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2.1. Materials

2.1.1. Proteins

- Lyophilized hen egg white lysozyme (Carl Roth GmbH & Co. KG, Karlsruhe, Germany, lots 29358838, 03783700 and 03676045)
- Lyophilized hen egg white lysozyme (product number: L7651, lot 114K7054), protamine sulfate (salt from salmon, grade X, lot 21H0519), bovine serum albumin fraction V (Sigma Aldrich Chemie GmbH, Steinheim, Germany)

2.1.2. Polymers

- Uncapped (RG 502H, lot 1005072 and RES-0254) and end-capped (RG 502, lot 1001933 and RG 502S, lot RES-0354) low molecular weight (intrinsic viscosity: 0.2 dl/g), fast degrading (D,L-lactide:glycolide 50:50) poly(D,L-lactide-co-glycolide) (PLGA) (Resomer, Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany)
- Polyvinylpyrrolidone (Kollidon 17 PF), poloxamer 188 (Lutrol F68) and 407 (Lutrol F127) (BASF Aktiengesellschaft, Ludwigshafen, Germany)
- Polyvinyl alcohol (Mowiol 4-88, Kuraray Specialities Europe GmbH, Frankfurt am Main, Germany)

2.1.3. Solvents

- Acetic acid, benzyl alcohol, propylene carbonate, trifluoroacetic acid, phosphoric acid (Merck KGaA, Darmstadt, Germany)
- Acetonitrile (HPLC Gradient Grade), chloroform, dimethyl sulfoxide (DMSO), hydrochloric acid (HCl) (Carl Roth GmbH & Co, Karlsruhe, Germany)
- Benzyl benzoate, ethyl acetate, glycofurol (tetraglycol) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- N-methyl-2-pyrrolidone (Pharmasolve, ISP Global Technologies Deutschland GmbH, Köln, Germany)
- 2-Pyrrolidone (Soluphor P) and polyethylene glycol 400 (Lutrol E400) (BASF Aktiengesellschaft, Ludwigshafen, Germany)
- Triacetin (Fluka, Chemie AG, Buchs, Switzerland)
- Triethyl citrate (TEC) (Citroflex-2, Morflex, Inc, Greensboro, North Carolina)
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2.1.4. Fatty acid ester
- Castor oil, peanut oil, safflower seed oil, sesame oil, soybean oil (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- Ethyl oleate (Fluka, Chemie AG, Buchs, Switzerland)
- Medium chain triglycerides (Miglyol 812N, Sasol Germany GmbH, Witten, Germany)

2.1.5. Surfactants
- Macrogol 15 hydroxystearate (Solutol HS 15), poloxamer 188 (Lutrol F68), poloxamer 407 (Lutrol F127), polyoxy 35 castor oil (Cremophor EL) (BASF Aktiengesellschaft, Ludwigshafen, Germany)
- Sodium dodecyl sulfate, polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monooleate (Tween 80), sorbitan monooleate (Span 80) (Carl Roth GmbH & Co. KG, Karlsruhe, Germany)
- Soy lecithins differing in the fatty acid composition (Lipoid S45 and S75, Lipoid GmbH, Ludwigshafen, Germany)

2.1.6. Other compounds
- Aluminium monostearate, magnesium hydroxide (Fluka, Chemie AG, Buchs, Switzerland)
- Anhydrous citric acid, histidine, potassium dihydrogen phosphate, sodium azide, sodium edetate, urea (Merck KGaA, Darmstadt, Germany)
- Glycine, imidazole, methionine, potassium chloride, D-saccharose, sodium chloride, sodium hydroxide, tris(hydroxymethyl)aminomethan hemisulfate (tris) (Carl Roth GmbH & Co. KG, Karlsruhe, Germany)
- Lactose, micrococcus lysodeikticus, N-acetyl cystein, trehalose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- Hydroxypropyl-ß-cyclodextrin (CAVASOL W7 HP, Wacker-Chemie GmbH, Burghausen, Germany)
- Dialysis bag with 2 cm diameter and a 100 kDa molecular weight cut-off (Spectra/Por CE membrane, Spectrum Europe B.V., Breda, Netherlands)
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2.2. Methods

