

3. Results and Discussion

3.1. Protein delivery with in situ forming microparticles - methodical aspects

The evaluation of in situ forming microparticles for the controlled parenteral delivery of proteins means primarily to monitor the stability of these fragile compounds during manufacturing, storage and release. Stability monitoring includes a quantitative but also a qualitative characterization of the protein in the formulation. A method to assay a protein and its derivatives is an acidic hydrolysis of a sample with hydrochloric acid at elevated temperatures followed by the quantification of the amino acids, which is often referred to as amino acid analysis. This advantage of this destructive procedure is the recovery of all protein-related material, including insoluble, otherwise irrecoverable protein material but it impedes the determination of the qualitative composition of the protein material. The capability of this method to quantitatively recover protein material was the basis for an evaluation of other widely used protein extraction procedures for conventional microparticles, the methylene chloride / water method and a technique based on PLGA-hydrolysis with sodium hydroxide (Sharif et al., 1995). A sophisticated extraction procedure could be an important tool for the monitoring of protein stability, because the separation of the protein from the inactive formulation ingredients would allow the characterization of the integrity of the protein. However, the evaluation of both extraction methods with microparticles of three different proteins revealed an underestimation of the total drug contents in most cases. Of course, this could be simply due to an incomplete extraction, but the conditions applied during extraction, like the organic / aqueous interface or the extreme pH, are deleterious for proteins, which might have led to the formation of irrecoverable protein fractions during both extraction procedures. The reason for the underestimation cannot be pinpointed using microparticle formulations of proteins, since irrecoverable protein fractions could have been already formed during the preparation of the microspheres (paragraph 1.2.2.2.1.). It is noteworthy, that the outcomes of these obviously unsuitable protein extraction methods were often utilized as actual drug loadings. This necessitates a deliberate interpretation of the corresponding release data, with regard to the reported completeness of the drug release from the formulations (paragraph 1.2.2.2.3.). The lack of suitable methods for protein extraction from biodegradable dosage forms required the development of a more sophisticated protocol. The usefulness of this method to extract the model protein, lysozyme, from in situ forming biodegradable implants and microparticles without affecting its structural integrity was investigated and is presented in this chapter.

However, the suitability of in situ forming microparticles for controlled protein delivery is not only determined by the stability of a protein within the formulations. Moreover, the drug release patterns are essential criteria to evaluate the overall performance of a delivery system and compare different formulation approaches. However, numerous in vitro release methods were described for dissolution testing of controlled parenteral delivery devices, which differ in the experimental setups (D'Souza and DeLuca, 2006) and the utilized release media. In the simplest case an in vitro release medium consists of a buffering agent and a preservative. However, often stabilizing agents like surfactants or other proteins are added to avoid adsorption of proteins to surfaces and interfaces. Although it is well known that commonly used buffers often destabilize proteins (Eberlein et al., 1994, Pèrez and Griebenow, 2003 or Sah and Bahl, 2005), the effect of the release medium composition on the dissolution characteristics has been rarely evaluated (Kim and Burgess, 2002). An in vitro release drug dissolution test should ideally describe the situation at the injection site as closely as possible. That this goes far beyond the choice of the pH and the osmolarity of the release medium can be understood, considering in vivo conditions like protein adsorption to the device or the formation of a connective tissue capsule around biodegradable dosage forms (Pistner et al., 1993), which cannot be mimicked ex vivo so easily. On the other hand, certain potentially deleterious conditions, like the presence of surfaces (air) or interfaces (e.g., glass, plastic), appear to be inevitable for in vitro testing and could therefore affect already released protein, which impacts the release performance, without being relevant to the in vivo situation. A proper in vitro characterization of the release performance of fragile drugs like proteins should therefore allow a differentiation between formulation-related effects and potential contributions arising from the dissolution test protocol. Experimental parameters were therefore screened in order to establish release conditions, under which the model protein, hen egg white lysozyme is physically and chemically stable.

3.1.1. Evaluation of dissolution conditions for the release of lysozyme

Previously, a setup for testing in vitro drug release of in situ forming biodegradable microparticles was developed (Luan et al., 2006b). There, drug-loaded formulations were put into dialysis tubes and subjected to a release medium-containing sealable test tube. The final assembly was stored in an orbital shaker at 37°C. The test tubes were stored in horizontal (lying) position in order to enhance protein diffusion through the dialysis membrane. This method was

appropriate for the testing of low molecular weight substances and small peptides, but the suitability for protein release was not tested, yet. Therefore, effects of the type of buffer, the buffer concentration, the pH of the medium and the addition of excipients on the stability of lysozyme in solution were investigated.

3.1.1.1. Agitation-induced lysozyme aggregation

Accelerated aggregation was previously observed upon agitation of protein solutions, even though the drugs were stabilized by PEGylation (Treuheit et al., 2002). The test tube position was investigated for its effect on the aggregation behavior of lysozyme, since it influenced the surface area of the buffer. It was estimated, that the surface area of lysozyme solutions was about eight-times higher in the horizontal compared to the vertical position (unshaken).

The aggregation behavior of lysozyme solutions in test tubes in horizontal position was studied using UV-transmittance or absorbance measurements during or after incubation. Besides the conventional buffer systems consisting of phosphate (pKa₂ 7.2) and citrate (pKa₃ 6.4) ions, a histidine buffer (pKa₂ 6.0) was selected. Due to its pKa₂ value, histidine is the most suitable amino acid buffer for conditions close to physiological pH-ranges at the administration sites, e.g. pH 6-7 in muscles (Kemp, 2005) or pH 7.3-7.6 in subcutaneous regions (Mellstroem et al., 2001). Furthermore, it was previously shown, that among different buffer types, which contained the same type and amount of surfactant, histidine buffer resulted in the highest recovery of recombinant human interferon- γ upon release from PLGA microparticles (Yang and Cleland, 1997). According to the relevant pKa₂ of histidine, the pH of all buffers was set to 6.

Lysozyme aggregated rapidly and in 100 mM phosphate buffers and citrate buffers (Figure 16). A decreased strength (50 mM vs. 100 mM) of the phosphate buffer showed only marginally less lysozyme aggregation, whereas the protein was most stable in histidine buffer. However, aggregation was obtained in all cases.

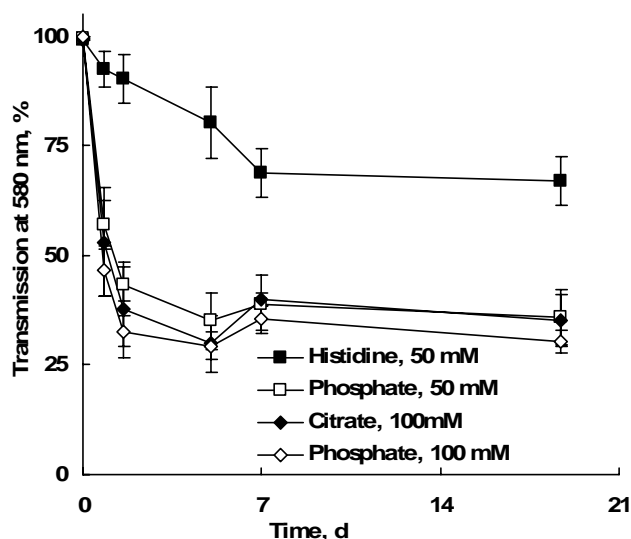


Fig. 16: Transmission at 580 nm over incubation time of lysozyme solutions in pH 6.0 buffers as a function of buffer type and concentration (200 $\mu\text{g}/\text{ml}$ lysozyme solutions containing 0.1 % sodium azide, horizontal test tube position, 80 rpm agitation, 37°C)

Tab. 2: Absorbance ($\lambda=281.2$ nm) in centrifuged test tubes after 19 days incubation of lysozyme solutions in different pH 6.0 buffer relative to the initial absorbance (200 $\mu\text{g}/\text{ml}$ lysozyme containing 0.1 % sodium azide as preservative, horizontal test tube position, 80 rpm agitation, 37°C)

Buffer	Absorbance, %
Histidine, 50 mM	54.2 (15.0)
Phosphate, 50 mM	2.6 (2.3)
Citrate, 100 mM	1.5 (4.0)
Phosphate, 100 mM	1.3 (1.1)

The results obtained from transmission measurements could be confirmed with the determination of the residual absorbances at 281.2 nm in centrifuged samples after 19 days incubation (Table 2). Thus, potential interferences, such as changes of the particle shape or size of the aggregates or protein adsorption to the test tubes, which would affect the UV-transmission results, could be excluded.

The differences in the aggregation behavior of lysozyme were primarily attributed to the buffer type (phosphate vs. histidine), whereas the phosphate buffer concentration between 50 mM and 100 mM had no significant effect.

In contrast to the lysozyme solutions stored in horizontal position (Table 2), loss of soluble lysozyme was marginal in phosphate and citrate buffer for up to 42 days, when the test tubes were incubated in vertical position (Table 3).

Tab. 3: Absorbance ($\lambda=281.2$ nm) in centrifuged test tubes after 42 days incubation of lysozyme solutions in different pH 6.0 buffer relative to the initial absorbance (200 $\mu\text{g/ml}$ lysozyme containing 0.1 % sodium azide as preservative, vertical test tube position, 80 rpm agitation, 37°C)

Buffer	Absorbance, %
Phosphate, 50 mM	98.4 (2.0)
Citrate, 100 mM	87.1 (3.6)
Phosphate, 100 mM	94.1 (1.9)

Thus, lysozyme aggregation was induced by agitation of the test tubes and not by the co-solutes, which just stabilized or destabilized the protein against agitation-induced aggregation. The sensitivity of the solubility of proteins (and other molecules) to the type and concentration of ions in their aqueous environment is not fully understood. Ions can show salting-in (chaotropic or destabilizing) or salting-out (kosmotropic or stabilizing) characteristics, which facilitated their classification according to the lyotropic series (Hofmeister, 1888). However, the physical background of this phenomenological classification of ions is complex and involves a number of contributing effects. Most theories focused on electrostatic or charge-dipole interactions between ions and proteins, such as binding or screening effects (Curtis et al., 2002a). However, it was recently discovered that only consideration of dispersion forces between ions and water, which are dependent on the size and the polarizability, could lead to a more accurate description of electrolyte solutions and thus to a better understanding of the interplay between electrolytes, like ion-protein or protein-protein interactions (Gitlin et al., 2006). In addition to electrostatic and dispersion forces, effects of the surface tension (water structure), an ion specific solvation energy

and of co- or counter-ion exclusion on protein-protein interactions are discussed (Bostroem et al., 2003).

The opportunity to adjust the aqueous environment of lysozyme and thus completely avoid agitation-induced aggregation led to further investigations of the role of co-solutes on the protein stability in the medium.

Effect of sodium azide as preservative

Preservatives like sodium azide are commonly added to in vitro release media to inhibit microbial growth during long term studies. However, their presence in the release medium has rarely been characterized with respect to potential effects on the stability of instable drugs (Kim and Burgess, 2002). In order to evaluate the effect of sodium azide on the stability of lysozyme in histidine buffer, the preservative concentration was varied between 0 % and 0.1 %.

A decrease of the absorbance at 281.2 nm was noticed in histidine buffers containing sodium azide during 33 days incubation, which corresponded to a lower amount of soluble lysozyme in the stored samples (Figure 17 and Table 4). The decrease was more pronounced at 0.1 % than with 0.02 % preservative concentration. However, the stored samples containing no preservative and one of the three samples with 0.02 % sodium azide showed a large increase of the extinction compared to the initial spectra. The reason for the increased absorbance was probably an insufficient preservation at and below 0.02 % sodium azide content, which facilitated microbial growth.

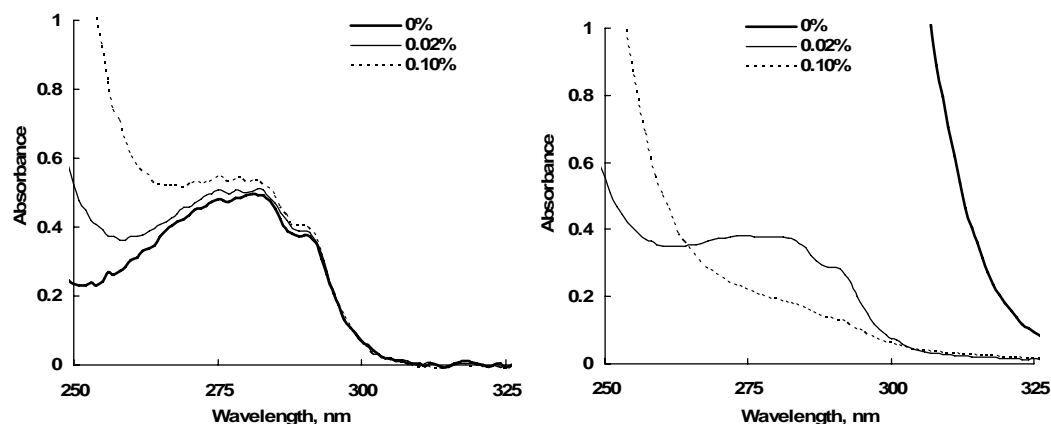


Fig. 17: UV-spectra of lysozyme solutions in 50 mM histidine buffers before (left) and after (right) 33 days incubation in dependence of the sodium azide concentration (200 µg/ml

lysozyme, horizontal test tube positions, 80 rpm agitation, 37°C). The spectra were representative for three individual samples.

Tab. 4: Absorbance ($\lambda=281.2$ nm) in centrifuged test tubes after 33 days incubation of lysozyme solutions different pH 6 media relative to the initial absorbance (~ 200 $\mu\text{g/ml}$ lysozyme, horizontal test tube position, 80 rpm agitation, 37°C)

Buffer	Absorbance, %
50 mM Histidine, 0 % NaN_3	772.3 (78.2)
50 mM Histidine, 0.02 % NaN_3	79.8 (23.4)
50 mM Histidine, 0.1 % NaN_3	33.7 (7.2)
0 % NaN_3	112.9 (4.4)
0.02 % NaN_3	77.1 (2.3)
0.1 % NaN_3	49.1 (2.8)

Similar to the lysozyme solutions in histidine buffer, unbuffered (no histidine) protein solutions (adjusted to pH 6) showed a sodium azide concentration-dependent decrease of the absorbance during incubation (Table 4). The relative absorbance after incubation decreased with increasing the preservative concentration. The stability of lysozyme solutions (pH 6) without additives led to the conclusion that sodium azide but also all buffer substances tested (Table 2 and Figure 16) are destabilizing factors for dissolved lysozyme.

In contrast to the markedly increased absorbances with the histidine buffered solutions of lysozyme, no such observation was made with unbuffered and unpreserved protein solutions, which pointed to histidine as the essential feature for enhanced microbial growth. This was further supported, when protein-free histidine buffer was stored for 35 days (Figure 18a). Unpreserved histidine buffer showed an increased absorbance, whereas preserved histidine (Figure 18b) and unpreserved imidazole buffer (Figure 19) showed no changes during incubation.

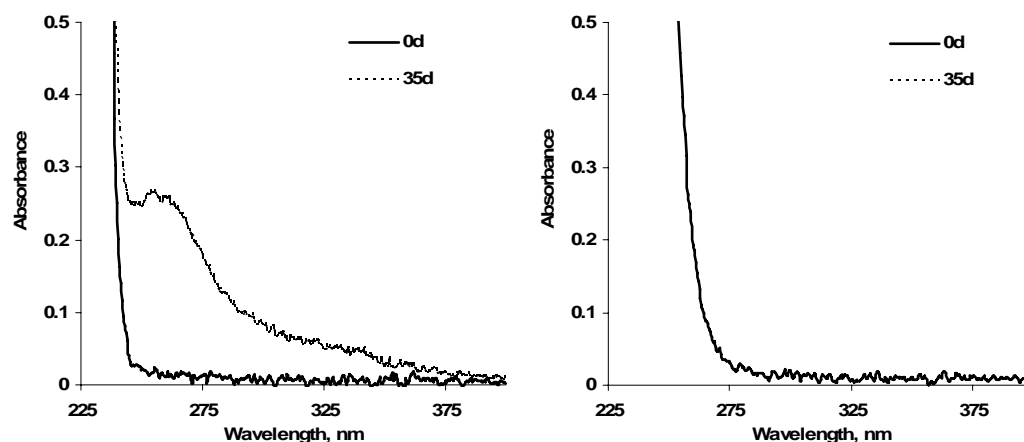


Fig. 18: UV-spectra of 50 mM histidine buffers (pH 7) before and after 35 day incubation at 50°C without (left) and with (right) 0.06 % sodium azide preservation.

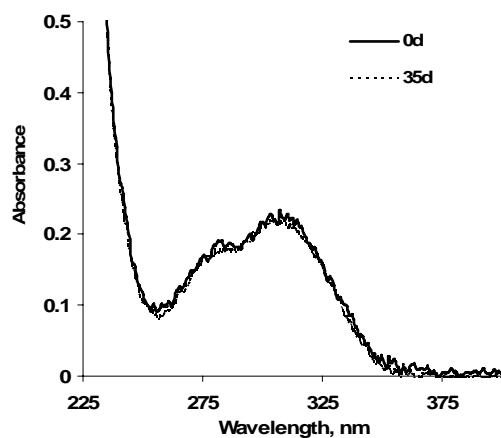


Fig. 19: UV-spectra of 50 mM imidazole buffer (pH 7) before and after 35 day incubation at 50°C without sodium azide preservation.

Histidine and imidazole feature the same heterocyclic structure (Figure 20). The basicity of the imidazole ring system is decreased in histidine due to the influence of the substituent. Imidazole could require less sodium azide compared to the amino acid, histidine, and was therefore further investigated as buffer substance.

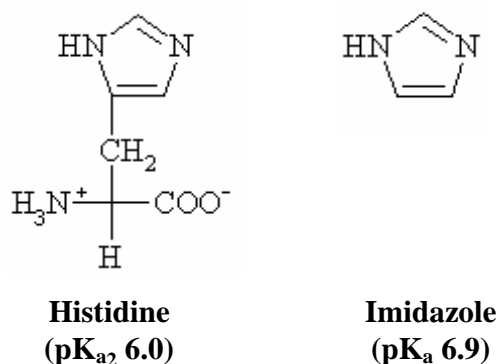


Fig. 20: Chemical structure of histidine and imidazole

Lysozyme aggregation in imidazole was more pronounced than in histidine buffer (Figure 21 and Table 5). Although a decrease of the sodium azide concentration from 0.1 % to 0.02 % decreased the agitation-induced aggregation of lysozyme, the destabilization was still more pronounced than for histidine buffer with the higher preservative content. A decrease of the concentration of imidazole in the buffer from 50 mM to 20 mM did not improve the stability of lysozyme. Thus, imidazole was no alternative to histidine buffer in order to decrease agitation-induced lysozyme aggregation through lower concentrations of sodium azide.

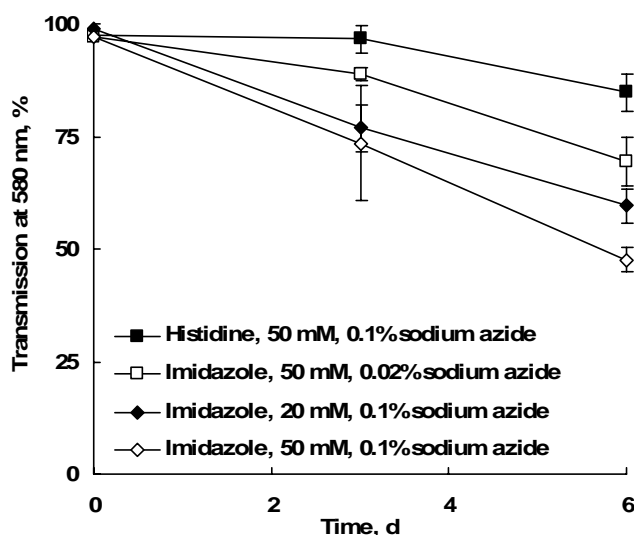


Fig. 21: Transmission at 580 nm of lysozyme solutions in pH 6.0 buffers over incubation time as a function of buffer type, concentration and sodium azide concentration (200 μ g/ml lysozyme, horizontal test tube position, 80 rpm agitation, 37°C)

Tab. 5: Absorbance ($\lambda=281.2$ nm) in centrifuged test tubes after 6 days incubation of lysozyme solutions in different pH 7 media relative to the initial absorbance; both normalized for buffer absorbance in case of imidazole (~ 200 $\mu\text{g/ml}$ lysozyme, horizontal test tube position, 80 rpm agitation, 37°C)

Buffer	Absorbance, %
50 mM Histidine, 0.1 % NaN_3	67.7 (3.6)
50 mM Imidazole, 0.1 % NaN_3	28.9 (9.7)
50 mM Imidazole, 0.02 % NaN_3	48.4 (7.7)
20 mM Imidazole, 0.1 % NaN_3	23.9 (13.1)

It could be concluded from this preliminary screening of potential release buffers that lysozyme aggregation could be minimized using the vertical test tube position or excluding any destabilizing co-solutes (buffer ions and sodium azide).

Water soluble formulation ingredients

A selection of excipients, potentially applicable as protein stabilizers or release modifier, was screened for their influence on lysozyme aggregation. The tested substances included surfactants (five non-ionic and one anionic), polyols, proteins, polymers and amino acids. All compounds were water soluble and, thus, could affect the susceptibility of lysozyme to agitation-induced aggregation. Lysozyme was therefore dissolved in solutions of the additives and the pH was adjusted to 6. Only negative influences could be revealed since lysozyme solutions without additive showed no aggregation during 33 days incubation (Table 4). A marked decrease of the transmission at 580 nm revealed aggregate formation and precipitation in polyoxyethylene (20) sorbitan monolaurate (Tween 20, HLB=16.7) and polyoxyethylene (20) sorbitan monooleate (Tween 80, HLB=15.0) containing solutions over time (Figure 22), which was in agreement with a destabilizing effect of Tween on protein solutions described previously (Treuheit et al., 2002). Nevertheless, Tweens are often improvidently added to protein release media (“PBT-buffer”) to actually stabilize proteins. The aggregation kinetics in the Tween samples appeared to be more complicated compared to the destabilization in buffer systems (Figure 22d or 16). Quantification of precipitates using transmission measurements interferes with size and shape of the particles,

which could depend on the precipitation conditions as well as on the association behavior of the protein (Tatford et al., 2004). This could be the reason, why in case of the Tween samples the transmission measurements indicated more lysozyme aggregation in Tween 80 than in Tween 20 samples, whereas an opposite trend was obtained from the absorbance measurements (Tween 20 > Tween 80) in the centrifuged samples (Figure 22 vs. Table 6).

Lysozyme appeared to aggregate in solutions containing polyethylene glycol (660) 12-hydroxystearate (Solutol HS 15, HLB~15) in the beginning of the incubation. However, after 30 days the aggregates appeared to be resolubilized, which was supported by the measured absorbance after incubation. Lysozyme aggregation in solutions containing the anionic surfactant, sodium dodecyl sulfate (SDS, HLB=40), polyoxyl 35 castor oil (Cremophor EL, HLB ~13) or poloxamer 188 (F68, HLB >24) was less pronounced and there was no correlation between the hydrophilic-lipophilic balance values (HLB) of the surfactants and the aggregation behavior of lysozyme.

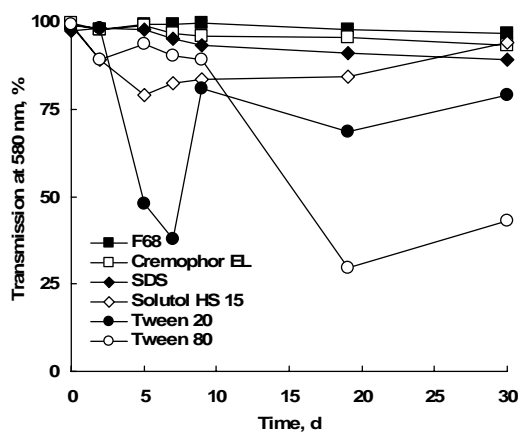


Fig. 22: Transmission at 580 nm during 30 days incubation of lysozyme solutions (pH 6) containing 0.1 % of surfactant (~200 $\mu\text{g/ml}$ lysozyme, horizontal test tube position, 80 rpm agitation, 37°C, n=1)

It had to be mentioned that the complex behavior of the positively charged lysozyme and anionic surfactants was beyond the scope of this experiment. However, the conditions applied here (lysozyme: 200 $\mu\text{g/ml}$ = 0.014 mM and SDS: 0.1 % = 3.5 mM) would correspond to a molar ratio of SDS to lysozyme of 250:1, where lysozyme was reported to be denatured (Glassman and Molnar, 1951 and Lad et al., 2003).

Tab. 6: Absorbance relative to the initial absorbance ($\lambda=281.2$ nm) in centrifuged test tubes after 30 days incubation of lysozyme solutions (pH 6) containing additives (~ 200 $\mu\text{g/ml}$ lysozyme, horizontal test tube position, 80 rpm agitation, 37°C , $n=1$)

Additive	Concentration	Absorbance, %
Poloxamer 188 (F68)	0.1 %	90.7
Cremophor EL	0.1 %	89.7
Sodium dodecyl sulfate (SDS)	0.1 %	96.5
Solutol HS 15	0.1 %	107.8
Tween 80	0.1 %	72.1
Tween 20	0.1 %	44.7
Saccharose	1 %	95.4
Trehalose	1 %	99.3
Lactose	1 %	99.0
Hydroxypropyl- β -cyclodextrin (HP β CD)	1 %	93.4
Bovine serum albumin (BSA)	1 %	121.5*
Polyvinyl pyrrolidone (PVP)	0.1 %	98.2
Polyvinyl alcohol	0.1 %	96.3
Methionine	20 mM	99.1
Glycine	20 mM	89.4
Histidine	20 mM	520.0**

* UV-interference (colorization during storage)

** UV-Interference by microbial growth

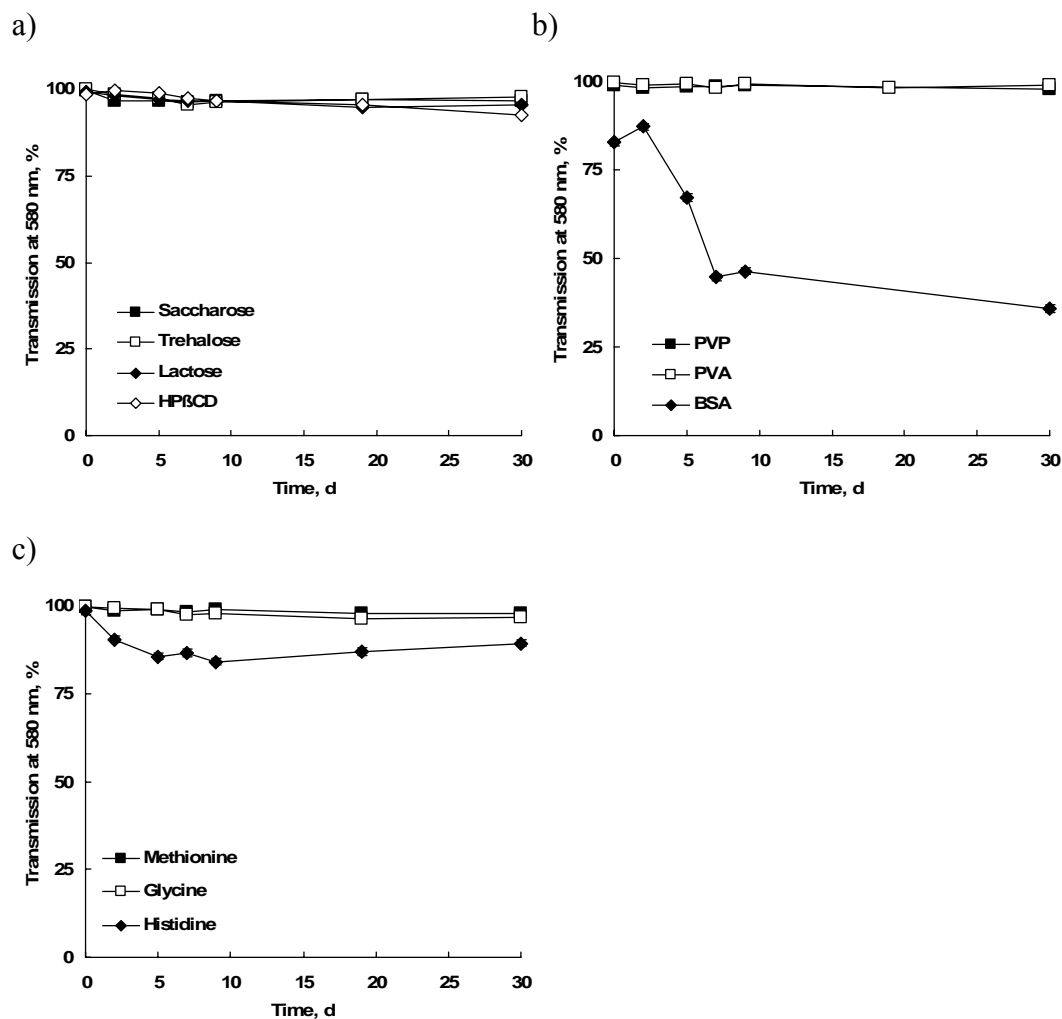


Fig. 23: Transmission at 580 nm during 30 days incubation of lysozyme solutions (pH 6) containing a) 1 % of a polyol, b) 0.1 % polymer / 1 % BSA or c) 20mM of an amino acid ($\sim 200 \mu\text{g/ml}$ lysozyme, horizontal test tube position, 80 rpm agitation, 37°C , $n=1$)

The aggregation behavior was not affected by the investigated polyols (Figure 23a and Table 6) and also not by the polymers polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) (Figure 23b and Table 6).

