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# Preparation and characterisation of Poly (D,L-lactic-co-glycolic acid) microspheres containing desferrioxamine

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**Abstract**

The iron chelator desferrioxamine (DFO) demonstrates antimalarial activity upon multiple or continuous parenteral administration. The aim of this study was to develop a controlled release system for the water-soluble drug DFO based on poly (D,L-lactic-co-glycolic acid) (PLGA). PLGA microspheres containing DFO were prepared using the water-in-oil-in-water (w/o/w) solvent evaporation technique. The influence of formulation parameters (internal phase volume and polymer concentration) on the microsphere characteristics (particle size, porosity, encapsulation efficiency of DFO) and DFO release profile were investigated. An increasing internal phase volume at a fixed polymer concentration resulted in an increasing volume weight mean diameter, an increasing porosity and a decreasing encapsulation efficiency. An increasing PLGA concentration at a fixed internal phase volume resulted in a decreasing porosity of the particles with an increased volume weight mean diameter and encapsulation efficiency of DFO. DFO was released from the microspheres in an initial burst (controlled by formulation parameters) followed by marginal release and a pulse release at the time the microspheres start to dissolve (20–30 days). The release of DFO was incomplete after complete dissolution of the microspheres. This is probably caused by the instability of DFO at low pH, generated upon degradation of the PLGA matrix. © 1997 Elsevier Science B.V.

*Keywords:* Poly (D,L-lactic-co-glycolic acid); Microspheres; Desferrioxamine; Water-in-oil-in-water emulsion; Controlled release; Malaria

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## 1. Introduction

Malaria is still one of the major infectious diseases in the tropics, resulting in about 1–2 million deaths (mainly children) each year (Wernsdorfer and Wernsdorfer, 1992). The prevention and treatment is hampered by an increasing spread of multi-drug resistant strains of *Plasmodium falciparum*, urging the need for new chemotherapeutic strategies (Clyde, 1987; Postma et al., 1996). Iron-chelating agents, such as desferrioxamine (DFO), have demonstrated antimalarial activity in vitro and in vivo, probably by depriving the parasite of essential iron (Chabantchik, 1995; Mabeza et al., 1996). The application of DFO in antimalarial therapy, however, is greatly limited by its low half-life in plasma ($t_{1/2} = 5–10$ min) and poor absorption following oral administration (Keberle, 1964). Multiple or continuous parenteral administration is therefore required to obtain the therapeutic effect.

The aim of this study was to investigate whether microspheres based on poly (D,L-lactic-co-glycolic acid) (PLGA) could serve as drug delivery systems for the controlled release of the water-soluble drug DFO. PLGA copolymers have been widely studied as drug delivery systems because of their biodegradable and biocompatible characteristics (Lewis, 1990). Several studies report on the encapsulation of hydrophilic drugs into the microspheres using the water-in-oil-in-water (w/o/w) solvent evaporation technique (Ogawa et al., 1988; Conway and Oya Alpar, 1996). Different release profiles and release rates were achieved by varying both process and formulation parameters such as mixing rate, volume of internal and organic phase and polymer concentration (Cohen et al., 1991; Sturesson et al., 1993; Sah et al., 1995).

In the present study, PLGA microsphere characteristics (size and size distribution, porosity and encapsulation efficiency of DFO) were studied as a function of internal phase volume and polymer concentration. Moreover, the DFO release profiles were studied.

## 2. Materials and methods

### 2.1. Materials

D,L-Lactic-co-glycolic acid (PLGA) w/w% 50/50, intrinsic viscosity 0.58 dl/g, was obtained from Purac Biochem. B.V. Gorinchem, The Netherlands. Poly (vinylalcohol) (PVA 87–89% hydrolysed, $M_n$ 13–23 kDa) was supplied by Aldrich, Milwaukee, USA. DFO was obtained as the mesylate salt, Desferal, from Ciba Geigy AG, Basel, Switzerland. Dichloromethane was obtained from Merck, Darmstadt, Germany. Acetonitrile, HPLC grade was purchased from Biosolve B.V. Barneveld, The Netherlands.

### 2.2. Methods

#### 2.2.1. Molecular weight of PLGA

The molecular weight of PLGA was determined by gel permeation chromatography on a system consisting of a Waters 510 HPLC pump and 410 differential refractometer (Waters Associates, Milford, MA) with three thermostated (35°C) Shodex KF series columns (KF 800P $4.6 \times 10$ mm, precolumn; KF 801 $8 \times 300$ mm, exclusion limit $1.5 \times 10^3$, KF 80M $8 \times 300$ mm, exclusion limit $2 \times 10^7$; SHOWA Denko, Tokyo, Japan). Chloroform was used as the mobile phase. The flow rate was 1.0 ml/min. The columns were calibrated using polystyrene standards of known molecular weights. The chromatograms were analysed with Millennium 2010 V. 2.0 software (Waters Associates, Milford, MA).

