Novel strategies and technologies for the aseptic microencapsulation of pharmaceutical compounds

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Novel strategies and technologies for the aseptic microencapsulation of pharmaceutical compounds

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presented by

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Background and purpose

Biodegradable microspheres have been intensively studied as delivery systems for low and high molecular weight bioactive compounds throughout the last two decades. Considered primarily for parenteral and pulmonary administration, they offer a number of potential benefits: Controlled and sustained release of the encapsulated bioactive material, protection of the non-released material from degradation and physiological clearance, and adjuvancy for encapsulated immunoreactive compounds. Biodegradable microspheres are of special interest for the formulation of fragile, highly active molecules with relatively short biological half-life such as peptides and proteins, or of molecules that require targeted delivery into cells or the nucleus such as oligonucleotides and DNA [1].

Poly(lactic acid), PLA, and poly(lactic-co-glycolic acid), PLGA, are the most frequently employed materials in the formulation of biodegradable microspheres. These polyesters are highly biocompatible and degrade at controlled rates into the metabolic products lactic acid and glycolic acid [2,3]. PLA and PLGA microspheres with microencapsulated bioactive compounds are generally produced by either of three basic processes or their numerous variants, which are the so-called solvent extraction/evaporation, phase separation (coacervation) and spray drying. Spray-drying is a continuous process with high throughput, but may not be compatible with temperature-sensitive compounds. Yields for small batches are moderate [4], and achievable particle sizes limited by the specifications of the particular equipment. Phase separation, a batch process, yields relatively high encapsulation efficiencies for water-soluble compounds, but is impaired by organic process solvent residues in the microspheres [5]. Furthermore, it is not well suited for producing microspheres in the low micrometer range. Finally, solvent extraction/evaporation offers mild processing conditions and good control of the particle size from tens or hundreds of micrometers down to the nanometer range, but requires thorough selection of materials and encapsulation conditions to yield efficient encapsulation and low solvent residues (see Chapter I).

The considerable number of microsphere formulations emerging from the labs brought forth the need to prepare such particles in larger quantities and safe and reliable quality for clinical trials and commercialisation. Lab scale pro-
duction processes frequently are inappropriate for the economic and well-controlled production of larger amounts of microspheres. In addition, parenteral administration requires a sterile product. PL(G)A microspheres cannot be terminally sterilised by heat due to the low glass transition temperature of these polymers, while the use of ethylene oxide is not generally approved and \(\gamma\)-irradiation has been shown to result in radiolytic polymer chain scission, altering the drug release [6,7]. In addition, damage to the encapsulated bioactive compound may occur through irradiation [6]. As a result, aseptic manufacturing of microspheres is favoured over terminal sterilisation by \(\gamma\)-rays.

The aim of this study was, therefore, to develop processes for the preparation of drug-loaded microspheres that are especially well suited for aseptic production and scale-up. The process had to match the following features: (i) continuous processing; (ii) simple, sterilisable and easy-to-clean equipment; (iii) isolation of the processed materials from the environment. The continuous process should be adequate to prepare quantities suitable for clinical trials, i.e. microsphere quantities in the 10 gram range.

First, a method for dispersing an aqueous solution of the bioactive compound in an organic solution of the biodegradable polymer (W/O) to yield the starting material for microsphere preparation was developed. Then, variants of the solvent extraction/evaporation and spray-drying processes, which are the most frequently employed methods for the microencapsulation of peptides and proteins, were developed. Finally, the W/O-dispersion and solvent extraction processes were combined, and the suitability of the combined process to aseptically prepare microspheres was assessed.

References


Abstract

The development and production of poly(lactic-co-glycolic acid) (PLGA) microspheres for controlled parenteral drug delivery faces two main hurdles in an industrial setting, namely the necessity of preparing a sterile particulate product and the challenge of scale-up of relatively complex processes. As PLGA is sensitive to heat ($T_g \approx 40^\circ C$) and $\gamma$-rays (radiolytic chain scission), PLGA microspheres cannot be terminally sterilised by heat and $\gamma$-irradiation is detrimental to the product quality. Therefore, manufacturing under aseptic GMP conditions is the method of choice. In this work we developed new methodologies of emulsification and microsphere formation, which are particularly suited for aseptic processing and scaling-up. The novel processes were developed and assessed by microencapsulating bovine serum albumin (BSA), as a model protein drug, into PLGA and evaluating various process and formulation parameters.

Chapter I reviews the various existing techniques to prepare microspheres by solvent extraction/evaporation, the most frequently used method for microencapsulation. Initial lab-scale experiments are frequently performed in simple beaker/stirrer set-ups, which are not appropriate for the production of amounts suitable for clinical trials and market introduction. More sophisticated technologies are required allowing for economic, robust, well-controllable and aseptic production of microspheres. Microsphere preparation by solvent extraction/evaporation can be subdivided into four major sub-steps, namely (i) incorporation of the bioactive compound, (ii) formation of the microdroplets, (iii) solvent removal and (iv) harvesting and drying the particles. For the formation of microdroplets, stirring, static mixing, extrusion through needles, microfabricated microchannel devices or membranes, and dripping using electrostatic forces and ultrasonic jet excitation were examined. Removal of the biodegradable matrix's solvent may be achieved by evaporation, possibly assisted by heat or reduced pressure, or by extraction, for which aqueous or organic solutions may be employed.

Commonly, the first step in preparing drug-loaded microspheres is the formation of a dispersion of the bioactive compound in a solution of the biodegradable polymer. For the encapsulation of proteins, this is frequently achieved
Abstract

by ultrasonic emulsification of an aqueous protein solution in an organic solution of the polymer using a standard ultrasonic probe and a small vessel. In chapter II, a flow-through ultrasonic cell is presented for this purpose, based on exciting a steel jacket, which transmitted the sound waves via pressurised water to a glass tube installed inside the jacket. This set-up prevents contamination of the sonicated dispersion with metallic particles eroded from the sonotrode as well as microbial contamination from the environment. To characterise the novel system, vegetable oil-in-water emulsions, which constitute a standard model system for evaluating emulsification equipment, were chosen. The starting materials were fed into the ultrasonic cell as coarse pre-emulsions. During passage through the cell, the emulsion mean droplet diameter was decreased by two orders of magnitude yielding Sauter diameters of 0.5 \( \mu \text{m} \) and below with very good repeatability. Increasing the residence time in the ultrasonic field and the sonication power both lowered the emulsion mean diameter, while higher disperse phase viscosity and interfacial tension tended to increase the droplet sizes. The energetic efficiency amounted to a rather low 10\%, which was ascribed to the complex mechanism of energy transfer.

In chapter III, a static micromixer, consisting in essence of an array of microchannels was evaluated for the formation of BSA-loaded PLGA microspheres by solvent extraction. The mixer's simple set-up along with its small size, easy handling and suitability for continuous production makes it well suited for aseptic processing. Scale-up is easily feasible through parallel installation of a sufficient number of micromixers ("number-up"). The mean diameter of the microspheres was varied between 9 and 30 \( \mu \text{m} \) simply by modulating the flow rates of the mixed fluids. The microsphere size distributions were excellently reproducible and largely unaffected by the polymer solution concentration, polymer type and nominal BSA load, but depended on the type of polymer solvent. BSA encapsulation efficiencies were mostly in the region of 75 – 85\%.

In chapter IV, the ultrasonic flow-through cell and the micromixer were combined to prepare BSA-loaded microspheres. While the ultrasonic cell produced an emulsion of a BSA solution in a PLGA solution, the micromixer further processed this emulsion along with the extraction medium to form microspheres. The BSA-in-PLGA emulsions exhibited mean droplet sizes of
<700 nm. Their further processing into microspheres of 15 – 40 µm mean diameter resulted in approx. 70% BSA encapsulation efficiency. Batch-to-batch reproducibility was excellent. Microsphere batches produced under aseptic conditions to assure product sterility exhibited no microbial contamination when examined by a simplified sterility test.

Finally, chapter V describes a spray-drying technology potentially suitable for aseptic processing. The process consists of feeding a fluid through an ultrasonic atomiser, drying the spray under reduced pressure and collecting the particles in a liquid bath. Drying by mild vacuum instead of warm air, as employed in conventional spray-drying, and simple particle recovery render this method suitable for aseptic microsphere preparation. As in the previous chapters, PLGA microspheres engulfing BSA were studied as a model system. Particle yields of above 80% exceeded largely values found for conventional laboratory-scale spray-drying. BSA encapsulation efficiency mostly ranged in the region of 60%, with losses probably occurring through partitioning into the aqueous collection bath. Mean particle sizes ranged from 13 to 24 µm, depending on the polymer type and solvent; particle size distributions were excellently reproducible. The microspheres were found to be very porous and exhibited a pronounced 24 h burst release of above 50% of total dose, probably promoted by the porosity. However, when more concentrated polymer solutions were employed, burst release was significantly reduced to an average of 16%.
Zusammenfassung

Die pilot- und grossstechnische Produktion von Mikrosphären aus Poly(milch-co-glykolsäure) (PLGA) als parenteral verabreichtes System zur kontrollierten Wirkstoffabgabe hat zwei Hürden zu überwinden: Einerseits die Notwendigkeit, ein steriles partikuläres Produkt zu erzeugen und andererseits die Aufstufung relativ komplexer Prozesse. Da PLGA hitzeempfindlich reagiert (T\textsubscript{g} \approx 40°C), ist eine Sterilisation des Endprodukts über Hitze oder Dampf nicht möglich. Der Einsatz von \gamma-Strahlen zur Sterilisation kann zu einer Spaltungs der Polymerketten und damit zu einem negativ veränderten Freisetzungsverhalten des Wirkstoffs aus den Mikrosphären führen. Aus diesen Gründen ist die aseptische Produktion der Mikrosphären zu präferieren. Im Rahmen dieser Arbeit sollten Verfahren zur Emulgierung und zur Produktion von Mikrosphären entwickelt werden, die für eine aseptische Prozessführung besonders gut geeignet sind und sich einfach aufstufen lassen. Die entwickelten Verfahren wurden anhand der Verkapselung von bovinem Serumalbumin (BSA) als Modellprotein in PLGA-Mikrosphären charakterisiert und der Einfluss verschiedener Prozess- und Formulierungsparameter wurde untersucht.

falls unterstützt durch Wärmezufuhr oder Unterdruck – oder per Extraktion mittels wässriger oder organischer Lösungen aus den zuvor gebildeten Mikrotropfen entfernt werden.


Kapitel III beschreibt die Verwendung eines statischen Mikromischers zur Verkapselung von BSA in PLGA-Mikrosphären mittels Lösungsmittelextrak-
Zusammenfassung


Im Kapitel V wird schliesslich ein Sprühtrocknungsverfahren beschrieben, welches für eine aseptische Produktion von Mikrosphären gut geeignet erscheint. Eine die Wirksubstanz und Polymer enthaltende Lösung wird dabei mittels Ultraschallsprühkopf in einen unter verringertem Druck stehenden Glasbehälter zerstäubt und dabei getrocknet. Die entstehenden Partikel sedimentieren in ein Auffangbad, mit dem sie aus dem Behälter entnommen werden können. Die Verwendung von Unterdruck anstelle warmer Luft, wie in herkömmli-
Chapter I

Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology

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I.1 Introduction

Biodegradable microspheres are widely investigated delivery systems for bioactive compounds such as low molecular weight and macromolecular therapeutics, antigens or DNA. As such they may add substantially to the value of therapies and vaccinations. Considered for parenteral, pulmonary, oral or nasal administration, they are capable of providing sustained and controlled release of the encapsulated bioactive compound, while the non-released bioactive material may be protected from degradation and physiological clearance. For vaccines, microspheres may provide additional adjuvancy [1,2] and allow for direct targeting to professional antigen presenting cells [3]. Furthermore, they may be surface-modified to target specific cells [4] and tissues [5].

Owing to their excellent biocompatibility, the biodegradable polyesters poly(lactic acid), PLA, and poly(lactic-co-glycolic acid), PLGA, are the most frequently used biomaterials for the microencapsulation of therapeutics and antigens [6,7]. Other materials like proteins [5], polymer blends [8], polysaccharides such as chitosan [9], and lipids [10] have also been studied, though at a lower frequency. A large variety of bioactive compounds have been formulated into microspheres, among them antineoplastic drugs [11,12], narcotics [13], anaesthetic agents [14] as well as therapeutic peptides [15,16] and proteins [17,18], DNA [19,20], viruses [21] and bacteria-derived compounds [22,23]. Preparation technologies capable of producing larger amounts of microspheres in a safe, economic, robust and well-controlled manner are therefore required.

In accordance with the common practice in literature, in this paper, the terms "microspheres" and "microparticles" are used interchangeably. The same holds true for the terms "encapsulation" and "entrapment".

Abbreviations: ACN: acetonitrile; BSA: bovine serum albumin; CSTR: continuously stirred tank reactor; CV: coefficient of variation; DCM: dichloromethane; HPMC: hydroxypropylmethylcellulose; OVA: ovalbumin; PEG: poly(ethylene glycol); PLA: poly(lactic acid); PLGA: poly(lactic-co-glycolic acid); PMMA: poly(methyl methacrylate); PTFE: poly(tetrafluorethylene); PVA: poly(vinyl alcohol); PVP: poly(vinyl pyrrolidone); rhGH: recombinant human growth hormone; sCT: salmon calcitonin; SDS: sodium do-decyl sulfate; SPG: Shirasu Porous Glass.
Microspheres have been prepared by various techniques, which feature partly competing, partly complementary characteristics. Many microencapsulation processes are modifications of the three basic techniques solvent extraction/evaporation, phase separation (coacervation) and spray-drying [24]. Spray-drying is relatively simple and of high throughput, but must not be used for highly temperature-sensitive compounds. Moreover, control of the particle size is difficult, and yields for small batches are moderate [25]. Coacervation is frequently impaired by residual solvents and coacervating agents found in the microspheres [26]. Furthermore, it is not well suited for producing microspheres in the low micrometer size range. The use of supercritical gases as phase separating agents was intensively studied to minimize the amount of potentially harmful residues in the microspheres, resulting in processes named, e.g., Precipitation with Compressed Antisolvent (PCA) [27], Gas or Supercritical fluid Anti-Solvent (GAS or SAS), and Aerosol Solvent Extraction System (ASES) [28]. Solvent extraction/evaporation neither requires elevated temperatures, nor phase separation inducing agents. Controlled particle sizes in the nano- to micrometer range can be achieved, but careful selection of encapsulation conditions and materials is needed to yield high encapsulation efficiencies and a low residual solvent content.

Microsphere preparation by solvent extraction/evaporation basically consists of four major steps: (i) dissolution or dispersion of the bioactive compound often in an organic solvent containing the matrix forming material; (ii) emulsification of this organic phase in a second, continuous (frequently aqueous) phase immiscible with the first one; (iii) extraction of the solvent from the dispersed phase by the continuous phase, which is optionally accompanied by solvent evaporation, either one transforming the droplets into solid microspheres; (iv) harvesting and drying of the microspheres (Fig. 1.1).

This article reviews the current state of the art in solvent extraction/evaporation based microencapsulation technology with a focus on process-related aspects. Issues like materials, microsphere formulation, choice of appropriate solvents or surfactants are not central aspects of this review, though technology and starting materials are interconnected and can by no means be
segregated completely. Both, well-established and more advanced technologies will be reviewed.

Bioactive compounds may be added to the solution of the matrix material by either co-dissolution in a common solvent, dispersion of finely powdered solid material, or emulsification of an aqueous solution of the bioactive comp-

**Figure I.1.** Schematic overview over the four principal process steps in microsphere preparation by solvent extraction / evaporation.

1. Incorporation of the bioactive compound
2. Droplet formation
3. Solvent removal
4. Microsphere harvest (and drying)

I.2 Incorporation of bioactive compounds

Bioactive compounds may be added to the solution of the matrix material by either co-dissolution in a common solvent, dispersion of finely pulverised solid material, or emulsification of an aqueous solution of the bioactive com-
pound immiscible with the matrix material solution [29]. Co-dissolution may require a co-solvent to fully dissolve the drug in the matrix containing solvent. Dispersion of the solid or dissolved bioactive material in the matrix containing solution may be achieved by ultrasonication [30], impeller or static mixing [31], high-speed rotor-stator mixing [32], or microfluidisation [30].

The microencapsulation of hydrophilic compounds by dispersion of their aqueous solution in an organic solution of the matrix material was more efficient with finer W/O-emulsions, i.e. at a lower ratio of bioactive material droplet size to microsphere diameter [32,33]. For the entrapment of bovine serum albumin (BSA) into poly(methyl methacrylate) (PMMA) microspheres, a ratio of less than 1:10 was suggested to yield protein loadings of >80% [32]. A higher target load of bioactive material is likely to decrease the encapsulation efficiencies of proteins and peptides in PLGA [33,34,35] and increase the 24 h ("burst") drug release [35,36], although some studies report the opposite, e.g., an increase in entrapment efficiency of ovalbumin (OVA) from 40 to 98% with an increase in actual OVA content from 7 to 16% (w/w) [37,38]. Increasing the volume fraction of the internal aqueous phase lowered the encapsulation efficiency due to droplet coalescence and increased probability of contact between the internal drug solution and the external extraction phase resulting in drug loss [39,40]; in addition, an increase in the burst release and microsphere porosity was reported [41,42].

In analogy, entrapment of solid protein particles also improved with decreasing particle size [32,43]. The particle size of drug powders can be reduced by either micronisation of the drug powder prior to its dispersion, or during the dispersion step itself [44,45], or by the use of excipients which are co-formulated with the drug so that the blended material dissolves in the matrix's solvent [46]. Finally, spherically shaped protein particles caused a trend towards more efficient encapsulation than irregular ones [32].

For efficient encapsulation of drugs dissolved in an aqueous phase to be dispersed in an organic matrix solution, stabilisation of the resulting W/O-emulsion may be required. When drug-free microparticles were prepared from emulsions consisting of plain water and PLA dissolved in dichloromethane (DCM) [47], increasing amounts of BSA added to the water as a surfactant sta-
bibilised the emulsions and decreased the pore sizes in the resulting microspheres; the latter observation was ascribed to the finer water droplets that were entrapped and left a corresponding void in the matrix. The addition of a surfactant (poloxamer) to the organic phase was found to be much less efficient. Similarly, the model substance indigocarmine was more efficiently entrapped with increasing BSA concentrations in the inner water phase [48]. Other substances, e.g., gelatine [49], poly(vinyl alcohol) (PVA) [35], ovalbumin [50] or combinations of sorbitan esters and polysorbates [51] have also been reported for the stabilisation of such W/O-emulsions. The selection of stabilisers for the W/O-emulsion has to be made with caution, as co-encapsulated surfactants can adversely affect drug encapsulation efficiency and release [48,52].

I.3 Droplet formation

The droplet formation step determines the size and size distribution of the resulting microspheres. Microsphere size may affect the rate of drug release, drug encapsulation efficiency, product syringeability, in vivo fate in terms of uptake by phagocytic cells and biodistribution of the particles after subcutaneous injection of intranasal administration. In the following, the main procedures used for droplet formation in microsphere production are described. Henceforth, the different types of mixtures of bioactive and matrix materials described above will, for simplicity, be referred to as drug/matrix dispersion.

I.3.1 Stirring

Stirring is the most straightforward method to generate droplets of the drug/matrix dispersion in the continuous extraction phase for subsequent solvent removal. In the simplest approach, extraction phase is filled into a vessel and agitated by an impeller. The drug/matrix dispersion is then added, drop-wise or all at once, under agitation at a speed sufficient to reach the desired droplet size.

Obviously, the impeller speed is the main parameter for controlling the drug/matrix dispersion's droplet size in the continuous phase. Increasing the mixing speed generally results in decreased microsphere mean size
as it produces smaller emulsion droplets through stronger shear forces and increased turbulence. The extent of size reduction that is attained depends on the viscosity of the disperse and continuous phases, the interfacial tension between the two phases, their volume ratio, the geometry and number of the impeller(s), and the size ratio of impeller and mixing vessel. For example, a 52 mm impeller installed in a 250 ml beaker of 65 mm inner diameter produced microsphere mean diameters decreasing from 38 to 14 μm with impeller speed increasing segmentially from 250 to 1600 rpm, using PLGA dissolved in DCM and an aqueous HPMC solution as disperse and continuous phases, respectively [53]. In addition to a smaller mean diameter, more vigorous mixing also resulted in lower microsphere polydispersity [53,56].

Increased viscosity of the drug/matrix dispersion yields larger microspheres because higher shear forces are necessary for droplet disruption [16,33,38,41,57]. For PLGA dissolved at 6.25, 12.5 and 25% in a mixture of acetonitrile (ACN) and DCM and dispersed in liquid paraffin, microsphere mean diameters of 36, 115 and 208 μm were obtained [57]. Such increase in drug/matrix dispersion viscosity, typically caused by higher concentration or molecular weight of the matrix material, may be desirable to restrict the migration of the drug to the continuous phase and thus improve its entrapment.

To prevent coalescence of the drug/matrix dispersion droplets, a surfactant or viscosity enhancing stabiliser such as PVA is generally added to the continuous phase. Increasing the stabiliser concentration frequently leads to decreased microsphere sizes [20,35,37,53,58]. For instance, when microspheres were prepared from PLGA dissolved in DCM and emulsified in an aqueous PVA solution, the mean diameter decreased from 8.3 to 3.7 μm when the PVA concentration was increased step-wise from 1 to 10% [37]. When HPMC was used as a stabiliser, an increase of its concentration in the continuous phase from 0.4 to 2.4% resulted in an almost linear decrease of the microsphere size from 29 to 13 μm along with a reduced width of the size distribution [53]. Higher stabiliser concentrations will yield a larger excess of material that adsorbs on the surface of newly formed droplets, thus preventing coalescence [35,53]. With macromolecular stabilisers, the viscosity of the continuous phase
will also increase, amplifying – for a given stirring rate - the shear forces acting upon the drug/matrix dispersion droplets and, thus, minimising their size. Reports about the impact of the volume ratio between drug/matrix dispersion and continuous phase on the size of the resulting microspheres are conflicting. Various studies reported a reduction in the mean microsphere size with decreasing continuous phase volume [16,37,59,60], while in other studies no significant effect was observed [53,54].

