2. Materials and Methods

2.1. Materials

The following chemicals were obtained from commercial suppliers and used as received.

Drugs

leuprolide acetate (leuprolide, Lipotec S.A. Barcelona, Spain).

Caffeine (Merck KGaA, Darmstadt, Germany).

Polymers

Poly(D,L-lactide) (PLA), Poly(D,L-lactide-co-glycolide) (PLGA) (Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany) (Table 4).

Table 4 Physicochemical properties of poly(lactide-co-glycolide) PLGA and poly(lactide) PLA.

Polymer / Properties	PLGA				PLA	
	RG 503H*	RG 502H	RG 503**	RG 502	R 203H	R 202H
Inherent viscosity *** (dl/g)	0.38	0.19	0.41	0.24	0.35	0.20
Acid number (mg KOH/g)	4	11			7	10

* H-series with free carboxyl termini

** Non-H-series with ester termini

*** 0.1% solution in chloroform, at 25 °C

All the data are from Boehringer Ingelheim Pharma GmbH & Co. KG.

Polyvinylpyrrolidone (PVP) Kollidon® 12 PF, 17 PF, Poloxamer 188 (Lutrol® F68) (BASF Aktiengesellschaft, Ludwigshafen, Germany).

Solvents

N-methyl-2-pyrrolidone (NMP) (Pharmasolve) (ISP customer service GmbH, Köln, Germany), dimethyl sulfoxide (DMSO), 2-pyrrolidone, benzyl alcohol, methylene chloride,

methanol, polyethylene glycol 400 (PEG 400), propylene carbonate triacetin (Merck KGaA, Darmstadt, Germany), benzyl benzoate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), triethyl citrate (TEC) (Morflex, Inc, Greensboro, North Carolina), acetonitrile, ethanol (Rotisolv® HPLC Gradient Grade, Carl Roth GmbH + Co, Karlsruhe, Germany).

Oils

Ethyl oleate (Merck KGaA, Darmstadt, Germany), medium chain triglyceride (MCT) (Miglyol 812 N®, Synopharm GmbH, Barsbüttel, Germany), peanut oil, sesame oil, soybean oil, safflower bean oil, and castor oil (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Surfactants

Poloxamer 188 (Lutrol® F68) (BASF Aktiengesellschaft, Ludwigshafen, Germany), polyvinyl alcohol (PVA, Mowiol 40-88, Clariant GmbH, Frankfurt am Main, Germany), sorbitan monooleate (Span 80) polyethylene sorbitan monooleate (Tween 80) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Buffering agents and inorganic salts

Potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, sodium chloride, and sodium azide (Merk KGaA, Darmstadt, Germany), mannitol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Viscosity increasing agents

Aluminum monostearate (AM) (Sigma Aldrich Chemie GmbH, Steinheim, Germany).

2.2. Methods

2.2.1. Preparation of the formulations

2.2.1.1. Microparticles prepared by the cosolvent method

350 mg PLGA and 88 mg leuprolide acetate were dissolved in a solvent mixture of 2.5 g methylene chloride and 0.5 g methanol. 574 mg PLA and 100 mg leuprolide acetate were dissolved in a solvent mixture of 4.0 g methylene chloride and 0.8 g methanol. The solution was emulsified into 800 ml (PLGA solution) or 1300 (PLA solution) 0.25% w/w PVA aqueous solution (external phase) using a homogenizer (Ultra-Turrax T 25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at 8000 rpm. The emulsion/suspension was stirred at 400 rpm for 2 h with a magnetic stirrer (Variomag® Electronicrührer, Multipoint HP 6, H+P Labortechnik GmbH, Oberschleissheim, Germany) to extract and evaporate the methylene chloride. The solidified microparticles were recovered by filtration and vacuum-dried for 1 d or freeze-dried (microparticles prepared with PVP or MCT addition).

The standard formulation of PLGA microparticles was kept as 20% drug loading based on PLGA plus drug, PLGA RG 503H as polymer carrier, 800 ml external aqueous phase, vacuum-drying, unless specifically mentioned.

The additives, PVP (K12 and K17) and MCT were added to the leuprolide-containing polymer solution. The theoretical content (%) was calculated based on the weight of polymer and leuprolide.