#### 2.2.2. Preparation of PLGA microspheres

PLGA microspheres were prepared using a water-in-oil-in-water (w/o/w) solvent evaporation technique essentially according to Sah et al., 1995. The procedure is schematically shown in Fig. 1. Briefly, PLGA was dissolved in dichloromethane (DCM, 20 ml) and DFO was dissolved in water (200 mM, pH $\pm$ 4). The DFO solution was added to the PLGA solution and the mixture was emulsified for 10 s with an ultraturrax stirrer (Ika-Werk type 18/10). Subsequently, the resulting primary emulsion was added to 200 ml of an aqueous PVA solution (1% PVA w/v, 193 mM

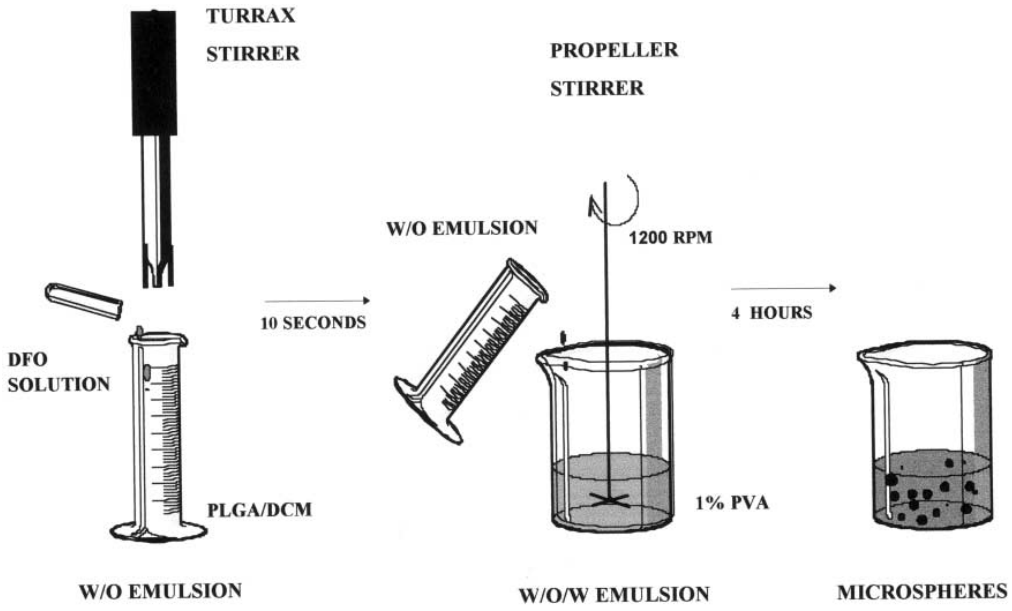


Fig. 1. Schematic presentation of the preparation of DFO PLGA microspheres by the water-in-oil-in-water solvent evaporation technique.

NaCl). After stirring the w/o/w emulsion (secondary emulsion) for four hours at room temperature with a propeller type stirrer, the microspheres were harvested by centrifugation. Non-encapsulated DFO was removed by washing the microspheres three times with water. After the third time, the microspheres were collected and freeze-dried.

The influence of internal phase volume and polymer concentration on the microsphere characteristics and DFO release profiles was studied by varying internal phase volume or polymer concentrations during preparation. In the first series of experiments, the volume of the DFO solution (internal phase) was 0.5, 1, 3 or 6 ml, while the polymer concentration and volume were fixed (1.5 g PLGA in 20 ml DCM). In the second series of experiments, the PLGA concentration was 1, 2, 3 or 4 g in 20 ml DCM while the internal phase was kept at a constant volume and concentration of DFO (1 ml, 200 mM DFO).

*2.2.3. Size and size distribution*

The average diameter (number weight diameter ($\Sigma nd/\Sigma n$) and volume weight diameter ($\Sigma nd^4/\Sigma nd^3$) and size distribution of the prepared microspheres were measured using a laser blocking technique (Accusizer™, model 770, Santa Barbara, CA, USA).

*2.2.4. Surface characteristics and porosity*

The surface characteristics of the microspheres were studied using Scanning Electron Microscopy (CamScan, Cambridge). The samples were prepared by sputter coating with Au. Of some selected samples the porosity and density of the microspheres were determined by mercury intrusion porosimetry (Micro Meritics type Autopore II 9220, Norcross USA).