2.2.1. Lysozyme characterization in aqueous medium

2.2.1.1. Lysozyme aggregation and adsorption – release media screening

The aggregation behavior of lysozyme solutions in various aqueous buffers was investigated either by following the UV-transmittance at 580 nm during incubation or alternatively by measuring the absorbance at 281.2 nm upon incubation in a horizontal shaker (GFL 3033, Gesellschaft für Labortechnik GmbH & Co. KG, Burgwedel, Germany) at 37°C and 80 rpm. The latter method was also applied to characterize lysozyme adsorption to interfaces. The samples were analyzed in quartz cuvettes with a UV-spectrophotometer (UV-vis scanning spectrophotometer 2101 PC, Shimadzu, Kyoto, Japan). For turbidity measurements, the samples were gently vortexed prior analysis. The UV-absorbance at 281.2 nm was either determined in the supernatants during incubation or in centrifuged samples at 3000 rpm for 5 minutes (Ecco-Praxa-2 centrifuge, Theodor Karow Laboratoriumseinrichtungen, Berlin, Germany) after completing the incubation period. In both cases the net absorbance at 281.2 nm in supernatants was obtained after normalization of the measured absorbance at 281.2 nm for the absorbance measured at 350 nm (alternatively: 400 nm).

2.2.1.2. Long-term stability of lysozyme in acetate buffer

The following four analytical assays were applied in order to quantitatively and qualitatively evaluate the stability of lysozyme in the release medium (33 mM acetate buffer, pH 5 containing 0.01% sodium azide) in presence or absence of formulation ingredients for 71 days.

2.2.1.2.1. Coomassie assay

A modified Bradford assay was used to quantify the amount of soluble lysozyme in the incubated samples. A ready-to-use dye-binding reagent formulation (Coomassie plus, Pierce, Rockford, USA) was added to withdrawn samples either in a ratio of 500 µl:500 µl (micro assay for 1-32 µg/ml lysozyme) or 1500 µl:50 µl (standard assay for 100-1000 µg/ml lysozyme). Net absorbance values (normalized against blank) at 595 nm were measured at 25°C with a diode array UV-spectrophotometer with a Peltier thermostatted cell holder (Agilent 8453, Agilent Technologies Inc., Palo Alto, USA). Properly diluted samples showed total absorbances (A595nm) of less than 0.9 (micro assay) or 1.3 (standard assay). Two separate
standard curves were prepared for the micro and the standard assay. The net absorbance was then used to calculate the corresponding lysozyme concentration on the basis of 2\textsuperscript{nd}-order polynomial standard curves, which facilitated a broadening of the concentration interval for each of the standard curves compared to linear curves.

2.2.1.2.2. HPLC

C4-RP-HPLC analysis of the samples was performed as follows. The HPLC apparatus (SCL-10A VP, Shimadazu, Japan) was equipped with a C4 reversed phase column (Eurosphere-100, 7 µm, 125x4 mm, Knauer, Berlin, Germany). Eluents used for HPLC consisted of water / acetonitrile / trifluoroacetic acid blends (phase A: 95/5/0.1, phase 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 sample were injected. Chromatograms obtained with a diode-array UV-detector (SPD M-10A, Shimadazu, Japan) was quantified based on a detection wavelength of 281 nm. The retention time was about 14 min.

2.2.1.2.3. Biological activity of lysozyme

The results were correlated to the biological activity of lysozyme, which was measured with a modified turbidimetric assay developed previously (Shugar, 1952). The concentration of active enzyme was thereby calculated from 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. 100 µl sample were added to 2.5 ml of the bacteria suspension (A\textsubscript{450} 0.7-0.6). 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.

2.2.1.2.4. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was conducted using pre-cast NuPAGE Novex 10 % Bis-Tris gels (15 well) with NuPAGE-MES [2-(N-morpholino)ethanesulfonic acid] running buffer (Invitrogen Ltd, Paisley, UK). Electrophoresis was conducted under native and reducing condition in a pre-cast mini-gel system (X-Cell SureLock Minicell with
Power Ease 500 power supply, Invitrogen Ltd, Paisley, UK). Buffers and samples were prepared according to standard protocol (NuPAGE Technical Guide, Invitrogen Ltd, Paisley, UK). A sample volume of 15 µl and 10 µl of an unstained marker (Precision Plus, Bio-Rad Laboratories, Inc., Hercules, USA) were loaded onto the gels. A Coomassie G-250 staining (SimplyBlue SafeStain, Invitrogen Ltd, Paisley, UK) was applied using the microwave protocol (SimplyBlue SafeStain protocol, Invitrogen Ltd, Paisley, UK).