Freeze-drying of an aqueous microparticle suspension

After filtration, microparticles (approx. 450mg) were suspended in 900 mg 16% (w/w) (20% in the case of the MCT addition) mannitol solution. The suspension was frozen at -40 °C for 2 h and freeze-dried (primary drying: chamber pressure 0.01 mbar over 24 h with a shelf temperature of -15 °C, second drying: chamber pressure 0.01 mbar with a shelf temperature of 20 °C for 12 h) (Gamma 2-20, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

2.2.1.2. Film casting

175 mg PLGA and 44 mg leuprolide acetate were dissolved in a solvent mixture of 1.25 g methylene chloride and 0.25 g methanol. 0.5 g solution was added to a glass vial (height: 65 mm; diameter: 17 mm). The vial was kept at ambient temperature for 24 h and then at 40 °C for another 24 h to evaporate the organic solvents.

2.2.1.3. In situ forming implants

380 mg PLGA and 20 mg leuprolide acetate were dissolved in 885 mg NMP by shaking the filled glass vial on a horizontal shaker (HS 501 Digital, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) for 30 min at ambient temperature.

2.2.1.4. In situ forming microparticles

In situ forming microparticles were prepared with a two-syringe system (Fig. 9).

One 1 ml syringe (Single-use syringe, B. Braun Melsungen AG, Melsungen, Germany) containing the internal polymer phase (a solution of 5 mg leuprolide acetate and 95 mg PLGA in 221 mg NMP, 15 mg leuprolide acetate and 85 mg PLA in 200 mg of NMP) was coupled with a connector (inner diameter: 1.4 mm) to another 1 ml syringe containing the same weight of external continuous phase (peanut oil containing 2% Span 80 and 2% aluminum monostearate or sesame oil containing 2% Span 80 and 2.5% aluminum monostearate). Emulsification was achieved by pushing the plungers of the syringes and hence the internal and external phases forward and backward 50 cycles at a speed of 2 cycles per second.

Cosolvent system

A solvent mixture of NMP and the other solvent (PEG 400, PC, TA, TEC, BA, or BB, 0-40% w/w based on total weight of solvents) was used to dissolve the leuprolide acetate and PLGA to consist of internal phase. The external phase used is 2% span 80 and AM in peanut oil. Emulsification was achieved in the same procedure as mentioned above.

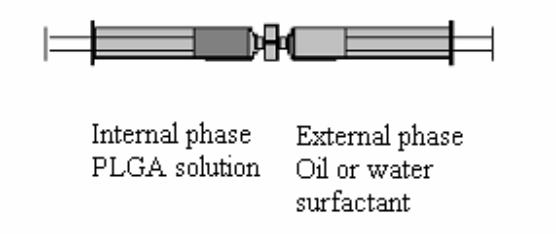

Fig. 9. Two syringe system for preparation of in situ forming microparticles.

2.2.2. Determination of encapsulation efficiency of conventional microparticles

UV assay

13 mg microparticles were added to 10 ml 0.1 N NaOH aqueous solution. After 12 h of shaking, the drug concentration in solution was determined by UV at 279 nm (UV-vis scanning spectrophotometer 2101 PC, Shimadzu, Kyoto, Japan). The encapsulation efficiency (100 x actual drug content/theoretical drug content) was calculated.

Reverse phase high performance liquid chromatography (RP-HPLC) assay

Leuprolide acetate-containing PLGA microparticles (approx. 10 mg) were suspended in a mixture of 10 ml phosphate buffer (1/30 M, pH 7.0) and 2 ml methylene chloride. The suspension was shaken for 24 h at ambient temperature on a horizontal shaker (HS 501 Digital, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). After centrifugation, the aqueous supernatant was collected. The concentration of leuprolide in the supernatant was analyzed by RP-HPLC (SCL-10A VP, Shimadazu, Japan), C18 Eurospher-100 column (150 X 4 mm, Knauer GmbH, Germany) (mobile phase: phosphate buffer (1/30 M, pH 7), acetonitrile 70:30 v/v; flow rate: 1.2 ml/min; UV detection at 280 nm) (n=2). The percent encapsulation efficiency is calculated as (actual drug loading / theoretical drug loading) * 100%. With formulations containing PVP or MCT, the encapsulation efficiency was calculated based on 100% PVP and MCT entrapment.

