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RESEARCH PAPER

Microsphere Preparation Using the Untoxic Solvent Glycofurol

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ABSTRACT

Purpose At laboratory scale, the most widely applied methods in therapeutic microencapsulation are based on emulsification using organic solvents. Here, glycofurol was proposed as non-toxic solvent to circumvent these inconveniences using a quasi-emulsion extraction method for the preparation of poly (lactide-co-glycolide) microspheres.

Methods Matrix polymer and lipophilic drug were dissolved in glycofurol, building the internal phase, and emulsified under stirring into various external phases before microspheres could be obtained and characterized for their pharmacotechnical properties.

Results Microspheres were spherical with particle diameters around 100 to 200 μm and also showed a monomodal particle size distribution. The internal sponge-like structure was related to an incomplete glycofurol extraction (residual content: 16.9% ± 1.6% of total particle mass), which is, however, no toxicological drawback. The encapsulation rate of several model compounds increased with rising partition coefficient (Ibuprofen: 1.9% ± 0.6%, Ritonavir: 11.2% ± 0.4%, Lopinavir: 14.0% ± 2.2%, Sudan III: 28.3% ± 0.4%) due to the decreasing solubility in the external phase. *In-vitro* release kinetics were varying from a complete release after 4 h for Ritonavir to 3 weeks for Sudan III.

Conclusion This new method was confirmed to be suitable for the preparation of microspheres with the use of a non-toxic solvent and to allow for the entrapment of lipophilic actives and their controlled release.

KEY WORDS glycofurol · microencapsulation · microspheres · quasi emulsion · solvent extraction

INTRODUCTION

The easiest and most frequently used methods for preparation of microparticles at laboratory scale are emulsification processes followed by solvent evaporation or extraction processes (1–5). O/W techniques are preferred to entrap lipophilic drugs. Matrix polymer and lipophilic drugs are dissolved in a water-immiscible solvent and are emulsified under stirring in an aqueous surfactant solution (PVA, poloxamer, e.g.). Organic solvents are removed from the emulsion by solvent extraction or solvent evaporation in order to obtain the desired microspheres (6).

The main drawback of this technique is the toxicity of frequently used organic solvents. Only solvents which are rated in classes II and III by USP and Pharm. Eur., respectively, are used for preparation methods of microspheres even though they have significant toxic potential. Thus, these preparation methods require an aftertreatment (lyophilisation, e.g.) to assure that residual solvent concentrations in microspheres are lower than the requested

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specific ppm limits (7,8). Furthermore, the analytical controls on these given specifications require extensive and expensive methods (9,10).

Therefore, an emulsion extraction method for the preparation of microspheres using a non-toxic solvent would be of great interest, because biodegradable microparticles are a frequently used dosage form for controlled parenteral drug delivery (11). Several efforts to discover less-toxic solvents were previously reported. For example, ethyl acetate, ethyl formate and methylethyl ketone were proposed as alternatives (12–14). Furthermore, an emulsification method based on an O/W-type emulsion solvent evaporation method using acetone as polymer solvent was developed (5). A different approach is the design of *in situ*- forming drug delivery systems using 2-pyrrolidone as polymer solvent (15) or simply based on oil phases (2).

Glycofurol is considered non-toxic (LD_{50} in mice i.v.—3.5 ml/kg body weight), and as it is used in parenteral pharmaceutical formulations as an injectable solvent, it is suitable as a polymer solvent for the preparation of microspheres (16–18). Here, we propose a microencapsulation method based on a quasi emulsion extraction with a hydrophilic external phase. The study was focused on setting process parameters such as surfactant concentration, temperature, viscosity of internal and external phase, stirring rate, extraction time and extraction volume (19–22). After developing a robust preparation process, diverse model compounds were encapsulated, and particles were analysed for their properties, such as size, encapsulation efficiency, and yield and drug release kinetics.

MATERIALS AND METHODS

Materials

PLGA RG 502 H was a kind gift of Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol and glycofurol were purchased from Sigma-Aldrich (Steinheim, Germany). Ibuprofen 50 was also a kind gift of BASF AG (Ludwigshafen, Germany). Polysorbate 80 was purchased from Caelo (Hilden, Germany), and PEG 1000 was purchased from Fagron (Barsbüttel, Germany). All other chemicals were of analytical grade.