In an attempt to predict the mean diameter of microspheres prepared in a so-called continuously stirred tank reactor (CSTR), an empirical equation was derived [61]. In a vast number of experiments, the size of PLGA and PMMA particles was correlated with reactor parameters and fluid properties using dimensional analysis. In agreement with previous reports, the equation predicted a strong correlation of the microsphere mean diameter with stirring speed, impeller diameter (decreased diameter) and polymer concentration (increased diameter), as well as moderate correlation with continuous phase viscosity (decreased diameter) and interfacial tension (increased diameter). Disperse and continuous phase volumes did not significantly influence microsphere size. The equation reproduced and predicted the microsphere diameter with good accuracy for different types of extraction fluids and for microspheres without and with protein loading. Also, in scaled-up equipment (from 1 to 3, 10 and 100 liters), the deviation of the predicted diameter from the experimentally obtained one was less than 20%. However, no prediction on the width of the particle size distribution could be made.

### 1.3.2 Static mixing

Static mixers consist of baffles or other flow obstacles installed in a tube. The baffle arrangement repeatedly splits and recombines the stream of fluid passing through the tube. Recombination occurs through impingement of the substreams, creating turbulence and inducing back-mixing.

In a comprehensive study, static mixers of different baffle design, length (4 - 76 cm) and diameter (0.6 - 2.5 cm) were examined for microsphere production involving concentrated solutions (18 and 30%, w/w) of PLGA and PMMA in DCM dispersed in aqueous PVA solutions [62]. Using continuous phase flow
rates of 36 to 320 l/h yielded microsphere mean diameters of 35 to 90 \( \mu \text{m} \). For each of the three mixer designs, an empirical equation relating microsphere size to fluid properties, mixer geometry and flow rate was derived by dimensional analysis. Correlation between the equations and experimental data was good, as was the predictive power, with the calculated mean diameter deviating less than 10\% from that experimentally determined. Analysis of the equations revealed that increasing the interfacial tension, polymer concentration and mixer diameter produced larger microspheres, while increasing the flow rate, continuous phase viscosity and length of the mixer resulted in smaller particles. Moreover, the authors concluded that the mean size of the microspheres would not change during scale-up if the flow velocity inside the mixer could be maintained. However, no statement about retention of the particle size distribution was made, which is of equal interest in a scale-up. For the three mixer designs studied, a ranking with respect to emulsification efficiency was established and explained with respect to baffle geometry. A comparison of the static mixers with a CSTR for emulsification efficiency revealed that static mixers generate the same degree of mixing at much lower Reynold numbers. Uniformity of the particle size distribution was not improved by static mixing. The authors concluded that static mixing scores over CSTR-based microencapsulation with respect to process continuity, mixing efficiency and scalability.

A convenient way to scale-up microencapsulation by static mixing is the parallel installation of several small-diameter mixers with outflows that are recombined downstream, rather than using a single mixer of larger diameter (Fig. 1.2) [63]. A pre-blending mixer preceding the mixer manifold ensures that a uniformly composed pre-emulsion of drug/matrix dispersion and extraction phase enters each mixer of the manifold. Furthermore, it was observed that the uniformity and symmetry of the microspheres' size distribution was improved by increasing the emulsion's residence time in the static mixer manifold, i.e. by increasing the manifold's length.

As an alternative to classical static mixing, a tube of very small diameter was suggested for the formation of an emulsion of the drug/matrix dispersion in the continuous extraction phase [64]. The two phases to be mixed were pumped through such a tube at flow rates high enough to yield Reynolds num-
bers exceeding values of 4,000 to induce intense turbulent mixing. As an example of conditions applicable for microsphere preparation, a 3.7 m long PTFE tube of 1.65 mm inner diameter and drug/matrix dispersion and continuous phase flow rates of 70 and 240 to 900 ml/min, respectively, are given. The resulting microsphere size distribution displayed rather polydisperse particles, i.e., with particle diameters ranging from below 10 to 200 μm.

Generally, the fact that the droplet size is a function of the flow rate constitutes a drawback in the use of static mixers for microencapsulation, because microsphere size and throughput cannot be controlled separately.

![Diagram](https://via.placeholder.com/150)

**Figure 1.2.** Parallel installation of several static mixers for scale-up of microsphere production. Adapted from [63].

### 1.3.3 Extrusion

Extrusion denotes feeding the drug/matrix dispersion through a single or a plurality of pathways directly into the continuous extraction phase. Upon leaving the pathway(s), discrete droplets of the drug/matrix dispersion are formed within the slowly flowing continuous phase, which also transports the droplets away from the site of their formation.
Extrusion is distinguished from static mixing by the droplet forming mechanism and the prevailing flow regime. In extrusion, the flow is mainly laminar and the droplets are formed directly at the site of introduction of the dispersed phase into the continuous phase and do not change their dimension thereafter (given that coalescence is negligible). On the contrary, static mixing relies mainly on turbulent flow, which constantly acts on the disperse phase and, thus, causes the size of the droplets to change over the whole length of the mixer. Therefore, extrusion is considered to allow for more uniform and better controlled microsphere sizes than static mixing.

1.3.3.1 Single pathway systems

The continuous injection of a drug/matrix dispersion (hydrocortisone/PLA co-dissolved in DCM) via a hypodermic needle into a coaxial stream of continuous extraction fluid (mineral oil) was studied for microsphere formation [65]. The microsphere size (mean diameter of 145-400 μm) was controlled by the needle diameter (510 and 710 μm) and by the flow rate of the mineral oil at the needle tip, with smaller particles being obtained from smaller needle diameters and higher oil flow rates. Downstream inlets were used to further add mineral oil for efficient extraction of the solvent independent of the flow rate at the needle tip. Particle size distributions were considerably polydisperse (CV = 15-40%), and the drug/matrix dispersion flow rate was 3.6 ml/h representing a very low process productivity.

In a slightly different approach, a stainless steel, blunt-ended needle was used to inject a solution of PLGA in DCM into a perpendicular flow of an aqueous PVA solution used as continuous phase [66]. With a PLGA solution flow rate of 30 ml/h, process productivity was considerably higher than with the aforementioned technique. Mean microsphere size varied between 68 and 295 μm (CV = 5-35%). Measures to decrease the mean particle diameter comprised increasing the continuous phase flow velocity, reducing the needle diameter (from 457 to 254 μm) and decreasing the adhesion between needle and polymer solution (e.g., by using PTFE or silicone coated needles [67]). The width of the size distribution narrowed when one of the two prominent forces prevailed, i.e. either the shear force exerted by the extraction phase on the
growing droplet, or the adhesion force between the droplet and the needle tip. Changing the angle between needle and extraction phase flow from 90 to 45° did not significantly influence the microsphere size distribution [67].

Generally, the single pathway extrusion systems have turned out to be unsuitable for the production of small microspheres (<50 μm), and their throughput was quite low. Scale-up may be feasible through parallel employment of a plurality of needles, which, however, might be difficult to implement without considerably perturbing the flow of the extraction phase and causing interactions between the outflows from the different needles.

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**Figure 1.3 a-d.** Multilamination micromixer. (a) Assembled micromixer. (b) Dismantled mixer with extracted mixing tool. (c) Close-up of the microchannel array. Channel width is 40 μm. (d) Formation and disintegration of fluid lamellae in the mixer’s outlet slit. (a)-(c) with kind permission of Institut für Mikrotechnik Mainz (www.imm-mainz.de); (d) reproduced from [68] with permission.
1.3.3.2 Multichannel systems

Recently, a micromixer consisting in essence of an array of fine channels (25 or 40 μm in width; 300 μm in depth; Fig. 1.3 a-c) was employed for microsphere preparation [68]. PLGA dissolved in DCM, into which an aqueous BSA solution was emulsified, and an aqueous PVA solution used as extraction phase were separately fed into the microchannel array from opposite sides and discharged through an outlet slit (60 μm wide), which was micromachined in the mixer housing's top plate perpendicular and central to the channel array (Fig. 1.3 b). Upon entering the outlet slit, alternating fluid lamellae of the two fluid phases formed. Owing to the much faster flow rate of the extraction fluid, the microsphere forming phase disintegrated into droplets (Fig. 1.3 d) [68,69]. The

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**Figure I.3 e.** Multilamination micromixer. (e) Control of the microsphere size by variation of the flow rates. Extraction phase flow rate was 1200, 900, 600 and 420 ml/h (size distributions from left to right); drug/matrix dispersion flow rate was adapted at 1/50 of the extraction fluid rate. Reproduced from [68] with permission.
mean microsphere diameter was tuned from 8 to 29 μm by simply varying the flow rates of the two fluids pumped through the mixer (Fig. 1.3 e). Relatively wide particle size distributions were obtained, e.g., ranging from 4 to 60 μm for a mean diameter of 16 μm. Interestingly, both the microsphere mean size and size distribution remained largely unaffected by varying PLGA solution concentrations (2 - 10%, w/w), drug load and polymer type. On the contrary, switching the polymer solvent from DCM to ethyl formate yielded considerably smaller microspheres (7 μm mean diameter instead of 16 μm for DCM), which was attributed to decreased interfacial tension. Scale-up can be comfortably achieved by so-called numbering-up, i.e., by employing a large number of micromixers in parallel. Owing to its simple design and because it may be easily sterilised, the micromixer was suggested for aseptic microsphere manufacturing [68].

Figure 1.4 a. Interfacial tension driven droplet formation using a microchannel device. (a) Experimental set-up. Adapted from [76].
Another simple and ingenious microchannel system, etched into a silicon chip (Fig. 1.4 a,b) [70], was examined intensively for the formation of monodisperse emulsions and, more recently, for the solvent evaporation-based preparation of uniform lipid microparticles [71]. The channels measure only a few micrometers in height and width and open up to a terrace that descends to a well through which the continuous phase slowly passes (Fig. 1.4 b,c). The device is covered by a glass plate to allow for observation by a camera system. The disperse phase, flowing out of the microchannel, spreads into the space between the terrace and the glass cover in a disk-like shape until it reaches the rim of the well. When flowing over the rim and into the well, interfacial forces contract the fluid to form a droplet (Fig. 1.4 c). The interfacial area of the disperse phase as
Figure 1.4 d. Interfacial tension driven droplet formation using a microchannel device. (d) Typical droplet size distributions obtained for the system triolein in aqueous SDS solution using three different microchannel geometries and two different continuous phase flow rates. Reproduced from [72] with permission.
spread on the terrace is large compared to that of the droplet in the well, driving the fluid to leave the terrace and adopt a spherical form. On a micrometer scale interfacial forces dominate over other forces like gravity, inertia and viscosity [70]. Therefore, droplet formation was governed by this single force only, leading to monodisperse droplets (CV < 5%) (Fig. 1.4 d) [72]. Droplets of a few up to 100 \( \mu \text{m} \) were produced [73,76]. Because the produced droplets were, in general, significantly larger than the channels' dimensions, production devices for low micrometer-scaled microspheres may be susceptible to clogging. Droplet size increased with channel height and terrace length, but was largely independent of channel width and length, though longer and narrower channels accommodated a wider range of disperse phase pressures still producing monodisperse droplets [74]. An empirical equation predicted the droplet size as a function of microchannel height and terrace length with good accuracy [75]. Unfortunately, the achievable throughput of such devices is limited to just a few millilitres per hour, even when using several hundred channels in parallel [76]. Increasing the throughput by augmenting the pressure applied to the disperse phase produced more polydisperse and larger droplets as interfacial tension no longer dominated over the viscous force.

I.3.3.3 Membranes

Microporous glass membranes of well defined pore size were used for nitrogen-driven extrusion of polystyrene dissolved in chloroform [77] and PLA/PLGA dissolved in DCM [78] into a continuous, slowly circulating aqueous surfactant solution, followed by subsequent solvent evaporation. This method, also named Shirasu Porous Glass (SPG) emulsification technique [79], produced very uniform PLGA microspheres of 1.2, 1.8 and 2.9 \( \mu \text{m} \) (number-averaged) mean diameter from membranes with pore sizes of 0.7, 1.1 and 2.4 \( \mu \text{m} \), respectively. Generally, the particles produced were slightly larger than the pores from which they were manufactured. The continuous phase preferably contained anionic surfactants like sodium dodecyl sulphate (SDS), while cationic and non-ionic surfactants (polysorbates) and protective colloids like PVA or poloxamer were inappropriate [78]; cationic surfactants interacted electrically...
Figure 1.5 a,b. Droplet formation from a micromachined membrane ("straight-through microchannel"). (a) Experimental set-up. (b) Principle of droplet formation. (a) adapted and (b) reproduced from [81] with permission.
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Figure I.5 c. Droplet formation from a micromachined membrane ("straight-through microchannel"). (c) Left: Droplets forming from a membrane with oblong pores. Right: Monodisperse droplets of soybean oil dispersed in an aqueous SDS solution formed from said oblong micropore device. Reproduced from [81] with permission.

with the negatively charged glass membranes, the non-ionic surfactants were soluble in both the aqueous phase and DCM so that they did not adsorb sufficiently at the interface, and PVA was assumed to partition to slowly to the interface upon droplet formation. Furthermore, uniform microspheres were only obtained when the aqueous continuous phase was pre-saturated with the polymer solvent. When progesterone was co-dissolved in the PLA/PLGA solutions to yield particles with a payload of up to 50%, no changes in the size and uniformity of the resulting microspheres was observed. A SPG membrane of larger pore size (5.2 \( \mu \text{m} \)) was also used to produce PLA microparticles [79]. PLA was dissolved in DCM at high concentrations of 10 to 20\% (w/w), along with dodecyl alcohol or hexadecane as co-surfactant, which were used to reduce the solution's hydrophilicity and, thereby, its wetting of the polar glass pores to yield more uniform microspheres. An aqueous solution of PVA and SDS was employed as continuous phase. The resulting microspheres were considerably larger (mean diameters of 10-25 \( \mu \text{m} \)) than the membrane pores and moderately polydisperse (CV = 10-15\%). No consistent relationship between particle size or polydispersity and polymer or co-surfactant concentrations was observed. Moreover, the microspheres were not perfectly spherical, but elliptical and hemispherical when made with dodecyl alcohol and hexadecane, respectively.
A hydrophilic polycarbonate membrane [80] and a micromachined silicon chip (Fig. 1.5 a,b) [81], both featuring uniformly sized pores or holes, have also been studied for emulsion formation. Although the emulsions were not used to form microspheres, an interesting insight into droplet formation with such devices was achieved. Membranes of both materials with 10 μm circular pores yielded polydisperse droplets of up to about 100 μm for the emulsification of soybean oil in an aqueous surfactant solution flowing parallel to the membrane. With the polycarbonate membrane [80], the droplet mean size (along with polydispersity) was lowered from approx. 70 to 20 μm by increasing the continuous phase flow velocity from 0.02 to 0.54 m/s. In agreement with observations on glass membranes [78], anionic surfactants were superior to non-ionic ones, while cationic surfactants hampered droplet formation. Silicon chips with oblong holes of 17.3 μm equivalent diameter yielded highly uniform (CV < 1.5%) droplets of 32.5 μm average diameter (Fig. 1.5 c) [81]. Here, droplet size and polydispersity remained unaffected by variations in the very low (0-9.2 mm s⁻¹) continuous phase velocity. Hence, it was concluded that the microdroplets detach spontaneously from the oblong channels due to instability of the elongated interface at the channel outlet without the need of the continuous phase shearing action. The productivity per channel plate (5,000 channels) amounted to 6.5 ml/h of disperse phase.

I.3.4 Dripping
I.3.4.1 Single droplet formation

Microspheres have been prepared by dripping 10 and 15% (w/w) solutions of poly(ethylene-co-vinyl acetate) in DCM, containing dispersed protein particles, from a needle into an electric field (Fig. 1.6) [82]. In this process, the forming droplets were detached from the needle by electrostatic forces. Particle collection and solvent removal occurred in a bath of cold (-75°C) methanol. The electric field was generated by connecting the needle to electric potentials of up to 4 kV and the collection bath to ground. Very large microspheres of 500 to 1500 μm average diameter were obtained, whereby the largest particles formed with voltage-free dripping. Droplets disrupted by the electric field upon detachment from the needle tip resulted in highly polydisperse size distributions, rang-
ing from 600 to 1200 μm or sometimes even from 200 to 1200 μm. Productivity was low with 30 ml/h of processed polymer solution. Dripping PLGA dissolved in ACN from a needle into a collection bath of light mineral oil in which a ring-shaped anode was submerged, resulted in much smaller microspheres of 50 – 100 μm mean diameter, using voltages of 1.25 – 1.85 kV [83].

Figure 6. Microsphere preparation by electrostatic dripping. Adapted from [82].

1.3.4.2 Jet excitation

The vibration of a liquid jet for its disruption into droplets was originally studied by Lord Rayleigh as early as in the late 19th century [84,85]. A longitudinal oscillation imposed on a liquid stream causes periodic surface instabilities, which break up the liquid into a chain of uniform droplets. Lord Rayleigh found that uniform droplets are produced from a range of excitation wavelengths corresponding to 7 to 36 times the liquid jet radius.

This principle was recently used to produce uniform PLGA microparticles [86,87]. A 5% (w/v) solution of PLGA in DCM was fed through a nozzle to form a cylindrical jet while the nozzle was excited by an ultrasonic transducer of ad-
justable frequency (Fig. 1.7 a). The particles were collected in 1% (w/v) PVA solution for solvent extraction/evaporation. Very uniform microspheres of 45 to 500 μm diameter were produced by jetting the polymer solution from nozzles of different orifice size (Fig. 1.7 b,c). Generally, 95% of the microspheres were within 1.5 μm of the average diameter. At fixed feed rate (2-3 ml/min; 60 μm nozzle), the microsphere size could be adjusted between 70 and 130 μm by decreasing the frequency from 70 to 19 kHz. Augmenting the feed rate at fixed excitation frequency from 2 to 3 ml/min resulted in a 30% increase in the

Figure 1.7 a. Microencapsulation by jet excitation. (a) Schematic representation of the process. Adapted from [86].
Figure I.7 b,c. Microencapsulation by jet excitation. (b) Size distributions and (c) SEM picture of PLGA microspheres produced by jet excitation. Scale bar in (c) represents 100 \( \mu \text{m} \). (b) and (c) reproduced from [87] with permission.

Microsphere diameter. Predetermined size distributions were obtained by switching the excitation frequency during production. Generally, the size of the microspheres was slightly larger than the diameter of the nozzle. Therefore, particle sizes below 25 \( \mu \text{m} \) are difficult to achieve with this technique as the pressure drop across the orifice opening rapidly increases as does the risk of orifice clogging. Scale-up is achieved using multi-orifice nozzles [e.g., 88]. Multi-
orifice nozzles with non-uniform openings were designed to yield desired microsphere size distributions [89].

The jet of drug/matrix dispersion may be surrounded by an annular stream of extraction fluid or any other suitable fluid immiscible with the drug/matrix dispersion (Fig. 1.7 a). The biphasic jet is then again vibrated and disintegrated into biphasic droplets [86,90]. The outer layer of fluid around the droplets of drug/matrix dispersion protected the latter from deformation upon impact with the collection/extraction fluid bath [91,92]. Feeding the outer stream at a higher velocity than the inner stream of drug/matrix dispersion stretched and thinned the latter due to the friction between the two phases. Subsequent vibration of the biphasic jet yielded uniform particles as small as 5 μm produced from a nozzle of much larger diameter [86]. The combined control of exciting frequency and annular sheath stream velocity allowed for a wide range of particle sizes manufactured from a single nozzle. The annular stream may alternatively be employed to dissolve a second matrix material, allowing for the manufacture of core/(multi)shell microspheres [90,92].

1.4 Solvent removal

In both, solvent extraction and evaporation, the solvent of the disperse phase, i.e., the drug/matrix dispersion, must be slightly soluble in the continuous phase so that partitioning into the continuous phase can occur leading to precipitation of the matrix material. In solvent evaporation, the capacity of the continuous phase is insufficient to dissolve the entire volume of disperse phase solvent. Therefore, the solvent must evaporate from the surface of the dispersion to yield sufficiently hardened microspheres. In solvent extraction, the amount and composition of the continuous phase is chosen so that the entire volume of disperse phase solvent can be dissolved.

Generally, a continuous phase that is a non-solvent for the microencapsulated bioactive compound is favourable. While for lipophilic compounds, aqueous solutions may be comfortably chosen, the use of hydrophobic, organic liquids as continuous phase for the encapsulation of hydrophilic compounds [e.g. 57,93,94] is more delicate. Hydrophobic extraction fluids may not be read-
ily removed from the final product, potentially causing undesired residues. Therefore, aqueous solutions are frequently used as continuous phase, even for the microencapsulation of hydrophilic compounds. Here, loss of bioactive compound is typically prevented by increasing the concentration of the matrix material solution; the resulting higher viscosity restricts the migration of the bioactive compound from the solidifying microspheres to the external phase by means of lowered diffusion and increased stability of the drug/matrix dispersion [33,48,95]. Other means of preventing loss of bioactive material into the continuous phase encompass the adaptation of the continuous phase pH to lower the solubility of the bioactive compound [50], or the addition of electrolytes to increase the osmotic pressure of the continuous phase [96,97,98].

The ideal rate of solvent removal depends on a variety of factors like the type of matrix material, drug and solvent, as well as the desired release profile of the microspheres. For example, fast microsphere solidification will be preferred if the drug easily partitions into the continuous phase. On the other hand, slow solidification favours denser over more porous microspheres, affecting the drug release.

1.4.1 Evaporation

The rate of volatile solvent removal from the solidifying microspheres can be controlled by the temperature of the microsphere dispersion. Higher temperatures will facilitate the evaporation of the solvent from the continuous phase and thereby maintain a high concentration gradient for the solvent between the microspheres and the continuous phase. In two similar studies on the encapsulation of BSA in a PLGA-PEG blend [99] and in pure PLGA [100], both dissolved in DCM and using an aqueous PVA solution as continuous phase, the influence of the temperature (4-42 °C) at which the resulting dispersion was stirred for 30 min was examined. Maintaining the temperature, the dispersion was thereafter diluted with additional continuous phase until a defined volume was attained. The PLGA microspheres tended to be larger when prepared at higher temperatures (38 and 42 °C), showed wider size distributions and decreased particle density compared to those prepared at lower temperatures (4-33 °C). As 38 and 42 °C are close to or even above the boiling point of the solvent DCM
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(b.p. ≈ 40 °C), these findings were attributed to very rapid microsphere solidification with insufficient mixing time to reduce droplet size. With PLGA, the morphology of the particle interior (honeycomb-like) and BSA encapsulation efficiency (53 to 63%) were unaffected by the preparation temperature while for the PLGA-PEG blend, BSA encapsulation appeared to be temperature-sensitive with a minimum efficiency of 15% obtained at 22°C, which steadily improved (up to 52%) for lower and higher temperatures (Fig. 1.8). For both polymers, the burst (24 h) release was highest at intermediate preparation temperatures, while values continuously decreased for higher and lower temperatures (Fig. 1.8). For the PLGA-PEG microspheres, these phenomena were explained by a fast skin formation at the extremes of temperature range studied, restricting BSA transport to the microspheres' periphery and loss of the protein. At high temperatures, rapid solvent evaporation obviously leads to fast solvent depletion in the microspheres. The authors' hypothesis for the low temperature effect was an increased DCM solubility in water.

