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RESEARCH PAPER

Development of PLGA-Based Injectable Delivery Systems For Hydrophobic Fenretinide

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ABSTRACT

Purpose Although efficient *in vitro*, fenretinide has not been successful clinically for either of the targeted indications—cancer prevention and dry age-related macular degeneration—because of various issues, such as low oral bioavailability. Therefore, controlled release carriers for parenteral delivery of fenretinide were developed.

Methods After examining the solubility profile of fenretinide, the drug was encapsulated in poly(lactic-*co*-glycolic acid) (PLGA) microparticles at 20% drug loading by an s/o/w methodology as well as into *in situ*-forming PLGA implants. The carrier morphology and drug release kinetics in an elevated polysorbate 80-containing release medium were studied.

Results Preformulation studies revealed increased fenretinide solubility in various PLGA solvents including N-methylpyrrolidone (NMP) and 1:9 *v/v* methanol:methylene chloride. Co-solvent emulsion methods resulted in low encapsulation efficiency. With a s/o/w method, fenretinide release rates from injectable microparticles were adjusted by the o-phase concentration of end-capped PLGA, the drug particle size, and the particle porosity. *In situ* implants from non-capped PLGA in NMP exhibited a continuous release of ~70% drug over 1 month.

Conclusions Injectable carriers for fenretinide were successfully prepared, exhibiting excellent drug stability. Based on the *in vitro* release properties of the different carriers, the preferred injection sites and *in vivo* release rates will be determined in future preclinical studies.

KEY WORDS controlled release · fenretinide · hydrophobic drug · *in situ* implant · s/o/w PLGA microparticle

INTRODUCTION

After initially being studied for delivery of hydrophobic drugs, e.g., contraceptives in the late 1970s and early 1980s (1,2), research on poly(lactic-co-glycolic acid) [PLGA] has mostly focused on delivery of proteins and peptides in the last decade (3,4). However, as recently summarized (5), there is an increasing number of hydrophobic compounds being discovered and evaluated as an active pharmaceutical ingredients (API). A significant percentage of them are not only hydrophobic, but also exhibit low oral bioavailability and are indicated for diseases that require chronic administration. Such drugs may benefit from the potential advantages of injectable PLGA delivery systems: 1) a higher bioavailability for BCS (6) class 3 or 4 compounds compared to oral administration, 2) a sustained drug release with constant plasma levels in the therapeutic window, 3) a reduced frequency of drug administration,

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which may increase compliance, and 4) if applicable, a local delivery at the site of application, e.g., to the brain (7) or to tumors by intratumoral injection (8).

Fenretinide is a hydrophobic drug which is structurally derived from vitamin A (9). Fenretinide has prominent anti-tumor effects including the induction of apoptosis in tumor cells (10). Due to its accumulation in fatty tissue such as the mammary gland (9) and its low elimination rates from this compartment (11), fenretinide was suggested for the treatment of breast cancer. The chemopreventive and chemotherapeutic usage in other types of cancer has been addressed so far in more than 30 phase 1 to phase 3 clinical studies (12). Moreover, a less studied application of fenretinide is in the treatment of dry age-related macular degeneration (AMD) and Stargardt disease, which presently is being evaluated in a phase 2 clinical study (12). Although caused by different mechanisms, both diseases phenomenologically lead to lipofuscin deposits containing toxic vitamin A byproducts and, finally, geographic atrophy of the retina (13). By interfering with vitamin A transport proteins, fenretinide reduces lipofuscin deposition (14).

Fenretinide shows very poor oral bioavailability due to low solubility and low permeability (likely BCS class IV). As a consequence, the high oral doses that are required have occasionally precluded dose escalation in experimental tumor therapy due to the high number of capsules to be taken (15). Therefore, one approach suggests embedding the drug in a lipid matrix that forms chylomicron-like particles for increased intestinal absorption by the lymphatic pathway (16). Intravenous injections may be an alternative route of fenretinide administration, which, however, is challenging because of the low aqueous solubility of pure drug. Conjugates of fenretinide with water-soluble polymers like polyvinylalcohol (17) or polyglutamic acid (18) as well as drug encapsulation in micelles from block copolymers (19) have been studied to increase drug solubility, enable intravenous administration, and improve targeting of tumors by the enhanced permeability and retention (EPR) effect.

For application in both tumor therapy and ocular diseases like AMD, a controlled-release formulation, which provides drug release over a longer period of time, would be advantageous. Two PLGA-based formulation approaches were developed in this study, namely, microparticles (5) and *in situ*-forming implants (20). *In situ*-forming implants solidify in the body after injection due to polymer precipitation after extraction of the biocompatible solvent (21) into the surrounding tissue.

To the authors' knowledge, fenretinide-controlled delivery systems based on polymer matrix systems have not been reported to date. Therefore, an initial characterization of physicochemical properties of fenretinide relevant to microencapsulation processes and release experiments was carried out. Adjustment of fenretinide release rate was achieved by controlling the morphology of two carrier systems, microparticles and *in situ* implants, both based on degradable PLGA. Particularly notable challenges with this drug included a strong susceptibility to photo-oxidation (requiring special handling) and the lack of water-solubility.

MATERIALS AND METHODS

Materials

Fenretinide [N-(4-hydroxyphenyl)retinamide] was provided by Merck & Co., Inc. and was stringently handled under light protection in all steps of the study (Whitehouse Station, NJ, USA). Poly(D,L-lactic-co-glycolic acid) [PLGA] of medical grade quality (Resomer® RG 502H, inherent viscosity [i.v.] 0.20 dl/g; RG 503, i.v. 0.42 dl/g) was purchased from Boehringer Ingelheim Chemicals, Inc. (Petersburg, VA, USA). Detergents used in this study were polyvinyl alcohol (PVA, M_w 10 kDa, 80% hydrolyzed) and Polysorbate 80 (Tween® 80, SigmaUltra) both from Sigma-Aldrich (St. Louis, MO, USA). All solvents and other chemicals were HPLC or USP grade or higher.

Fenretinide Solubility and Hansen Solubility Parameter

For determining the solubility of fenretinide in organic solvents with a large dissolving power, practical solubility was measured by placing tightly locking test tubes containing drug were placed on an analytical balance (AG285, Mettler-Toledo, Inc., Columbus, OH, USA) followed by stepwise addition of the respective solvent. For aqueous media with low drug solubility, drug was suspended in 15 ml medium and incubated at 37°C on a horizontal shaker for 3 days. Solubility studies in PVA solution were performed at two extreme temperatures potentially relevant for solvent evaporation, 4°C and 30°C, in glass bottles on a low-speed magnetic stirrer. After filtering out undissolved drug (0.2 μm membrane filter), the filtered drug solutions were analyzed by HPLC (see section "Encapsulation Efficiency, Recovery Assay, and HPLC Analysis"). If required, drug solutions were concentrated by lyophilization and extraction with acetonitrile.

The Hansen solubility parameters for fenretinide were estimated by the 'Yamamoto molecular breaking' group contribution method from its structure by the HSPiP software, 3rd edition (C. Hansen, S. Abbott) (22). From the experimental data on good solvents, the Hansen solubility parameters of fenretinide were additionally obtained by fitting.

