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Research paper

# Recombinant human erythropoietin (rhEPO) loaded poly(lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics

Beate Bittner[a], Michael Morlock[a], Hans Koll[b], Gerhard Winter[b], Thomas Kissel[a,*]

[a]*Philipps-University, Marburg, Germany*
[b]*Boehringer Mannheim GmbH, Penzberg, Germany*

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**Abstract**

Recombinant human erythropoietin (EPO) and fluorescein isothiocyanate-labelled dextran (FITC-dextran) loaded biodegradable microspheres were prepared from poly(lactide-co-glycolide) (PLG) by a modified spray-drying technique. This microencapsulation method was compared with the water-in-oil-in-water (w/o/w) double-emulsion method. As expected, microsphere morphology, particle size and particle size distribution strongly depended on the production process. The spray-drying method was found to have a number of advantages compared to the w/o/w double-emulsion technique. The content of residual dichloromethane (DCM) in the final product was significantly lower in case of the microspheres prepared by spray-drying. Concerning EPO loaded microspheres, spray-drying yielded higher encapsulation efficiencies. Although the microspheres obtained by spray-drying are subjected to intensive mechanical and thermal stress during the preparation, the amount of aggregates of EPO in PLG microspheres were not increased compared to the w/o/w technique. Depending on the manufacturing method, addition of cyclic DL-lactide dimers (referred to as monomers in the following) affected the in vitro release profiles of EPO and FITC-dextran from PLG microspheres. Using differential scanning calorimetry it was shown that these low molecular weight substances only seem to be present inside the microspheres produced by spray-drying. DL-Lactide significantly reduced the initial burst release of both EPO and FITC-dextran. While the following release period of EPO was not affected by the DL-lactide content, a more linear FITC-dextran release pattern could be achieved. It can be concluded that the spray-drying technique provides a number of advantages compared to the w/o/w method. The modulation of protein release using low molecular weight additives is of particular interest for parenteral depot systems. © 1998 Elsevier Science B.V.

*Keywords:* Erythropoietin; Parenteral protein delivery; Microencapsulation techniques; Poly(lactide-co-glycolide)

## 1. Introduction

Erythropoietin (EPO) is a glycoprotein hormone of $M_r$ 30.4 kDa. The protein is mainly produced in the kidneys and plays an important role in stimulating the red cell proliferation and their differentiation in the bone marrow. EPO is clinically used for the treatment of anemia associated with chronic renal failure [1,2]. The protein is administered in cases of neoplastic diseases and chronic inflammation [3].

EPO and fluorescein isothiocyanate-labelled dextran (FITC-dextran) loaded microspheres were prepared using a modified spray-drying technique and a water-in-oil-in-water (w/o/w) double-emulsion method. The spray-drying process has been used for the encapsulation of both hydrophilic and hydrophobic substances into biodegradable poly-

* Corresponding author. Department of Pharmaceutics and Biopharmacy, Philipps-University, Ketzerbach 63, D-35032 Marburg, Germany. Tel.: +49 6421 285880; fax: +49 6421 287016;
e-mail: kissel@mailer.uni-marburg.de

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mers [4,5]. Depending on drug solubility in the organic solvent, the drug can be introduced into the process either dissolved or dispersed into a suitable solvent for biodegradable polymers. Frequently, dichloromethane (DCM) is used as solvent. In a single step, the feed is transferred from a fluid state into a dried microparticulate form [6]. Manufacturing of microspheres under aseptic conditions is also possible. Microsphere characteristics are depending on different process parameters such as inlet temperature, spray rate of the feed, flow rate, polymer and drug concentration in the organic solvent [7], organic solvent [8], and the polymer utilized [9,10]. The microencapsulation of hydrophilic macromolecules by a spray-drying technique was mostly limited to inexpensive 'model proteins', such as bovine serum albumin [8].

The spray-drying technique has been used for the microencapsulation of Parodel® into biodegradable polyesters [11]. This microencapsulation method was transferred to the production scale for Parodel LAR. The process includes $\gamma$-sterilization, which can be prohibitive for proteins [12].

The w/o/w double-emulsion (w/o/w) method has been described by Ogawa et al. in 1988 for the encapsulation of leuprorelide acetate into poly(lactide-co-glycolide) microspheres [13]. To prepare microspheres by the w/o/w double-emulsion technique, an aqueous solution of the hydrophilic drug is emulsified into an organic solution of the polymer. Usually, DCM is selected as organic solvent, but other solvents like ethylacetate or methylethyl ketone have also been investigated [14]. This primary w/o emulsion is then injected into a second water phase containing stabilizers, such as polyvinylalcohol, PVA. Subsequently, the solvent is removed by extraction or evaporation and the microspheres are collected by filtration or centrifugation.

Numerous studies have been carried out to vary the different process parameters to optimize the w/o/w double-emulsion technique. The influence of e.g. shear force [15], drug loading, polymer molecular weight [16], polymer composition [17], volume of the inner water phase, volume of DCM, residual solvents [18], temperature [19], and terminal $\gamma$-irradiation [20] were subject of recent investigations.

EPO is like almost all therapeutically relevant proteins susceptible to aggregation to a certain degree. We studied in detail particle morphology, particle size and particle size distribution, effective drug loading levels, and glass transition temperatures of the microspheres containing both EPO and FITC-dextran. FITC-dextran was encapsulated into the microspheres since its $M_r$ of 38.9 kDa is quite similar to that of EPO, but it is less prone to aggregation. Furthermore, we investigated aggregation behavior of EPO. Since the desired release pattern for both drugs would be characterized by a continuous profile over a period of up to 1 month, the release profiles of EPO and FITC-dextran were determined as a function of the manufacturing process.

A variation of polymer composition by blending with low molecular weight additives was carried out by several investigators to improve microsphere degradation and drug release profiles [21–23]. Commercially available polymers like poly(lactide-co-glycolide), prepared by ring opening polymerization [24] contain small amounts of the residual monomers which may have an influence on microsphere characteristics. These low molecular weight impurities may modulate in vitro release profile and stability of a protein. Thus, a further objective of our work was to determine the effect of different amounts of DL-lactide monomers enclosed in the microspheres on microsphere characteristics and protein stability. These low molecular weight additives were introduced in the particles both by spray-drying and by the w/o/w double-emulsion method.

