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Development of an in situ forming PLGA drug delivery system I. Characterization of a non-aqueous protein precipitation

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ABSTRACT

The incorporation of the model protein hen egg white lysozyme into liquid in situ forming poly(lactide-*co*-glycolide) (PLGA) implant or microparticle formulations was investigated. Ternary solvent blends of dimethyl sulfoxide (DMSO), ethyl acetate and water were used to adjust the protein solubility in order to facilitate the incorporation of either dispersed or dissolved protein into the polymer solution. Lysozyme formed large gel particles when dispersed directly in the polymer solution. These formulations had a pronounced initial release. Non-aqueous precipitation of lysozyme from solutions in DMSO with ethyl acetate led to a reversible aggregation without loss in biological activity. Lysozyme could be incorporated in a finely dispersed state through an in situ precipitation by non-solvent or polymer addition. Non-aqueous precipitation could thus be utilized to manufacture biodegradable in situ forming drug delivery systems containing homogeneously distributed and bioactive protein.

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1. Introduction

The limited ability of proteins to pass biological membranes favors administration via parenteral routes. Biodegradable microparticles, implants and in situ systems based primarily on poly(lactide-*co*-glycolide) (PLGA) polymers have been used as extended release parenteral drug delivery systems for the delivery of macromolecular drugs (Johnson et al., 2000; Pèrez et al., 2002a; Ghaderi and Carlfors, 1997; Castellanos et al., 2001; Kim and Park, 2001; Park et al., 1998; Bittner et al., 1998).

Microparticles have a patient-friendlier administration when compared to solid implants but their manufacturing is costly and complex. A potential problem during preparation of microparticles by conventional microencapsulation processes (e.g. solvent evaporation or organic phase separation techniques) is the denaturation of the dissolved protein at solvent interfaces (Pèrez et al., 2002a,b; Ghaderi and Carlfors, 1997; Van de Weert et al., 2000; Perez-Rodriguez et al., 2003; Lu and Park, 1995; Krishnamurthy et al., 2000; Kang et al., 2002). Preparation-induced loss of soluble protein fraction can consequently result in incomplete protein release (Morlock et al., 1997; Kim and Park, 1999) and loss in therapeutic activity.

The native secondary structure of proteins is kinetically trapped in protein suspensions in organic solvents (Griebenow

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and Klibanov, 1996). Microencapsulation procedures based on dispersed protein particles were developed, where the exposure of dissolved protein to water/organic solvent interfaces was reduced (Castellanos et al., 2001; Kim and Park, 2001) or completely avoided (Schwendeman et al., 1998; Carrasquillo et al., 2001). However, the properties of the microparticles (e.g. encapsulation efficiency, release) were strongly dependent on the particle size of the dispersed protein (Costantino et al., 2004; Morita et al., 2001). Conventional milling, aqueous precipitation, spray drying (Costantino et al., 1995), freeze drying (Morita et al., 2001; Morita et al., 2000), spray freeze drying (Costantino et al., 2000, 2001, 2002) and techniques with supercritical fluids (Winters et al., 1996; Thiering et al., 2000; Moshashaèe et al., 2000) have been used to obtain protein particles of varying size. However, milling/grinding exposes proteins to high shear, which can result in loss of biological activity (Platz et al., 1994; Wolf et al., 2003). Protein particles prepared by aqueous precipitation with inorganic salts, non-solvents, polyelectrolytes or non-ionic polymers have to be downstream purified and dried/freeze dried before particles of a reduced size can be obtained. Drying methods, such as spray drying, exert stress to proteins due to exposure to a large solution surface and the application of elevated temperatures. Spray freeze drying and freeze drying are complicated and expensive manufacturing techniques. Stabilizers are usually necessary to protect the protein against dehydration- and rehydration-induced structural changes in all three drying methods (Prestrelski et al., 1993; Morita et al., 2000). Removal of stabilizers, often hydrophilic substances (e.g. polyols), further complicates the micronization process (Maa and Hsu, 1997) but often avoids an initial high drug release (Perez-Rodriguez et al., 2003; Jaganathan et al., 2005). Supercritical fluid processes can be used for protein particle size reduction but are also complex and costly.

A simpler alternative to conventional microparticles/implants is in situ forming implant formulations (ISI) (Dunn and Tipton, 1997). ISI formulations consist of solutions of a biodegradable polymer (e.g. PLGA) in a biocompatible organic solvent with dissolved or dispersed drug. After administration, the polymer precipitates in contact with physiological fluids and forms a solid implant in situ, entrapping the drug. The drug is then released over extended periods of time from the matrix. However, the high viscosity of the polymer solution complicates the administration through hypodermic needles and a rapid initial release (burst) is often observed before polymer precipitation.

To overcome these limitations, in situ forming biodegradable microparticle systems (ISM) were developed (Bodmeier, 2000), whereby a drug-containing PLGA solution is emulsified into either an oily or aqueous external phase. The inner polymer phase of the emulsion hardens after injection in contact with body fluids, thus forming microparticles. The emulsion systems have a lower viscosity and thus easier injectability and a lower burst effect because of an external oil phase.

Proteins have been extensively studied in aqueous media, but only to a limited extent in non-aqueous media. Although the dissolution of proteins in organic solvents was associated with a loss of secondary structure (Jackson and Mantsch, 1991; Knubovets et al., 1999), lysozyme retained its biological activity after being recovered from solutions in DMSO (Park et al., 1998). Most proteins have a relatively high solubility in DMSO (Chin et al., 1994; Houen, 1996). Ovalbumin and chymotrypsin could be precipitated from DMSO solutions with the non-solvent ethyl acetate (Chang et al., 1991). However, the biological activity of the precipitated material was not investigated.

With in situ forming systems, proteins come in contact with organic solvents, which are required for the dissolution of PLGA. In this study, lysozyme was used as model protein and was incorporated into organic PLGA solutions. Lysozyme and PLGA were investigated separately or in combination in the ternary solvent system DMSO, ethyl acetate and water. The influence of formulation and process parameters on lysozyme distribution, solvation state and the integrity of recoverable protein was studied and related to in vitro release profiles of the protein-containing in situ formulations.