The bovine serum albumin-containing sample (BSA), however, showed a decreased transmission directly upon addition of lysozyme to the BSA solution. Thus, an interaction of lysozyme and BSA was indicated. This was unexpected considering a previous report (Filipe and Gosh, 2005). There, an interaction between both proteins was seen at pH 9 but not at pH 7 and pH 4.7, which includes the investigated pH in this work (pH 6). They proposed an electrostatic interaction due

to the isoelectric points (pI) of both proteins (lysozyme = 11.0 and BSA = 4.7). However, according to the pI-values an interaction should be feasible within the entire pH-range between 4.7 and 11. Two differences in the experimental setup could explain why an interaction was not seen in the previous study. Firstly, the solution concentration of bovine serum albumin was 50-times lower than in the previous study (0.2 mg/ml versus 10 mg/ml), which might decrease the probability for collisions and thus slow down the aggregation rate. Secondly, Filipe and Gosh investigated 50 mM buffer at pH 7 (tris) and pH 4.7 (phosphate), whereas in the present study the protein solutions were adjusted to pH 6 with either diluted sodium hydroxide or hydrochloric acid and thus remained unbuffered. The presence of salts can destabilize (Figure 16) but also stabilize proteins by shielding charges or by being preferentially excluded from proteins. However, BSA solutions were not further investigated due to its potential association with lysozyme and its own stability issues. A colorization of the release medium during incubation (never observed with lysozyme alone) interfered with the UV-measurements after 30 days (Table 6).

As observed with the 50 mM buffers, histidine induced lysozyme aggregation even at a concentration as low as 20 mM (Figure 23c). Again, elevated absorbances after 30 days pointed again to a stability problem of unpreserved histidine solutions (Table 6). Unpreserved glycine and methionine containing protein solutions did neither induce lysozyme aggregation nor showed any destabilization of lysozyme comparable to histidine solutions.

3.1.1.2. Lysozyme adsorption to surfaces and interfaces

In situ forming microparticle formulations consist of one phase, that contains drug, biodegradable polymer (e.g. PLGA) and solvent, which is emulsified with either oil (oil-in-oil ISM) or water (oil-in-water ISM). PLGA and the oil for injection, utilized in oil-in-oil formulations, are not soluble in or miscible with water. Thus, an interface or surface with the buffer would be formed, where amphiphilic proteins could be adsorbed. Following the previously developed dissolution setup, the utilization of a dialysis membrane for protein release was investigated.

Polymer surface

Protein-PLGA interactions are known to be an important cause of incomplete in vitro release (van de Weert et al., 2000a). The reason for the adsorption of proteins to the polymer under in vitro conditions was attributed to hydrophobic or electrostatic forces, while the latter was of special importance for proteins with high isoelectric points ($pI \sim 11$), like lysozyme (Park et al., 1998). However, a relevance of this interaction for the in vivo release was previously questioned (van de Weert et al., 2000a), with the argument that components of biological fluids may compete with the protein for the polymer surface. Furthermore, it was intended here to use lysozyme as a model compound for protein drugs of similar molecular weights. Thus, stability issues resulting from special characteristics of lysozyme, like its strong basicity, were less relevant for the overall goal of this work to evaluate in situ forming microparticles for the suitability to deliver protein drugs in a controlled manner.

Test tubes filled with lysozyme solutions of different buffers containing RG 503H powder were incubated in horizontal position in order to evaluate the deleteriousness of the PLGA surface for the protein. The decrease of the lysozyme concentration in the buffer was rapid in unpreserved phosphate and preserved histidine buffer (Figure 24). An influence of the buffer type on the extent of protein loss could not be concluded for the pH 7 media, due to the rapidness of the adsorption / aggregation process, when both deleterious conditions were combined. Interestingly, lysozyme dissolved in acetate buffer (pH 5) was not affected by any of both stresses. The reason for the stability of lysozyme could be attributed to the elevated conformational stability of lysozyme at pH 5 (Claudy et al., 1992). A decreased dissociation of PLGA ($pK_a(\text{lactic acid}) = 3.86$ and $pK_a(\text{glycolic acid}) = 3.83$; $pK_a = 3.85$ used as average for PLGA) was probably not responsible for this effect, due to the small absolute change of the degree of protonization of PLGA (acid / base ratio: 99.9 / 0.1 to 93.3 / 6.7) involved in a pH change from 7 to 5.

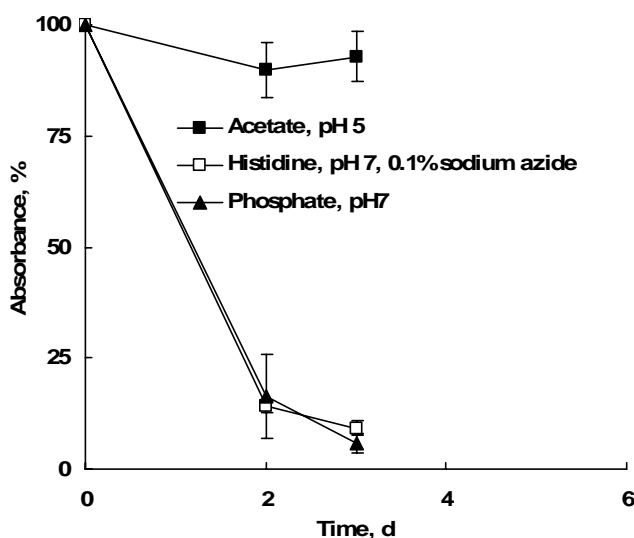


Fig. 24: Relative UV-absorbance at 281.2 nm of centrifuged lysozyme solutions in 33 mM buffers incubated in presence of 50 mg RG 503H per vial over 3 days as a function of buffer type and pH (500 μ g/ml lysozyme, horizontal test tube positions, 80 rpm agitation and 37°C)

The reversibility of the agitation- and adsorption-induced lysozyme loss was investigated with a PLGA particles (wetted powder) containing lysozyme solution in a phosphate buffer pH 7 with 0.1 % sodium chloride and 0.01 % sodium azide. The test tubes (n=9) were incubated in horizontal position for 2 days before they were centrifuged and the supernatant, containing the residual soluble fraction of lysozyme, replaced with 33 mM phosphate buffer pH 7 containing either 1M NaCl, 6 M urea or 0.1 % sodium dodecyl sulfate (SDS). The test tubes (3 x 3) were analyzed after additional incubation for 18 hours. According to Park et al., 1998, sodium chloride replaced electrostatically bound lysozyme from the PLGA surface. Instead of guanidine hydrochloride used in the previous report, uncharged urea was applied as denaturant, which should disrupt non-covalent aggregates without affecting ionic interactions. The sodium dodecyl sulfate concentration of 0.1 % was slightly lower than used previously (5 mM = 0.14 %). However, both concentrations could be expected to be above the critical micelle concentration (Nakamura et al., 1998) and thus without a risk to form insoluble lysozyme-SDS “complexes” (salts). The amount of soluble lysozyme in the removed supernatant was about 38 % after the incubation for 2 days. Attributing the loss of soluble protein to adsorption only would lead to an amount of 5.8 μ g lysozyme adsorbed per mg polymer, which was in good agreement with the 11

$\mu\text{g}/\text{mg}$ adsorbed to blank PLGA microparticles (Nam et al., 2000). Thus, the contribution from the agitation of the solution on loss of soluble protein could be less pronounced compared to adsorption.

Incubation with one molar sodium chloride containing phosphate buffer had no effect on the recovery of lysozyme, whereas denaturation with urea could redissolve some of the aggregated / adsorbed lysozyme (Table 7). SDS was slightly more efficient than urea but in general, none of the recovery media was suitable to completely redissolve aggregated / adsorbed lysozyme. This was somewhat unexpected with respect to the more than 90 % recovery reported for microencapsulated lysozyme (Park et al., 1998). However, the experimental conditions were not comparable, due to different incubation conditions and a potential effect of the involved microencapsulation process on the protein (paragraph 1.2.2.2.1.).

Tab. 7: Evaluation of solution conditions in order to redissolve lysozyme aggregates / adsorbed lysozyme obtained after 2 days incubation of the solutions with 50 mg of PLGA (RG 502H) particles after 18 h incubation under the recovery conditions ($\sim 500 \mu\text{g}/\text{ml}$ lysozyme, 33 mM phosphate buffer pH 7 containing 0.1 M sodium chloride and 0.01 % sodium azide, horizontal test tube position, 80 rpm agitation, 37°C)

Absorbance after incubation, %	Adsorbed or precipitated lysozyme / mass of PLGA, $\mu\text{g}/\text{mg}$	Recovery of lost lysozyme, %		
		1 M NaCl	6 M urea	0.1 % SDS
38 (8.6)	5.8 (0.8)	0.0 (0.8)	11.2 (1.5)	18.2 (4.2)

Besides a potential denaturation during adsorption and aggregation or due to the applied recovery conditions, the insufficient recovery of insoluble lysozyme would even impede a quantitative interpretation of the release profiles.

One opportunity to reduce adsorption to PLGA, which is initiated by electrostatic interactions, is the addition of salts to the medium, which compete with lysozyme for the negative charges on the polymer surface (Park et al., 1998). Indeed, the concentration of lysozyme in phosphate buffer containing sodium chloride decreased at a lower rate in presence of 0.1 M and 0.5 M sodium chloride (Figure 25), which confirmed the reason for interactions causing loss of soluble protein. However, the decrease of the soluble protein within 3 days incubation fraction could not be prevented completely.

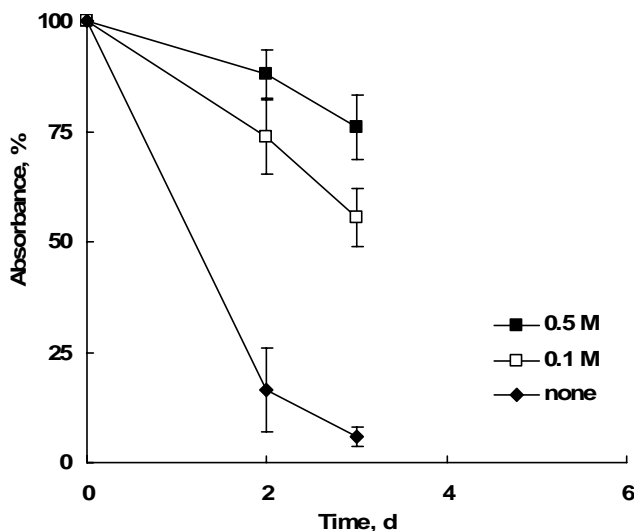


Fig. 25: Relative UV-absorbance at 281.2 nm of centrifuged lysozyme solutions in 33 mM phosphate buffers (pH 7) incubated in presence of 50 mg PLGA (RG 503H) per vial over 3 days as a function of sodium chloride concentration (500 $\mu\text{g}/\text{ml}$ lysozyme, horizontal test tube positions, 80 rpm agitation and 37°C)

The adsorption / aggregation behavior of lysozyme in presence of potassium chloride (KCl) was investigated in tris-buffer. The concentration of KCl was varied in a broader range, from 0 to 0.75 M. The cation was changed to potassium, since potassium could be a slightly better stabilizer for lysozyme compared to sodium (Hofmeister, 1888). Comparable to the unpreserved phosphate buffer and RG 503H, a relatively large improvement of the aggregation behavior was obtained with the addition of the first salt portion, 0.15 M KCl in the present case (Figure 26). Although, a total KCl concentration of 0.3 M further resulted in even less loss of soluble lysozyme, a further increase of the salt concentration to 0.5 M showed no additional benefit. The loss of soluble protein after 3 days incubation even increased again upon an increase of the KCl concentration to 0.75 M, which might be due to a salting-out effect (Poznanski, 2006) or could be due to an increased sensitivity of the protein to agitation-induced stress at elevated salt concentrations. Complete stabilization could not be obtained with salt addition, which illustrated the limited suitability of the approach to just decrease electrostatic interactions of lysozyme and PLGA.

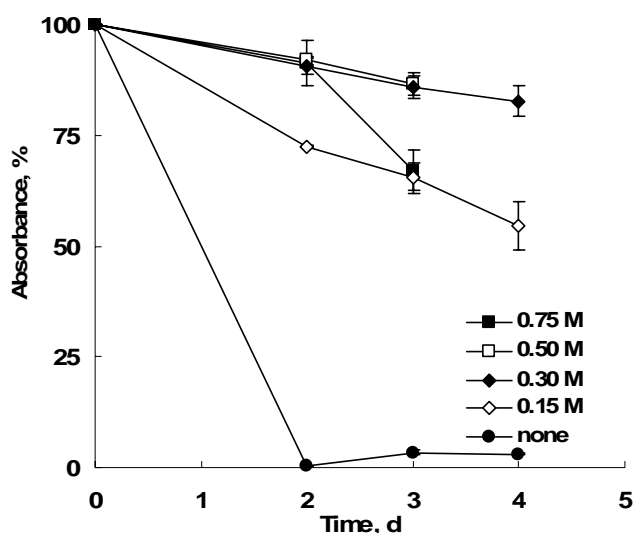


Fig. 26: Relative UV-absorbance at 281.2 nm of centrifuged lysozyme solutions in 33 mM tris-sulfate buffer pH 7 containing 0.01 % sodium azide incubated in presence of 50 mg RG 502H per vial for up to 4 days as a function of potassium chloride concentration (500 $\mu\text{g}/\text{ml}$ lysozyme, horizontal test tube positions, 80 rpm agitation and 37°C)

Another approach to keep lysozyme in solution could be the addition of surfactant. Sodium dodecyl sulfate (SDS) and Solutol HS 15 were selected as examples of an anionic and a non-ionic surfactant. Both substances were able to stabilize lysozyme against agitation-induced aggregation upon 30 days incubation in water. Lysozyme adsorbed rapidly and completely to PLGA (100 mg RG 503H) in preserved histidine buffer. The non-ionic surfactant (Solutol HS 15) was not able to prevent the soluble protein fraction from a rapid decrease, whereas the anionic SDS solubilized lysozyme efficiently (Figure 27). This is in agreement with the initiation of lysozyme adsorption to the PLGA surface by electrostatic interactions.

The problem of a solubilization of released lysozyme by SDS addition to the release medium would be the denaturation of the protein caused by surfactant concentrations, which were necessary for solubilization (Glassman and Molnar, 1951 and Lad et al., 2003). This would impede the characterization of potential effects of the formulation, the in situ forming microparticle systems, on the biological integrity of the protein. Furthermore, it could be expected that a solubilization effect of lysozyme by SDS would mainly affect the released protein fraction and less likely the fraction of lysozyme inside the PLGA matrix, where the surfactant would have to diffuse to.

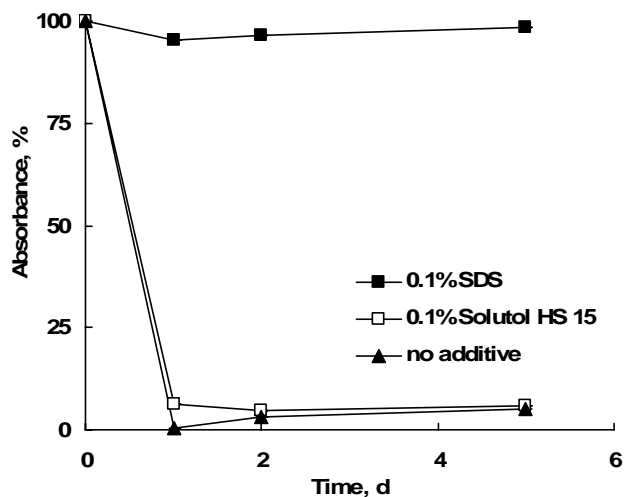


Fig. 27: Relative UV-absorbance at 281.2 nm of centrifuged lysozyme solutions in 50 mM histidine buffers pH 7 containing 0.1 % sodium azide incubated in presence of 100 mg RG 503H per vial over 5 days as a function of surfactant (250 $\mu\text{g/ml}$ lysozyme, horizontal test tube positions, 80 rpm agitation and 37°C)

Sesame oil interface

In situ forming microparticles can be formulated as oil-in-water or oil-in-oil systems, where the non-aqueous polymer solution is either emulsified with an aqueous or an organic liquid phase. In the latter case the organic phase can consist of oil and an optional stabilizer (e.g. surfactant), which are suitable for injection. Sesame oil is an example of a suitable oil phase. It is water immiscible and thus forms an interface with aqueous media. Proteins are prone to occupy surfaces and interfaces, due to their amphiphilic character. Therefore, the exposure of sesame oil (with and without stabilizing excipients) to lysozyme solutions was investigated for its effect on the stability of lysozyme.

The decrease of soluble lysozyme in aqueous solutions exposed to the interface to sesame oil containing 2 % Span 80 was fairly small (Figure 28). No soluble lysozyme was lost in preserved histidine buffer and in acetate buffer during 6 days of incubation with the oil phase, whereas some, probably agitation-induced, loss of lysozyme occurred in the phosphate buffer. Obviously, the hydrophobic interface to the oil phase was much less problematic for lysozyme than the negatively charged surface of PLGA. It was even indicated, that agitation in presence of the oil

was less deleterious than agitation without (Figure 28 vs. Figure 16 and Table 2). The oil phase swam on top of the buffer due to the lower density and hence decreased the area the buffer was exposed to air. A “stabilizing effect” of the oil, with respect to the agitation-induced aggregation of lysozyme at the buffer surface, could thus be explained with a higher stability of lysozyme upon exposure to a sesame oil-water interface compared to a buffer surface. However, an advantageous contribution from the co-incorporated water-insoluble surfactant (HLB 4.3) could not be excluded.

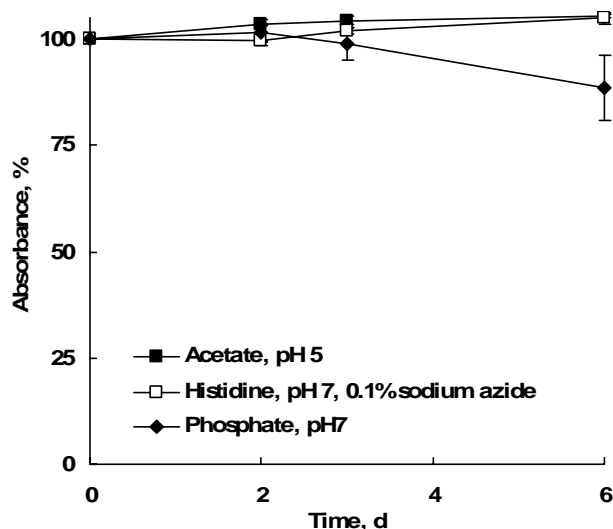


Fig. 28: Relative UV-absorbance at 281.2 nm of centrifuged lysozyme solutions in 33 mM buffers incubated in presence of 300 mg sesame oil containing 2 % Span 80 per vial over 6 days as a function of buffer type and pH (500 μ g/ml lysozyme, horizontal test tube positions, 80 rpm agitation and 37°C)

The substitution of the Span 80 portion in the oil phase with aluminum stearate did not affect the stability of lysozyme in acetate buffer, whereas lysozyme was markedly affected in tris-buffer (Figure 29). Whether the decreased solution stability of lysozyme was due to the aluminum / the stearate ion presence or other effects was not elucidated. However, a decreased solubility of lysozyme-fatty acid salts was described previously (Yoo et al., 2000).

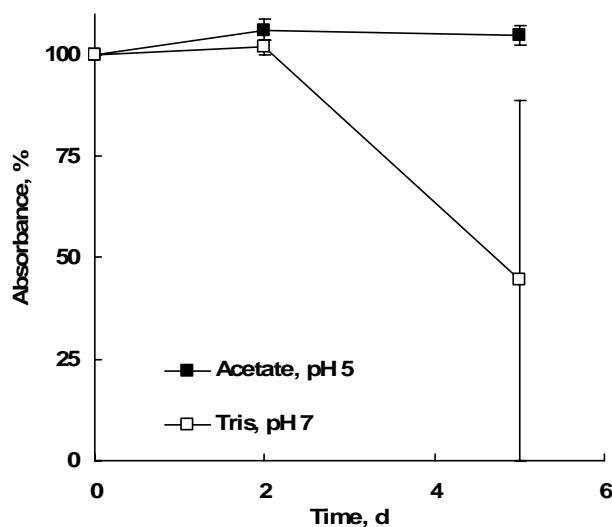


Fig. 29: Relative UV-absorbance at 281.2 nm of centrifuged lysozyme solutions in 33 mM buffers containing 0.01 % sodium azide incubated in presence of 300 mg sesame oil containing 2 % aluminum stearate per vial over 5 days as a function of buffer type and pH (500 $\mu\text{g}/\text{ml}$ lysozyme, horizontal test tube positions, 80 rpm agitation and 37°C)

Dialysis bag method

The previously developed dissolution methodology applied a dialysis bag to accommodate the formulation. Thus, the drug was allowed to diffuse through the dialysis membranes, whereas the excipients were separated from the release medium. Thus, sampling through an exchange of the external release medium was convenient, since it saved an additional centrifugation step.

To investigate an influence of this method on the rate or extent of lysozyme recovery, a molecular weight cut-off (MWCO) of the cellulose ester membranes of 100,000 Da was selected to facilitate rapid and complete passage of lysozyme (14,450 Da) through the dialysis bag. Therefore, 0.5 g protein solution was placed into dialysis bags, subjected to test tubes filled with 8 g release medium and incubated. As a comparison, the lysozyme solution was directly injected into the release medium, which contained an empty dialysis bag.

A retarded increase of the lysozyme concentration in the external medium was observed for the samples, where the lysozyme solution was placed inside the dialysis bags (Figure 30). This indicated that the dialysis membrane was a diffusion barrier for lysozyme, which would slightly

affect the release kinetics. Lysozyme adsorption to the cellulose ester membrane was not indicated, since the protein, which was incubated in presence of an empty bag, appeared to be unaffected.

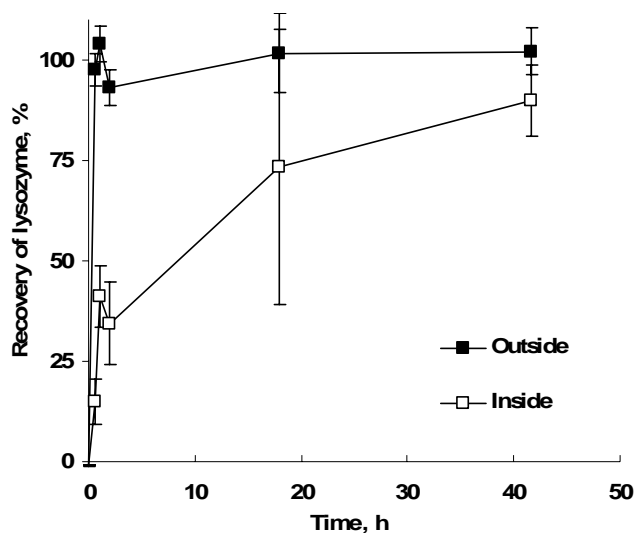


Fig. 30: Relative concentration of soluble lysozyme in the medium measured with Coomassie plus assay. “Inside”: 0.5 g of a 1 mg/ml lysozyme solution in 33 mM acetate buffer (pH 5) containing 0.01 % sodium azide was placed in a dialysis bag (MWCO 100,000 Da), which was closed and subjected to test tubes filled with 8 g of the same buffer. “Outside”: 0.5 g of a 1000 μ g/ml lysozyme solution in 33 mM acetate buffer (pH 5) containing 0.01 % sodium azide was directly injected into the buffer containing an empty dialysis bag (horizontal test tube positions, 80 rpm agitation and 37°C).

In a comparable experiment, a dispersion of lysozyme in the polymer solvent triacetin was placed either inside or outside the dialysis bag and agitated in horizontal test tube position. Oppositely to the aqueous solution, the recovery of protein upon direct injection of the dispersion was incomplete (Figure 31). Only 65 % of lysozyme was recovered. This had to be further investigated, since it could be important for the protein release from corresponding in situ formulations. Upon injection of the lysozyme / triacetin dispersion (0.25 g of 10 mg/g) into acetate buffer (8 g) and dissolution of the triacetin, the protein was fully recovered (99.4 \pm 1.6, n=6). Although the amount of applied dispersion differed (0.5 vs. 0.25 g), the triacetin concentration in both experiments was below the solubility limit in water (5 % and 3 % < 6.2 %).

The major difference was that the dialysis bag-containing sample was agitated more strongly (horizontal test tube position), which could have acted as additional source of stress to protein aggregation and precipitation at the initially present triacetin-buffer interface. However, the presence of the dialysis bag surface could have also contributed to the decreased recovery. More importantly, the release from the dialysis bag was affected significantly. Only about 20 % of lysozyme was released over 48 hours. The release testing was conducted without a dialysis bag due to the retardation of the release.

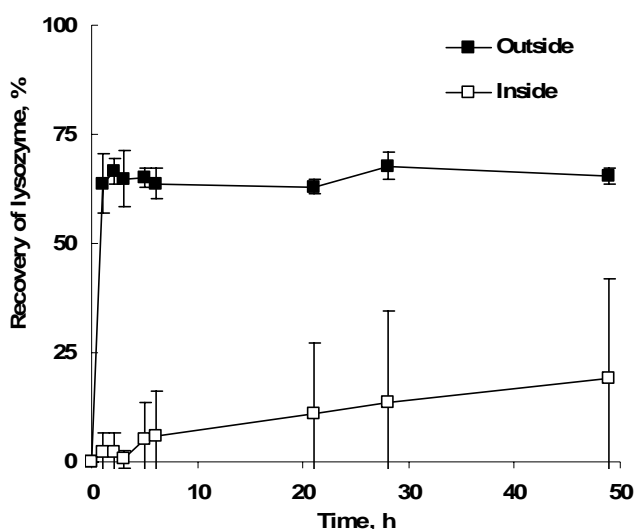


Fig. 31: Relative concentration of soluble lysozyme in the medium measured with Coomassie plus assay. “Inside”: 0.5 g of a 10 mg/g lysozyme dispersion in triacetin was placed in a dialysis bag (MWCO 100,000 Da), which was closed and subjected to test tubes filled with 10 g of 33 mM acetate buffer (pH 5) containing 0.01 % sodium azide. “Outside”: 0.5 g of the lysozyme dispersion in 33 mM was directly injected into acetate buffer (pH 5) containing 0.01 % sodium azide containing an empty dialysis bag (horizontal test tube positions, 80 rpm agitation and 37°C).

3.1.1.3. Long term stability of aqueous lysozyme solutions

Stability in acetate buffer

The screening of potential release conditions revealed, that the main causes for a loss of soluble lysozyme during the dissolution assay are adsorption of lysozyme to the PLGA surface as well as agitation-induced aggregation. Especially the electrostatic interactions between the positively charged lysozyme, with its particularly high isoelectric point, and the negatively charged PLGA resulted in irrecoverable losses of the protein.

The obtained results for lysozyme suggested that a reduction of these interactions requires the application of unphysiologically conditions, such as addition of 0.1 % of the anionic surfactant dodecyl sulfate (SDS), an increase of the ionic strength of the medium with 0.3 M KCl or a decrease of the pH to around 5, where lysozyme has its stability optimum. Solubilization by SDS causes itself lysozyme denaturation and was therefore not the first choice for the evaluation of in situ microparticles for protein release. The pH adjustment to the stability optimum was selected, due to the complete recovery of soluble lysozyme upon short term incubation with PLGA powder compared to the salt addition. Therefore, 33 mM sodium acetate buffer pH5 containing 0.01 % sodium azide was further investigated.

The solution stability of lysozyme in pH 5 acetate buffer was excellent over 70 days, when the test tubes were incubated at 37°C in vertical position (Figure 32), whereas the concentration of lysozyme decreased by about 14 % in the horizontal position (Table 8). A slight difference between both positions was also seen between the biological activities of the enzyme. However, the HPLC results were close and no aggregates could be observed visually or were detectable with polyacrylamide gel electrophoresis (PAGE) in the horizontally incubated sample (Lane 4, Figure 33).

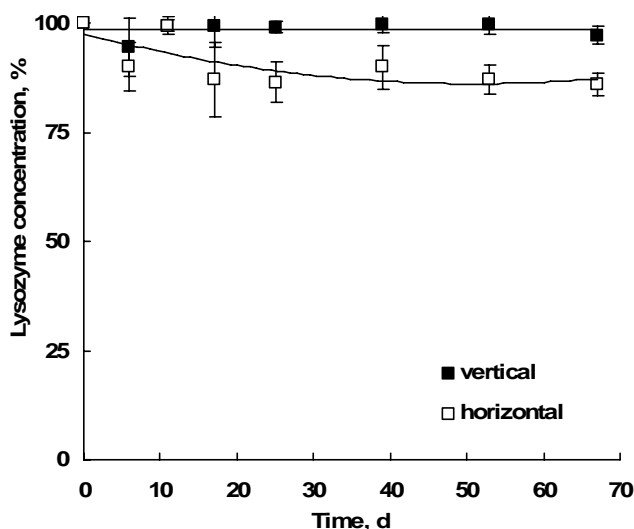


Fig. 32: Relative absorbance (Coomassie plus assay) of lysozyme solutions in 33 mM acetate buffer pH 5 over incubation time as a function of test tube position (500 $\mu\text{g/ml}$ lysozyme in buffer containing 0.01 % sodium azide, 80 rpm agitation, 37°C)

Tab. 8: Incubation of 500 $\mu\text{g/ml}$ (5000 $\mu\text{g/ml}$) lysozyme solutions in 33 mM acetate buffer pH 5 containing 0.01 % sodium azide or alternatively in 0.1 N HCl at 37°C (4°C) for 67 days in dependence of the test tube position. Relative values related to initial concentrations (0d).