## 2. Materials and methods

### 2.1. Materials

Poly(DL-lactide-co-glycolide) (PLG) with a monomer ratio of lactide/glycolide 50:50 and a weight average molecular weight $M_r$ = 33 kDa and cyclic dimers of DL-lactic acid (referred to as monomers, DL-lactide, or lactic acid, respectively) were purchased from Boehringer Ingelheim (Ingelheim, Germany). EPO ($M_r$ 30.4 kDa) was used as 0.186% aqueous solution (Boehringer Mannheim, Germany). FITC-dextran ($M_r$ 38.9 kDa, Sigma, Germany) was used as hydrophilic model drug. All other materials used were of analytical grade.

### 2.2. Preparation of microparticles

#### 2.2.1. Spray-drying using w/o-emulsions

1400 mg of the polymer (without and with 0.5%, 1%, and 5% (w/w) DL-lactide addition) were dissolved in 28 ml of dichloromethane p.a. (DCM, Riedel de Haen). Subsequently, 1600 $\mu$l of an aqueous EPO or FITC-dextran solution (containing 7.0 mg EPO or FITC-dextran) were emulsified into the polymer solution using an ultra turrax (Janke & Kunkel, Germany) at 20 000 rpm for 60 s at room temperature. Microspheres were obtained by spray-drying the polymer-drug solution through a 0.7 mm nozzle of a Büchi 190 Mini spray-dryer. The process parameters were set as follows: inlet temperature (46°C), outlet temperature (36°C), aspirator setting (20 — maximum scale), pump setting (6 ml/min), spray flow (700 Nl/min). The microspheres were subsequently lyophilized (Edwards Freeze Dryer Modulyo, 15 h, −50°C, 3.3 mbar) and stored at 4°C under desiccation.

#### 2.2.2. w/o/w double-emulsion method

Microspheres were prepared by a w/o/w double-emulsion technique, as reported previously [25]. Briefly, 700 mg of PLG (without and with 0.5%, 1%, and 5% (w/w) DL-lactide addition) were dissolved in 2.33 ml of DCM in a 10 ml polypropylene syringe. 800 $\mu$l of an aqueous EPO or FITC-dextran solution (containing 3.5 mg EPO or FITC-

dextran) were emulsified into the organic solution using an ultra turrax homogenizer (Janke & Kunkel, Germany) operating at 20 000 rpm for 30 s at room temperature. This w/o emulsion then was rapidly injected into 300 ml of an aqueous PVA solution containing 0.1% (w/v) PVA. The homogenization was carried out using an ultra turrax homogenizer S 25 N-25 F (Janke & Kunkel, Germany) at 8000 rpm for 30 s. For the removal of DCM the resultant w/o/w emulsion was stirred continuously for 3 h. The microspheres were subsequently collected by centrifugation (10 min, 5000 rpm), washed with 400 ml of water using a glass sinter funnel (pore width 16–40 $\mu$m) and subsequently lyophilized (Edwards Freeze Dryer Modulyo, 15 h, −50°C, 3.3 mbar). The final product was stored at +4°C under desiccation.

### 2.3. Total EPO content

Briefly, 20 mg of the microspheres were dissolved in 1 ml of 1 N sodium hydroxide by shaking for 20 min in an Eppendorf shaker (type 5432, Eppendorf, Germany). The samples reacted at room temperature under light protection for 4 h to ensure complete hydrolysis of the polymer. Afterwards, they were centrifuged at 13000 rpm for 2 min (Eppendorf type 5415 C). 900 $\mu$l of the supernatants were measured spectrophotometrically at 280 nm (UV 160, Shimadzu) using an EPO calibration curve. To carry out the calibration, different amounts of EPO were added to about 20 mg of placebo microspheres. Each sample was assayed in triplicate.

### 2.4. Total FITC-dextran content

Total drug content of the FITC-dextran loaded microspheres was analyzed by an extraction method [26]. Briefly, 20 mg of the FITC-dextran loaded microspheres were dissolved in 0.5 ml of DCM, followed by addition of 4 ml of PBS buffer solution pH 7.4 (8 g NaCl, 0.2 g KCl, 0.24 g $KH_2PO_4$, 1.81 g $Na_2HPO_4.2H_2O$, 0.1 g Tween-20, 0.5 g $NaN_3$ and 1000 ml aqua dem.) and agitation in a rotating bottle apparatus for 24 h at 30 rpm and 37°C (Rotatherm, Liebisch, Germany). After separating the two phases, the FITC-dextran concentration in the aqueous phase was determined fluorimetrically (excitation: 493 nm, emission: 515 nm; Fluorispec, Fluorescence spectrometer, Model SF-100, Baird-Atomic).

### 2.5. Aggregation of EPO

EPO aggregates were determined by vertical, discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [27] and immunolabelling and visualization of bound antibodies after electrotransfer to nitrocellulose membrane. Briefly, microspheres were dissolved in DMSO at 5 mg/ml and directly loaded onto the gel. After electrophoresis, the gel was blotted electrophoretically onto a nitrocellulose membrane (Western blot). For detection of the protein transferred to the membrane by immunostaining, unspecific binding sites were first blocked by incubation of the membrane in TBS-buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl) containing 5% (w/v) skimmed milk powder. Afterwards, the membrane was incubated in TBS-buffer + 5% skimmed milk powder containing a biotinylated polyclonal antibody directed against EPO at a concentration of 1 $\mu$g/ml. After several washes with TBS-buffer and TBS-buffer + 0.05% Tween-20®, the membrane was incubated in TBS-buffer + 5% (w/v) skimmed milk powder containing streptavidin conjugated alkaline phosphatase (0.75 U/ml). After several washing steps, bound alkaline phosphatase was detected by incubation of the membrane in a color developing buffer (100 mM Tris-HCl, pH 8.8; 5 mM $MgCl_2$, 100 mM NaCl) containing 0.37% (v/v) BCIP (50 mg/ml in DMF) and 0.4% (v/v) NBT (75 mg/ml in 75% DMF).

### 2.6. Determination of residual solvents using gas chromatography

To determine the amount of residual solvents, a gas chromatography system equipped with flame ionization detector (FID) attached to a headspace sampler (HP 5890, Hewlett Packard) was used. As reported earlier [27], the microspheres were dissolved in a standard solution (10 mg of n-heptane in 10 ml of DMF). The temperatures were selected as follows: oven temperature 80°C, injector temperature 150°C and detector temperature 200°C. The column was packed with 5% of Carbowax 20M on Carbopack B. Each microsphere batch was analyzed in duplicate.

