# 2 Materials and Methods

## 2.1 Materials

Poly(D,L-lactide) (PLA RESOMER® grade R 203 and R 202H); Poly(D,L-lactide-co-glycolide) (PLGA 50:50 RESOMER® grade RG 502, RG 503, RG 502H, RG 503H and RG 504 and PLGA 75:25 RESOMER® grade RG 752 and RG 755) (Boehringer Ingelheim Pharma KG, Ingelheim, Germany); lidocaine base and hydrochloride**,** peanut oil, sesame oil, corn oil, polysorbate 80 (Tween® 80) and benzyl benzoate (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); Miglyol 812 (Sasol germany GmbH, Witten, Germany); N-methyl-2-pyrrolidinone (NMP), sorbitan monooleate (Span® 80) and benzyl alcohol (Merck-Schuchardt OHG, Hohenbrunn, Germany); polyethylene glycol (PEG 400), dimethyl sulfoxide (DMSO), 1,4-dioxane, acetic acid and ethyl acetate (Merck KGaA, Darmstadt, Germany); 2-pyrrolidone and block copolymer of polyethylene and polypropylene (Lutrol F 68) (BASF AG, Ludwigshafen, Germany); triacetin (Eastman Chemical Company, Kingsport, USA).

## 2.2 Methods

### 2.2.1 Solubility and miscibility

*Polymer solubility:* Glass vials were filled with 400 mg of different PLGA polymers and 10 ml solvent, sealed with cap and aluminium foil and agitated at 165 rpm for 48 hours at room temperature in a horizontal shaker (HS 501 digital, IKA Labortechnik, IKA® Werke GmbH & Co. KG, Staufen, Germany). A visual analysis was made (soluble = single clear phase; partially soluble = two phases, one bigger than the other; insoluble = two phases, equal size). During the procedure no evaporation of the solvent occurred. A very low concentration was chosen because the aim in this part of the study was only to know whether the polymers could be dissolved or not.

*Solvent/oil miscibility:* Glass vials were filled with 5 ml solvent and 5 ml oil sealed with cap and aluminium foil and agitated at 165 rpm for 24 hours at room temperature in a horizontal shaker (HS 501 digital, IKA Labortechnik, IKA® Werke GmbH & Co. KG, Staufen, Germany). A visual analysis was made after shaking and 8 hours after shaking.

### 2.2.2 PLGA dissolution time in organic solvents

PLGA RG 752 (200 mg, 500 mg or 800 mg) and different organic solvents were filled into 2 ml glass vials. The vials were then agitated at 165 rpm and room temperature in a horizontal shaker (HS 501 digital, IKA Labortechnik, IKA® Werke GmbH & Co. KG, Staufen, Germany). The time until complete dissolution of the polymer was measured. The tested polymer concentrations were 10%, 25% and 40% w/v, based on solution.

#### 2.2.3 PLGA solution viscosity

The viscosity of PLGA solutions was measured with a rotational rheometer with cone-plate geometry (Haake Mess–Technik GmbH & Co. KG., Karlsruhe, Germany) at a shear rate of 20 $s^{-1}$ during 30 s (22±0.5°C). Solutions prepared with ethyl acetate were not measured because of a too rapid solvent evaporation.

### 2.2.4 Milling of PLGA powder

PLGA (as received from the supplier) was milled in a ball mill (Retsch MM2000, Retsch GmbH & Co. K.G., Haan Germany) under nitrogen cooling for 5 min to reduce its particle size. The milled particles were classified into different particle size fractions by sieving.

### 2.2.5 Lyophilization of PLGA solution into PLGA-sponges

*Reconstituable solid PLGA systems:* PLGA (2% w/v, based on the solvent) and lidocaine base or hydrochloride (40% w/w based on PLGA) were dissolved in 1,4-dioxane or acetic acid. The solutions were poured in Petri dishes Duroplan® *(*100 mm diameter*,* Carl-Roth GmbH & Co. KG, Karlsruhe, Germany) or 2 ml plastic syringes (Terumo, Leuven, Belgium), frozen in a freezer (Galenkamp super cold 85, Haake Mess–Technik GmbH & Co. KG., Karlsruhe, Germany) at -70°C and then freeze-dried at 0.040 mbar and – 40°C for 72 h (primary drying) and at 0.040 mbar and 10°C for 10 h (secondary drying) (Gamma 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The freeze-dried PLGA-sponges were then stored in a desiccator.

*Implants:* The polymer (5%, 10%, 20% or 30% w/v based on solvent) and lidocaine base or hydrochloride (10%, 25% or 40% w/w based on polymer mass) were dissolved in 1,4-dioxane or acetic acid. 1-2 ml solution was placed in polypropylene moulds (rack of 6 or 9 mm diameter). The samples were frozen in a freezer (Galenkamp super cold 85, Haake Mess – Technik GmbH u. Co, Karlsruhe, Germany) at -70°C (slow freezing) or with liquid nitrogen (fast freezing) and then freeze-dried at 0.040 mbar and – 40°C for 72 h (primary drying) and 0.040 mbar and 10°C for 10 h (secondary drying) (Gamma 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Some implants were cured at different temperatures (22°C, 30°C, 50°C, 65°C and 70°C) under vacuum for 24 h (Heraeus oven VT 5042 EKP, Hanau, Germany coupled with a chemistry hybrid pump, Vacuubrand GmbH, Wertheim, Germany). The freeze-dried samples were then stored in desiccators.

