Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
European Journal of Pharmaceutics and Biopharmaceutics 61 (2005) 181–187
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

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

Sergio Freitas, Beat Rudolf, Hans P. Merkle, Bruno Gander*

Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH), ETH Hönggerberg, Zürich, Switzerland

Received 23 December 2004; accepted in revised form 4 May 2005
Available online 11 July 2005

Abstract

Final sterilisation of drug-loaded polymeric microspheres is problematic as dry heat or steam sterilisation are not applicable, and γ-irradiation may result in radiolytic scission of the polymer chains, and potentially damage the bioactive compound. Therefore, aseptic production is the method of choice to obtain a sterile product. A novel process for the production of microspheres is introduced based on the principle of double emulsion–solvent extraction. The process uses a flow-through ultrasonic cell for the preparation of the primary emulsion, in combination with a static micromixer for the production of the double emulsion. Because of its small scale, the equipment is readily accommodated in a laminar air-flow cabinet or an isolator. Thanks to the low technical complexity and easy handling of the process, only minimal manual interventions is required. Finally, the possibility for in-place cleaning and sterilisation makes the equipment and process well suited for aseptic microsphere preparation. Microspheres were prepared from poly(lactic-co-glycolic acid) (PLGA), and bovine serum albumin (BSA) served as model protein for microencapsulation. The BSA-in-PLGA (w/o) emulsions produced by the ultrasonic flow-through cell exhibited mean droplet sizes of <700 nm. 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. The presented technology offers great potential for aseptic microsphere production for batch-sizes suitable, e.g. for clinical investigations. Complete validation of product sterility would, however, demand more extended tests.

Keywords: PLGA microspheres; Solvent extraction; Ultrasonic emulsification; Flow-through sonication; Static micromixer; Aseptic processing

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 dry 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 [1–3]. 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 unsuitable for scaling-up and aseptic manufacturing as it involves a series of manual interventions [4,5].

* Corresponding author. Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH), ETH Hönggerberg, Wolfgang-Pauli-Strasse 10, 8093 Zürich, Switzerland. Tel.: +41 44 6337312; fax: +41 44 6331314.
E-mail address: bruno.gander@pharma.ethz.ch (B. Gander).

0939-6411/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2005.05.004

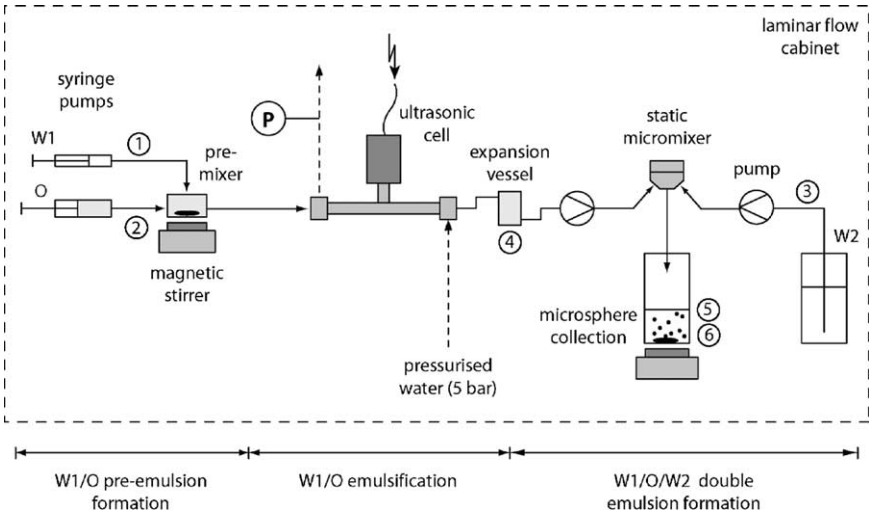

Fig. 1. Schematic overview of the microsphere preparation process, consisting of three main steps: (i) W_1/O pre-emulsification, (ii) W_1/O emulsion homogenization, and (iii) $W_1/O/W_2$ double emulsion formation. The numbers (1)–(6) indicate the sampling points for sterility testing (see text for details).

Although a vast number of modified processes have been developed [6], only very few of them have been specifically designed for aseptic microsphere preparation, with some of them requiring specialised, cost-intensive equipment [7].