Condition variable(s)	Coomassie recovery, %	HPLC, recovery, %	Activity, %
Horizontal	86.1 (2.6)	94.2 (5.8)	94.1 (2.3)
Vertical	97.2 (2.0)	101.9 (4.7)	105.7 (3.3)
4°C, vertical	101.9 (1.7)	96.7 (2.8)	99.2 (2.3)
5000 $\mu\text{g/ml}$, vertical	101.1 (5.5)	106.7 (4.2)	99.6 (2.4)
0.1 N HCl, horizontal	75.7 (5.9)	1.4 (2.3)	3.7 (0.4)
0.1 N HCl, vertical	86.0 (4.2)	1.8 (0.1)	5.6 (1.9)

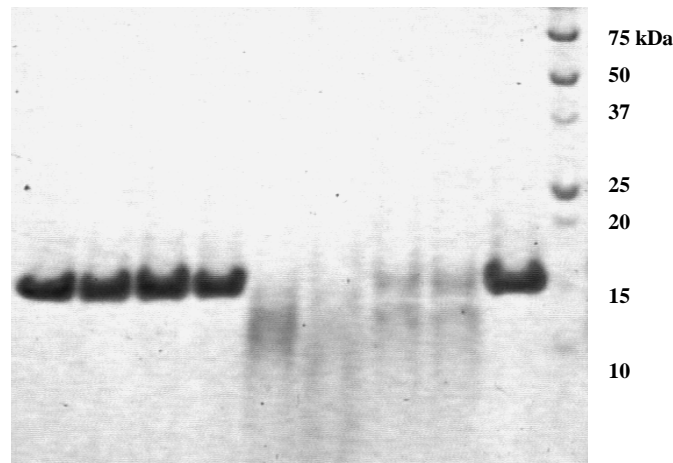


Fig. 33: Non-reducing (native) PAGE of lysozyme solutions after 67 days of incubation. Lanes: 1) 4°C; vertical, 2) 5000µg/ml (1/10 dilution); 3) vertical; 4) horizontal; 5 and 6) 0.1 N HCl, vertical; 7 and 8) 0.1 N HCl, horizontal; 9) standard (546 µg/ml); 10) molecular weight marker

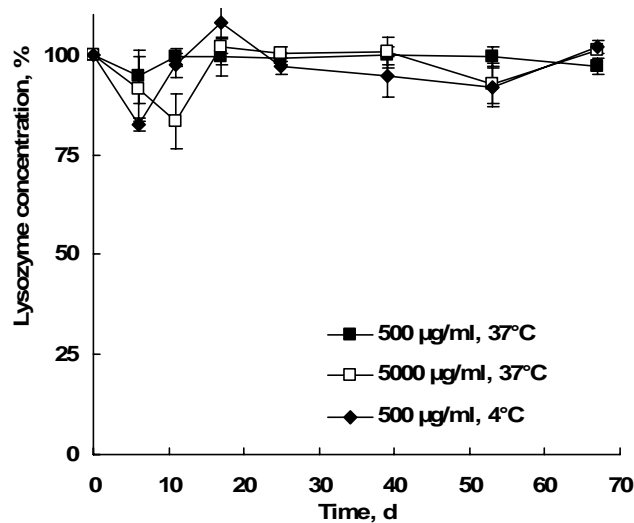


Fig. 34: Relative absorbance (Coomassie plus assay) of lysozyme solutions in 33 mM acetate buffer pH 5 containing 0.01 % sodium azide over incubation time as a function of lysozyme concentration and storage temperature / agitation (vertical test tube position)

Increased protein concentrations could lead to an accelerated aggregation (Treuheit et al., 2002). Therefore, a 10-times higher lysozyme concentration (5 mg/ml) was incubated. No difference in

the stability was seen compared to the more diluted 500 $\mu\text{g/ml}$ samples (Figure 34 and Table 8). Both solutions were stored in vertical position in the incubation shaker at 80 rpm and 37°C. Storage of the protein solutions in acetate buffer at 4°C (static) did not cause lysozyme aggregation or conformational changes and thus facilitated storage of samples in a refrigerator before analysis (Figure 34 and Table 8). It was even indicated, that storage at 4°C could be beneficial for lysozyme. HPLC analysis revealed two lysozyme-related peaks in the standard solution, a large peak at a retention time of about 14 minutes and a smaller one at 13.5 minutes (Figure 35a). The ratio of the peak areas (peak at 14 min / peak at 13.5 min) decreased from 15 (Figure 35a) to about 2.0-2.2 upon incubation in acetate buffer at 37°C for 67 days (Figure 35b-d). An increase of the peak area of a smaller peak was previously seen with a comparable HPLC method but was not identified (Schaefer et al., 1999). The decrease of the peak ratio was slower in the samples stored at 4°C (Figure 36), where a ratio of 10.5 was obtained after 67 days (Figure 35f). The apparent temperature dependence and the peak ratio of 2.0-2.2 after equilibration were in a fair agreement with the racemization behavior of lysozyme (Tomizawa et al., 1994a and b). The reported ratio (native + 101-succinimide lysozyme) to racemized lysozyme obtained upon incubation at 40°C in pH 5 acetate buffer was about 1.7. Although Tomizawa and coworkers used ion exchange and not reversed phase HPLC, native and racemized species of smaller peptides could be distinguished with RP-HPLC previously (Geiger and Clarke, 1987).

Incubation of lysozyme solutions in hydrochloric acid was conducted to reveal the analytical features of (partially) hydrolyzed protein. Protein hydrolysis occurs at elevated acidity, which develops in matrices of PLGA during polymer degradation. In contrast to lysozyme in acetate buffer pH 5, the protein was almost completely lost upon incubation in 0.1 N HCl (Figure 35f and g). It is known that peptide bonds can be hydrolyzed under acidic conditions. Accordingly, lower molecular weight fragments occurred in the electrophoresis (Figure 33, Lanes 5-8).

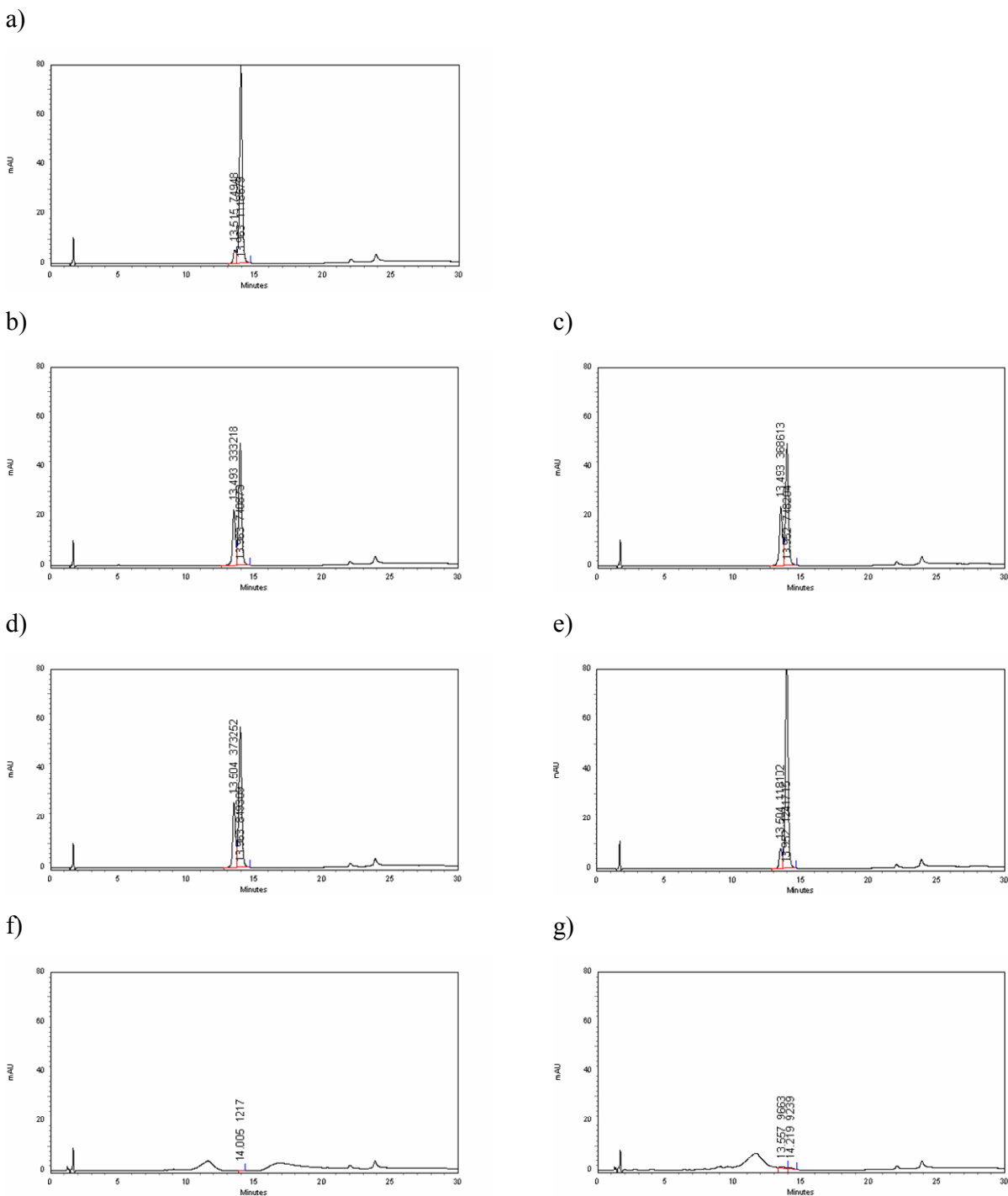


Fig. 35: HPLC chromatograms of 500 $\mu\text{g/g}$ lysozyme solutions (d: 1/10 solution of 5 mg/g) after 67 days (a: 0 day) incubation in 33 mM acetate buffer, pH 5 containing 0.01 % sodium azide (f, g: 0.1 N HCl) in horizontal (b, e, f) or vertical (c, d, g) position at 37°C (e: 4°C) and 80 rpm (e: static)

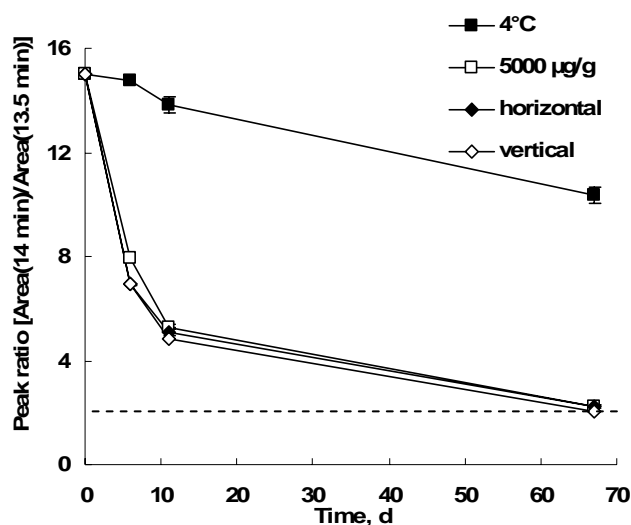


Fig. 36: Ratio of peak areas of the peaks at 14.0 min and 13.5 min retention time from HPLC analysis of lysozyme solutions (500 µg/g or 5000 µg/g) in 33 mM acetate buffer pH 5 containing 0.01 % sodium azide in dependence of the incubation time in vertical (horizontal) position at 37°C (4°C). Peak ratio at equilibration according to Tomizawa et al., 1994a (- - -).

Interestingly, the protein fragments interacted with the dye in the Coomassie reagent resulting in apparent lysozyme concentration between 76 and 86 % after 67 days (Figure 37), which did not correlate with the actual lysozyme concentration measured with HPLC and the biological activity assay (Table 8). Furthermore, the apparent increase of the lysozyme concentration between day 6 and 17 in the vertically stored samples could be due to a different response of denatured protein to the dye in the Coomassie reagent. The dye usually changes its color in response to the protein concentrations. However, binding to a protein occurs primarily at basic and aromatic amino acid residues, especially arginine. The exposure of previously buried amino acid residues would therefore affect the dye binding effect and thus the apparent concentration. Similar effects of denaturation on the optical behavior (e.g., UV-extinction or fluorescence emission) are used to differentiate non-native from native protein derivatives (Herberhold et al., 2003).

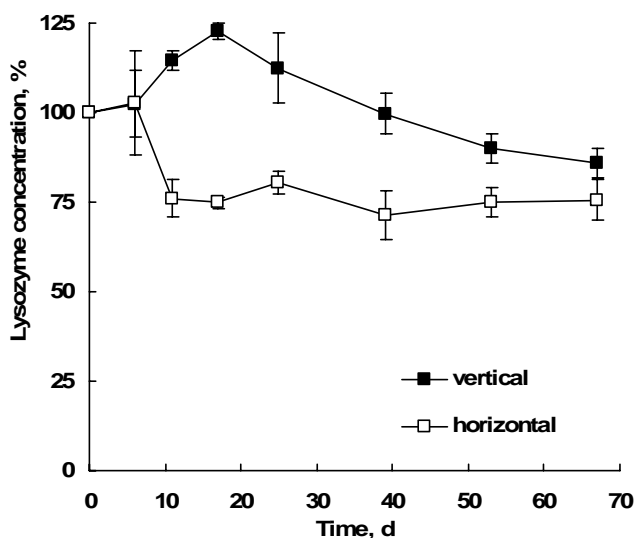


Fig. 37: Relative absorbance (Coomassie plus assay) of lysozyme solutions in 0.1 N HCl over incubation time as a function of the test tube position

Assuming a correlation between the peak ratio of lysozyme in HPLC chromatograms and its chemical stability, storage at 4°C appeared to be most suitable to retain the native structure of lysozyme in an aqueous solution for prolonged time intervals. The vertical position showed a slightly better performance compared to agitation of the test tubes in horizontal position at 37°C.

Incubation of lysozyme solutions in presence of PLGA

Lysozyme in the pH 5 acetate buffer was exposed to PLGA, which was identified as the most critical stress factor for the protein. The short term exposure of lysozyme to the surface of PLGA (50 mg RG 503H) led to a decrease of the protein concentration in acetate buffer by only 7 % (Figure 24). However, longer incubation times were investigated to establish a suitable sampling schedule and to elucidate the effect of PLGA degradation on lysozyme.

Therefore, lysozyme solutions in the acetate buffer were incubated with 100 mg uncapped short-chain PLGA (RG 502H). Only 83 % of the initial lysozyme concentration was measurable after 3 days incubation in the horizontal test tube position (Figure 38). In comparison to the previous results this meant a slightly more than twofold increase of the loss of soluble lysozyme (17 % vs. 7 %), which was in agreement with the higher amount (100 mg vs. 50 mg) and with the type of

the used PLGA (RG 502H vs. RG 503H). RG 502H has a lower molecular weight than RG 503H and thus, it has an increased amount of carboxylic acid groups per mass unit.

The lysozyme concentration decreased within 14 days to 57 % for the horizontally and 76 % for the vertically stored samples. The difference between the incubation methods could be attributed to the additional stress due to the enhanced agitation stress in the horizontal test tube position. However, the difference was mainly due to one of the three samples. The continuous decrease of the lysozyme concentration in that time period was likely caused by adsorption of lysozyme to PLGA. The progression of the adsorption up to day 14 was probably caused by the increasing number of free carboxylic acid groups due to the degradation of PLGA. This was in accordance with an increased adsorption of lysozyme to partially degraded PLGA microparticles reported previously (Nam et al., 2000). Interestingly, the lysozyme concentration in the buffer stabilized and even increased again to 80 % for the horizontally and 96 % for the vertically stored samples. This indicated that adsorbed lysozyme could be resolubilized upon degradation of the absorbing polymer into soluble oligomers and monomers.

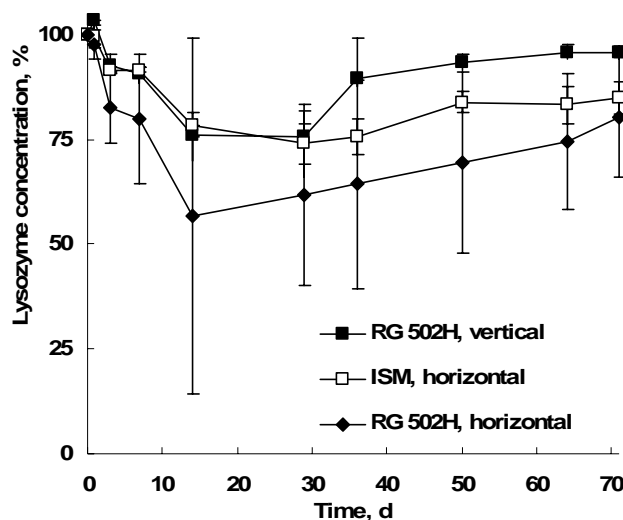


Fig. 38: Relative absorbance (Coomassie plus assay) of lysozyme solutions (500 $\mu\text{g/ml}$) in 33 mM acetate buffer pH 5 containing 0.01 % sodium azide upon incubation in presence of 100 mg RG 502H or 0.5 g of a blank in situ microparticle emulsion (30 % RG 502H in DMSO emulsified with sesame oil, phase ratio 1:1) as a function of test tube position (80 rpm agitation, 37°C)

HPLC analysis and the activity assay after 71 days incubation (Table 9) supported that the increase of the concentration measured with the Coomassie plus assay was not be caused by denaturation and lysozyme hydrolysis like seen in 0.1 N HCl (Figure 37). A fair correlation of the three independent assays was obtained. All three quantification methods confirmed the better recovery of lysozyme from the vertically stored test tubes, which pointed to a contribution of agitation-induced aggregation. However, no sign for aggregation was detected with electrophoresis in the horizontally stored samples (Figure 39, Gel 2, Lanes 3-6).

Tab. 9: Incubation of 500 µg/ml lysozyme solutions in 33 mM acetate buffer pH 5 containing 0.01 % sodium azide at 37°C for 71 days in dependence of the test tube position. Relative values related to initial concentrations (Coomassie plus assay at 0 d).

Condition variable(s)	Coomassie recovery, %	HPLC, recovery, %	Activity, %
RG 502H, horizontal	80.4 (14.3)	91.0 (37.1)	78.5 (27.9)
RG 502H, vertical	95.7 (1.7)	103.4 (13.0)	108.6 (5.2)
ISM (RG 502H), horizontal	84.8 (3.9)	80.9 (2.5)	84.9 (1.3)
RG 502, horizontal	71.9 (12.2)	84.0 (20.1)	85.9 (11.7)
RG 502, vertical	91.0 (0.5)	100.3 (11.1)	106.5 (4.2)
Sesame oil, horizontal	90.6 (6.1)	75.2 (3.0)	91.1 (19.4)
Sesame oil, vertical	95.4 (4.8)	100.3 (12.1)	100.2 (5.0)

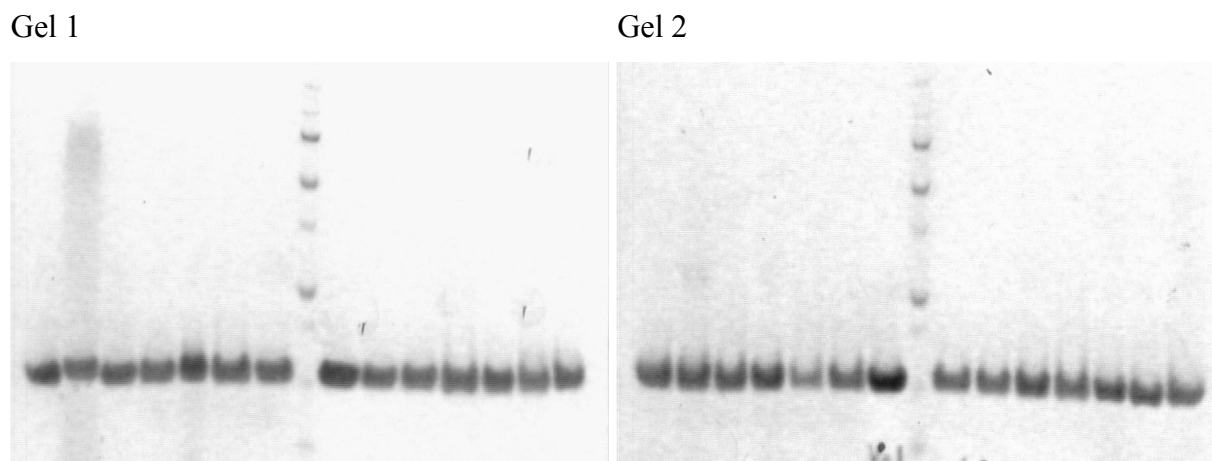


Fig. 39: Non-reducing (native) PAGE of lysozyme solutions after 71 days of incubation. Gel 1: Lanes: 1-3) sesame oil, vertical, 4-6) RG 502H, vertical; 7) precipitate of RG 502H, vertical (sample 1); 8) molecular weight marker (10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa); 9) standard (822 $\mu\text{g/ml}$); 10-12) RG 502, vertical; 13) precipitate of RG 502, vertical (sample 1); 14 and 15) ISM, horizontal (samples 1 and 2); Gel 2: Lanes: 1) ISM, horizontal (samples 3) 2) precipitate of ISM, horizontal (sample 1); 3-5) RG 502H, horizontal; 6) precipitate of RG 502H, horizontal (sample 1); 7) standard (822 $\mu\text{g/ml}$); 8) molecular weight marker (10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa); 9-11) RG 502, horizontal; 12) precipitate of RG 502, horizontal (sample 1); 13-15) sesame oil, horizontal

The decrease of the concentration of soluble lysozyme over the first 14 days was less pronounced when the protein solutions were incubated in presence of a blank ISM formulation (250 mg of a 30 % RG 502H solution in DMSO emulsified with 250 mg sesame oil) compared to incubation with RG 502H (Figure 38). However, the amount of PLGA in the ISM (75 mg) was lower. Neither the recovery nor the activity of lysozyme was differentiable between incubation with ISM or PLGA only after 71 days incubation (Table 9). A very slight dimer band was seen upon electrophoresis with a precipitate remaining in the ISM sample (Figure 39, Gel 2, Lane 2). There was no indication of a lysozyme dimer in the ISM samples although a dimer could be expected to be water-soluble (Figure 39, Gel 1, Lanes 14 and 15 and Gel 2, Lane 1). The overall lack of multimer bands could be due to the low amount of formed aggregates in all samples and the

limited resolvability of lost material with anionic surfactant (Table 7), which is essential for electrophoresis (lithium dodecyl sulfate PAGE).

End-capped or esterified PLGA (RG 502) was used in order to further investigate the adsorption of lysozyme to PLGA. Initially, this PLGA-type would not possess electrostatic charges, whereas during hydrolysis of the polyester free carboxylic acid groups are formed. The decrease of the lysozyme concentration in presence of capped was slower than with the uncapped polymer (Figure 40 vs. Figure 38), which was in agreement with a slower degradation of capped PLGA of comparable molecular weight (Tracy et al., 1999). Interestingly, the concentration of lysozyme was almost unaffected throughout the incubation period in the vertical test tube position, although protein adsorption was expected to occur upon the formation of free carboxylic acids during polymer hydrolysis. All analytical assays confirmed the retention of the lysozyme concentration in vertical and the decrease of the concentration in the horizontal test tube position during incubation (Table 9). Whether the solution conditions (pH or polymer degradants) or agitation-induced aggregation led to an unexpected adsorption behavior was not further investigated. Only monomeric lysozyme was found on electrophoresis gels (Figure 39, Gel 1, Lanes 10-13 and Gel 2, Lanes 9-12).

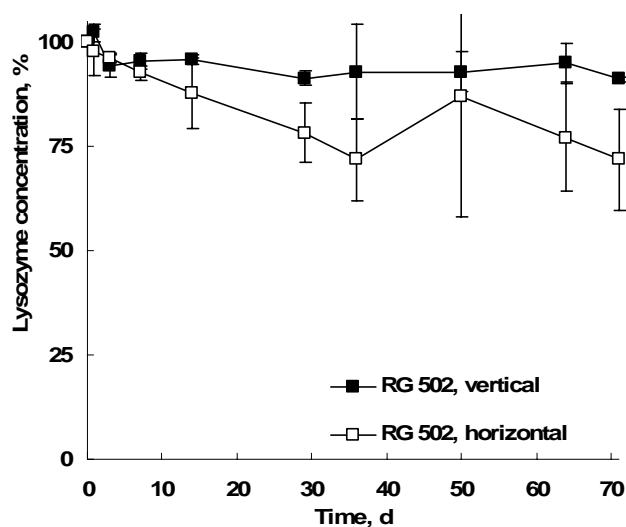


Fig. 40: Relative absorbance (Coomassie plus assay) of lysozyme solutions in 33 mM acetate buffer pH 5 in presence of 100 mg RG 502 over incubation time as a function of test tube position (500 μ g/ml lysozyme containing 0.01 % sodium azide, 80 rpm agitation, 37°C)

Independent of the test tube position, the lysozyme concentrations appeared to slightly decrease within the first 7 days upon incubation with sesame oil (Figure 41). Thereafter, the concentrations were fairly stable at about 90-95 %. HPLC analysis and the activity assay, however, indicated a slightly higher recovery and full biological activity for the samples incubated in vertical position (Table 9). This was in agreement with the minor effect of the sesame oil-water interface on lysozyme obtained previously (Figure 28).

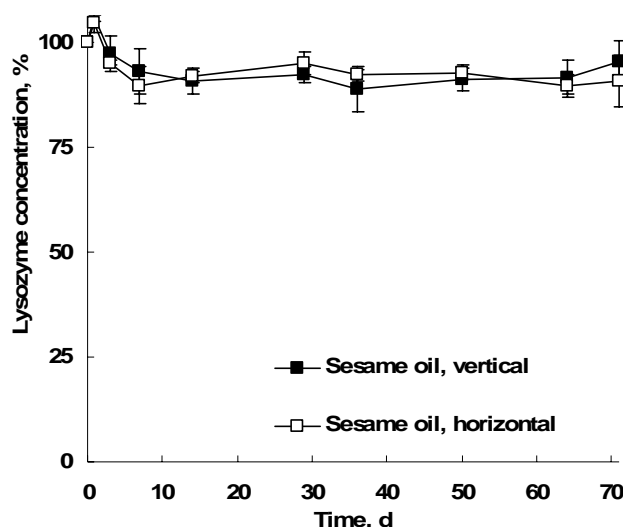


Fig. 41: Relative absorbance (Coomassie plus assay) of lysozyme solutions in 33 mM acetate buffer pH 5 in presence of 300 mg sesame oil over incubation time as a function of test tube position (500 $\mu\text{g}/\text{ml}$ lysozyme containing 0.01 % sodium azide, 80 rpm agitation, 37°C)

It could be concluded that lysozyme could be completely recovered after 71 days without loss of biological activity when the solutions in 33 mM acetate buffer pH 5 were incubated in vertical position.

3.1.2. Polymer degradation

Protein release from dosage forms based on PLGA is controlled by diffusion through water-filled pores and erosion of the polymeric matrix (paragraph 1.2.2.2.3.). The polyester can only start to erode, when the hydrolytic degradation passed the point where soluble and thus

diffusible oligomers occur. The degradation process of PLGA can be understood as a diffusion-reaction mechanism, which is controlled by the hydrolysis rate constants of the ester bonds, the diffusion coefficient of water within the polymer matrix, the diffusion coefficients of degradation products within the polymeric matrix and their solubility. All other factors, such as temperature, additives, pH, buffering capacity, morphology / porosity of the polymer matrix, affect the degradation behavior of the polyester through effects on the four main factors (Vert et al., 1997). In order to get release data which would be also meaningful for other proteins, the alignment of the in vitro conditions to the stability characteristics of lysozyme were evaluated for potential effects on the hydrolysis of the release-controlling polymeric carrier.

3.1.2.1. Influence of buffer (ion species, ionic strength and pH)

The stability of PLGA was investigated in acetate buffer pH 5 and in tris buffer pH 7 containing 0.5 M potassium chloride. The acetate buffer showed the lowest amount of lysozyme adsorption to the PLGA surface, whereas the tris-buffer was investigated as alternative system offering a neutral pH. Additionally, tris-buffer without potassium chloride was used in order to resolve the effect of the salt addition on polymer hydrolysis.

The degradation of RG 502H (weight average molecular weight, $M_w = 10850$ g/mol and number average molecular weight, $M_n = 5611$ g/mol) in all three buffers was comparable (Figure 42). Typical pseudo-first-order degradation patterns were obtained, which could be seen from the linear decay between 0 and 21 days in the corresponding semi-log plots (Figure 43). The decrease of M_n was less pronounced compared to M_w . Thus, the polydispersity (PD), which is the ratio M_w / M_n and is commonly used to describe the molecular weight distribution of the polymer, decreased (Figure 44). The addition of 0.5 M potassium chloride to the tris-buffer did not affect the degradation profiles of RG 502H. This was in agreement with a previous report, where sodium chloride addition up to 0.6 M did not impact the degradation behavior of a comparable PLGA grade (Schmitt et al., 1994). The degradation of the polyester was independent of the nominal medium pH. This was different to previous reports, which revealed a degradation rate minimum for PLA at about pH 4 - 4.5 (de Jong et al., 2001 and Schliecker et al., 2003a). Below the stability optimum the degradation rate increased with the concentration of protons, whereas at pH-values above 4-4.5 the rate increased with the hydroxide ion concentration.