When salmon calcitonin (sCT) was encapsulated into PLGA using a temperature gradient to remove the solvent, hollow microspheres with porous walls were obtained [101]. An aqueous solution of sodium oleate was used as continuous phase and the temperature of the resulting dispersion was increased from 15 to 40°C. A rapid temperature increase within 30 min led to particles with a large empty core and a thin wall, while a gradual or a stepwise increase over 200 min resulted in increased wall thickness. Peptide incorporation, however, was largely unaffected by the solvent removal conditions. The formation of the hollow core, which was not found when the solvent was removed by extraction, was attributed to the slow removal of methanol in the evaporation process; methanol was used as co-solvent for the dissolution of sCT in the polymer solvent DCM.

As an alternative to elevated temperatures, reduced pressure is sometimes used to promote the evaporation of the solvent, as in the encapsulation of lidocain [14] or albumin [102] in small (0.7 – 1.2 μm) PLA microspheres. In both studies, an aqueous PVA solution was employed as the continuous phase. Evaporation of the polymer solvent DCM was accomplished within 6 h at 760 mm Hg or 2 h at 460 or 160 mm Hg at 25°C. Irrespective of the encapsu-
lated drug, i.e., lidocain or BSA (lidocain was co-dissolved in the polymer solution for encapsulation and BSA was dissolved in an aqueous phase, which was subsequently emulsified in the organic polymer solution), both the microsphere mean size and encapsulation efficiency decreased at reduced pressure, whereas the drug release profile remained unaffected. With the encapsulation of progesterone in PLA, however, drug release was slower for microspheres prepared at reduced pressure (200 mmHg) as compared to those manufactured at atmospheric pressure [103]. The slow solvent removal at atmospheric pressure favoured the formation of a crystalline over an amorphous polymer matrix, which prevailed at reduced preparation pressure. In the amorphous state, data indicated a molecular dispersion of polymer and drug, lowering the release rate of the latter. Drug encapsulation efficiency was not affected by the mode of solvent removal.

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**Figure 1.8.** Influence of the temperature, at which the drug/matrix dispersion's solvent is evaporated, on the encapsulation efficiency (●) and 24 h burst release (○) in the microencapsulation of the model protein BSA in a PLGA-PEG polymer blend. Adapted from [99].
1.4.2 Liquid extraction

Solvent extraction is frequently performed as a two-step process: First, the drug/matrix dispersion is mixed with a small amount of continuous phase to yield an emulsion of desired droplet size (distribution). Then, further continuous phase and/or additional extraction agents are added at an amount sufficient to absorb the entire solvent leaching from the solidifying microspheres. Nonetheless, a patent application [104] teaches a one-step solvent extraction process. Without prior emulsification step, the drug/matrix dispersion is immediately homogenised with such a quantity of continuous phase that is capable of dissolving the total amount of disperse phase solvent at once. However, this process requires careful settings of the physicochemical parameters during the homogenisation step in order to yield homogenously dispersed particles.

A number of publications have reported that the drug substance can be more efficiently retained in the microspheres, if the amount of continuous phase strongly exceeds that theoretically necessary for dissolving the disperse phase solvent [e.g. 105]. The rapid formation of a skin on the microspheres’ periphery reduces the loss of drug to the continuous phase, which is of special importance when the latter is a good solvent for the drug. For example, the use of 10-fold the amount of fluid necessary to extract all the disperse phase solvent is suggested for the encapsulation of substances that are sparingly to freely soluble (> 10 mg/ml) in the continuous phase [105].

Rather than adding the entire amount of continuous phase at once, it may be added continuously over an extended period of time. However, in a system composed of aqueous BSA dispersed in a solution of PLA in DCM and 0.05% aqueous PVA solution, stirred at constant rate in a beaker for 30 min, further addition of continuous phase at constant rates ranging from 1.5 to 9 ml/min exerted no significant influence on the microspheres’ characteristics [99]. Likewise, in a similar process using a fixed addition rate but different final volumes of continuous phase, no significant influence on the resulting sCT-loaded microspheres was observed [101]. A continuously operated alternative to the batch-mode metering of continuous phase into a beaker consists in introducing further continuous phase or additional extraction-promoting agents through a series of feed streams into a continuous flow of dispersed nascent
microspheres [64]. A conduit featuring a number of down-stream inlets can be employed for this purpose. A static mixer installed at the entrance of the conduit may be used for emulsion formation.

Figure 1.9. Combining solvent extraction and evaporation by using a mixed solvent composed of ACN and DCM for the encapsulation of different model proteins in PLGA. Flow sheet of the encapsulation process [57].

A combination of solvent evaporation and extraction is suggested to improve the economic efficiency of the microencapsulation process [64]. After emulsion formation, a sufficient quantity of an extraction fluid is added to induce skin formation on the microspheres' periphery while the remaining solvent is
removed by evaporation. The brief skin-forming extraction step prior to evaporation minimises the loss of drug during the following evaporation procedure while the volume of extraction fluid consumed is reduced as compared to an extraction process alone.

The two steps of solvent extraction and evaporation may be combined by using a mixed solvent system [57]. For example, a system has been studied consisting of an aqueous protein solution, which was dispersed in a solution of PLGA in a mixture of ACN and DCM (Fig. I.9). The drug/matrix dispersion was emulsified in liquid paraffin containing sorbitan mono-oleat. The production vessel was then purged with air and thereafter put stepwise under reduced pressure (300/50 mmHg). The moderately polar DCM is extracted by the paraffin, whereas the strongly polar ACN, which is not soluble in paraffin, is evaporated during the purging and evacuating steps. BSA and lysozyme were very efficiently encapsulated, i.e. at 93 and 91%, respectively, while lower values were obtained for gelatin (71%) and a decapeptide (25-46%). After the extraction of DCM, the remaining ACN is miscible with the water dissolving the protein causing precipitation of BSA and lysozyme, while the decapeptide remained dissolved enhancing its potential to escape encapsulation.

Solvent extraction, evaporation and a combined procedure were compared for the entrapment of ovalbumin (OVA) in PLGA microspheres [106]. Aqueous OVA solution was intensely homogenised in a solution of PLGA in DCM. The drug/matrix dispersion was further emulsified in either water (solvent evaporation), a 1:1 water-methanol mixture (combined mechanism) or solely methanol (extraction) as continuous phases, using poly(vinyl pyrrolidone) (PVP) as an emulsifier. OVA entrapment was approx. 10% with the combined and the pure extraction processes, but only 7.5% with the evaporation method. As DCM is much more soluble in methanol than in water, the presence of the alcohol led to faster solvent removal and thus improved drug entrapment.

Two patents [107,108] teach methods for in-process reprocessing and recycling of the continuous phase to minimise waste. A portion of continuous phase rich in disperse phase solvent is repeatedly or continuously withdrawn from the suspension of nascent microspheres, deprived of part of the solvent and re-fed to the microsphere suspension. Solvent removal is achieved by ex-
posing the said portion of continuous phase to either a gas separation membrane [107] on which a vacuum is applied, or to an absorption fluid of high dissolution capacity for the disperse phase solvent, using a liquid-liquid column [108].

1.5 Microsphere harvest and drying

Separation of the solidified microspheres from the continuous phase is usually done either by filtration or centrifugation. The particles may then be rinsed with appropriate liquids to remove adhering substances such as dispersion stabilisers or non-encapsulated drugs. Rinsing may involve elevated temperatures or the use of extraction agents to reduce the amount of residual solvent in the microspheres [109]. Finally, the microspheres are dried either at ambient conditions or under reduced pressure, heat or by lyophilisation to yield a free-flowing powder. The drying procedure removes not only continuous phase and wash fluid adhering to the microspheres' surface, but also traces of solvents and continuous phase from the interior of the particles. Thus, the conditions and rate of drying influence the amount of solvent and moisture residue [110], microsphere morphology and porosity as well as drug re-crystallisation inside the spheres, and are therefore likely to affect the release behaviour of the final product.

1.6 Conclusions

The widespread interest in microencapsulated drugs brought forth the need to prepare such particles in larger quantities and in sufficient quality suitable for clinical trials and commercialisation. The most frequently described solvent extraction/evaporation-based technology using simple beaker/stirrer set-up is inappropriate for producing larger amounts of microspheres in an economic, robust and well-controlled manner. Static mixers warrant continuous production and simple scale-up, while the extrusion through porous membranes or microchannels, integrated in small-scaled equipment that is easy to operate and sterilise, additionally offers improved control of the microsphere size distribution as
compared to classical mixing processes. Further, jet excitation is powerful in combining productivity and microsphere size control. Solvent removal by evaporation may be accelerated using elevated temperatures or reduced pressure. The rapid solvent extraction may require relatively large amounts of processing fluids and their subsequent recycling. Therefore, combined extraction and evaporation represents a compromise in terms of both time- and waste-efficient microsphere production.

References


Chapter II

Continuous contact- and contamination-free ultrasonic emulsification – a useful tool for pharmaceutical development and production

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II.1 Introduction

Emulsification and homogenisation are common unit operations in the pharmaceutical, cosmetic, food, chemical and other industries. A number of mechanical processes are employed to produce emulsions, among them stirring, toothed disc dispersing (often referred to as homogenising or rotor-stator dispersing), colloid milling and high-pressure homogenisation [1]. Ultrasonic emulsification has been studied for many decades [2,3] and gathered increasing interest recently [4,5,6]. Studies comparing ultrasonic emulsification with rotor-stator dispersing [6,7] found ultrasound to be competitive or even superior in terms of droplet size and energetic efficiency. Microfluidisation (a type of high-pressure homogenisation) was found to be more efficient than ultrasound, but less practicable with respect to equipment contamination and aseptic processing [6].

A straightforward way to produce an emulsion by ultrasound is by immersion of a sonotrode either into the mixture of all components or into the continuous phase and adding gradually the phase to be dispersed during sonication. This procedure works well for small batches, but scale-up is difficult. As the intensity of ultrasound in a liquid decreases rapidly with the distance to the sonotrode, larger volumes may not be well homogenised. Results may be improved by stirring the volume or moving around the sonotrode [6]. However, continuous systems forcing the fluids to be sonicated through a small volume in the vicinity of the sonotrode are preferred. Such systems may consist of a flow-through beaker [8], a flow channel with ultrasonic transducers and reflectors installed in the walls [9] or a flow channel into which one or more sonotrodes protrude [4].

Still, existing ultrasonic processing systems are not well suited for the production of, e.g., pharmaceutical emulsions for the delivery of therapeutic agents, fat emulsions for parenteral nutrition or liposomes. Such pharmaceutical products require manufacturing equipment that can be readily cleaned and sterilised, and which offers the possibility of aseptic production. Furthermore, as ultrasonic emulsification is mainly driven by cavitation, ions or particles are emitted into the product by cavitational abrasion of the sonotrode. Frequently,
such sonotrodes consist of metallic alloys, leading to critical product contamination.

The objective of this study was to evaluate a novel ultrasonication concept for the production of pharmaceutical dispersions using different vegetable oils in water as model system. The equipment basically consists of a glass tube, through which the fluid mixture is pumped, surrounded by a jacket filled with pressurised water for conduction of the sound waves. Ultrasound is transmitted to the system by a sonotrode attached to the jacket. In addition to the preparation of oil-in-water emulsions, the system was also found useful for the preparation of biodegradable nanoparticles using the solvent extraction/evaporation method. The novel methodology is highly advantageous for continuous, contact-free emulsification and homogenisation. The process can be run in a closed system to prevent any environmental contamination of the product, and thus provides an opportunity for aseptic manufacturing. Future work will implement this process in the preparation of drug-loaded biodegradable microspheres, completing our group's portfolio of aseptic microencapsulation technologies [10,11].

II.2 Materials and methods

II.2.1 Design of the ultrasonic flow-through cell and experimental set-up

The ultrasonic flow-through cell consisted of a cylindrical steel jacket, in which a glass tube of 2 mm inner diameter for conducting the emulsion was installed (Fig. II.1). A sonotrode fixed to a piezoelectric transducer (24 kHz, UIP250, Dr. Hielscher, Teltow, Germany) was welded to the outside of the steel jacket to provide ultrasonic vibration. Through the space between the glass tube and the jacket, pressurised water was passed for sound conduction [12]. For the experiments described here, water pressure was maintained between 4.5 – 5.5 bar.

The continuous phase, fed by a double-piston pump (L-6000, Hitachi-Merck, Darmstadt, Germany), and the disperse phase, fed by a syringe pump (syritol 12, Heinzerling Medizintechnik, Rotenburg a.d. Fulda, Germany), were
Figure II.1. Photograph (a) and design (b) of the ultrasonic flow-through cell.
pre-mixed in a 3 ml glass cell by means of a cross-shaped magnetic stirrer (Fig. II.2). For the oil-in-water emulsions, two such mixing cells were connected in series, while for nanoparticle production, a single cell was found sufficient. The pre-mixed coarse emulsion was transported to the ultrasonic flow-through cell, where it was further homogenised.

Figure II.2. Experimental set-up used for the production of oil-in-water emulsions.

Sonication power was controlled by the amplitude of the transducer's oscillation. To quantify the power consumed for emulsification, the power intake of the high frequency generator driving the transducer was recorded using a standard household power monitor (PowerMonitor pro, Conrad Electronic, Hirschau, Germany). For 100, 80 and 60% of the maximum amplitude, the power intake amounted to 32, 25 and 17W, respectively. Assessment of the actual power transferred to the sonicated emulsion is usually done by measuring the heat taken up by the emulsion, which for the present ultrasonic flow-through cell would have been difficult to do with reasonable accuracy. Still,
it is reasonable to assume that the power consumption by the generator should be proportional to that delivered to the emulsion [13,14].

The residence time of the emulsion in the ultrasonic field, $t_R$, was calculated from the dead volume of the flow-through cell (0.53 ml) divided by the respective emulsion flow rate. Residence times were in the range of 7 to 50 s.

**II.2.2 Preparation of oil-in-water emulsions**

Olive and linseed oil (Ph.Eur./Ph.H.VIII grade) were obtained from Hänseler (Herisau, Switzerland), soybean oil from Sigma Chemie (Buchs, Switzerland) and Tween 40, used as emulsifier, from Fluka (Buchs, Switzerland). Water was of NANOpure-quality (Barnstead, Dubuque, USA).

The three vegetable oils were emulsified in water containing 3% (w/w) Tween-40 (HLB 15.6). After pre-filling the experimental equipment with the aqueous solution, the magnetic stirrer of the pre-mixing cell and the ultrasonic flow-through cell were activated. Thereafter, oil was injected into the pre-mixer. The product of the first 10 min of processing was discarded to let the process reach steady-state. During product collection, a sample of 0.5 ml was taken directly from the US cell outlet for droplet size analysis. For each set of parameters, production was repeated three times.

The dynamic viscosity of the oils was determined by means of a cone/plate rotational viscosimeter (VT 550/ PK 100, Haake, Karlsruhe, Germany) using a 1° cone. The interfacial tension between the different oils and the aqueous surfactant solution was measured using a droplet volume tensiometer (DVT30, Krüss, Hamburg, Germany).

**II.2.3 Preparation of PL(G)A nanoparticles**

End-group capped poly(lactic acid) (PLA) of approx. 0.2 dl/g inherent viscosity (Resomer® R202) and end-group uncapped 50/50 poly(lactic-co-glycolic) acid (PLGA) of approx. 0.38 dl/g inherent viscosity (Resomer® RG503H) were purchased from Boehringer-Ingeheim (Ingelheim, Germany). Synthesis grade dichloromethane (DCM) was from EGT Chemie (Scharlau, Switzerland). Poly(vinylalcohol) (PVA, Mowiol® 4-88), used as dispersion
stabiliser, was kindly donated by Kuraray Specialities (Frankfurt/M., Germany). Water was of NANOpure-quality.

Nanoparticles were produced using a modified solvent extraction/evaporation process [15]. PLA or PLGA was each dissolved in DCM at 2 and 5% concentrations. Water containing 0.5% (w/w) PVA was used as continuous phase. After pre-filling the equipment with continuous phase, the magnetic stirrer of the pre-mixing cell and the ultrasonic flow-through cell were activated, and polymer solution was injected into the pre-mixing cell. The flow rates of the polymer solution and continuous phase were set at a 1:8 ratio for all experiments. The product of the first 5 min of processing was discarded to let the process reach steady-state. Thereafter, the dispersion of nascent nanoparticles was collected for 30 min in a beaker pre-filled with 500 ml of continuous phase and gently stirred for a further 60 min to extract and evaporate the polymer solvent. For each set of parameters, two nanoparticle batches were prepared.

Particle collection for subsequent SEM analysis was done by centrifuging the nanoparticle dispersion at 5,000 rpm for 5 min. The resulting pellet was re-dispersed twice in purified water and centrifuged. Finally, the washed nanoparticles were re-dispersed in 500 µl of purified water and freeze-dried.

**II.2.4 Size measurement of oil droplets and PL(G)A nanoparticles**

The size distribution of the oil-in-water emulsions and the nanoparticle dispersions was determined by laser light scattering (Mastersizer X, Malvern, Worcestershire, UK) using a Mie diffraction model taking into account the refractive indices of the oils, PLGA and water. All size distributions are presented in the volume-weighted mode. Following the common usage in the literature, the oil-in-water emulsions were characterised by the surface-moment average of the size distribution, D[3,2], also called Sauter diameter, while for the nanoparticles, the diameter calculated from the volume-moment average of the size distribution, D[4,3], was chosen as characteristic mean diameter.
II.3 Results

II.3.1 Oil-in-water emulsions

The coarse emulsions produced by the two serial pre-mixers were compared with the emulsions further processed in the ultrasonic flow-through cell by light microscopy. The pre-emulsions exhibited oil droplets measuring mostly from 50 to 200 μm (Fig. II.3 a,b) and strongly tending to coalesce. After processing in the ultrasonic cell, the droplet size was reduced by a factor of

Figure II.3. Light microscope micrographs of coarse, pre-mixed (a,b) and post-ultrasonication (c,d) emulsions. (c) and (d) show emulsions processed at 25 W and 32 W sonication power, respectively. Arrows in (c) point at single larger droplets. Emulsions were prepared with 20% (v/v) olive oil (stained with Fat Red 7B) in water; residence time $t_R = 13$ s. Bars represent 100 μm.
approximately 100, yielding mean diameters of less than 1 \( \mu m \) (Fig. II.4). While occasional oil droplets of 5 to 10 \( \mu m \) could be observed in emulsions processed at an ultrasonic power of 25 W (Fig. II.3 c, arrows), practically no droplets were microscopically visible at 32 W, i.e. at full power (Fig. II.3 d).

The repeatability of the oil-in-water emulsification at full sonication power (32 W) was generally good irrespective of the residence time, the oil-to-water ratio and oil type. As an example, for six batches of olive oil in water emulsions prepared under identical conditions at maximum power virtually superimposed, mono-modal distributions were obtained (Fig. II.4).

![Droplet size distribution](image)

**Figure II.4.** Droplet size distribution for repeated production of a 20% (v/v) olive oil in water emulsion. Emulsion production was repeated six times under identical conditions \( (t_R = 13 \text{ s}, 32 \text{ W sonication power}) \).

When sonication power was decreased from 32 W to 25 W, the Sauter diameter increased from 0.54 to 0.73 \( \mu m \) at likewise slightly increased batch-to-batch variability (Fig. II.5). The larger droplet mean diameter resulted from an increased span rather than a shift of the size distribution. At 32 W, the 10 and
90% percentiles of the droplet size distribution amounted to 0.26 and 1.88 \( \mu \text{m} \) whereas at 25 W values of 0.30 and 3.69 \( \mu \text{m} \) were obtained. A further decrease of the sonication power to 17 W yielded a very inhomogeneous emulsion with a large number of macroscopically visible droplets of > 1 mm and therefore, this experiment was not repeated. Sonication below 17 W did not afford proper emulsification.

\[
\begin{array}{c|c|c}
P [\text{W}] & D_{3.2} [\mu \text{m}] &  \\
--- & 25 & 0.73 \pm 0.05 \\
--- & 32 & 0.54 \pm 0.01 \\
\end{array}
\]

**Figure II.5.** Change of the droplet size distribution of a 20% olive oil in water emulsion with sonication power: 25 W (---) and 32 W (—). Residence time \( t_R = 25 \) s. For both power levels, the resulting emulsion Sauter diameter \( D_{3.2} \) is given as mean of three batches \( \pm \) standard deviation.

The residence time of the emulsions in the ultrasonic flow-through cell was controlled by proportionally varying the flow rate of both the oil and water. The oil content of the emulsion was altered by varying the oil flow rate and keeping the water flow rate constant. Irrespective of the oil content, it was observed that the mean droplet size decreased with longer residence time of the emulsion in the sonic field (Fig. II.6, left panel). The droplet size reduction
occurred in a degressive manner, i.e. increasingly longer residence times were required to reduce the mean droplet diameter by a given decrement. For none of the curves, the diameter could be reduced below that displayed for the longest respective residence time; a more prolonged sonication did not further improve results. Therefore, 0.65, 0.50 and 0.47 \( \mu \text{m} \) represent the limiting Sauter diameters achievable for 33, 20 and 11% olive oil-in-water emulsions with the present ultrasonic flow-through cell. The variability for repeated production was generally low as reflected by standard deviations of generally below 0.03 \( \mu \text{m} \).

