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Research paper

Preparation of protein loaded poly(D,L-lactide-co-glycolide) microparticles for the antigen delivery to dendritic cells using a static micromixer

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Abstract

The cellular immune response against tumors, viruses, or intracellular bacteria requires adequate antigen delivery to professional phagocytes, their processing and the presentation of antigenic peptides to T-cells. Biodegradable microparticles to enhance antigen phagocytosis and the response of cytotoxic lymphocytes have been proposed. The aim of the present study was to formulate poly(lactide-*co*-glycolide) (PLGA) microparticles using a *w/o/w* solvent evaporation procedure in order to obtain suitable vehicles for vaccination. Bovine serum albumin bearing fluorescein isothiocyanate (FITC–BSA) was used as a model antigen. For microparticle preparation a static micromixer was employed. Microparticles of 2–3 μm can be produced with good reproducibility by applying high flow rates at the micromixer. Microparticles with a smooth surface and only one pore were observed using scanning electron microscopy (SEM). Confocal laser scanning microscopy (CLSM) allowed localisation of the FITC–BSA near the surface of the microparticle. Microencapsulation of FITC–BSA did not altered the polymer characteristics, as determined by measuring the glass transition temperature. Additionally we could determine residual methylene chloride, employed as solvent in microparticle preparation, to be less than 1/1000 of the USP and Ph. Eur. limit. The microparticles described herein were able to deliver the model antigen to human dendritic cells (DC).

Keywords: Static micromixer; Microparticles; PLGA; Residual solvent; Dendritic cells; Vaccination

1. Introduction

The goal of immunological treatment of cancer or viral infection is to initiate a cellular immune response. The induction of an efficient cellular immune response requires the uptake of soluble antigens, their processing, and presentation by antigen presenting cells. Dendritic cells (DC) are the most potent antigen presenting cells. Data provided by early clinical pilot studies indicate that DC vaccination can induce immunological as well as clinical responses in selected patients with advanced cancer [1]. Activated DC present the antigens to initiate immunological reactions, e.g. the stimulation of antigen specific T-helper reaction and of cytotoxic lymphocytes (CTL), that are able to attack virus infected cells or tumor cells [2]. One precondition for vaccination with DC will be appropriate delivery of antigens to the DC [3,4]. In many cases this cannot be achieved by soluble antigens comprehensively. Therefore, vehicles, such as biodegradable microparticles, are used to facilitate phagocytosis, to attain a long-lasting stimulation by sustained antigen release from the microparticles and to induce an adequate T-cell response [4–6]. In order to achieve an optimal phagocytosis particle sizes <5–10 μm should be prepared [5,7].

The presented experiments show the preparation and characterisation of biodegradable microparticles from poly (llactide-*co*-glycolide) [PLGA], which will be used for the phagocytosis experiments later on. The preparation of the microparticles follows a *w/o/w* solvent evaporation method, using a static micromixer for the secondary emulsion. Its usefulness for protein encapsulation by means of a *w/o/w* procedure has been shown [8]. There are a number of advantages of using a micromixer: The size of the produced droplets can be controlled easily by changing the flow rates of both phases [9,10]. It is resistant against organic solvents and is without moving parts, whose abrasion could contaminate

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the product. It can be easily sterilized, which is of great importance concerning the usage of the microparticles in phagocytosis experiments. The continuously working micromixer is usable on laboratory scale due to its small dead volume, as well as for large scale production, using several mixers in parallel flow (number-up). Concerning the simple scaling-up this is of great advantage compared to conventional mixers for laboratory scale. In comparison with rotor stator mixers the micromixer is of higher efficiency, i.e. similar particle sizes can be obtained with much less energy input. This is important with respect to the low thermostability of the products [11].