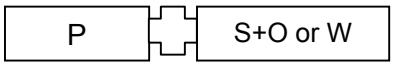
### 2.2.6 Reconstitution of solid PLGA systems into in situ implant or microparticle (ISM) systems

*In situ implants:* 100 mg polymer powder (as received) was placed in one syringe (1 ml syringe, Henke-Saas, Wolf GmbH, Tuttlingen, Germany) and 900

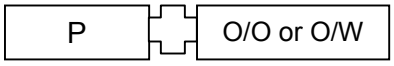
mg solvent in a second syringe. The two syringes were connected with a polypropylene connector. The PLGA solution (10% w/w, based on solution) was formed by pushing the plungers forward and backward at a rate of 0.5 cycle/s. The dissolution time was taken as the number of cycles required for complete dissolution of the polymer (as indicated by a clear polymer solution). The following parameters were investigated: type of solvent (NMP, triacetin, 2-pyrrolidone and ethyl acetate), type of polymer (R 203, RG 502, RG 503, RG 504, RG 502H, RG 503H and RG 752), polymer concentration (10%, 25% and 40% w/w), polymer treatment (milled PLGA particles with a size from 50 µm to 500 µm; PLGA sponges prepared by lyophilization), syringe size (2 ml from Terumo, Leuven, Belgium and 5 ml from B. Braun Melsungen AG, Melsungen, Germany), mixing speed (0.25, 0.5, 1 and 2 cycles/s).

*In situ forming O/W- and O/O-microparticles:* The same procedure as above was used with the following modifications: first syringe: 50 mg, 100 mg or 150 mg PLGA particles or sponge (resulting in a 10%, 20% or 30% w/w polymer solution, respectively); second syringe: for O/W-ISM [350 mg water/350 mg solvent (triacetin, benzyl alcohol and ethyl acetate)], for O/O-ISM [350 mg sesame oil/350 mg solvent (2-pyrrolidone, DMSO and NMP)]. The maximal polymer concentration, which could be prepared, was 10% w/w for triacetin and 20% w/w for benzyl alcohol, at higher concentrations the polymer precipitated. In addition to PLGA particle size/ PLGA sponge and type of solvent, the effect of the following process parameters on the dissolution time was studied:
*Mixing method:* The number of cycles necessary to dissolve the PLGA polymer (polymer as received, milled particles with a particle size < 160 µm, PLGA-sponge) in benzyl alcohol for the O/W-systems and in DMSO for the O/O-systems (at a 20% w/w polymer concentration) was investigated by the following mixing methods. Method A: Syringe 1 - polymer, syringe 2 - solvent and external phase (water or oil) (not mixed); Method B: Syringe 1 - polymer, syringe 2 - solvent and external phase (water or oil) (mixed, emulsion); Method C: Syringe 1 - polymer dispersed in external phase (water or oil), syringe 2 – solvent.

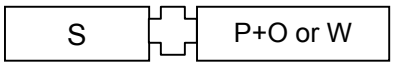
- Method A → phase ratio (1:1)




- Method B → phase ratio (1:1)




- Method C → phase ratio (1:1, 1:2, 1:3, 1:4)




*Mixing cycles*: Syringe 1: 50.0 or 150.0 mg PLGA powder or sponge (to give a final polymer solution concentration of 10% or 25% w/w); syringe 2: for O/W-ISM [450.0 mg ethyl acetate / 448.2 mg water + 1.8 mg Poloxamer 188], for O/O-ISM [445.5 mg 2-pyrrolidone + 4.5 mg Tween® 80 / 441.0 mg peanut oil + 9.0 mg Span® 80]. The contents of the two syringes were mixed to dissolve the polymer and to form the ISM emulsion. The size of 100 emulsion droplets was measured immediately after 10, 25 and 50 mixing cycles with a light microscope (Axioskop, Carl Zeiss Jena, GmbH, Jena, Germany coupled with an imaging software Easy measure, INTEQ Informationstechnik GmbH, Berlin, Germany) and again after 2 h standing in order to determine the short term stability of the ISM-emulsions.

### 2.2.7 Differential scanning calorimetry (DSC)

*Freezing behavior:* The freezing cycle of the lyophilization process for the preparation of the PLGA sponges was simulated by DSC (Mettler DSC 821e equipped with a thermal analysis data system; Mettler Toledo AG, Giessen,

Germany). Solutions of different PLGAs in acetic acid or 1,4-dioxane were prepared at concentrations of 10%, 20% and 30% w/w and accurately weighed (100-140 mg) in aluminium pans (Al-Crucibles, 70 µl), sealed, cooled from 25°C to -70°C, and heated to 25°C at a rate of 5°C/min.