For a fixed residence time, the emulsion mean droplet diameter increased with the oil content. In agreement with the findings for varied sonication power, the decrease of the mean droplet size with increasing residence time is caused by a narrowing span of the size distribution. For an 11% olive oil in water emulsion

\[ \text{Figure II.6. Left panel: Sauter diameter as a function of the residence time in the ultrasonic field for 11\% (\textcolor{red}{\textbullet})}, 20\% (\textcolor{blue}{\textbullet}) \text{ and } 33\% (\textcolor{purple}{\textbullet}) \text{ emulsions of olive oil in water. Error bars are for repeated productions (n} = 3). \text{ Right panel: Influence of the residence time on the droplet size distribution of 11\% (v/v) olive oil in water emulsions: 28 s (---), 14 s (--) \text{ and } 7 \text{ s (-----)}. Sonication power was set to 32 W for all experiments.} \]
and residence times of 7, 14 and 28 s, size ranges of 0.30 - 2.98, 0.24 – 1.67 and 0.23 – 1.39 μm (10 - 90% percentiles) were obtained (Fig. II.6, right panel). All droplet size distributions were mono-modal. For the 33% olive oil emulsions and the most shortly sonicated 20% emulsion, very few macroscopically visible oil droplets of >1 mm in diameter were observed. These droplets were not detectable by laser light scattering. Sonicating the 33% emulsion for only 11 s resulted in an increased occurrence of large droplet of > 1 mm; therefore, this experiment was excluded from droplet size determination.

Finally, vegetable oils of different viscosity and interfacial tension towards the water phase were compared. When olive oil with the highest viscosity and interfacial tension was exchanged for soybean oil, which has slightly lower viscosity and interfacial tension, almost no reduction in the droplet size was observed (Tab. II.1). Linseed oil, whose viscosity and interfacial tension are much lower than those of olive oil, yielded a clearly decreased Sauter diameter of 0.47 μm compared to 0.62 μm for olive oil.

<table>
<thead>
<tr>
<th>Oil type</th>
<th>Viscosity [mPas]</th>
<th>Interfacial tension [mN m⁻¹]</th>
<th>Sauter diameter, D[3,2] [μm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linseed oil</td>
<td>43.9</td>
<td>1.32</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>59.3</td>
<td>2.20</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>Olive oil</td>
<td>72.3</td>
<td>2.65</td>
<td>0.62 ± 0.01</td>
</tr>
</tbody>
</table>

*Interfacial tension between the different vegetable oils and an aqueous solution of 3% Tween 40.*

**II.3.2 PL(G)A Nanoparticles**

Nanoparticles with a mean diameter of 0.49 μm were readily prepared from a 2% PLGA solution in DCM at 32 W sonication power (Tab. II.2). The size
distribution was mono-modal with a slight tailing (Fig. II.7 a). Nanoparticle sizes extended from 0.18 to 0.76 μm according to the 10 and 90% percentiles. Repeatability of the production process was good for all preparations examined, as reflected by superimposed size distributions (not shown) and only minor variability in the mean particle diameter (Tab. II.2). Lowering the emulsion's residence time in the sonic field from 14 to 7 s had only a minor impact on the nanoparticle size. A reduction of the sonication power from 32 to 25 W, however, resulted in a significant increase of the mean particle size from 0.49 to 0.7 μm, caused by a more pronounced tailing of the size distribution curve (Fig. II.7 a). A less prominent, though significant increase in the mean particle size from 0.49 to 0.6 μm was found when using a 5% instead of a 2% PLGA solution. Finally, the more hydrophilic PLGA was exchanged for the more hydrophobic and lower molecular weight PLA without noticeable changes in particle mean size and size distribution.

<table>
<thead>
<tr>
<th>Polymer type</th>
<th>Polymer concentration</th>
<th>Sonication power</th>
<th>Residence time</th>
<th>Mean particle diameter, D[4,3]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 50:50</td>
<td>2 [% (w/w)]</td>
<td>32 [W]</td>
<td>14 [s]</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>PLGA 50:50</td>
<td>2</td>
<td>32</td>
<td>7</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>PLGA 50:50</td>
<td>2</td>
<td>25</td>
<td>14</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td>PLGA 50:50</td>
<td>5</td>
<td>32</td>
<td>14</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>PLA</td>
<td>2</td>
<td>32</td>
<td>14</td>
<td>0.48 ± 0.00</td>
</tr>
</tbody>
</table>

* PLGA 50:50 was Resomer® RG 503H; PLA was Resomer® R202

No differences could be observed in the morphology of the different particles prepared from 2% polymer solutions. They all exhibited perfectly spherical shapes and smooth surfaces (Fig. II.7 b). The particles made from the
5% PLGA solution, however, were less spherical, showed slightly wrinkly surfaces, and fusions of two or - less frequently - more particles were observed (Fig. II.7 c).

When particles were produced from a 2% PLGA solution using water saturated in DCM, no difference could be seen in terms of morphology and particle size compared to particles made using non-saturated water (not shown).

---

**Figure II.7.** PLGA nanoparticles. (a): Size distribution of particles prepared at a polymer concentration/sonication power of 2%/32 W (—), 5%/32 W (—–), and 2%/25 W% (—––); residence time $t_R = 14$ s. (b),(c): SEM pictures of particles prepared of 2 and 5% polymer solutions, respectively. Residence time $t_R = 14$ s; sonication power was set to 32 W. Bars represent 1 μm.
II.4 Discussion

II.4.1 Working principle of the ultrasonic flow-through cell

The key innovation of the ultrasonic flow-through cell examined in this study was the transmission of sonic waves from an ultrasound emitting source (sonotrode) to the liquid mixture to be emulsified via a pressurised transmission fluid. The transmission fluid surrounded a glass tube through which the emulsion flowed. Using this set-up, direct contact between the sonotrode and the emulsion was prevented, avoiding contamination of the emulsion with ions or particles eroded from the metallic sonotrode by cavitation. Furthermore, as the glass tube is the only part of the ultrasonic cell coming into contact with the emulsion, cleaning and aseptic production is facilitated. Prior to production, the tube can be sterilised by any suitable means and easily installed into the cell under aseptic conditions. At the end of production, the tube can be removed from the cell and either cleaned or exchanged for a new one.

Cavitation is a well described phenomenon in the interaction of high-intensity ultrasound with liquids [16]. Very briefly, above a specific sound intensity, microbubbles form in the liquid during the low-pressure phase of the sonic oscillation. After oscillating for several pressure cycles accompanied by an overall bubble growth, these microbubbles will collapse resulting in a shock wave with local pressure and temperature of up to 1000 bar and several thousand Kelvin [16]. However, when a liquid is pressurised above a specific pressure threshold, which depends on temperature and the physicochemical characteristics of the fluid, cavitation will not occur. For the experiments accomplished in this study, pressures of above 4.5 bar were found to be sufficient to suppress cavitation in the transmitting water. Thus, acoustic energy was efficiently transferred via the glass tube into the emulsion. As the latter was not pressurized, cavitation occurred, resulting in break-up of the oil droplets.

Ultrasonic emulsification was described as a two-step process [17,18]. First, instable interfacial waves form at the oil-water interface, which results in the eruption of rather large oil droplets (approx. 50 – 100 μm) into the water phase. Second, the shock waves of cavitation events in the close vicinity of the coarse oil droplets will cause their disruption into much finer droplets. In viscous fluids, the formation of instable interfacial waves is compromised. Thus, a pre-
mixing step producing a coarse emulsion, which can be readily broken up further by cavitation, is beneficial for such substances. In our experiments with the ultrasonic flow-through cell, emulsions of vegetable oils in water could not be prepared without pre-mixing. Pre-mixing is indeed crucial when using the flow-through cell, as compared to a sonotrode-beaker set-up, as the latter will provide streaming and, thus, macroscopic mixing which does not occur in the flow-through cell.

### II.4.2 Oil-in-water emulsions

Optical micrographs of the emulsions prepared at 25 and 32 W power output (Fig. II.3 c,d) mirrored well the droplet size distributions obtained by laser light scattering (Fig. II.5). Larger droplets of 5 to 10 \( \mu \text{m} \) were observed microscopically and by light scattering analysis for the emulsions prepared at 25 W sonication power, but not for the preparation treated with 32 W.

The emulsion droplet mean size decreased both with increasing sonication power and residence time, in agreement with findings in the literature [6,7,19]. Both measures increase the energy transferred to the emulsion by enhancing the absolute number and, for increased power, also the intensity of the cavitation events, resulting in a more effective droplet size reduction. However, an optimal power input exists above which coalescence will become predominant resulting in a re-increase of the droplet mean diameter [16]. The narrowing of the droplet size distribution observed with both increased sonication power and residence time results from the susceptibility of larger droplets to be broken up by cavitation, while smaller droplets are more resistant or even stable. Enhancing the number of cavitation events by either increased power or residence time predominantly affects larger oil droplets and leads to an accumulation of fine droplets (given that coalescence is comparatively slow).

An increased oil content in the emulsion yielded, at fixed sonication power and residence time, larger oil droplets, which is agreement with earlier studies [7]. As cavitation occurs predominantly in the continuous aqueous phase [16], a larger fraction of oil requires more cavitation events per unit volume emulsion. Therefore, more concentrated oil-in-water emulsions require longer residence time to yield a desired mean droplet size. Nonetheless, the
limiting droplet diameter increased with the oil content even under prolonged sonication. This is probably due to intensified coalescence in the concentrated emulsions as well as to changes in the sound conduction and cavitation properties of the liquid mixture. Moreover, higher oil contents would possibly have required increasing amounts of surfactant for stabilisation.

In emulsions with high oil content, few macroscopically visible oil droplets survived the sonication process. As these droplets were considerably larger than those produced by the pre-mixer, they must have formed by accumulation and coalescence of coarse pre-emulsion droplets in the inlet tube or the nodes of the ultrasonic cell. Repeatedly passing the emulsion a second or more times through the cell abolished this problem.

When different vegetable oils were compared, as could be expected from literature [6,16], the least viscous linseed oil yielded a reduced Sauter diameter. The break-up of low viscosity droplets will require less vigorous cavitation shock waves than needed for more viscous ones, promoting break-up. However, a decrease in the emulsion droplet mean diameter was not observed when olive oil was exchanged for soybean oil, which has an intermediate viscosity. While the interfacial tension between the aqueous surfactant solution and the olive (2.65 ± 0.23 mN/m) and soybean oils (2.20 ± 0.18 mN/m) was found to be similar, that of linseed oil, however, was significantly lower (1.32 ± 0.20 mN/m). Thus, for linseed oil, reduced interfacial tension and viscosity may have added to result in simplified droplet disruption, while for soybean oil these parameters were not sufficiently different from those of olive oil to exert a significant effect.

The energy density, i.e. the energy input per unit volume of emulsion, was estimated from the power dissipated per unit volume of emulsion divided by the emulsion flow rate. In the production of a 20% vegetable oil-in-water emulsion, energy densities of around $10^8$ Jm$^{-3}$ were required to yield Sauter diameters of approximately 0.4 - 0.5 µm [19]. For the ultrasonic flow-through cell examined here, the power consumption of the high frequency generator was measured instead of the power dissipated in the emulsion. An energy consumption of approx. $10^9$ J m$^{-3}$ was found to yield a Sauter diameter of 0.5 µm. Relating this energy consumption with that required for emulsification, an energetic efficiency of approx. 10% was obtained. Therefore, the energy
transfer in the flow through cell is less efficient than in a classical system of a sonotrode installed in a beaker (26% – 75%) [13,14]. The lower efficiency of the ultrasonic cell could be expected from the more complex mechanism of energy transfer. In the beaker set-up, the sound emitting sonotrode is in direct contact with the emulsion, while in the flow-through cell, the sonotrode excites the steel jacket, which in turn transfers the energy to the pressurised water and the glass tube, which then finally excites the emulsion. Moreover, the mode of excitation is different in both systems. In the beaker system, the sonotrode basically performs a longitudinal vibration, whereas the steel jacket of the flow-through cell transforms the longitudinal oscillation of the attached sonotrode into a transversal oscillation. Finally, as the volume sonicated in the present cell is very small (0.53 ml), the energy expended for exciting the apparatus itself largely outweighs the energy expended for emulsification. With slightly to moderately larger diameter glass tubes or a manifold of parallel small tubes installed in the cell’s steel jacket, the sonicated volume would increase, probably without much impact on the overall energy consumption.

11.4.3 PL(G)A nanoparticles

Solvent extraction/evaporation employing ultrasonic emulsification is a common process for the preparation of biodegradable PLA/PLGA micro- and nanoparticles [15,20]. Briefly, it consists of forming an emulsion of an organic solution of the polymer in usually an aqueous continuous extraction phase. A drug to be encapsulated may be co-dissolved or dispersed in the polymer solution. The organic solvent is then either extracted by the continuous phase (solvent extraction), diffuses into the same and evaporates to the environment (solvent evaporation), or is removed by a combination of both. The size of the resulting particles is mainly determined by the emulsification conditions, but other factors like polymer concentration, viscosity, the ratio of dispersed to continuous phase do also play a role. Although ultrasound is frequently used to generate emulsions for nanoparticle preparation, high-pressure homogenisation or rotor-stator dispersion are equally employed.

In the present study, we demonstrate the feasibility of nanoparticle preparation in the ultrasonic flow-through cell, opening the road to nanoparticle
production under aseptic conditions. For PLA/PLGA nanoparticles, emulsion formation in the ultrasonic flow-through cell was fast, as the reduction of the residence time from 14 to 7 s did not markedly increase the particle diameter. In analogy to the oil-in-water emulsions, particle sizes increased at decreased sonication power, though the differences in size distribution were less pronounced than observed for the oil emulsions. By increasing the polymer solution concentration, and thereby its viscosity, larger particles were produced. Interestingly, no impact on the particle size was observed when PLGA was substituted by PLA having roughly half the inherent viscosity, while in the literature a correlation between polymer inherent viscosity and nanoparticles size was reported [15]. Obviously, viscosity is not the only physicochemical parameter governing emulsification, as already noted for the oil-in-water emulsions. Factors like interfacial tension and suitability of the employed surfactants are equally important, especially with respect to droplet coalescence.

The deformed and partially fused nanoparticles observed for the 5% PLGA solution probably resulted from rapid solvent deprivation and particle solidification, reducing the time available for droplet break-up (larger and fused particles) and shape rearrangement.

II.5 Conclusions

In the present study, a novel technology for the continuous treatment of fluid mixtures with ultrasound was characterised using vegetable oil-in-water emulsions as a model system. The flow-through equipment consisted of a glass tube for the conduction of the sonicated fluid, which was installed in a steel jacket excited by a transducer and filled with pressurised water for the transmission of the sound waves. Its design makes this apparatus well suited for aseptic processing, an important issue in pharmaceutical development and production. The mean droplet diameter of oil-in-water emulsions could be decreased by two orders of magnitude starting from coarse pre-emulsions and yielding Sauter diameters of 0.5 μm and below. The reduced efficiency of sound energy transfer compared to directly contacting sonotrode and fluid might be
improved by scaling-up the cell. Furthermore, the ultrasonic flow-through cell was found to be well suited for emulsion-solvent extraction/evaporation based production of biodegradable polymeric nanoparticles. Future research will be directed towards scaling-up the process and increasing the power input to yield even finer emulsions. In addition, the suitability of the cell for the preparation of water-in-oil emulsions, e.g. for further processing into drug-loaded microspheres, will be studied.

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References


Chapter III

Solvent extraction employing a static micromixer: a simple, robust and versatile technology for the microencapsulation of proteins

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III.1 Introduction

With an increasing number of proteins emerging as therapeutic agents, protein delivery has become a central topic in pharmaceutics. As many of those proteins are rapidly degraded in the gastro-intestinal tract or cannot pass the intestinal epithelia, their parenteral delivery is preferred over oral administration. Due to their short in vivo half-life, repeated injections or intravenous infusion are often required to achieve sufficiently high plasma levels [1]. Parenteral drug delivery systems providing sustained release as well as protection of the non-released protein from degradation are therefore advantageous. Microspheres consisting of the biodegradable polymers poly(lactic acid), PLA, and poly(lactic-co-glycolic acid), PLGA, have been shown to provide these characteristics [2] while degrading to toxicologically acceptable products.

The most common technologies for the preparation of drug-loaded microspheres are solvent extraction or evaporation, coacervation and spray-drying [3]. Solvent extraction/evaporation is the most frequently used method for the encapsulation of proteins into PLA/PLGA microspheres (e.g. [2, 4-7]) due to its mild processing conditions and the use of comparatively safe processing agents. A critical step during the process is the formation of a W/O/W double emulsion when a pre-formed dispersion of an aqueous protein solution in an organic polymer solution (W/O) is emulsified in a second aqueous solution (referred to as the extraction fluid). The commonly employed lab bench procedure for this second emulsification step consists of two sub-steps: Firstly, the addition of the pre-formed protein/polymer dispersion (W/O) into a small amount of extraction fluid under vigorous agitation using a magnetic, propeller or turbine stirrer to form a double emulsion (W/O/W); secondly, the addition of a large amount of extraction fluid under gentle stirring to harden the microdroplets and form the microspheres. This technique encompasses a number of drawbacks. Process scale-up is difficult and may impose a lengthy trial-and-error approach to adjust the hydrodynamic conditions of larger reactors to the desired microsphere size distribution. While suitable mean diameters may be readily achieved at reasonable effort [8], control over the distribution's profile may be more difficult to achieve, but is crucial for the release kinetics of the entrapped protein and the in vivo fate of the microspheres. Equally challenging is the ad-
justment of the hydrodynamics in the production vessel when changes in polymer concentration, polymer type or polymer solvent are needed. Moreover, solvent extraction/evaporation is typically a batch process and therefore difficult to automate. However, for aseptic production of protein-loaded microspheres at large scale an automated process would be a must.

A number of approaches were previously proposed for improved control of microsphere size distribution and to allow for continuous production. Static mixers installed in tubes [9] feature continuous production, but fail to offer much improved microsphere size distribution control as compared to conventional stirred tank reactors. However, formation of single droplets of the protein/polymer dispersion by extrusion through, e.g., needles, orifices, membranes or channels and their simultaneous or subsequent exposure to the extraction fluid seem to be more advantageous over systems relying on bulk mixing. As bulk mixing depends on chaotic turbulent flow patterns for disruption of the protein/polymer dispersion into droplets it is difficult to obtain well controllable and narrow size distributions. In this context, a quasi-continuous process was proposed featuring the drug/polymer solution to be fed by a needle into a co-current stream of extraction fluid [10]. In a similar approach, a stream of extraction fluid flowing perpendicularly to the needle was employed [11]. Furthermore, an electrostatic field was applied to form microdroplets of a protein/polymer dispersion when fed through a needle [12]. However, none of these approaches proved able to produce microspheres with mean diameters of less than approximately 70 µm and very broad size distributions were found. Further, microporous glass membranes were used to extrude a polymer solution into an extraction fluid [13]. The produced microspheres were nearly monodisperse with their mean size varying between 1 and 3 µm, depending on the pore size of the microporous membrane. Recently, a promising but rather complex technology was developed using a jet of polymer solution which was pumped through a fine orifice, disrupted by vibration to equally sized droplets and subsequently collected in an extraction fluid [14]. Spheres of monomodal distribution with adjustable sizes in the range of 25 to 500 µm could be produced. The size of the microspheres was further decreased when the polymer solution jet was additionally surrounded by an annular mantle of a downward
carrier fluid stream to accelerate and thin the jet by frictional forces. As a result, microspheres as small as 1 μm with monomodal distribution were obtained.

Here, a new technology is introduced to prepare microspheres by means of a static micromixer. The micromixer was previously used for the dispersion of immiscible fluids [15], to produce pharmaceutical creams [16], as chemical reactor [17] and for fluid extraction [18]. It is small-sized, features a simple set-up, lacks moving parts, can be run continuously and is easy to sterilise. Thus, the micromixer is ideally suited for automation and aseptic manufacturing. Furthermore, it offers good size distribution control and tolerates a wide range of ingredients and excipients. Scale-up from the laboratory bench to the industrial plant is feasible simply through the parallel installation of a sufficient number of individual units (“number-up”). Thus, by multiplication of units, laboratory bench and large-scale production can be made on the same micromixer, with considerable advantages for development and registration, and the adaptation to different batch sizes can be made by simply adding or removing mixing units.

The scope of this work was to evaluate the potential of the static micromixer for the microencapsulation of a model protein, bovine serum albumin (BSA), in PLGA microspheres by solvent extraction. For this purpose the effect of various process and formulation parameters on the characteristics of the resulting microspheres was studied with a special focus on particle size distribution.

III.2 Materials and methods

III.2.1 Materials

Various poly(lactic-co-glycolic acid) type polymers (Resomer® RG502H, RG503, RG503H and RG752) were obtained from Boehringer-Ingeheim (Ingelheim, Germany). The physico-chemical properties of the polymers are detailed in the results section. Bovine serum albumin (BSA, fraction V) was from Fluka (Buchs, Switzerland). Poly(vinylalcohol) (PVA, Mowiol® 4-88) and polysorbate 20 were from Hoechst (Frankfurt/M., Germany) and Hänseler (Herisau, Switzerland), respectively. Dichloromethane (DCM) and ethyl formate (EF) were
of synthesis grade and obtained from EGT Chemie (Scharlau, Switzerland) and Fluka, respectively.

**III.2.2 Experimental set-up**

The experimental set-up used to prepare the microspheres consisted of two HPLC piston pumps (L-6000, Hitachi-Merck, Darmstadt, Germany; 10 ml/min maximum flow) connected in parallel to a T-type valve to sum up their flow capacities, a syringe pump (KDS100, kdScientific, New Hope, USA), the static micromixer (featuring two exchangeable mixing tools with channel widths of 25 and 40μm, both made of silver on a copper support), that was purchased from the Institut für Mikrotechnik Mainz (IMM), Mainz, Germany, and equipped with a stainless steel outlet tube of 120 mm length and a standard glass beaker (Fig. III.1). By means of the piston pumps the extraction fluid was fed into one of the two inlets of the micromixer, while the previously prepared protein/polymer dispersion was fed into the other inlet by means of the syringe pump. Upon contact between the protein/polymer dispersion and the extraction fluid nascent microspheres were formed inside the micromixer. For final hardening and product collection the resulting dispersion was transferred into the beaker containing 150 ml extraction fluid.