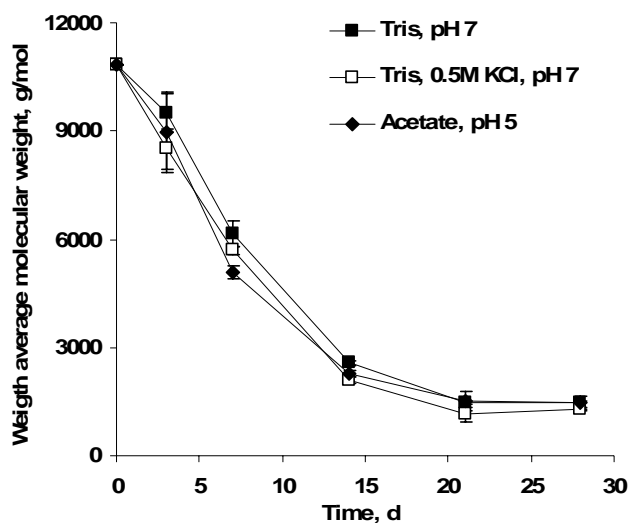


Fig. 42: Weight average molecular weight (M_w) of RG 502H as a function of time in dependence of the release medium (33 mM buffers, tris buffers containing 0.01 % sodium azide, 35 mg RG 502H, no buffer change)

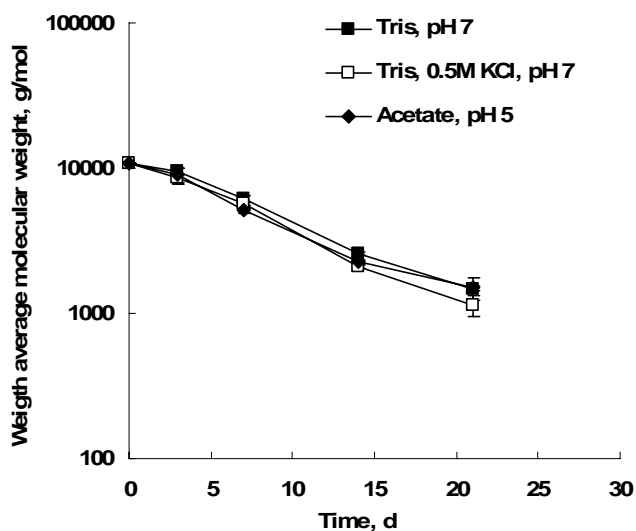


Fig. 43: Semi-log plot of the weight average molecular weight (M_w) of RG 502H as a function of time in dependence of the release medium (33 mM buffers, tris buffers containing 0.01 % sodium azide, 35 mg RG 502H, no buffer change)

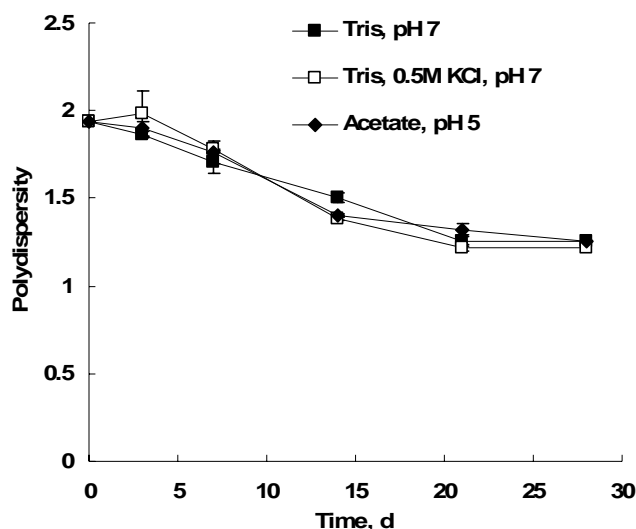


Fig. 44: Polydispersity of RG 502H as a function of time in dependence of the release medium (35 mg RG 502H, no buffer change)

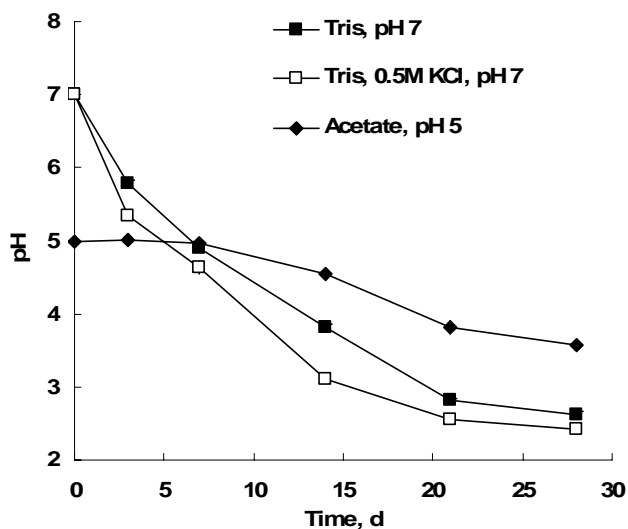


Fig. 45: pH of medium upon incubation of RG 502H as a function of time in dependence of the buffer type, pH and ionic strength (35 mg RG 502H per vial, no buffer change)

This could be explained with the actual pH conditions in the medium. The pH decreased in the tris-buffers from pH 7 to 2.5 within 28 days, whereas it just decreased from 5 to 3.6 in the acetate buffer (Figure 45). Using the arithmetic mean \pm standard deviation as an evaluation tool, the actual pH-values, measured during the incubation period, were comparable for all buffers

(acetate: 4.5 +/- 0.6, tris: 4.5 +/- 1.7 and tris / KCl: 4.2 +/- 1.8). The higher standard deviations of the average pH of the tris buffers expressed the higher pH decrease. The lower robustness of the pH in the tris-buffer media was due to lower actual buffer strengths in comparison to the acetate buffer. Although all three buffers had a nominal strength of 33 mM, the medium pH in relation to the pKa value of the buffer compound led to different actual buffer strengths. Acetate buffer pH 5 is 0.25 pH units above its pKa (4.75). Thus, this buffer pH was slightly more robust to acid than to base addition. Oppositely, tris has a pKa of 8.06. Thus, a pH 7 tris buffer, where the ratio of [tris-H⁺] to [tris-base] is about 11.5:1, would be easily exhausted upon further addition of acid and is therefore unsuitable as a pH 7 buffer.

Thus, it is very important to consider the real medium conditions during incubation (actual strength of a buffer and the actual pH) in order to compare PLGA (PLA) degradation behaviors as a function of the medium pH.

3.1.2.2. Medium exchange

Although a perfectly buffered in vitro release medium might not correspond to the actual in vivo conditions (Heidemann et al., 2002), a possibility to reduce changes of the pH in insufficiently buffered media is the utilization of large buffer volumes (Park et al., 1995b) or the repeated exchange of the incubation medium. In order to evaluate the effect of medium exchange on the degradation of PLGA the 33 mM tris-buffers were studied because of their lower actual buffer strength.

The exchange of the tris-buffer without potassium chloride decreased the degradation rate of PLGA slightly (Figure 46), which was in agreement with an increase of the pH-minimum from pH 2.6 in the unchanged (Figure 45) to pH 3.4 in the exchanged samples (Figure 47). However, the pH-minimum in the 0.5 M potassium chloride containing tris-buffer was also elevated from pH 2.4 (Figure 45) to pH 3.0 (Figure 47) through repeated exchange of the medium but no change of the degradation behavior of PLGA occurred (Figure 48).

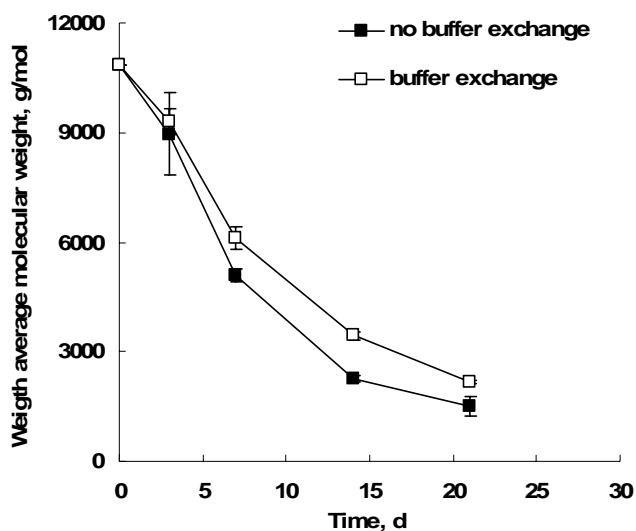


Fig. 46: Weight average molecular weight (M_w) of RG 502H as a function of time in dependence of buffer exchange for 33 mM tris buffer pH 7 containing 0.01 % sodium azide as preservative. The weight of PLGA per vial was 35 mg for the unchanged and 75 mg for the changed buffers.

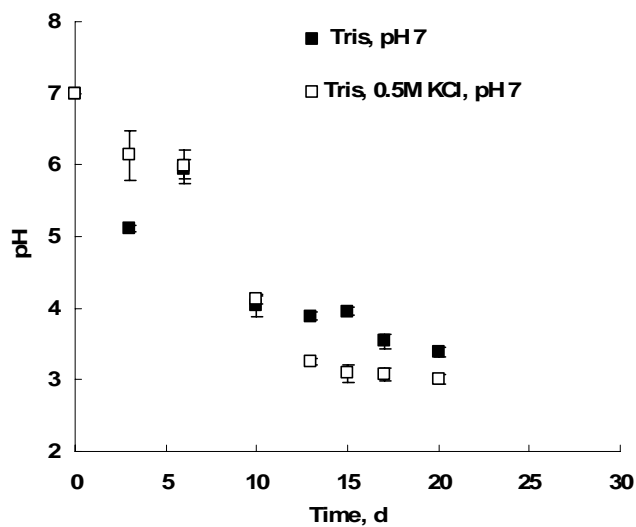


Fig. 47: pH of medium exchanged at days 3, 6, 10, 13, 15 and 17 of the incubation of RG 502H as a function of time and dependence of potassium chloride addition (75 mg polymer per vial)

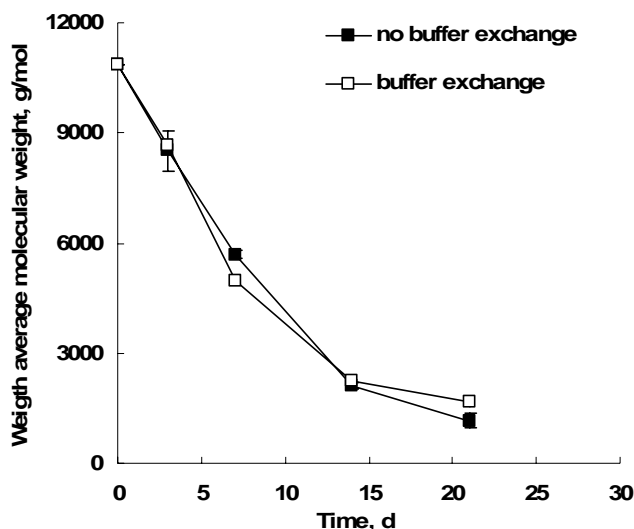


Fig. 48: Weight average molecular weight (M_w) of RG 502H as a function of time in dependence of buffer exchange for 33 mM pH 7 tris buffer with 0.5 KCl, containing 0.01 % sodium azide. The initial weight of PLGA was 35 mg for the unchanged and 75 mg for the changed buffers.

Correspondingly, the comparison of the exchanged samples revealed a slightly slower PLGA degradation in the tris-buffer without potassium chloride (Figures 49 and 50), whereas the polydispersity appeared to be less affected (Figure 51). An accelerated degradation at high ionic strength was previously attributed to a higher solubility of PLGA in presence of sodium ions (ionic strength > 0.154) (Makino et al., 1986). Competition of protons and metal ions for carboxylate groups would correlate with the lower pH-values in potassium chloride-containing tris-buffer compared to the buffer without salt (Figure 47). However, different pH-values were also observed in the unchanged buffers (Figure 45), where no difference between PLGA degradation in tris- and tris / KCl-buffer was observed. Furthermore, a slower PLGA degradation in a saturated (~ 6 M) sodium chloride solution was previously reported (Dong et al., 2006), which would also contradict the explanation of Makino. Thus, the reason for the observation remained unclear.

Despite the small differences found in the exchanged media, the most important criterion was the correlation of the in vitro with in vivo degradation rates of PLGA. The obtained degradation rates for all buffers (exchanged: -0.08 to -0.09 d^{-1} , unchanged: -0.10 to -0.11 d^{-1}) were in between in

vitro ($-0.05 \pm 0.01 \text{ d}^{-1}$) and in vivo ($-0.13 \pm 0.05 \text{ d}^{-1}$) degradation rates reported for RG 502H, previously (Tracy et al., 1999).

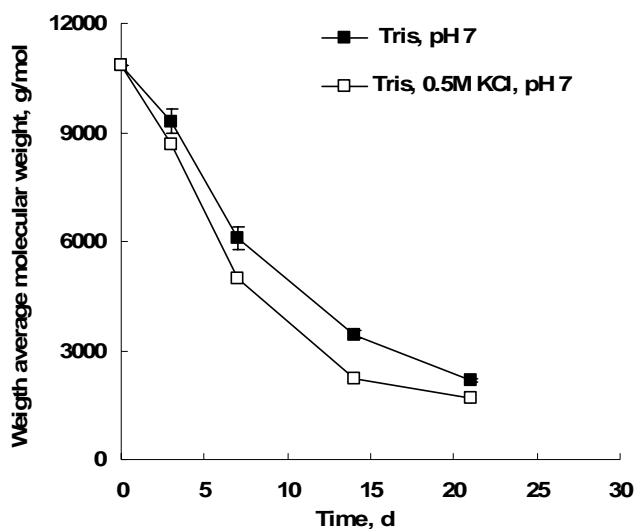


Fig. 49: Weight average molecular weight (M_w) of RG 502H as a function of time in dependence of potassium chloride addition to 33 mM tris-buffer pH7 containing 0.01 % sodium azide (75 mg polymer per vial, buffer exchange)

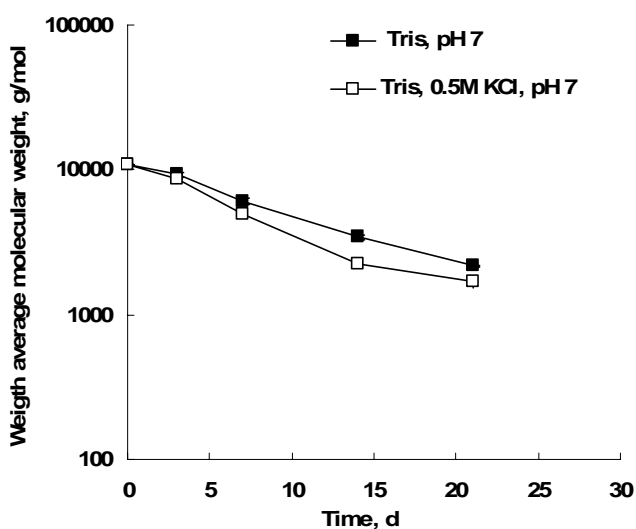


Fig. 50: Semi-log plot of the weight average molecular weight (M_w) of RG 502H as a function of time in dependence of potassium chloride addition to 33 mM tris-buffer pH7 containing 0.01 % sodium azide (75 mg polymer per vial, buffer exchange)

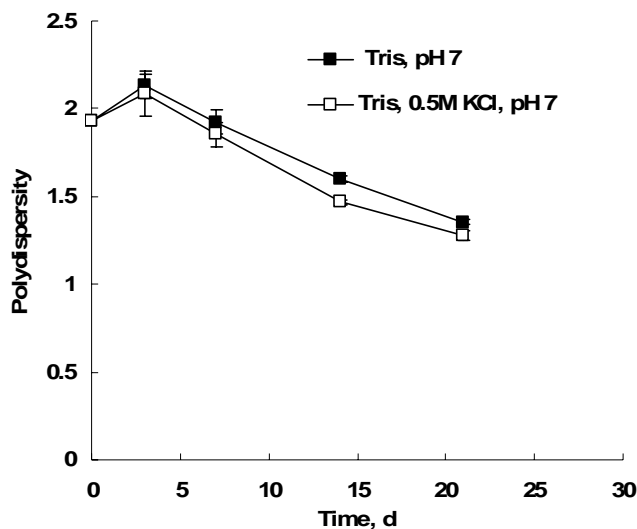


Fig. 51: Polydispersity of RG 502H as a function of time in dependence of potassium chloride addition to 33 mM tris-buffer pH7 containing 0.01 % sodium azide (75 mg polymer per vial, buffer exchange)

3.1.3. Protein separation method

A separation / extraction method for proteins should be able to fully recover the active compound from a dosage form. The critical point with proteins is to avoid deleterious extraction conditions, which lead to irrecoverable drug fractions or affect the quality (biological activity) of the protein and could thus lead to misleading conclusions.

3.1.3.1. Alkaline drug extraction

In order to evaluate the solution stability of lysozyme under the extraction conditions, the protein was first incubated in 0.5 M sodium hydroxide containing 0.1 % SDS (400 $\mu\text{g}/\text{ml}$). The initial absorbance decreased only slightly to 92 \pm 2 % during 2 days incubation (Figure 52), which indicated that the formation of aggregates was only a minor problem under this condition.

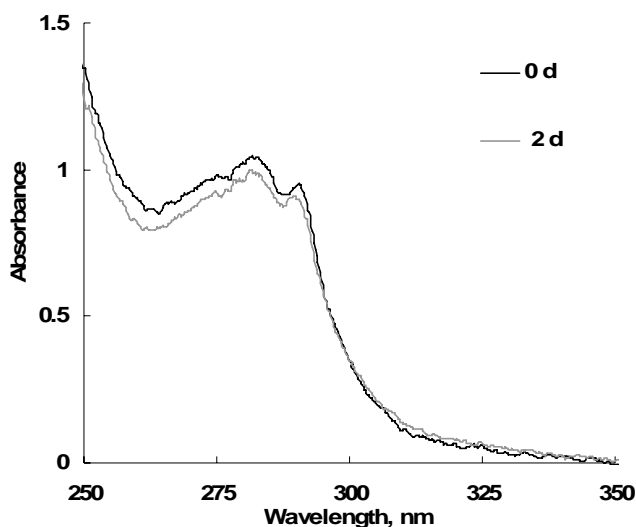
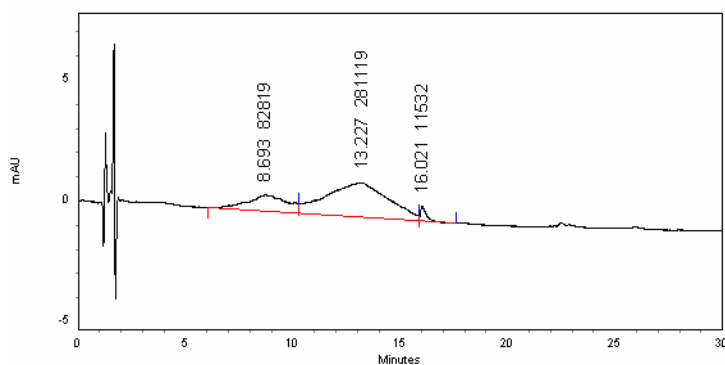


Fig. 52: UV-spectra of lysozyme in 0.5 M sodium hydroxide solution containing 0.1 % sodium dodecylsulfate before and after 2 days incubation (400 $\mu\text{g/ml}$ lysozyme, 80 rpm agitation, 37°C). The spectrum at 2 days was representative for three individual samples.

An alkaline extraction of lysozyme-containing PLGA solutions (30 % RG502H) based on the water-miscible polymer solvent 2-pyrrolidone resulted in acceptable protein recoveries, independent of how the drug was incorporated into the formulation (polymer dissolved in the drug / solvent dispersion: 99.5 \pm 1.1 % or drug dispersed in the polymer solution: 96.9 \pm 7.2 %). This was in agreement with a more or less quantitative recovery reported previously for protein-containing biodegradable microparticles (Sharif and O'Hagan, 1995).

However, a qualitative analysis of the neutralized samples (pH adjusted to \sim 7 with 0.5 N HCl / 0.1 % SDS) with HPLC was impeded due to a significant shift of the retention times and a peak broadening in the chromatograms of SDS-containing samples compared to aqueous lysozyme solutions (Figure 53 vs. Figure 35a). Furthermore, the denaturation of lysozyme by SDS-solubilization impeded a determination of the biological activity of the recovered material (paragraphs 1.1.2.2.2. and 3.1.1.1.).

a)



b)

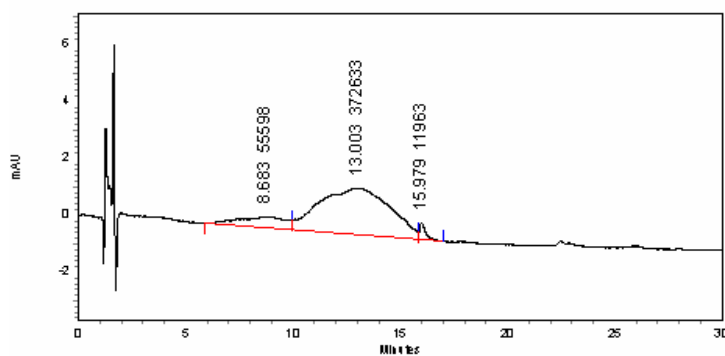


Fig. 53: HPLC chromatograms of a) filtrated lysozyme solution in 0.5 N NaOH / 0.5 N HCl 1:1 containing 0.1 % SDS and b) lysozyme extracted from 30 % RG502H in 2-pyrrolidone dissolved in 0.5 N NaOH / 0.5 N HCl 1:1 containing 0.1 % SDS

The alkaline extraction procedure was also investigated with a PLGA (30 % RG 502H) solution based on the water-immiscible solvent triacetin. A viscous polymer mass remained at the bottom of the vial during 3 days incubation. This indicated an increased resistance of the polymer to alkaline hydrolysis, when shielded by the hydrophobic solvent. Additionally, a precipitation was observed in drug-containing samples but not in the blanks, which pointed to lysozyme aggregation.

It was concluded that the alkaline extraction would facilitate the quantitative analysis of protein contents in dry microparticles or polymer solutions (in situ implants) based on water-miscible solvent. However, limitations regarding the qualitative analysis of protein contents or the extraction of in situ implants based on water-immiscible solvents led to the evaluation of an alternative methodology.

3.1.3.2. Recovery with organic solvents

The shortcomings of the alkaline extraction procedure and the incomplete recovery reported for the protein partitioning method (methylene chloride / water interface, Sharif and O'Hagan, 1995) suggested the development of a more suitable separation procedure. A repeated washing with a protein non-solvent was previously used to separate BSA from PLGA microparticles (Castellanos et al., 2001). Accordingly, lysozyme was intended to be separated from other ingredients in in situ formulations. A protein non-solvent had to be selected, which could dissolve PLGA, would be miscible with PLGA solvents and with the external oil phase of oil-in-oil in situ forming microparticles. Preferentially, the non-solvent should be environmentally friendly and volatile to facilitate the complete removal of the solvent prior to the re-dissolution of lysozyme upon water or buffer addition.

3.1.3.2.1. Lysozyme solubility in organic solvents

Proteins usually have a limited solubility in non-aqueous media. Among the solvents, which could be used to dissolve PLGA for formulate in situ forming delivery systems or extract the drug, the highest solubility of lysozyme was reported for DMSO (Chin et al., 1994: > 10 mg/ml and Houen, 1996: > 100 mg/ml). However, the solubility data for proteins in organic solvents is rare (Stevenson, 2000) and depends on experimental parameters such as the protein net charge, which is a function of the isoelectric point and the solution pH prior lyophilization. An increasing net charge was reported to result in an increased solubility (Bromberg and Klibanov, 1995)

Therefore, the solubility of lysozyme in dimethyl sulfoxide (DMSO), polyethylene glycol 400 (PEG 400), 2-pyrrolidone (2P), N-methyl-2-pyrrolidone (NMP), glycofurol (GF), propylene carbonate (PC), ethyl acetate (EA), triacetin (TA) and benzyl alcohol (BA) was determined at room temperature. Acetate buffer (pH 5) was added to the samples after 18 hours or 7 days equilibration in order to obtain aqueous samples for HPLC analysis. The first five solvents are completely water-miscible, whereas the latter four showed miscibilities with water of 22 (PC), 7.7 (EA), 6.2 (TA) and 4 % (BA). Thus, monophasic or biphasic systems formed upon addition of buffer (mass ratio 1:1). Lysozyme (~30 mg / 3 g solvent) was completely dissolved in DMSO and 2P after 7 days (Table 10). The amount of protein dissolved in 2P increased between 18 hours and

7 days incubation, which was attributed to a retarded dissolution process. In contrast, a decrease of the solubility in this period was obtained with the NMP-samples. Lysozyme partially precipitated upon dilution of the filtrated NMP solutions with acetate buffer, which was not observed with the protein solutions in DMSO and 2P. A very small amount of lysozyme could be dissolved in PEG 400, whereas no protein was detectable in GF. After an extraction time of 16 hours, almost no protein could be detected in the aqueous phase of the biphasic systems formed with the water-immiscible solvents. In PC and benzyl alcohol, lysozyme appeared to be soluble to some extent. Although no lysozyme was measurable in the aqueous phase of the benzyl alcohol / buffer system, precipitated protein was observed at the interface between the buffer and benzyl alcohol. No lysozyme was detected upon extraction of ethyl acetate and triacetin. UV-spectra of the centrifuged protein dispersions in the organic solvents also suggested very low solubilities in these solvents. However, inconspicuous losses at the interface, which would lead to an underestimation of the protein solubility, could not be certainly excluded.

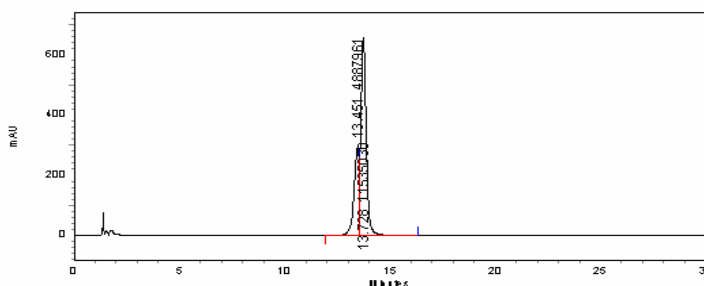
Tab. 10: Amount of lysozyme dissolved in PLGA solvents after 18 hours or 7 days equilibration under agitation (100 strokes/min in horizontal shaker) at room temperature quantified with HPLC upon filtration of drug dispersions (0.45 μ m nylon) and dilution or extraction for 16 hours with 33 mM sodium acetate buffer pH 5 containing 0.01 % sodium azide (mass ratio 1:1) (undiluted injection at 7d for PEG 400 and GF)

Solvent	Lysozyme dissolved, mg/g	
	18 h	7 d
Dimethyl sulfoxide (DMSO)	n.d.	> 11.84
2-Pyrrolidone (2P)	1.74	> 9.89
N-Methyl-2-pyrrolidone (NMP)*	1.71	0.65
Polyethylene glycol 400 (PEG 400)	0.00	0.01
Glycofurol (GF)	0.00	0.00
Propylene carbonate (PC)	0.01	0.01
Ethyl acetate (EA)	0.00	0.00
Triacetin (TA)	0.00	0.00
Benzyl alcohol (BA)*	0.00	0.00

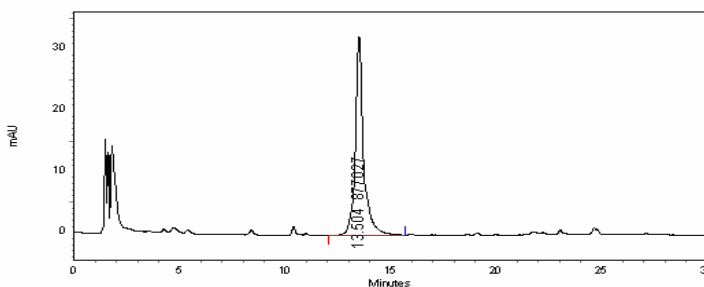
*Precipitation observed upon contact with acetate-buffer; n.d.: not determined

The HPLC chromatograms of the diluted incubated samples in 2P or NMP featured a decrease in the peak ratio (peak area of peaks at retention times 13.7 min/13.5 min), as observed with stored lysozyme solutions in acetate buffer (paragraph 3.1.1.3.). The ratio decreased to 2.8 at 18 hours and 2.5 at 7 days (Figure 54a) in 2P. In NMP the originally smaller peak at 13.5 min dominated already after 18 hours incubation (peak ratio 0.13), whereas after 7 days a single peak at 13.5 min appeared (Figure 54b). Small additional peaks occurred in the DMSO sample just before 13.5 min (Figure 54c). However, a peak ratio of 15.2 was comparable to freshly prepared aqueous solutions of lysozyme.

a)



b)



c)

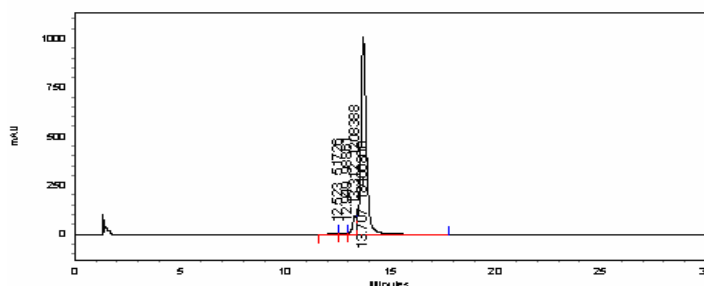


Fig. 54: HPLC chromatograms in aqueous dilutions (1:1) of a) 2-pyrrolidone, b) N-methyl-2-pyrrolidone and c) DMSO solutions of lysozyme after 7 days equilibration.