Furthermore, fluorescein isothiocyanate marked bovine serum albumin (FITC–BSA) used as model protein showed the distribution of the protein in the microparticle by means of confocal laser spectroscopy. The encapsulation efficiency, the thermal behaviour of the polymer after microencapsulation of FITC–BSA, the surface structure of the microparticles, as well as the residual solvent have also been investigated. The potential of microparticles, prepared and characterised in this way, to deliver the model antigen to antigen presenting cells (APC) was studied using monocyte derived dendritic cells generated by the cytokines granulocyte Macrophase Colony Stimulating Factor (GM-CSF) and IL-4. Moreover, a relation between droplet size of the primary emulsion, protein distribution inside the microparticles, and particle morphology shall be examined in this paper.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) [PLGA, Resomer RG 502H, inherent viscosity 0.18 dl/g, end group uncapped statistic copolymer with 50:50 lactide:glycolide ratio] was a gift from Boehringer Ingelheim (Ingelheim, Germany). Bovine serum albumin, bearing fluorescein isothiocyanate groups (FITC–BSA) was purchased from Sigma (Taufkirchen, Germany). The used poly(vinyl alcohol) was Mowiol® 4-88 with 12% residual acetate groups (Hoechst, Frankfurt, Germany). Methylene chloride, trifluoroacetic acid (TFA), acetonitrile (ACN), and all other chemicals were of analytical grade.

2.2. Principle of a static micromixer and experimental set-up

The micromixer (Institut für Mikrotechnik, Mainz, Germany) consists of a mixing element, made using an etching technique (so-called LIGA technique [9]), which is fixed in a two-part housing. Via two inlets in the lid the phases to be mixed pass to the mixing element, where they approach each other in microchannels. Liquid lamellas are formed, which flow perpendicular from the inlet to the outlet slot in the lid of the mixer and form the emulsion (Fig. 1).

An HPLC pump (Masterkron 4, Kronlab, Sinsheim, Germany) with possible flow rates between 0.1 and 200 ml/min and a syringe pump (TSE 540200, Technical and Scientific Equipment GmbH, Bad Homburg, Germany) were

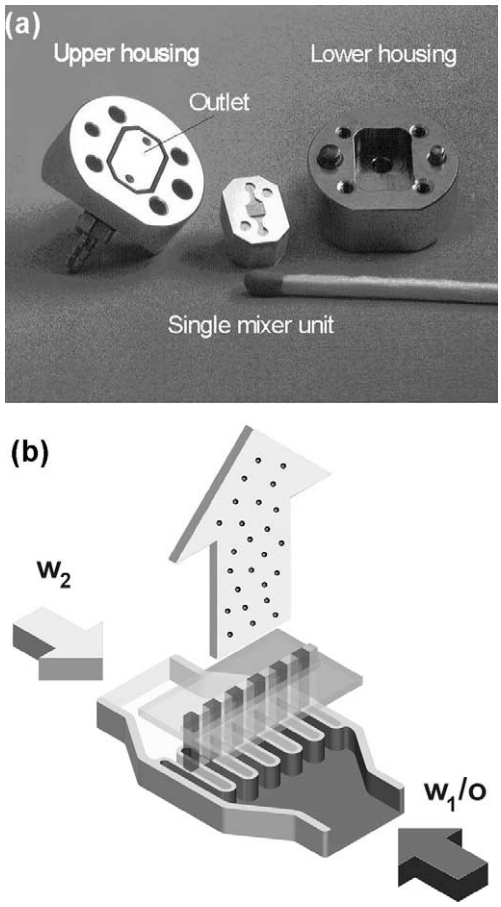

Fig. 1. Construction and mode of operation of the standard slit interdigital micromixer. (a) Left: upper housing with two inlets and the outlet slit in the middle; center: inlay with interdigital mixing structure; right: lower housing with cutout for the inlay. (b) Mixing principle: fluids to be mixed flow into mixing channels, formation of a fluid lamellae and disintegration into droplets. (Figures with kind permission of Institut für Mikrotechnik Mainz).

connected to the two inlets of the micromixer via HPLC tubings and fittings (Fig. 2). The tubing connected to the outlet of the micromixer ended up in a beaker on a magnetic stirrer. The mixing tool inside the micromixer housing was made from silver on a copper support with channel widths of 25 μm. To start the emulsification process, first the HPLC pump was started, pumping the evaporation media (w_2) at a flow rate up to

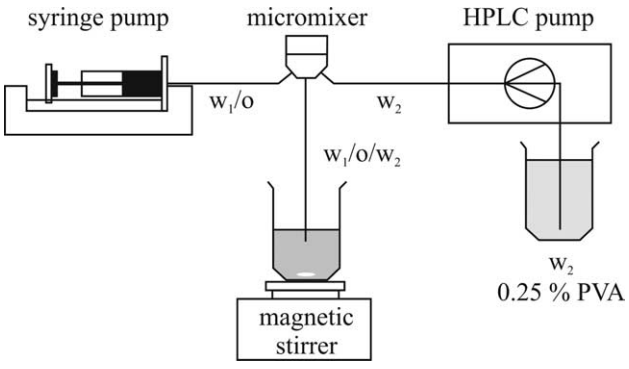

Fig. 2. Experimental setup for microparticle production using a static micromixer.