Microsphere Preparation

Solvent Evaporation Method

An amount of 200 mg PLGA RG 502 H and 20 mg drug was dissolved in 3 ml methylene chloride, which built the internal phase. The internal phase was emulsified under stirring with a three-blade propeller at 400 rev./min over 2 h in 100 ml 0.1% PVA solution, which built the external phase. An O/W emulsion was obtained, and solvent evaporation was performed by magnetic stirring at 600 rev./min overnight. While evaporating, the emulsion droplets solidified, and microspheres were obtained. Thereafter, microspheres were filtered (cellulose nitrate filter, Whatman®, pore size 5 µm), washed extensively with distilled water and dried overnight under vacuum in a desiccator.

Solvent Extraction Method

An amount of 200 mg PLGA RG 502 H and 20 mg drug were dissolved in 4 ml ethyl acetate. This solution was added drop by drop with a syringe to 20 ml of a 0.1% PVA solution, and an emulsion was formed by stirring with a three-blade propeller.

The emulsion was stirred at 400 rev./min for 3 min and was then poured into a solution of 300 ml 0.01% PVA, which built the external phase. During this extraction step, emulsion droplets precipitated, and microspheres were obtained. Obtained microspheres were stirred overnight with a magnetic stirrer at 600 rev./min until curing of microspheres. Afterwards, microspheres were filtered (Cellulose nitrate filter, Whatman®, pore size 5 µm), washed extensively with distilled water and dried overnight under vacuum in a desiccator.

Glycofurol Extraction Method (GEM)

An amount of 100 mg PLGA RG 502 H and 20 mg drug were dissolved in glycofurol, which built the internal phase. The internal phase was then emulsified under stirring with a three-blade propeller at 400 rev./min over 8 h at 40°C in 100 ml of the external phase, and a quasi-emulsion was obtained. Thereafter, 200 ml of distilled water was added slowly to the solvent system under stirring at 400 rev./min., whereupon polymer precipitated, and microspheres were obtained. Finally, microspheres were filtered (Cellulose nitrate filter, Whatman®, pore size 5 µm), washed extensively with distilled water and dried overnight under vacuum in a desiccator.

Drop Shape Analysis

The interfacial tension and the miscibility between glycofurol and various external phases were measured by the Contact Angle Measuring Instrument Easy Drop by Kruess® (Hamburg, Germany) with the pendant drop method.

Scanning Electron Microscopy

Microspheres were cooled with liquid nitrogen and then milled with a pestle. Fractured microspheres were fixed on supports with double-faced adhesive tape, and coated with gold using a gold sputter module. Afterwards, external and internal morphology of microspheres were analyzed by scanning electron microscopy (Hitachi S-2460N, Tokyo, Japan) at 15 kV.

Confocal Laser Scanning Microscopy

The extraction medium, external or internal phase was stained by fluorescein sodium during the preparation process. A Nikon® Eclipse Ti A1 Laser Scanning Confocal Imaging System (Düsseldorf, Germany) equipped with a modular laser system and a Nikon® microscope was used to investigate the internal structure of microspheres. For imaging, dry microspheres were dispersed in miglyol 812 and fixed on glass slides.

Residual Glycofurol Content

Residual glycofurol content was analysed in blank microspheres. Samples of 20 mg were dissolved in 1 ml acetone, and then polymer was reprecipitated by 4 ml of distilled water. Thereafter, the solution was centrifuged at 6000 rev./min for 30 min, and the supernatant was further analysed. Four milliliter of an ammonium cobaltothiocyanate solution and 4 ml methylene chloride were added to the sample. This mixture was centrifuged, and the methylene chloride phase was taken to analyse residual glycofurol content by UV at 620 nm on a Shimadzu spectrophotometer (23). The glycofurol calibration curve was linear from 0.1 to 0.75 g/100 g (r^2=0.9764). All experiments were made in triplicate.