*2.2.5. Analysis of DFO by HPLC*

The concentration of DFO was determined by HPLC analysis essentially according to the method described by Horst van der et al., 1986. Briefly, standard solutions of DFO (1.5 mM) and $FeCl_3.6H_2O$ (3 mM) were prepared in water. To different volumes of the DFO standard solution (maximum of 0.5 ml) 0.5 ml $FeCl_3$ was added and the volume was adjusted to 1 ml with water. A total volume of 50 $\mu$l of the standard solutions

Table 1
Recovery of DFO (%) for different batches of PLGA microspheres

| Internal phase volume (ml) | Polymer (% g/ml) | DFO recovery (%) | | | |
|---|---|---|---|---|---|
| | | PVA fraction | Wash fractions | Microspheres | Total |
| 0.5 | 7.5 | 28.1 ± 6.8 | 9.7 ± 2.2 | 44.6 ± 23.3 | 82.4 ± 32.3 |
| 1 | 7.5 | 30.6 ± 0.6 | 25.2 ± 5.1 | 23.0 ± 5.0 | 78.8 ± 10.7 |
| 3 | 7.5 | 86.3 ± 1.3 | 5.8 ± 0.3 | 2.5 ± 0.4 | 94.6 ± 2.0 |
| 6 | 7.5 | 87.5 ± 0.5 | 5.5 ± 1.0 | 0.9 ± 0.3 | 93.9 ± 1.8 |
| 1 | 5 | 71.6 ± 1.4 | 16.3 ± 0.8 | 9.5 ± 1.4 | 97.4 ± 3.6 |
| 1 | 10 | 16.6 ± 1.6 | 7.7 ± 2.0 | 67.2 ± 11.4 | 91.5 ± 15.0 |
| 1 | 15 | 13.3 ± 2.3 | 0.7 ± 0.3 | 73.3 ± 11.5 | 87.3 ± 14.1 |
| 1 | 20 | 5.5 ± 2.3 | 0.5 ± 0.3 | 71.8 ± 16.6 | 77.8 ± 19.2 |

The batches were prepared in duplicate.

was injected onto a RP 18 column (Merck, Darmstadt, Germany). The mobile phase consisted of phosphate buffer (20 mM $KH_2PO_4$, 2 mM EDTA, pH 6.6) and acetonitrile (85:15, v/v) and the flow rate was 1.0 ml/min. DFO forms a complex with Fe(III) with an absorption maximum around 440 nm. The DFO calibration curve was linear from 5 to 800 $\mu$M.

The amount of DFO in the samples was determined in the same way. The core loading of DFO in the microspheres was determined as follows. Approximately 50 mg (accurately weighed) of DFO microspheres was dissolved in 500 $\mu$l DCM. After addition of 500 $\mu$l of water, the two-phase system was vortexed for 2 min. Following centrifugation, the upper layer containing the DFO was removed. This extraction was repeated two times and the concentration of DFO in each fraction was measured.

*2.2.6. Encapsulation efficiency of DFO*

The percentage encapsulation efficiency of DFO is defined as follows: Encapsulation efficiency (%) = 100 × (recovered $\mu$mol DFO per g microspheres/loaded $\mu$mol DFO per g polymer).

*2.2.7. In vitro release studies*

The release of DFO from the microspheres was established by suspending DFOloaded microspheres (100 mg) in 1 ml of phosphate-buffered saline (PBS; 10 mM $KH_2PO_4$, 0.9% NaCl, 0.02% sodium azide, pH 7.2). The sample tubes were incubated at 37°C under continuous shaking. Periodically, the microspheres were centrifuged and the supernatant was removed. The pellet was resuspended in 1 ml of PBS again. The samples were stored at −20 or 4°C before analysis. The amount of DFO released from the microspheres was determined by HPLC analysis as described before.

*2.2.8. Stability of DFO at pH 7.2 and 2.5*

DFO (3.4 mM) was dissolved in PBS (pH 7.2) and the solution was incubated at 37°C. For stability at low pH, the pH was adjusted to 2.5 with D,L-lactic acid (Eur. Pharm BP, Janssen) and the solution was incubated at 4 or 37°C. Samples were taken for 6–8 days and the concentration of DFO in the samples was determined.

## 3. Results

### 3.1. Microsphere characteristics

*3.1.1. Recovery of DFO*

The recovery of DFO (%) in the microspheres, the PVA fraction and in the washfractions for the different batches of PLGA microspheres, is shown in Table 1. The overall drug recovery was about 75–95%.

*3.1.2. Size and size distribution*

A representative example of the volume-weight diameter distribution of a batch of PLGA microspheres containing DFO (7.5% PLGA, 6 ml DFO) is shown in Fig. 2.

Fig. 3a and b show the effect of internal phase volume and polymer concentration on particle size. An increasing volume of the aqueous DFO solution (internal phase volume) at a fixed PLGA concentration and volume (7.5% PLGA; 20 ml DCM) resulted in an increased volume weight mean diameter (from 27 to 48 $\mu$m) whereas the number weight diameter was not affected (Fig. 3a). This means that the particle size distribution broadened with an increasing internal phase volume. When the concentration of PLGA in DCM was increased at a fixed internal phase volume (1 ml), the volume weight mean diameter also increased (from 27 to 88 $\mu$m), while the number weight mean diameter was not affected, again indicating that the distribution broadened (Fig. 3b).