Microparticle Preparation

The encapsulation of fenretinide was performed at a theoretical loading of 20% (w/w). For the standard cosolvent method, 125 mg of drug were dissolved in 2.5 ml of a mixture of methylene chloride (MC) and the cosolvent in a glass tube, followed by the addition of 500 mg Resomer® RG 503 to form the o-phase. For the solid-in-oil-in-water technique (s/o/w), varying amounts of the polymer were dissolved in 2.5 ml MC with subsequent drug suspension (10.000 rpm, 60 s; Tempest I.Q.^{2}, Virtis, Gardiner, NY, USA). In some cases, fenretinide (non-hygroscopic) was micronized in an agate mortar on dry ice and subsequently dried at 37°C to constant weight. Concentration units during microparticle formulation were practically expressed based on percentage of polymer weight added to the solvent volume approximately assuming equivalent density, i.e., 20% PLGA denotes a polymer solution prepared with 500 mg PLGA and 2.5 ml MC. The o-phase or s/o-phase, respectively, was then emulsified in 5 ml of 5% (w/v) PVA solution by vortexing for 20 s and poured into 75 ml of a magnetically stirred 0.5% (w/v) PVA solution for solvent evaporation at room temperature. After 3 h, particle fractions were collected on test sieves (Newark Wire Cloth Company, Clifton, NJ, USA), washed with water, and lyophilized. The weight of the dried particles in each fraction was determined on an analytical balance.

In Situ Implants

Fenretinide was dissolved in N-methylpyrrolidone (NMP) to obtain a 100 mg/ml stock solution. Different amounts of PLGA polymer were placed in 5 ml glass vials, and the required volumes of drug stock solution and additional pure NMP were added (37°C) to obtain solutions with different polymer concentrations (see section "Microparticle Preparation" for explanation of concentration units) and a final loading of 7 mg fenretinide/200 µl *in situ* implant forming solution. The resulting drug+polymer/NMP solutions were slowly injected with 1-ml syringes (BD 309602, Becton Dickinson, Franklin Lakes, NJ, USA) and 23G needles (BD 305193) into the release medium to form implants. The precise amount of injected drug solution was determined by balancing the weight of the syringe before and after injection.

Scanning Electron Microscopy and Digital Photography

For analysis by scanning electron microscopy (SEM), the microparticles or implant samples were sputtered with gold (Desk II, Denton Vacuum, Moorestown, NJ, USA). Cross sectioning of implants was performed with a razor blade after lyophilisation of the samples. Micrographs were taken with a Hitachi S3200 (Pleasanton, CA, USA) or a Philips XL 30 ESEM (Bothell, WA, USA). Digital photography (EOS 350D, Canon, Krefeld, Germany) was used to document the macroscopic appearance of the implants after 32 days incubation in release medium.

Encapsulation Efficiency, Recovery Assay, and HPLC Analysis

In order to determine the encapsulation efficiency (EE) or the remaining drug in microparticle pellets from the release study, the lyophilized samples were dissolved in 0.5 ml of tetrahydrofuran (THF), and PLGA was precipitated by the addition of 9.5 ml of ethanol. After centrifugation (5 min, 16,100 g, Centrifuge 5415D, Eppendorf, Hamburg, Germany), the samples were analyzed on a Waters HPLC system (1525 pumps, 717plus Autosampler, 2487 Dual λ absorbance detector) with a Nova-Pak® C18 column. The observed limit of quantification corresponded to a sample concentration of 0.1 µg/ml.

Release Studies

Different experimental configurations were evaluated for release studies of microparticle samples under sink conditions. The highest reproducibility and practical use was realized with a procedure in which 5 mg of microparticles were placed in 50-ml plastic tubes and suspended in 50 ml of a sterilized solution of 1% (v/v) Polysorbate 80 in phosphate-buffered saline (PBS) at pH 7.4. The samples were incubated at 37°C on a rocking platform shaker (25 rpm, VWR, West Chester, PA, USA) without exchanging the buffer until finally being removed at predefined time points ($n=3$) and filtered through a 0.45-µm membrane filter and both the pellet after lyophilisation and the filtrate being analyzed for drug content.

Drug release from *in situ*-forming implants was evaluated after injecting the implants inside filter bags with a 1-µm pore size (NMO1SBF cut to size, Midwest Filter Corporation, Lake Forest, IL, USA), which were placed in 50-ml plastic tubes containing 45 ml of a sterilized solution of 1% (v/v) Polysorbate 80 in PBS, pH 7.4. In the case of *in situ* implants, 35 ml of the release medium was frequently withdrawn for HPLC analysis and replaced by fresh medium, which was necessary to maintain sink conditions due to higher amount/ rate of released drug.

RESULTS

Fenretinide Physicochemical Properties

In order to follow a rational microencapsulation approach, the solubility of fenretinide was first determined

Table I Solubility of Fenretinide in Selected Organic Solvents (S), Solvent Miscibility with Water, and Hildebrand as well as Hansen Solubility Parameters

Solvent (S)	Solubilities			Hildebrand[c]	Hansen[d]		
	Drug/S (mg/ml)[a]	S/Water (%)[b]	Water/S (%)[b]	δ_t ($MPa^{1/2}$)	δ_d ($MPa^{1/2}$)	δ_p ($MPa^{1/2}$)	δ_h ($MPa^{1/2}$)
Methylene chloride (MC)	2.5	1.32	0.20	20.3	17.0	7.3	7.1
Ethyl acetate (EA)	21	8.70	3.30	18.2	15.8	5.3	7.2
Chloroform	7	0.80	0.20	19.0	17.8	3.1	5.7
Methanol (MeOH)	36	miscible		29.7	14.7	12.3	22.3
Ethanol (EtOH)	47	miscible		26.6	15.8	8.8	19.4
1-Propanol (1-Prop)	36	miscible		24.6	16.0	6.8	17.4
2-Propanol (2-Prop)	37	miscible		23.5	15.8	6.1	16.4
Acetone	100	miscible		20.1	15.5	10.4	7.0
Acetonitrile (ACN)	3.8	miscible		24.6	15.3	18.0	6.1
Tetrahydrofurane (THF)	>200	miscible		19.4	16.8	5.7	8.0
Dimethylformamide (DMF)	>99	miscible		24.8	17.4	13.7	11.3
Dimethylsulfoxide (DMSO)	>95	miscible		26.6	18.4	16.4	10.2
N-Methylpyrrolidone (NMP)	150	miscible		22.9	18.0	12.3	7.2

[a] Solubility as determined at room temperature (see section "Fenretinide Solubility and Hansen Solubility Parameter"); [b] Solubility in % (w/w) at 20°C (methylene chloride: 25°C) according to (39); [c] Values according to (40); [d] Values according to the HSPiP software, 3rd edition

in aqueous and organic solvents and organic solvent mixtures. The drug showed limited solubility in useful water-immiscible carrier solvents for encapsulation like MC or ethyl acetate. However, the solubility in some fully water-miscible organic solvents was considerably higher (Table I). Methanol and THF were selected as cosolvent candidates, and the dissolving power of their mixtures with MC was evaluated (Table II). Improved drug solubility (>140 mg/ml) in just 8:92 v/v methanol/methylene chloride was observed, indicating methanol is a very efficient cosolvent for fenretinide. By contrast, THF was much less efficient, requiring 20% cosolvent to attain a solubility of >100 mg/ml.