**Figure III.1.** Experimental set-up for the production of BSA-loaded microspheres by solvent extraction employing a micromixer.
11.2.3 Preparation of the microspheres

Solutions of BSA in PBS (pH = 7.4) and of PLGA in EF or DCM were prepared at various concentrations. BSA solution was added to 3-4 ml of polymer solution at specific ratios R (given as mg BSA solution per mg polymer solution) and finely dispersed for about 15 s at 50 W using a 20 kHz, 6 mm ultrasound probe (VibraCell VC50T, sonics and materials, Danbury, USA). The nominal drug load of the microspheres to be prepared, LD, i.e. the nominal amount of drug entrapped in the particles, mD, divided by the amount of polymer employed, mP, was calculated by:

\[ L_D = \frac{m_D}{m_P} = R \cdot \frac{w_D}{w_P} \]  

where \( w_D \) and \( w_P \) denote the weight fraction of the drug and the polymer in their respective solutions.

The protein/polymer dispersion was transferred into a 20 ml single-use syringe (Discardit II, Becton-Dickinson, Basel, Switzerland), which was installed in the syringe pump. Aqueous PVA and polysorbate 20 solutions (0.5% w/w each) were used as extraction fluids. After micromixing and collection in the glass beaker, the particles were allowed to harden in the extraction fluid under gentle stirring and at room temperature, either for 30 min (EF) or 90 min (DCM). The product was harvested by filtration over a 0.2 \( \mu \)m pore cellulose acetate filter (Sartorius, Göttingen, Germany), washed twice with 150 ml of deionised water, and finally dried for 24 h at 20 mbar and room temperature. All experiments were conducted in an air-conditioned room at 20 °C.

11.2.4 Particle size distribution

Samples of approximately 30 mg of dried microspheres were dispersed in 12 ml of de-ionised water and subsequently treated with ultrasound for 5 s (VibraCell VC50T, sonics and materials, Danbury, USA; 50 W, 20 kHz, 6 mm probe). Microsphere size was determined by laser light scattering (Mastersizer X, Malvern, Worcestershire, UK, equipped with a 100 mm lens) using a Fraunhofer diffraction model for the analysis of the raw data. All size distributions are presented in the volume-weighted mode and characterised by their 10, 50 and 90% undersize diameters \( d(v,0.1) \), \( d(v,0.5) \) and \( d(v,0.9) \). The 50% un-
dersize diameter is referred to as the mean diameter, and \( d(v,0.1) \) and \( d(v,0.9) \) are used to characterise the span of the distribution.

**III.2.5 Encapsulation efficiency**

Samples of approximately 40 mg of dried microspheres were dissolved in 4 ml of DCM, and BSA was separated from the dissolved polymer by filtration over a 0.2 \( \mu m \)-pore regenerated cellulose filter (Sartorius, Göttingen, Germany). The filter was washed once with 6 ml of DCM to remove remaining polymer. BSA was then extracted from the filter into 10 ml of PBS by shaking for 3 h. The resulting solution was diluted to 25 ml and analysed fluorimetrically (Cary Eclipse, Varian, Mulgrave, Australia) at an excitation wavelength of 278 nm and an emission wavelength of 347 nm. The encapsulation efficiency \( \varepsilon \) was expressed as the ratio of the amount of BSA extracted from the microspheres to the amount of BSA employed for the preparation of the spheres. This protocol was validated for accuracy. Typical deviations observed for repeated analysis were below 1% of the averaged encapsulation efficiency.

**III.2.6 In vitro burst release**

The release of BSA from the microspheres was determined using approximately 20 mg of dried microspheres in 4.5 ml glass vials containing 4 ml PBS and placed in an overhead mixer (Heidolph Reax 2, Heidolph, Schwabach, Germany) revolving at 60 rpm and incubated at 37 °C. For complete dispersion of the microspheres prior to the experiment the vials were shaken vigorously for a few seconds. At predefined time points, the vials were centrifuged at 3700 rpm for 5 min (sigma 204, Sigma, Osterode/Harz, Germany), and 1.5 ml of the clear supernatant was taken for fluorimetric assay and replaced by fresh PBS. The amount of BSA released was expressed as fraction of the experimentally determined total BSA content.

**III.2.7 Scanning electron microscopy (SEM)**

Samples of dried microspheres were placed on a double-sided adhesive tape and coated with 6 nm of platinum using a planar magnetron sputter-coater (MED 010, Bal-Tec, Balzers, Liechtenstein). The micrographs were taken on a
III.3 Results

The micromixer developed by IMM features a stainless steel housing consisting of a top and a bottom plate (Fig. III.2 a,b). The actual mixing tool, made of silver (or nickel) on a copper support, is embedded in this housing [17]. The mixing tool basically consists of an array of channels (25 or 40 \(\mu\)m in width and 300 \(\mu\)m in depth) etched into the metallic substratum and separated by corrugated walls (Fig. III.2 c). The channels are alternately accessible from each of the two inlet distribution funnels. The two fluids to be mixed are fed from the opposite inlet funnels into the array of channels and mixing of the two liquids occurs once they reach the outlet slit (60 \(\mu\)m in width), situated perpendicular in the middle of the channel array in the housing's top plate (Fig. III.2 b). When the two fluids are discharged through this outlet slit, alternating fluid lamellae are formed [17]. Given the fact that the flow velocities are sufficiently high, these lamellae disintegrate into droplets of one of the liquids dispersed in the second (Fig. III.2 d).

<table>
<thead>
<tr>
<th>Mixer channel width [(\mu)m]</th>
<th>Extraction fluid flow rate [ml/h]</th>
<th>Ratio of flow rates [1/50]</th>
<th>Polymer solution conc. [5%]</th>
<th>Nominal drug load [%]</th>
<th>Polymer solvent</th>
<th>Polymer type</th>
</tr>
</thead>
<tbody>
<tr>
<td>* 40</td>
<td>600</td>
<td>1/50</td>
<td>5</td>
<td>5</td>
<td>DCM</td>
<td>RG503H</td>
</tr>
<tr>
<td>1 25, 40</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2 *</td>
<td>420 - 1200</td>
<td>1/50 - 1/12.5</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>3 *</td>
<td>*</td>
<td>2 - 10</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>4 *</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5 *</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>DCM / EF</td>
<td>various</td>
</tr>
</tbody>
</table>
Five series of experiments were conducted to characterise the performance of the micromixer for solvent extraction in terms of microsphere size, yield and encapsulation efficiency. Throughout the study BSA was used as a model protein. In each series, one process or formulation parameter was varied while the remaining parameters were kept constant. The studied parameters were (i) micromixer channel width, (ii) flow velocity of extraction fluid and ratio of extraction fluid flow to protein/polymer dispersion flow, (iii) concentration of polymer solution, (iv) microsphere nominal drug load and (v) type of polymer and polymer solvent (Tab. III.1). Selected batches of the microspheres were further
examined with respect to the in vitro release of BSA and the morphology of the microspheres.

III.3.1 Repeatability of the microsphere preparation and influence of micromixer channel width

Three batches of microspheres were produced using the mixing tool with 40 μm wide channels (Fig. III.3 a,b) and four batches using the tool of 25 μm channel width (Fig. III.3 c), keeping all other parameters unchanged. In essence, all resulting particle size distributions exhibited a practically monomodal shape, with a small fraction of very fine particles (0.5 – 3 μm) in all batches.

For the three batches prepared with the 40 μm tool, the size distributions of the microspheres were practically identical when measured before filtration and drying (Fig. III.3 a). After drying, one of the three samples exhibited a marginal increase in particle size (Fig. III.3 b, solid line), indicating a tendency of the spheres to slightly aggregate upon filtration or drying. Possibly for the same reason, a very small fraction of larger particles (> 100 μm) was observed among the dried spheres. Nevertheless, for the rest of the study all size distributions were determined with dried microspheres only. According to the d(v,0.1) and d(v,0.9) diameters, typically, 80% of the volume weighted particle diameters of the three batches ranged from 7 to 36 μm. This size range was confirmed by SEM (Fig. III.8 a). The mean diameters of the two batches unaffected by drying were almost identical (16.1 and 16.2 μm), whereas that of the mentioned third batch was slightly larger (18.3 μm). The product yield of the three batches was high and reproducible, ranging from 93.6 to 95.3%. The encapsulation efficiency of BSA amounted to 75.9 ± 4.8%.

Using the 25 μm tool, the reproducibility of particle size distribution was less satisfactory than with the 40 μm tool (Fig. III.3 c). Two out of the four batches exhibited size distributions similar to those obtained using the 40 μm tool, with mean diameters of 16.0 - 17.0 μm, d(v,0.1) of 7.5 and d(v,0.9) of 35.5 μm. The other two batches, however, featured slightly smaller mean diameters (13.5 and 14.0 μm) and a narrower distribution (d(v,0.1) = 6.5 and
Figure III.3. Reproducibility of microsphere size distributions. (a) channel width of 40 μm, particle size before filtration and drying; (b) channel width of 40 μm, size of dried particles; (c) channel width of 25 μm, size of dried particles. All other preparation conditions were identical to Table III.1 standard conditions.
\( d(v,0.9) = 24.4 \ \mu m \). This discrepancy was found for both the non-dried and dried microspheres and, thus, cannot be attributed to agglomeration upon drying. Microsphere yields in all four cases were close to 95% as also found with the 40 \( \mu m \) tool. Encapsulation efficiency was slightly higher but at moderately increased variability (79.0 ± 7.6%).

### III.3.2 Influence of extraction fluid flow rate and flow rate ratio

As described elsewhere [15], the droplet size formed by the micromixer can be controlled by the flow velocities of the two fluids pumped into the mixer. In a first series of experiments, the flow rate of the extraction fluid was varied from 420 to 1200 ml/h (Fig. III.4), while maintaining a 1/50 ratio \( \Phi \) of the flow rates between the protein/polymer dispersion and the extraction fluid. When increasing the extraction fluid flow rate in the mentioned range, the mean diameter of the microspheres was decreased from 29.0 to 8.8 \( \mu m \). The lowering of the mean diameter was degressive, i.e. increasingly larger extraction fluid flow rates were required to reduce the mean diameter by a given decrement. The shape of the size distribution was unaffected by the decrease in the distribution's mean diameter and, therefore, both \( d(v,0.1) \) and \( d(v,0.9) \) were shifted in parallel to the mean diameter. For example, for the lowest (\( F = 420 \) ml/h) and highest (\( F = 1200 \) ml/h) extraction fluid flow rate, the particle size range \( d(v,0.1) \) to \( d(v,0.9) \) was 14.9 – 50.2 \( \mu m \) and 3.5 – 17.1 \( \mu m \), respectively. Again, a small fraction of fine particles was observed in all preparations. Interestingly, both the microsphere yield (91.2 – 96.4%) and the encapsulation efficiency (75.1 ± 1.7%) remained essentially unaffected by \( F \) (Fig. III.4).

In a second series of experiments, the influence of the ratio \( \Phi \) of the protein/polymer dispersion flow rate to that of the extraction fluid on the microspheres' properties was examined (Tab. III.2). The extraction fluid flow rate was kept at 600 ml/h, while that of the protein/polymer dispersion was adapted to achieve \( \Phi \)-values of 1/50, 1/25 and 1/12.5. Principally, an increase in \( \Phi \) would improve the productivity as the output of microspheres per unit mass extraction fluid and, thus, per time would be raised. An increase in \( \Phi \) from 1/50 to 1/12.5 augmented slightly the microspheres' mean diameter from 18.4 to 23.5 \( \mu m \).
Yield (102.9 ± 1.0%) and encapsulation efficiency (74.5 ± 0.9%) were independent from the flow rate ratio $\Phi$.

*Figure III.4. Microsphere characteristics as a function of the extraction fluid flow rate $F$. Upper panel: particle size distributions obtained at $F = 1200, 900, 600, 420$ ml/h (from left to right). Lower panel: microsphere mean diameter, $d(v,0.5)$ ($\bullet$), encapsulation efficiency, $\varepsilon$ ($\square$).*
Table III.2. Influence of the ratio between the protein/polymer dispersion flow rate and extraction fluid flow rate on the characteristics of the microspheres. The extraction fluid flow rate \( F \) was kept at 600 ml/h.

<table>
<thead>
<tr>
<th>Ratio of flow rates</th>
<th>Microsphere mean diameter ( d(v,0.5) ) [( \mu \text{m} )]</th>
<th>Yield [%]</th>
<th>Encapsulation efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/50</td>
<td>18.4</td>
<td>103.8</td>
<td>73.5</td>
</tr>
<tr>
<td>1/25</td>
<td>19.5</td>
<td>101.9</td>
<td>74.8</td>
</tr>
<tr>
<td>1/12.5</td>
<td>23.5</td>
<td>103.0</td>
<td>75.3</td>
</tr>
</tbody>
</table>

III.3.3 Influence of polymer concentration

Batches of microspheres were produced at various polymer solution concentrations, \( \omega_p \), from 2 to 10\% (w/w) (Fig. III.5). The W/O dispersion ratio \( R \) was adapted as to yield a nominal drug load \( L_d \) of 5\%, constantly using a 5\% (w/w) BSA solution (equation 1). Here, the microspheres' mean diameter varied randomly between 17.1 and 21.1 \( \mu \text{m} \) and the shapes of the size distribution curves were almost superimposed, with \( d(v,0.1) = 8.2 \pm 1.0 \mu \text{m} \) and \( d(v, 0.9) = 37.8 \pm 4.4 \mu \text{m} \). Yields were 95.6\% on the average and varied considerably (± 7.2\%). BSA encapsulation efficiency tended to increase from approximately 65 to 80\% for the range of polymer concentrations examined (Fig. III.5). Nevertheless, because the deviations from the trendline were found to be quite large in case of the 2\% and 4\% solutions, this trend is speculative.

III.3.4 Influence of nominal drug load

Microspheres with nominal BSA loads of 5, 10 and 15\% were produced (Tab. III.3). Basically, the nominal drug load in microspheres can be adjusted by varying either the BSA solution concentration, \( \omega_d \), while maintaining the W/O dispersion ratio \( R \), or \( R \), while maintaining \( \omega_d \) (equation 1). For the first three batches displayed in Tab. III.3, \( \omega_d \) was increased to augment the drug load from 5 to 15\%, whereas for the forth batch \( R \) was increased to bring the nominal load
Chapter III

Figure III.5. Microsphere characteristics as a function of the polymer solution concentration $w_P$. Upper panel: particle size distributions for $w_P = 2\%$ (---), $4\%$ (----), $6\%$ (-----), $8\%$ (-----), $10\%$ (-----). Lower panel: microsphere mean diameter, $d(v, 0.5)$ ( ), encapsulation efficiency, $\varepsilon$ (■).
from 10 to 15%. None of these changes markedly affected the particle size distribution with the mean diameter remaining almost identical (16.8 ± 0.9 μm). Yields were similarly high for all batches (97.5 ± 1.8%), while an increase in BSA encapsulation efficiency from 73.5 to 83.4% was observed when the drug load \( L_D \) was augmented from 5 to 10%. An increase to 15% nominal load did not further improve the efficiency of encapsulation. Equally, no marked difference was found between the two batches with the 15% load, one prepared from 15% BSA solution at \( R = 0.05 \), the other from 10% BSA solution at \( R = 0.075 \).

<table>
<thead>
<tr>
<th>Concentration of BSA solution</th>
<th>Protein/polymer dispersion ratio</th>
<th>Nominal drug load</th>
<th>Microsphere mean diameter ( d(v,0.5) ) [μm]</th>
<th>Yield [%]</th>
<th>Encapsulation efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.05</td>
<td>5.0</td>
<td>16.2</td>
<td>96.5</td>
<td>73.5</td>
</tr>
<tr>
<td>10.0</td>
<td>0.05</td>
<td>10.0</td>
<td>16.6</td>
<td>96.4</td>
<td>83.4</td>
</tr>
<tr>
<td>15.0</td>
<td>0.05</td>
<td>15.0</td>
<td>18.1</td>
<td>96.8</td>
<td>83.6</td>
</tr>
<tr>
<td>10.0</td>
<td>0.075</td>
<td>15.0</td>
<td>16.2</td>
<td>100.1</td>
<td>81.1</td>
</tr>
</tbody>
</table>

### III.3.5 Influence of polymer solvent and polymer type

When ethyl formate (EF) was used in exchange for the generally employed polymer solvent dichloromethane (DCM, Tab. III.4), the microspheres' mean diameter was lowered from 16.9 to 7.1 μm, \( d(v,0.1) \) from 7 to 2 μm and \( d(v,0.9) \) from 36 to 18 μm. Microsphere yield, however, was similar (approximately 95%) with both polymer solvents. Very importantly, BSA encapsulation efficiency attained only 60.4 ± 7.5% with EF as compared to 75.9 ± 4.8% with DCM. In view of achieving reproducible particle size distributions and controlling the particle size, EF performance was as good as DCM (Fig. III.6). For three microsphere batches prepared with EF under identical conditions size distributions were virtually the same (Fig. III.6) with only minute variations of the mean
### Table III.4. Influence of the polymer solvent and polymer type on microsphere characteristics.

<table>
<thead>
<tr>
<th>Polymer type</th>
<th>Polymer solvent</th>
<th>Microsphere mean diameter $d(v,0.5)$ [µm]</th>
<th>Yield [%]</th>
<th>Encapsulation efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG503H</td>
<td>DCM</td>
<td>16.9 ± 1.2</td>
<td>94.6 ± 0.9</td>
<td>75.9 ± 4.8</td>
</tr>
<tr>
<td>RG503H</td>
<td>EF</td>
<td>7.1 ± 0.3</td>
<td>96.5 ± 1.9</td>
<td>60.4 ± 7.5</td>
</tr>
<tr>
<td>RG503</td>
<td>DCM</td>
<td>14.3</td>
<td>98.6</td>
<td>55.3</td>
</tr>
<tr>
<td>RG502H</td>
<td>DCM</td>
<td>19.9</td>
<td>95.4</td>
<td>64.0</td>
</tr>
<tr>
<td>RG752</td>
<td>DCM</td>
<td>17.7</td>
<td>96.0</td>
<td>49.6</td>
</tr>
</tbody>
</table>

### Figure III.6. Particle size distributions of microspheres prepared with ethyl formate as polymer solvent. $F = 900, 600, 420$ ml/h (from left to right). At $F = 600$ ml/h, three batches were produced under identical conditions.
diameter (Tab. III.4). The distributions were practically monomodal with only a small fraction of very fine particles, as observed with DCM. EF was equally suited to control the microsphere size by means of the extraction fluid flow rate. Mean diameters varied from 11.6 to 4.9 μm by increasing the extraction fluid flow from 420 to 900 ml/h (Fig. III.6).

Finally, microspheres were prepared of polymers other than the generally used RG503H (Tab. III.4). When compared to RG503H, RG503 features a comparable inherent viscosity but no carboxylic end-groups, RG502H a lower inherent viscosity (0.17 compared to 0.36 dl/g for RG503H), and RG752 a different lactic acid to glycolic acid ratio (75/25 compared to 50/50 for RG503H). Concerning microsphere size, RG503 yielded a modestly smaller, RG502H a moderately larger, and RG752 a similar mean diameter, as compared to RG503H. RG502H offered a much wider particle size distribution ranging from 5 to 51 μm, which must be attributed to the strong tendency of the RG502H spheres to agglomerate upon collection on a filter and drying. Yields were generally comparable (95 – 99%) irrespective of the polymer type. For all newly introduced polymer types, BSA encapsulation was strongly decreased as compared to RG503H (76%) and followed the order RG503H > RG502H > RG503 > RG752, with the latter featuring even less than 50% efficiency.

III.3.6 BSA release from the microparticles

Six batches of microspheres, with two of them prepared under identical conditions, were further examined for their in vitro release behaviour. In all cases, a burst release within the first 24 h without significant subsequent release during the following 20 days was observed. For the two batches prepared under "standard" conditions, burst release was comparable with a value of 39% of the total dose (Fig. III.7). The increase in the extraction fluid flow from 600 to 1200 ml/h slightly increased the burst release of the yielded smaller particles (45%) whereas the increase in the flow rate ratio $\Phi$ from 1/50 to 1/12.5 decreased the burst release to 25%. The use of a more concentrated polymer solution ($w_p = 10\%$) markedly reduced (9%), and increasing the nominal drug load ($L_D = 15\%$) markedly increased (72%) the burst release as compared to the "standard" microspheres (39%).
Figure III.7. BSA release within the first 24h from various microsphere formulations as a fraction of total dose. (1) Two batches of the standard formulation (see table 1): $F = 600 \text{ ml/h}, \Phi = 1/50, w_p = 5\%, L_D = 5\%$. For batches 2 – 5, one process parameter was altered at a time as follows: (2) $F = 1200 \text{ ml/h}$; (3) $\Phi = 1/12.5$; (4) $w_p = 10\%$; (5) $L_D = 15\%$ ($w_D = 15\%$).

Furthermore, the same six batches of microspheres were examined for their morphology. Scanning electron micrographs in all cases revealed spherical particles with smooth surfaces and only a few small pores (Fig. III.8). In some cases crater-like dents were observed possibly caused by neighbouring particles during filtration. Darker spots possibly indicate pores situated closely below the microspheres' surface. The six batches virtually show the same surface morphology with the microspheres prepared from the more concentrated polymer solution exhibiting a slightly less and the microspheres with increased nominal drug load a slightly more porous surface.

III.4 Discussion

The microspheres produced by micromixing exhibited essentially monomodal size distributions. Nonetheless, although formed from equally sized
microchannels, the microspheres were far from being monodisperse. This finding as well as the fraction of fine particles observed in all batches might arise from inhomogeneous droplet formation due to turbulent flow sections in the mixer’s outlet as was previously postulated, based on the observation of a nonlinear pressure-loss in the micromixer as a function of volume flow [17]. In ex-
periments, the authors examined whether the fine particles resulted from fragmentation of nascent microspheres upon entering the collection bath. However, no evidence for such fragmentation was found.

The size of microdroplets produced by the micromixer basically depends on three parameters [15], i.e. (i) width of the microchannels, (ii) absolute flow rates of the fluids to be mixed and (iii) their flow rate ratio. By transition from the channel array to the outlet slit alternating lamellae of the two fluids are being formed, which exhibit periodical flow velocity gradients due to a parabolic velocity profile in the microchannels (Fig. III.2 d). These gradients cause the lamellae configuration to become unstable so that the lamellae of the one fluid transform into droplets, whereas those of the other fluid coalesce to form a continuous phase. The smaller the width of the microchannels, the smaller the lamellae and, thus, the theoretical droplet size. Furthermore, an increase in the flow rates while maintaining the flow rate ratio \( \Phi \) augments the velocity gradients and further destabilises the lamellae, thereby yielding smaller droplets. Finally, if the flow rate ratio is less than unity, i.e. one fluid is fed at lower flow rate than the other and, thus, enters the outlet slit at a lower velocity, hydrodynamic focussing occurs: Due to frictional forces, the fluid fed at the lower rate is accelerated resulting in a thinning of its lamellae and, consequently, in a reduction of particle size.