*3.1.3. Surface characteristics and porosity*

PLGA microspheres containing DFO were spherical as shown by SEM analysis (Fig. 4). The porosity of the microspheres dependend on the formulation parameters; large pores could be seen in microspheres prepared at low polymer concentrations or high internal phase volumes (Fig. 4a and b), while particles with a marginal porosity were obtained at high polymer concentrations or low internal phase volumes (Fig. 4c and d).

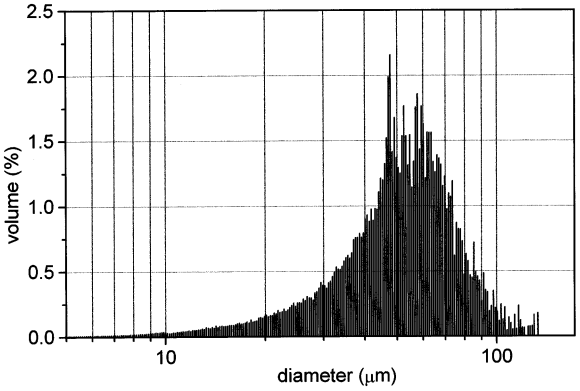


Fig. 2. Volume weight diameter distribution of PLGA microspheres (7.5% PLGA/6 ml DFO) prepared using the w/o/w solvent evaporation technique.

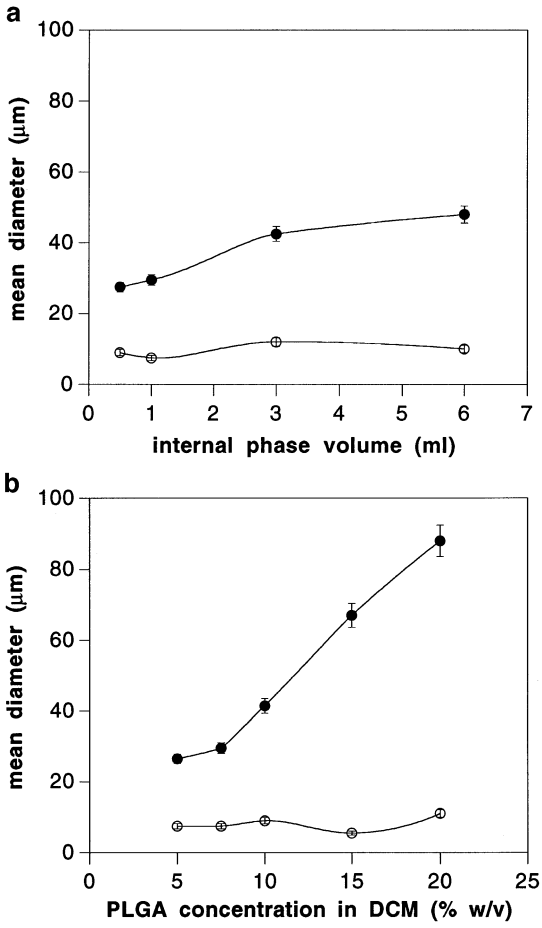


Fig. 3. Effect of the internal phase volume (a) and PLGA concentration (b) on the mean particle size of DFO-loaded PLGA microspheres. (○) number weight mean, (●) volume weight mean. The batches were prepared in duplicate.

The pore volume and pore size distribution of some selected samples were quantified by mercury intrusion porosimetry (Table 2 and Fig. 5). In line with SEM analysis, a high internal phase volume resulted in microspheres with large pores. The porosity of the microspheres decreased with an increasing polymer concentration. For highly porous particles, pores with a diameter between 1 and 7 $\mu$m were detected, whereas for particles with a low porosity, the diameter of the pores ranged from 0.005 to 0.1 $\mu$m.