Providing estimates on the physicochemical properties of new compounds is of major interest for a rational formulation development for economic reasons. Based on the determined solubilities, good solvents for fenretinide were defined as those solvents capable of dissolving ~100 mg/ml or more. When comparing the Hildebrand solubility parameter δ_t of good solvents with those of poor solvents, no correlation between δ_t values and the dissolving power could be established (Table I). This illustrates that the Hildebrand theory that was developed primarily for non-polar, non-hydrogen-bonding interactions between solvents and compounds is not so useful for fenretinide. By including such additional interactions as provided by the Hansen theory of solubility parameters, solubility parameters of fenretinide (δ_d; δ_p; δ_h ($MPa^{1/2}$): 17.8; 6.7; 8.8) were estimated by a group contribution method by means of the HSPiP software. The graphical plot as depicted in Fig. 1 illustrates some clustering of good solvents in the Hansen space. When calculating the Hansen solubility parameter of fenretinide by a fit of the experimental data for pure good solvents (δ_d; δ_p; δ_h ($MPa^{1/2}$): 17.1; 10.8; 12.2), a clear shift particularly in the contributions from polar and hydrogen-binding forces towards higher values was observed. With the only exception of MC that was wrongly suggested as a good solvent, the fit allowed differentiation between good and poor solvents.

Drug solubility in aqueous media is particularly important for two media types - detergent solutions used during

Table II Solubility[a] of Fenretinide in Methylene Chloride : Cosolvent Mixtures

Methylene chloride : cosolvent ratio	Drug solubility (mg/ml)	
	Cosolvent MeOH	Cosolvent THF
10 : 0	2.5	2.5
9.6 : 0.4	53	–
9.5 : 0.5	62	22
9.4 : 0.6	87	–
9.2 : 0.8	>140	–
9 : 1	>150	44
8.75 : 1.25	–	57
8.5 : 1.5	–	69
8.25 : 1.75	–	86
8 : 2	–	103
0 : 10	36	>200

[a] Solubility as determined at room temperature (see section "Fenretinide Solubility and Hansen Solubility Parameter")

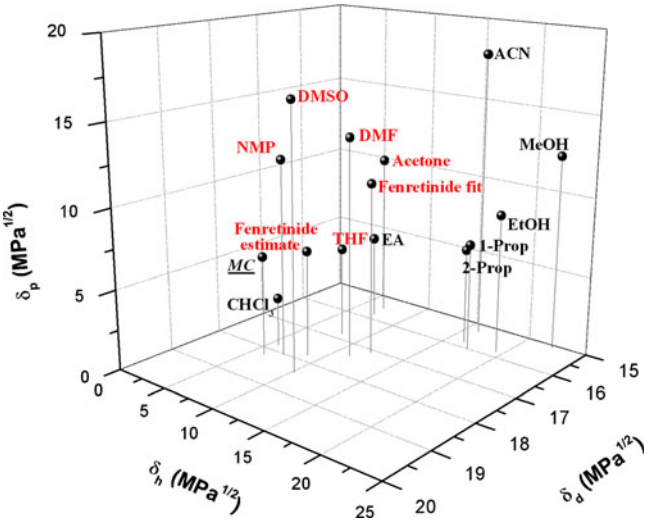

Fig. 1 Plot of Hansen solubility parameters including contributions of dispersive (δ_d), polar (δ_p), and hydrogen-bonding forces (δ_h). Good solvents are in red. For fenretinide, the Hansen solubility parameters were either estimated from its structure by a group contribution method or fitted based on the values of good solvents (HSPiP software). According to the fit, methylene chloride (MC) was wrongly expected to be a good solvent. For explanation of solvent abbreviations see Table I.

the microparticle preparation and media employed in release studies. Two extreme temperatures for solvent evaporation techniques, 4°C and 30°C, were chosen to detect the range of drug solubility in aqueous PVA solutions and showed very low fenretinide solubility at both temperatures (Fig. 2a). Thus, the loss of drug to the water-phase (w-phase) driven by its solubility in aqueous PVA solutions was not expected to be a concern when maximizing encapsulation efficiency. By contrast, another detergent, Polysorbate 80, strongly increased the fenretinide solubility (Fig. 2b) and was considered to serve for drug solubilization in release studies.

Impact of the Microencapsulation Method on the PLGA Microparticle Properties

Based on the solubility characteristics of fenretinide, two microencapsulation methods were selected, namely, the o/w cosolvent and s/o/w techniques. Although the cosolvent was kept at a minimum level required to completely dissolve the drug, low EE of only 30–40% was observed for both cosolvents, THF and methanol. Increasing the speed of polymer precipitation at the surface of nascent microparticles by higher polymer concentration in the o-phase, e.g., for Resomer® RG 503 an increase from 16 to 20%, did not have a large impact on the EE (32.6% *vs.* 35% for methanol as cosolvent). Due to the poor EE by cosolvent techniques, the s/o/w-technique was used in all further experiments.

Increasing the polymer concentration is known to often result in alterations towards a denser particle matrix structure (23), which may be associated with a lower burst and a more prolonged release profile. When the PLGA concentration (Resomer® RG 503) in the o-phase was increased from 15 to 25%, a shift in the particle size distribution toward the larger size fractions was observed (Fig. 3). Therefore, along with a further increase in polymer concentration to, e.g., 35%, broader particle size fraction of 20–63 μm or 20–90 μm was collected to ensure a significant yield of particles. The EE occasionally varied from batch to batch and mostly was in the range of 70–80%. The overall yield of microparticles was typically 80–85%. Some of the missing material was observed to stick to the stirring bar, due to aggregation of nascent particles in the early stage of their hardening, including variable amounts of drug.