In this study, BSA-loaded PLGA microspheres were prepared by means of a $W_1/O/W_2$-double emulsion solvent extraction method [4]. A flow-through ultrasonic cell for the preparation of the primary W_1/O emulsion was combined with a static micromixer for the preparation of the $W_1/O/W_2$-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 technology 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.

2. Materials and methods

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.

2.2. Equipment and process

BSA-loaded PLGA microspheres were prepared using a recently developed micromixer-based double-emulsion solvent extraction method [8]. The equipment comprises three serially connected subunits (Fig. 1). First, an aqueous solution of BSA (W_1) and a solution of PLGA

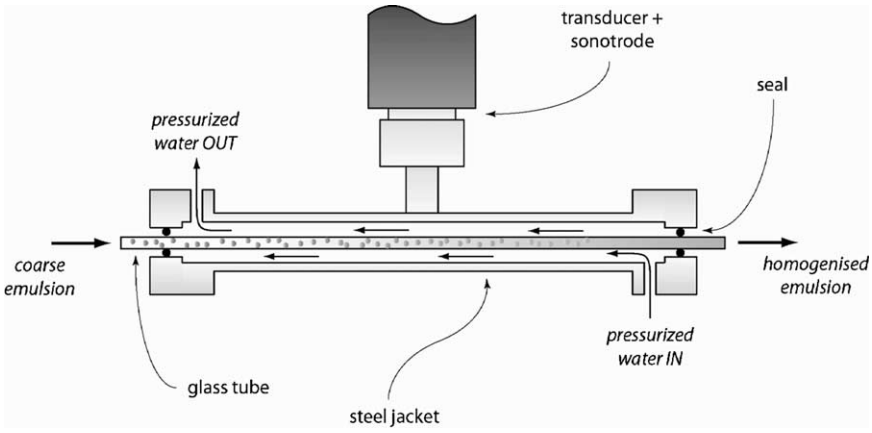

Fig. 2. Design of the ultrasonic flow-through cell for the contact-free preparation of the W_1/O emulsion.

in an organic solvent (O), both fed by syringe pumps, are pre-mixed in a 1-ml rubber-sealed glass cell by means of a magnetic stirrer. Second, the resulting coarse W_1/O-emulsion is transported into a flow-through ultrasonic cell (Dmini250, Dr Hielscher, Teltow, Germany; Fig. 2) for homogenisation. The homogenised emulsion is further transported to a 3 ml rubber-sealed glass receptacle used as expansion vessel to decouple the W_1/O-emulsification from the following process step. Third, the W_1/O-emulsion is further emulsified in an aqueous extraction fluid (W_2) using a static micromixer (SSIMMAg40T35, Institut für Mikrotechnik Mainz (IMM), Mainz, Germany; Fig. 3A). The resulting W_1/O/W_2 double emulsion is collected in a glass vessel and stirred gently. Upon extraction of the polymer solvent into the extraction fluid W_2, solid microspheres with encapsulated BSA are formed.

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. The glass tube is inserted into a steel mantle, which is excited by an attached 20 kHz-sonotrode (Fig. 2). The open space between the glass tube and steel mantle is filled with pressurised water (minimum 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. 3B). 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 (Fig. 3C) [11]. The size of the droplets can be readily controlled by varying the flow velocities of the two fluids [8].

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) (W_1). The two solutions were processed by the ultrasonic cell as described above. The portion of W_1/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 (W_2) for DCM and EF, respectively. Flow rates were set at 4, 45 and 360 ml/h for