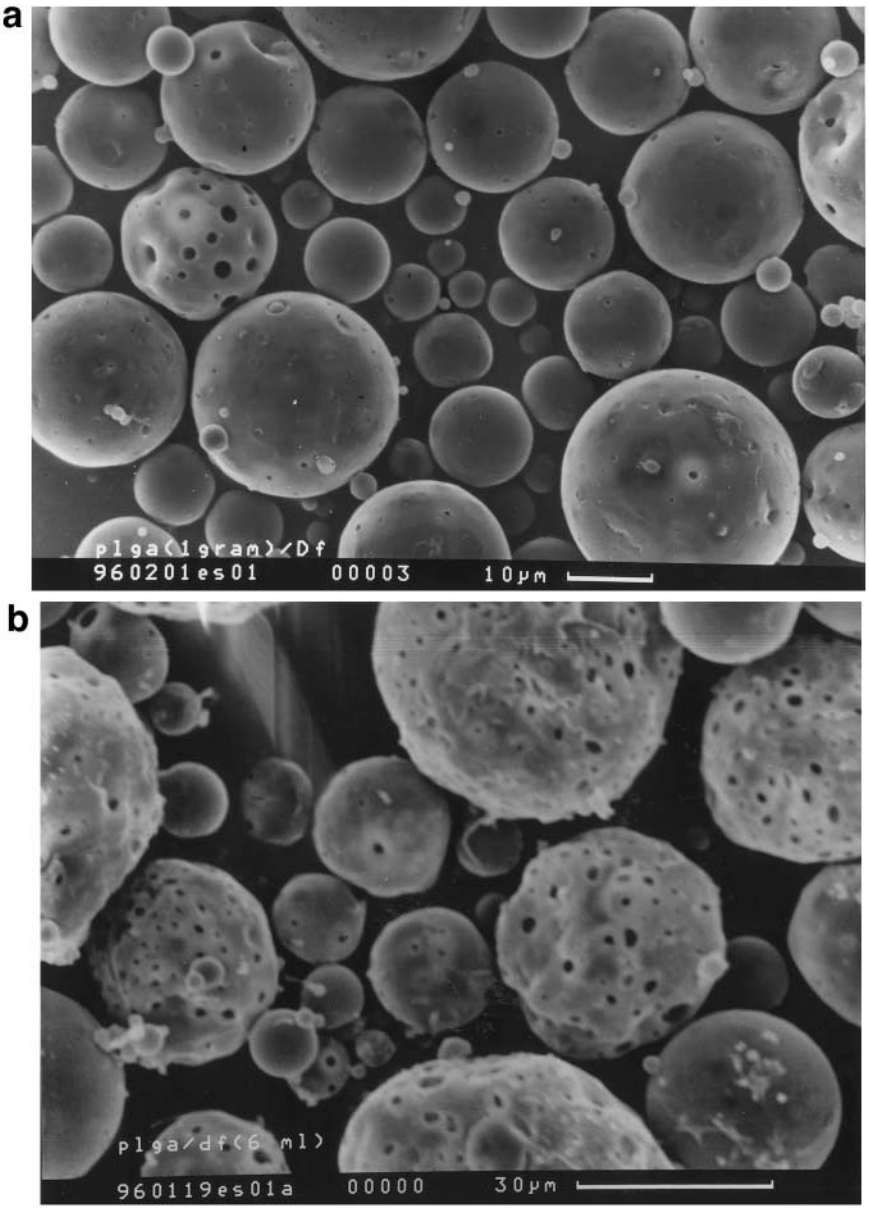


Fig. 4. (a–d) Representative scanning electron micrographs of DFO PLGA microspheres with different formulation parameters. (a) 5% PLGA/1 ml DFO; (b) 7.5% PLGA/6 ml DFO; (c) 15% PLGA/1 ml DFO; (d) 7.5% PLGA/0.5 ml DFO.

### *3.1.4. Encapsulation efficiency of DFO*

Fig. 6a and b show the effect of internal phase volume and polymer concentration on the core loading and encapsulation efficiency of DFO, respectively. An increasing internal phase volume resulted in both a decreasing encapsulation efficiency and core loading (Fig. 6a). On the other hand, an increasing PLGA concentration resulted in an increasing encapsulation efficiency of DFO up to 70% (Fig. 6b). The core loading