Differential Scanning Calorimetry

An amount of approximately 5 mg microspheres of each batch was inserted into aluminium pans with holes. Thermal analysis was accomplished in a Pyris 1 differential scanning calorimeter (Perkin Elmer®, Waltham, Massachusetts, USA). Samples were cooled to −60°C, and temperature was held for 10 min. Samples were then heated from −60 to 75°C at 50°C$\,$min^{-1}. A temperature of 75°C was held for 2 min, and samples were again cooled to −60°C at 50°C$\,$min^{-1}. A temperature of −60°C was held again for 10 min, and then samples were heated from −60 to 150°C at 50°C$\,$min^{-1}. Thermal data were taken from first and second heating cycle using the supplied software.

Particle Size Measurement

Size of all microspheres was measured by a laser diffraction system (Helos, Sympatec®, Clausthal, Zellerfeld, Germany). Obtained microspheres were suspended in 0.2% aqueous Tween 80 solution (40 ml) and were sonicated for 30 sec., and 1 ml of this suspension was taken for measurement and for volume distribution.

Encapsulation Efficiency

Encapsulation efficiency was analysed with a direct method by disintegrating microparticles with a 0.1N sodium hydroxide +5% SDS solution.

Ibuprofen concentration of disintegrated microspheres was analysed by high performance liquid chromatography (HPLC) with a modification from an earlier-described method (24). The setup was as follows: RP-18 column (LiChrospher® 100, Merck, Darmstadt, Germany); eluent: methanol:water:acetic acid (600:377:23); flow rate 0.8 ml/min. Samples of 20 µl were injected into the column, followed by UV detection at 264 nm. Ritonavir, Lopinavir and Sudan III were used as alternative lipophilic model compounds in order to identify details on the encapsulation mechanism. Also, their encapsulation efficiencies were analysed directly as described before. Sudan III concentration was determined by an HPLC method described by Nagase *et al.* (25), and concentrations of Ritonavir and Lopinavir were measured by an HPLC method described by Takahashi *et al.* (26), which was suitable for both drugs. A RP-18 column (LiChrospher® 100, Merck, Darmstadt, Germany) was used, and samples of 20 µl were injected into the column. All experiments were made in triplicate, and values were given as percentage ±SD.

In Vitro Release Studies

Five to 10 mg of drug-loaded microspheres were suspended in 100 ml of phosphate buffer pH 7.4 containing 0.7% sodium dodecylsulfate in closed Erlenmeyer flasks and kept under continuous shaking (80 rev./min) at 37°C. Samples of 1.0 ml were taken at specified time intervals, and removed volume was replaced with fresh buffer. The amount of Ibuprofen, Ritonavir, Lopinavir and Sudan III in the particular sample was analysed by high performance liquid chromatography (HPLC) based on standard methods described before during the determination of the encapsulation efficiency (24–26). All measurements were performed in triplicate.

RESULTS

In initial experiments, the polymer containing glycofurol solution was dispersed under stirring into various external phases (Table 1). A good miscibility of internal and external phase, e.g. glycofurol and glycerol are miscible in all

Table 1 Experimental Series Summarising the Feasibility of Microsphere Preparation

Experimental Series	External Phase	Microsphere Formation
1	PVA -/ Glycerol solutions	−
2	Poloxamer -/ Glycerol solutions	−
3	Methylcellulose solutions	−/+
4	Glycerol -/ Distilled water ; Glycerol-/Methylcellulose solutions	−
5	Castor oil, Dimeticon, Glycerol, PEG	−
6	Glycerol, PEG 1000 (90/10)	+

proportions, led to spontaneous diffusion of glycofurol towards the external phase followed by nanosphere formation. Consequently, diffusion rate of the internal solvent had to be lowered in order to obtain larger particles. Various external phases with varying compositions and viscosities were tested to delay solvent diffusion from internal towards external phase (Table 1). To reconstruct the particle formation process, interfacial tension between internal phase and external phase or water was determined by drop tensiometer. While all phases were miscible, glycofurol diffusion into the Glycerol/PEG phase was delayed as measured by a drop tensiometer (data not shown). A glycerol/ PEG mixture with a viscosity of 334 mPas (series 6) was determined as suitable external phase, while a 1% methylcellulose solution with a viscosity of 291 mPas (series 3) enabled for emulsification but failed