2.2.2. Preparation of in situ formulations

2.2.2.1. In-situ implants (ISI)

2.2.2.1.1. Lysozyme dispersions

The protein (as received or ground) was dispersed in the protein nonsolvent (PLGA solvent) under vortexing before PLGA was dissolved in the dispersion. For 1 g of an in situ implant formulation with a PLGA concentration of 30%, for example, 12 mg lysozyme was dispersed in 692 mg protein nonsolvent / PLGA solvent (e.g., triacetin) under gentle vortexing (MS-1 minishaker, IKA-Werke GmbH & CO. KG, Staufen, Germany) for 5 minutes. Then, 296 mg PLGA were added, which led to a final lysozyme / PLGA / solvent composition of 1.2/ 29.6/ 69.2 corresponded to a 4 % (based on polymer) lysozyme-containing 30 % PLGA solution. The formulations were stored in a desiccator until complete polymer dissolution was obtained.

2.2.2.1.2. Lysozyme solutions / in situ precipitated lysozyme dispersions

The protein was dissolved in DMSO or DMSO / water mixtures under gentle vortexing before PLGA or PLGA solvents, such as ethyl acetate or triacetin (protein nonsolvents), were added. Exemplified with a solvent system based on DMSO, ethyl acetate and water, 1 g of the corresponding in situ implant formulations with PLGA concentrations of 30% would be prepared as follows: 12 mg lysozyme (as received) was dissolved in 692 mg DMSO or in 519 mg DMSO (DMSO / ethyl acetate 75/25) or in 529 mg DMSO / water (10.75:1) under intermittent vortexing for 0.5-2 h (MS-1 minishaker, IKA-Werke GmbH & CO. KG, Staufen, Germany). Then, 173 mg (DMSO / ethyl acetate / water 75/25) or 163 mg (DMSO / ethyl acetate / water 70.5/23.5/6) ethyl acetate and subsequently 296 mg PLGA were added and then stored in a desiccator until complete polymer dissolution was obtained. 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. One dose was equal
to 0.25 g of the in situ implant formulation. If not stated otherwise, formulations, differing from the example in the solvent composition (solvent types or ratios), polymer concentration or drug loading were prepared accordingly.

2.2.2.2. In-situ microparticle systems (ISM)

Lysozyme-containing polymer solutions (phase 1) were filled into 1 ml single-use syringes (B. Braun Melsungen AG, Melsungen, Germany); sesame oil (phase 2) (0.25 g) was filled into a second syringe. The syringes were coupled with a connector (1.4 mm inner diameter, Transcoject-Gesellschaft für medizinische Geräte GmbH & Co. KG, Neumünster, Germany) and the two phases were emulsified by back-and-forth movement of the syringe plungers. If not stated otherwise, the formulations were prepared from a phase ratio 1:1w/w (total weight of one dose: 0.5 g) at a mixing speed of 1 cycle per second. 50 mixing cycles were applied.