2. Materials and methods

2.1. *Materials*

Poly(lactide-co-glycolide) (PLGA; uncapped low molecular weight 50:50 PLGA; Resomer RG 502H, Boehringer-Ingelheim, Germany); lyophilized hen egg white lysozyme (M_w 14.3 kDa), dimethyl sulfoxide (DMSO), acetonitrile (HPLC grade), sodium hydroxide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); ethyl acetate (EA), trifluoroacetic acid, sodium azide, potassium dihydrogen phosphate, acetic acid (Merck KGaA, Darmstadt, Germany); Micrococcus lysodeikticus, sesame oil (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); triacetin (TA; Fluka, Chemie AG, Buchs, Switzerland).

2.2. *Methods*

2.2.1. *Phase diagrams*

Turbidimetric titrations with visual endpoint determination were conducted at room temperature in order to determine phase boundaries of protein solubility. The phase diagram of the solvent system was determined by dropwise addition of water or ethyl acetate to blends of DMSO/ethyl acetate or DMSO/water. Compositions at the apparent phase boundaries were repeatedly evaluated at room temperature in order to exclude temperature effects during mixing. Solutions of either 40% PLGA (RG 502H) in DMSO/ethyl acetate or 2.7% lysozyme in DMSO/water (27 mg lysozyme in 1 g of DMSO/water) were titrated under gentle agitation with their respective non-solvents water and ethyl acetate until phase separation occurred in order to estimate the position of the phase boundary (all ratios and percentages are expressed as w/w). In subsequently performed titrations, the concentration of lysozyme was adjusted to take the dilution through non-solvent addition into account.

2.2.2. *Preparation of in situ formulations*

2.2.2.1. *Lysozyme-containing polymer solutions (in situ implants, ISI)*. The formulations were prepared as follows. 12 mg lysozyme were dissolved in: formulation 1: 692 mg DMSO (“DMSO only”); formulation 2: 519 mg DMSO prior

to addition of 173 mg ethyl acetate ("DMSO/ethyl acetate 75/25") or formulation 3: 529 mg DMSO/water (10.75:1) prior to addition of 163 mg ethyl acetate ("DMSO/ethyl acetate/water 70.5/23.5/6"). The protein was dissolved under intermittent vortexing for 0.5–2 h. Finally, 296 mg PLGA were added. The resulting lysozyme/PLGA/solvent system composition of 1.2/29.6/69.2 corresponded to a 4% (based on polymer) lysozyme-containing 30% PLGA solution.

2.2.2.2. *In situ microparticle systems (ISM)*. Lysozyme-containing polymer solutions (0.25 g) were filled into 1 ml single-use syringes (B. Braun Melsungen AG, Melsungen, Germany); sesame oil (external phase) (0.25 g) was filled into a second syringe. The syringes were coupled with a connector (1.4 mm inner diameter) and the two phases (phase ratio of 1:1, 0.5 g formulation) were emulsified by back-and-forth movement (50 mixing cycles) of the syringe plungers at a mixing speed of 1/s.

2.2.3. *Lysozyme separation from non-aqueous systems*

A separation method was developed to recover lysozyme from organic solvents, in-situ implant and in-situ microparticle formulations.

The separation was carried out as follows: 0.1–0.25 g of a formulation was weighed into 2 ml Eppendorf vials and ethyl acetate was added up to the mark. The vials were gently vortexed and then centrifuged at $16060 \times g$ for 30 min (Heraeus Biofuge 13 Haemo, Heraeus Instruments, Osterode, Germany). Approximately 1.5 ml of the supernatant was removed and the washing cycle was repeated twice more. The protein precipitate was then dried under vacuum (30 min) to remove residual ethyl acetate and was then redissolved in 8 g release medium (33 mM sodium acetate buffer pH 5 containing 0.01% sodium azide).

The vacuum-dried lysozyme pellet, which was separated from a lysozyme solution (2.7%) in DMSO, was evaluated by IR-spectroscopy and compared to the spectrum obtained with a lysozyme solution in DMSO (5.4%). Furthermore, the IR-spectrum was compared with spectra obtained from lysozyme, which separated extracted from dispersion (1%) in ethyl acetate, and with lyophilized lysozyme (as received). FTIR-spectra were generated with an Excalibur 3100 FTIR spectrophotometer (Varian Inc., Palo Alto, USA). The spectra were collected using a horizontal ATR accessory with a single reflection diamond crystal (Pike MIRacle, Pike Technologies, Madison, USA). 500 scans at 2 cm^{-1} resolution were averaged and spectral contributions coming from water vapor in the light pass were subtracted using Varian software (Resolution Pro 4.0). Finally, the spectra were treated with a 13-point smoothing function.

The separation method was then used to determine the solubilities of lysozyme in the polymer solutions after 2 d equilibration of the formulations at room temperature (desiccator). Supernatants of lysozyme-containing polymer solutions were obtained by filtration of the protein solutions/dispersions through 0.45 μm PVDF filter cartridges (3 ml Captiva cartridge, Varian Inc., Palo Alto, CA, USA). Lysozyme was separated from the supernatants and was quantitatively analyzed.

2.2.4. *Particle size determination of precipitated lysozyme in PLGA solutions*

2.2.4.1. *Optical light microscopy*. The formulations were observed with an optical light microscope (Axioscope, Carl Zeiss Jena GmbH, Jena, Germany). The particle size of precipitated lysozyme was determined by averaging the diameter of 100 particles with image analysis software (EasyMeasure, Inteq Informationstechnik GmbH, Berlin, Germany).