The analysis of the microparticle shape and surface structure by SEM revealed a significant amount of broken,

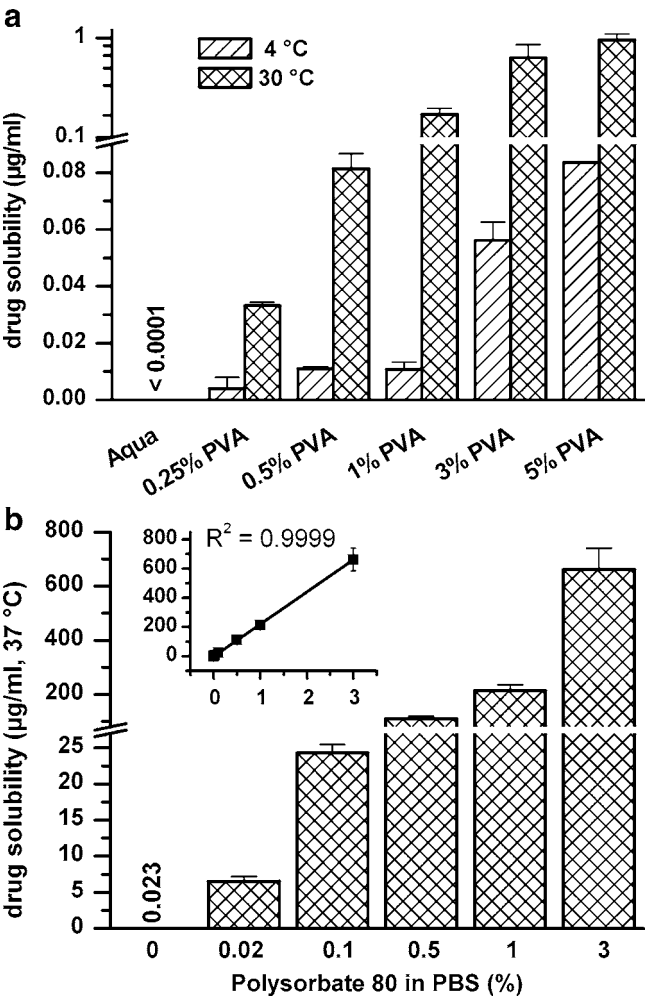

Fig. 2 Solubility of fenretinide in aqueous media. **a** Effect of temperature and polyvinyl alcohol (PVA) concentration (w/v) on drug solubility. **b** Drug solubility as a function of Polysorbate 80 concentration (v/v) in phosphate-buffered saline (PBS) at 37°C. Inset: Linear correlation between Polysorbate 80 concentration and drug solubility. ($n = 3$, median, range).

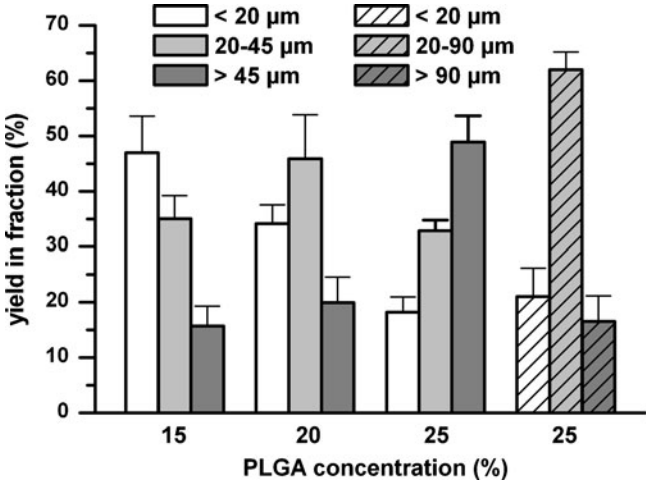

Fig. 3 Particle size distribution as determined by the mass yield of fenretinide-loaded s/o/w microparticles in different sieved fractions depending on the concentration of PLGA (Resomer® RG 503) in the o-phase ($n = 3$, median, range).

collapsed, or poorly formed particles at low polymer concentrations (15% PLGA). Higher polymer concentrations (25% PLGA) resulted in smoother particle surfaces, although a certain number of microparticles exhibited an ellipsoid shape with an occasional surface perforation due to spiky drug needles (Fig. 4a–c). In the employed medium with an elevatated detergent concentration, a faster release from microparticles with lower polymer concentration and imperfect polymer coatings of drug crystals was observed compared to those with a higher polymer concentration and fully embedded drug (Fig. 4d).

In the next series of experiments, a further increase in the polymer concentration from 25 to 35% did not result in a more spherical shape of the particles (Fig. 5a–c). Non-encapsulated ends of drug needles were still present in few cases, even though the drug used in these experiments had a crystal size <20 µm, and the size range of the collected main fraction was extended to 20–90 µm. The release rates for the 20–90 µm fraction of the 25% PLGA formulation (Fig. 5d) were in good agreement with the release rates for the 20–45 µm fraction (Fig. 4d), at least during the first week of the study. In the following weeks, the 20–90 µm samples showed an ongoing fenretinide release, whereas the 20–45 µm particles exhibited a lag phase. Higher polymer concentrations (30%) resulted in a slower release, and the release rate was not strongly affected for particles prepared above this concentration at 35%. In conclusion, the 25% PLGA formulation was expected to provide the required drug release over 2–4 weeks if <20 µm drug crystals were used.

However, the apparent size of drug crystals is not an invariable property of a specific substance but is largely influenced by various process parameters during crystallization. For a more robust microparticle formulation,

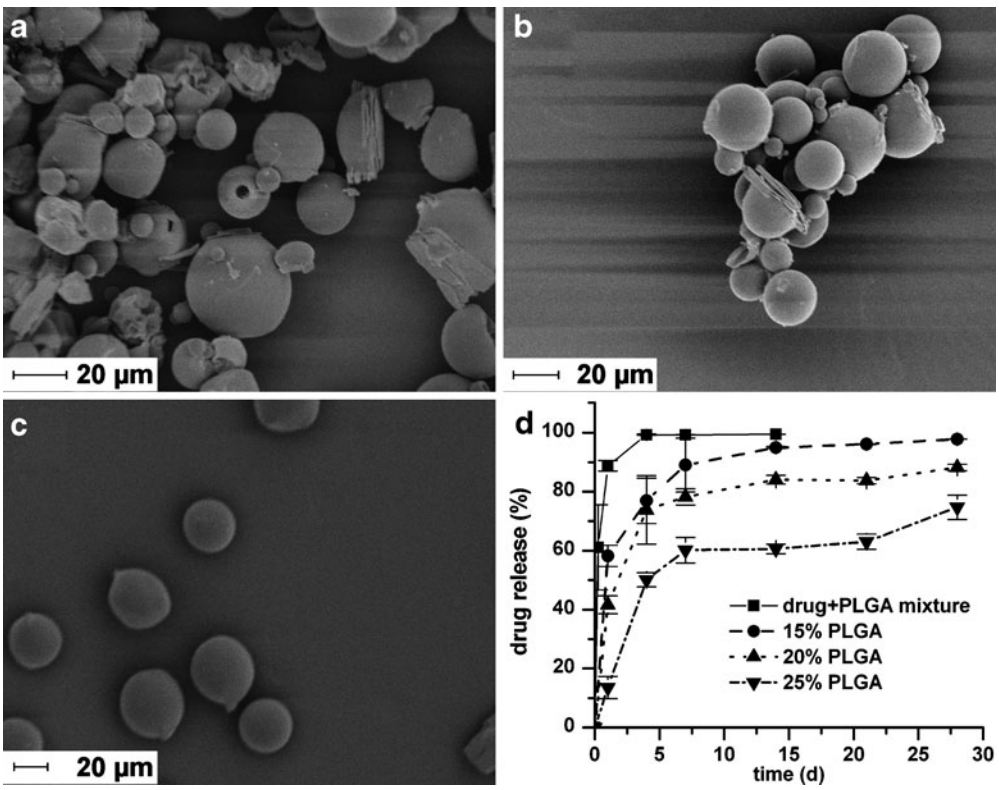

Fig. 4 Effect of the PLGA (Resomer® RG 503) concentration in the o-phase on the particle morphology and fenretinide release behavior. SEM images are displayed for **a** 15% PLGA (EE: 74.8 wt.%), **b** 20% PLGA (EE: 75.1 wt.%), and **c** 25% PLGA (EE: 57.1 wt.%); **d** Drug release of different microparticle formulations was compared to a physical mixture of fenretinide and PLGA in 1% (v/v) Polysorbate 80/PBS pH 7.4 at 37°C ($n = 3$, median, range). All particles were prepared with the s/o/w method, and a size fraction of 20–45 μm was used.