Contrary to findings on the dispersion of immiscible fluids [15], it was found that for the microsphere production the microchannel width did not significantly affect particle size. Of the four microsphere batches produced using the 25 \( \mu \)m tool, two exhibited similar and two only slightly smaller particle sizes than those obtained with the 40 \( \mu \)m tool. A possible explanation could be the low flow rate ratio \( \Phi \) of 1/50 chosen for these experiments, which may dominate the flow regime at the mixer’s outlet slit and renders the impact of the channel width secondary. However, in agreement with the above-mentioned dispersions of immiscible fluids [15], particle sizes deceased markedly with increasing extraction fluid flow rate \( F \), which may be used to control effectively the size distribution of the microspheres. Additionally, a slight but still significant increase in particle size was observed at an increased flow rate ratio \( \Phi \). However, this effect of
increased $\Phi$ can be easily counteracted by a moderate increase in $F$. An increase in $\Phi$ is of interest to improve the productivity of the process.

Importantly, the size distribution was found to be essentially unaffected by changes in the formulation of the microspheres like polymer solution concentration, polymer type and nominal BSA load. In the literature, the nominal drug load generally is not reported to have an impact on particle size. However, studies using standard stirred vessel techniques report increased microsphere sizes with increased polymer solution concentration [5,7,19] or enlarged inherent viscosity of the polymer [20]. This is explained by the increased viscosity of the respective organic solutions requiring higher shear forces for fine dispersion. This demand can be met by increasing the stirring rate, but the increment necessary to respond adequately to the increased viscosity is difficult to determine. In case of the micromixer, the used pumps are flow rate controlled and respond automatically to increased viscosities by increasing pressure. Therefore, the flow velocity gradients of the fluid lamellae formed in the micromixer remain unchanged and so do the microsphere sizes. A significant decrease in particle size was, however, observed when DCM was replaced by the more water-soluble EF. This might be explained by the decreased interfacial tension between water and EF as compared to water and DCM, which possibly gives rise to a reduction in droplet size [21]. Moreover, the impact of the interfacial tension between the two fluids and the mixer's material is not yet fully understood but strongly assumed to be of relevance [Hardt, S., IMM, 2001, Personal communication]. Further studies are ongoing to understand this phenomenon.

BSA encapsulation efficiencies were highly satisfactory, with values ranging between approximately 65 and 80% for RG503H dissolved in DCM. Batch-to-batch reproducibility of the encapsulation efficiency was less satisfactory, with standard deviations of 5 to 7.5%. Such variations, however, are not uncommon in literature [5,22]. Neither the extraction fluid flow rate $F$, nor the flow rate ratio $\Phi$ had any impact on the encapsulation efficiency. The variations among the different batches of these experiments were even smaller than those found for the repeatability experiments. The encapsulation efficiency was slightly improved at higher polymer solution concentrations, which can be attributed to the increased viscosity of the polymer solution and a faster formation of
a gel-like layer at the periphery of the microdroplets [7,22]. Both phenomena restrict the migration of the engulfed BSA towards the extraction fluid. Finally, the encapsulation efficiency increased considerably when the nominal drug load was increased from 5 to 10%, with no further marked improvement at L_D = 15%. Literature data on the dependence of encapsulation efficiency on nominal BSA load is quite inconsistent. Some authors report decreased efficiency at increased nominal drug load [7,22], others describe the opposite [5]. Several explanations are available to understand these observations: On the one hand, a higher nominal loading increases the concentration gradient between inner aqueous phase and the extraction fluid which would promote the loss of BSA. Conversely, as BSA features surface-active properties, a higher BSA load could stabilise the primary W/O emulsion, which would improve encapsulation [23]. Factors like the quality of the primary emulsion, use of surfactants and polymer solution concentration are further factors that may affect the efficiency of BSA encapsulation. When EF was used in exchange for DCM the encapsulation efficiency decreased markedly. This may be explained by the considerably larger miscibility of EF and water, which might promote diffusion of BSA from the inner aqueous phase to the extraction fluid.

The exchange of the end-group uncapped PLGA 50:50 (RG503H) for other PLGA types strongly decreased encapsulation efficiency. It is believed that a more hydrophilic polymer would lead to improved interactions between polymer and BSA [5]. From this point of view it seems logical, that the end-group capped PLGA 50:50 (RG503) and PLGA 75:25 (RG752) exhibited decreased encapsulation efficiencies as compared to the uncapped and thus more hydrophilic RG503H. Moreover, the lower encapsulation efficiency in PLGA 75:25 versus PLGA 50:50, both carrying capped end-groups, must be ascribed to the increased hydrophobicity of the former. On the other hand, the lower molecular weight end-group uncapped PLGA 50:50 (RG502H) was expected to yield higher encapsulation efficiency than its higher molecular weight analogue, because of the larger number of carboxylic end-groups per unit mass polymer. This expectation was, however, not fulfilled, which may be explained by the strong tendency of RG502H microspheres to agglomerate upon filtration and block the filter, so that the microspheres were substantially longer exposed to
the washing water. Under such conditions, a larger part of the BSA embedded just close to the surface of the microspheres may have been released into the washing water.

Finally, microsphere yield was basically unaffected by any variation in formulation or process parameters and constantly numbered approximately 90 - 100%.

The release profiles featuring a burst release within the first 24 h without subsequent release during the following 20 days were unsatisfactory. However, such profiles are not uncommon in literature [24,25] and attributed to non-covalent protein aggregation and protein adsorption on the polymer backbone. It was found that exposure of a protein to an air/liquid interface combined with high shear forces led to protein aggregation [26]. The dispersion of the aqueous BSA solution in the organic polymer solution by ultrasonication could have led to such aggregation due to the presence of high shear forces in addition to an oil/water interface.

The burst release of 39% of total dose found for the microspheres prepared under “standard” conditions is rather large. This might be attributed to pores possibly present closely under the smooth surface. These pores might be rapidly accessible to the release medium through degradation and mechanical disruption of the polymer cover. Thus a large surface area would be in contact with the release medium, giving rise to fast dissolution of BSA. The increase of the burst release to 45% for the smaller particles prepared at an extraction fluid flow of 1200 ml/h has to be attributed to the larger specific surface of the smaller particles. The reduction of the burst release to 25% upon increase of the flow rate ratio to 1/12.5 might be caused by the reduced concentration gradient between inner aqueous phase and extraction fluid during preparation of the particles as the protein/polymer dispersion per unit mass is in contact with less extraction fluid. A reduced gradient would retard the diffusion of BSA from the centre to the periphery of the solidifying microsphere where later on it would be easier accessible to the release medium. The markedly reduced burst release found for the 10% polymer solution is probably caused by its larger viscosity inhibiting the coalescence of the dispersed BSA solution droplets. Thus, BSA is more finely dispersed in the polymer matrix and less prone to dissolution
by the release medium. Moreover, the increased viscosity generally restricts the diffusion of BSA from the centre to the periphery of the solidifying microsphere. Finally, a strongly increased burst release was found for a nominal drug load of 15%. As much more BSA is present in the polymer matrix it is more likely that the BSA inclusions closely neighbour each other. Upon dissolution of the BSA close to the microspheres' surface further BSA situated deeper in the microspheres would be rapidly accessible to the release medium.

Among the various approaches to improve the control of microsphere size distribution in solvent extraction, extrusion through a glass membrane [13] and the vibrating nozzle technique [14] seem to be the most promising ones. However, the use of glass membranes [13] for the emulsification of protein/polymer dispersions into extraction fluid imposes some drawbacks. For instance, the size of the microspheres can only be controlled by the pore size of the membrane. The preparation of very fine particles necessitates glass membranes with equally small-sized pores, being highly susceptible to clogging upon contact with polymeric W/O-dispersions. Nevertheless, a clear advantage of the membrane extrusion technique lies in the very narrow size distributions that can be obtained.

The vibrating nozzle technique [14] offers a number of powerful features: Production of monodispersed microspheres, easily adjustable particle size, and feasibility within a wide range of materials and concentrations. It is believed that the static micromixer is a powerful alternative when monodispersity is not needed. The micromixer-based procedure is simple, cheap and space-saving. Moreover, as the fluids to be mixed can be perfectly contained within a closed installation, micromixing is ideally suited for the aseptic processing of particulate polymeric therapeutics embedding difficult to sterilise peptides, proteins, antigens or oligo- and polynucleotides. Furthermore, lack of moving parts and simple set-up simplify disinfection or sterilisation of the micromixer prior to processing.

Nevertheless, the micromixer offers potential for further improvements. Re-engineering of the micromixer's outlet section to avoid turbulent flow should reduce the span of the particle size distribution and prevent the formation of undesired fine particles. As particles below 5 \( \mu \text{m} \) or even in the sub-micron range
are desirable for a number of applications, re-engineering of the micromixer should strongly consider this objective. A test reaction performed in the micromixer indicated that improved mixing can be achieved by reduction of the mixer's outlet slit [17]. This measure might reduce the particle size, though maybe at the risk of higher particle polydispersity. A second measure would be the reduction of the channel width below 25 \( \mu \text{m} \). However, from the data it is questionable whether this will have a pronounced effect. Narrower channels may even render the mixer to become more susceptible to clogging. A further measure to reduce particle size and, especially, polydispersity might be the redesign of the zone of transition from the channel array to the outlet slit, replacing sharp edges by smoother geometries thus avoiding the creation of turbulent eddies.

III.5 Conclusions

In this work the potential of a static micromixer consisting of an array of microchannels was examined with respect to the encapsulation of proteins in PLGA microspheres by solvent extraction. Being small-sized, of simple set-up, suitable for continuous production and easy to sterilise, the micromixer seems to be ideally suited for automated and aseptic production. Scale-up is easily feasible through the employment of a sufficient number of mixers in parallel. The mixer proved to produce monomodal particle size distributions with excellent reproducibility, which were found to be very robust towards changes in the formulation of the microspheres. Moreover, the particle mean diameter could be varied in a considerable range by modulating the flow rate of the fluids to be mixed. High yields and encapsulation efficiencies were obtained which were unaffected by the modulation of the flow rates. Further research will be directed towards the production of microspheres in the low micron and sub-micron range as well as narrower size distributions.
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References


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Chapter IV

Flow-through ultrasonic emulsification combined with static micromixing for aseptic production of microspheres by solvent extraction

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IV.1 Introduction

Administration of biodegradable microspheres for drug and antigen delivery is primarily via the parenteral route. Thus, product sterility has to be assured. Standard laboratory procedures yield microbiologically contaminated particles [1,2]. Microbial contamination mostly originates from human cutaneous flora and non-sterilised equipment. To yield a sterile product, two approaches may be followed: (i) terminal sterilisation of the product or (ii) aseptic manufacturing.

The most common matrix materials used to formulate biodegradable microspheres for parenteral drug delivery are poly(lactic acid), PLA, and poly(lactic-co-glycolic acid), PLGA. PLA and PLGA microspheres cannot be sterilised by heat or steam, because of the low glass transition temperature of these polymers, while the use of ethylene oxide may result in toxic residues. Therefore, γ-irradiation remains the only acceptable sterilisation method. However, it has been shown that γ-irradiation causes radiolytic chain scission resulting in polymer molecular weight reduction, and thus alters drug release [1,2,3]. Moreover, damage to the active principle may occur [2]. As a result, aseptic microsphere manufacturing should be favoured over terminal sterilisation of PLA and PLGA microspheres.

Very commonly, microencapsulation is based on the so-called solvent extraction method. Lab-scale experiments are frequently performed in a simple beaker/stirrer set-up, which is not well suitable for aseptic manufacturing as it involves a series of manual operations and is entirely unsuitable for scaling-up [4,5]. Although a vast number of modified processes have been developed [6], only very few of them have been specifically designed for aseptic microsphere preparation, requiring specialised, cost-intensive equipment [7].

In this study, BSA-loaded PLGA microspheres were prepared by means of a W1/O/W2-double emulsion solvent extraction method [4]. A flow-through ultrasonic cell for the preparation of the primary W1/O emulsion was combined with a static micromixer for the preparation of the W1/O/W2-double emulsion. This equipment offers a number of advantages with respect to aseptic microsphere preparation, e.g., small scale, low complexity, moderate cost equipment and easy handling, cleaning and assembling. Therefore, the proposed technol-
ogy appears suitable for cost-efficient aseptic production of drug-loaded microspheres in amounts suitable, e.g., for use in clinical studies. The prepared microspheres were characterised with respect to particle size, drug loading and, for those prepared under aseptic conditions, for absence of microbial contamination.

IV.2 Materials and methods

IV.2.1 Materials

Uncapped, 35 kDa poly(lactic-co-glycolic acid), PLGA (Resomer® RG503H) was purchased from Boehringer-Ingelheim (Ingelheim, Germany). Bovine serum albumin (BSA, fraction V) was from Fluka (Buchs, Switzerland). Poly(vinyl alcohol) (PVA, Mowiol® 4-88) and polysorbate 20, used as dispersion stabilisers, were obtained from Kuraray Specialities (Frankfurt/M., Germany) and Hänseler (Herisau, Switzerland), respectively.

IV.2.2 Experimental set-up

BSA-loaded PLGA microspheres were prepared using a recently developed micromixer-based double-emulsion solvent extraction method [8]. The experimental set-up consisted of three serially connected subunits (Fig. IV.1). First, an aqueous BSA solution (W₁) and a solution of PLGA in an organic solvent (O), both fed by syringe pumps, were pre-mixed in a 1 ml rubber-sealed glass cell by means of a magnetic stirrer. Second, the resulting coarse W₁/O-emulsion was transported into a flow-through ultrasonic cell (Dmini250, Dr. Hielscher, Teltow, Germany; Fig. IV.2) for homogenisation. The homogenised emulsion was further transported to a 3 ml rubber-sealed glass receptacle used as expansion vessel to decouple the W₁/O-emulsification from the following process step. Third, the W₁/O-emulsion was further emulsified in an aqueous extraction fluid (W₂) using a static micromixer (SSIMMAg40T35, Institut für Mikrotechnik Mainz (IMM), Mainz, Germany; Fig. IV.3 a). The resulting W₁/O/W₂ double emulsion was collected in a glass vessel and stirred gently. Upon extraction of the polymer solvent into the extraction fluid W₂, solid microspheres with encapsulated protein were formed.
Figure IV.1. Schematic overview of the microsphere preparation process, consisting of three main steps: (i) $W_1/O$ pre-emulsification, (ii) $W_1/O$ emulsion homogenisation, and (iii) $W_1/O/W_2$ double emulsion formation. The numbers 1 to 6 indicate the sampling points for sterility testing (see text for details).
The working principle of the ultrasonic cell was described in detail elsewhere [9,10]. Briefly, the cell consists of a glass tube through which the emulsion is transported and which is shielded by a steel mantle. The steel mantle is excited by an attached 24 kHz-sonotrode (Fig. IV.2). The open space between the glass tube and steel mantle is filled with water pressurised to a minimum of 5 bar for transmission of the sound waves from the mantle to the glass tube. Inside the glass tube, cavitation occurs resulting in emulsion droplet break-up. The power consumption of the high frequency generator driving the ultrasonic transducer was recorded, and the sonication time was calculated from the volume of the glass tube (0.53 ml) divided by the emulsion flow rate.

The static micromixer for preparing the double emulsion is described in detail elsewhere [8]. In brief, the mixer consists, in essence, of an array of interdigitated 40 μm-wide channels, alternately fed from opposite sides with the two fluids to be mixed (Fig. IV.3 b). In the middle of the channel array, the two fluids are discharged through a 60 μm-wide outlet slit machined into the mixer housing’s cover plate, which yields a stream of alternating fluid micro-lamellae. The lamellae of the more slowly flowing fluid disintegrate into droplets
Figure IV.3. Static multilamination micromixer for the preparation of the \( W_1/O/W_2 \) double emulsion. (a) Mixer with dismantled inlay. The inlay contains an array of microchannels. (b) SEM close-up of the microchannel array. (c) Principle of droplet formation from fluid lamellae upon transition of two fluids from the channel array to the outlet slit in the mixer housing's top part. (a and b) with kind permission of the Institut für Mikrotechnik Mainz (IMM).
(Fig. IV.3 c) [8,12]. The size of the droplets can be readily controlled by varying the flow velocities of the two fluids [8].

**IV.2.3 Microsphere preparation**

PLGA was dissolved in dichloromethane (DCM) or ethyl formate (EF) at 6% (w/w) (O) and BSA in PBS at 5% (w/w) (W1). The two solutions were processed by the ultrasonic cell as described above. The portion of W1/O emulsion discharged from the ultrasonic cell during the first 5 min of processing was discarded to let the process equilibrate. Aqueous 0.5% (w/w) solutions of PVA or polysorbate 20 were used as extraction fluids (W2) for DCM and EF, respectively. Flow rates were set at 4, 45 and 360 ml/h for the W1-, O- and W2-phases, respectively. The dispersion of nascent microspheres discharged from the micromixer was collected in a glass vessel pre-filled with 500 ml of extraction fluid and stirred gently for 30 (EF) or 120 min (DCM) for further hardening. The microspheres were collected over a 0.2 μm pore-size filter, washed with 50 ml of de-ionised water and dried at 20 mbar and room temperature for 24 h. For each set of preparation parameters, three microsphere batches (400-500 mg/batch) were prepared and analysed.

**IV.2.4 Droplet size distribution of the W1/O emulsion**

Samples of the W1/O emulsions were taken from the expansion vessel placed between ultrasonic cell and micromixer, diluted 250-fold with DCM saturated with water, and analysed by laser light scattering (Mastersizer X, Malvern, Worcestershire, UK) using the Mie diffraction model. The refractive indices of the BSA solution and diluted polymer solution were approximated by those of water and DCM. The emulsion droplet size distributions are presented in the volume-weighted mode and characterised by the mean diameter calculated from the volume-moment average of the size distribution, D[4,3].

**IV.2.5 Microsphere size distribution**

Dispersions of the microspheres in the extraction fluid were analysed by laser light scattering (see above). Here, the Fraunhofer model was chosen for raw data analysis.
IV.2.6 Protein content in the microspheres

The BSA content in the microspheres was quantified as described elsewhere [8]. Briefly, samples of 40 mg of dried microspheres were dissolved in DCM. BSA was separated from the dissolved polymer by filtration over a 0.2 μm pore-size regenerated cellulose filter. After elution of the protein from the filter into PBS, the resulting solution was analysed fluorimetrically. The encapsulation efficiency was expressed as the ratio of the amount of BSA extracted from the microspheres to the amount of BSA employed for their preparation. Each microsphere batch was assayed in duplicate.

IV.2.7 Aseptic microsphere production and sterility testing

To evaluate the feasibility of the described technology for aseptic manufacturing, a number of microsphere batches were prepared under aseptic conditions and tested for sterility.

Syringes, pre-mixing cell, expansion vessel, glassware, tubing, as well as the dismantled micromixer and the glass tube of the ultrasonic cell (Fig. IV.2) were steam-sterilized, while the remaining parts of the equipment were surface-disinfected with 70% ethanol. The experimental set-up was set together in a laminar air-flow cabinet and finally purged with 70% ethanol.

The BSA and PLGA solutions were subject to sterile filtration over 0.2 μm cellulose acetate and PTFE filters, respectively, while the PVA and polysorbate solutions (extraction fluids) were steam-sterilised at 121°C and 2 bar for 15 min. For validation of the sterilisation methods, all solutions involved in the process were loaded with a defined bioburden of 1:1-mixtures of Bacillus subtilis and Candida albicans. Volumes of 10 ml of both BSA and PLGA solutions were separately loaded with $7 \times 10^6$ CFU/ml of the microbial mixture to achieve a bioburden of $10^7$ CFU/cm² for sterile filtration on 7 cm² cross-section membrane filters. Typically, 10 ml of PLGA solution was used to produce one microsphere batch. The extraction fluid was loaded with $10^6$ CFU per 100 ml of the microbial mixture. All contaminated solutions were found to be sterile after sterile filtration or steam sterilisation (for sterility testing method see below).

During microsphere production, sterility tests were performed with the BSA and PLGA solutions, the extraction fluid (Fig. IV.1, sampling points
to \( \circ \)), the \( W_1/O \) emulsion \( (\circ) \), the hardened microspheres \( (\circ) \) and the extraction fluid waste \( (\circ) \). Aliquots of the BSA solution (5 ml), the extraction fluid and extraction fluid waste (25 ml each) were mixed with casein-peptone-soypeptone (CASO) broth, incubated for 24 h at ambient conditions, filtered using a Milliflex 100 system (Millipore, Massachusetts, USA), and the filters incubated with tryptic soy broth (TSB). Incubation conditions were 30-35 °C, 14 days for bacterial growth, and 20-25 °C, 14 days for growth of yeast and moulds. The filters were checked daily for colony growth. The PLGA solution was tested by filtering first over a sterile 0.2 \( \mu m \) regenerated cellulose filter, then re-hydrating the filter \[13\], and incubating it in CASO broth for 24 h. Then the broth was further processed as described above. Samples of the \( W_1/O \)-emulsion were diluted (1:6, v/v) in sterile ethyl formate and processed in analogy to the PLGA solution. The microspheres dispersed in the extraction fluid were collected on a sterile 0.2 \( \mu m \) cellulose acetate filter. One portion of the collected particles was mixed with CASO broth, incubated for 24 h, and the broth/particle-mixture was further processed using the Millipore system as described above. Another portion was dissolved in 5 ml of sterile EF and processed further as described for the PLGA solution.

Positive controls were performed by direct incubation of the test bacteria and yeast (88 CFU of each) in TSB. The controls showed colony growth after three days of incubation. Sterility and control tests were carried out in triplicate.