25 ml/min. After this, the syringe pump was used to transport the primary w_1/o emulsion with 1/25 of the flow rate used for the w_2 phase.

2.3. Preparation of the microparticles

Microparticles were prepared using a $w_1/o/w_2$ double emulsion solvent evaporation procedure. Solutions of 10 mg/ml FITC–BSA in 10 mM Tris buffer pH 7.0 (w_1 phase) and 5% PLGA (w/w) in methylene chloride (o phase) were prepared. For the primary w_1/o emulsion, 200 μl of w_1 were dispersed in 4 g of the o phase using an Ultra Turrax® T25 homogenizer equipped with an S 25 N—10 G dispersing tool (Jahnke & Kunkel, Staufen, Germany) at 24.000 rpm for 1 min, corresponding to a protein load of 1%. Emulsification was carried out in a 10 ml polyethylene syringe without a stem. After that the syringe was immediately placed into the syringe pump. The secondary emulsification of w_1/o in a 0.25% (w/v) solution of PVA (w_2 phase) was carried out using the micromixer as described above. The resulting $w_1/o/w_2$ emulsion was collected in a beaker and stirred for 3 h for solvent evaporation. The microparticles were collected by centrifugation ($6200 \times g$, 10 min, Heraeus Minifuge RF, Kendro, Langenselbold, Germany), washed three times with distilled water, and lyophilized for 20 h (Alpha 1-4, Christ, Osterode, Germany). The microparticles were stored at 4 °C in the dark.

2.4. Particle size distribution

Samples of the lyophilized particles were suspended in distilled water and particle size distribution was determined by laser diffraction (LS 230, Coulter). The raw data were analysed using the Mie theory (refraction index described by [11]) and depicted in volume-weighted plots.

2.5. Encapsulation efficiency

Encapsulation efficiency was evaluated using a method adapted from one used for HPLC sample preparation [12]. Briefly, 10 mg microparticles were dissolved in 500 μl of 90% ACN/0.1% TFA/9.9% water. The PLGA was precipitated by adding 750 μl 0.1% TFA and separated by centrifugation at $2800 \times g$ for 10 min. The protein concentration of the supernatant was analysed by measuring the absorption at 275 nm (UV-2101 PC, Shimadzu Scientific Instruments, Columbia, USA). A standard curve was obtained by dissolving 10 mg PLGA in the ACN/TFA/water mixture and adding different volumes of FITC–BSA stock solution prior to the precipitation step.

2.6. Differential scanning calorimetry

For differential scanning calorimetry (DSC) measurements (DSC 821^e, Mettler Toledo, Giessen, Germany), samples of about 5 mg were accurately weighed and sealed in aluminium pans. Curves were taken from 15 to 95 °C with a heating rate of 5 °C/min. The raw data were collected and the glass transition temperature (T_g) was determined using a sigmoidal Boltzmann fit.

2.7. Residual solvent

Lyophilization of the samples was performed in vials suitable for gas chromatography (GC) and sealed with GC caps. Samples were analysed for residual methylene chloride by GC-headspace (Agilent GC-6890N, Waldbronn, Germany) coupled with mass spectrometry (Hewlett–Packard MS-5970, Agilent) on a HP5MS column using the selective ion monitoring (SIM) mode. In our test assay, samples were compared to a negative control (empty vial) and a 1 ppm standard solution of methylene chloride in water. For quantification, $M^{+\cdot}$ ($m/z = 84$) was used, taking $^{15}N_2$ from the air ($m/z = 30$) as internal standard.

2.8. Protein distribution inside the microparticles

Protein distribution was analysed by confocal laser scanning microscopy (LSM 510 meta, Axiovert 100, Zeiss, Germany). Microparticles were suspended in water and spread on a cover slip, where they were allowed to dry prior to analysis. Fluorescein was excited by a 488 nm argon laser.

2.9. Scanning electron microscopy (SEM)

Samples of the lyophilized microparticles were placed on aluminium specimen stubs using conductive double adhesive tabs (Plano, Wetzlar, Germany) and sputtered with 10 nm of gold (SCD030 Sputter, Balzers, Liechtenstein). The micrographs were taken with a field emission scanning electron microscope (S-4000, Hitachi, Krefeld, Germany).