### 2.7. Particle size determination

Size distribution of the microspheres was analyzed by dispersing ca. 50 mg of the samples in 5 ml of an aqueous solution of Tween-20® (0.1%). The measurements were carried out by laser light scattering using a Malvern MastersizerX (Malvern Instruments, UK). The 300-mm lens utilized covered a particle size range of 1.2–600 $\mu$m. The weighted average of the volume distribution $D$[4.3] was used to describe the particle size. Each sample was measured in triplicate.

### 2.8. Scanning electron microscopy (SEM)

Cross-sections of the microspheres were obtained by sticking the microspheres to aluminum pans and by freezing these samples in fluid nitrogen. The distinct microspheres were cut using a razorblade. Subsequently, the samples were lyophilized (Edwards Freeze Dryer Modulyo, 24 h, −50°C, 3.3 mbar). These pretreated microparticles were dried in vacuum to remove residual water and subsequently sputter-coated with a gold layer at 25 mA in argon atmosphere at 0.3 hPa for 2 min (Edwards/Kniese Sputter Coater S 150).

The coating procedure was repeated three times. Particle size and surface structure of the microspheres were determined using a scanning electron microscope in vacuum (0.001 mbar) at a voltage of 25 kV (Hitachi S501, Hitachi Denshi, Japan).

### 2.9. In vitro release of EPO

Forty milligrams of the microparticles were suspended in 4 ml of PBS buffer, pH 7.4. The samples were agitated in a rotating bottle apparatus (Rotatherm, Liebisch, Germany) at 30 rpm and 37°C. At defined time intervals, the buffer was withdrawn after centrifugation and 4 ml of fresh medium were added to the samples. The amount of EPO released was detected carrying out size exclusion chromatography (autosampler AS 2000a, L-6000 pump, Merck-Hitachi, Merck, Germany, columns: TSK pre column and TSK G3000 SWXL, Tosohaas). Total EPO content in the release buffer was measured fluorimetrically (excitation: 280 nm, emission: 340 nm; Fluorescenz-Spectrometer F-1000, Merck-Hitachi). The flow rate was 0.7 ml/min using a mobile phase of phosphate buffer containing 0.3 M NaCl. The peak-areas of EPO were calibrated using standards of known concentrations.

### 2.10. In vitro release of FITC-dextran

In vitro release studies of FITC-dextran loaded microspheres were carried out by suspending ca. 40 mg of microparticles in 4 ml of PBS buffer, pH 7.4. The samples were agitated in a rotating bottle apparatus with 30 rpm at 37°C. At predetermined time intervals, the buffer was withdrawn after centrifugation and replaced by 4 ml of fresh medium. Concentration of FITC-dextran in the supernatant was determined fluorimetrically (Section 2.4).

### 2.11. Differential scanning calorimetry

Glass transition temperatures ($T_g$) were measured using a differential scanning calorimeter DSC7 (Perkin Elmer, Germany) [28]. Polymer samples (about 5 mg) were sealed in aluminum pans and heated twice under nitrogen atmosphere. The resulting thermograms covering a range of −10°C to 80°C were recorded at heating rates of 10°C/min. The second run was used for $T_g$ calculation. Calibration of the system was performed using indium and gallium standards.

## 3. Results and discussion

### 3.1. Particle yield

The yield of isolated microspheres varied significantly depending on the microencapsulation process. Since for laboratory scale production often small amounts of microspheres in the milligram range are needed, one advantage of the w/o/w technique is the high yield of about 70% (Table 1), allowing the encapsulation of expensive drugs, such as EPO.

The yield of microspheres obtained using the spray-drying technique is usually lower (ca. 40%). This is attributed to the geometry of the spray-drying equipment leading to a loss of microspheres. Embryonic microspheres, not completely dried, form a film on the walls of the drying chamber, lowering the yield. In our work, we used ca. 1400 mg of PLG generating satisfactory particle yields, whereas for laboratory scale usually about 3 g of a polymer or even more are spray-dried for the preparation of biodegradable microspheres [29,30]. The yields obtained on larger scale equipment can reach 80–90% under production conditions [31].

### 3.2. Microsphere size and morphology

For microspheres prepared by the w/o/w technique, the weighted average of the volume distribution ($D$[4. 3]) varied between 29 and 44 $\mu$m, whereas microspheres prepared by spray-drying had an average diameter of ca. 10 $\mu$m (Table 1). Particle size distribution of the microspheres produced

Table 1

Properties of the microspheres prepared from PLG (monomer ratio of lactide/glycolide 50:50; $M_r$ = 33 kDa)

| Formulation | Microsphere preparation technique | Yield (EPO) (%) | Yield (Dex.) (%) | EPO encaps. eff. (%) | FITC-Dex. encaps. eff. (%) | $D$[4. 3] (EPO) ($\mu$m) | $D$[4. 3] (FITC-Dex.) ($\mu$m) |
|---|---|---|---|---|---|---|---|
| PLGA | w/o/w | 80.0 | 72.3 | 75.97 ± 1.16 | 69.67 ± 5.56 | 41.16 ± 1.79 | 34.47 ± 1.73 |
| PLGA + 0.5% lactide | w/o/w | 78.6 | – | 85.16 ± 0.38 | – | 28.68 ± 0.03 | – |
| PLGA + 1% lactide | w/o/w | 75.7 | – | 73.24 ± 0.00 | – | 30.78 ± 0.19 | – |
| PLGA + 5% lactide | w/o/w | 75.7 | 71.1 | 96.34 ± 1.52 | 82.80 ± 3.05 | 43.63 ± 1.27 | 29.77 ± 0.30 |
| PLGA | SDR | 35.0 | 41.6 | 96.72 ± 5.75 | 86.42 ± 0.99 | 10.30 ± 0.04 | 7.92 ± 0.09 |
| PLGA + 0.5% lactide | SDR | 37.1 | – | 100.96 ± 0.02 | – | 7.12 ± 0.06 | – |
| PLGA + 1% lactide | SDR | 45.0 | – | 100.19 ± 0.75 | – | 9.02 ± 0.05 | – |
| PLGA + 5% lactide | SDR | 40.7 | 36.3 | 97.90 ± 0.77 | 81.60 ± 8.04 | 8.23 ± 0.05 | 10.12 ± 1.26 |

Effects of the manufacturing methods, i.e. w/o/w double-emulsion technique (w/o/w) and the spray-drying method (SDR), on particle yield, encapsulation efficiency, and average particle size measured by laser light scattering. Comparison between rhEPO (EPO)- and FITC-dextran (Dex.) loaded microspheres.