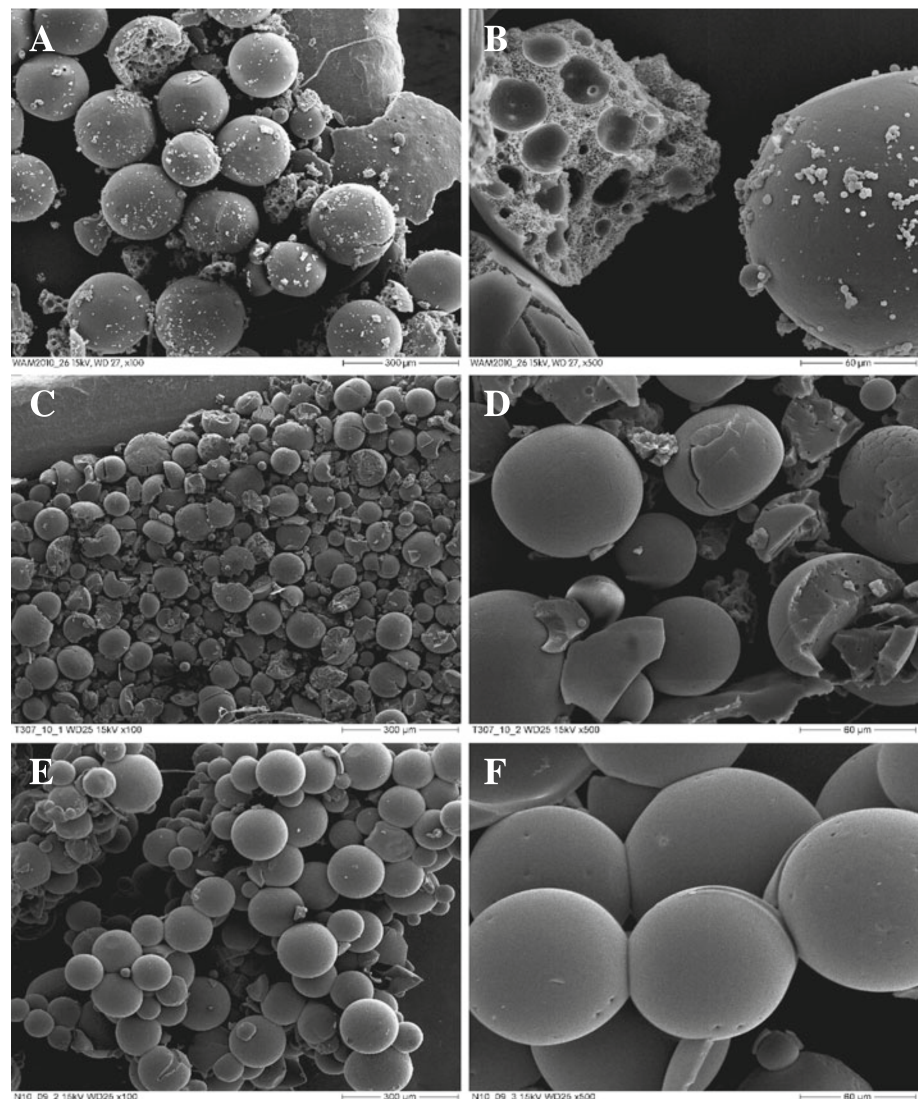

Fig. 1 Scanning electron microscopic images of microspheres produced by glycofurol extraction method (A, B); by solvent evaporation (C, D); or by solvent extraction (E, F).