2.2.4.2. *Photon correlation spectroscopy*. Samples were measured with a Malvern Zetasizer 3000 HS (Malvern Instruments Ltd., United Kingdom) at 20 °C. Lysozyme was dispersed in triacetin to serve as a comparison to the precipitated samples. A refractive index of 1.4305 was determined for triacetin (Carl-Zeiss refractometer, Jena, Germany). A viscosity of 17.0 mPas was determined for triacetin at 20 °C ± 0.2 °C (Rheostress RS 100, Haake Messtechnik GmbH, Karlsruhe, Germany).

Gelled lysozyme particles, which formed upon addition of 30% RG 502H to solutions of the protein in DMSO, were measured undiluted in order to avoid dilution artifacts (state of solvation). A refractive index of 1.4900 was used for lysozyme gel particles in the solution of 30% RG 502H in DMSO. This value was measured for a solution of 15% lysozyme in DMSO with a refractometer (Carl-Zeiss refractometer, Jena, Germany). Accordingly, the refractive index of 1.4767 was determined for the continuous phase (30% RG 502H in DMSO). The viscosity of the 30% RG 502H solution in DMSO was 215.6 mPas at 20 °C ± 0.2 °C (Rheostress RS 100, Haake Messtechnik GmbH, Karlsruhe, Germany).

Protein dispersions in 30% RG 502H solutions in DMSO/ethyl acetate/water (75/25/0 and 70.5/23.5/6) were diluted (about 40 times) with triacetin (protein non-solvent/polymer solvent) shortly before analysis. A refractive index of 1.59 was used for the precipitated lysozyme particles in 30% RG 502H solutions in DMSO/ethyl acetate/water 75/25/0 and 70.5/23.5/6.

2.2.5. *Drug release*

0.25 g of the ISI- and 0.5 g of the ISM-formulations were injected into screw cap sealed test tubes filled with 8 g of 33 mM pH 5 sodium acetate buffer containing 0.01% sodium azide as preservative. The pH of the release medium of 5 was chosen according to the stability optimum of lysozyme (Claudy et al., 1992). The vials were incubated in vertical position in a horizontal shaker (Gemeinschaft für Labortechnik, Burgwedel, Germany) at 80 rpm and 37 °C ($n = 3$). The release medium was carefully removed with a pipette or decanted and replaced with 8 g fresh buffer at each sampling time-point. Drug release was used as an indication for the evaluation of the homogeneity of the drug distribution within in situ formulations.

2.2.6. *Protein characterization*

2.2.6.1. *Quantification of total soluble protein*. Lysozyme concentrations in release samples were quantified by Coomassie plus assay (Pierce, Rockford, USA), whereas lysozyme separated from formulations was characterized by HPLC (SCL-10A VP, Shimadazu, Japan) using a C4 reversed phase column (Eurosphere-100, 7 μm, 125 mm × 4 mm, Knauer, Berlin, Germany). The solvent system consisted of

water/acetonitrile/trifluoroacetic acid (A: 95/5/0.1, B: 5/95/0.1, v/v). A linear gradient method was applied (0-22-27-28 min 15-60-60-15%B) at a flow rate of 1 ml/min and a temperature of 25 °C. 25 μl samples were injected. Quantification of lysozyme was based on a linear standard curve obtained in the range of 10–1000 μg/g ($R^2 = 0.999$, S/N@10 μg/g ≥ 10, theoretical plates ≥6000, asymmetry ≤2).

2.2.6.2. Biological activity of lysozyme. The biological activity of separated lysozyme was measured with a modified turbidimetric assay (Shugar, 1952). The concentration of active enzyme was correlated with the linear rate of the absorbance decrease at 450 nm of a Micrococcus lysodeikticus cell suspension in 66 mM phosphate buffer (pH 6.24) at 25.0 °C. The initial absorbance of the filtrated cell suspension was adjusted to values between 0.6 and 0.7. 100 μl of the aqueous lysozyme solution was added to 2.5 ml of the bacteria suspension. The turbidity was measured for 2 min each 6 s and the slope of the linear portion was used for the quantification of the amount of active lysozyme in the sample based on a freshly prepared standard curve (0–35 μg/g). The samples were bracketed with standards after each third sample. A diode array UV-spectrophotometer with a Peltier thermostatted cell holder (Agilent 8453, Agilent Technologies Inc., Palo Alto, USA) equipped with a UV-Chemstation biochemical analysis software was used for data collection.

3. Results and discussion

Ternary solvent blends of dimethyl sulfoxide (DMSO), ethyl acetate and water were used to adjust the protein solubility in order to facilitate the incorporation of either dispersed or dissolved protein into the polymer solution. The pharmaceutically acceptable solvent DMSO was used because of its ability to dissolve both the model protein (lysozyme) and the biodegradable polymer (PLGA). The GRAS-listed biocompatible ethyl acetate (Royals et al., 1999; Kranz et al., 2001) dissolves the polymer but is a non-solvent for the protein. Ethyl acetate was used in order to allow adjustments of the protein solubility. Additionally to DMSO and ethyl acetate, water was introduced into the solvent system since preliminary investigations showed decreased dissolution times for lysozyme in DMSO in the presence of small amounts of water (from about 2 h to less than 0.5 h). This would be desirable for formulations, which require reconstitution prior to administration, e.g. where protein and polymer have to be stored separately from the solvent systems due to storage instability. On the other hand, water could alter the protein release patterns of in-situ forming drug delivery systems through an accelerated phase inversion of the PLGA solutions (Brodbeck et al., 1999).