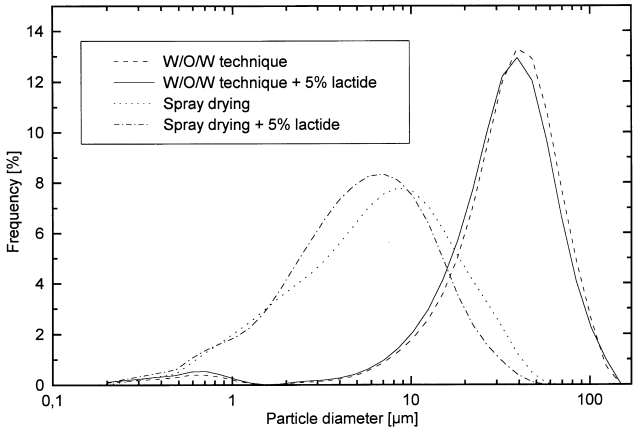


Fig. 1. Laser light scattering measurements of the microspheres prepared by the w/o/w double-emulsion technique and by spray-drying. Influence of monomer addition on particle size distribution.

using the w/o/w technique was narrower than that of spray-dried microspheres (Fig. 1). These size distributions are representative for both microencapsulation processes on the laboratory scale. In both cases, monomer addition did not influence particle size and particle size distribution significantly. In this context, it has to be pointed out that particle size distribution in the dry state could be different from the particle size distribution of a microsphere suspension in water due to a possible swelling of the microsphere matrix.

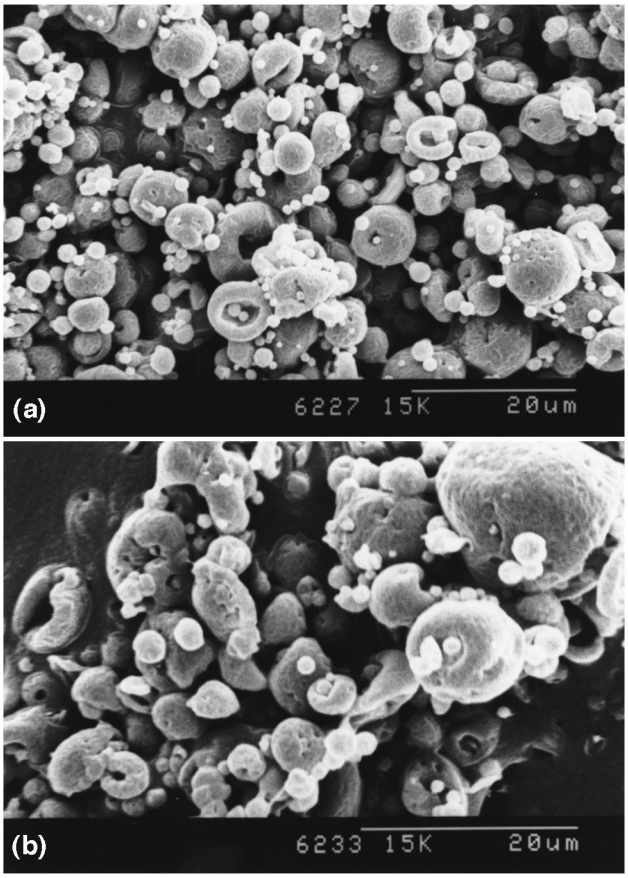


Fig. 2. Scanning electron micrographs of EPO loaded microspheres prepared by spray-drying in the absence (a) and in the presence (b) of DL-lactide monomers.

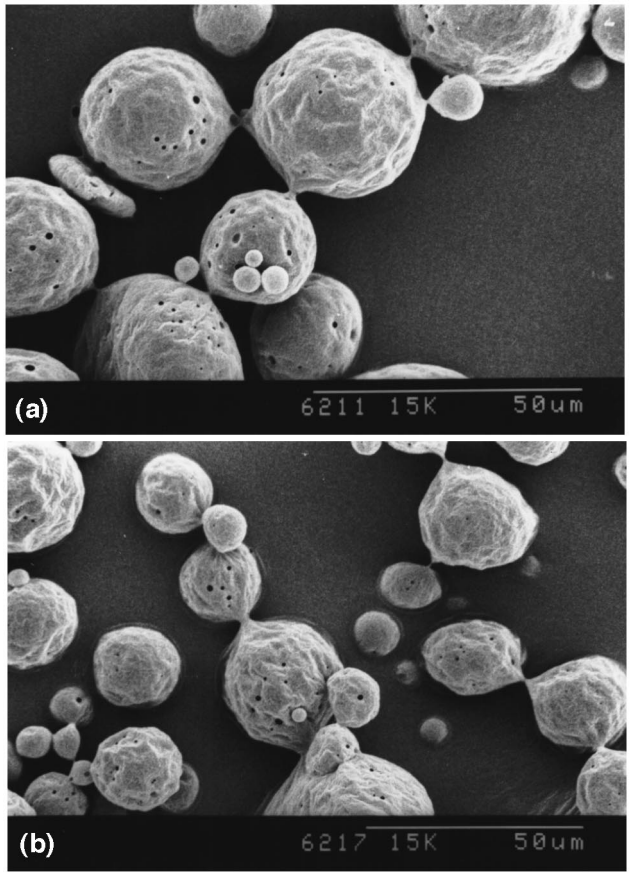


Fig. 3. Scanning electron micrographs of EPO loaded microspheres prepared by the w/o/w double-emulsion technique in the absence (a) and in the presence (b) of DL-lactide monomers.

A number of deformed particles could be observed amongst the microspheres prepared by spray-drying (Fig. 2). This collapsed structure is caused by the rapid evaporation of the organic solvent.

SEM micrographs of the particles prepared by the w/o/w double-emulsion procedure with and without addition of 5% lactide showed no difference in the surface structure (Fig. 3). The microspheres were spherical, wrinkled and a number of pores was observed on their surface. These pores were most likely caused by dissolution of surface located drug particles into the water during microsphere formation and by the internal aqueous phase.

Independent of a monomer addition cross section micrographs of the FITC-dextran loaded particles obtained by the w/o/w double-emulsion method showed microspheres with a porous internal structure (Fig. 4).