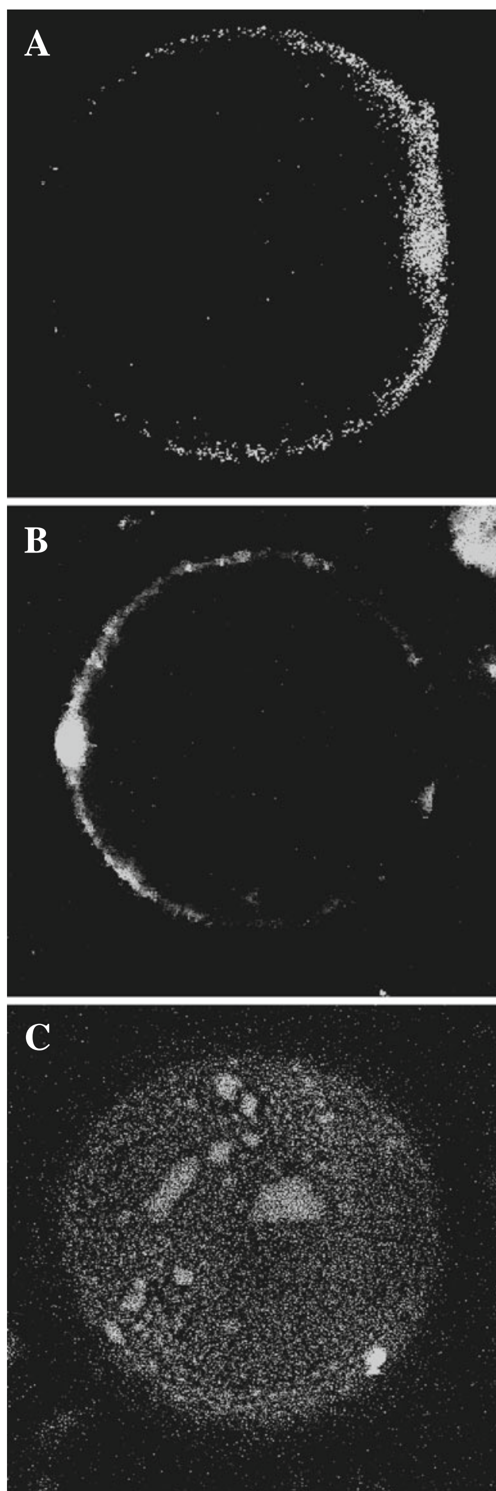

Fig. 2 Confocal laser scanning microscopic images of representative particle cross-sections: No signals were observed form inside the particle matrix when the extraction medium (**A**) or the outer phase (**B**) was containing fluorescein; however, significant fluorescence was detected when inner glycofurol phase was stained (**C**).

in terms of filterability. Thus, glycerol/ PEG mixture as external phase was chosen as basis formulation for further experiments due to an adequate viscosity of the external phase and a given filterability of the solvent system. Microspheres appeared spherical with a smooth surface (Fig. 1a). The internal structure of glycofurol microspheres appeared sponge-like with pores of varying diameter (Fig. 1b), whereas the internal and external structure of microspheres produced by conventional methods for comparison looked relatively dense beside the usual solvent evaporation and solvent extraction pores (Fig. 1c-f).

Staining different phases of the quasi-emulsion indicated that the origin of these internal droplets was related to an incomplete glycofurol extraction. Fluorescein in the external or extraction phase did not lead to significant signals from inside the matrix (Fig. 2a, b), while staining of the internal glycofurol phase showed clear droplet-shaped structures within the matrix (Fig. 2c).

Residual glycofurol was detectable during DSC analysis (endothermic peak at around 126°C). Transition temperatures of PLGA generally decreased from around 43°C to around 27°C in the presence of glycofurol, while, quantitatively, the residual solvent content was of 16.9±1.6 mg per 100 mg sample. Microspheres showed monomodal particle size distributions with no major influence by the entrapment of the various model compounds (Fig. 3). When determining the encapsulation rates for several model compounds, such as Ibuprofen, Lopinavir, Ritonavir, the entrapment was found to be relatively low (Table 2). When correlating the obtained encapsulation rates of these lipophilic model drugs with their respective log P, a linear relation was observed (Fig. 4). Details of the encapsulation mechanism using this new technique were elucidated while preparing the primary emulsion containing Sudan III, which then diffused from