*Glass transition temperature (Tg):* The glass transition temperature of the polymers and implants was determined by DSC (Mettler DSC 821e equipped with a thermal analysis data system; Mettler Toledo AG, Giessen, Germany). The samples were accurately weighed (5-10 mg) in aluminum pans (Al-Crucibles, 40 µl), sealed, heated from 5°C to 100°C, quenched to – 40°C and reheated to 100°C at a rate of 10°C/min.

### 2.2.8 Thermogravimetric analysis (TGA)

The samples (approx. 20 to 40 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) during heating from 50°C to 130°C at a heating rate of 20°C/min followed by holding at 130°C for 15 min. The amount of residual solvent was the difference between the initial and the final sample weight. The percentage of weight loss was calculated as the ratio of weight of residual solvent to the initial sample weight multiplied by 100.

### 2.2.9 X-ray diffraction (XRD)

Wide angle X-ray scattering measurements were carried out with a Philips PW 1830 X-ray generator with a copper anode (Cu K$\alpha$ radiation, $\lambda$ = 0.154418 nm, 40 kV, 20 mA) fixed with a Philips PW 1710 diffractometer (Philips Industrial & Electron-acoustic Systems Division, Almelo, The Netherlands). The scattered radiation was measured with a vertical goniometer (Philips PW 1820) (Philips Industrial & Electron-acoustic Systems Division, Almelo, The Netherlands). The scanning rate used was of 0.02° 2$\theta$ $s^{-1}$ over the range of 4-40° 2$\theta$.

### 2.2.10 Scanning electron microscopy (SEM)

The PLGA-sponges obtained after lyophilization were immersed in liquid nitrogen, cut with a razor blade to expose their inner structure, fixed on a sample holder and coated for 120 s with gold-palladium under an argon atmosphere using a gold sputter chamber (sputter coater device 040, Balzers Union, Lichtenstein). The samples were then observed with a scanning electron microscope (Philips SEM 515, Type PW 6703, Philips Industrial Electronics N.V., Eindhoven, The Netherlands).

### 2.2.11 Mechanical properties

The weight and height (micrometer caliper) of the implants were measured. The strength of the implants was determined with a texture analyzer (TA-XT plus, Stable Micro Systems, Winopal GmbH, Ahnsbeck, Germany). The implant was placed under a cylindrical probe with a flat surface (6 mm diameter), compressed to 75% of the initial implant height (mode, force in compression; load cell, 50 kN; speed, 1 mm/s) and then held for 60 s. The strength is defined as the force necessary to compress the sample to 75% of the initial sponge height ($force_{max}$). The percentage of elasticity is calculated as the force after 60 s holding ($force_{hold}$) divided by the force before holding ($force_{max}$) multiplied by 100.

$$\% \text{ elasticity} = \frac{\text{Force}_{\text{max}}}{\text{Force}_{\text{hold}}} \times 100$$

### 2.2.12 Lidocaine Release

The implants were placed in 25 or 50 ml borosilicate glass test tubes with screw-cap (Schütt Labortechnik GmbH, Göttingen, Germany), which were filled completely with phosphate buffer pH 7.4 in order to immerse the implants. The test tubes were then agitated at 37°C and 68 rpm (horizontal shaker, GFL 3033, Gesellschaft für Labortechik GmbH & Co. KG, Burgwedel, Germany) (n=3). 5 or 10 ml release medium were withdrawn at predetermined time points and

replaced with an equivalent volume of fresh buffer. The amount of lidocaine released was determined UV-spectrophotometrically (UV-VIS scanning spectrophotometer, UV – 2101 PC, Shimadzu, Kyoto, Japan) at 271 nm.

### 2.2.13 Swelling, water uptake and mass loss of the implants

*Swelling*: Implants (6 mm diameter) were placed in 60 ml Nalgene® polypropylene containers filled completely with phosphate buffer pH 7.4. The containers were then agitated at 68 rpm and 37°C (horizontal shaker, GFL 3033, Gesellschaft für Labortechik GmbH & Co. KG, Burgwedel, Germany) (n=3). The implants were removed at different time points, wiped slightly with absorbent paper, weighed and measured (diameter and height) with a macroscope (Carl Zeiss Jena, GmbH, Jena, Germany) coupled with a imaging software (Easy measure, INTEQ Informationstechnik GmbH, Berlin, Germany). The implants were then put back in the medium.

*Water uptake and mass loss studies*: implants (9 mm diameter) were immersed in phosphate buffer pH 7.4 at 37°C at the conditions described above. Implants removed at different time points, were frozen (Freezer Galenkamp super cold 85, Haake Mess–Technik GmbH u. Co., Karlsruhe, Germany) at -70°C and then freeze-dried at 0.040 mbar and – 40°C for 48 h (primary drying) and 0.040 mbar and 10°C for 10 h (secondary drying) (Gamma 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and subsequently weighed. The water uptake was the difference between the wet weight at time t and the initial or the dry weight at time t; the mass loss was the difference between the initial and the dry weight at time t.

### 2.2.14 Follow-up stability

PLGA-sponges were stored in a desiccator during two and half years in order to found if any drug recrystallization occurred. The samples were analyzed by DSC as explained in 2.2.7, glass transition temperature.