2.10. Delivery of FITC–BSA loaded microparticles to human dendritic cells

Monocytes were isolated from human blood by isopycnic separation on Ficoll™ (Amersham Biosciences, Uppsala, Sweden). Dendritic cells were generated in vitro by culturing monocytes in the presence of IL-4 (RD Systems, 500 U/ml)and GM-CSF (RD Systems, 800 U/ml) for 5 days as described elsewhere [13,14]. These are so-called immature dendritic cells, which are still able to take up particles by phagocytosis. Lyophilized microparticles were resuspended in PBS buffer and 15 μg FITC-labeled particles were added per ml cell suspension (10^6 cells/ml). Within 30 min phagocytosis was completed and fluorescent cells were determined by flow cytometry using the Cellquest Software (FACScalibur, BectonDickson).

3. Results and discussion

3.1. Particle size distribution and encapsulation efficiency

On using a static micromixer for preparing the secondary emulsion, the particle size of the resulting microparticles can

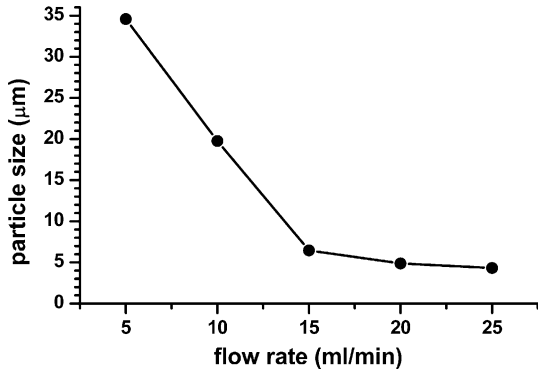

Fig. 3. Influence of the flow rate of w_2 on the modes of the microparticle sizes.

easily be controlled by the flow rates of the phases to be mixed. Other possible parameters, such as the width of the outlet slot, the type of polymer, the viscosity of the polymer solution, and the concentration of the emulsifier have only minor influence [11]. Thus, in the presented results only the flow rate of the w_2 phase was changed, whereas the volume stream of the primary emulsion always was 1/25 of w_2. An increasing flow rate resulted in a hyperbolic decrease of the particle size (Fig. 3). Moreover, the particle size distribution was clearly increased (Fig. 4). At high flow rates an increasing deviation from the Gaussian normal distribution occurred. A good reproducibility of the particle sizes could be achieved, as shown for 25 ml/min (Table 1). A further increase in the volume stream in order to achieve even smaller particles was not reasonable due to several reasons. First an increase of the flow rate from 20 to 25 ml/min resulted only in a small decrease of the particle size. Second the higher flow rate caused a wider distribution of the particle sizes. Moreover, the used micromixer is limited to flow rates up to 30 ml/min. Therefore, a safety factor was applied to the flow rates and a maximum of 25 ml/min was adjusted.

The microparticles were examined with respect to their encapsulation efficiency. There was no dependency between applied flow rate and the extent of encapsulated protein (Fig. 5). For 25 ml/min, the encapsulation efficiency was between 74 and 84% with a mean of about 79%, as shown in Table 1.

As a standard condition for all further experiments the flow rate was adjusted to 25 ml/min for w_2, because the particle size

Table 1
Particle size and encapsulation efficiency of 8 batches at a flow rate of 25 ml/min

	Median	Range/2
Particle size (µm)	3.06	0.87
Encapsulation efficiency (%)	78.7	5.0

distribution was still acceptable and the resulting microparticles showed an optimum size for the phagocytosis experiments. This could be shown in experiments using immature dendritic cells (see also Section 3.5.) [15].

3.2. Influence of protein encapsulation on PLGA characteristics

The PLGA (Resomer 502H), which has been used for the described experiments, is of amorphous state. The glass transition temperature (T_g) is an important property to characterise this state and can be determined by means of differential scanning calorimetry (DSC). Exceeding this temperature the polymer passes from a glassy state to a rubber-like state. This may cause microparticle agglutination and change in the release behaviour.

Because of the low molecular weight of Resomer 502H, which is reflected in its low inherent viscosity, T_g will be only slightly above body temperature. Lowering T_g, by loading the polymer matrix with other molecules, could be critical with respect to the stability of the microparticles during storage and their behaviour in vivo (or in the cell culture, respectively).