### *3.3. DSC measurements*

DSC measurements demonstrated the successful incorporation of DL-lactide only into particles prepared by

spray-drying (Fig. 5). Glass transition temperatures ($T_g$) were decreased depending on the amount of DL-lactide added to the feed. Microspheres prepared by the w/o/w method, however, showed $T_g$s independent of the addition of DL-lactide. This can be explained by the escape of low molecular weight additives into the aqueous PVA phase during the w/o/w process. In contrast, the lactide monomers remain inside the microparticles when using a spray-drying technique.

### 3.4. Encapsulation efficiency of the microspheres

The efficiency of encapsulation of EPO and FITC-dextran was determined by comparing the total amount of drug present in the microspheres with the theoretical drug loading.

Our results demonstrate the successful encapsulation of a protein as an aqueous solution by spray-drying. This microencapsulation technique yielded higher encapsulation efficiencies (96–100%) than the w/o/w double-emulsion method (74–96%) (Table 1). This result can be explained, as already mentioned in case of the encapsulation of DL-lactic acid, by a better suitability to encapsulate hydrophilic drugs by spray-drying.

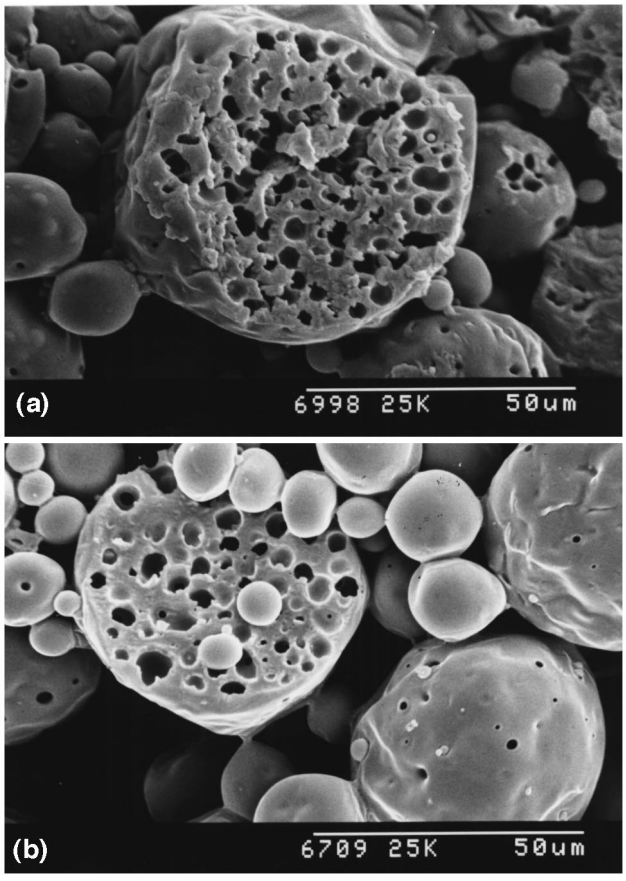


Fig. 4. Cross-section micrographs of FITC-dextran loaded microparticles prepared by the w/o/w double-emulsion technique in the absence (a) and in the presence (b) of DL-lactide monomers.

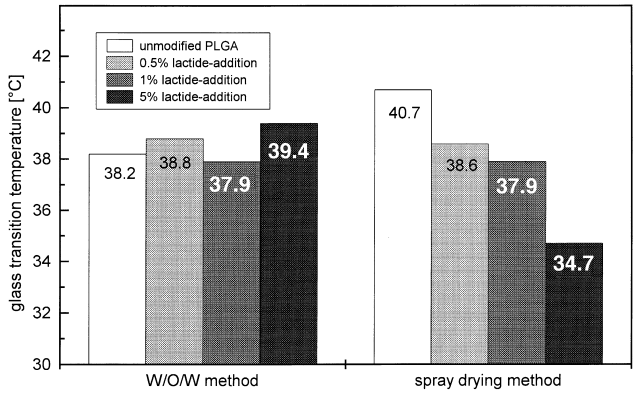


Fig. 5. Differential scanning calorimetric measurements of EPO loaded microspheres prepared by the w/o/w double-emulsion technique and by spray-drying. Effect of different amounts of lactic acid on glass transition temperatures.

The loss of hydrophilic substances into the aqueous phase was also observed by Kamei et al. [32], when they had prepared microspheres by an in-water-drying method. The drug loading efficiency decreased with an increase in the low molecular weight fraction inside the polymer. They hypothesized that during microsphere preparation the drug is leached together with low molecular weight impurities in the external water phase. This loss of low molecular weight substances does not occur during preparation of the microspheres by spray-drying because the feed is spray-dried in a single operation step and no external water phase is present. Thus, the spray-drying process is favorable for the incorporation of hydrophilic drugs such as polypeptides or proteins in biodegradable polymers.

Independent of the preparation method, FITC-dextran loaded microspheres showed no difference in the effective drug loading level (70–79%) (Table 1).

### 3.5. Influence of the manufacturing method on the aggregation of EPO

During microsphere preparation, EPO is subjected to different stress conditions which may induce aggregation or even protein denaturation. Covalent EPO dimerization and aggregation can be rationalized on the basis of a disulfide shuffling of thiol groups of individual EPO molecules resulting in new intermolecular disulfide bonds [33]. Emulsification of the primary w/o emulsion by ultrasound has been identified as the main step inducing covalent aggregation in the case of EPO loaded microspheres prepared by the w/o/w double-emulsion method. The use of a rotor-stator homogenizer may also induce the formation of aggregates. Other manufacturing steps and process parameters such as stirring of the double-emulsion, collection of the microspheres and the final lyophilization seem to have less influence on the aggregation of EPO [25].

In this study, for the first time, EPO was microencapsulated as w/o-emulsion in conjunction with the spray-drying technique. Apart from shear forces due to homogenization

of the w/o emulsion, other process parameters can lead to an increase in the amount of aggregates, e.g. spray-air contact in the nozzle and elevated temperatures (46°C). Thus, in contrast to the w/o/w technique, an increase in the formation of aggregates should be expected.

Surprisingly, it was found that microspheres prepared by spray-drying did not yield high molecular weight aggregates, whereas those prepared by the w/o/w double-emulsion method showed high molecular weight aggregates of EPO remaining in the slots of the SDS-gel (Fig. 6). These results indicate that spray-drying operates under unexpectedly mild process conditions which may allow the native encapsulation of sensitive proteins.

