/ml)	Quantity of BSA taken in the sample (µg)	Cumulative quantity to be added to the total quantity (µg)	Total quantity of BSA (µg)	Corrected concentration (µg/ml)	Released BSA (%)
0	0	0	0	0	0	0
0.008	9.16496	9.16496	0	183.3	9.165	22.341
0.017	8.97407	8.97407	9.16496	188.65	9.4323	22.993
0.033	8.69918	8.69918	18.139	192.12	9.6061	23.416
0.05	8.13028	8.13028	26.8382	189.44	9.4722	23.09
0.067	7.72472	7.72472	34.9685	189.46	9.4731	23.092
0.083	7.32723	7.32723	42.6932	189.24	9.4619	23.065
0.17	7.36093	7.36093	50.0204	197.24	9.862	24.04
0.5	7.02155	7.02155	57.3814	197.81	9.8906	24.11
1	7.24978	7.24978	64.4029	209.4	10.47	25.522
2	6.73477	6.73477	71.6527	206.35	10.317	25.15
3	6.218	6.218	78.3875	202.75	10.137	24.711
4	5.98418	5.98418	84.6055	204.29	10.214	24.899
5	5.74378	5.74378	90.5897	205.47	10.273	25.043
6	5.37211	5.37211	96.3334	203.78	10.189	24.837
24	5.07204	5.07204	101.706	203.15	10.157	24.76
48	4.10056	4.10056	106.778	188.79	9.4394	23.01
168	9.77427	9.77427	110.878	306.36	15.318	37.34
336	4.70005	4.70005	120.652	214.65	10.733	26.163

BSA. PCL particles. no membrane. pH 1.2 - series 1						
Time (h)	Concentration (µg/ml)	Quantity of BSA taken in the sample (µg)	Cumulative quantity to be added to the total quantity (µg)	Total quantity of BSA (µg)	Corrected concentration (µg/ml)	Released BSA (%)
0	0	0	0	0	0	0
0.008	4.01263	4.01263	0	80.253	4.0126	11.538
0.017	10.07818	10.07818	4.01263	205.58	10.279	29.555
0.033	10.13089	10.13089	14.0908	216.71	10.835	31.156
0.05	9.81109	9.81109	24.2217	220.44	11.022	31.692
0.067	9.38369	9.38369	34.0328	221.71	11.085	31.874
0.083	9.28122	9.28122	43.4165	229.04	11.452	32.929
0.17	9.74853	9.74853	52.6977	247.67	12.383	35.607
0.5	8.67255	8.67255	62.4462	235.9	11.795	33.914
1	8.14251	8.14251	71.1188	233.97	11.698	33.637
2	8.11286	8.11286	79.2613	241.52	12.076	34.722
3	7.65704	7.65704	87.3742	240.51	12.026	34.578

4	7.1749	7.1749	95.0312	238.53	11.926	34.293
5	6.63712	6.63712	102.206	234.95	11.747	33.778
6	6.1443	6.1443	108.843	231.73	11.586	33.315
24	0	0	114.99	114.99	5.749	33.315
48	0	0	114.99	114.99	5.749	16.531