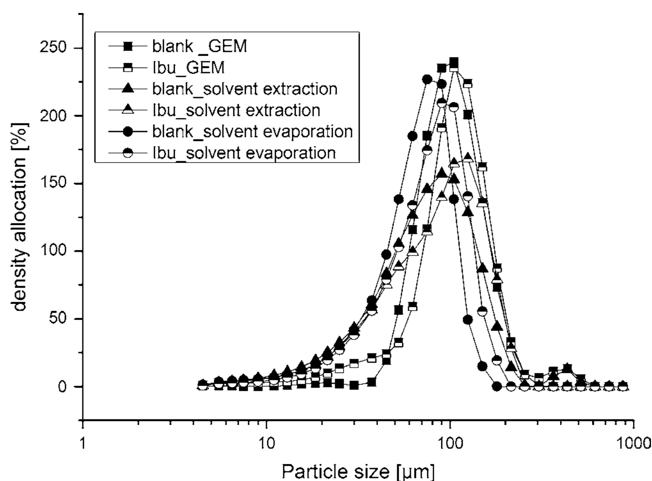

Fig. 3 Particle size distributions of microspheres prepared by the glycofurol extraction method, solvent evaporation method or solvent extraction method.

Table 2 Characteristics of Microspheres After Solvent Extraction, Solvent Evaporation And Glycofurol Extraction

Trial	Preparation method	Encapsulation rate [%]	Yield [%]
Ibuprofen–microspheres	Solvent extraction	63.9 ± 5.5	63.8 ± 2.7
Ibuprofen–microspheres	Solvent evaporation	62.2 ± 5.8	62.8 ± 0.4
Ibuprofen–microspheres	GEM	1.9 ± 0.6	47.8 ± 10.3
Ritonavir–microspheres	GEM	11.2 ± 0.4	47.5 ± 23.1
Lopinavir–microspheres	GEM	11.3 ± 2.2	48.9 ± 12.0
Sudan III–microspheres	GEM	28.3 ± 0.4	56.7 ± 17.6

the internal phase towards the external phase during the emulsification step. Due to its relatively good solubility in both internal and external phase, Sudan III was equally distributed, which leads to drug loss during the hardening step. Whether the addition of water during the solvent extraction step is leading to a partial flux back into the particle matrix remains unclear. Microsphere formulations with Ritonavir, Lopinavir and Sudan III were tested for their *in vitro* drug release (Fig. 5), while Ibuprofen was considered not worth testing due to the low encapsulation rate. The release of Ritonavir (log P: 4.68±0.56) was dominated by a burst effect, and a complete release was observed after 4 h. Lopinavir (log P: 5.18±0.92) release showed also an initial burst effect of about 30% followed by a sustained release. Sudan III (log P: 6.85±0.45) release started with an 8 h lag time followed by a biphasic release. The more lipophilic the drug, the smaller its burst effect to the point of an approximately zero-order controlled release behaviour. In general, the release of encapsulated drugs was controlled by a diffusion mechanism since microspheres were observed to remain nearly intact over the entire test period (Fig. 6). At day 0, microspheres appeared spherical with a smooth surface and a porous internal structure (Fig. 6a), while after one day of *in-vitro* drug release the surface of microspheres became slightly roughened (Fig. 6b). After 7 and 21 days, surface roughness became more dominant; however, a real microsphere deformation was not observed (Fig. 6 C+D).

DISCUSSION

Microencapsulation by solvent evaporation or solvent extraction processes is very popular for the preparation of biodegradable microspheres. The main drawback of conventional emulsification methods for the preparation of biodegradable microspheres is the toxicity of the prevalently used standard organic solvents.

The proposed glycofurol extraction method is based on known physicochemical phenomena frequently encountered in micro- and nanoencapsulation techniques. The process is similar to the principle of the spontaneous emulsion solvent diffusion described for nanoparticle preparation with water-miscible organic solvents (35–37). Initially, various external phases were tested for the preparation of microspheres; however, the complete miscibility of internal and external phase and the fast diffusion rate of the internal phase towards the external phase led to nanoparticle formation. Based on the fast diffusion of glycofurol across the interface between glycofurol and the external phase, an increased viscosity was considered to be helpful to enable microsphere