As can be seen in Table 2 loading the microparticles with 1% FITC–BSA did not lead to a change of T_g, compared to the pure polymer and the non-loaded microparticles.

In contrast to our findings the loading of PLGA microparticles with 5% BSA caused a depression of T_g by 3 °C as described in the literature [16]. Higher loads resulted in stronger depression of T_g in this study. This is due to the small water amount, which is bound to the protein and acts as plasticizer on PLGA.

However, we could not find this effect in our formulation. It is assumed that it results from the smaller load of the microparticles. Another explanation might be that our

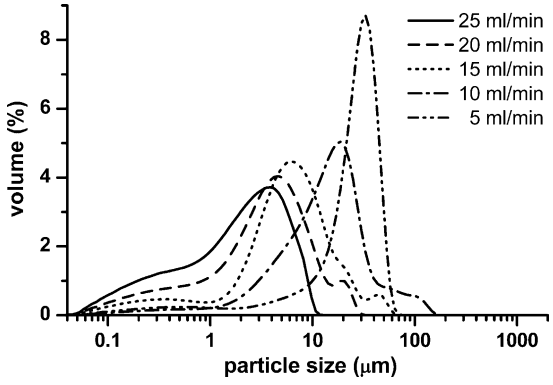

Fig. 4. Particle size distribution at different flow rates used for w_2 in the micromixer.

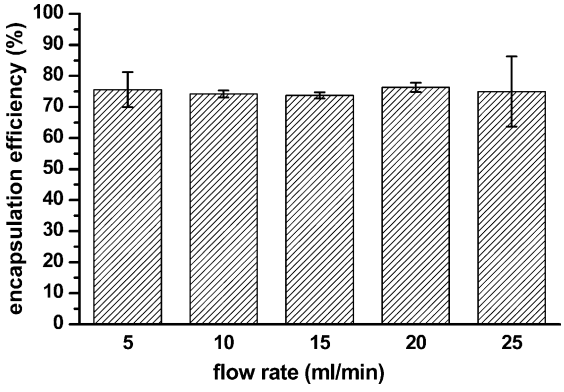

Fig. 5. Dependency of the encapsulation efficiency on the flow rate (triple determination, median and range).

Table 2
Glass transition temperature of PLGA and PLGA-microparticles

	Glass transition temperature (°C)		
	Pure PLGA	Blank particles	FITC–BSA loaded particles
Sample 1	42.49	43.14	42.99
Sample 2	42.80	43.18	43.01
Sample 3	42.40	42.63	42.89
Median	42.49	43.14	42.99
Range	0.40	0.55	0.12

lyophilization procedure is more effective, leaving less water inside the microparticles.

3.3. Residual solvent

During the preparation of the microspheres, methylene chloride was used as solvent for PLGA. It is well known, that methylene chloride has a high acute toxicity, e.g. central nervous system effects, heart rhythm disturbance, respiratory paralysis. The chronic exposure to low doses is said to have a carcinogenic effect. Therefore, methylene chloride is classified as a class 2 solvent in Ph. Eur. and limited to 600 ppm in pharmaceutical formulations (USP: 600 ppm also). As a result, attention has been paid to find possibilities, which avoid methylene chloride during microencapsulation in PLGA [17–20].

Using a semiquantitative method we examined the amount of methylene chloride finally remaining in the product under the respective method of preparation. The amount was determined to be smaller than 0.6 ppm (Table 3), which is less than 1/1000 of the USP and Ph. Eur. limit. Thus, we may conclude that a method avoiding methylene chloride is not necessary.

3.4. Surface structure and protein distribution inside the microparticles

Using high resolution scanning electron microscopy (SEM) we analysed the surface structure of the microparticles and confirmed the particle size determined by laser diffraction. As depicted in Fig. 6, the microparticles showed a spherical shape and an even surface. Single pores could be found in particles >1 μm. The pores were generated by volatilising water from the w_1 phase during the lyophilization. Cracks in the surface and ellipsoid particle shape, visible in Fig. 6(b), were artifacts due to the high energy of the electron beam at large magnifications. This happens, since the used PLGA has a low T_g (Section 3.2) and becomes rubbery.