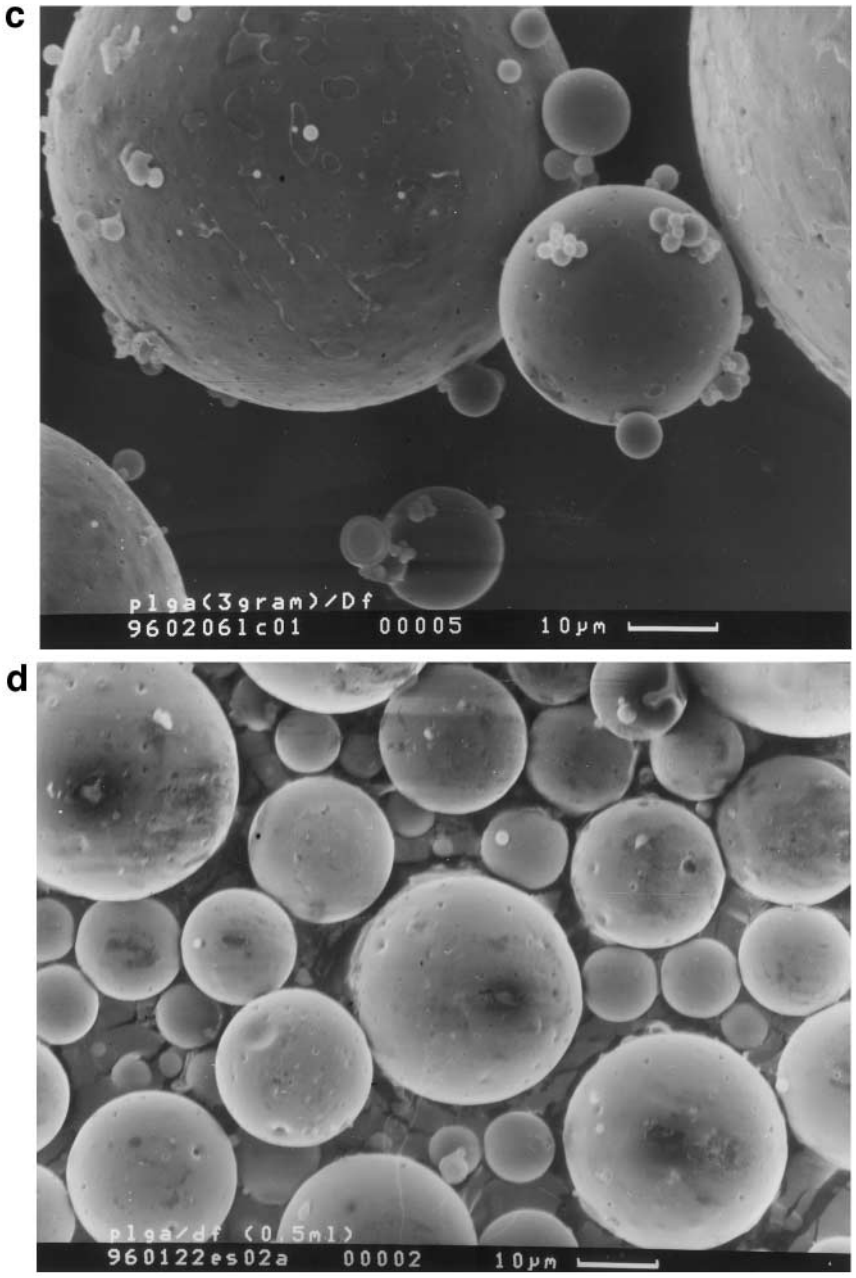


Fig. 4.

increased up to 10% PLGA but decreased at higher concentrations of PLGA. The nonencapsulated DFO was almost exclusively present in the PVA phase (Table 1).

### 3.2. In vitro release studies

The release profiles of DFO from the microspheres as a function of internal phase volume

Table 2
Pore volume (ml/g) and average pore diameter ($\mu$m) of PLGA microspheres as a function of internal phase volume (ml) and polymer concentration (g/ml)

| Volume DFO (ml) | Concentration PGLA (% g/ml) | Pore volume (ml/g) | Average pore diameter ($\mu$m) |
|---|---|---|---|
| 0.5 | 7.5 | nd[a] | nd |
| 1 | 7.5 | nd | nd |
| 3 | 7.5 | 1.89 | 0.22 |
| 6 | 7.5 | 0.76 | 1.57 |
| 1 | 5 | 1.05 | 0.13 |
| 1 | 10 | nd | nd |
| 1 | 15 | 0.32 | 0.03 |
| 1 | 20 | 0.18 | 0.02 |

[a]Not determined because not enough material was available.

and polymer concentration are shown in Fig. 7a and b. Three phases can be distinguished in the release profile of the different batches. First, a burst release (within 2 h) is observed from 3 to 95% of the encapsulated amount of DFO. Secondly, DFO is released at a very low rate (about 1.5–5% of the encapsulated amount) until day 20. In the third phase between 20 and 30–35 days, a pulse release (1–10% of the encapsulated amount) occurred which coincides with the dissolution time of the microspheres (visual inspection). Suprisingly, the total release of DFO (burst + sustained + pulse release) did not reach 100% after complete dissolution of the microspheres.

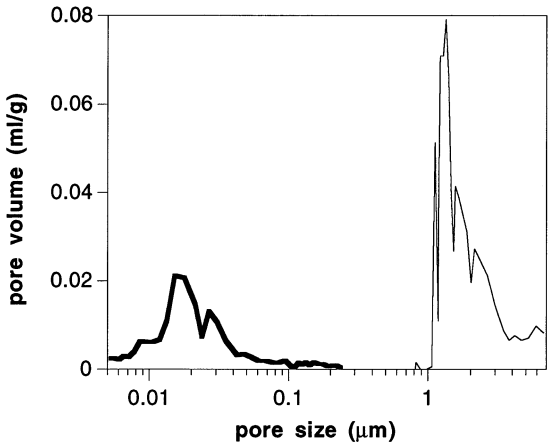


Fig. 5. Pore size distribution of PLGA microspheres containing DFO. 20% PLGA/1ml DFO (thick rule), 7.5% PLGA/6 ml DFO (light rule).

The initial burst release was highly dependend on the formulation parameters. When the internal phase volume decreased from 6 to 0.5 ml, the burst release decreased from 95 to 40% (Fig. 7a). An increasing PLGA concentration from 5 to 20% resulted also in a decreased burst release, from 40 to 3% (Fig. 7b). The other two phases were not affected by the formulation parameters.