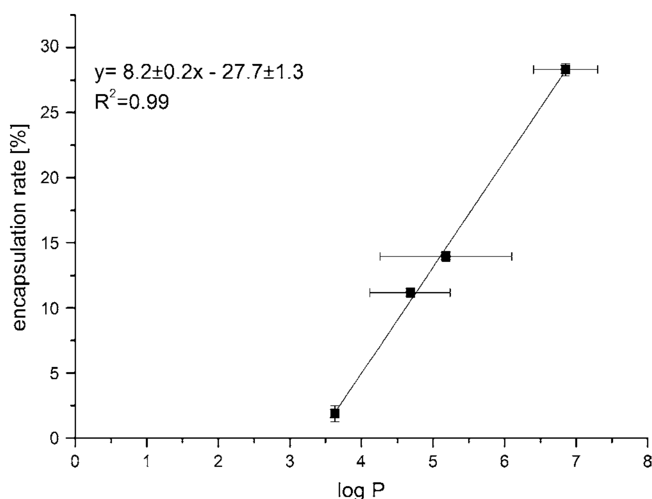

Fig. 4 Correlation of log P (according to Ref. 27–34) of the encapsulated drugs *versus* the respective encapsulation rates.

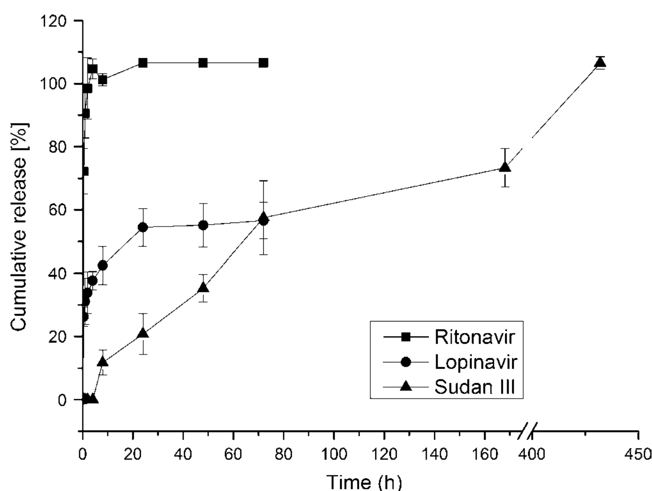

Fig. 5 *In vitro* release kinetics of different drug-loaded microspheres (Ritonavir, Lopinavir, Sudan III) ($n = 3$).

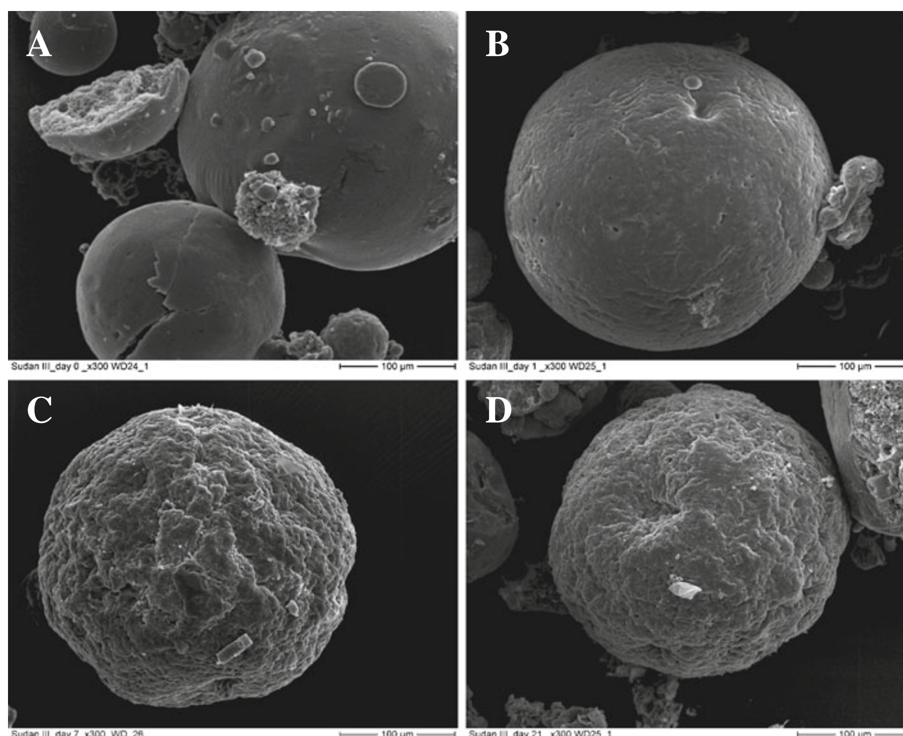

Fig. 6 Representative scanning electron microscopic images of Sudan III microspheres during *in-vitro* release sampled after 0, 1, 7, and 21 days (**A**-**D**).