Overall, the solubility of lysozyme was in the sequence DMSO \geq 2P > NMP \gg PC, PEG 400 \geq TA, EA. The solubility in BA was not further investigated.

3.1.3.2.2. Miscibility of solvents

The miscibility of solvents was investigated, which allowed selecting a suitable solvent to separate of lysozyme from other formulation ingredients. Therefore, the solvent should be miscible with the additives to be extracted from the formulations. The miscibilities / solubilities of selected PLGA solvents with water and various oils were determined through titration under agitation with visual endpoint examination. Generally, water-miscible solvents (Table 11, upper part) showed limited miscibility with the oil phases except for NMP, which was completely miscible with all tested liquids. Ethyl acetate, benzyl alcohol and benzyl alcohol were completely miscible with the oils but not with water. All other solvents showed limited or no solubility in the oils, except for castor oil. The solubility of DMSO appeared to be affected by the water content in the solvent. Already small amounts of water appeared to decrease the miscibility of DMSO with the oil phases. From the perspective of the oils, castor oil showed a unique behavior among the triglycerides, which was due to the relatively hydrophilic nature of the predominant fatty acid, ricinoleic acid (12-hydroxy-9-octadecenoic acid). Similar miscibilities were obtained for peanut, safflower seed and soybean oil, which are triglyceride mixtures with comparable compositions of fatty acids (mainly C18:2 and C18:1). Equal or slightly higher solvent uptake was obtained with the medium chain triglycerides (MCT or Miglyol 812), which are triesters of octanoic and decanoic acid.

Although NMP and benzyl alcohol were broadly miscible with the oils, they could not be used to wash-off polymer and oil (water) from in situ formulations due to the solubility of lysozyme in these solvents. Although it was not tested whether lysozyme was soluble in benzyl benzoate, it had a much higher boiling point compared to ethyl acetate (323°C vs. 77°C). This would hamper the evaporative removal of the solvent after the washing. Additionally, ethyl acetate was completely miscible with DMSO, glycofurol, N-methyl-2-pyrrolidone, 2-pyrrolidone, triacetin, benzyl alcohol and benzyl benzoate. Thus, it was selected as protein non-solvent, which could wash-off the polymer, other solvents and the oil phase from ISM emulsions.

The determined miscibilities also facilitated the selection of appropriate polymer solvent / oil phase combinations to form stable ISM emulsions. A higher miscibility could lead to premature polymer precipitation in the syringe prior administration.

Tab. 11: Miscibilities / solubilities of polymer solvents in water or oily phases (fatty acid ester).

Polymer solvent	Water %	Ethyl oleate %	Triglycerides				
			MCT 812 %	Castor oil %	Peanut oil %	Safflower oil %	Soy bean oil %
PEG 400	100	< 2	< 1	7	< 2	< 1	< 2
DMSO							
0.49 % water cont.	100	< 2	< 2	100	2	< 2	< 2
0.01 % water cont.	100	n.d.	7	100	4	4	n.d.
2-Pyrrolidone	100	3	5	100	7	7	8
Glycofurof	100	13	100	100	12	14	14
N-Methyl-2-pyrrolidone	100	100	100	100	100	100	100
Propylene carbonate	22*	6	17	9	2	4	4
Ethyl acetate	8	100	100	100	100	100	100
Triethyl citrate	7	100	100	100	20	26	24
Triacetin	6	23	34	7	9	11	11
Benzyl alcohol	4**	100	100	100	100	100	100
Benzyl benzoate	< 1	100	100	100	100	100	100

n.d.: not determined; * from Lexikon der Hilfsstoffe; ** 1996; from Merck Index, 12th edition, 1996

3.1.3.2.3. Lysozyme separation with ethyl acetate

Separation of lysozyme from solutions or dispersions in organic solvents

Proteins have been extensively studied in aqueous but only to a limited extent in non-aqueous media. The dissolution of proteins in organic solvents was associated with a loss of secondary structure (Jackson and Mantsch, 1991 and Knubovets et al., 1999). However lysozyme could restore its native structure (biological activity) after being recovered through dilution of its solution in DMSO with aqueous medium (Chang et al., 1991). It has never been investigated

whether native lysozyme could be recovered from an organic solution using a non-aqueous precipitation step.

Fourier transform infrared spectroscopy (FTIR) was used to identify potential effects of the extraction procedure on lysozyme. The maximum of the amide I band ($1720\text{-}1580\text{ cm}^{-1}$) of lysozyme occurred at a higher wavenumber in DMSO compared to the aqueous protein solution (Figure 55). The shift of the amide I band and a distortion of the amide II ($1580\text{-}1480\text{ cm}^{-1}$) bands indicated denaturation of the protein in DMSO, which was in agreement with the previous reports. In contrast to organic solutions, proteins are kinetically trapped, hence conformationally stable in non-aqueous dispersions (Griebenow and Klibanov, 1996). Accordingly, the conformation of lysozyme dispersed in ethyl acetate was not affected during the extraction procedure as revealed by superimposing FTIR-spectra of extracted, dry protein in comparison with the original lyophilized material (Figure 56).

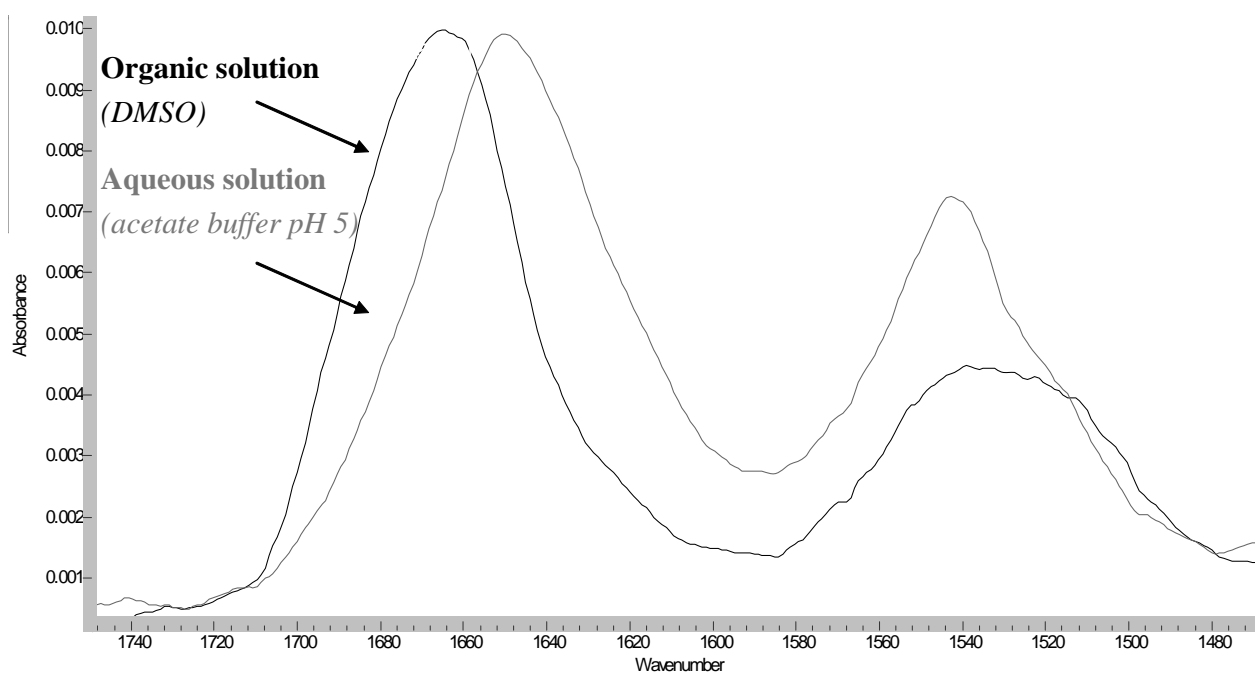


Fig. 55: Amide I and II region of FTIR-spectra of lysozyme in DMSO and in aqueous solution (33 mM acetate buffer pH 5 containing 0.01 % sodium azide)

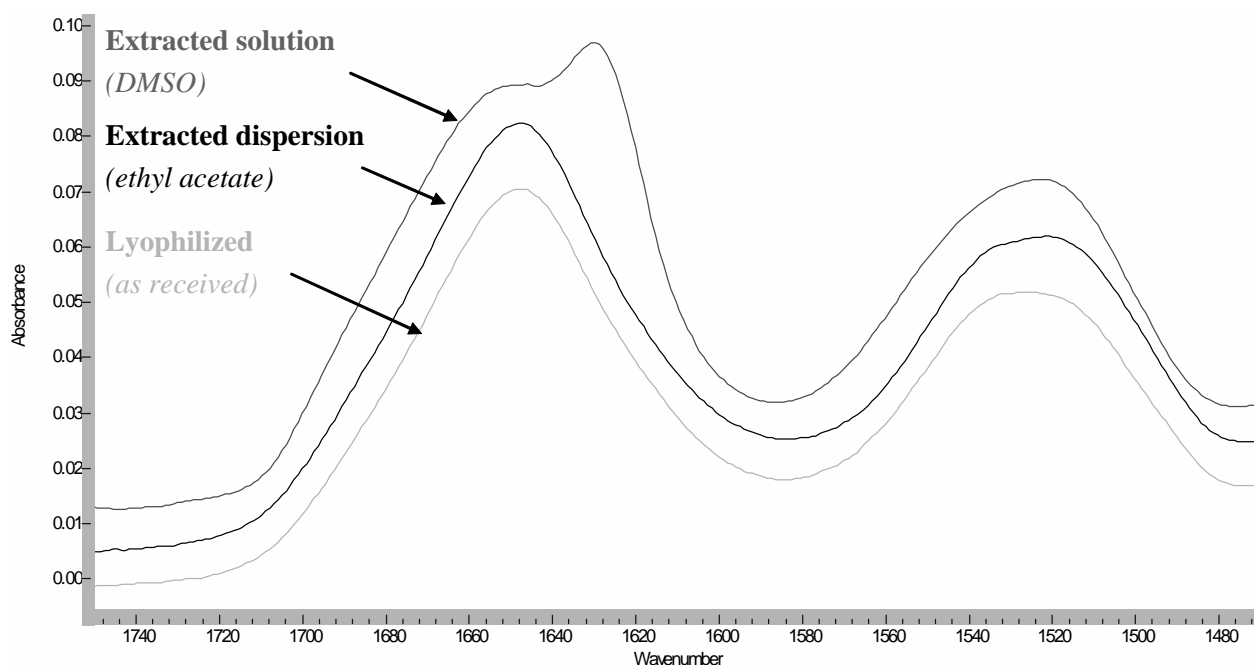


Fig. 56: Amide I and II region of FTIR-spectra of dry lysozyme extracted (triplicate washing with ethyl acetate and 30 min vacuum-drying) from a DMSO solution or an ethyl acetate dispersion in comparison to the excipient-free lyophilized powder

Two other proteins, chicken egg ovalbumin and bovine pancreatic chymotrypsinogen, were previously precipitated from DMSO solutions by ethyl acetate addition (Chang et al., 1991). The quality of the precipitated denatured proteins was not further investigated. The question thus was whether lysozyme would be trapped in the denatured or would restore its native conformation upon desolvatization from DMSO. The FTIR-spectrum of lysozyme extracted from the solution in DMSO showed two interesting features. An additional band at 1628 cm^{-1} appeared which was assigned to an intermolecular β -sheet formation (Jackson and Mantsch, 1991) and reflected protein aggregation. Except for the intermolecular interaction in the extracted protein pellet, the position of the amide I region shifted back from 1665 cm^{-1} in the DMSO solution (Figure 55) to 1647 cm^{-1} (Figure 56) upon precipitation / desolvatization with ethyl acetate. Thus, reversibly aggregated native protein was obtained during extraction of the protein solutions in DMSO.

Investigation of redissolved protein pellets obtained after extraction of lysozyme from various organic solvents revealed that lysozyme could be almost completely recovered and was fully active in most cases (Table 12). Thus, precipitation of lysozyme from non-aqueous solutions appeared to lead to reversible aggregates.

Tab. 12: Total recovery and relative activity (based on HPLC recovery) of lysozyme separated from 1 % protein containing solvent systems (triplicate washing with ethyl acetate, 0.5 min vacuum drying redissolution in acetate buffer)

Lysozyme / solvent system	Total recovery (HPLC), %	Relative activity, %
DMSO solution	95.9 (1.4)	101.3 (2.8)
2-Pyrrolidone solution / dispersion	89.5 (2.2)	105.8 (0.9)
Benzyl alcohol solution / dispersion	87.4 (3.3)	92.4 (2.5)
Benzyl benzoate dispersion	95.6 (0.3)	95.0 (1.7)
Triacetin dispersion	97.7 (0.5)	103.3 (3.9)
Ethyl acetate dispersion	71.4 (39.7)	96.0 (3.5)

The separation method was therefore suitable to extract lysozyme from organic solvents without affecting its conformational stability.

Separation of lysozyme from in situ implants (effect of wetting/drying)

As for the solution in DMSO, the additional band, which reflected lysozyme aggregation, also occurred upon extraction from a 30 % PLGA solution in DMSO (Figure 57). However, the amide I band position in the FTIR-spectrum of lysozyme, which was extracted from the polymer solution, did not superimpose the spectrum of the lyophilized powder as seen with the extracted protein solution in DMSO (Figure 56). This might indicate retention of the denatured conformation during desolvatization, maybe due to an increased amount of residual DMSO.

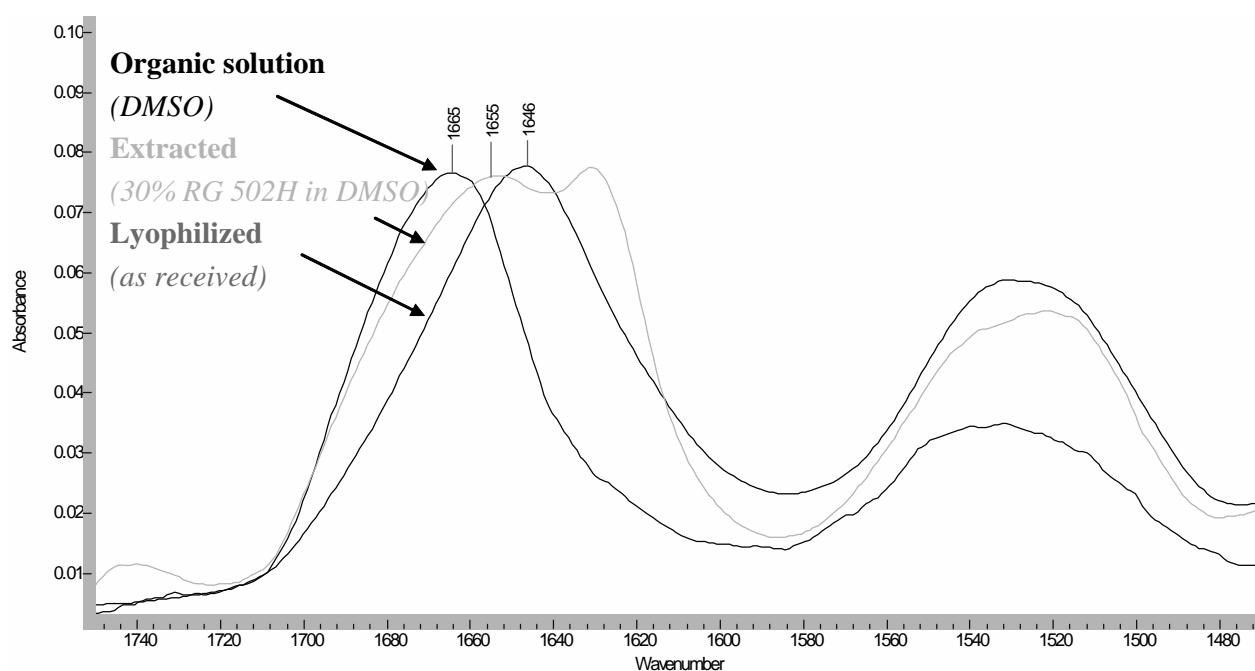


Fig. 57: Amide I and II region of FTIR-spectra of lysozyme dissolved in DMSO or extracted from a 30 % RG 502H solution in DMSO in comparison to the excipient-free lyophilized powder

Further evaluation of lysozyme extracted from the 30 % PLGA solution in DMSO and redissolved in acetate buffer revealed complete recovery of fully active material (Table 13). Full protein recovery was also obtained with a higher concentrated 50 % PLGA solution in ethyl acetate containing dispersed lysozyme. Polyacrylamide gel electrophoresis (PAGE) of both samples confirmed that aggregates, which formed during the extraction of DMSO solutions of the protein, were fully reversible (Figure 58).

Tab. 13: Total recovery and relative activity (based on HPLC recovery) of lysozyme separated from polymer solutions with 4 % drug loading based on PLGA (RG 502H)

In situ implant formulation	Total recovery (HPLC), %	Relative activity, %
30 % PLGA in DMSO	100.6 (9.8)	100.6 (6.3)
50 % PLGA in ethyl acetate	99.9 (3.5)	94.5 (0.6)

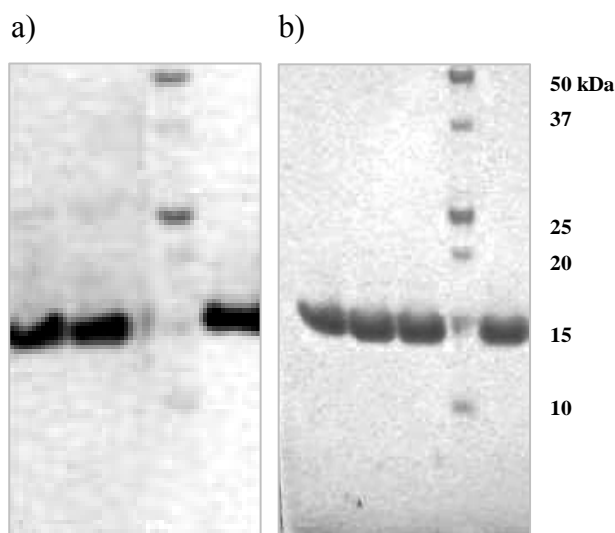


Fig. 58: Native polyacrylamide gel electrophoresis of lysozyme redissolved in acetate buffer (pH 5) after extraction from a) 30 % PLGA (RG 502H) solution in DMSO; Lanes: 1) and 2) extraction samples 1 and 2; 3) marker; 4) lysozyme solution (488 µg/ml) and b) 50 % PLGA (RG 502H) solution in ethyl acetate; Lanes: 1-3) extraction samples 1-3; 4) marker; 5) lysozyme solution (250 µg/ml)

The extraction method with ethyl acetate was also evaluated for its suitability to separate lysozyme from formulations during the release testing. The effect of residual moisture on the extraction result was mimicked by wetting dry lysozyme-containing implants obtained from 50 % RG 502H solutions in ethyl acetate with release medium for 90 min (acetate buffer pH 5). The wetted implants were either extracted directly or after an additional vacuum- or freeze-drying step to remove residual aqueous medium. The resulting recoveries of the dry (wetted) implants were compared to the recovery obtained with the corresponding in situ implant (50 % RG 502H in ethyl acetate containing 4 % lysozyme, based on PLGA).

The analysis of the redissolved extracted protein pellet on the same day revealed decreased total recoveries for the vacuum- and freeze-dried wetted implants (Table 14). The values increased with redissolution time. Complete recovery was obtained after 2 days for the vacuum-dried sample, whereas 2-8 days were needed with the lyophilized sample. Lysozyme could be completely recovered on the day of separation upon extraction of the in situ implants and the solid implants, which were wetted but not additionally dried. The activities of recovered enzyme were comparable between all samples.

Tab. 14: Total recoveries (%) after extraction of in-situ implants (ISI), wetted solid implants, wetted and subsequently vacuum-dried (2 hours at room temperature) or lyophilized (2 days) solid implant in dependence of the incubation time during re-dissolution (4°C). The solid implants were prepared by vacuum-drying the ISI formulation (100 mg of 50 % RG 502H in ethyl acetate with 4 % drug loading, based on polymer) at ambient temperature for 1h. The solid implants were wetted with 0.25 g acetate buffer pH 5 for 90 min before drying or extraction.

Extraction experiment	0d	1d	2d	8d	Activity (rel. to 8d)
In situ implant	93.9 (8.8)	90.5 (6.9)	97.5 (2.4)	99.9 (3.5)	94.5 (0.6)
Wetted implant	99.8 (7.5)	93.6 (9.0)	93.5 (8.2)	97.2 (9.1)	92.9 (1.8)
Vacuum-dried, wetted implant	85.6 (11.2)	93.0 (1.7)	102.3 (1.4)	99.9 (1.5)	93.6 (2.1)
Lyophilized, wetted implant	41.9 (15.4)	71.4 (22.2)	92.5 (10.1)	99.3 (1.1)	93.5 (3.0)

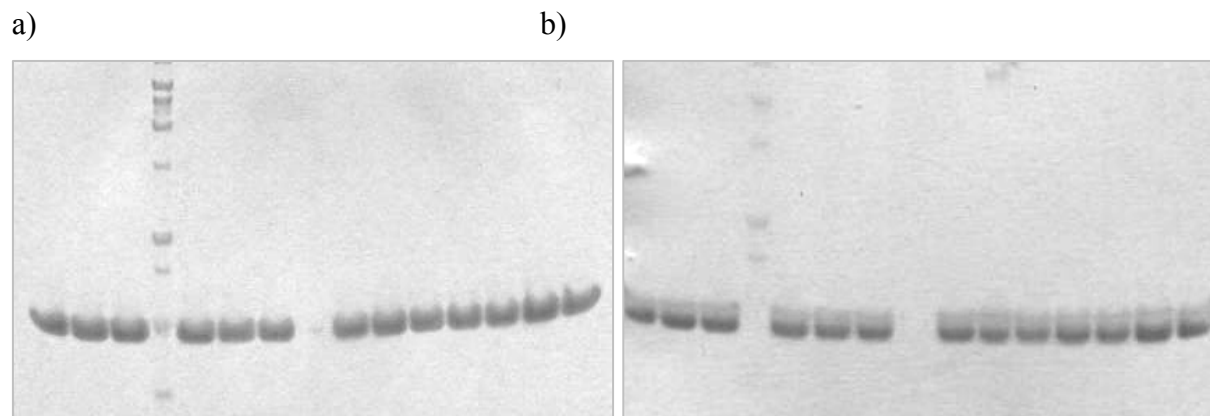


Fig. 59: a) Native and b) reducing polyacrylamide gel electrophoresis of lysozyme after redissolution of the extracted protein pellet in acetate buffer (pH 5) for 8 days; Lanes: 1-3) ISI; 4) molecular weight marker (10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa); 5-7) wetted implant; 8) blank; 9) lysozyme solution (250 µg/g), 10-12) wetted and vacuum dried implant; 13-15) wetted and lyophilized implant

The retarded redissolution of the protein pellet could be attributed to a sustained dissolution of reversible aggregates, which could have formed during the drying step in presence of PLGA.

This would imply that the intermediately formed buffer-ethyl acetate interface in the wetted but undried sample were less deleterious for the integrity of lysozyme than the conditions during the dehydration step. However, no sign for aggregates was found with PAGE after redissolving the protein pellets for 8 days (Figures 59a and b).

Separation from in situ microparticle emulsions

The utilization of ethyl acetate also facilitated the removal of the oil phase and thus the extraction of in situ microparticle emulsions due to the suitable miscibility characteristic (Table 11). Three in situ emulsions differing in the solvent composition (DMSO: protein and PLGA solvent; ethyl acetate: protein nonsolvent / PLGA solvent and water: protein solvent / PLGA nonsolvent) were extracted to evaluate the extraction methodology. Complete recovery of lysozyme was obtained from the ISM emulsions independent of the solvent system and thus of the physical state (dissolved or dispersed) of lysozyme (Table 15).

Tab. 15: Total recovery and relative activity (based on HPLC recovery) of lysozyme separated from in situ microparticle emulsions [0.25 g 30 % PLGA (RG 502H) in solvent system with 4 % drug loading (based on polymer) emulsified into 0.25 g sesame oil, 50 mixing cycles at 1/s]

Solvent system	Total recovery (HPLC), %	Relative activity, %
DMSO (protein solution)	99.2 (10.3)	94.2 (11.1)
DMSO:ethyl acetate 75:25 (protein dispersion)	103.5 (4.3)	88.8 (4.4)
DMSO:ethyl acetate:water 70.5:23.5:6 (protein dispersion)	108.3 (7.9)	90.8 (2.9)

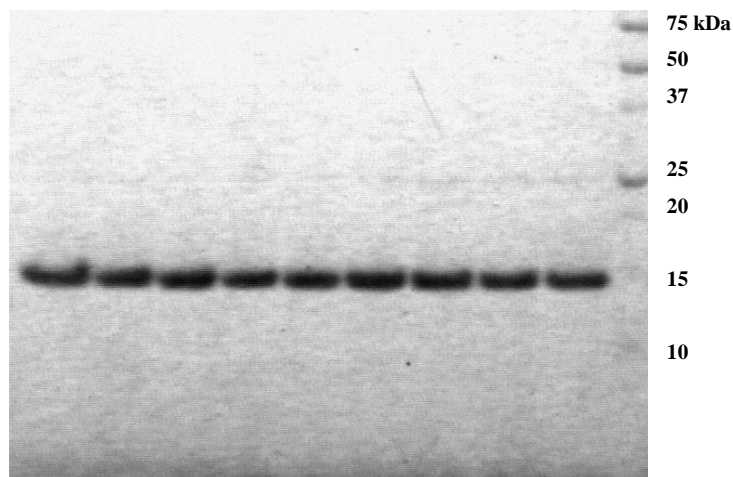


Fig. 60: Native polyacrylamide gel electrophoresis of lysozyme extracted from ISM emulsions based on different solvent systems; Lanes: 1-3) DMSO; 4-6) DMSO:ethyl acetate 75:25; 7-9) DMSO:ethyl acetate:water 70.5:23.5:6; 10)marker

No sign of aggregates occurred in polyacrylamide gel electrophoresis (Figure 60). The extracted protein was monomeric. The full activity of lysozyme extracted from the ISM based on DMSO indicated that the ISM manufacturing process did not cause irreversible changes of the protein. Dissolved proteins are known to denature and aggregate at interfaces. However, the oil / oil interface appeared to be less attractive and deleterious for lysozyme than oil / water interfaces such as methylene chloride / water for example (van de Weert et al., 2000c and Pérez et al., 2002a, 2003). Whether the slight loss of the activity for the ISM-emulsions containing dispersed lysozyme was due to a analytical error or due to preparation induced protein changes was not further investigated. An effect of the oil / oil interface could be excluded since the protein was in the dispersed state and should thus be kinetically trapped. However, it was further elucidated whether the in situ protein precipitation during the preparation of the protein containing polymer solution could affect the protein integrity (upon PLGA / ethyl acetate addition).

Lysozyme appeared to be unaffected by the manufacturing of in situ forming implants / microparticles.

3.2. Formulation parameters of in situ forming drug delivery systems

The formation of microparticles out of in situ microparticle emulsions or of solid implants out of in situ implant formulations is based on the solidification of the polymer (PLGA) solution phase through exchange of the biocompatible organic solvent with water or through leaching of the polymer solvent. The phase separation process determines the morphology of the solidified system and thus the release characteristics. The dynamics of phase inversion is influenced by the affinities between polymer, organic solvent and the precipitating medium (Thomasin et al., 1998). In case of in situ implants, the precipitating medium for the water-insoluble PLGA is the release buffer in the in vitro and body fluids in the in vivo situation. Thus, phase separation is mainly a function of the solvent, the polymer type and its concentration (Strahtmann et al., 1975 and Strathmann and Kock, 1977), although formulation ingredients, such as drug or additives, can also have an effect (Brodbeck et al., 1999a). With in situ forming microparticles, the miscibility of the polymer solvent with the external (oil or water) phase needs to be also considered. Already before the contact with the aqueous media, polymer solvent could diffuse into the external oil or water phase during the preparation of the in situ microparticle emulsions. Thus, the polymer solution could be concentrated or even be precipitated prior to injection.

Formulation and process parameters were investigated for their potential influences on the release of the model protein lysozyme from in situ microparticle emulsions. The focus was put on oil-in-oil systems were investigated due to their potential to decrease the initial release of hydrophilic drugs (Kranz and Bodmeier, 2007). In the simplest case the internal phase of lysozyme-containing in situ microparticle emulsions consists of protein dissolved or dispersed in a solution of a biodegradable polymer (PLGA). The formulation parameters with a potential influence on the release of lysozyme from in situ systems include solvent type (water miscible or immiscible), the polymer type (end-group functionality), the polymer concentration, the drug (loading, dissolved/dispersed state and supplier) and the presence of release modifiers. In situ forming biodegradable microparticles were compared with corresponding in situ implant formulations in order to facilitate identification of advantages and disadvantages.