Moreover, addition of monomers does not seem to affect

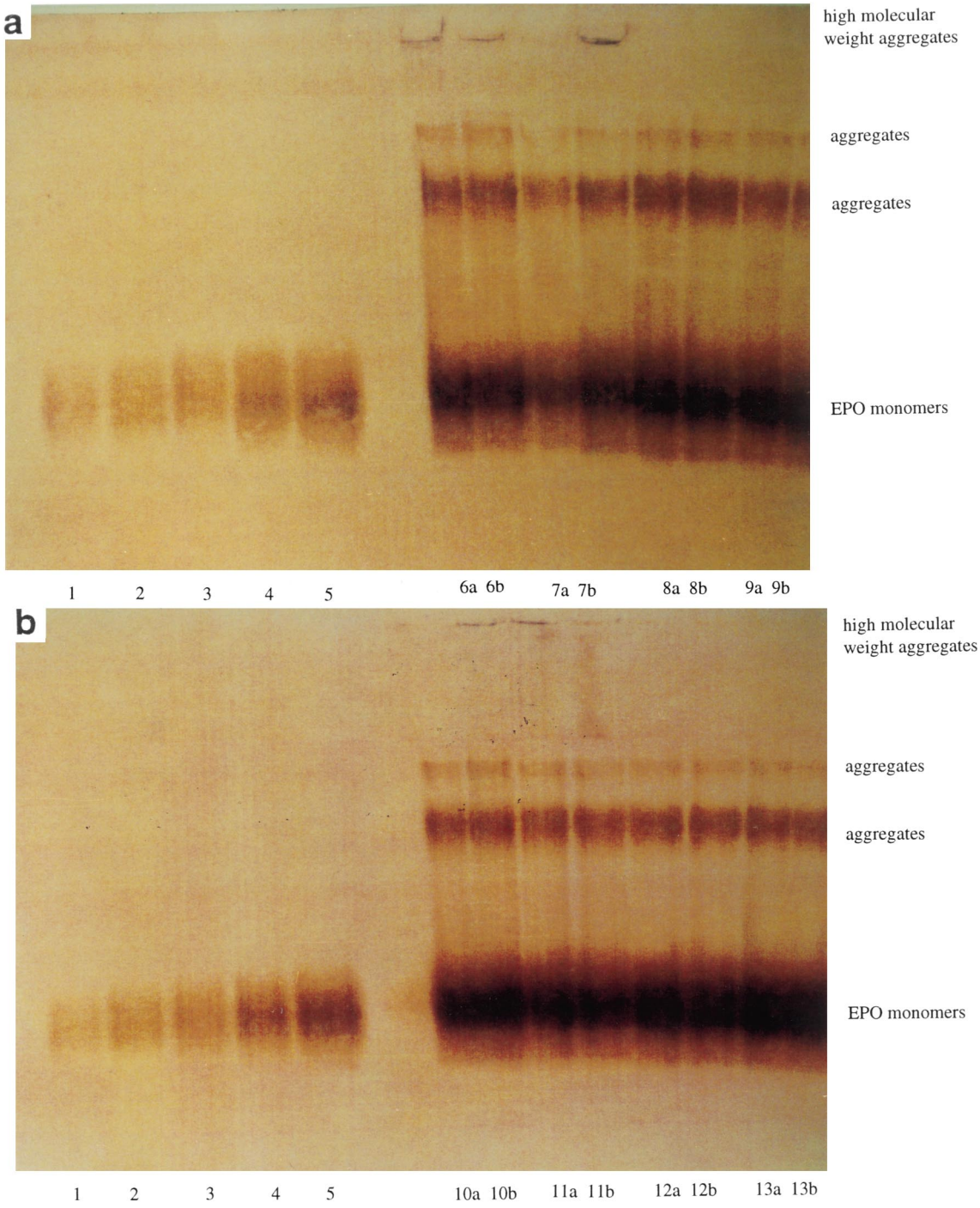


Fig. 6. (a,b) SDS-PAGE of EPO incorporated in PLG microspheres. The influence of the preparation method and the amount of DL-lactic acid inside the particles on the formation of dimers, oligomers and higher molecular aggregates. Each sample was assayed twice. EPO-Standards: 0.025 (1), 0.05 (2), 0.1 (3), 0.25 (4), and 0.5 μg (5); Microsphere batches: w/o/w technique (w/o/w) (6), w/o/w 0.5% DL-lactide addition (11), w/o/w 1% DL-lactide addition (7), w/o/w 5% DL-lactide addition (10), spray-drying (SDR) (8), SDR 0.5% DL-lactide addition (12), SDR 1% DL-lactide addition (13), SDR 5% DL-lactide addition (9).

the amount of aggregates formed inside the dry particles. Independent of the quantity of DL-lactic acid added to the microspheres, all batches showed a similar aggregation tendency for encapsulated EPO. Since DL-lactide was not encapsulated into the microspheres during the w/o/w process, an aggregation of the protein was not induced. The monomer is present in the particles prepared by spray-drying, but the volume of the aqueous phase seems to be too low for an induction of high molecular weight aggregates.

### 3.6. Residual solvents

The content of residual DCM inside the microspheres was analyzed using gas chromatography. Evaporation of DCM is influenced by the manufacturing method [34], the pretreatment of the polymer used for the preparation of the particles, and chiefly by the subsequent drying step by lyophilization or under vacuum after microsphere preparation [35]. In our study, the content of residual solvents in EPO loaded microspheres was determined after lyophilization. Particles prepared by spray-drying and by the w/o/w double-emulsion technique both fulfill the requirements of the USP XXIII for volatile impurities, which specifies a residual DCM content $<$500 ppm [36].

Comparing both microencapsulation techniques, the w/o/w double-emulsion method led to a residual DCM content in the EPO loaded microparticles more than 2 times higher than in the microspheres prepared by spray-drying (Fig. 7). The larger surface area of the spray-dried particles and the elevated temperature (46°C) during microsphere preparation may be responsible for the lower amount of residual DCM inside these microspheres. Thus, with respect to the content of residual DCM in microspheres the spray-drying process in combination with lyophilization seems to offer an interesting alternative to the frequently used w/o/w double-emulsion technique.

Thoma and Schlütermann [37] prepared chinocain loaded microspheres by spray-drying and by a solvent evaporation technique without a further drying procedure. They also found that the spray-dried particles had a lower amount of residual DCM inside the microsphere matrix.

As also illustrated in Fig. 7, addition of DL-lactide had an influence on the final content of residual DCM. Not only the microspheres prepared by spray-drying but also the particles produced by the w/o/w double-emulsion technique showed a significant decrease in DCM content due to the monomer addition to the organic polymer solution.