2.2.3. Lysozyme extraction from nonaqueous systems  

2.2.3.1. Protein separation with ethyl acetate

The separation of lysozyme from nonaqueous suspensions and solutions in absence or presence of PLGA or an oil phase (in case of in situ microparticles) was carried out as follows: 0.10 – 0.25 g of a sample was weighed into 2 ml Eppendorf vials and ethyl acetate (protein nonsolvent / polymer solvent) was added up to the mark. The vials were gently vortexed and then centrifuged at 16060 g for 30 min (Heraeus Biofuge 13 Haemo, Heraeus Instruments, Osterode, Germany). Approx. 1.5 ml of the supernatant was removed and the washing cycle was repeated twice more. The protein precipitate was then dried under vacuum for 30 min (Heraeus oven VT 5042 EKP, Hanau, Germany coupled with a chemistry hybrid pump, Vacuubrand GmbH, Wertheim, Germany) to remove residual ethyl acetate. The dry protein pellet was then dissolved in 8 g release medium (33 mM sodium acetate buffer pH 5, which contained 0.01 % sodium azide). The quality and quantity of the recovered lysozyme could then be characterized with analytical methods described above (paragraph 2.2.1.2.). Additionally, the dry protein pellets was subjected to Fourier-transform infrared-spectroscopy (FTIR) to compare it with lyophilized lysozyme (as received) or lysozyme suspended or dissolved in aqueous or nonaqueous media. FTIR-spectra were generated with an Excalibur 3100 FTIR spectrophotometer (Varian Inc., Palo Alto, USA). Samples were placed on a horizontal ATR accessory with a single reflection diamond crystal (Pike MIRacle, Pike
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Technologies, Madison, USA). 500 scans at 2 cm\(^{-1}\) resolution were averaged and spectral contributions from water vapor were subtracted using Varian software (Resolution Pro 4.0). Finally, the spectra were smoothed with a 13 point smoothing function.

2.2.3.2. Alkaline extraction

As comparison to the nonaqueous extraction, lysozyme was extracted from a 30 % PLGA solution in the water-miscible 2-pyrrolidone. Masses of about 0.25 g (n=3) were injected into screw cap sealable test tubes filled with 8 g 0.5 N sodium hydroxide (NaOH) solution containing 0.1% sodium dodecyl sulfate. The tubes were incubated at 37°C in laying position in a horizontal shaker (GFL 3033, Gesellschaft für Labortechnik GmbH & Co. KG, Burgwedel, Germany) at 80 rpm for 2 days. The protein concentration in the solution was determined with an UV-spectrophotometer (UV-VIS scanning spectrophotometer 2101 PC, Shimadzu, Kyoto, Japan) at 281.2 nm or was analyzed with a C4-RP-HPLC (method described in paragraph 2.2.1.2.2.) after neutralization with 0.5 N. The actual drug loading was compared to the theoretical drug loading.

2.2.4. Lysozyme extraction of in situ implants wetted with release medium

In order to evaluate the capability of the nonaqueous separation method (paragraph 2.2.3.1.) to extract lysozyme from wetted implants (0.1 g each, n=3), 0.25 g release medium (33 mM sodium acetate buffer pH5 containing 0.01 % sodium azide) was added to 1 hour vacuum dried (Heraeus oven VT 5042 EKP, Hanau, Germany coupled with a chemistry hybrid pump, Vacuubrand GmbH, Wertheim, Germany) in situ implants based on 50% RG 502H in ethyl acetate (drug loading of 4 %, based on polymer). The wetted implants were either dried under vacuum at ambient temperature for 3 hours (Heraeus oven VT 5042 EKP, Hanau, Germany coupled with a chemistry hybrid pump, Vacuubrand GmbH, Wertheim, Germany), lyophilized for 2 days or were extracted without drying. For lyophilization, the samples were frozen in a freezer (Galenkamp super cold 85, Haake Mess – Technik GmbH u. Co, Karlsruhe, Germany) at -70°C and then freeze-dried in two steps: at 0.040 mbar / – 40°C for 24 h and at 0.040 mbar / 10°C for 16 h (Gamma 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The extracted protein was characterized in dependence of incubation time in the release buffer after extraction with Coomassie assay, HPLC, biological activity assay and PAGE according to the described methods (paragraph 2.2.1.2.).

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2.2.5. Lysozyme in PLGA solvents

The amount of lysozyme dissolved in PLGA solvents after 18 hours or 7 days equilibration at room temperature on a horizontal shaker (IKA HS501 digital, IKA-Werke GmbH & CO. KG, Staufen, Germany) at 100 rpm was determined after filtration through 0.45 mm polyamide membrane filter (NL 17, Schleicher & Schuell, Dassel, Germany). Except for PEG 400 and glycofurol, release medium (33 mM acetate buffer pH 5, containing 0.01% sodium azide) was added to all filtered samples in a ratio 1:1 (w/w) prior to quantification by HPLC analysis as described above (paragraph 2.2.1.1.2.2.). Additionally, lysozyme suspended or dissolved in selected organic was analyzed with FTIR and then compared to lyophilized lysozyme (as received) or with lysozyme dissolved in aqueous medium.