3.1. *The ternary solvent system: DMSO/ethyl acetate/water*

Ternary phase diagrams were determined for the solvent system DMSO/ethyl acetate/water with and without 2.7% lysozyme (equivalent to a 4% target loading based on PLGA only) or 40% PLGA. A high PLGA concentration of 40% was selected because the initial drug release (burst) from in situ

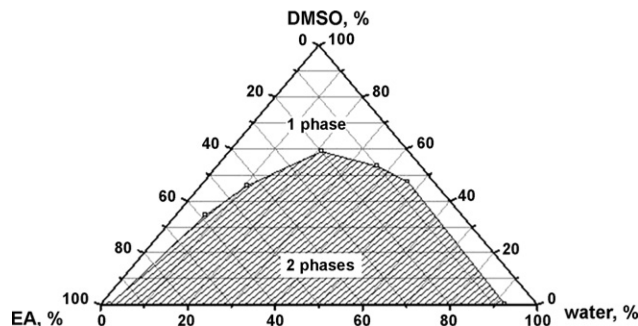

Fig. 1 – Phase diagram for DMSO, ethyl acetate (EA) and water mixtures.

forming systems decreases at higher polymer concentrations (Lambert and Peck, 1995). The phase diagrams should identify suitable conditions for lysozyme incorporation into the in situ drug delivery systems in dissolved or finely dispersed form in order to get good entrapment and a low initial release.

3.1.1. *DMSO/ethyl acetate/water mixtures*

The phase diagram of the ternary solvent system showed areas of miscibility (1 phase) and of immiscibility (2 phases) (Fig. 1). Water and ethyl acetate were completely miscible with DMSO, whereas only 8% ethyl acetate could be added to water and only 2% water to ethyl acetate before phase separation occurred. Compositions with DMSO contents higher than 60% were miscible independent of their ethyl acetate to water ratio.

3.1.2. *Lysozyme in DMSO/ethyl acetate/water mixtures*

The two phase region consisted of two regions, namely the miscibility gap of the ternary solvent mixture (similar to Fig. 1) and an additional region, where protein precipitation occurred (Fig. 2). The phase boundary for precipitation of lysozyme depended on the water content (0–8.5%) and was between ethyl acetate concentrations of 29–47%. A slow appearance of cloudiness accompanied by protein gelation was observed during titration, when the composition approached the boundary for protein precipitation. Both phenomena were in accordance with observations made during lysozyme crystallization close to the liquid–liquid phase boundary in aqueous systems (Galkin and Vekilov, 2000). However, whether cloudiness observed before protein precipitation was caused

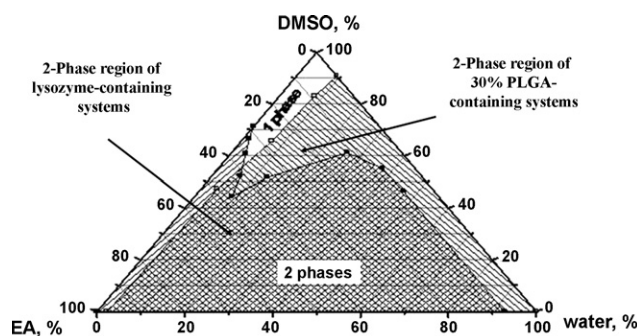

Fig. 2 – Phase diagram for DMSO, ethyl acetate (EA) and water mixtures containing 40% PLGA (open symbols) and 3% lysozyme (closed symbols).

by crystal- or simply by aggregate-formation was not further investigated since a precipitate could be obtained without gelation through continuous titration of the protein solutions with the non-solvent ethyl acetate under gentle agitation.

3.1.3. PLGA in DMSO/ethyl acetate/water mixtures

A one phase region in the phase diagram of 40% PLGA solutions was apparent at low water contents (Fig. 2). Dependent on the DMSO/ethyl acetate ratio, between 2 and 9% water could be incorporated into the polymer solutions before liquid–liquid phase separation occurred. The phase diagrams for PLGA and lysozyme revealed compositions at which the model protein could be incorporated into polymer solutions either in a dissolved or suspended state. DMSO/ethyl acetate ratios in the range between 71:29 and 60:40 resulted in protein suspensions. These suspensions could turn into protein solutions prior to PLGA coacervation/precipitation upon contact with aqueous media (release medium or tissue fluid).

3.2. Lysozyme incorporation into PLGA solutions

3.2.1. Solutions of PLGA in DMSO only

3.2.1.1. Visual examinations. The protein and PLGA could be incorporated into the ternary solvent mixtures in several ways. Dividing the solvents into portions to first separately dissolve protein and polymer before combining them was not possible because of the already high viscosity of 40% PLGA solutions (DMSO: 957 ± 103 mPas, ethyl acetate: 20,121 ± 546 mPas). Solutions of 0, 3.4, 7.1 and 9.6%, w/v, lysozyme in DMSO had viscosities of only 1.5, 2.4, 4.4 and 5.4 mPas at 20 °C, respectively. This was in contrast to Chin et al. (1994), who observed formation of a viscous lysozyme gel in DMSO, at concentrations around 5% w/v. The reason for the discrepancy could be due to the used protein material or the dissolution process. Lyophilized lysozyme gelled immediately after coming in contact with DMSO. Without sufficient agitation, the semisolid particles sedimented during the dissolution process followed by gelling at the bottom of the vials, which impeded further protein dissolution.

Therefore, the following three methods to prepare lysozyme containing biodegradable in situ forming implant and microparticle systems were investigated. Lysozyme was (1) dispersed in PLGA solution (“dispersed”), (2) mixed with PLGA powder prior to DMSO addition (“co-dissolution”) and (3) lysozyme was dissolved in DMSO prior to PLGA addition (“dissolved”). High shear mixing was not used due to potential stability problems. Large agglomerated gel particles of up to 400 μm in size (Fig. 3a) were observed when lysozyme was added stepwise to a 40% PLGA solution under vigorous vortexing. The high viscosity of the polymer solution made it difficult to disperse the quickly swelling lyophilized protein.

DMSO addition to a PLGA/lysozyme powder mixture (prepared by vortexing) resulted in gel particles of up to 100 μm (Fig. 3b). Although the distribution of lysozyme was improved when compared to the dispersion method, a homogenous mixture was not obtained.