In the case of the w/o/w double-emulsion method, DL-lactide is not incorporated into the microspheres due to partitioning and extraction into the aqueous PVA solution. During this escape of the monomer in the aqueous phase, an evaporation of the organic solvent seems to be facilitated.

Microspheres prepared by spray-drying showed a different behavior. As shown by the DSC measurements, DL-lactide is co-encapsulated in the microspheres, reducing the glass transition temperature of the microspheres. The particle surface becomes more flexible due to the plastisizing effect of DL-lactide. The diffusion rates of DCM from the microspheres during drying and lyophilization will therefore be increased leading to lower residual amounts of this solvent. Moreover, the affinity of the solvent to the polymer or polymer/monomer mixture has to be taken into account in this context.

### 3.7. Influence of the manufacturing method on in vitro drug release profile

The release profile of polypeptides or proteins from a lipophilic polymer like PLG is usually described as follows [16,38]. In the first phase, protein located at or near the surface of the polymer matrix is released, while during the second phase, the so called ‘pore diffusion phase’, molecules diffuse through a network of newly generated water filled pores. In the third release phase, protein diffusion from PLG microspheres is controlled by the degradation of the polymeric matrix.

In the case of EPO loaded microspheres prepared by the w/o/w double-emulsion method, only one release phase could be observed, namely the initial burst [25]. This observation may be attributed to the formation of aggregates [33] after water penetration into the microspheres matrix. These aggregates are no longer available for a release. In our study, spray-dried microspheres showed a similar release behavior (Fig. 8) and the spray-drying process did not change this general pattern. The amount of EPO released after this initial burst was negligible.

The addition of DL-lactide monomers to the feed led to a significant reduction in the initial EPO burst. During the first 24 h, spray-dried microspheres without and with 0.5% DL-lactide addition, respectively, showed an initial release of about 15% of the encapsulated EPO. The initial burst of the particles with 1% lactide addition was decreased to approximately 10% and 5% lactic acid addition led to an initial release of only 7%. Due to their acidic character, DL-lactide may lower the pH value inside the microspheres. Further-

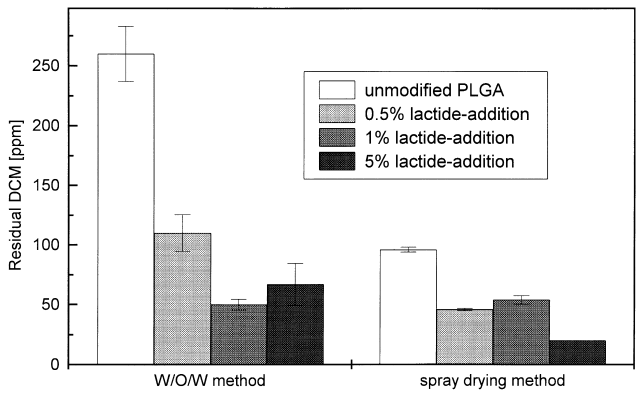


Fig. 7. Residual solvents in microspheres in dependence on manufacturing method and the amount of lactide monomers. The measurements were carried out using a gas chromatography system with flame ionization detector (FID).

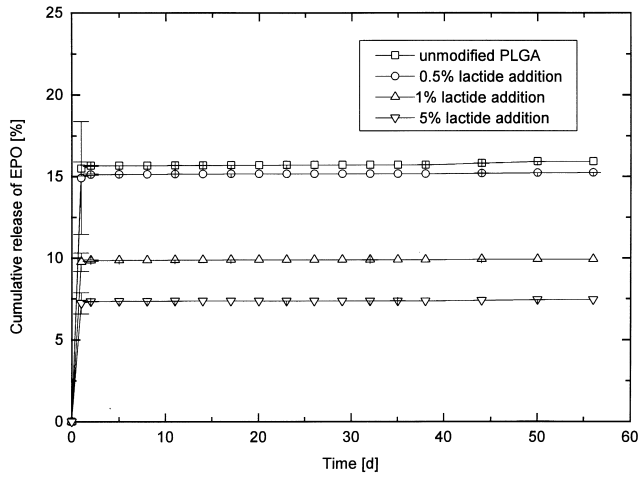


Fig. 8. Release profile of EPO from the spray-dried poly(lactide-co-glycolide) microspheres over a period of 50 days. The amount of EPO released was determined by size exclusion chromatography (SEM).

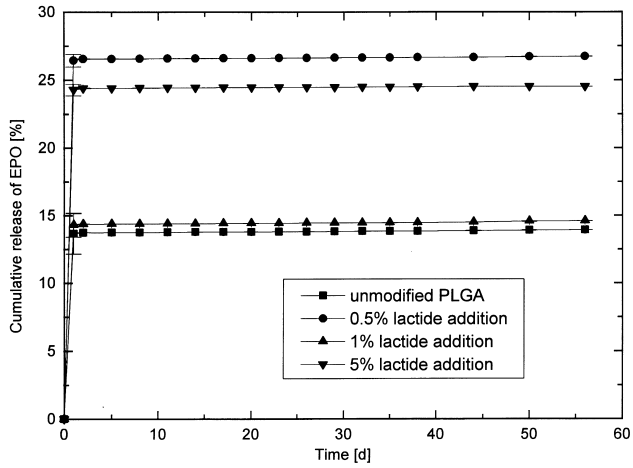


Fig. 9. Release profile of EPO from the poly(lactide-co-glycolide) microspheres prepared by the w/o/w double-emulsion technique over a period of 50 days. The amount of EPO released was determined by size exclusion chromatography (SEM).

more, the increasing number of carboxylic endgroups may cause a more hydrophilic environment in the microsphere matrix and therefore a pronounced water uptake. Both effects may lead to the formation of aggregates which are no longer released into the buffer solution.

In previous experiments [27], an animal study was performed using female sheep. EPO loaded microspheres were prepared from ABA triblock copolymers [28] to determine the amount of active EPO released from the particles. A quantification of the amount of active EPO was not possible reliably, since the formation of EPO antibodies induced after 10 days interfered with the determination of the protein by radioimmunoassay.