2.2.6. Lysozyme amount dissolved in in situ implants

The amounts of dissolved lysozyme in in situ implants based on 30 % PLGA (RG 502H and RG 502S) in DMSO, DMSO / triacetin 50:50 or triacetin were determined after 2 d equilibration of the systems at ambient temperature (~22°C) in a desiccator. A 2 g sample of each formulation was transferred into 2 ml Eppendorf vials and subjected to centrifugation at 16060 g for 30 min (Heraeus Biofuge 13 Haemo, Heraeus Instruments, Osterode, Germany). Obtained supernatants were filtered through 0.45 μm poly(vinylidene fluoride) (PVDF) filter cartridges (3 ml Captiva cartridge, Varian Inc., Palo Alto, CA, USA) and, after overnight standing in a desiccator, the clear filtrates were divided into three sub-samples before lysozyme was extracted from each sub-sample using the described separation method (paragraph 2.2.3.1.).

2.2.7. Phase diagrams of lysozyme and PLGA in ternary solvent blends

Turbidimetric titrations with visual endpoint determination at room temperature were conducted in order to component ratios, where phase separation occurred. The phase boundaries were determined for ternary solvent blends of DMSO / ethyl acetate / water and DMSO / triacetin / water or for 40 % PLGA (RG 502H or RG 502S) and 2.7 % lysozyme-containing solutions in the ternary solvent systems, respectively. Phase diagrams of the solvent systems were determined by dropwise addition of water or ethyl acetate (triacetin) to blends of DMSO / ethyl acetate (triacetin) or DMSO / water.
Compositions at the apparent phase boundaries were repeatedly evaluated in order to exclude temperature effects during mixing of the solvents. About 1 g solution of either 40 % PLGA (RG 502H or RG 502S) in DMSO / ethyl acetate (triacetin) or 2.7 % lysozyme in DMSO / water (27 mg lysozyme in 1 g of DMSO / water) were titrated under gentle agitation with their respective nonsolvents water and ethyl acetate (triacetin) 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.

2.2.8. Storage stability of lysozyme in DMSO / triacetin blends

The stability of lysozyme in presence or absence of 30 % PLGA (RG 502H, RG 502S in DMSO only) was investigated in blends of DMSO and triacetin.

Preparation of lysozyme-containing DMSO / triacetin blends

Approx. 2.5 g of approx. 67 mg lysozyme-containing DMSO / triacetin blends (protein content of 2.7 %) were prepared. Dissolution in DMSO was conducted through application of intermittent vortexing starting directly upon contact of DMSO with the exactly weighed amount of lysozyme for approximately 2 h to avoid protein sedimentation and caking of gelled protein particles. The DMSO / triacetin blend (50/50) was prepared by precipitation of lysozyme from a pre-prepared 5.4 % protein solution in DMSO through dropwise addition of triacetin under vortexing. The triacetin / DMSO (50/50) system was prepared by adding lysozyme to triacetin under vortexing to obtain a concentration of 5.4 % prior to the addition of DMSO. The dispersion of lysozyme in triacetin was prepared by a vortex mixing.

Preparation of lysozyme-containing PLGA solutions (in-situ implants, ISI)

Lysozyme (as received) was incorporated with PLGA into DMSO / triacetin systems (100/0, 50/50 and 0/100) to obtain 5 g of in-situ implant formulations. The resulting formulations contained 30 % PLGA and 4 % protein (based on polymer). In case of the DMSO sample, lysozyme (about 60 mg) was first dissolved in DMSO (3.5 g) before PLGA (1.5 g) was added slowly under gentle vortexing. For the DMSO / triacetin (50/50) sample, the lysozyme portion (60 mg) was again dissolved in DMSO (1.75 g) prior to the incorporation of PLGA (1.5 g). Triacetin (1.75 g) was added after the polymer was
completely wetted. The triacetin sample was prepared by dispersing lysozyme (as received; 60 mg) in triacetin (3.5 g) before PLGA (1.5 g) was added. Complete polymer dissolution was obtained upon standing in a desiccator at ambient temperature.