Table 3
Semi quantitative analysis of residual methylene chloride in PLGA-microparticles by GC–MS

	AUC[a]	Calculated amount of methylene chloride (ppm)[b]
0 ppm standard	0.82	0.014
1 ppm standard	89.36	1.000
Sample 1	39.81	<0.6(0.445)
Sample 2	25.77	<0.6(0.288)

[a] Raw data were standardised by relating the area under the curve (AUC) from the peak of $M^{\ddagger}$ to the peak of $^{15}N_2$ and corrected for the sample mass.
[b] Amount of residual solvent was calculated by relating the sample's AUC to the 1 ppm standard.

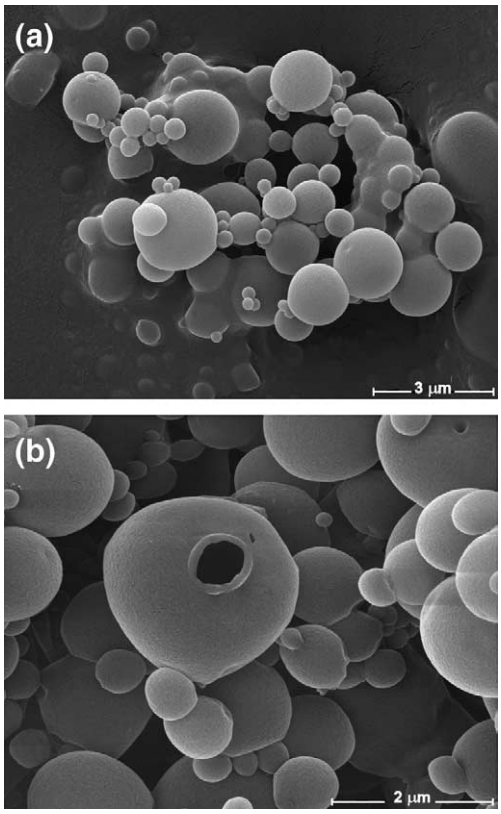

Fig. 6. Scanning electron micrographs of FITC–BSA loaded microparticles produced at a flow rate of 25 ml/min.

The use of FITC–BSA as a model protein enabled us to study protein distribution inside the microparticles by confocal laser scanning microscopy (CLSM). As depicted in Fig. 7(b), the protein was located close to the surface of the microparticle. Fluorescence intensity measurements along the arrow clearly indicated that no FITC–BSA could be found in the center of the microparticle (Fig. 7(c)). Comparing the microparticle images of light microscopy and CLSM we assumed, that nearly every particle visible in Fig. 7(a) contained FITC–BSA (Fig. 7(b)).

From SEM and CLSM data we can conclude, that the obtained microparticles from our preparation procedure are microcapsules, i.e. they have a large central hole and typically only one pore. The model protein is adsorbed to the walls of the microcapsules inside and/or outside the particles. The existence of all protein on the outer side of the particle would implicate, that the $w_1/o/w_2$ procedure failed because the two phases w_1 and w_2 merged. But in this case, encapsulation efficiency would be expected to be lower than described (Section 3.1.).

However, we have to attribute the appearance of microcapsules to the primary emulsion procedure. Using an Ultra-Turrax, w_1 droplets dispersed in the methylene chloride phase