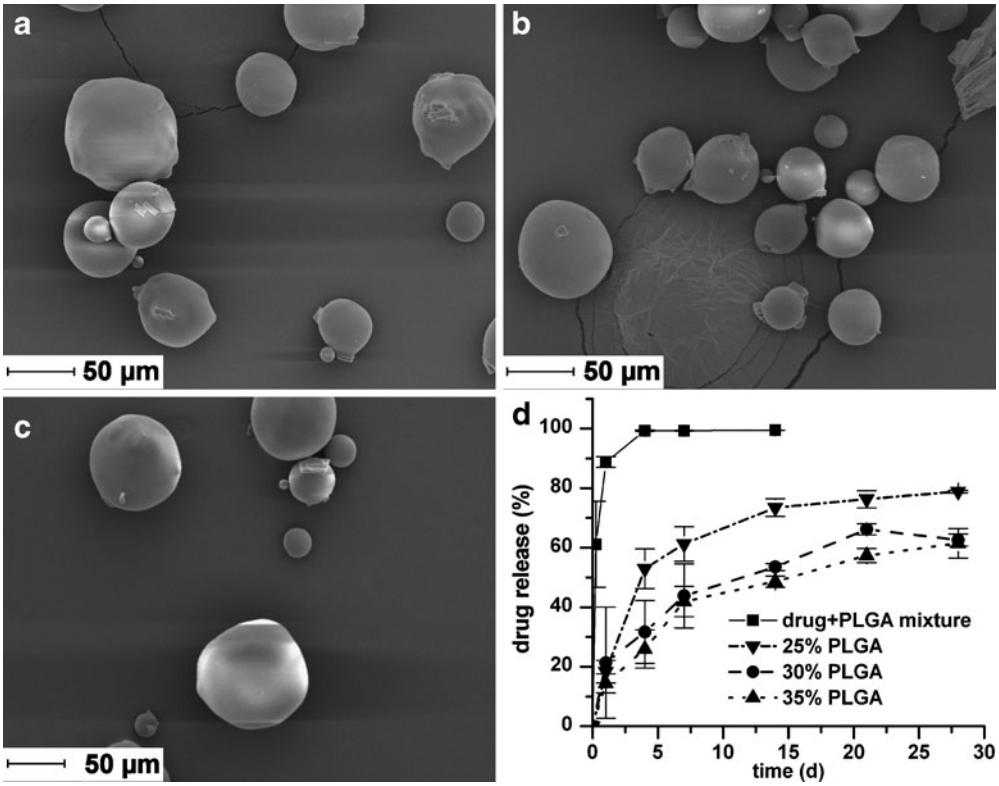

Fig. 5 Effect of the PLGA (Resomer® RG 503) concentration in the o-phase on the particle morphology and fenretinide release behavior. SEM images are displayed for **a** 25% PLGA (EE: 79.6 wt.%), **b** 30% PLGA (EE: 74.3 wt.%), and **c** 35% PLGA (EE: 64.8 wt.%); **d** Drug release of different microparticle formulations was compared to a physical mixture of fenretinide and PLGA in 1% (v/v) Polysorbate 80/PBS ($n = 3$, median, range). All particles were prepared with the s/o/w method, and a size fraction of 20–90 μm was used.

ground fenretinide was encapsulated into microparticles with 25% PLGA in the o-phase in the next set of experiments. This procedure resulted in particles with a perfect spherical shape (Fig. 6). However, a small portion of non-encapsulated drug that may contribute to a burst release could not be removed from the surface during the washing procedure. As expected, the embedding of smaller, likely well-separated drug crystals into the polymer matrix

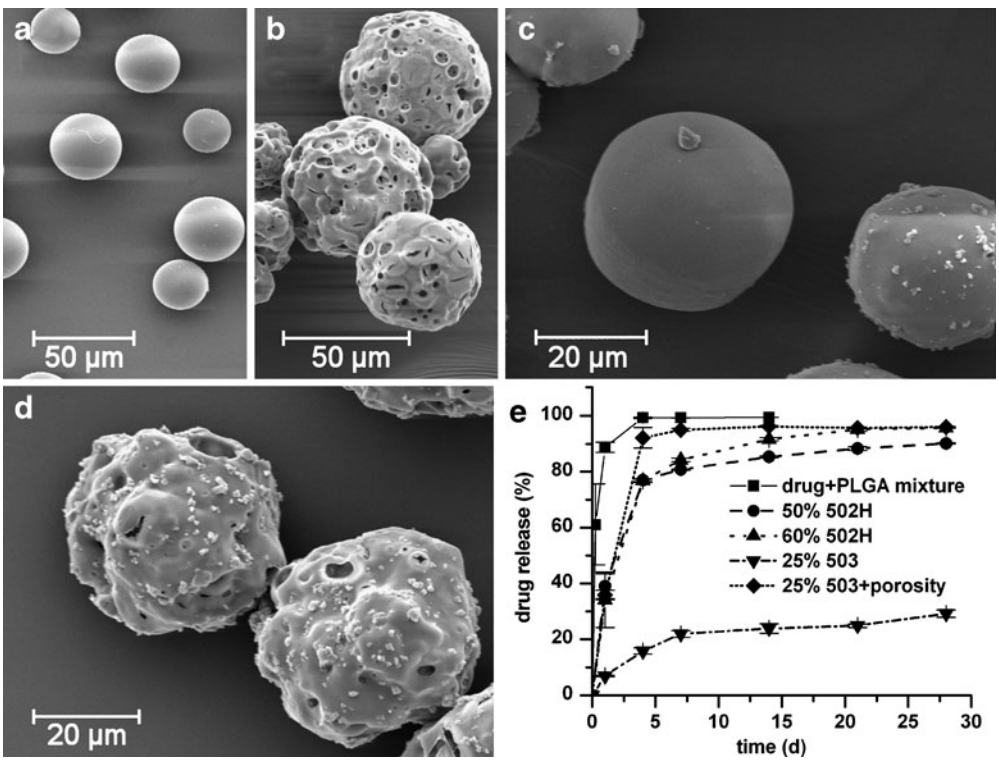

Fig. 6 Microencapsulation of ground fenretinide. SEM of **a** non-porous blank particles, **b** porous blank particles (w_1: 5X PBS), **c** non-porous drug-loaded particles, and **d** porous drug-loaded particles. Samples a-d were prepared with 25% Resomer® RG 503 in the o-phase. Panel **e** shows the release of drug from formulations based on ground drug including 50% RG 502H (EE: 85.3 wt.%), 60% RG 502 (EE: 57.1 wt.%), 25% RG 503 (EE: 76.8 wt.%), and 25% RG 503 + porogens (EE: 87.9 wt.%) in 1% (v/v) Polysorbate 80/ PBS pH 7.4 at 37°C. All data for 20–63 μm particles ($n = 3$, median, range).