### 3.3. Stability of DFO at pH 7.2 and 2.5

The stability of DFO was studied at pH 7.2 (37°C) and pH 2.5 (4 and 37°C) (Fig. 8). DFO was stable for at least 8 days at pH 2.5 and 4°C, while the concentration of DFO decreased to 80% at pH 7.2 and 37°C. However, when the pH was decreased to pH 2.5 at 37°C, DFO degraded rapidly ($T_{1/2} = 2.5$ days).

## 4. Discussion

The present study demontrates that the w/o/w solvent evaporation technique is a simple and suitable technique to encapsulate a highly water-soluble drug such as DFO, with a good batch to batch reproducibility with respect to the particle characteristics (size and size distribution, porosity, encapsulation efficiency of DFO and DFO release profile).

An increasing volume of the internal phase at a fixed polymer concentration and volume, resulted in a somewhat higher volume weight mean diame-

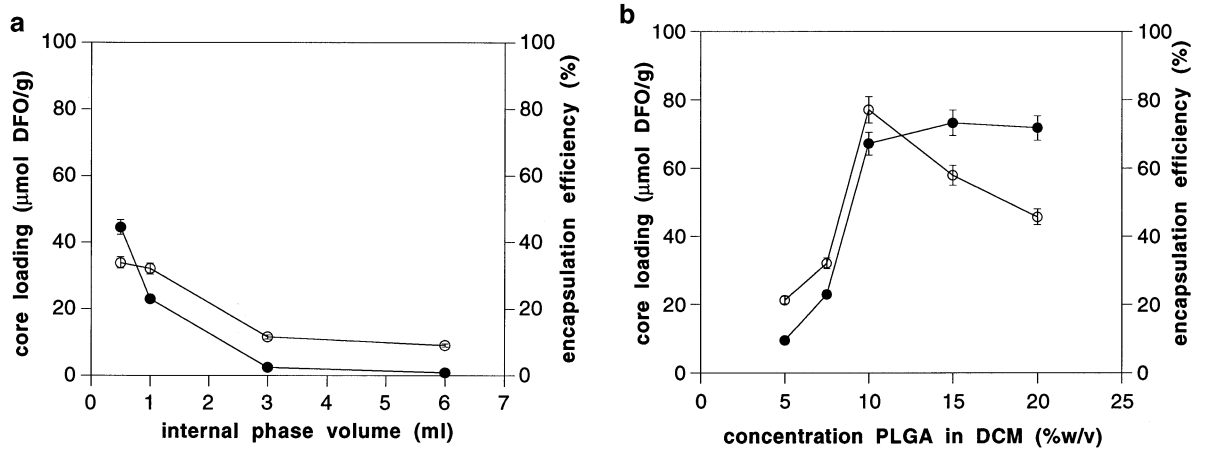


Fig. 6. Effect of internal phase volume (a) and PLGA concentration (b) on the encapsulation efficiency of DFO (%) (○) and the core loading ($\mu$mol DFO per g PLGA microspheres) (●). The batches were prepared in duplicate.

ter of the microspheres (Fig. 3a), an increasing porosity (Table 2 and Fig. 4b) and a decreasing encapsulation efficiency of DFO (Fig. 6a). Similar trends regarding an increasing particle size with increasing internal phase volume were also observed by Jeffery et al., 1993. However, no obvious explanation is available. The observed increase in porosity of the microspheres and simultaneously decrease in encapsulation of DFO can be explained by a demixing of the primary emulsion during the formation of the secondary emulsion. This is in agreement with results obtained by Alex and Bodmeier, 1990 for the highly water-soluble drug pseudoephedrine HCl. The presence of a direct relationship between the stability of the primary emulsion and the final structure of the microspheres was demonstrated by Nihant et al., 1994.

When the PLGA concentration was increased at a fixed internal phase volume, nonporous particles (above 10% PLGA, Table 2 and Fig. 4c) with an increased size (Fig. 3b) and an increased encapsulation efficiency of DFO (Fig. 6b) were formed. At increasing polymer concentrations, the viscosity of the polymer solution is increased, resulting in larger droplets of the secondary emulsion and thus an increased particle size after evaporation of the organic solvent (Spenlehauer et al., 1986; Sturesson et al., 1993). The increased viscosity of the organic phase probably also stabilises the film around the internal water droplets, preventing the formation of pores and loss of drug (Alex and Bodmeier, 1990).