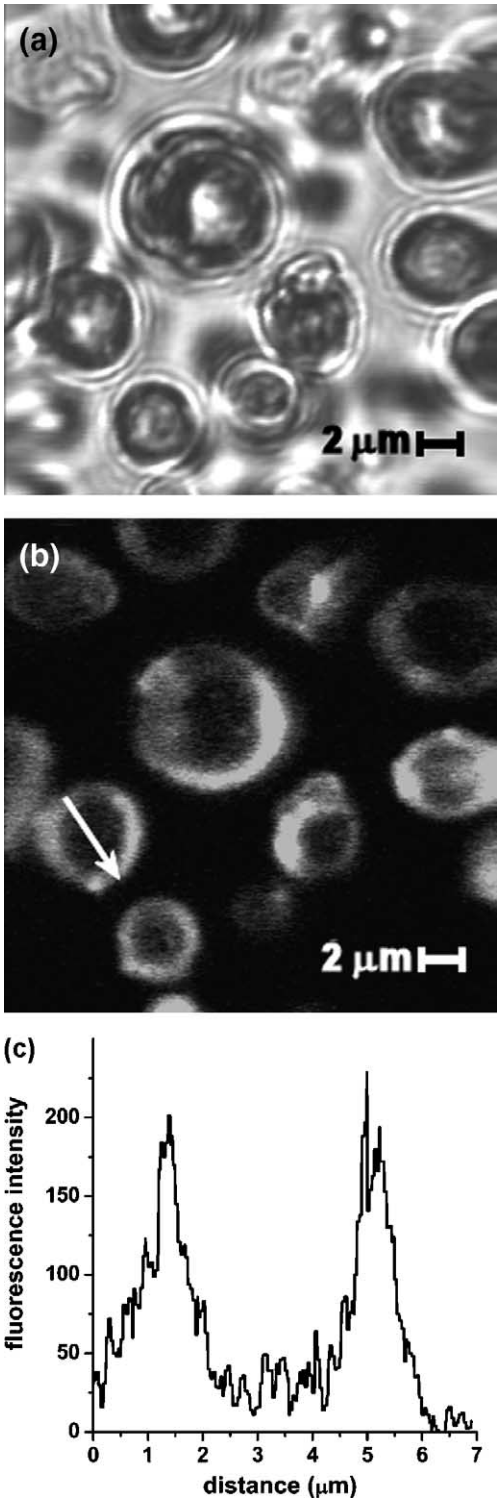

Fig. 7. Protein distribution inside the microparticles. (a) light microscopy, (b) confocal laser scanning microscopy, (c) fluorescence intensity along the arrow in Fig. 7(b).

are likely to have nearly the same size as the resulting microparticles produced at 25 ml/min flow rate in the micromixer. Therefore, only one w_1 droplet can be encapsulated into one microparticle, resulting in a localization of the model protein close to the surface. Furthermore, the particles smaller than 1 μm do not contain FITC–BSA and have no

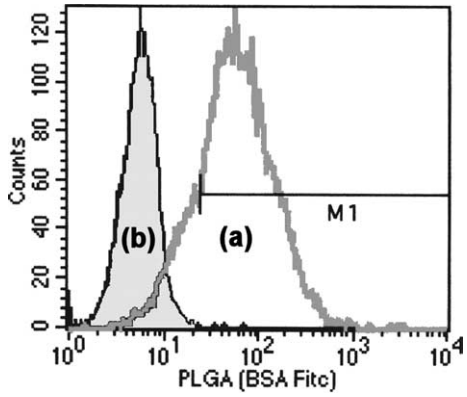

Fig. 8. Protein delivery into human immature dendritic cells by PLGA microparticles. Comparison of cell fluorescence following (a) phagocytosis of FITC–BSA loaded microparticles compared to (b) nontreated cells by flow cytometry.

pores, because the primary emulsion was not adequate to be encapsulated by very small particles. For preparing microspheres instead of microcapsules, more than one primary emulsion droplet has to be encapsulated into one microparticle. If the size of the resulting microparticles should be kept at 3 μm, the droplets of the primary emulsion have to be in the nanometer range. This is not achievable with the Ultra-Turrax. However, we could show that the microcapsules described herein were suitable vehicles for protein delivery to DC.

3.5. Delivery of model antigen to human dendritic cells

In a phagocytosis experiment we examined if the microparticles described herein are really able to deliver potential antigens into human dendritic cells. Cells having phagocytized FITC–BSA loaded microparticles will show intensive fluorescence. As shown in Fig. 8, we could find an increase in FITC-positive cells following coincubation with microparticles. So there is a good agreement between particle size ($<$5–10 μm) and the ability for an uptake by DC. Furthermore, we could not observe a loss in cell viability due to the treatment of dendritic cells with microparticles. So we may conclude, that microparticles, prepared with the micromixer at 25 ml/min, are efficiently ingested by human dendritic cells and could be applied for the delivery of bacterial, viral, or tumor antigens to dendritic cells.

4. Conclusion

Using a static micromixer, microparticles which are suitable as antigen delivery devices to phagocytes can be produced. These particles are ingested efficiently by dendritic cells with only a weak effect on cell viabilty. In view of residual solvents, microparticles conform to Ph. Eur. and USP can be prepared without the necessity of a solvent free methodology.

In general, there is a good correlation between scanning electron microscopy and confocal laser scanning microscopy data. Microparticles showing a smooth surface and typically

only one pore will be capsules, bearing the encapsulated protein close to the surface.

As a next step, a tumor antigen will be encapsulated instead of the model protein and the cellular response will be studied in order to develop an improved cancer immunotherapy based on autologous dendritic cells.

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