formation as described elsewhere (38). The solvent diffusion rate can be lowered by a smaller solubility of the internal phase in the external phase or by a slower miscibility rate of internal and external phase based on different viscosities (39). The viscosity range allowing for a microparticle formation appeared to be >300 mPas at which the solvent extraction, however, does not occur in the reasonable time. The subsequent solvent extraction step triggered by the addition of water is responsible for the particle hardening and simultaneously limits the solubility of the drug in the external phase. However, besides the viscosity, the filterability of the external phase is a highly important factor which is hardly manageable with other external phase compositions. Methylcellulose solutions were similarly able to reduce nanoparticle formation at lower viscosities and led, however, to a failure of the filtration step. The reasons for these observations are not clear yet but seem to be related to the incomplete glycofurol extraction and subsequent insufficiently solidified particle matrices.

One aspect to be addressed is the relative high residual solvent content. Although it is not of toxicological relevance, its influence on the particle properties is not negligible, especially in terms of lowering the glass transition temperature. The low glass transition temperature did not lead to a distinct melting of the polymer matrix during *in-vitro* drug release, as the general spherical shape of the microspheres remained similar, but may have a significant influence on long-term storage. With decreasing lipophilicity of the encapsulated model compounds it is probable that they preferentially interact with glycofurol than with the matrix polymer, which has a significant influence on the encapsulation rate as well as the release rate. The linear correlation between logP and the encapsulation rate correlation further supports this assumption and that encapsulation is mainly triggered by the lipophilicity of the drug as it is reported for other techniques where the solvent extraction step lowers the drug entrapment (38). The high solubility of the drugs in the external phase appeared as a major drawback and will require further research. The drug release was triggered by a diffusion process, as no signs of erosion were observed prior to complete release. In the initial step of the release, microspheres start to swell, and residual glycofurol diffuses out of the particle matrix. Again, the most lipophilic compounds undergo the highest retention due to stronger polymer-drug interactions, which results in a variable burst release in dependency of the lipophilic properties of the model drug. It also can be concluded that low glass transition temperatures of GEM microspheres have only little influence on the particle matrix during *in-vitro* drug release.

Putting this technique in the context of existing approaches, only few solvents have been proposed as an alternative to develop processes for the preparation of biodegradable microspheres. Ethyl acetate, ethyl formate and methylethyl ketone (12–14) were proposed as solvents with less toxicity. These solvents are indeed an advantage in contrast to the well-established dispersed phase solvent

methylene chloride, but a significant toxicity remains. Acetone has been reported in literature as polymer solvent for the entrapment of vitamin B12, and microsphere formation has been carried out dependent on the glycerol content of the dispersed phase (5). An adequate glycerol content of the dispersed phase has to be adjusted in order to control microsphere formation by phase separation as well as by solvent diffusion. Nevertheless, acetone is categorised in ICH class 3 and still owns a toxic potential. Further approaches using low toxicity solvents dealt mainly with *in-situ*-forming drug delivery systems (2,15). However, these examples possess properties of semi-solid formulations, while microspheres, in contrast, are solid.

The use of glycofurol has been reported for microencapsulation using an external oil phase, which, however, did not allow the entrapment of lipophilic drugs (16), and it remained the problem of the removal of external phase. This limited number of solvent alternatives underlines the potential and necessity to develop the well-known encapsulation techniques towards non-toxic procedures.

CONCLUSION

Microspheres can be successfully prepared by this new method with glycofurol as a non-toxic solvent for the inner organic phase. The encapsulation of lipophilic drugs is mainly triggered by their solubility in the external phase, and the *in-vitro* drug release behaviour depends on the lipophilicity of the entrapped drug. This method is a distinct progress for the use of polyester microspheres in the context that an additional step for the removal of harmful organic solvents can be avoided. Therefore, we believe the glycofurol extraction method is an attractive alternative for the preparation of biodegradable microspheres using the non-toxic solvent glycofurol for the entrapment of lipophilic drugs.

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