The samples (with and without PLGA) were then stored in glass vials, which were placed into desiccators at room temperature or additionally at 4°C or at 40°C (DMSO only samples without PLGA). The weight of the vials was followed during storage and no weight increase / loss was observed. After vortexing, n=3 samples (0.1 to 0.3 g) were withdrawn from the each formulation. The protein-containing solvent systems were investigated after 1, 7, 20 and 48 d and the in-situ implants after 6, 20, 48, 66, and 98 d of storage. The protein was separated from the samples and then analyzed with HPLC, the activity assay and PAGE according to the methodology described above (paragraphs 2.2.1.2. and 2.2.3.1.).

2.2.9. Drug release

0.25 g of in situ implants and accordingly 0.5 g of in situ microparticle formulations with a phase ratio of 1:1 were injected into screw cap sealable 12 ml test tubes (VWR International GmbH, Darmstadt, Germany) filled with 8 g of release medium (33 mM pH 5 sodium acetate buffer containing 0.01 % sodium azide as preservative). The sealed tubes 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. Quantitative and qualitative analysis was performed with the Coomassie assay, HPLC and/or the biological activity assay described above (paragraph 2.2.1.2.). Quantification of lysozyme and protamine sulfate was performed using the Coomassie assay (paragraph 2.2.1.2.2.) to determine the total protein content and the HPLC assay (paragraph 2.2.1.2.2.) to quantify the concentration of lysozyme only. Oppositely to the Coomassie assay with lysozyme only, only net absorbances $[A_{595\text{ nm}(protein)}-A_{595\text{ nm}(blank)}]$ below 0.5 were used in the standard curve. This area could be well described by a linear relationship between total amount of protein and net absorbance. The maximum concentration ranges were thus 0-25 µg/g for lysozyme and 0-12 µg/g for protamine sulfate. The standard curve and the contribution of each protein to the total net absorbance ($A_{595\text{ nm}}$) was calculated using multiple-linear-regression of net absorbance data obtained from solutions of lysozyme and protamine sulfate in release medium at various concentration ratios. Knowing the
concentration of lysozyme from the HPLC analysis, the concentration of protamine could then be calculated.

2.2.10. **Study of flow behavior and viscosity**

The flow behavior of solvents and lysozyme solutions (3, 6 and 9 %, w/w) were investigated with a fixed shear stress of 10 Pa for 120 s using a computer-linked rheometer equipped with a plate-cone setup of 60 mm diameter (1° angle) (Rheostress RS 100, Haake Messtechnik GmbH, Karlsruhe, Germany) at 20 +/- 0.2°C. Concentrated PLGA solutions (30 or 40 % RG 502H or RG 502S) in DMSO, ethyl acetate and triacetin were characterized with a plate-cone setup of 20 mm diameter (4° angle).

The viscosity values were calculated through averaging the viscosity values obtained from RheoWin Pro software (Thermo Haake GmbH, Karlsruhe, Germany) after equilibration (constant viscosity).

2.2.11. **Milling of lysozyme powder**

Lysozyme (as received) was milled in a ball mill (Retsch MM2000, Retsch GmbH & Co. K.G., Haan Germany) under nitrogen cooling for 2 x 10 min to reduce its particle size.

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

2.2.12.1. **Optical light microscopy**

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).

2.2.12.2. **Photon correlation spectroscopy**

Samples were measured with a Malvern Zetasizer 3000 HS (Malvern Instruments Ltd., United Kingdom) at 20°C. Lysozyme dispersed in triacetin served as a comparison to samples containing precipitated protein. A refractive index of 1.59 was used for the suspended particles and 1.4305 for triacetin. A viscosity of 17.0 mPas was determined for triacetin at 20 +/- 0.2°C (Rheostress RS 100, Haake Messtechnik GmbH, Karlsruhe, Germany).

Gelled / coacervated protein particles, which formed during preparation of in situ implant formulations based on 30 % RG 502H in DMSO, were measured undiluted in order to avoid
dilution artifacts (state of solvation). A refractive index of 1.49 was used for the coacervated lysozyme particles, which corresponded to the refractive index of a 15% lysozyme solution in DMSO (measured with a Carl-Zeiss refractometer, Jena, Germany). A refractive index of 1.4767 was measured 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 ±/− 0.2°C (Rheostress RS 100, Haake Messtechnik GmbH, Karlsruhe, Germany).