The choice of the polymer solvent significantly affects the phase separation of polymer solutions, which determines the morphology and finally the drug release from in situ formulations (Brodbeck et al., 1999). The most important characteristic thereby is the affinity of the polymer solvent to the precipitating aqueous medium. The investigations of parameters with a potential

effect on the release were therefore separated according to water-miscibility of the polymer solvent used in the in situ formulations.

3.2.1. In situ systems based on water-immiscible polymer solvent

Glyceryl triacetate (triacetin) was chosen as PLGA solvent with limited water-miscibility (paragraph 3.1.3.2.2., Table 11). The GRAS-listed substance is biocompatible (Hem et al., 1975) and may even have advantageous properties as parenteral nutrient (Bailey et al., 1991). An important question for proteins is the stability under non-aqueous conditions. In order to circumvent protein denaturation during microencapsulation, proteins could be encapsulated in the dispersed state, where they are kinetically trapped (Griebenow and Klibanov, 1996). As protein non-solvent, triacetin could be therefore advantageous.

3.2.1.1. Macroscopic appearance of in situ forming systems

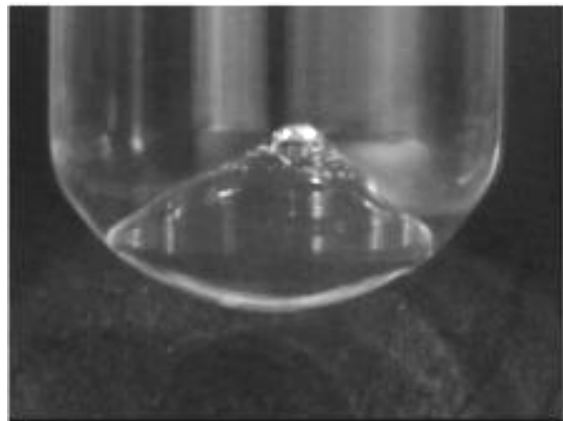
The limited affinity of polymer solution based on water-immiscible solvents, such as triacetin, for the precipitating aqueous medium impedes a rapid phase separation process. Instead, the main driving force for polymer precipitation in triacetin systems was solvent leaching (out-diffusion) with a subsequent polymer concentration increase. Accordingly, the volume of drug-free in situ implants (0.25g, n=3) based on triacetin decreased upon incubation in release medium (Figures 61 and 62). Although the appearance changed from a transparent to a turbid gel within the first day, the highly viscous gel-like consistency did not change, which might be attributed to a slow phase separation occurring at the surface of the implant only (Brodbeck et al., 1999). The 6 % miscibility / solubility of triacetin in water (paragraph 3.1.3.2.2., Table 11), meant that the amount present in the implant (175 mg) would be completely miscible with the 8 g release medium and the aqueous medium was even replaced at days 1, 2, 5, 9 and 14. A correspondingly decreased rate of mass transport was therefore not expected.

No significant differences were seen between the uncapped (RG 502H) and end-capped (RG 502) polymer type, with the possible exception of day 14 (Figures 61 and 62). In contrast to smooth surface with the capped polymer, a rougher surface with polymer-skinned bubbles was observed with the uncapped polymer type (Figure 61), which could result from the generation of water-soluble degradation products of PLGA. This probably caused increased water ingress along the developing osmotic gradient. The capped polymer is more hydrophobic and degrades slower than

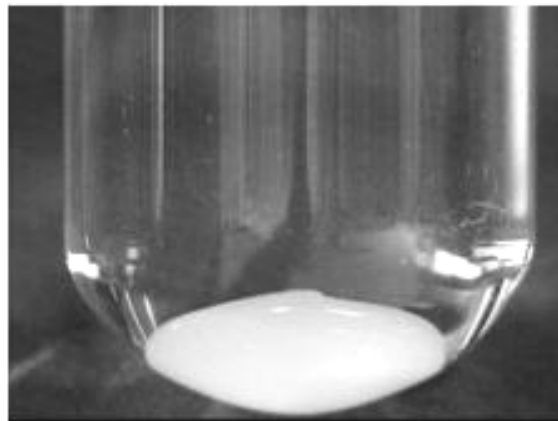
the uncapped type. It was previously shown for these polymer types, that the onset of the release of water-soluble degradation products was shifted from 7 days to 21 days by the end-capping of PLGA (Tracy et al., 1999).

In correspondence with the in situ implants, a fluffy layer was found with the corresponding in situ microparticle formulation (emulsified into sesame oil, phase ratio 1:1) on the same day (Figure 63). There, one larger bubble could still be observed at day 34. However, the most obvious difference to the in situ implant formulations was observable in the beginning of the incubation. The emulsion of polymer solution and sesame oil were bulkier, due to the additional oil phase (2 x 0.25 g injection volume) but lost the predominant part during days 1 and 2 to separation of a coalesced sesame oil bubble from the system. The reason for the separation is the low density of the oil. Sesame oil has a density of about 0.9 g/ml only, whereas for triacetin alone has already a density of about 1.2 g/ml.

0 d



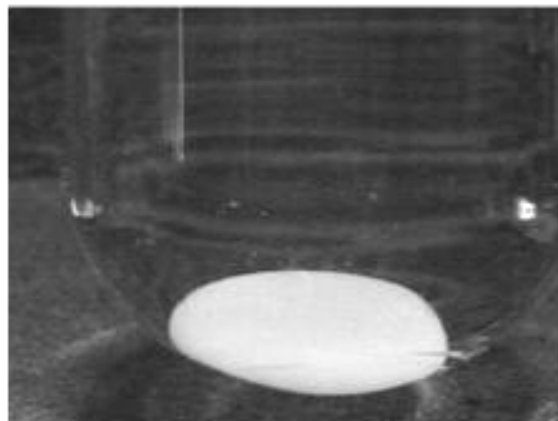
1 d



5 d



9 d



14 d



34 d



Fig. 61: Drug-free solutions (0.25 g) of 30 % uncapped PLGA (RG 502H) in triacetin (in situ implants) upon incubation in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 2, 5, 9 and 14)

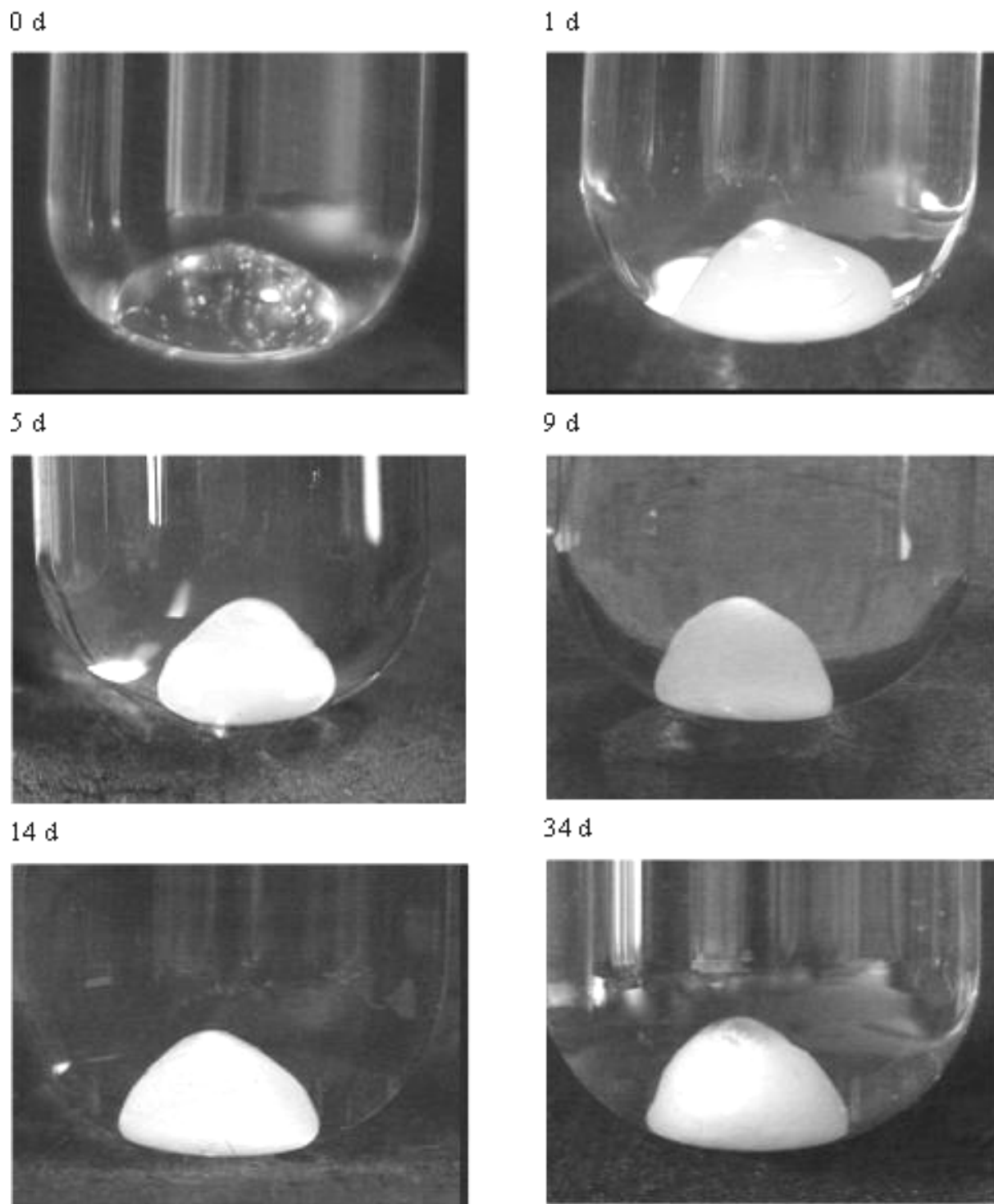


Fig. 62: Drug-free solutions (0.25 g) of 30 % capped PLGA (RG 502) in triacetin (in situ implants) upon incubation in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 2, 5, 9 and 14)

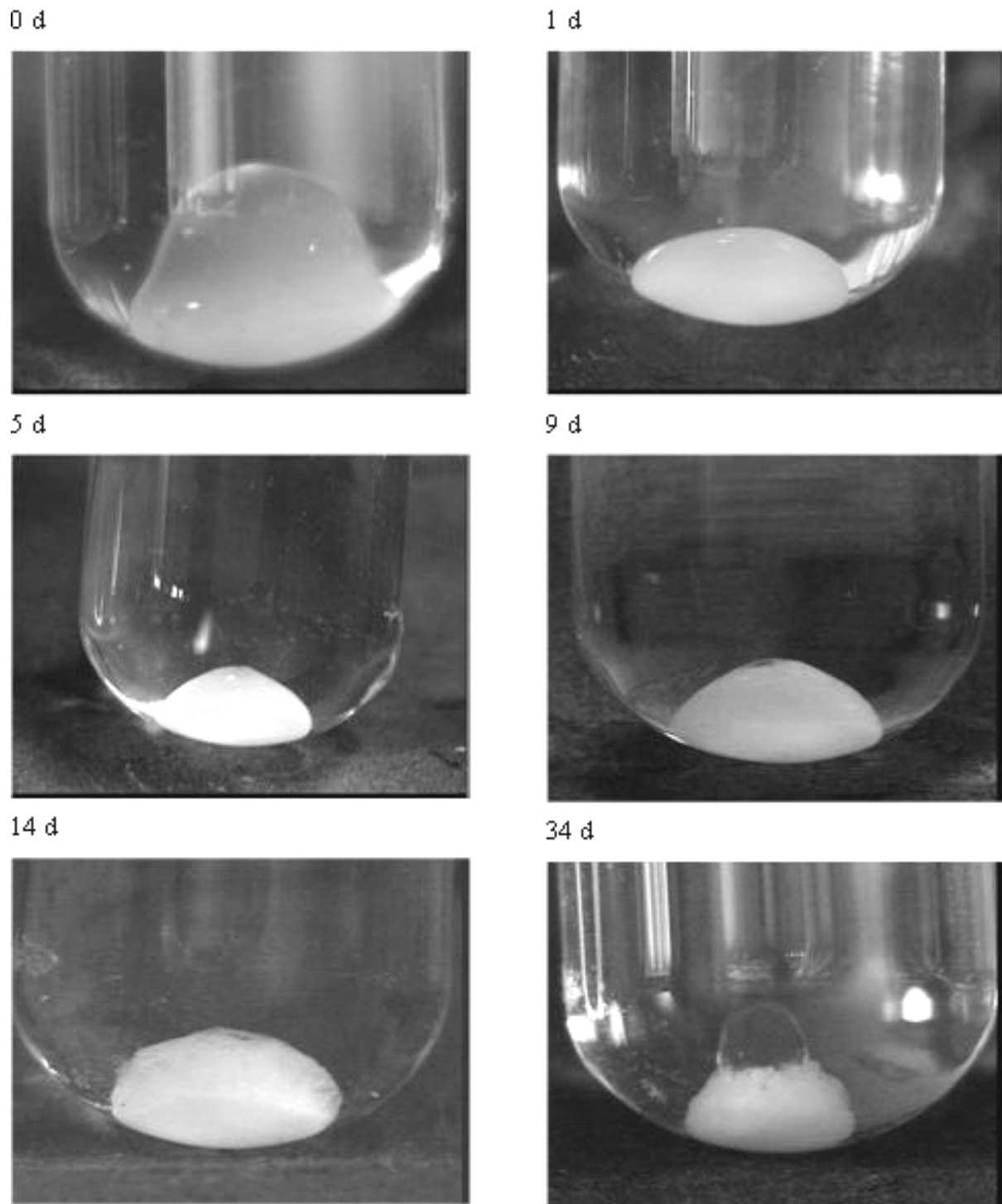


Fig. 63: Drug-free in situ microparticle formulation consisting of 30 % uncapped PLGA (RG 502H) solution in triacetin (0.25 g) emulsified into sesame oil (0.25 g) as a function of incubation time in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 2, 5, 9 and 14)

3.2.1.2. *In vitro* drug release of lysozyme from *in situ* formulations based on triacetin

3.2.1.2.1. Effect of drug particle size

Triacetin is a non-solvent for lysozyme. Therefore, lysozyme needed to be dispersed in the polymer solution of the *in situ* systems. For the microencapsulation of dispersed drug, the particle size of drug in relation to the microparticle size is important, since drug particles with immediate access to the microsphere surface would be released in an initial burst (Costantino et al., 2004). For proteins, particle size reduction can require complex protocols in order to guarantee the stability of susceptible drugs during micronization (Herceg et al., 2005 and paragraph 1.2.2.2.1.). Milling and homogenization are common micronization procedures. Two milling / grinding methods (ball mill, mortar treatment) and Ultra-Turrax homogenization were investigated regarding their capability to reduce the particle size of commercially available lyophilized lysozyme powder. The particle size of untreated lyophilized lysozyme powder could be reduced from 13.0 μm to 3.3 μm through a mortar and pestle treatment (Table 16). Milling with a nitrogen-cooled ball mill lead to particle sizes of about 2.2 μm . The ball milled product was fairly charged after the treatment, which complicated the handling of the resulting powder and led to significant material losses.

Lyophilized lysozyme (as received) did not take up water during the mortar treatment as seen from unchanged loss on drying (LOD) values ($n=1$). The LOD increased slightly upon ball milling. This was probably due to condensation of water vapor in the nitrogen-cooled mill, as indicated by ice formation outside the milling chamber. The condensation could not be avoided under ambient humidity conditions.

Tab. 16: Particle size and resulting loss on drying (LOD) of lysozyme in dependence of the micronization method

Method	Particle size, μm	LOD, %
Lysozyme (as received)	13.0 +/- 7.7	6.4
Mortar treatment for 5 min	3.3 +/- 1.1	5.8
Ball milling for 10 min	2.2 +/- 0.7	10.2

An opportunity to avoid extensive contact of a lyophilized protein powder with ambient conditions is to disperse it directly in the organic solvent or the polymer solution and homogenize

the dispersion using an Ultra-Turrax. Although the data suggested a trend to smaller particle sizes when the dispersions of lysozyme in two solvents were homogenized for one minute at 8000 rpm or 24000 rpm (Table 17), the effect was small. A longer homogenization time would probably lead to a further reduction of the particle size but it was noticed that the temperature of the dispersion increased significantly during the homogenization. An increase of the viscosity of the continuous phase did also not affect the resulting particle size (TEC: 35 cP and ethyl acetate: 0.5 cP, both at 25°C).

Tab. 17: Particle size of lysozyme upon Ultra-Turrax treatment (1 min) of protein dispersions in ethyl acetate and triethyl citrate (50 mg in 2 g solvent)

Method	Particle size, μm
Lysozyme (as received)	13.0 +/- 7.7
Ethyl acetate, 8000 rpm	11.4 +/- 7.4
Triethyl citrate, 8000 rpm	9.3 +/- 9.6
Triethyl citrate, 24000 rpm	8.5 +/- 6.9

Applying the agate mortar and pestle treatment prior to incorporation of lysozyme particles into the formulation did not affect the release profiles of the in situ microparticles (Figure 64). The reason for the robustness of the formulation to changes in the protein particle size in the observed range could be attributed to the larger dimensions of the in situ compared to conventional microparticles. As shown above (Figure 63), the in situ microparticle emulsion separated into the sesame oil and the polymer solution phase after immersion into release medium. Thereby, the viscous polymer solution phase appeared to coalesce and form a single in situ depot at the bottom of the vertically incubated test tube.

An impact of the particle size of lysozyme on the in vitro release of in situ microparticles could be excluded. However, reducing the particle size of lysozyme could still be advantageous to prevent drug sedimentation and thus guarantee formulation homogeneity upon storage.

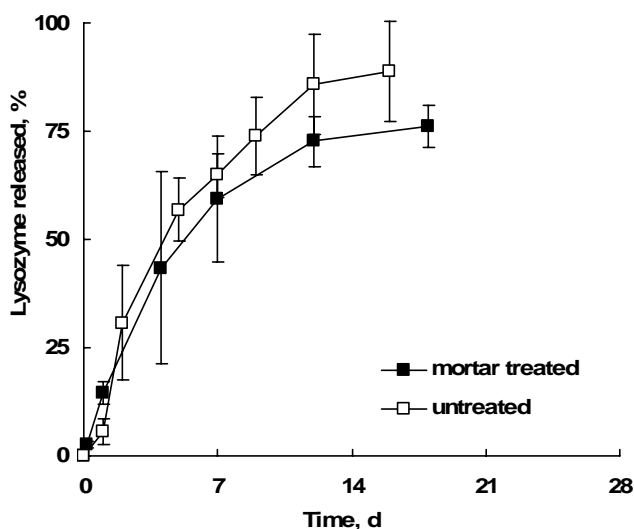


Fig. 64: Lysozyme release from in situ microparticles (30 % RG 502H in triacetin emulsified into sesame oil) as a function of lysozyme milling

3.2.1.2.2. Effect of polymer concentration

The rate of lysozyme release from in situ implants decreased with increasing PLGA (RG 502H) concentration (Figure 65), which was in agreement with previous reports (Shah et al., 1993 and Al-Tahami et al., 2006). The increase of the polymer concentration from 10 % over 20 % to 30 % affected the dissolution profiles especially within the first 2 to 3 days (Figure 65). The slower initial release with a higher polymer concentration could be attributed to the increased viscosity of the polymer solution. The initial release of lysozyme was previously attributed to diffusion through the triacetin-based in situ implants (Brodbeck et al., 1999). An increased viscosity of the in situ implants would therefore decrease the diffusivity of lysozyme.

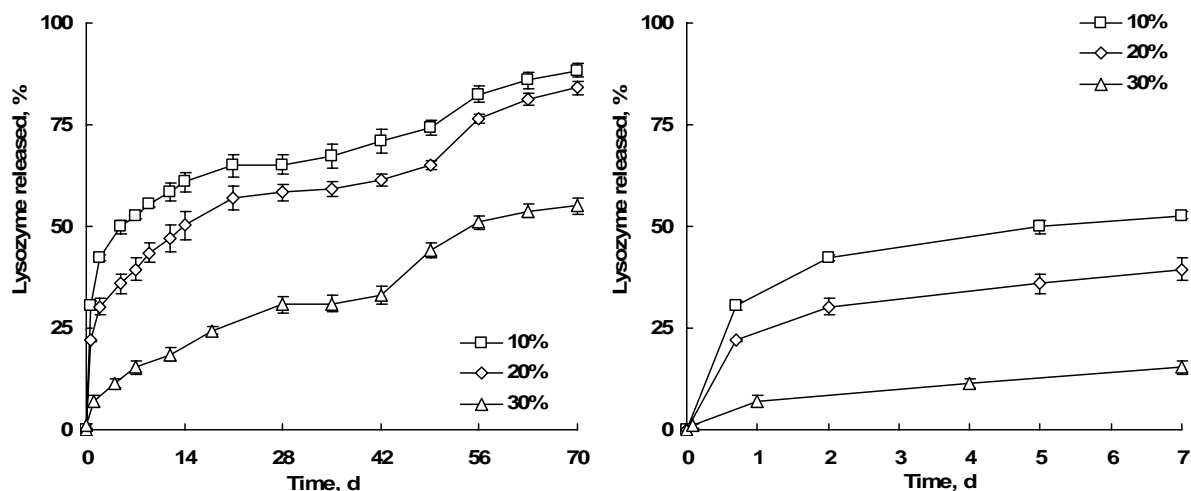


Fig. 65: Lysozyme release from in situ implants (RG 502H in triacetin, 4 % drug loading based on polymer) as a function of the polymer concentration; a) total release period and b) release period between 0–7 days

Normalization of the profiles to the lysozyme fraction released at day 7 visualized that the relative release rates between 7 and 70 days were independent of the initial polymer concentration (Figure 66). This could be attributed to the formation of comparable implant morphologies after leaching of triacetin and hardening of the implants. This assumption was reasonable, since a polymer concentration-dependent finger-to-sponge transition of the morphology of the implanted matrix would not be expected for triacetin with its low affinity to the precipitating aqueous medium. Previously, a 10 % substitution of the water-miscible solvent N-methyl-2-pyrrolidone by triacetin was already sufficient to decrease the phase inversion rate of a PLGA solution (40 %) in a degree, which caused a transition of the resulting membrane morphology from finger- to sponge-type (Graham et al., 1999).

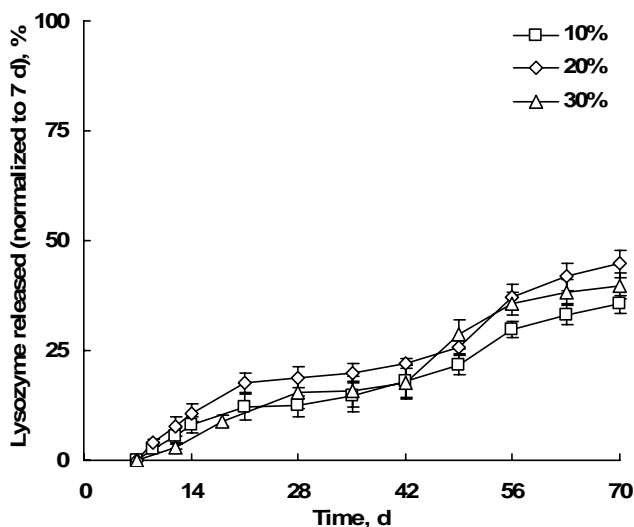


Fig. 66: Lysozyme release from in situ implants (RG 502H in triacetin, 4 % drug loading based on polymer) between 7 and 70 days (normalized to 7days) as a function of the polymer concentration

Beside the initial release also the amount of lysozyme unrecovered after 70 days decreased with increasing polymer concentration. The similar release behavior after the initial release phase of all polymer concentrations (Figure 66) indicated that the initially released lysozyme and the unrecovered drug amount could be related to each other. Lysozyme was better entrapped at a higher polymer concentration, but the incorporated material was not released thereafter. Very likely this was due to the formation of irrecoverable (insoluble) aggregates during incubation.

The release profiles appeared to consist of four phases, the polymer concentration-dependent diffusion phase in the beginning (Figure 65), followed by an apparently triphasic pattern thereafter (Figure 66). The release phase between 7 and 21 days could be attributed to erosion of the hardened implants due to polymer degradation. The end of this phase matched perfectly with the reported in vitro mass loss pattern of the same uncapped, short-chain PLGA (Tracy et al., 1999). Thereafter, almost no lysozyme was released from the in-situ implants. The appearance of a release phase after about 42 days of incubation was unexpected. According to the current opinion (e.g. Yeo et al., 2004a) a release beyond an incubation of one month would be unexpected for matrices consisting of fast-degrading, low molecular weight PLGA like RG 502H. Thus the incubation periods are mostly too short to facilitate the detection of this release phase. However, indications for the presence of such a late protein release from PLGA matrices

can be found, mostly for conventional microparticles (e.g. Lu and Park, 1995 or Wei et al., 2004). The occurrence of this release phase could be attributed to lysozyme entrapped in a slowly degrading surface layer of the implants, which is caused by faster polymer degradation in the core of PLGA matrices due to autocatalysis (Li et al., 1990).

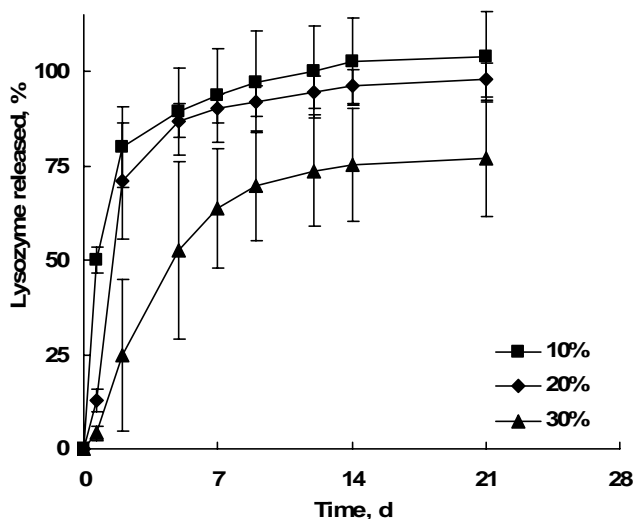


Fig. 67: Lysozyme release from in situ microparticles (RG 502H in triacetin emulsified into sesame oil, 4 % drug loading based on polymer) as a function of the polymer concentration

Similarly to the in situ implants, the rate of lysozyme release from in situ microparticles decreased with increasing PLGA (RG 502H) concentration (Figure 67). The formulations were only incubated for 21 days due to the relatively fast release. In contrast to the in situ implants, complete release was obtained with the formulations based on the 10 % and 20 % PLGA solutions. Only 77 % could be recovered with the system of the 30 % polymer solution but this was still 22 % more than recovered from the corresponding in situ implant. The large error bars of the formulations indicated some variability of the drug loadings of the individual samples. However, the difference between the individual samples of the in situ microparticle formulation with 30 % PLGA in the internal phase originated from day 2 (Figure 68). In analogy to the in situ implants, this could suggest a contribution from a different degree of initial entrapment on the total recovery. The occurrence of a late release phase with these in situ microparticle formulations was not investigated.

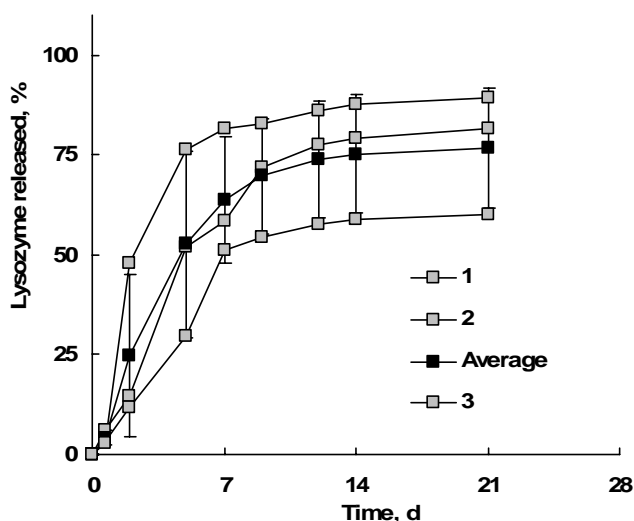


Fig. 68: Lysozyme release from the individual in situ microparticle formulations based on 30 % RG 502H in triacetin emulsified into sesame oil, 4 % drug loading based on polymer

3.2.1.2.3. Comparison of uncapped and capped PLGA

Lysozyme release from in situ implants consisting of 4 % (based on polymer) lysozyme-loaded 30 % polymer solutions in triacetin was dependent on the PLGA type (Figure 69). The release of lysozyme appeared to be faster with the capped PLGA (RG 502) compared to the uncapped type (RG 502H). This was unexpected, since the 30 % solution of uncapped PLGA in triacetin had a lower viscosity (RG 502H: 8.8 Pas and RG 502: 14.1 Pas) and was therefore expected to have a higher diffusivity during the initial release phase. Furthermore, the degradation of the more hydrophilic uncapped polymer should be faster than the corresponding capped type (intrinsic viscosity of 0.2 dl/g from Boehringer Ingelheim, Tracy et al., 1999).