IV.3 Results and discussion

IV.3.1 Process and microsphere characteristics

Pre-mixing of the BSA solution in the PLGA-solution produced aqueous droplets of a size of typically < 200 \( \mu m \) \[9\]. Pre-mixing was necessary to ensure that the composition of the \( W_1/O \)-feed entering the ultrasonic flow-through cell remained constant. Processing of the coarse pre-emulsion by the ultrasonic cell reduced the mean droplet size of the \( W_1/O \)-emulsion to 620 - 670 nm (Tab. IV.1). The droplet size distributions were monomodal and relatively narrow with negligible batch-to-batch variations (Fig. IV.4). The mean droplet size of the \( W_1/O \)-emulsion was 620 nm when the maximum sonication power of
30 W and a sonication time of approx. 40 s were used. The droplet size increased slightly to 670 nm when the sonication power was lowered to 23 W (75% of maximum) or the sonication time shortened to 23 s (Tab. IV.1). The latter was achievable by increasing the flow rates of the BSA- and PLGA-solutions by 2/3 from respectively 4 and 45 ml/h to 6.6 and 75 ml/h. Exchanging the polymer solvent DCM by EF did not influence the mean droplet size of the W/O emulsion (Tab. IV.1). Interestingly, the homogenisation of the W/O-emulsion in the ultrasonic flow-through cell operated at 30 W during 40 s was as efficient as a previously used batch-wise processing with a 20 kHz ultrasonic probe immersed in a 10 ml glass vessel and working at 50 W for 20 s [8], which yielded W/O-emulsion droplet sizes of approx. 600 nm (data not shown).

Figure IV.4. Size distributions of W/O emulsions prepared by the ultrasonic flow-through cell (•, n = 2) and of microspheres prepared from these emulsions employing the static micromixer (—, n = 3). For further preparation parameters see Tab. IV.1 (batch DC1).
Table IV.1. Characteristics of BSA-loaded microspheres prepared under different conditions of primary emulsion (W₁/O) and double emulsion (W₁/O/W₂) formation. Data are given as mean of three microsphere batches ± S.D.

<table>
<thead>
<tr>
<th>Batch ID</th>
<th>Sonication power</th>
<th>Sonication time</th>
<th>Polymer solvent</th>
<th>Mean W₁/O droplet diameter D[4.3] [μm]</th>
<th>Mean microsphere diameter D[4.3] [μm]</th>
<th>Theoret. drug loading [%]</th>
<th>Encapsulation efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC1</td>
<td>30</td>
<td>39</td>
<td>DCM</td>
<td>0.62 ± 0.05</td>
<td>36.4 ± 0.8</td>
<td>5.6</td>
<td>73.0 ± 3.6</td>
</tr>
<tr>
<td>DC2</td>
<td>23</td>
<td>39</td>
<td>DCM</td>
<td>0.67 ± 0.03</td>
<td>38.9 ± 1.7</td>
<td>5.6</td>
<td>72.5 ± 1.2</td>
</tr>
<tr>
<td>DC3</td>
<td>30</td>
<td>23</td>
<td>DCM</td>
<td>0.67 ± 0.05</td>
<td>30.9 ± 0.3</td>
<td>5.6</td>
<td>67.1 ± 2.5</td>
</tr>
<tr>
<td>EF1</td>
<td>30</td>
<td>40</td>
<td>EF</td>
<td>0.63 ± 0.03</td>
<td>14.8 ± 0.2</td>
<td>6.0</td>
<td>70.2 ± 3.7</td>
</tr>
</tbody>
</table>

Between the ultrasonic cell and the micromixer, an expansion vessel had to be installed to decouple the fluid processing in the ultrasonic cell, which occurred at ambient pressure, from the fluid processing in the micromixer, occurring at a slightly higher pressure due to the pressure drop in the mixer. An increased pressure during the ultrasonication of the W₁/O-emulsion reduced the homogeneity of the emulsion, because of less efficient cavitation. Enhanced pressure increases the threshold for the onset of cavitation, resulting in a decreased number of cavitation events per unit volume of emulsion [14].

Microsphere formation in the micromixer occurred through extruding the W₁/O-emulsion through an array of interdigitated microchannels that were fed from the opposite side by the extraction fluid, W₂, and further through a fine gap (the micromixer’s outlet slit). Specifically, W₁/O-droplets in the W₂-phase formed by friction forces exerted on the slowly flowing W₁/O-lamellae by the nine-fold faster flowing W₂-lamellae. This W₁/O-droplet formation was accompanied by simultaneous solvent extraction giving birth to microspheres of relatively broad size distribution (Fig. IV.4), as previously found in earlier experiments with this type of mixer [8]. Batch-to-batch reproducibility was highly satisfactory. Microsphere sizes were not affected by the slight changes of the W₁/O-emulsion.
droplet size upon reduction of the sonication power (Tab. IV.1). The reduction of the sonication time by increasing the flow rates of the W1- and O-solutions, in turn, necessitated to increase the flow-rate of the extraction fluid, W2, from 360 to 420 ml/h in order to maintain stable emulsification in the static micromixer, resulting in a decrease of the microsphere mean size from 36 μm to 31 μm. Microsphere sizes were reduced substantially, i.e., from above 36 to 15 μm when DCM was exchanged for EF. Both observations are in agreement with earlier experiments using this type of micromixer [8]. An explanation for the decreased microsphere size with the use of EF may be the lower interfacial tension between EF and the aqueous extraction fluid, which may have facilitated droplet break-up.

The microencapsulation of BSA by this continuous process yielded encapsulation efficiencies (approx. 70%; Tab. IV.1), which were similar to those obtained previously with the same micromixer in combination with a batch-mode preparation of the W1/O-emulsion using a standard ultrasonic probe immersed in a glass vessel [8]. The encapsulation efficiency was not significantly influenced by the small differences in the W1-droplet sizes (620-670 nm) of the primary W1/O-emulsion, nor by the exchange of polymer solvent, although EF produced much smaller microspheres than DCM. For similar experiments with EF, in which a standard ultrasonic probe and the micromixer were used, the attained encapsulation efficiency was only 60% and varied largely between the batches [8].

**IV.3.2 Aseptic production**

The combination of ultrasonic flow-through cell and static micromixer offers specific advantages for aseptic processing of drug-loaded microspheres. The entire production unit is small and fits readily into a laminar-flow cabinet or an isolator. The static micromixer consists basically of only three parts, which all can be heat-sterilised. The glass tube which conducts the W1/O emulsion can be dismantled from the ultrasonic cell and heat-sterilised, as all other tubing as well. Thanks to the low complexity of the equipment, the few sterilised parts can be readily assembled in a Class 100 containment. The unit allows for continuous production requiring minimum manual handling, which is a prerequisite for
cost-efficient aseptic production, and obviates contact between the processed materials and the environment.

None of the samples taken for sterility testing from the starting materials, intermediate and final products showed microbial contamination. For sterility testing of the microspheres, we firstly incubated them directly to check for surface contamination. The absence of surface contaminations was confirmed by sterility testing of the extraction fluid waste. Secondly, the microspheres were dissolved in EF, the obtained solutions sterile filtered, the filters re-hydrated and incubated to test for microorganisms entrapped in the core. Although the use of solvents like DCM for microsphere production could have reduced the risk of core contamination by killing vegetative forms of bacteria, yeasts and moulds, the more resistant spores could have survived this process [1,13,15].

In conclusion, the primary goal of this work was to obtain first information on the feasibility of aseptic microsphere preparation combining the flow-through ultrasonic cell and the multilamination micromixer. We are well aware that the sterility tests performed are by no means sufficient to validate product sterility on a GMP-level. Further work will have to include testing for anaerobe microorganisms. In addition, the method used for testing the sterility of the microsphere cores needs further validation work with respect to the microbial toxicity of EF, which was employed for the dissolution of the microspheres.

References


Chapter V

Ultrasonic atomisation into reduced pressure atmosphere – envisaging aseptic spray-drying for microencapsulation

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V.1 Introduction

The embedding of pharmaceutical compounds into bioerodible microspheres has gained considerable interest over the last decade as it opens the door to a variety of therapeutic applications. Drugs can be released from such microparticles in a sustained and controlled fashion, while non-released drug is protected from degradation [1]. Furthermore, microparticles may be surface-modified for targeting specific tissues or cells [2]. Various materials have been used for such microspheres, amongst them lipids, proteins and a vast number of biodegradable polymers. The most frequently used biodegradable polymers are the polyesters poly(lactic acid), PLA, and poly(lactic-co-glycolic acid), PLGA, as they are well tolerated and degrade to toxicologically harmless products.

Besides solvent extraction/evaporation and phase separation (coacervation), spray-drying is the method most frequently employed for microsphere preparation. Standard laboratory spray-dryers consist of a pneumatic spray nozzle, an aspirator coupled with a heating to provide hot air, a drying chamber, a system for recovering the dried particles and a system clearing the exhaust air, e.g., a bag filter. Separation of particles from the heat-providing and transporting stream of air commonly occurs in a cyclone, although filter systems may also be used. Both methods of particle separation incorporate specific drawbacks. Filtration requires continuous removal of the collected product from the filter in order to keep the pressure drop constant. A cyclone does not work equally efficient for different particle sizes and airflows. Especially particles of less than 5 μm in diameter are inefficiently separated so that a considerable part of the product may be lost with the exhaust air [3]. Moreover, small particles tend to deposit on the cyclone wall, which is typically caused by electrostatic interaction [4]. Nonetheless, variations in cyclone and receiver design, improvement in the exhaust air management by using a vacuum filter system and the use of dual- or anti-static cyclone set-ups may all contribute to reduced product loss [3]. Generally, product loss becomes less pronounced with larger batch sizes [5].

Aseptic microencapsulation is increasingly demanded because numerous biodegradable materials, such as PLA or PLGA, cannot be heat-sterilised
and sterilisation by gamma rays may harm the encapsulated drug and degrade the polymer [6]. However, aseptic preparation of microspheres by conventional spray-drying is difficult to achieve. The stream of drying air has to be passed through a sterilisation filter resulting in a considerable pressure drop, which might negatively affect the dryer's performance in terms of particle separation. Moreover, particles deposited on the cyclone wall might have to be recovered by washing [3,4] which is most delicate for aseptic production.

To make aseptic spray-drying more economic and feasible in laboratory environments, we developed a process consisting of an ultrasonic spray-head for the disruption of a solution into microdroplets, a vessel kept under reduced pressure for drying of the microdroplets and a bath of a non- or antisolvent for collection and if necessary final desolvation of the resulting particles. The velocity of the microdroplets ejected from an ultrasonic nozzle is one to two orders of magnitude smaller than in pressurised nozzles, so that the drying chamber can be of considerably shorter dimensions. Thus, the apparatus can be readily installed in a laminar flow cabinet or isolator. Reduced pressure as the driving force for the evaporation of the solvent from the microdroplets obviates the need for large quantities of sterile hot air. A cyclone or filter-system is not needed as the formed particles settle into the collection fluid, which can be comfortably withdrawn from the apparatus. Additionally, the collection bath can be used to surface-modify the particles, e.g. by adsorption of a substance dissolved in the bath.

Microsphere production using an ultrasonic spray-head equipped to a standard laboratory spray-dryer is reported in literature [5]. However, as the drying (warm air) and particle collection mechanisms remained unaltered, the system offers little benefit with respect to aseptic preparation. Furthermore, direct ultrasonic spraying into an antisolvent without previous drying is reported [7,8].

The aim of this study was to evaluate the potential of the novel spray-drying process for the preparation of protein-loaded PLA and PLGA microparticles. Bovine serum albumin (BSA) was chosen as model protein and the influence of various process and formulation parameters on the characteristics of the resulting microparticles was studied.
V.2 Materials and methods

V.2.1 Materials

End-group uncapped 14.6 kDa PLA (Resomer® R202H; molecular weights given are weight-averaged according to [9]), end-group uncapped 13.7 kDa and 35 kDa PLGA50:50 (Resomer® RG502H and RG503H) and end-group capped 41.2 kDa PLGA50:50 (Resomer® RG504) were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Bovine serum albumin (BSA, fraction V) was from Fluka (Buchs, Switzerland) and polysorbate-20 from Hänself (Herisau, Switzerland). Synthesis grade dichloromethane (DCM) and ethyl acetate (EA) were from EGT Chemie (Scharlau, Switzerland), ethyl formate (EF) was obtained from Fluka. Purified water was obtained from a NANOpure ultrapure water system (Barnstead, Dubuque, USA).

Figure V.1 a. Sketch of the vacuum spray-dryer.
V.2.2 Preparation of microspheres

Solutions of BSA in PBS (pH = 7.4) and of PLA / PLGA in EF, EA or DCM were prepared at various concentrations. BSA solution was added to 6-8 ml of polymer solution at specific ratios \( R \) (given as mg BSA solution per mg polymer solution) and finely dispersed for about 10 s at 50 W using a 20 kHz, \( \Omega6 \) mm ultrasound probe (VibraCell VC50T, sonics and materials, Danbury, USA). The nominal drug load of the microspheres, \( L_D \), i.e. the nominal amount of drug to be entrapped in the particles, \( m_D \), divided by the amount of polymer employed, \( m_p \), was calculated by:

\[
L_D = \frac{m_D}{m_p} = R \cdot \frac{w_D}{w_p} \tag{1}
\]
where \( w_D \) and \( w_P \) denote the weight fraction of the drug and the polymer in their respective solutions.

The protein/polymer dispersion was transferred into a 20 ml single-use syringe and by means of a syringe pump (syritol 12, Heinzerling Medizintechnik, Rotenburg a.d. Fulda, Germany) fed to a 100 kHz ultrasonic spray head (US1, Lechler, Metzingen, Germany) installed in a glass vessel (Fig. V.1). The vessel was kept under reduced pressure controlled precisely by a vacuum pump (LVS 610T ecoflex, Ilmvac, Ilmenau, Germany), to evaporate the solvent from the fine droplets formed by the ultrasonic head. A throttle valve was installed between syringe and spray head to prevent the protein/polymer dispersion from being uncontrollably drawn into the glass vessel. The resulting microspheres settled into 1.5 l of aqueous polysorbate-20 solution (0.5% w/w) filled into the bottom of the glass vessel for collection and harvest of the spheres. At the end of product atomisation, the microspheres were allowed to remain dispersed in the collection fluid for 5 min while the spray head was purged with pure solvent. The microsphere dispersion was then drained from the glass vessel and passed through a 90 \( \mu \)m sieve to remove potential large-scaled matter. The microspheres were separated from the fluid by filtration over a 0.2 \( \mu \)m pore size cellulose acetate filter (Sartorius, Göttingen, Germany), washed with 200 ml of deionised water, and finally dried at 20 mbar and room temperature for 24 h. All experiments were conducted in an air-conditioned room at 20 °C. For each formulation or set of parameters, two batches were produced.

The height of the glass vessel could be varied in two steps from 400 to 550 to 700 mm by adding cylindrical extensions to the main glass body (Fig. V.1 a) to adapt the residence time of the nascent microspheres in the low pressure atmosphere as desired. All described experiments were conducted in the 550 mm high version. Furthermore, the ultrasonic spray head was equipped with an inlet for purge air intake. The air leaves the spray head through an annular gap encircling the atomisation tip (Fig. V.1 b), thereby shaping the spray ejected from the tip to a 30° cone. The flow of air could be controlled by a needle valve. Finally, a steel capillary (0.4 mm inner diameter) was installed inside the feed tube of the spray head to reduce dead volume.
Chapter V

V.2.3 Particle size distribution

The particle size distribution was determined directly after preparation when the particles were still dispersed in the collection fluid and again after drying of the microspheres. In the latter case, the particles were re-dispersed in 0.5% (w/w) polysorbate-20 solution and subsequently treated with ultrasound for 5 s (6 mm probe, 20 kHz, 50 W). Microsphere size was determined by laser light scattering (Mastersizer X, Malvern, Worcestershire, UK, equipped with a 100 mm lens) using a Fraunhofer diffraction model for the analysis of the raw data. All size distributions are presented in the volume-weighted mode with the mean diameter $D[4,3]$ being calculated from the volume-moment average of the size distribution.

V.2.4 BSA encapsulation efficiency

Samples of approximately 40 mg of dried microspheres were accurately weighted and dissolved in 5 ml of DCM. BSA was separated from the dissolved polymer by filtration over a 0.2 μm pore-size regenerated cellulose filter (Sartorius, Göttingen, Germany). The filter was washed with additional 5 ml of DCM to remove remaining polymer. BSA was eluted from the filter into 10 ml of PBS by shaking for 3 h. The resulting solution was diluted to 25 ml and analysed fluorimetrically ($\lambda_{ex} = 278$ nm, $\lambda_{em} = 347$ nm; Cary Eclipse, Varian, Mulgrave, Australia). The encapsulation efficiency $\varepsilon$ was expressed as the ratio of the amount of BSA extracted from the microspheres to the amount of BSA employed for their preparation. Each microparticle batch was assayed in duplicate.

V.2.5 Scanning electron microscopy (SEM)

Samples of dried microspheres were placed on a double-sided adhesive tape and coated with 6 nm of platinum. The micrographs were taken on a Hitachi S-700 (Nissei Sangyo, Ratingen, Germany) electron microscope.

V.2.6 In vitro BSA release

For each formulation examined, samples of approximately 30 mg each were taken from two different microsphere batches. After their accurate weighting, the samples were added to glass vials containing 3 ml of PBS along with
0.02% (w/w) sodium azide and 0.05% (w/w) polysorbate 20. The vials were placed in an overhead shaker (Heidolph Reax 2, Heidolph, Schwabach, Germany) revolving at 60 rpm and incubated at 37 °C. At predefined time points, the vials were centrifuged at 3700 rpm for 5 min (sigma 204, Sigma, Osterode/Harz, Germany), and 1 ml of the clear supernatant was taken for fluorimetric assay as described above, while the missing volume was replaced by fresh buffer. The amount of BSA released was expressed as a fraction of the experimentally determined total BSA content. Each microsphere batch was assayed in duplicate.

V.3 Results and discussion

Six series of experiments were conducted to characterise the performance of the novel spray-drying process. In each series, one formulation or process parameter was varied while the remaining parameters were kept constant (Tab. V.1). To assess the reproducibility of microsphere characteristics for repeated production, nine identical batches were prepared choosing standard conditions as defined in Tab. V.1. The nine batches were analysed for

<table>
<thead>
<tr>
<th>Feed rate [ml/h]</th>
<th>Vessel pressure [mbar]</th>
<th>Atomisation power [% of max.]</th>
<th>Polymer solution conc. [%]</th>
<th>Nominal drug load [%]</th>
<th>Polymer solvent</th>
<th>Polymer type</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>30</td>
<td>700</td>
<td>30</td>
<td>5</td>
<td>EF</td>
<td>RG503H</td>
</tr>
<tr>
<td>1</td>
<td>30 - 120</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>*</td>
<td>500 - 900</td>
<td>30 - 90</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>3</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>5, 8</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>4</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>5, 15</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>various</td>
<td>*</td>
</tr>
<tr>
<td>6</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>various</td>
</tr>
</tbody>
</table>

Table V.1. Experimental design: extent of variation of process and formulation conditions. * stands for standard conditions as listed in the first row.
their particle size distribution and six of them further for yield and protein encapsulation.

**V.3.1 Product yield**

For the batches produced to assess repeatability, the yield amounted to 83.7 ± 4.7% (Fig. V.2 a). Repeatability of the production was satisfactorily reflected by the moderate standard deviation. Yields above 80% by far exceed values usually obtained in conventional spray-drying equipment for microsphere batches as small as those prepared here (200 - 300 mg), with values reported in the literature amounting to 35 - 60% [4,10,11]. In conventional spray-drying equipment, product is mainly lost through the exhaust air and by adherence to the cyclone wall. Therefore, the exchange of the pressurised nozzle of a conventional spray-dryer by an ultrasonic spray-head did not improve the yield [5].

In the novel spray-dryer some product is lost through particle attachment to the vessel wall and via the vacuum system. However, owing to the narrow-angled spray-cone of the ultrasonic spray-head and the lack of significant air movement in the dryer, only a very small fraction of microspheres actually came into contact with the vessel. Moreover, as only a few litres per hour of air and evaporated polymer solvent were withdrawn by the vacuum pump, only very few particles could have escaped by this way.

Vessel pressure and fluid feed rate did not affect product yield (Fig. V.3), which for all experiments amounted to 80 - 85%. However, feed rates could only be varied within a rather narrow range; an unlimited increase in the feed rate will not be feasible. Under controlled conditions, the liquid leaving the spray-head's feed capillary spreads on the atomising tip (Fig. V.1 b) and forms a thin film. Capillary waves are formed on this film excited by the tip's oscillation. The waves become unstable and their crests are ejected as fine droplets [12,13]. Uncontrolled atomisation occurs when the feed rate becomes too high to allow for the fluid to evenly spread on the atomisation tip, leading to the formation of large-scaled, undefined droplets.

Excessive decrease of the vessel pressure causes rapid solvent evaporation prior to atomisation, so that the solution may become too viscous
Figure V.2. Repeatability of microsphere preparation (a,b) and microsphere recovery (c). a: Product yield $Y$ (●) and BSA encapsulation efficiency $\varepsilon$ (●) of six microsphere batches prepared under identical conditions (standard conditions, Table V.1). The horizontal lines represent the mean values of $Y$ and $\varepsilon$; error bars for $\varepsilon$ are for repeated analysis ($n = 2$). b: Particle size distribution of the six batches detailed in panel A plus a further three prepared identically (but not analysed with respect to $Y$ and $\varepsilon$). c: Particle size distribution of a batch (prepared at standard conditions), measured once directly in the collection fluid and once after drying and re-dispersion.
Figure V.3. Product yield Y (●), BSA encapsulation efficiency ε (●) and mean particle diameter D[4,3] (○) as a function of vessel pressure p (a) and feed rate F (b). All other conditions were standard conditions (Table V.1). Error bars are for batch-to-batch variability (n = 2).
Table V.2. Influence of atomisation power on microsphere characteristics. Values are
given as mean of two microsphere batches ± error of the mean. First row gives data for
standard conditions (see Table V.1; n = 6, mean ± S.E.M.).

<table>
<thead>
<tr>
<th>Atomisation power</th>
<th>Product yield</th>
<th>BSA encapsulation efficiency</th>
<th>Microsphere mean diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>[W (% of maximum)]</td>
<td>[%]</td>
<td>[%]</td>
<td>[μm]</td>
</tr>
<tr>
<td>2.4 (30)</td>
<td>83.7 ± 4.7</td>
<td>59.5 ± 4.9</td>
<td>18.25 ± 1.05</td>
</tr>
<tr>
<td>4.8 (60)</td>
<td>72.8 ± 9.8</td>
<td>54.2 ± 13.7</td>
<td>18.62 ± 1.18</td>
</tr>
<tr>
<td>7.2 (90)</td>
<td>50.4 ± 5.7</td>
<td>54.8 ± 4.0</td>
<td>17.54 ± 0.64</td>
</tr>
</tbody>
</table>

for spreading and defined spraying. Within the limits mentioned above, an in¬
creased feed rate counteracts excessive solvent evaporation. In addition, with
decreased vessel pressure the risk for vapour bubble formation in the feed tube
increases, resulting in unstable feed conditions and uncontrolled spraying. The
latter effect was observed when the low feed capillary was de-installed and the
spray head’s dead volume thereby increased.