2.2.3. In vitro release

2.2.3.1. Conventional microparticles

With PLGA microparticles, microparticles (approx. 10 mg, accurately weighed) were suspended in 6 ml phosphate buffer (1/30 M, pH 7.0, 0.01% w/w Tween 80 and 0.01 % w/w sodium azide). The suspensions were incubated in glass test tubes at 37 °C in an incubation shaker (GFL 3033, Gesellschaft für Labortechnik GmbH & Co. KG, Burgwedel, Germany) (85 rpm). The samples were centrifuged at predetermined time points. 5 ml supernatant was collected and replaced. The leuprolide concentration was determined by UV at 279 nm (n=2) In the case of PVP-containing microparticles, the leuprolide concentration was determined by RP-HPLC (method described in section 2.2.4). The UV-absorbance of leuprolide solutions at 37°C did not change during sample intervals up to 7 d (longest sampling span) confirmed by reverse phase high performance liquid chromatography RP-HPLC (data not shown).

With PLA microparticles, the drug left in the polymer matrix rather than in the release medium was analyzed for all sampling points after 30 d because of leuprolide degradation in the release medium after long time periods. 13 mg microparticles were suspended in 6 ml of phosphate buffer (1/30 M, pH 7.0, and 0.05 % w/w sodium azide). The mixtures were incubated at 37 °C in an incubation shaker (85 rpm). During the first 30 days, 5 ml release medium was separated and replaced. The leuprolide concentration was analyzed by UV at 279 nm (n=3). After 30 days, at each time interval, microparticles were separated from the release medium and washed with hexane and water and filtered. Thereafter, they were suspended in a mixture of methylene chloride (2 ml) and phosphate buffer (1/30 M, pH 7.0, 0.05% sodium azide, 8 ml) (n=3). After shaking overnight, the leuprolide concentration in the aqueous solution was analyzed by RP-HPLC.

2.2.3.2. Film

6 ml phosphate buffer (1/30 M, pH 7.0, 0.01 % w/w sodium azide) were added to the film-containing vials and shaken at 37 °C in an incubation shaker. 3 ml of medium was taken and replaced at predetermined time points. The leuprolide concentration was determined by UV at 279 nm (n=3).

2.2.3.3. In situ forming implants

0.3 g drug-containing polymer solution was injected into 6 ml phosphate buffer (1/30M, pH 7.0) through a 20 gauge needle within 10 sec and shaken at 37 °C in an incubation shaker. 5 ml release medium was taken and replaced at determined time points. The leuprolide concentration was determined by UV at 279 nm (n=3). The influence of the solvents on the UV adsorption was diminished by blank formulation.

2.2.3.4. In situ forming microparticles

With PLGA-ISM, approx. 0.2 g emulsion was injected into dialysis bags (6 cm long, 2.2 cm wide, molecular weight cut off 12-14,000 Daltons, Medicell International Ltd, London, United Kingdom). The dialysis bags were placed into 10 ml phosphate buffer (1/30 M, pH 7.0, 0.01% sodium azide) at 37 °C in an incubation shaker (n=3). 8 ml medium were taken and replaced at predetermined points. The leuprolide concentration was determined by UV at 279 nm.

With PLA-ISM, the drug left in the polymer matrix rather than in the release medium was analyzed for all sampling points after 30 d because of leuprolide degradation in the release medium after long time periods. Approx. 0.15 g emulsion was injected into 10 ml phosphate buffer (1/30 M, pH 7.0, 0.05% sodium azide). The mixtures were incubated at 37 °C in an incubation shaker. During the first 30 d, 8 ml release medium was sampled and replaced. The leuprolide concentration was analyzed by UV at 279 nm (n=3). After 30 d, at each time interval, microparticles were separated from the release medium, washed with hexane and water, filtered and suspended in a mixture of 2 ml methylene chloride and 8 ml release medium (n=3). After shaking overnight, the leuprolide concentration in the aqueous solution was analyzed by RP-HPLC (SCL-10A VP, Shimadazu, Japan), C18 Eurospher-100 column (150 X 4 mm, Knauer GmbH, Germany) (mobile phase: phosphate buffer (1/30 M, pH 7), acetonitrile 70:30 v/v; flow rate: 1.2 ml/min; UV detection at 280 nm).

2.2.4. Particle and droplets size characterization

Optical Light Microscope

The microparticles and emulsions were observed under an optical light microscope (Axioskop, Carl Zeiss Jena GmbH, Jena, Germany). The particle size distribution of the formulation was evaluated following the observation of 100 particles and then calculated using an imagine software analysis (EasyMeasure, INTEQ Informationstechnik GmbH, Berlin, Germany).