PLGA addition to lysozyme solutions in DMSO resulted in a homogenous dispersion of almost transparent droplets of less than 10 μm after dissolution of the polymer (Fig. 3c). A clear appearance of the resulting dispersions was probably due to a small difference between the refractive indices of the dispersed (lysozyme solution/gel in DMSO) and the continuous phase (PLGA solution in DMSO). However, the presence of dispersed material was unexpected from the individual phase diagrams for lysozyme and PLGA in DMSO; this was attributed to a phase incompatibility.

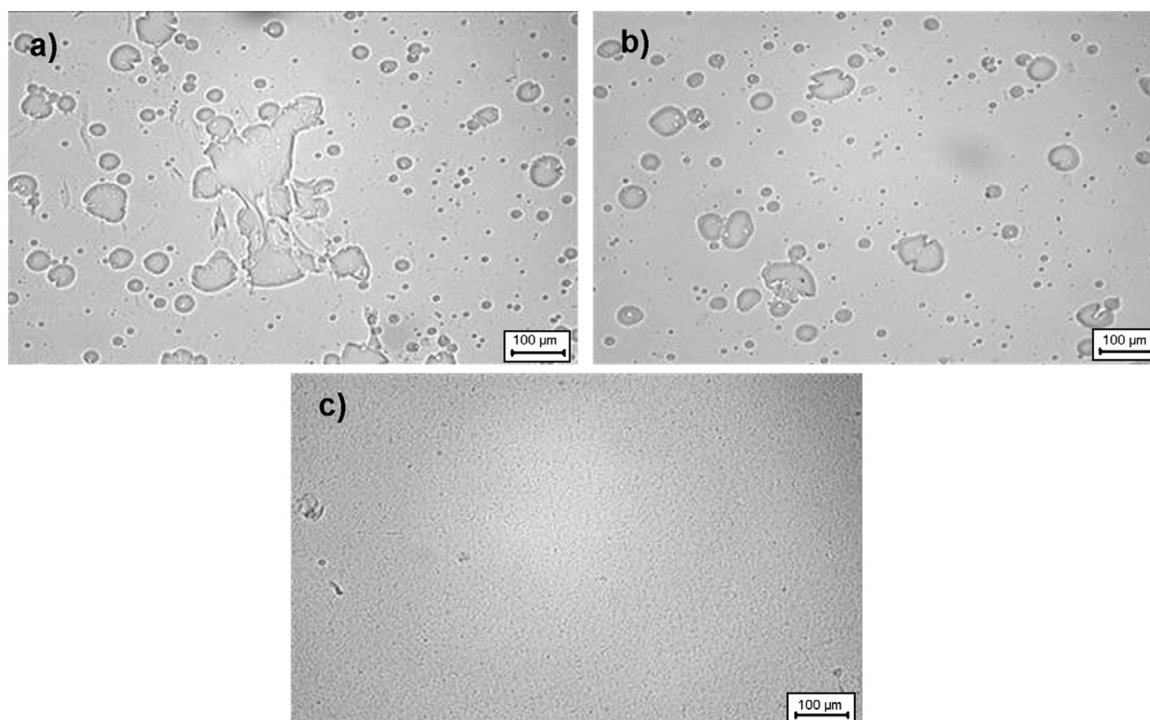

Fig. 3 – Microscopic appearance of 2.7% lysozyme containing 40% PLGA solutions depending on preparation technique (a) lysozyme to PLGA solution, (b) DMSO to PLGA/lysozyme blend and (c) PLGA to lysozyme solution.

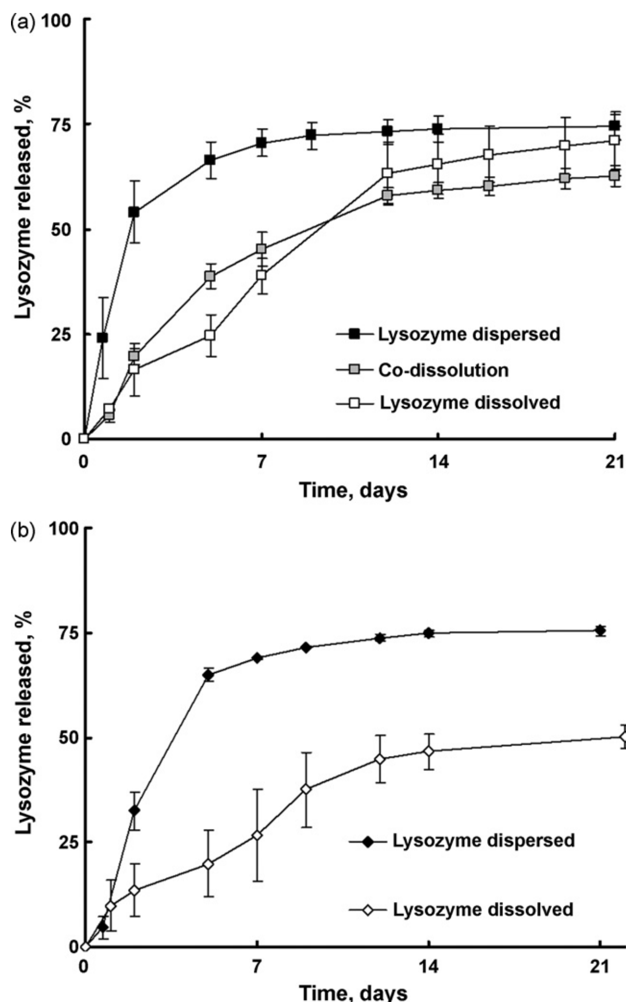

Fig. 4 – Lysozyme release as a function of preparation method: (a) in situ microparticle formulations [40% PLGA solutions in DMSO containing 4% lysozyme (relative to polymer) emulsified into sesame oil] and (b) corresponding in situ implants.