Protein suspensions in 30% RG 502H solutions in DMSO / ethyl acetate / water (75/25/0 and 70.5/23.5/6) were diluted (40 times) with triacetin (protein nonsolvent / 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/ EA/ water 75/25/0 and 70.5/23.5/6. Because of dilution with triacetin, the refractive index and viscosity were used accordingly.

2.2.13. Solvent miscibilities

First, a broad range of ratios of investigated solvent pairs (PLGA solvents and water or fatty acid esters) was prepared in glass test tubes in order to approximate the miscibilities before turbidimetric titrations with visual endpoint determination were repeatedly conducted at room temperature. Approx. 1 g of deionized water or a fatty acid ester was accurately weighed into a 5 ml glass test tube followed by dropwise addition of organic solvent under vigorous vortexing until phase separation occurred. The sealed samples were stored in desiccators and repeatedly evaluated in order to identify the amount of organic solvent, which was just miscible with the water or oil phase.

2.2.14. Degradation study of PLGA (RG 502H)

PLGA (RG 502H as received) was incubated in aqueous media for up to 4 weeks in order to evaluate a potential effect of the choice of the release medium and the influence of buffer exchange on the degradation of the polyester. For each sampling time-point, n=3 samples of 35 to 75 mg PLGA in 8 g buffer were prepared. The vials were incubated in a horizontal shaker (Gemeinschaft für Labortechnik, Burgwedel, Germany) at 37°C and 80 rpm. At predetermined time-points, the buffer was discarded and the samples lyophilized according to the described method (paragraph 2.2.4.). The samples were then stored at 4°C before analysis. The residual PLGA mass was then dissolved in chloroform (~2 mg/ml), filtered through 0.45 μm polyamide filter membranes (Schleicher & Schuell, Dassel, Germany) and then injected into a HPLC apparatus (SCL-10A, Shimadzu, Tokyo, Japan).
which was equipped with a gel permeation chromatography column (PLgel 5 μm MIXED-D, 7.5 × 300 mm; Polymer Laboratories GmbH, Darmstadt, Germany). The injection volume was 50 μl and the mobile phase consisted of chloroform containing 0.1 % (v/v) triethlylamine. The flow rate was 1 ml/min and the column temperature 40°. Refractive index detection was applied (RID-10A, Shimadzu, Tokyo, Japan). The number (Mn) and weight (Mw) average molecular weight and the polydispersity index (PI) were calculated by Cirrus GPC software (Polymer Laboratories GmbH, Darmstadt, Germany) using 9 polystyrene standards with a peak average molecular weight ranging from 580 to 299,400 (Polymer Laboratories GmbH, Darmstadt, Germany). The molecular weight of the sample at time-point \( t \) was expressed relative to the initial molecular weight of the polymer.

### 2.2.15. Injection force measurements

The evaluation of the injectability of formulations was based on measurements with a computer-assisted texture analyzer (TA.XTplus, Stable Micro Systems, Vienna Court, UK). 0.25 g drug-free 30% PLGA (RG 502H and RG 502S) solutions in triacetin or 0.5 g of the corresponding in situ microparticle emulsion with sesame oil (phase ratio 1:1, 50 mixing cycles at 1/s) were ejected from 1 ml syringes (Injekt-F, B. Braun Melsungen AG, Melsungen Germany), which were coupled with 20 G (1.5 inch length) hypodermic needles. The plunger of the syringe was placed in contact with a cylindrical probe (10 mm diameter). A 50 N load cell was used and the injection speed was set to 0.5 mm/s. A trigger force of 0.1 N, a maximum force of 20 N and a break sensitivity of 100 N were set. The injection force-over plunger displacement curves were monitored using Texture Expert Exceed software (Stable Micro Systems, Vienna Court, UK).

### 2.2.16. Thermogravimetric analysis (TGA)

Lysozyme samples (approx. 10 to 25 mg) were placed in alumina crucibles for TGA. The weight loss was measured on a Mettler TC 15- TA controller coupled with a Mettler TG50 thermobalance (Mettler Toledo AG, Giessen, Germany). Initial heating from ambient to 105°C was performed at a heating rate of 20°C/min followed by holding at 105°C for 45 min. The absolute loss on drying (LOD) was then calculated as the difference between the initial and the sample weight after 40 min at 105°C. The percentage of weight loss was the ratio of LOD and the initial sample weight multiplied by 100%.