Laser Diffraction (LD)

Microparticles were suspended in 0.1% Tween 80 aqueous solution and the size distribution was determined by laser diffractometry (LD) (LS 230, Beckman Coulter GmbH, Krefeld, Germany). Unless otherwise mentioned, the particle size of the microparticles was in the range of 5 µm to 40 µm for all investigated batches.

2.2.5. Microparticle morphology characterization

ISM emulsions were injected into 0.1 % Tween 80 containing phosphate buffer (1/30 M, pH 7.0) under stirring. After 5 hours of stirring, the formed microparticles were filtered and vacuum-dried.

Scanning electron microscopy (SEM) was used to image the surface and internal morphology of the microparticles. To investigate the inner structure, the microparticles were dispersed in a solvent-free glue UHU® (UHU GmbH & Co. KG, Baden, Germany). After drying in a desiccator, the hardened matrix was frozen in liquid N_2, followed by cutting with a razor blade. Samples were sputtered under an argon atmosphere with gold to a thickness of 8 nm (SCD 040, Bal-Tec GmbH, Witten, Germany), and were then observed with a scanning electron microscope (S-4000, Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

2.2.6. Differential Scanning Calorimetry (DSC)

DSC was used to quantitate MCT in the microparticles and to determine the glass transition temperature (T_g) of the PLGA. The sample (approx. 5 mg) was weighed in an aluminum pan (Al-Crucibles, 40 µl) and then analysed (DSC 821, Mettler Toledo AG, Giessen, Germany). In a typical analysis, the sample was cooled to –70 °C, followed by heating to 70 °C, cooling to 25 °C, and finally reheating to 70 °C at a constant rate of 10 °C/min. The T_g was determined during the second heating run.

The amount of MCT in the microparticles was quantified from the melting enthalpy of MCT, which was approx. 92.2 J/g. MCT entrapment was calculated by comparing the melting enthalpies of MCT-containing microparticles with that of pure MCT.

2.2.7. Investigation of the emulsion formation in ISM

Emulsification was carried out by using a two-component syringe system (Fig. 8). 30% (w/w) RG 503H solutions were prepared in various solvents (NMP, 2-pyrrolidone, DMSO, propylene carbonate, TEC, triacetin) using as internal phase. Oils (castor oil, ethyl oleate, MCT, safflower oil, soybean oil, sesame oil and peanut oil containing 2% Span 80) or water containing 1% Lutrol F68 were used as the external continuous phase. Each 0.25 g of above liquid was kept into one 1ml syringe and two syringes were coupled with a connector (inner diameter 1.4 mm). Emulsification was achieved by pushing internal phase into external phase forward and backward 50 cycles at the speed of 2 cycles per second. The formed emulsion were characterized visually and observed under the optical light microscope.

2.2.8. Comparison of diffusion of solvents into the external phase

2 ml 20% (w/w) RG 503H solutions in various solvent mixtures and the same volume of peanut oil containing 2% w/w Span 80 and 2% w/w aluminum monostearate were added to a glass vial (volume 5 ml, outer diameter 19 mm, height 40 mm; Fisher Scientific GmbH, Schwerte, Germany) (Fig. 10) (n=3). The glass vials were sealed and shaken on a horizontal shaker (HS 501 Digital, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at 200 rpm. At predetermined time points, the height of the PLGA solutions was measured and the volume was

calculated. The viscosity of the polymer phase was measured after removal of the upper oil/solvent phase at the end of the experiment.

The diffusion of oil into the polymer solution was negligible because of the high viscosity of the PLGA solution and the insolubility of PLGA in the oil. This was confirmed by ^{1}H nuclear magnetic resonance (NMR).

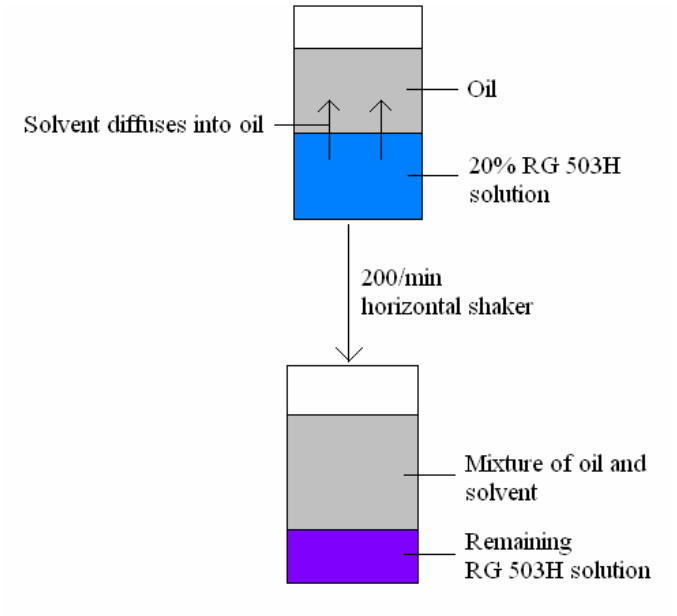

Fig. 10. Experiment set-up to determine the diffusion rate of solvent from polymer solution into different oily external phases.