3.2.1.2. Effect of the protein incorporation method on in vitro drug release. The state of the protein in the polymer solutions affected the in vitro drug release of corresponding in situ microparticle and in situ implant systems (Fig. 4a and b). Increased release rates were obtained with both in situ formulations, if large protein aggregates were present in the PLGA solution. The effect of particle size on the initial release from in situ forming microparticles could be explained with decreased encapsulation efficiencies (Morita et al., 2001). However, in situ implant formulations were also affected. A contribution of a redistribution process was indicated by noticing lysozyme particle accumulation at the bottom of the polymer solutions upon standing. As previously reported, sedimentation of large drug particles could alter the drug distribution in polymer solutions (Maa and Hsu, 1997). Average diffusion pathways from aggregates to the implant surface could be shortened due to protein particles near the surface, which were released more rapidly. The drug release was sensitive to the distribution of the protein in the in situ formulations and was thus indicative for formulation inhomogeneities.

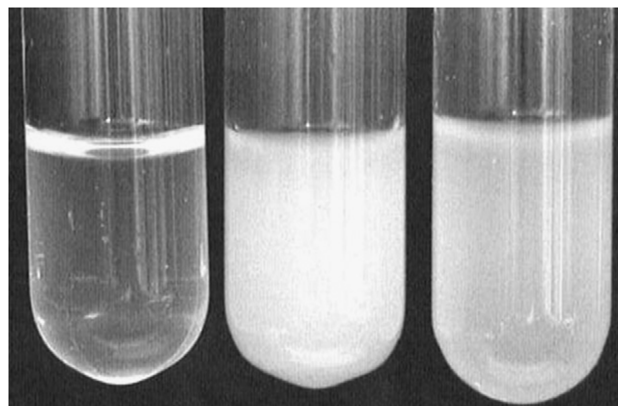

Fig. 5 – Appearance of 4% lysozyme (based on polymer) containing 30% PLGA solutions differing in solvent ratios DMSO/ethyl acetate/water (from left to right: 100/0/0, 75/25/0 and 70.5/23.5/6).

3.2.2. Solutions of PLGA in DMSO/ethyl acetate/water blends

3.2.2.1. Visual examinations. A dependence of the turbidity of lysozyme-containing PLGA solutions on the solvent ratio DMSO/ethyl acetate/water was clearly seen, when 30% PLGA solutions were investigated (Fig. 5). However, even with DMSO only, a pronounced turbidity developed in 40% PLGA solutions and hampered an optical differentiation of the solvent ratios.

The protein precipitated upon addition of the non-solvent ethyl acetate (DMSO/ethyl acetate/water 75/25/0). The turbidity decreased upon addition of water (DMSO/ethyl acetate/water 70.5/23.5/6) at the same DMSO/ethyl acetate ratio, which indicated that the precipitate consisted of the protein and not the polymer. These compositions were selected from the single phase regions of the individual protein and polymer phase diagrams (Fig. 2). The apparent turbidity of the combined protein/PLGA system indicated a shift of the phase boundary for the protein in concentrated PLGA solutions. A quantification of the dissolved protein fraction in the supernatant of the dispersions confirmed this. The fraction of dissolved lysozyme in 1.6% protein-containing formulations was reduced to $0.242 \pm 0.019\%$, $0.151 \pm 0.075\%$ and $0.002 \pm 0.001\%$ for DMSO/ethyl acetate/water ratios 100/0/0, 70.5/23.5/6 and 75/25/0, respectively.

Besides the previously described solvent-induced precipitation (Chang et al., 1991), the protein could therefore also be precipitated through the addition of PLGA. The polymer competed with the protein for the common solvent DMSO. The position of the protein solution in the phase diagram prior to the addition of polymer thereby affected the extent of precipitation (being higher with higher ethyl acetate and lower water contents).

3.2.2.2. Lysozyme separation method. In order to evaluate entrapped protein quantitatively and qualitatively, a recovery method had to be developed, which allows the separation of lysozyme from other formulation excipients (solvents and

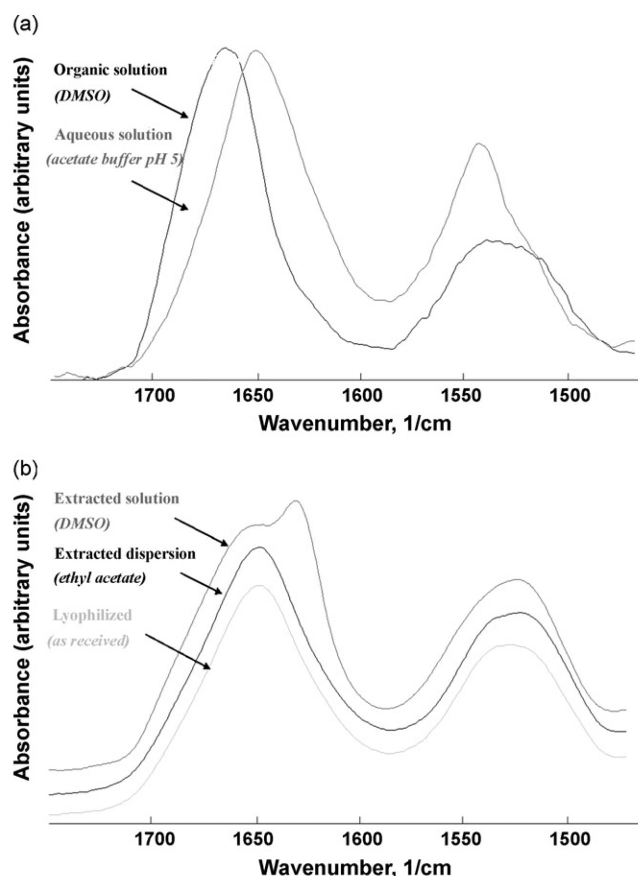

Fig. 6 – Amide I and II region of FTIR-spectra of (a) (5.4%) lysozyme in DMSO and of an aqueous lysozyme solution (33 mM acetate buffer pH 5 containing 0.01% sodium azide) and (b) dried lysozyme pellets after separation from a 2.7% lysozyme-containing solution in DMSO, a 1% dispersion in ethyl acetate (EA) and lyophilized lysozyme (as received) as comparison.