Table V.3. Influence of nominal drug load and polymer (RG503H) solution concentra¬
tion on microsphere characteristics. Values are given as mean of two microsphere
batches ± error of the mean. First row gives data for standard conditions (see Table
V.1; n = 6, mean ± S.E.M.).

<table>
<thead>
<tr>
<th>Nominal drug load</th>
<th>Polymer solution concentration</th>
<th>Product yield</th>
<th>BSA encaps. efficiency</th>
<th>Microsphere mean diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>[%]</td>
<td>[%]</td>
<td>[%]</td>
<td>[%]</td>
<td>[μm]</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>83.7 ± 4.7</td>
<td>59.5 ± 4.9</td>
<td>18.25 ± 1.05</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>81.4 ± 1.0</td>
<td>31.5 ± 0.3</td>
<td>17.49 ± 0.23</td>
</tr>
<tr>
<td>15</td>
<td>8*</td>
<td>83.8 ± 1.7</td>
<td>54.9 ± 1.8</td>
<td>29.16 ± 1.95</td>
</tr>
</tbody>
</table>

* For the 8% (w/w) polymer solution, the feed rate was increased from 30 to 60 ml/h.
Table V.4. Influence of polymer type and polymer solvent on microsphere characteristics. Values are given as mean of two microsphere batches ± error of the mean. First row gives data for standard conditions (see Table V.1; n = 6, mean ± S.E.M.).

<table>
<thead>
<tr>
<th>Polymer type</th>
<th>Polymer solvent</th>
<th>Product yield [%]</th>
<th>BSA encaps. efficiency [%]</th>
<th>Microsphere mean diameter D[4,3] [μm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG503H</td>
<td>EF</td>
<td>83.7 ± 4.7</td>
<td>59.5 ± 4.9</td>
<td>18.25 ± 1.05</td>
</tr>
<tr>
<td>RG503H</td>
<td>EA</td>
<td>81.9 ± 1.4</td>
<td>60.2 ± 1.1</td>
<td>15.08 ± 0.81</td>
</tr>
<tr>
<td>RG503H</td>
<td>DCM</td>
<td>74.1 ± 0.1</td>
<td>50.8 ± 1.6</td>
<td>23.91 ± 0.37</td>
</tr>
<tr>
<td>RG502H</td>
<td>EF</td>
<td>84.4 ± 0.3</td>
<td>74.0 ± 1.5</td>
<td>12.74 ± 0.18</td>
</tr>
<tr>
<td>R202H</td>
<td>EF</td>
<td>68.5 ± 1.0</td>
<td>36.6 ± 2.0</td>
<td>13.45 ± 0.37</td>
</tr>
<tr>
<td>RG504</td>
<td>EF</td>
<td>84.6 ± 0.5</td>
<td>27.5 ± 2.2</td>
<td>20.67 ± 0.41</td>
</tr>
</tbody>
</table>

Product yield decreased from above 80% to approximately 50% when the relative atomisation power was increased from 30 to 90% (Tab. V.2). It seems that the increased oscillation amplitude impaired the spreading of the fluid on the atomisation tip of the spray-head. At 60 and 90% relative atomisation power, large scaled droplets were ejected from the spray-head and formed a film on the surface of the collection fluid, which disintegrated into large flakes. The flaky matter was removed by a 90 μm sieve prior to yield determination.

Increase of the nominal drug load from 5 to 15% did not affect product yield (Tab. V.3). When the concentration of the polymer solution was increased from 5 to 8%, the feed rate had to be increased from the standard 30 to 60 ml/h to achieve effective atomisation and a yield of approximately 84% was obtained (Tab. V.3). At 30 ml/h, pre-atomisation solvent evaporation led to a pronounced increase in the solution’s viscosity and spray head malfunction.

The polymer and solvent type generally exerted no significant influence on product yield, except for the particles made from RG503H dissolved in DCM (74%) and those from R202H in EF (68%, Tab. V.4). In case of RG503H in DCM, the high vapour pressure of the solvent might again have resulted in pre-
atomisation evaporation, leading to uncontrolled atomisation. In fact, flaky material of >90 \( \mu m \) was observed.

**V.3.2 Microsphere size and morphology**

The reproducibility of the particle size distribution for repeated production was highly satisfactory. For nine batches of microspheres prepared under identical conditions, the size distribution curves were virtually superimposable (Fig. V.2 b). The mean diameter averaged 18.25 ± 1.05 \( \mu m \). According to the 10 and 90% undersize diameters, the majority of the particles ranged between 5 and 35 \( \mu m \). Generally, given a fixed liquid, the droplet size produced by an ultrasonic spray-head is a function of the exciting frequency [12]. Thus, using a spray-head with a higher frequency, smaller droplets and therefore particles could be produced.

Particle size distributions were identical before and after harvesting and drying of the particles irrespective of the process and formulation conditions employed (Fig. V.2 c). In fact, only the microspheres prepared of the polymer RG502H exhibited agglomeration upon harvesting and drying. This finding, already observed in a different study [14], might be attributable to water uptake by the comparatively polar polymer, leading to particle softening and cohesion.

Atomisation power did not have significant influence on particle size (Tab. V.2). With increasing vessel pressure, the size distribution shifted slightly but steadily to smaller particle sizes, i.e. from a mean diameter of ~23 \( \mu m \) at 500 mbar to ~16 \( \mu m \) at 900 mbar (Fig. V.3 a). The droplets ejected from the spray-head will shrink upon solvent evaporation until a solid layer forms at the surface, determining the solidifying particle's size. Further solvent removal creates pores inside the particles but will not significantly alter their dimension. As solvent evaporation is slower at higher pressure, the solid layer formation is delayed and particle shrinkage more extended.

With increasing feed rate, a significant shift of the particle size distribution to larger sizes was observed (Fig. V.3 b). The mean particle diameter increased from ~18 \( \mu m \) at 30 ml/h to ~32 \( \mu m \) at 120 ml/h. With increasing feed rate, collision and subsequent coalescence of droplets in close vicinity of the atomising tip becomes more likely, yielding larger particles. Collision between droplets
might be diminished by purging the spray-head with air (Fig. V.1 b), so that the
droplets are carried away from the atomising tip more rapidly. Actually, with air
intake, the mean particle size at $F = 30$ ml/h was reduced from $\sim 18$ to
$\sim 13.5$ $\mu$m, however at the cost of a reduced yield, amounting to 67% instead of
more than 80% found without air intake. The additional volume of air that has to
be withdrawn by the vacuum pump causes a greater loss of particles via the
exhaust air. In a study where the same type of ultrasonic atomiser was installed
in a standard spray-dryer for the production of BSA-loaded microspheres [5],
much smaller particles of approximately 10 $\mu$m mean diameter were found at
comparable experimental conditions (RG503 in DCM, $w_p = 5\%$, $F = 120$ ml/h).
This might partly be due to the massive air-flow used in their study.

Drug load did not affect particle size while an increase in polymer solu-
tion concentration from 5 to 8% increased the mean particle size from $\sim 18$ to
$\sim 29$ $\mu$m (Tab. V.3). Larger particle sizes for more concentrated polymer solu-
tions are reported both for conventional [11] as well as ultrasonic spray-drying
[5], which has to be attributed to the increased viscosity of the more concen-
trated solutions. Moreover, for the 8% polymer solution, a bimodal particle size
distribution was obtained; besides the main peak, a small fraction of smaller
particles (1 - 5 $\mu$m) was observed (not shown). We assume that for the more
viscous solution, droplet emission from the capillary waves was accompanied
by the formation of so-called satellite droplets.

When the polymer solvent EF was exchanged by EA or DCM the mean
particle size was slightly lowered ($\sim 15$ $\mu$m) by EA, but increased ($\sim 24$ $\mu$m) by
DCM (Tab. V.4). For the 5% solution in DCM, a bimodal particle size distribution
with a minor peak between 1 and 5 $\mu$m was observed (not shown). A possible
explanation for the increase in mean particle diameter with decreasing boiling
point of the polymer solvents ($T_b = 77$, 54 and 40°C for EA, EF and DCM, re-
spectively) could be a more rapid solidification of the microparticles' surface,
which might lead to larger particles as detailed above. Moreover, a more pro-
nounced evaporation of the solvent prior to atomisation increases the polymer
solution viscosity. Finally, the surface tension and density of the liquid do also
have an impact on the mean droplet size produced by an ultrasonic atomiser
[12]. The bimodal size distribution observed with DCM might again be ascribed
to increased solution viscosity due to assumed pre-atomisation solvent evaporation, resulting in satellite droplets.

Figure V.4. Dependence of the particle size distribution on the polymer type: R202H (-- -), RG502H (-----), RG504 (——) and RG503H (—). All other conditions were standard conditions (Table V.1).

When the polymer RG503H was exchanged by the moderately higher molecular weight RG504, a slight increase in the mean particle diameter (Tab. V.4) but almost no change in particle size distribution (Fig. V.4) was found. However, the low molecular weight polymers RG502H and R202H yielded smaller mean particle diameters of about 13 µm (Tab. V.4) and a narrow size distribution (approximately 6 to 23 µm, according to the 10 and 90% undersize diameters; Fig. V.4). As the molecular weight of RG502H and R202H is only half of that of RG503H, the reduced viscosity of their solutions might explain the observed decrease in particle size.
Scanning electron microscopy revealed that the particles produced by the new method were of spherical shape, but exhibited numerous surface pores (Fig. V.5 a) and a very porous interior (Fig. V.5 b). Particle porosity may be caused by fast evaporation of the solvent from the droplets as described above. However, no significant influence of the vessel pressure on particle porosity could be observed. Atomisation power and feed rate did not influence morphology and porosity either (pictures not shown). Generally, less diluted polymer solutions and smaller droplet sizes may reduce porosity.

Figure V.5. SEM micrographs of the microspheres. (a): Surface pores. (b): Porous interior. (a) made of RG503H, (b) made of RG502H; all other conditions were standard conditions (Table V.1).

V.3.3 BSA encapsulation efficiency

For the microsphere batches produced under identical conditions, the mean BSA encapsulation efficiency was 59.5% and the standard error of the mean 4.9% (Fig. V.2 a). This contrasts to the 80-90% BSA encapsulation efficiency reported for ultrasonic atomisation into a flow of warm air [5]. The moderate encapsulation efficiency for BSA produced by our new spray-drying method is likely due to BSA loss into the aqueous solution wherein the particles
were collected. Such loss may be reduced when a non-solvent for the compound to be encapsulated is used as collection fluid, which is subject of ongoing studies.

The process parameters of vessel pressure, feed rate (Fig. V.3) and atomisation power (Tab. V.2) did not significantly affect the BSA encapsulation efficiency, which generally varied between 50 and 60%.

When the nominal BSA load of the microparticles was increased from 5% to 15%, the encapsulation efficiency dropped from ~60% to ~32% (Tab. V.3). As detailed above, loss of BSA will mainly occur through partitioning into the aqueous collection bath, which is more pronounced for higher nominal loadings due to the higher concentration gradient between the microparticles and the surrounding fluid. However, when microparticles with a nominal drug load of 15% were produced from an 8% rather than 5% polymer solution, the encapsulation efficiency re-increased to approximately 55% (Tab. V.3). A more concentrated polymer solution may produce less porous particles and the higher viscosity of the solution will limit BSA droplet coalescence in the emulsion sprayed, both limiting BSA losses.

The use of EA instead of EF as polymer solvent did not affect BSA encapsulation (~60%), whereas DCM reduced the encapsulation efficiency (~51%, Tab. V.4). The different polymer types (dissolved in EF) affected BSA encapsulation efficiency in the following order: RG502H > RG503H > R202H > RG504, with a satisfactory 74% efficiency found for RG502H (Tab. V.4). This order correlates with the polarity of the polymers, which seems reasonable as increased polymer polarity affords stronger protein-polymer interactions thereby improving protein encapsulation [15].

V.3.4 In vitro BSA release

For all batches of microspheres prepared so far, the burst release (BSA release within the first 24 h) was unacceptably high exceeding 50% of total dose. The strongly porous particles may have readily taken up water from the release buffer, resulting in quick dissolution of the encapsulated BSA [16,17]. Atomisation power, feed rate and vessel pressure as well as the solvent type
Figure V.6. BSA release from the microspheres. (a): 24 h burst release dependent on polymer solution concentration \( w_P \) and nominal drug load \( L_D \). At least two batches of each microsphere formulation were examined, represented by the double/triple bars; error bars are for repeated analysis (\( n = 2 \)). (b): BSA release from microspheres prepared at \( w_P = 5\% \) (▲) and \( w_P = 8\% \) (●); all other conditions were standard conditions (Table V.1). Error bars are for batch-to-batch variability (\( n = 2 \)).
did not significantly influence BSA burst (data not shown) like they did not affect porosity. Increasing nominal drug $L_D$ from 5% to 15% augmented the average burst release from $\sim58\%$ to $\sim68\%$, while an increase in the polymer solution concentration $w_P$ from 5% to 8% (at $L_D = 15\%$) re-decreased the average burst to approximately 56% (Fig. V.6 a). Therefore, three additional microsphere batches were produced at $w_P = 8\%$ and the standard $L_D = 5\%$. For these batches, average BSA burst release strongly diminished to approximately 16% (Fig. V.6 a). The particles made of the more concentrated polymer solutions will feature less pores, thereby limiting instantaneous BSA dissolution.

The microsphere batches exhibiting high burst releases showed almost no further release of BSA during the following 21 days (Fig. V.6 b). Such release profiles are not uncommon in literature [18,19] and attributed to non-covalent protein aggregation [18] and protein adsorption on the polymer backbone [20]. It was found that protein exposure to oil/water or air/water interfaces combined with high shear force led to protein aggregation [21,22]. In our experiments, the dispersion of the aqueous BSA solution in the organic polymer solution by ultrasonication could have led to such aggregation. Milder dispersion conditions (e.g. reduced ultrasonic intensity) and the use of stabilisers [23] may reduce the detrimental effect.

The microspheres prepared at $w_P = 8\%$ and $L_D = 5\%$ showed a slightly more sustained release from a mean 16.1% of total BSA dose released on day 1 to a mean of 22.3% released on day 21. The higher viscosity of the more concentrated polymer solution might have reduced the shear forces acting upon the protein during ultrasonic dispersion.

V.4 Conclusions

In this work, ultrasonic atomisation into reduced pressure atmosphere was evaluated for the encapsulation of the model protein BSA in PLGA microspheres. Simple particle recovery and vacuum instead of hot air for drying the particles makes this method well suited for aseptic microsphere preparation as compared to conventional spray-drying. High particle yields but solely moderate BSA encapsulation efficiencies were obtained. The majority of particles was
very porous and exhibited an unacceptably high BSA burst release. However, 24 h bursts of particles prepared from more concentrated polymer solutions were strongly decreased. Further research will be directed at non-aqueous collection fluids to improve encapsulation of water-soluble compounds and to smaller and less porous particles.

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References


Discussion and outlook

The objective of the present work was to develop microencapsulation processes that are well suited for (i) the aseptic preparation of drug-loaded biodegradable microspheres, typically made from PL(G)A, and (ii) the scaling-up of the laboratory process to a pilot-scale process. The second aspect was not further investigated in the context of this study, because it would have caused relatively large investment costs; nonetheless, scaling-up was taken into consideration in the design of the new processes. At the beginning of the processes design, some critical criteria for enabling aseptic manufacturing were defined. They included:

(i) avoidance of open vessels to isolate the processed materials from the environment
(ii) feasibility of continuous processing and minimisation of manual manipulation
(iii) use of simple, sterilisable and easy-to-clean equipment
(iv) avoidance of warm drying air and pressurised air for liquid atomisation (as needed in conventional spray-drying)

Various process and formulation parameters were studied in the developed processes, and their influence on microsphere size distribution, morphology, protein loading and protein release were assessed. Bovine serum albumin encapsulated in PL(G)A was employed as a model system. The developed processes included: (i) a contact-free continuous emulsification in a novel ultrasonic flow-through cell for preparing primary W/O-emulsions that can be further processed into microspheres, or for preparing O/W-emulsions for direct fabrication of nanoparticles; (ii) a solvent extraction process for microsphere preparation that is based on micro-scale flow in a static micromixer; (iii) an ultrasonic atomisation process into low pressure atmosphere for microsphere preparation by spray-drying.

The continuous ultrasonic flow-through cell proved to be well suited to prepare both oil-in-water and water-in-oil emulsions. The droplet size of the disperse phase was effectively reduced by two or more orders of magnitude. The reproducibility of the resulting droplet size distribution was highly satisfactory. Emulsions of a BSA solution in a solution of PLGA prepared by the flow-through
ultrasonic cell were successfully further processed into BSA-loaded microspheres. Polymeric nanoparticles were prepared by oil-in-water emulsification and subsequent solvent extraction. However, not being the main focus of this thesis, this method of nanoparticle preparation was not further investigated, although various features appear highly attractive for further exploration such as the avoidance of PVA for use as stabiliser, the possibility to entrap proteins and other drugs, the feasibility to obtain sterile nanoparticles or the processing of other materials such as lipids or other polymers.

The particular benefit of this method to produce emulsions resides in the contact-free use of ultrasound. High-shear homogenisers of the Polytron®-type are difficult to clean and sterilise and thus constitute an important source of product contamination. Direct sonication by immersion of a sonotrode in the emulsion may lead to the emission of metallic particles or ions by action of cavitation. Moreover, both of these set-ups are inconvenient for fully segregating the processed materials from the environment. In contrast, the here developed flow-through ultrasonic cell features contact-free action and simple exchange and sterilisation of the parts in contact with the product, which both strongly facilitate aseptic processing.

The present flow-through cell has its limitations with respect to the power transferred to the emulsion. Scaling-up the process by installation of a manifold of parallel glass tubes in the cell would probably improve largely the energetic efficiency, as the ratio of energy expended for exciting the apparatus to that actually resulting in emulsification would be enhanced. However, it would be desirable to improve also the absolute power input to yield even finer emulsions.

BSA-loaded PL(G)A microspheres were reproducibly prepared employing the IMM multilamination micromixer. The mixer offers comfortable control of the microsphere size by simply varying the flow rates of extraction fluid and polymer solution and robustness towards changes in microsphere formulation parameters, which leaves size distributions essentially unaffected. Scale-up should be easily achievable by using a sufficient number of mixers in parallel ("number-up"). The mixer's small size and simple design facilitate cleaning, sterilisation and assembling, which makes the mixer attractive for aseptic microsphere preparation. We were able to demonstrate that the combined used of
the ultrasonic flow-through cell and micromixer yielded microspheres that passed a simplified sterility test.

The IMM micromixer wins over conventional vessel/stirrer set-ups, which offer poor control of microsphere size. Moreover, scale-up often is delicate and mostly achievable only via trial and error. Classical tube-installed static mixers are comparable to the micromixer in terms of microsphere size control and scalability, but they are difficult to clean and thus not well suited for aseptic processing. Extrusion through microdevices or membranes offers much better control of the microsphere size than the IMM micromixer, but suffer generally from low productivity as very slow flow velocities are required. Further, cleaning and sterilisation of extrusion-operated microdevices is difficult, because of the very small size and complex geometries.

The most significant drawback in using the IMM micromixer for microsphere preparation resides in the considerable polydispersity of the resulting particles, despite the seemingly well-defined, laminar flow regime inside the mixer. This may possibly be ascribed to non-optimal design of the mixer's outlet slit that exhibits an abrupt transition from a wide slit at the level of the channel array to a very fine tube towards the mixer's outlet. This transition is likely to transform the initially well-defined fluid lamellae into a turbulent chaotic flow.

Ultrasonic atomisation into a reduced pressure atmosphere allowed us to reproducibly prepare BSA-loaded microspheres, which, however, were rather polydisperse. The use of rather concentrated polymer solutions is required to yield particles of acceptable porosity and burst release. Very satisfactory product yields were obtained, even for very small batch sizes, exceeding by far those found with conventional spray-drying.

The novel spray-drying process offers considerable advantages over conventional spray-drying using warm-air and pressurized air-operated nozzles, if aseptic preparation is considered. The novel procedure obviates the need to sterilise large streams of warm drying air. The gentle spray produced by the ultrasonic atomiser allows the drying microspheres to settle slowly into a liquid bath, which can be comfortably withdrawn from the apparatus. Therefore, microsphere harvesting requires only minimum manual interference while conventional spray-drying often requires manual rinsing of the equipment to recover
sufficient amounts of the microspheres, which is unacceptable for aseptic processing.

Ultrasonic atomisation into a reduced pressure atmosphere tended to produce rather porous microspheres, which could be improved by using more concentrated polymer solutions and suitable polymer solvents. The choice of the collection fluid has to be done with care to avoid dissolution and loss of entrapped material. Finally, the mean particle size can only be controlled by the atomiser's operating frequency.

In summary, the novel technologies developed in the present study represent promising approaches towards aseptic production of drug-loaded biodegradable microspheres. However, the sterility testing performed in this study is by no means sufficient to ensure product sterility on a GMP-level. Especially, the method to dissolve/disintegrate the microspheres to get access to possible encapsulated microbial contaminants would require thorough validation to exclude erroneously positive sterility results by harming the entrapped microbes. The encapsulation of peptides and proteins necessitates stabilisation measures to protect them from the harsh conditions during microsphere preparation and degradation (aqueous-organic interfaces combined with shear forces, pH-change upon degradation) and prevent their adsorption to the matrix-forming polymer. Like in many other studies reported in literature, incomplete protein release from the microspheres was observed in the present study and ascribed to protein adsorption and aggregation. However, the required stabilisation measures do not solely depend on the processing conditions, but also strongly on the nature of the encapsulated compound, matrix material(s), microsphere formulation and solvent type. Thus, any stabilisation measure requires case-specific optimisation. Defining stabilisation strategies for the newly developed techniques was therefore beyond the scope of this work.
Curriculum Vitae

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Graduation from high-school "Johannes-Althusius-Gymnasium", Emden, Germany

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Publications


**Patents**

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