2.2.9. ^{1}H nuclear magnetic resonance (NMR)

^{1}H NMR measurements were performed at the Institute of Chemistry: Physical and Theoretical Chemistry (Free University Berlin, Germany) together with W. Wang. The spectra of oil, solvent and solutions were obtained with an NMR spectrometer (AMX500, Bruker) by dissolving samples in $CDCl_3$. ^{1}H NMR chemical shifts in chloroform was referenced relative to $CHCl_3$ (δ_H = 7.24 ppm).

2.2.10. Solvent diffusion into the release medium in ISM-systems

Approx. 0.2 g ISM emulsion was injected into dialysis bags. The dialysis bags were placed into 10 ml phosphate buffer (1/30 M, pH 7.0, 0.01% sodium azide) at 37 °C in an incubation shaker (n=3). 8 ml medium were taken and replaced at predetermined points. The concentration of NMP and triacetin in the release medium was analyzed by RP-HPLC (SCL-10A VP, Shimadazu, Japan), C18 Eurospher-100 column (150 X 4 mm, Knauer GmbH, Germany) (mobile phase: distilled water:acetonitrile 85:15 v/v; flow rate: 1.0 ml/min; UV detection at 230 nm for NMP and 220 nm for triacetin).

2.2.11. Viscosity measurements

The viscosity was measured by a computer interfaced rheometer (Rheostress RS 100, Haake MessTechnik GmbH, Karlsruhe, Germany) at 23 °C ± 0.2. The viscosity of solvents and oils were studied by using a plate and cone configuration 60 mm diameter/1° angle with a fixed shear rate (60 /s). The viscosity of PLGA solutions were measured by a plate and cone configuration 20 mm diameter/4 ° angle with a fixed shear rate (20 /s).

2.2.12. Injectability

The injection force of the formulations was determined by texture analyzer (Stable Micro System®, Vinna Court, UK). 400 mg of testing fluid (PLGA/NMP solution or emulsion) were kept in 1 ml syringes coupled with 20G needles. The plunger of the syringe was placed n contacted with a load cell. The injection speed was set as 100 mm/min and injection distance was 18 mm. the injection force (n=3) was calculated by software (Texture Expert Exceed Version 2.12).

2.2.13. Influence of the injection techniques on the in situ forming systems

250 mg of leuprolide loaded PLGA solution (prepared as in 2.2.2.) was injected directly (in situ forming implants) or after emulsification with same weight of sesame oil containing 2% w/w

Span 80 and 2.5% w/w AM (ISM) through a 1 ml syringe with different techniques into 6 ml of phosphate buffers.

i. 20 G needle, injected in 10 sec.
ii. 20 G needle, injected in 2 sec.
iii. 24 G needle, injected in 2 sec.

At predetermined time intervals, 5 ml of release medium were refreshed. Concentration of leuprolide in medium was quantified by UV at 279 nm.

2.2.14. In vivo evaluation

Male adult New Zealand rabbits with a mean body weight of 2000-2250 g were employed in this study. The injection site (back of rabbit neck) was marked and shaved with a hair cutter. The ISM emulsion (approx. 0.5 g, RG 503H, 80% w/w NMP, 20 w/w triacetin) was injected subcutaneously through a 23-gauge needle. The targeted dose was set at 75 µg/kg/day for 30 days. The syringes were weighed before and after dosing to determine the exact injected dose. On days 0, 0.25, 1, 3, 5, 7, 10, 14, 21, 28, 35, 42, approx. 1.5 ml blood sample was collected from the marginal ear vein into a vial containing 150µl sodium citrate solution (0.0129 M).

Serum was separated by centrifugation for 10 min at 3900 g and frozen at – 30 °C for future measurement. Samples were analyzed in duplicate of testosterone levels by radioimmunoassay (RIA) using a commercial kit (IBL-Testosterone ELISA, IBL Immuno-Biological Laboratories, Hamburg, Germany).