PLGA) without affecting the stability of the protein. A method with ethyl acetate was chosen due to its ability to mix with/dissolve all excipients (PLGA, PLGA solvents and sesame oil) but not lysozyme. The primary advantage was its volatility, which allowed fast removal under vacuum from the solid protein prior to redissolution of the protein pellet in an aqueous medium. The contact of dissolved lysozyme to deleterious conditions like water/organic mixtures (Griebenow and Klibanov, 1996) or interfaces (Van de Weert et al., 2000) was thus avoided. Although lysozyme was precipitated under non-aqueous conditions previously (Chang et al., 1991), the biological activity of the precipitated material has not been investigated.

Fourier transform infrared spectroscopy (FTIR) was used to identify potential effects of the extraction procedure on lysozyme. The maximum of the amide I band (1720–1580 cm^{-1}) of lysozyme occurred at a higher wavenumber in DMSO compared to the aqueous protein solution (Fig. 6a). The shift of the amide I band and a distortion of the amide II bands (1580–1480 cm^{-1}) indicated denaturation of the protein in DMSO, which was in agreement with the previous reports (Jackson and Mantsch, 1991; Knubovets et al., 1999). In contrast to organic solutions, proteins are kinetically trapped, hence conformationally stable in non-aqueous dispersions (Griebenow and Klibanov, 1996). Accordingly, the conformation of lysozyme dispersed in ethyl acetate was not affected as revealed by superimposing FTIR-spectra of extracted, dry protein in comparison with the original lyophilized material (Fig. 6b). However, extraction of lysozyme from the DMSO solutions involved a non-aqueous precipitation. The FTIR-spectrum of dried lysozyme pellets, obtained after protein precipitation in the DMSO solutions, showed two interesting features. The additional band at 1628 cm^{-1} could be attributed to an intermolecular β-sheet formation, which reflected protein aggregation (Griebenow and Klibanov, 1996). Except for the intermolecular interaction in the extracted protein pellet, the position of the amide I band shifted back from 1665 cm^{-1} (DMSO solution, Fig. 6a) to 1647 cm^{-1} (lysozyme as received, Fig. 6b) upon precipitation/desolvatization with ethyl acetate. Quantitative and qualitative characterization of lysozyme after redissolution of the protein pellet in acetate buffer showed that lysozyme could be almost completely recovered from the DMSO solutions (95.9 ± 1.4) with full activity (101.3 ± 2.8, based on total recovery). It was therefore concluded that reversibly aggregated, native lysozyme was obtained during extraction of the protein solutions in DMSO.

Separation of lysozyme from the 30% PLGA-containing formulations having different DMSO/ethyl acetate/water ratios resulted in full recoveries of completely biologically active protein, independent of the solvent ratio (Table 1). Lysozyme separation from the corresponding in situ forming microparticle formulations showed also no considerable loss of protein and no marked decrease in biological activity (Table 2).

The recovery method resulted in good recoveries of fully active lysozyme. Additionally, it could be concluded that the protein remained intact during the formulation process.

3.2.2.3. *Particle size of lysozyme*. In order to evaluate the particle size of precipitated protein, 4% lysozyme (based on polymer)-containing 30% PLGA solutions in DMSO/ethyl acetate/water (100/0/0, 75/25/0 and 70.5/23.5/6) were com-

Table 1 – Lysozyme recovered from biodegradable in situ implants based on 30% PLGA in DMSO/ethyl acetate (EA)/water mixtures (4% drug loading)

DMSO/EA/water ratio	Recovery (%)	Activity (%)
100/0/0	98.7 (3.6)	96.5 (3.0)
75/25/0	105.0 (10.5)	94.5 (5.6)
70.5/23.5/6	92.1 (2.6)	96.4 (1.0)

Table 2 – Lysozyme recovered from biodegradable in situ microparticles based on 30% PLGA solutions in DMSO/ethyl acetate (EA)/water mixtures (4% drug loading) and sesame oil

DMSO/EA/water ratio	Recovery (%)	Relative activity (%)
100/0/0	99.2 (10.3)	92.7 (2.0)
75/25/0	103.5 (4.3)	91.8 (2.2)
70.5/23.5/6	108.3 (7.9)	98.4 (8.5)