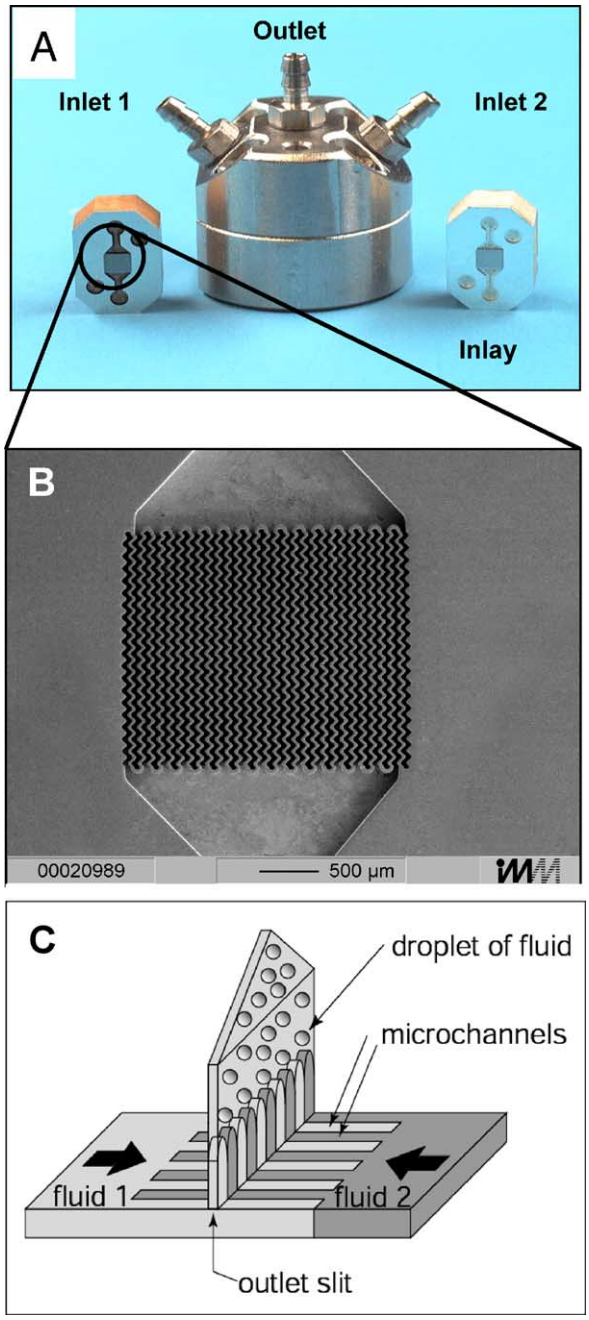

Fig. 3. Static multilamination micromixer for the preparation of the W_1/O/W_2 double emulsion. (A) Mixer with dismantled inlay. The inlay contains an array of microchannels. (B) SEM close-up of the microchannel array. (C) Principle of droplet formation from fluid lamellae upon transition of two fluids 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).

the W_1-, O- and W_2-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.

2.4. Size distribution of the W_1/O emulsion droplets and the final microspheres

Samples of the W_1/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 ($n_D = 1.333$) and DCM ($n_D = 1.424$), respectively. 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, $D[4,3]$.

Dispersions of the microspheres in the extraction fluid were also analysed by laser light scattering, however, with the Fraunhofer model being used for raw data analysis, as the microspheres had diameters above 5 μm, considered as critical for this calculation model. Further, the dried microspheres were analysed by scanning electron microscopy (Hitachi S-700, Nissey Sangyo, Rattingen, Germany) after having placed the particles on double-sided adhesive tape and coated with 6 nm of platinum (planar magnetron sputter-coater MED 010, Bal-Tec, Balzers, Liechtenstein).

2.5. 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.

2.6. 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. 2) were steam-sterilized, while the remaining parts of the equipment were surface-disinfected with 70% ethanol. The equipment was assembled in a laminar air-flow cabinet and finally purged with 70% ethanol.

The BSA and PLGA solutions were sterile-filtered 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 7×10^6 CFU/ml of the microbial mixture to achieve a bioburden of 10^7 CFU/cm^2 for sterile filtration on 7 cm^2 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 sterilization (for sterility testing method see below).

During microsphere production, sterility tests were performed with the BSA and PLGA solutions, the extraction fluid (Fig. 1, sampling points (1)–(3)), the W_1/O emulsion (4), the hardened microspheres (5) and the extraction fluid waste (6). Aliquots of the BSA solution (5 ml), the extraction fluid, and the extraction fluid waste (25 ml each) were each mixed with casein-peptone-soypeptone (CASO) broth, incubated for 24 h at ambient conditions, and filtered using a Milliflex 100 system (Millipore, Massachusetts, USA). The Milliflex filters were incubated with tryptic soy broth (TSB) at 30–35 °C for 14 days to test for bacterial growth, and at 20–25 °C for 14 days to test 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 μm regenerated cellulose filter, then re-hydrating the filter [12], 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 analogously to the PLGA solution. The microspheres dispersed in the extraction fluid were collected on a sterile 0.2 μm 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 3 days of incubation. Sterility and control tests were carried out in triplicate.