Stability of the EPO monomer inside the PLG microspheres during in vitro release was investigated by SDS-PAGE [25,27]. It was found that the amount of EPO aggregates increased as a function of incubation time in the release buffer. Aggregation of EPO may be influenced by the pH value of the buffer medium: a decrease in the pH may lead to an increase in EPO aggregates due to the acidic IEP of EPO. From these points it can be concluded that the addition of lactide decreases the pH during water uptake and, thereby, significantly increases the number of EPO aggregates. Consequently, the initial burst release of EPO from the microspheres was reduced under in vitro conditions.

EPO loaded microspheres produced by the w/o/w technique with 5% monomer addition showed an initial release of about 25%. A similar initial protein burst was observed when preparing the particles with 0.5% DL-lactide addition, whereas the EPO amount released from the microspheres with a 1% and without any lactide addition, respectively, was ca. 14% (Fig. 9).

Although DL-lactide was not incorporated in these microspheres, the initial drug burst has to be interpreted in context with the addition of these low molecular weight excipients. It is possible that the loss of DL-lactide into the aqueous PVA solution leads to a movement of EPO molecules to the surface of the particles. Therefore, EPO particles prepared with 0.5% and 5% monomer addition showed the highest initial burst release.

The relatively low initial amount of EPO released from the microspheres prepared by addition of 1% of DL-lactide (about 15% during the first 24 h) is probably due to a lower effective EPO encapsulation compared to the microsphere formulations prepared with 0.5% and 5% addition of DL-lactide, respectively (Table 1).

Concerning the microspheres prepared by the w/o/w double-emulsion technique, the relatively high initial release of EPO can be interpreted by the porous inner structure of the PLG matrix. Other studies also demonstrated that an increase in the number of pores inside the particles was accompanied by an increase in the initial burst [16,39–41]. The diffusion of EPO from the microsphere surface at

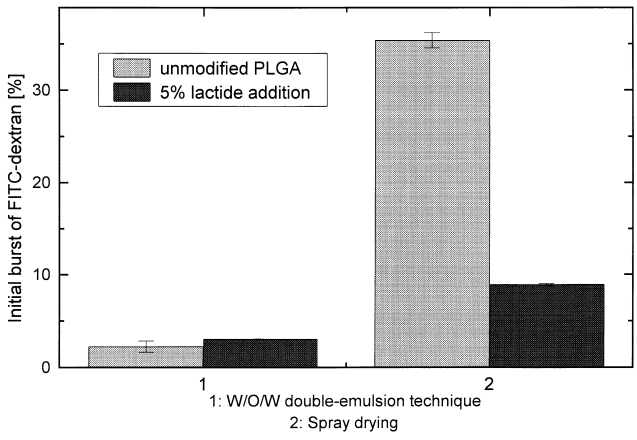


Fig. 10. Initial release of FITC-dextran from poly(lactide-co-glycolide) microspheres prepared by spray-drying and by the w/o/w double-emulsion technique within the first 24 h. Spray-drying (1), w/o/w technique (2).

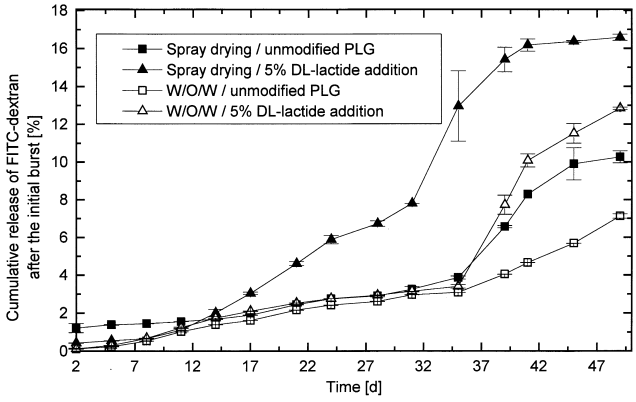


Fig. 11. Release of FITC-dextran from poly(lactide-co-glycolide) microspheres prepared by spray-drying and by the w/o/w double-emulsion technique after the initial burst over a period of 50 days. The amount of FITC-dextran released was determined fluorimetrically.

early times is facilitated and the initial drug release is increased.

FITC-dextran loaded microspheres produced by the w/o/w double-emulsion technique with and without the addition of DL-lactide, respectively, hardly showed any differences in the release pattern, whereas monomer addition led to a significant decrease in the initial FITC-dextran burst in case of the spray-dried microspheres (Fig. 10). Moreover, the continued release of FITC-dextran from the spray-dried microspheres was strongly affected by the addition of DL-lactic acid. The particles prepared without monomer addition showed a lag phase in which the model drug was released at a negligible rate, whereas FITC-dextran release from the microspheres with 5% DL-lactide addition was almost linear (Fig. 11).

Since FITC-dextran does not aggregate in contrast to EPO, the differences in the in vitro release profiles can be explained by a significant decrease of the glass transition temperature caused by monomers inside the spray-dried microspheres which plasticise the polymer matrix. At early time points of the in vitro release process, pores which have already been preformed are blocked and the release rate is strongly reduced. At later time points, a new porous structure is generated by the erosion of the matrix, leading to an enhanced release of FITC-dextran.

## 4. Conclusions

Our comparative study of EPO microencapsulation using the spray-drying method and the w/o/w double-emulsion technique in conjunction with PLG has demonstrated several unexpected features of these microencapsulation methods.

EPO microparticles prepared by spray-drying yielded a higher entrapment of the protein, a lower content of high molecular weight EPO aggregates and lower residual amounts of DCM than those prepared by the w/o/w technique. Moreover, addition of lactide monomers drastically reduced the initial drug burst. The relatively low particle yield obtained by spray-drying is clearly influenced by the geometry of the equipment and cannot be improved by optimization of the process conditions.

The main advantage of the w/o/w double-emulsion technique can be seen in maintaining acceptable encapsulation yields and efficiencies. Aggregation of EPO and residual DCM were found to be higher than in case of the spray-drying process.

The in vitro release of EPO from PLG microspheres was influenced only in the initial release phase by the encapsulation technique, whereas the entire FITC-dextran release profile was affected depending on the manufacturing process. Interactions between EPO and the polymer and formation of insoluble EPO aggregates during the initial release phase have most likely contributed to the unfavorable in vitro release profiles. To overcome this problem, faster swelling biodegradable polymers would be clearly desirable, allowing a more continuous release of this sensitive protein from parenteral delivery systems.

Our studies highlight the importance of protein stability and compatibility with biodegradable parenteral delivery systems. The aggregation behavior of proteins is not completely understood and extrapolations from model compounds may not be feasible for therapeutically relevant proteins.

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