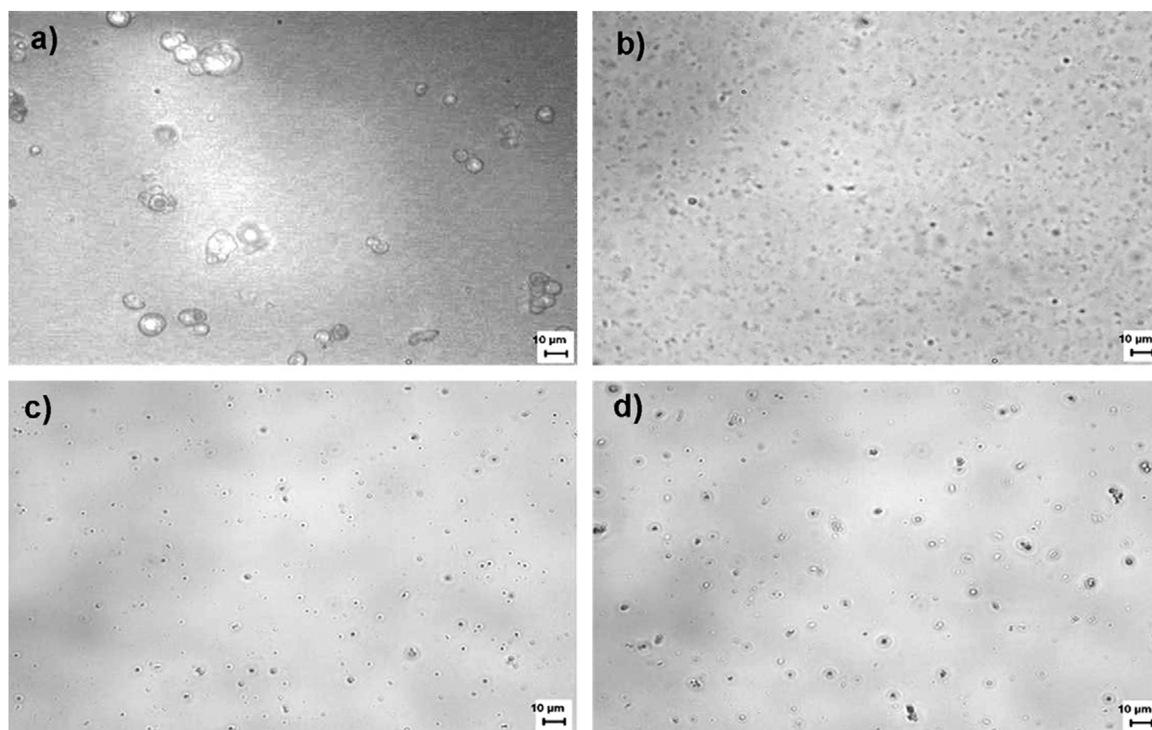

Fig. 7 – Microscopic pictures of (a) lyophilized (as received) lysozyme dispersed in sesame oil and precipitated lysozyme in 30% PLGA solutions in DMSO/ethyl acetate/water ratios of (b) 100/0/0, (c) 75/25/0 and (d) 70.5/23.5/6 (400×).

pared to a dispersion of lyophilized lysozyme (as received) in the protein non-solvent sesame oil. The particle size of lyophilized lysozyme dispersed in sesame oil was around $11.2 \pm 5.6\,\mu m$ (measured with light microscopy, Fig. 7a). This was larger than the upper limit of detection ($3\,\mu m$) for photon correlation spectroscope (PCS). The size of lysozyme particles formed in PLGA solutions in DMSO could not be determined with PCS as well. This was probably due to comparable refractive indices of dispersed and continuous phases after liquid–liquid phase separation, since microscopic pictures of the protein particles (Fig. 7b) suggested a mean size, which was comparable to the size of precipitated lysozyme particles in polymer solutions in DMSO/ethyl acetate/water (75/25/0 and 70.5/23.5/6) (Fig. 7c and d). The average diameters of these two DMSO/ethyl acetate/water systems obtained from three PCS measurements were 2.2 ± 0.2) and $2.2 \pm 0.3\,\mu m$ (PCS measurement), respectively. Although broad size distributions were indicated by high polydispersity indices of 1.0, large particles comparable to the dispersed raw material ($\sim 11\,\mu m$) were not observed upon microscopic evaluation.

3.2.2.4. *Effect of DMSO/ethyl acetate/water ratio on in-vitro drug release.* The release of lysozyme from all investigated in situ formulations was incomplete (Figs. 4 and 8), which was in accordance with a previous report on lysozyme-containing in situ implants based on *N*-methyl-2-pyrrolidone (Brodbeck et al., 1999). There, the incomplete release was attributed to protein, which is located in the polymer rich phase after the solidification of the in situ implants. Other, more likely reasons include specific but also non-specific adsorption of the positively charged lysozyme to the negatively charged PLGA, as suggested elsewhere (Park et al., 1998).

However, lysozyme release from in situ microparticles was only slightly affected by the investigated DMSO/ethyl acetate/water ratios (Fig. 8), although the addition of polymer solvents with limited water affinity (ethyl acetate) was expected to alter the phase separation of the polymer and thus the release (Brodbeck et al., 1999; Graham et al., 1999). Ethyl

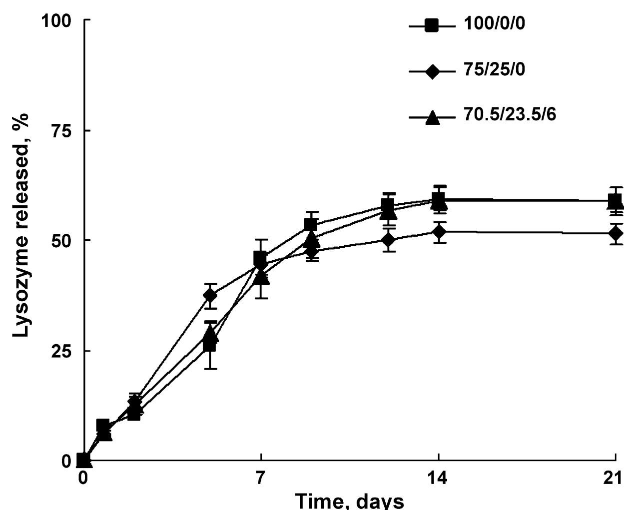

Fig. 8 – Lysozyme release from in situ microparticles differing in the solvent composition of the polymer solution phase [40% PLGA solutions containing 4% (relative to polymer) lysozyme, emulsified into sesame oil].

acetate could be partially removed from the polymer solution phase during emulsification into sesame oil. Ethyl acetate is completely miscible with sesame oil. The residual amounts of ethyl acetate and the incorporated water quantity were insufficient to alter the release of lysozyme.

On the other hand, the superimposing release patterns of the three formulations and hence the absence of an accelerated initial release seen for inhomogeneous formulations (Fig. 4), supported that the in situ protein precipitation during manufacturing led to homogenous lysozyme dispersions in PLGA solutions.

4. Conclusions

Hen egg white lysozyme dissolved in DMSO was precipitated under predominantly non-aqueous conditions with the protein non-solvent ethyl acetate. Native, reversible aggregates formed during the precipitation. The precipitate showed full biological activity when redissolved in aqueous medium. Application of the non-aqueous precipitation as a recovery method allowed complete recovery of fully active lysozyme. Deleterious conditions like water-organic solvent mixtures or interfaces could be completely avoided. Application as an in situ precipitation step during manufacturing facilitated the homogenous incorporation of lysozyme into in situ forming biodegradable microparticle and implant formulations. This in situ precipitation could avoid costly or harmful protein micronization methods.

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