As with the uncapped polymer, the observed end of the almost linear release phase from the in situ implants based on the capped polymer type was in good agreement with the reported completion of mass loss of incubated microparticles consisting of the same polymer (Tracy et al., 1999). The end of the erosion-controlled release phases of the in situ implants at day 21 for the uncapped and day 42 for the capped polymer, matched exactly the two-times faster degradation rate of the uncapped polymer, observed by Tracy et al. This indicated that the release of lysozyme could be ascribed to a similar release mechanism.

The onset of a late release phase, which was seen with the in situ implants based on the uncapped polymer, was not observed. Whether or not this phase would also occur with the capped polymer type at $2 \times 42 \text{ days} = 84 \text{ days}$ was not elucidated.

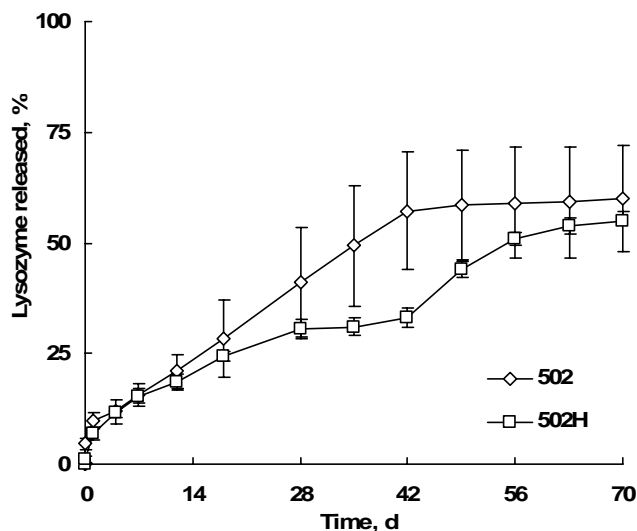


Fig. 69: Lysozyme release from in situ implants consisting of 4 % lysozyme-loaded 30 % PLGA solutions in triacetin as a function of the polymer type

Instead of fundamental mechanistic differences, the apparently faster release with the capped polymer implant would thus be attributed to a higher recovery of lysozyme after the completion of the (first) polymer erosion-controlled release phase. This could be due to an increased adsorption of positively charged proteins to the more hydrophilic and negatively charged uncapped PLGA, as also shown for recombinant human bone morphogenetic protein-2 (Woo et al., 2001a).

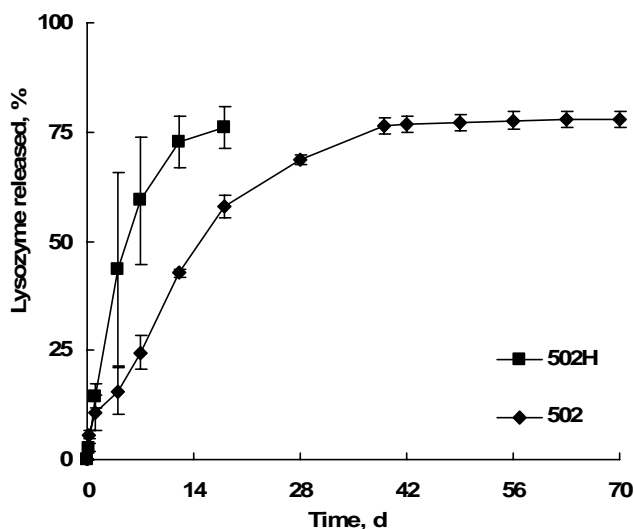


Fig. 70: Lysozyme release from in situ microparticles consisting of an emulsion of 4 % lysozyme-loaded 30 % PLGA solutions in triacetin with sesame oil as a function of the polymer type

In contrast to the in situ implants, the release from the in situ microparticles was in the anticipated sequence, considering the viscosities and erosion rates of both polymers. The formulation with the uncapped released faster than with the capped polymer type (Figure 70). Protein adsorption appeared to be less pronounced compared to the in situ implants, which could be due to the generally faster release of the in situ microparticle formulations compared to the in situ implants. Thus, most of the protein payload was released from the in situ microparticle systems before the erosion phase of the polymer occurred. This could indicate that the adsorption of positively charged lysozyme increased during the erosion phase of the polymer, which could be rationalized considering the increasing number of soluble PLGA-degradation products with free carboxylate groups.

3.2.1.2.4. Comparison of in situ microparticle with in situ implant systems

The release was faster with the in situ microparticle formulations compared to in situ implants independent of the polymer concentration (Figure 71) or whether uncapped or capped PLGA (RG 502) was used in the formulation (Figure 72). Most of the release with the in situ microparticles occurred before the erosion periods of both polymers would be expected (Tracy et al., 1999).

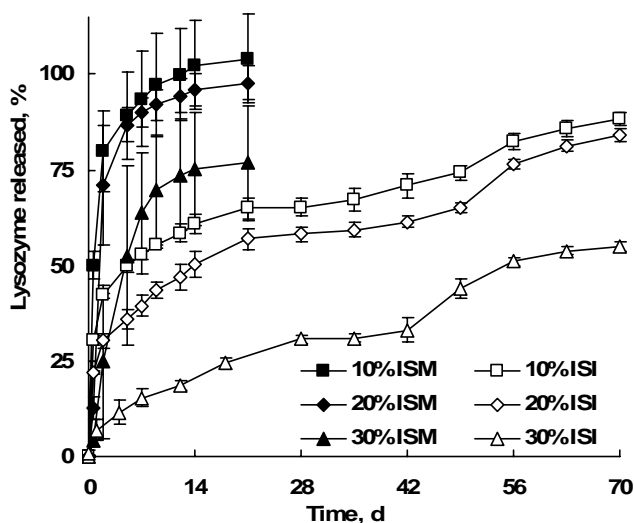


Fig. 71: Lysozyme release from in situ implants (ISI) and microparticles (ISM with sesame oil, phase ratio 1:1) based on PLGA (RG 502H) in triacetin as a function of the polymer concentration

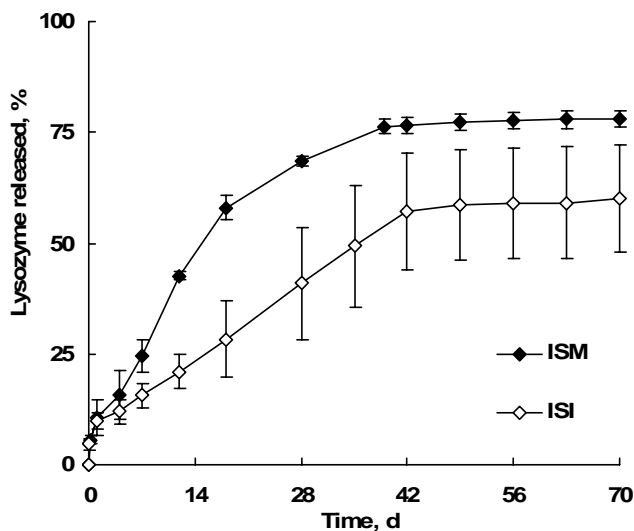


Fig. 72: Lysozyme release from an in situ implant (ISI) and an in situ microparticle (ISM) formulation (sesame oil, phase ratio 1:1) based on 30 % PLGA (RG 502) in triacetin

The faster release could not be explained with a lower particle size of the in situ microparticle systems compared to the in situ implants. The in situ microparticle emulsion was characterized by

a separation of the oil phase from the drug-containing polymer solution shortly after emulsification (paragraph 3.2.1.1., see also paragraphs 3.3.4. and 3.3.5.). The result was an implant-like drug depot on the bottom of the vial and not microparticles.

As argument against a particle size effect also served the release profiles resulting from different ratios of drug-containing polymer solution to sesame oil (Figure 73). Blending of an in situ implant (ISI) with only 10 % sesame oil already resulted in the acceleration of the release. No further increase of the release rate was achieved increasing the sesame oil proportion to 50 %. The oil was likely incorporated into the triacetin-based polymer solution phase instead of representing the continuous phase. This also excluded that the accelerated release would be due to unencapsulated drug particles being released from the oil phase.

The blending of the drug-containing polymer solutions with sesame oil was not expected to influence the viscosity of the PLGA solution and hence increase the diffusivity of lysozyme. Only about 2–4 % of triacetin could be incorporated into vegetative oils with comparable fatty acid composition (peanut, soybean and safflower oil).

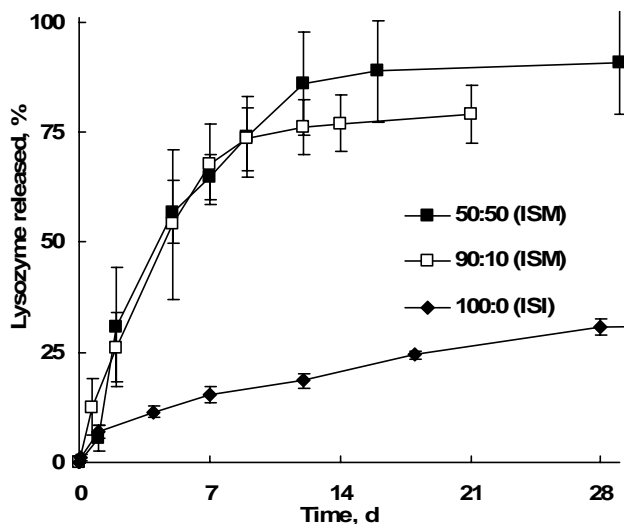


Fig. 73: Lysozyme release from in situ formulations based on 30 % PLGA (RG 502H) in triacetin as a function of the internal (in situ implant, ISI) to external (sesame oil) phase ratio

The faster release from the sesame oil containing implant-like depot was therefore attributed to an assisted mass transfer of lysozyme to the surface of the viscous polymer solution phase through incorporation of the lower density liquid sesame oil. As suggested previously (Maa and

Hsu, 1997), density-driven protein enrichment at or near the surface of polymer solutions affected the drug distribution in resulting conventional microparticles. Although highly viscous, the redistribution of drug could also occur in triacetin-based PLGA solutions. The slow phase separation process of these formulations facilitates a prolonged time interval for migration before hardening of the implants. In situ implants based on even 50 % RG 502 in triacetin were still of a gel-like consistency after 7 days incubation in release medium (Brodbeck et al., 1999).

Accordingly, sedimentation of drug particles (density of 30 % RG 502H in triacetin 1.27 g/ml at ambient temperature; lysozyme density: 1.46 g/ml, as calculated from Fischer et al., 2004) or creaming of aqueous droplets consisting of protein dissolved in polymer-poor phase (approximately 1 g/ml - density of water) could be involved into the mass transfer through the polymer solution / polymer-rich phase and thus to the release mechanism from in situ implants and in situ microparticles. The transport would also show the observed dependence on the viscosity of the continuous phase considering Stokes' law. Incorporation of sesame oil into the in situ implant formulation would add an additional immiscible low density (0.92 g/ml; The Merck Index, 12th edition, 1996) compound, which would tend to migrate to the top of the depot. A faster migration through the polymer solution / polymer-rich phase would be expected for sesame oil due to the higher density difference between the polymer-containing and the oil phase (same particle size). The accelerated lysozyme release could thereby be explained by collision of droplets and drug particles or aqueous (polymer-poor phase) and oily droplets. The large standard deviations with the ISM-formulation could reflect an insufficient control of the droplet size of the oil phase, which would also affect the migration velocity according to Stokes' law.

This hypothesis was supported, when in situ implants based on triacetin were emulsified with the same volume of air (not mass as for sesame oil). A separation of the dispersed air from the drug depot could be observed as with the sesame oil (Figure 74). Although air has a much lower density (0.0012 g/ml) compared to sesame oil, the acceleration of the release achieved was comparable to the effect of sesame oil within the first 2 days (Figure 75). Oppositely to the in situ microparticle system with sesame oil, the release decreased already after two days incubation. This could be attributed to a more rapid completion of the separation process for air compared to sesame oil.

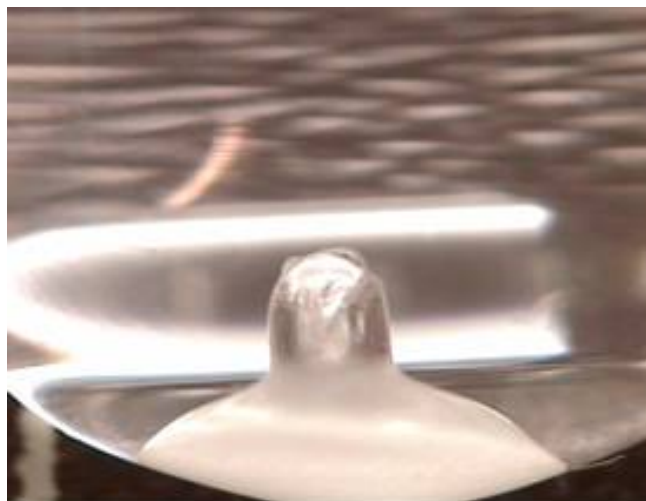


Fig. 74: In situ implant based on 30 % PLGA (RG 502H) in triacetin containing-dispersed air (phase ratio 1:1, v/v) after 1 day incubation in release medium

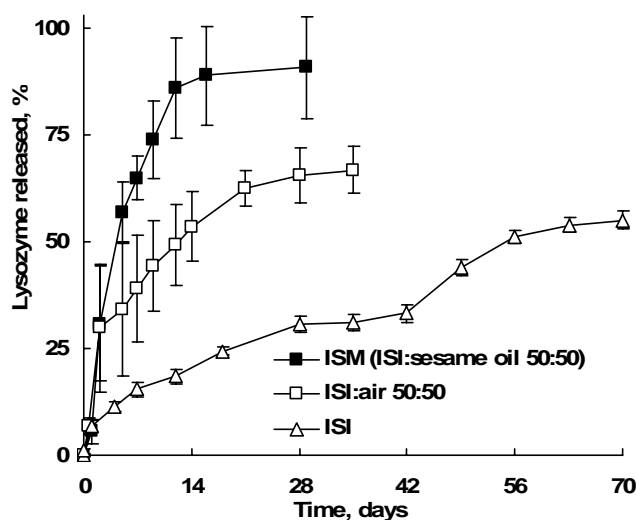


Fig. 75: Lysozyme release from an in situ implant (ISI), an in situ microparticle (ISM) formulation (sesame oil, phase ratio 1:1) and an in situ implant emulsified with air (volume ratio 50:50) based on 30 % PLGA (RG 502H) in triacetin

The incorporation of sesame oil into in situ implants (ISM) based on triacetin resulted in a faster release of dispersed lysozyme. The drug release from the in situ microparticle formulations was more continuous and led to overall higher recoveries of lysozyme.

3.2.1.2.5. Qualitative characterization of released and unreleased lysozyme

The conformational integrity of lysozyme during release was investigated with in situ microparticles and in situ implants based on solutions of 30 % RG 502H in triacetin. The dissolution test was conducted before the optimized release conditions (low agitation) were applied (paragraph 3.1.1.3.). Thus, the high shear exerted on the formulations in horizontal test tube position led to an increased initial release (Figure 76) compared to the release in vertical test tube position (Figure 75). The in situ microparticle emulsions were thereby more affected by the stronger agitation compared to the corresponding in situ implants. This was probably due to an easier breaking-up of the polymer solution phase due to the presence of sesame oil droplets in the viscous gel, as suggested in the previous paragraph. More importantly, the release profiles of active and total lysozyme were similar, which showed that only biologically active lysozyme was released from the in situ microparticle system and the in situ implant within 25 days.

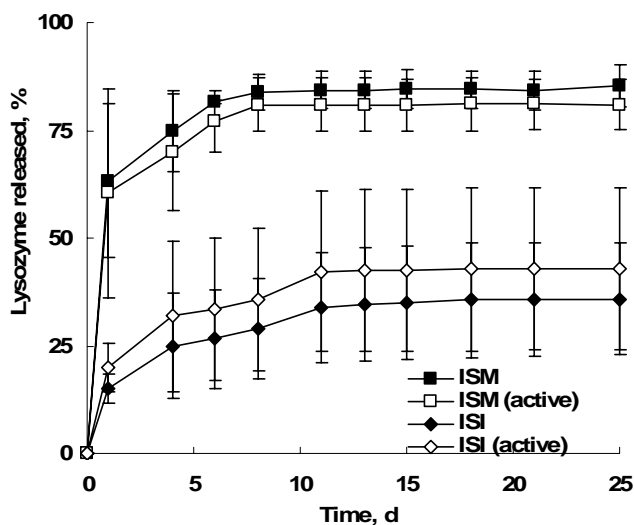


Fig. 76: Lysozyme release from 4 % protein-containing in situ microparticles (30 % RG 502H in triacetin emulsified with sesame oil containing 2 % Span 80, phase ratio 1:1) and corresponding in situ implants (horizontal test tube position)

An extraction of lysozyme after 25 days could not recover the complete payload of the in situ systems. The amount recovered after the dissolution study was increased by 5 % (from 85 % to 90 %) for the in situ microparticle and by 12 % (36 % to 48 %) for the in situ implant system. The biological activity of the recovered protein was similar for both formulations (ISM: 53 % and

ISI: 54 % based on total recovery with HPLC). The recovered lysozyme material after 25 days incubation was supposed to represent the fraction occurring during the late release phase (paragraph 3.2.1.2.2.). A very weak band of a lysozyme dimer band was seen with native PAGE, which disappeared under reducing conditions (Figure 77). This might indicate the formation of some covalent aggregates upon storage. However, it could not be concluded that aggregation was the main reason for the generation of irrecoverable protein due to the lack of bands of lysozyme multimers. Lysozyme fragments at lower molecular weights than the native protein were also not found, which argued against proteolysis. The unrecovered lysozyme fraction could therefore be lost at due to adsorption to PLGA. Although only small amounts of lysozyme adsorbed onto the surface of PLGA particles in a comparable setup (paragraph 3.1.1.3.), the surface area to drug ratio can be expected to be much higher inside the polymer matrix.

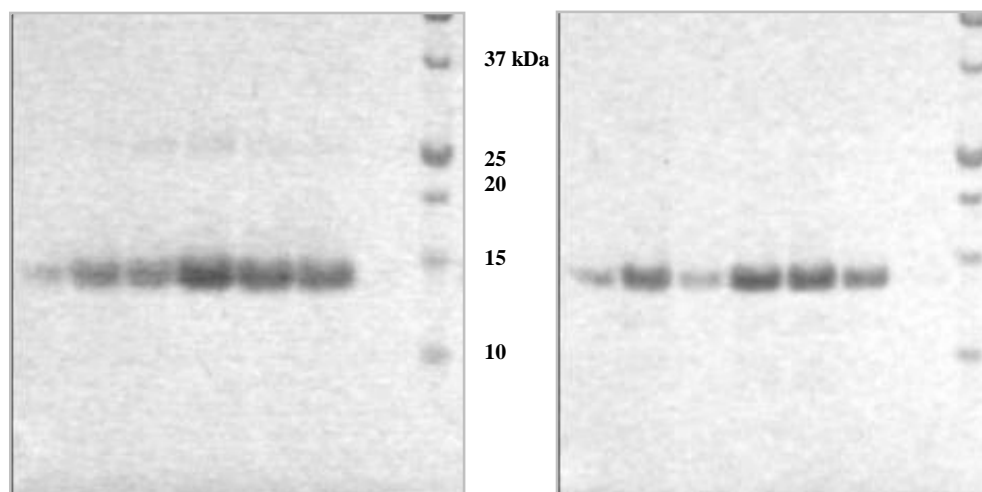


Fig. 77: a) Native and b) reducing PAGE of lysozyme extracted after 25 days release; Lanes: 1-3) in situ microparticle systems, 4-6) in situ implants, 7) drug-free in situ microparticle system and 8) molecular weight marker.

3.2.2. In situ systems based on water-miscible polymer solvents

3.2.2.1. N-methyl-2-pyrrolidone

N-Methyl-2-pyrrolidone (NMP) is one of the most popular PLGA solvents for in situ forming dosage forms due to its biocompatibility (Luan and Bodmeier, 2006b). The only two in situ products commercially available on the German market (paragraph 1.2.1., Table 1) comprise NMP.

3.2.2.1.1. Lysozyme stability in NMP

Lysozyme-loaded in situ implant formulations based on NMP were already investigated (Graham et al., 1999). However, the stability of lysozyme in NMP was not considered. Thus, the stability of lysozyme in NMP was investigated utilizing the developed separation procedure. A change of the ratio between the native peaks was observed in HPLC-chromatograms of lysozyme upon short-term contact with 2-pyrrolidone (2P) and NMP and lysozyme precipitation could be observed, when the solution in NMP was diluted with aqueous medium (paragraph 3.1.3.2.1.). In order to investigate the stability of lysozyme in DMSO on longer term, 2.7 % lysozyme was incorporated into NMP, which would correspond to an in situ formulation with 40 % polymer concentration and 4 % drug loading (based on PLGA). The concentration was above the solubility of lysozyme in NMP (Table 10). Thus, the protein was only partially dissolved. The effect of the presence of two additives, the chelating agent sodium edetate and the potential antioxidant N-acetyl cysteine (both in a concentration of 1 %), on the changes of lysozyme were investigated. Sodium edetate was not completely soluble in NMP.

In agreement with the previous observation (paragraph 3.1.3.2.1.), the peak ratio in HPLC chromatograms of lysozyme decreased rapidly after coming in contact with NMP (Figure 78). The changes appeared to be independent of the additives. The decrease was much faster than the change in the peak ratio observed in acetate buffer. If existent, the point of equilibration in the NMP systems appeared to be at much lower peak ratios (≤ 0.2) compared to the aqueous solutions of lysozyme (~ 2). The changes could be due to racemization, as presumably with the aqueous solutions, but could also be caused by a chemical reaction with the solvent. The reaction of a peptide with a polymeric derivative of NMP and 2P, polyvinyl pyrrolidone, was reported previously (D'Souza et al., 2003). However, the mechanism behind the changed peak ratio of lysozyme in the organic solution / dispersion in NMP was not elucidated.

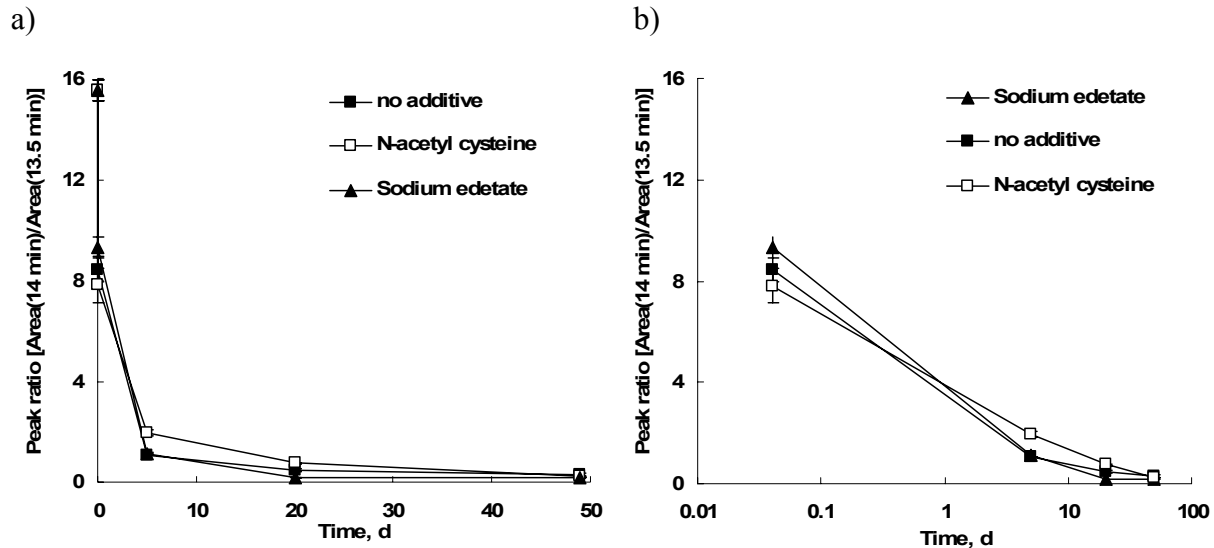


Fig. 78: a) Linear and b) semi-log plot of the peak ratio (14.0 min / 13.5 min retention time) change in HPLC-chromatograms of lysozyme extracted from 2.7 % protein containing NMP in dependence of incubation time at room temperature (desiccated).

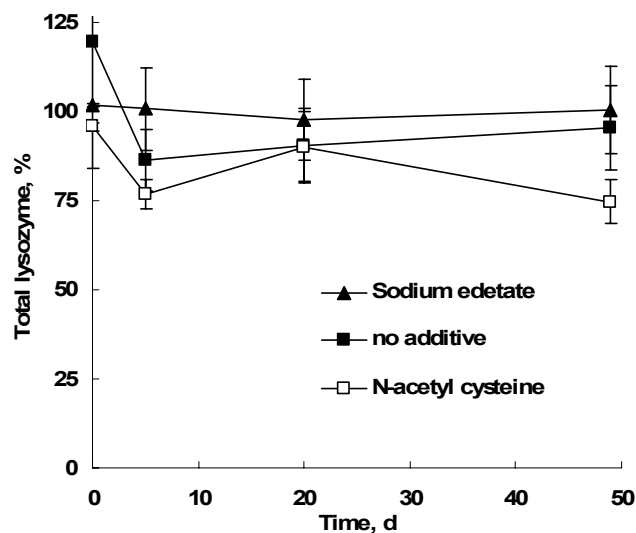


Fig. 79: Lysozyme recovery relative to the theoretical value after separation from 2.7 % protein containing NMP solutions / dispersions in dependence of formulation additives as function of storage time at ambient temperature in a desiccator

The total recovery of lysozyme dissolved / dispersed in NMP appeared to decrease for the samples containing no additive or 1 % N-acetyl cysteine, whereas the recovery from the sample containing 1 % sodium edetate appeared to be more complete (Figure 79). The lowered recoveries were accompanied with the development of an additional peak at 13 min for all samples and a peak shoulder (>14 min retention time) in the HPLC-chromatograms of neat NMP and the N-acetyl cysteine containing sample. Excluding the additional features from the quantification the differences between the samples were slightly more pronounced (Figure 80).

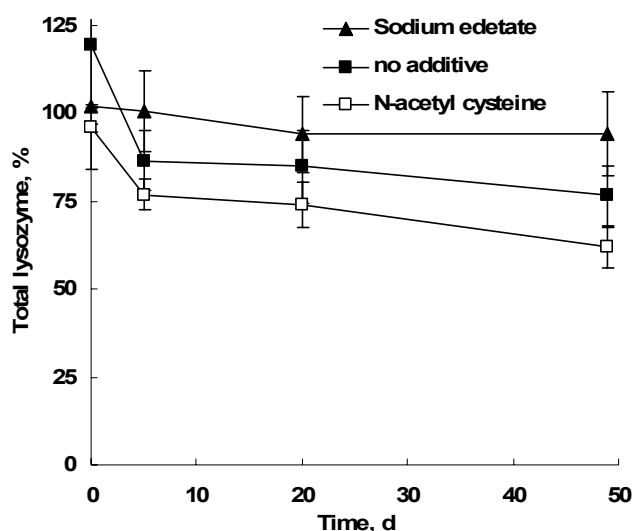


Fig. 80: Lysozyme recovery under exclusion of the peak shoulder (>14 min) relative to the theoretical value after separation from 2.7 % protein containing NMP solutions / dispersions in dependence of formulation additives (1 % additive concentration) as function of storage time at ambient temperature in a desiccator

As indicated by the relatively high standard deviations and the high initial recovery of lysozyme from neat NMP, the sample homogeneity affected the resulting recovery. The dispersed portion of lysozyme amount in the three formulations consisted of gelled particles, which sedimented and caked on the bottom of the vial. However, a decrease of the recovery was consistent with the electrophoresis result (Figure 81a). Lysozyme aggregated in NMP, but a fewer aggregates were seen in the sodium edetate containing sample. The partial disappearance multimer bands upon electrophoresis under reducing conditions suggested the presence of disulfide cross-linked aggregates (Figure 81b).

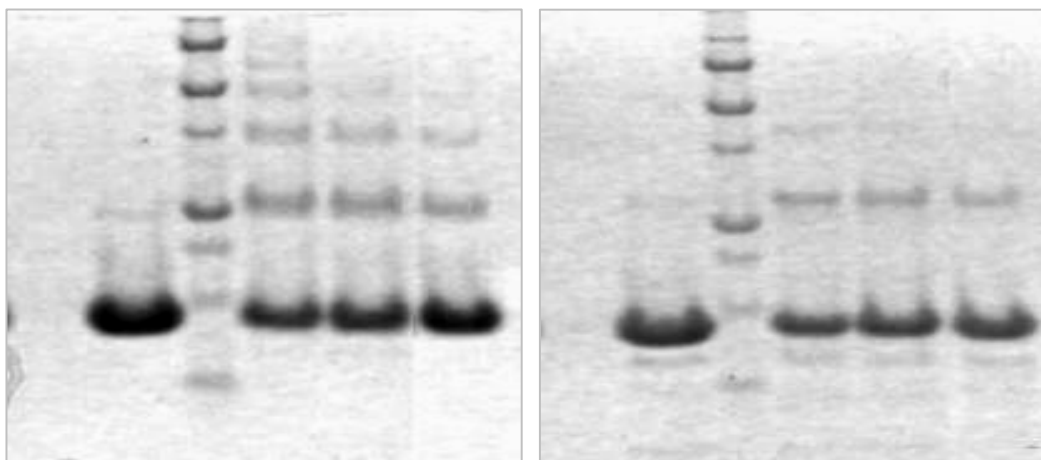


Fig. 81: a) Native and b) reducing PAGE of lysozyme recovered from solutions / dispersions in NMP after 49 days incubation at room temperature (desiccated); Lanes: 1) blank, 2) freshly prepared aqueous lysozyme standard (1018 µg/ml), 3) molecular weight marker, 4) NMP without additive, 5) NMP containing 1 % N-acetyl cysteine, 6) NMP containing 1 % sodium edetate

The sample homogeneity would not be expected to affect the relative activity of a sample since the actually measured activity was normalized for the total recovery in the same sample. The results again confirmed the sequence of lysozyme stability: NMP / 1 % sodium edetate > NMP > NMP / 1 % N-acetyl cysteine (Figure 82). Thus, sodium edetate dissolved / dispersed in NMP appeared to stabilize lysozyme against aggregation and loss of activity, whereas N-acetyl cysteine appeared to induce lysozyme aggregation and promoted its deactivation in NMP. No correlation appeared to exist between the relative activity and the shift of the peak ratio, which suggested that the derivative corresponding to increasing peak possessed a considerable biological activity.