All batches showed a burst release which was dependent on the formulation parameters. The high initial burst release of drug from microspheres is most probably caused by leaching of drug through water-filled pores and has been reported before for water-soluble drugs (Bodmeier et al., 1989, 1991; Niwa et al., 1993). The observed burst release of DFO was related with the observed decreasing porosity of the particles and could be reduced to 3% at 20% PLGA and 1 ml DFO solution (Fig. 7b). The marginal sustained release of DFO in the following twenty days is most likely caused by a low solubility of DFO in the PLGA matrix or by drug-matrix interactions. DFO contains a primary amino group, and interactions between amino groups of entrapped compounds and free carboxyl groups of the free polymer in the matrix was suggested before (Bodmeier et al., 1989; Sturesson et al., 1993). The core loading of DFO varied from 20 to 80 $\mu$mol DFO per g microspheres. GPC analysis showed that the $M_n$ of PLGA was around 25 000 g/mol, corresponding with 40 $\mu$mol free carboxylic acids per gram polymer. This indicates that initially almost an equal molar ratio of carboxylic acid endgroups and amino groups is present. Upon degradation, the amount of carboxylic acid end groups increases which would facilitate the interaction

between DFO and the matrix and therefore hamper the release. The pulse release observed between day 20 and 30 results from matrix dissolution. However, the release of DFO was incomplete following complete dissolution of the microspheres. Park et al., 1995 demonstrated that the pH can decrease dramatically to pH 3 in the release medium of degrading microspheres, while the pH in the matrix can even be lower. In a stability experiment, it was shown that DFO was rather labile at pH 2.5 and 37°C ($T_{1/2} = 2.5$ days) (Fig. 8). On the other hand, DFO was more or less stable during incubation at 4°C and at 37°C, pH 7.2. This indicates that the incomplete release of DFO from the microspheres can be explained by the degradation of DFO due to the low pH in the release medium and/or degrading matrix.

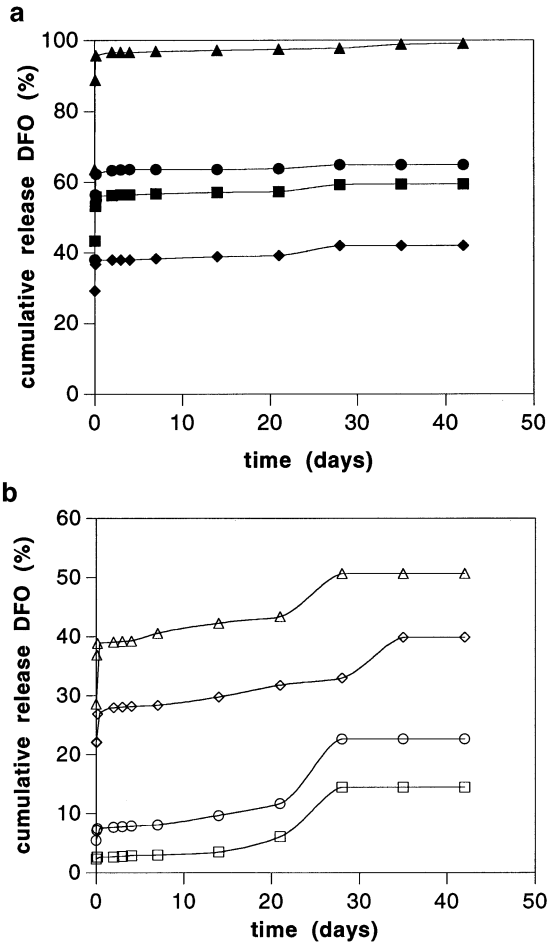


Fig. 7. Effect of internal phase volume (7a) and PLGA concentration (7b) on the release pattern of DFO. 0.5 ml (▲), 1 ml (●), 3 ml (■), 6 ml (◆), 5% (△), 10% (◇), 15% (□), 20% (○).

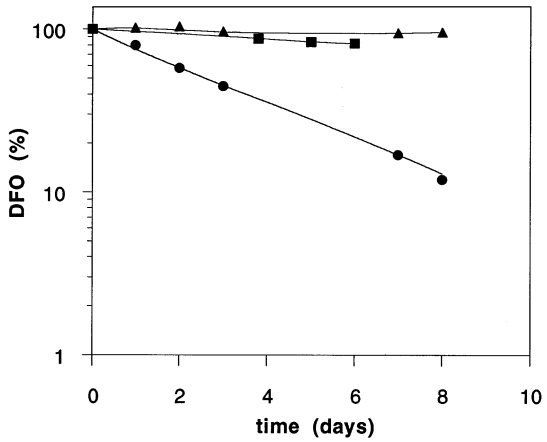


Fig. 8. Stability of DFO. pH 7.2 at 37°C (■), pH 2.5 at 4°C (▲), pH 2.5 at 37°C (●).

## 5. Conclusions

This study shows that the water-soluble drug DFO can be encapsulated into PLGA microspheres using the w/o/w solvent evaporation technique with a high encapsulation efficiency ($\pm$ 70%). The release profile of DFO was characterised by an initial burst release (depending on the formulation parameters) and marginal sustained release, probably due to ionic drug-matrix interactions. A pulse release was observed upon dissolution of the microspheres, however, release of DFO was incomplete suggesting that part of the DFO was degraded at the low pH generated upon degradation of the PLGA matrix.

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