3. Results and discussion

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 μm [9].

Table 1
Characteristics of BSA-loaded microspheres prepared under different conditions of primary emulsion (W_1/O) and double emulsion ($W_1/O/W_2$) formation. Data are given as mean of three microsphere batches ± SD

Batch ID	Sonication power (W)	Sonication time (s)	Polymer solvent	Mean W_1/O droplet diameter $D[4,3]$ (μm)	Mean microsphere diameter $D[4,3]$ (μm)	Theoretical drug loading (%)	Encapsulation efficiency (%)
DC1	30	39	DCM	0.62 ± 0.05	36.4 ± 0.8	5.6	73.0 ± 3.6
DC2	23	39	DCM	0.67 ± 0.03	38.9 ± 1.7	5.6	72.5 ± 1.2
DC3	30	23	DCM	0.67 ± 0.05	30.9 ± 0.3	5.6	67.1 ± 2.5
EF1	30	40	EF	0.63 ± 0.03	14.8 ± 0.2	6.0	70.2 ± 3.7

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 (Table 1). The droplet size distributions were monomodal and relatively narrow with negligible batch-to-batch variations (Fig. 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 (Table 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_1/O emulsion (Table 1). Interestingly, the homogenisation of the W_1/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_1/O-emulsion droplet sizes of approx. 600 nm (data not shown).

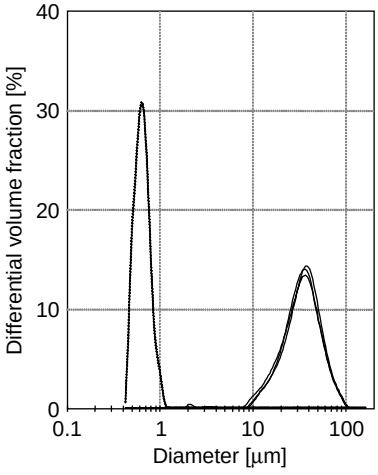

Fig. 4. Size distributions of W_1/O emulsions prepared by the ultrasonic flow-through cell (•••, $n=2$) and of microspheres prepared from these emulsions employing the static micromixer (—, $n=3$). For further preparation parameters see Table 1 (batch DC1).

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 W_1/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 [13].

Microsphere formation in the micromixer occurred through extruding the W_1/O-emulsion through an array of microchannels interdigitated with microchannels that were fed from the opposite side by the extraction fluid, W_2, and further through a fine gap (the micromixer's outlet slit). Specifically, W_1/O-droplets in the W_2-phase formed by friction forces exerted on the slowly flowing W_1/O-lamellae by the nine-fold faster flowing W_2-lamellae. This W_1/O-droplet formation was accompanied by simultaneous solvent extraction giving birth to microspheres of relatively broad size distribution (Figs. 4 and 5), as found in previous experiments with this type of mixer [8]. Scanning electron

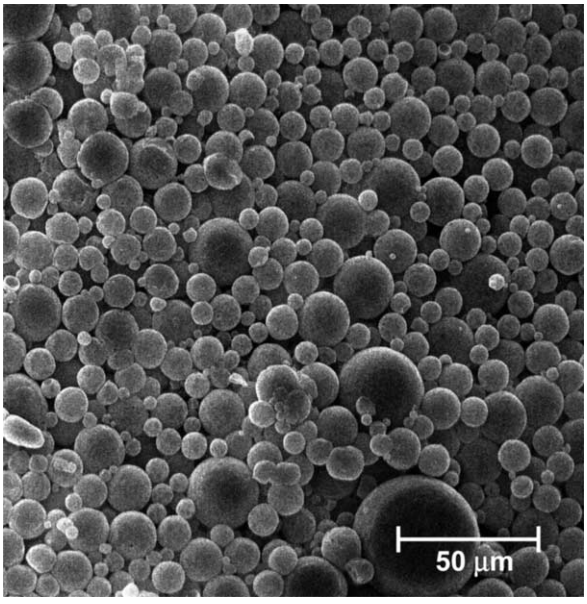

Fig. 5. Scanning electron micrographs of PLGA microspheres prepared in the processing unit illustrated in Fig. 1.