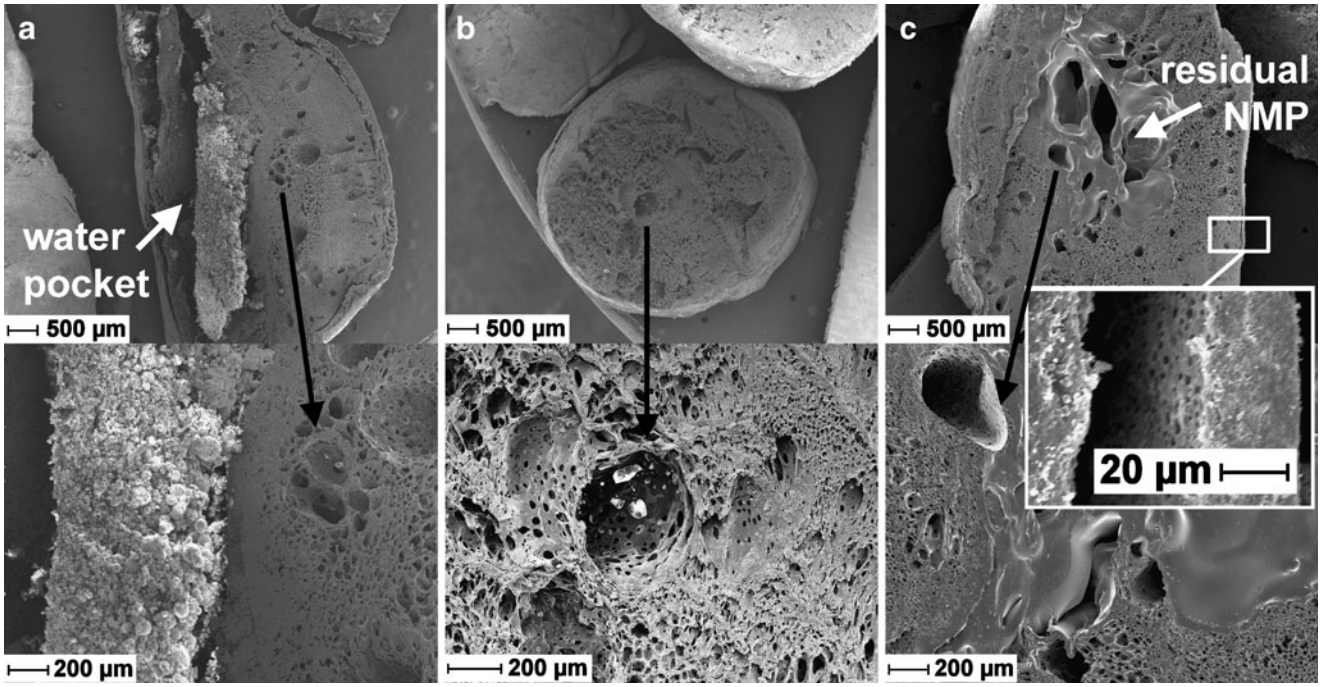

Fig. 7 Overview and details of the microstructure of 500 µl *in situ* implants after 3 days of incubation in PBS pH 7.4 at 37°C. Implants were prepared from **a** 15%, **b** 25%, and **c** 35% Resomer® RG 503 in NMP.

resulted in a slower drug release from 25% RG 503 microparticles (Fig. 6e). In order to increase release rates, porosity was induced by adding different concentrations of phosphate-buffered saline (PBS) in the inner water phase in a $(w_1+s)/o/w_2$ procedure. Finally, a formulation was selected that produced highly porous particles (w_1: 5X PBS), which exhibited increased release over 7 d. Additionally, Resomer® RG 502H with lower molecular weight and uncapped end groups was evaluated. However, fenretinide release rates could not be altered substantially by increasing the RG 502H concentration within its solubility range (Fig. 6e).

In Situ-Forming Implants

Besides microparticles, *in situ*-forming implants are an interesting alternative drug carrier concept. Since fenretinide was highly soluble in NMP (Table I), a solvent used in FDA-approved products (e.g., Eligard®), several formulations of drug and polymer in NMP were evaluated. First, unloaded implants were prepared with varying concentrations of RG 503 in NMP and lyophilized after 3 days (Fig. 7). The implant prepared using 10% RG 503 had collapsed and basically consisted of a shell with a hollow core rather than a matrix structure (data not shown). In the 15% PLGA implant, it appeared that a significant amount of water had entered the implant and resulted in a large pocket filled with spontaneously formed microparticles (Fig. 7a). In contrast, the implant with 35% PLGA in NMP consisted of a denser matrix with a thin shell (see insert of Fig. 7c). NMP diffusion out of the implant was limited; thus, some residual NMP was still noticeable in the core after 3 days of incubation. For both, the 25% and the 35% RG 503 formulations, no pores were observed on the outside of the implant.

When RG 502H was evaluated as the matrix for *in situ* implants, higher polymer concentrations of up to 70% were used because of the lower molecular weight of this PLGA grade. A clear relationship between the polymer concentration, i.e., the density of the precipitated matrix and the release profile was detected for RG 503 with the slowest release observed for the 35% RG 503 formulation (Fig. 8). Surprisingly, no such systematic effect of increasing RG 502H concentrations on fenretinide release rates was observed. However, Fig. 8 indicates a lower burst and desirable slow and continous release profile of fenretinide from RG 502H compared to RG 503 in the drug-solubilizing release medium.

Considerable differences in the shape of the implants were apparent at the end of the release study (day 32). All RG 502H samples exhibited a significantly swollen, occasionally translucent matrix (Fig. 9). Some residual yellow drug aggregates were still encapsulated in the implant core, which appeared to be comparatively less swollen. By contrast, the RG 503 samples showed some swelling but maintained the irregular shape that was initially formed during the *in situ* precipitation/encapsulation process.

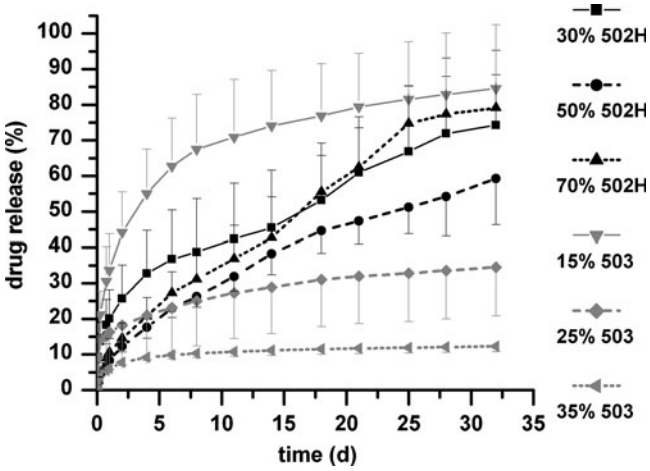

Fig. 8 Effect of PLGA type and concentration on the release of fenretinide from *in situ* forming implants in 1% v/v Polysorbate 80/ PBS pH 7.4 at 37°C ($n = 3$–5, median, range).