micrographs revealed spherical microspheres with smooth surfaces (Fig. 5). Batch-to-batch reproducibility was highly satisfactory. Microsphere sizes were not affected by the slight changes of the W_1/O-emulsion droplet size upon reduction of the sonication power (Table 1). The reduction of the sonication time by increasing the flow rates of the W_1- and O-solutions, in turn, necessitated to increase the flow-rate of the extraction fluid, W_2, 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 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%; Table 1), which were similar to those obtained previously with the same micromixer in combination with a batch-mode preparation of the W_1/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 W_1-droplet sizes (620–670 nm) of the primary W_1/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].

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 accommodates readily in a laminar air-flow cabinet or an isolator. The static micromixer consists basically of only three parts, which all can be heat-sterilised. The glass tube that conducts the W_1/O emulsion can be dismantled from the ultrasonic cell and heat-sterilised, as it can be done with all the tubing. 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 pre-requisite for cost-efficient aseptic production, and obviates contact between the processed materials and the environment.

All the samples taken for sterility testing from the starting materials, intermediate and final products were free of 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 [1,12,14].

We finally would like to emphasize that 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 evidence product sterility. Further work will have to include, for example, conducting the process with media fill and testing for anaerobe microorganisms. In addition, the method used for sterility testing of the interior of the microsphere needs further validation work with respect to the microbial toxicity of EF, which was employed to dissolve the microspheres.

References

[1] A. Gèze, M. Venier-Julienne, J. Cottin, N. Faisant, J. Benoit, PLGA microsphere bioburden evaluation for radiosterilization dose selection, J. Microencapsul. 18 (2001) 627–636.
[2] D. Mohr, M. Wolff, T. Kissel, Gamma irradiation for terminal sterilization of 17β-estradiol loaded poly-(D,L-lactide-co-glycolide) microspheres, J. Control. Release 61 (1999) 203–217.
[3] A. Hausberger, R. Kenley, P.P. De Luca, Gamma irradiation effects on molecular weight and in vitro degradation of poly(D,L-lactide-co-glycolide) microparticles, Pharm. Res. 12 (1995) 851–856.
[4] P. O'Donnell, J. McGinity, Preparation of microspheres by the solvent evaporation technique, Adv. Drug. Deliv. Rev. 28 (1997) 25–42.
[5] Y. Yang, T. Chung, X. Bai, W. Chan, Effect of preparation conditions on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method, Chem. Eng. Sci. 55 (2000) 2223–2236.
[6] S. Freitas, H.P. Merkle, B. Gander, Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology, J. Control. Release 102 (2005) 313–332.
[7] M. Tracy, Development and scale-up of a microsphere protein delivery system, Biotechnol. Prog. 14 (1998) 108–115.
[8] S. Freitas, A. Walz, H.P. Merkle, B. Gander, Solvent extraction employing a static micromixer: a simple, robust and versatile technology for the microencapsulation of proteins, J. Microencapsul. 20 (2003) 67–85.
[9] S. Freitas, G. Hielscher, H.P. Merkle, B. Gander, Continuous contact- and contamination-free ultrasonic emulsification—a useful tool for pharmaceutical development and production, Ultrasonics Sonochem. 12 (in press).

[10] S. Freitas, B. Gander, N. Lehmann, Verfahren und Durchflusszelle zur kontinuierlichen Bearbeitung von fliessfähigen Zusammensetzungen mittels Ultraschall. German Patent Application 102 43 837, 2002.
[11] V. Haverkamp, W. Ehrfeld, K. Gebauer, V. Hessel, H. Löwe, T. Richter, C. Wille, The potential of micromixers for contacting of disperse liquid phases, Fresen. J. Anal. Chem. 364 (1999) 617–624.
[12] F. Furkert, A validated test for sterility of microparticles made of poly(lactide-co-glycolide), Pharm. Ind. 60 (1998) 905–907.
[13] O. Behrend, H. Schubert, Influence of hydrostatic pressure and gas content on continuous ultrasound emulsification, Ultrason. Sonochem. 8 (2001) 271–276.
[14] H. Toguchi, Sterility assurance of microspheres, J. Control. Release (1999) 51–55.