DISCUSSION

Requirements for Fenretinide Carriers

Fenretinide is a drug with very low oral bioavailability (likely BCS class IV). Therefore, high daily doses of 200 mg/day in chemoprevention (corresponding to 2 capsules of drug formulation) (24,25) and up to 4000 mg/m^2/day in a Phase 1 study on neuroblastoma in children (15) were evaluated. Since only marginal amounts of the orally administered drug are absorbed, it remains unclear which parenteral daily dose would be useful for long-term injectable controlled-release dosage forms with much higher bioavailability. Recent publications with intravenously injected drug-polymer conjugates or micelle systems followed the general expectation that much lower parenteral doses will be required, and, therefore, injections of 0.2 (17) to 1 mg (19) of fenretinide equivalents in mice were used. However, when considering the low volumes that are possible for intramuscular or subcutaneous injection and the desired timeframe of sustained release over 1 to 4 weeks, a high loading of the drug carrier was set as a desired formulation criteria in the present study. The delivery of an average daily dose of ~0.15 mg by some *in situ* implants over periods as long as 5 weeks (Fig. 8) fits the dosing rate employed previously (17). However, a conclusion on clinical efficacy is not possible without comprehensive *in vivo* evaluation.

Solubility Issues Impact Microencapsulation Strategies

Hydrophobic drugs such as fenretinide are most commonly encapsulated in PLGA microparticles by o/w emulsion techniques (5). Due to the low aqueous solubility, the drug escape to aqueous PVA solutions used as external water phase can be considered as marginal (Fig. 2a), e.g., a total of 6 µg for standard batches with 125 mg drug. Fenretinide solubility in commonly employed o-phase solvents was rather poor (Table I), compared to the required level of at least 100 mg/ml. Still, the data from the preformulation

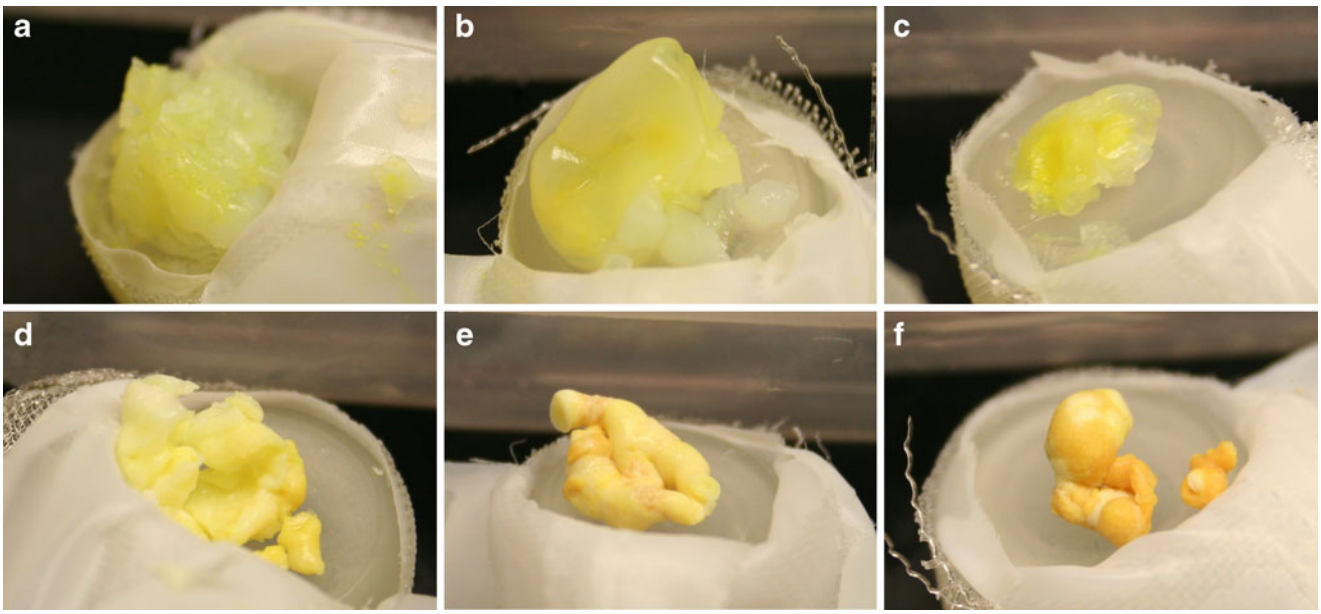

Fig. 9 Representative shapes of fenretinide-loaded implants at day 32 of the release study in 1% (v/v) Polysorbate 80/ PBS pH 7.4 at 37°C. Implants were prepared from **a** 30%, **b** 50%, and **c** 70% Resomer® RG 502 in NMP or from **d** 15%, **e** 25%, and **f** 35% Resomer® RG 503 in NMP. The relative intensity of the yellow color roughly reflects the drug content remaining in the implant.

analysis allowed a more precise determination of the Hansen solubility parameter compared to a rough estimation from the chemical structure by the group contribution method (Fig. 1). However, erroneous inclusion of MC in the group of good solvents by software fitting illustrates that additional, specific interactions which possibly may be relevant for high drug solubility are not included in the Hansen parameters.

Due to the absence of relevant drug solubility in commonly used organic solvents (Table I), two alternative encapsulation techniques have been suggested, i.e., o/w cosolvent methods and the s/o/w-technique. Methanol and THF were selected as cosolvent candidates because they showed a high dissolving power for fenretidine, a high vapor pressure that will ease their removal from the hardening bath and, for methanol, some evidence of successful usage as a cosolvent for microencapsulation (26). The extent of effects of methanol as a cosolvent for fenretinide was surprising, since methanol had only 15% of the dissolving power of THF (Table II). As a straight line connecting the two points that correspond to the solvents' Hansen solubility parameters of methylene chloride and methanol closely passes the fitted value of fenretinide, some increase in solubility for methanol/methylene chloride mixtures may be expected from Fig. 1.

Since water-miscible cosolvents will partition into the water phase during the emulsification procedure (27), drug dissolved in this eluting cosolvent may be lost to the external phase, thus, explaining the low EE. A faster solidification of particles as induced by employing higher polymer concentrations (28) showed some minor improvements on EE, but was not expected to be a feasible approach that could compete with loading levels expected for the s/o/w method.

Microencapsulation by s/o/w Technique and Interpretation of Release Data

By the s/o/w preparation method, microparticle fractions of a particle size suitable for injection were obtained with good reproducibility in all cases (Fig. 3). However, only for higher polymer concentrations such as 25% Resomer® RG 503, leading to an increased viscosity of the polymer solution, a suitable embedding of unmicronized drug needles was achievable (Fig. 4). At the same time and in good agreement with data from the literature (29), a shift towards larger particle size fraction resulted from such higher polymer concentrations (and higher polymer solution viscosity) (Fig. 3). Additionally, the burst and subsequent release rates were reduced (Fig. 4d) due to a better encapsulation of drug needles in polymer, longer diffusion lengths, and an expected lower inner porosity of the particles (23).

The penetration of microparticles by drug needles even in 25 to 35% PLGA formulations (Fig. 5) was most likely due to flocculation of individual drug particles <20 μm, which could not be separated during the s/o suspension procedure. This perforation of the polymer shell provided a fast access of release medium to the whole payload in the particle. When employing micronized drug in the 25% RG 503 formulation (Fig. 6), lower release rates were observed as expected, indicating that the drug has been well encapsulated and distributed in the polymer and was released gradually by diffusion and/or bulk erosion of the matrix. The contribution of erosion to the drug release typically occurs when a critical molecular weight is reached. Therefore, the induction time to polymer mass loss obviously is shorter for PLGA with shorter polymer chains (30,31). Moreover, water uptake as a precondition for degradation is higher in PLGA with free carboxyl end groups, such as Resomer® RG 502H. Because RG 502H has about half of the inherent viscosity of RG 503 (0.20 *vs.* 0.42 dl/g for the employed polymer batches), the concentration of RG 502H in the o-phase was increased to 50 or 60%. Although a much faster release was observed for both RG 502H formulations, as desired in principle (Fig. 6), RG 502H allowed less control of the *in vitro* release rate of fenretinide by changes in the polymer concentration.

Higher water uptake into particles can also be achieved by introducing macropores in the matrix such as by addition of osmotically active additives like salts in the inner water phase in $w_1/o/w_2$ techniques (5). As proof-of-concept, a highly porous microparticle formulation was selected for fenretinide. The release from this formulation was linear and much faster as compared to non-porous 25% RG 503 particles, but still slower when compared to that from a physical mixture of fenretinide and PLGA.

Due to the drug's extremely low solubility of ~20 ng/ml in PBS, the design of a suitable release assay under sink conditions was challenging. For example, for 5 mg particles encapsulating a total of 1 mg fenretinide, 500 L of PBS would be required for sink conditions in a closed vessel set-up. This large volume, besides having quantification issues, can be considered to be irrelevant in terms of handling and reproducibility. As drugs are often released faster *in vivo* than *in vitro* (32), there are several approaches to increase drug solubility and/or release rates *in vitro*, such as alcoholic release media, higher temperatures, altered pH values, or substances that increase the polymer hydrolysis (5). Polysorbate 80, commonly used at low concentrations to improve wetting of polymers, resulted in a linear increase in fenretinide solubility as a function of increasing Polysorbate 80 concentration (Fig. 2b, inset). These data indicate that the drug associates with, and was solubilised by, Polysorbate 80 micelles. The common micellar solubilization was strengthened by a multitude of diffusion studies (data not shown), where fenretinide (MW: 391 Da) dissolved in Polysorbate 80-containing release vehicles (micelle

size up to 20 nm (33)) did neither adsorb nor permeate through membranes with, e.g., 50 kDa (~6 nm) pores, but easily went through 1 μm pores.

Therefore, a configuration for release studies from microparticles was selected where microparticles were dispersed in 50 ml tubes with medium of elevated detergent concentration (1% Polysorbate 80), which was chosen as low as possible but still high enough to allow sink conditions over the entire study without media exchange. This procedure helped to avoid possible particle loss during medium replacement or alterations of particles during repeated centrifugation and resuspension. The principal goal of the *in vitro* release analysis was to determine which formulations provided continuous and long-term release. Due to the elevated detergent concentration and employed volumes, it is reasonable to expect that the release kinetics from the microparticles will be slower when evaluated *in vivo*.

Fenretinide-Loaded *In Situ*-Forming Implants

Besides preformed microparticles which typically are prepared in costly industrial processes, *in situ*-forming implants have attracted substantial interest due to easy handling, reduced needle size and injection volume, and low manufacturing costs (5,34). In contrast to conventional implantable rods with dense matrices, *in situ* implants are often characterized by a highly macroporous structure (Fig. 7). This structure is formed by polymer precipitation due to solvent exchange, i.e., efflux of NMP as polymer carrier solvent and influx of water as non-solvent to the polymer (35). Both the initial efflux of NMP as well as the formation of a large internal interface between the drug-loaded PLGA bulk and the water-filled interconnective pore structure may increase diffusion-governed controlled release. In contrast to microparticles, no aqueous carrier is required during injection of *in situ* implants and thus, a higher mass of drug per injection may be administered, and thus a longer-lasting release may be achievable for a fixed injection volume. This point may be of relevance when incorporation of sufficient drug in other types of depot formulations is challenging.

The reverse relationship between the fenretinide release and the polymer concentration for RG 503 implants (Fig. 8) correlates well with differences in matrix porosity (Fig. 7). The observed variability in release can be justified by the irregular implant shapes formed *in vitro* with different diffusion lengths, porosities, and surface-area-to-volume ratios. For high RG 503 concentrations, as expected, a long lag phase occurred after the burst release phase. In contrast, more hydrophilic RG 502H matrices allowed a slow and continuous drug release out of the polymer matrix, consistent with the absence of a lag phase from this low molecular-weight polymer with free carboxyl groups (30,31). The observed order in release rates from RG 502H formulations, i.e., 30% > 50% < 70% suggested an interplay of multiple contributions to the release mechanism. Besides the level of macroporosity and water/drug diffusion rates, which would be expected to have reduced release rate as initial PLGA/NMP concentration was increased, other affected mechanisms, e.g., reduced diffusion of water-soluble acid-degradation products (36), leading to increased autocatalytic polymer degradation might be involved in release control. RG 502H implants as isolated at the end of the release study showed differences in their swollen shape (Fig. 9a–c). The 70% RG 502H implants (Fig. 9c) were smaller and appeared to have a less swollen core covered by a strongly swollen shell. Drug encapsulated in this shell can be assumed to be more susceptible to drug release. These observations were consistent with the hypothesized slower efflux of acidic degradation products in the denser 70% RG 502H implants, resulting in faster local polymer degradation and drug release. Finally, it should be noted that photo- and thermolabile fenretinide was stable in all PLGA formulations, and no degradation products as previously characterized by stress tests (data not shown) were detected in both the loading assay and the release study.

CONCLUSIONS

This study provides an in-depth evaluation of fenretinide delivery from PLGA matrices *in vitro*. Both, preformed microparticles and *in situ*-forming implants allowed adjustment of fenretinide release rates depending on the morphology of the carrier. Since low bioavailability and, therefore, high oral doses have so far adversely impacted feasibility of fenretinide oral treatments, such parenteral depot formulations could be a reasonable alternative path to clinical development of this drug. Depending on the indication and the associated mechanism of drug action—e.g., a) prevention or treatment of different forms of cancer by direct intracellular induction of apoptosis or b) ophthalmic diseases with a reduction of vitamin A transport to the eye by intravascular blocking of transport proteins—both the most suitable site of administration and rate of drug release will have to be addressed in future preclinical and clinical studies.

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