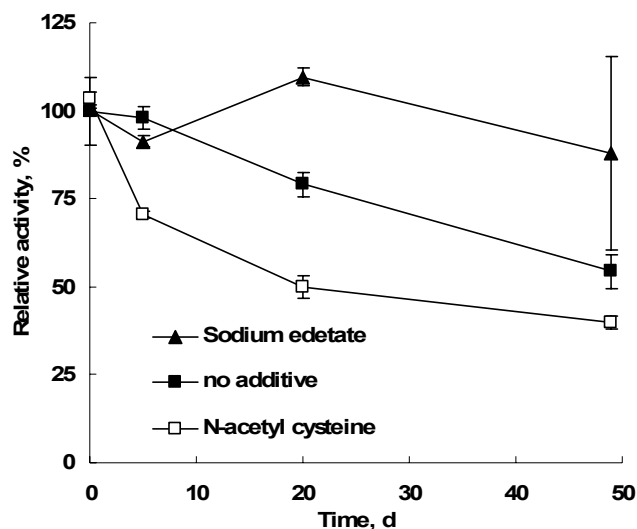


Fig. 82: Relative activity of lysozyme (active / total recovery, [%]) recoverable upon extraction of 2.7 % lysozyme solutions / dispersions in NMP in dependence of formulation additives (1 % additive concentration) as a function of storage time at ambient in a desiccator

3.2.2.1.2. Drug release from blends of DMSO and NMP

NMP was blended with the water miscible solvent DMSO in order to obtain homogenous in situ formulations through protein dissolution prior to polymer addition. DMSO was the better solvent for lysozyme (Table 10, paragraph 3.1.3.2.1.).

The release of lysozyme was dependent on the ratio of NMP to DMSO (Figure 83). An apparently biphasic pattern obtained with the DMSO based formulation turned into a burst-type release profile increasing the NMP portion of the solvent blend from 0 % to 50 %. Additionally, the total recovery of lysozyme after one month decreased as well upon the increase of the NMP fraction. The lowered recovery could be due to lysozyme aggregation, which was previously observed upon dilution of an NMP solution with water (paragraph 3.1.3.2.1.).

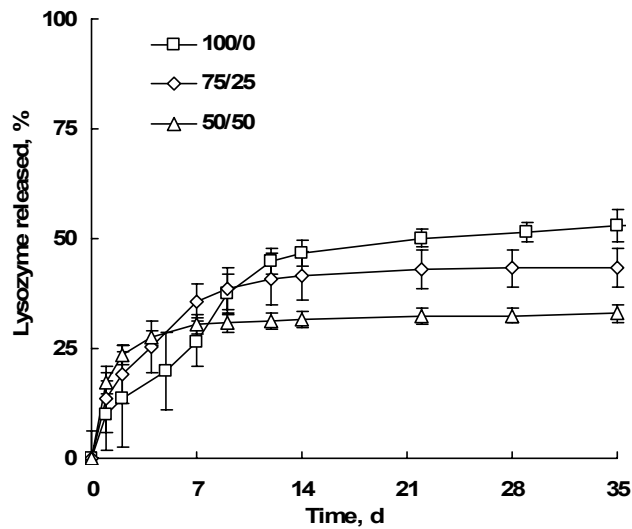


Fig. 83: Lysozyme release from in-situ implant formulations consisting of 40 % PLGA (RG 502H) solutions containing 4 % lysozyme (relative to polymer) as a function of the ratio DMSO to NMP

NMP appeared to be unsuitable as solvent for the delivery of lysozyme with in situ formulations due to the limited solubility and to the unsatisfying stability of the protein in contact with the solvent.

3.2.2.2. *In situ* systems based on dimethyl sulfoxide

Dimethyl sulfoxide (DMSO) is a water-miscible solvent for PLGA and, oppositely to triacetin, also for lysozyme (paragraph 3.1.3.2.1., Table 10). It is an ICH-class 3 solvent and is approved by the FDA for parenteral application for humans (Viadur, Bayer Health Care, Germany).

3.2.2.2.1. Macroscopic appearance

Characteristic morphological features of *in situ* formulations based on water-miscible solvents could be observed upon incubation in release medium. Oppositely to 30 % uncapped PLGA-containing triacetin-based *in situ* implants (Figure 61), the appearance of the polymer solutions in DMSO changed immediately after contact with the aqueous buffer (Figure 84). In contrast to hydrophobic polymer solvents such as triacetin (paragraph 3.2.1.1.), water-miscible solvents have a high affinity to the precipitating aqueous medium, which leads to a rapid increase of the polymer concentration in the polymer-rich phase and thus to a fast polymer solidification upon contact (Brodbeck et al., 1999). The irregular, non-spherical shape of the *in situ* implants after injection (0 and 1 day) supported a rapid solidification of PLGA. However, a volume increase of the implant could be observed between 1 and 5 days. This could be attributed to residual DMSO in the core of the solidified implants, which probably attracted water due to its osmotic activity. The swelling of the DMSO-based *in situ* implants contrasted the solvent leaching-induced shrinking observed with the formulations based on the water-immiscible triacetin. The scenario of an osmotically-triggered water uptake was in accordance with the reported mechanism behind macrovoid formation during polymer phase separation (McKelvey and Koros, 1996). Accordingly, the obviously hardened implants showed some large pores at days 5 and 9. The porous polymer network collapsed around day 14, which was attributed to a sufficiently decreased glass transition temperature due to the degradation of the polymer. The implants had a sticky semisolid consistency at this time-point. As polymer degradation continued the amount of residual polymer mass decreased. However, some polymer remained even after 34 days incubation in the release medium.

The 40 % uncapped PLGA-containing DMSO-based *in situ* implants did not solidify as fast as the 30 % polymer-containing formulations. The consistency was still gel-like after 1 day incubation (Figure 85), which reflected a slower phase separation process at the higher polymer

concentration. The increase of the implant volume appeared to be prolonged comparing the days 5 and 9. Interestingly, the implants were hollow after 14 days, which was attributed to a marked mass loss from the core between 12 and 14 days incubation. The polymer erosion phase is important for the release of large molecules like proteins, since the diffusivity through the continuous polymer phase decreases with molecular weight (Pitt., 1990). Accordingly, it was shown that the release of bovine serum albumin correlated fairly well with erosion of the polymer matrix (Shah et al., 1992).

Only the shell of the implant remained after 14 days, which in accordance with a slower degradation process due to the lack of autocatalysis (paragraph 1.2.1.1.2.). The material found after 34 days incubation of the 30 % RG 502H-containing formulations was probably also material from the slower degrading collapsed implant shell.

The antenna-like extension on top of the 30 % RG 502S-containing in situ implants, which remained from the injection step, indicated that the polymer (capped) underwent a rather fast solidification upon contact with the aqueous release medium (Figure 86). As seen with the formulations based on the uncapped polymer, a volume increase of the implants was observed between 1 and 5 days. The water ingress in that time period led to the formation of large pores, which were seen after 9 days. Although the degradation of the capped polymer type was expected to be 1.4-times slower than for the uncapped polymer (personal communication with Dr. Lietdke from Boehringer Ingelheim, Germany), the collapse of the 30 % polymer-containing implant occurred at the same time-point as observed with the uncapped polymer. In contrast to the 40 % in situ implants based on uncapped PLGA, the corresponding in situ implants based on capped PLGA showed no gel-like transparent appearance initially (Figure 87 vs. 85). This indicated that the polymer solidification with the more hydrophobic capped polymer type could be faster than with the more hydrophilic uncapped type. However, the formulations with 40 % capped polymer collapsed or lost mass later, between 14 and 34 days, than the uncapped polymer-based in situ implants, which was in agreement with the lower degradation rate of the capped polymer.

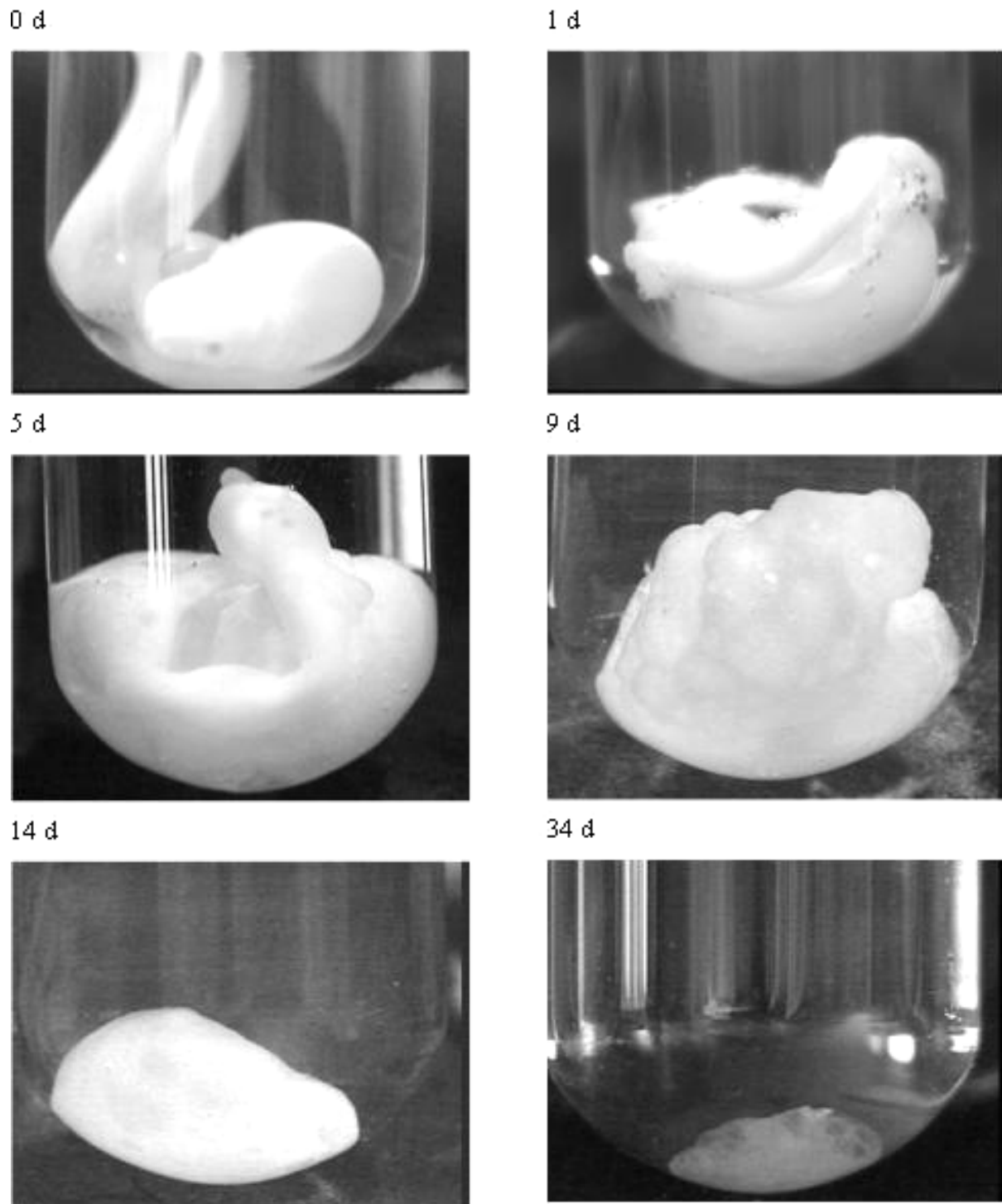


Fig. 84: Drug-free solution (0.25 g) of 30 % uncapped PLGA (RG 502H) in DMSO (in situ implant) upon incubation in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 2, 5, 9 and 14); representative sample of n=3 formulations

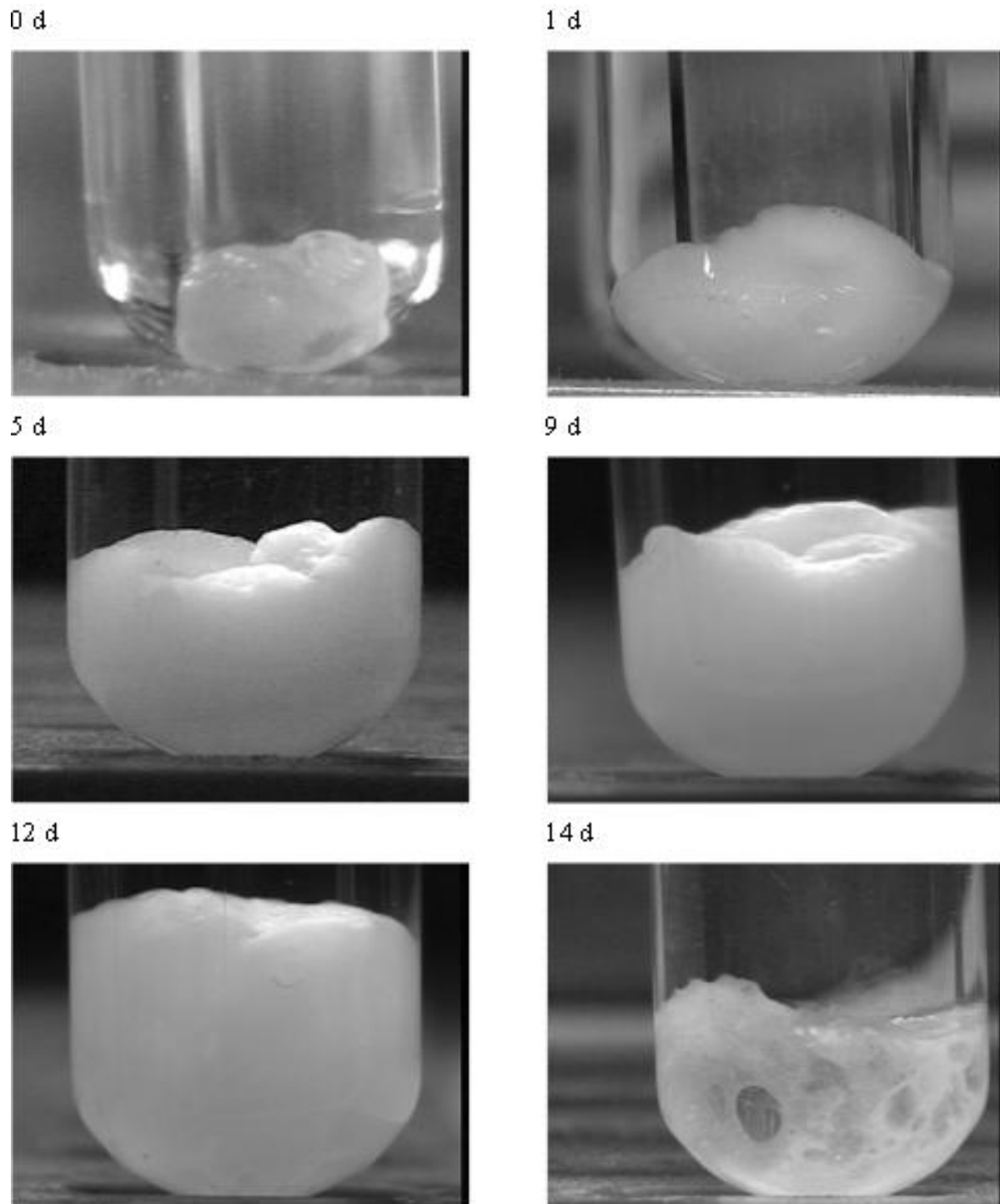


Fig. 85: Drug-free solutions (0.25 g) of 40 % uncapped PLGA (RG 502H) in DMSO (in situ implants) upon incubation in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 5, 9 and 12); representative sample of n=3 formulations

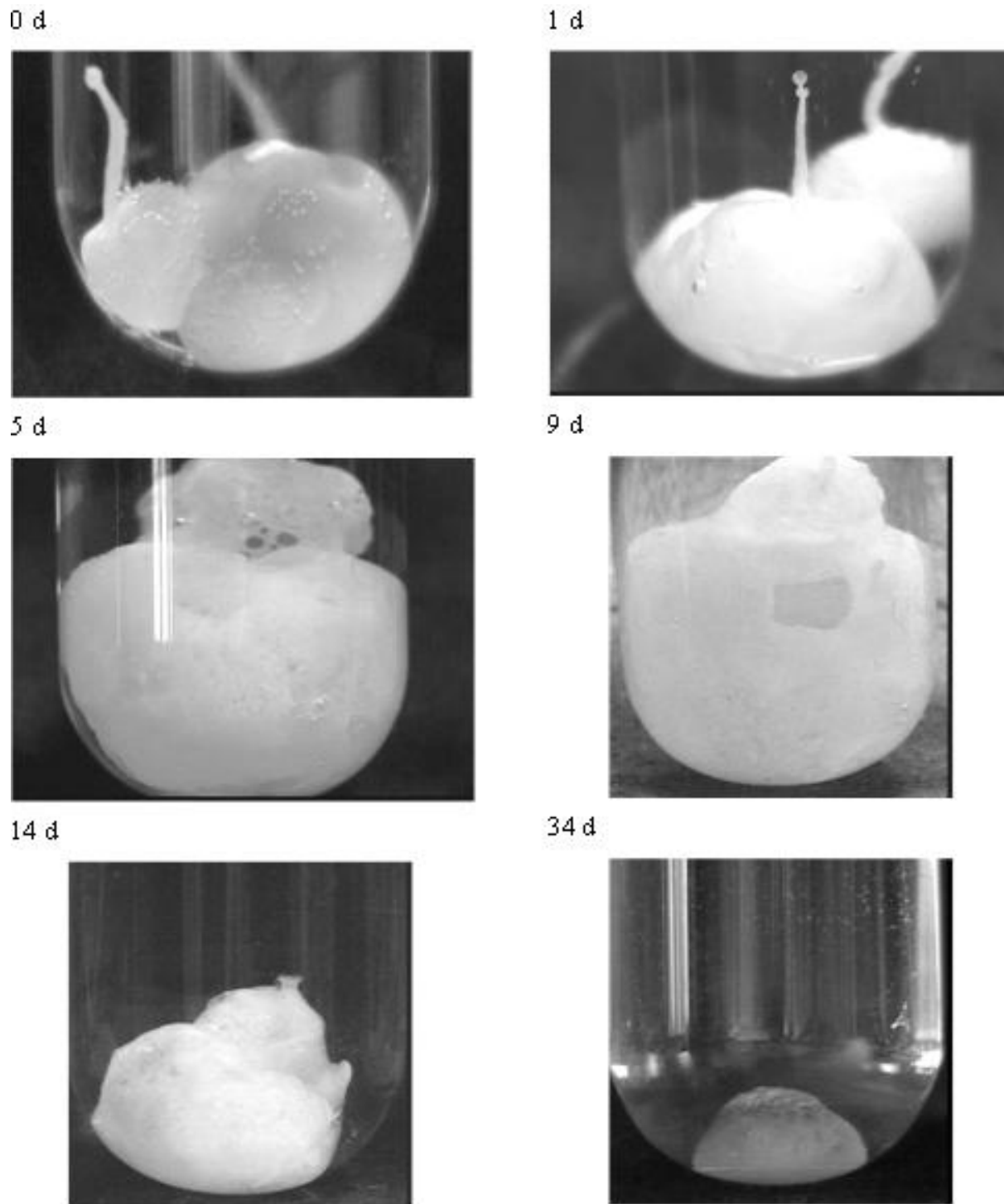


Fig. 86: Drug-free solutions (0.25 g) of 30 % capped PLGA (RG 502S) in DMSO (in situ implants) upon incubation in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 2, 5, 9 and 14); representative sample of n=3 formulations

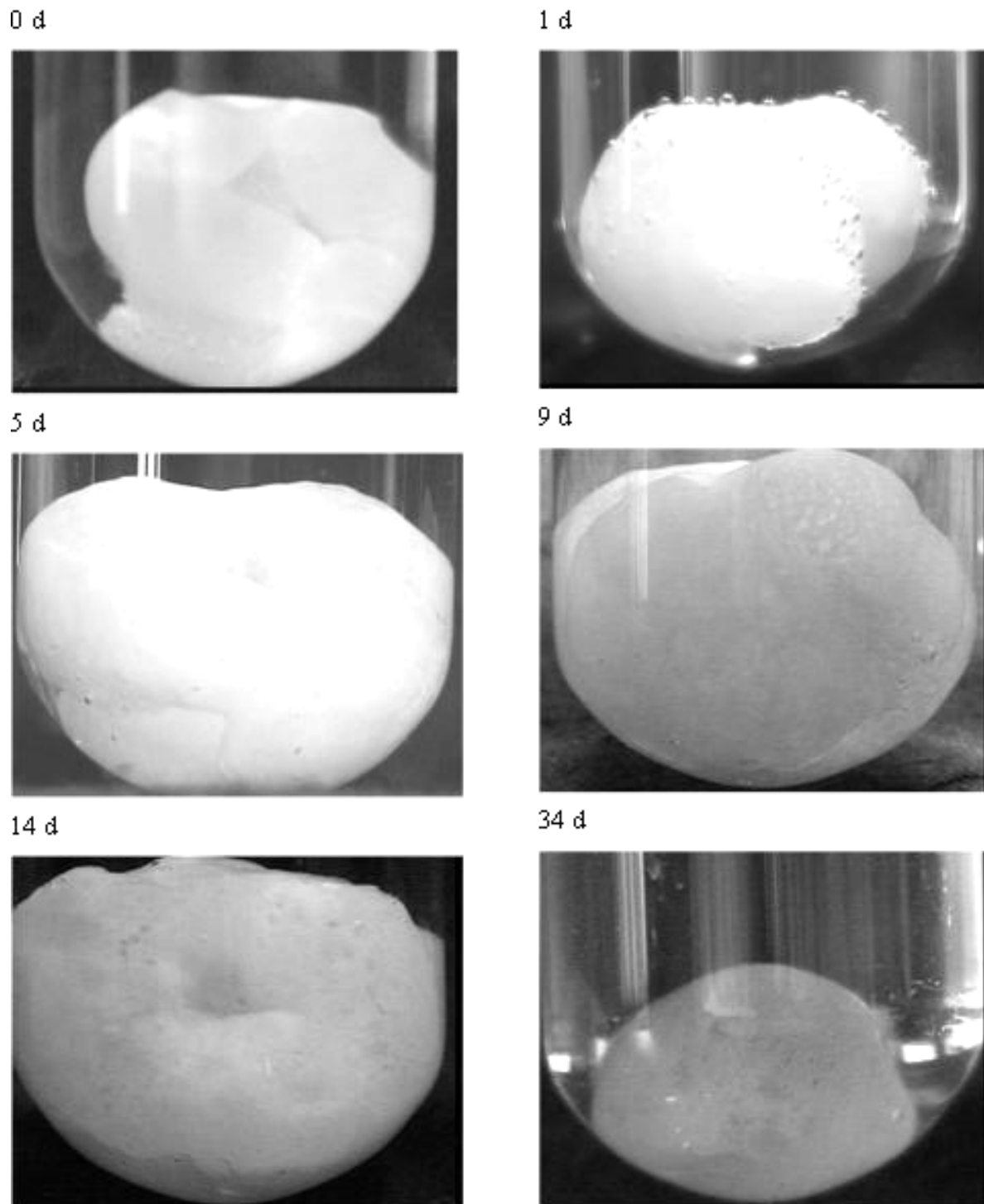


Fig. 87: Drug-free solutions (0.25 g) of 40 % capped PLGA (RG 502S) in DMSO (in situ implants) upon incubation in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 2, 5, 9 and 14); representative sample of n=3 formulations

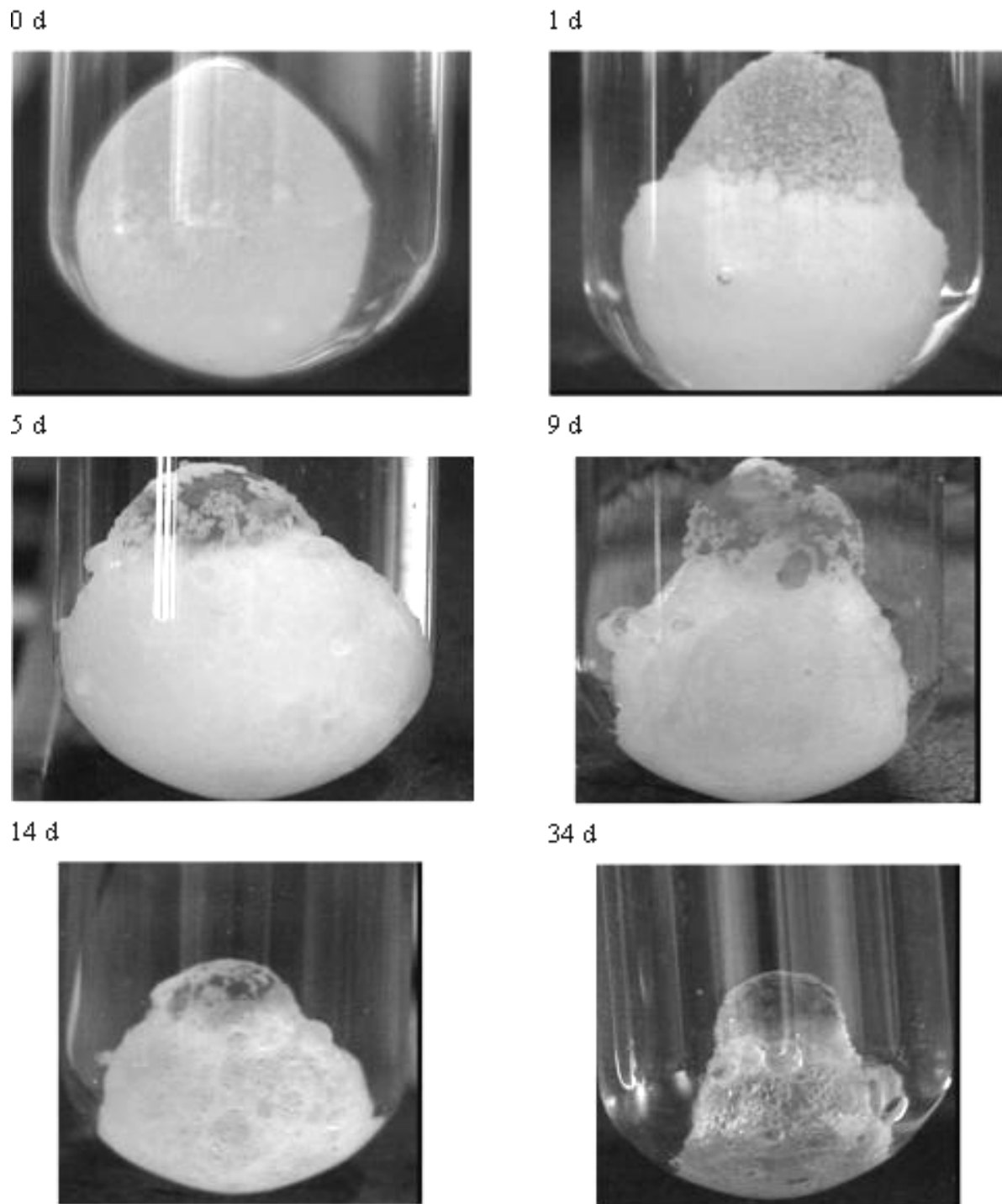


Fig. 88: Drug-free in situ microparticle formulation consisting of 30 % uncapped PLGA (RG 502H) solution in DMSO (0.25 g) emulsified into sesame oil (0.25 g) as a function of incubation time in release medium at 37°C (acetate buffer pH 5 containing 0.01 % sodium azide, horizontal test tube position in shaker at 80 rpm, medium replacement at days 1, 2, 5, 9 and 14); representative sample of n=3 formulations

The in situ microparticle emulsion formed a spherical droplet upon injection into the release medium (Figure 88). Polymer phase separation occurred predominantly at the bottom of the droplet, which was attributed to the separation of the dense polymer solution from the oil phase. The phases were expected to be immiscible with each other, since less than 2 % of vegetative oils with comparable fatty acid composition (peanut, soybean and safflower oil) could be incorporated into DMSO (paragraph 3.1.3.2.2. table 11). Oppositely to the triacetin-based formulations, a small skin of PLGA formed on top of the oil droplet, which apparently hindered the oil to buoying upwards to the surface of the release medium. However, the volume increase between 1 and 5 days and the decrease between 9 and 14 days as well as the presence of residual material after 34 days were comparable with the corresponding in situ implants. The entrapment of the droplet over 34 days supported that the residuals stem from a slower degrading implant shell.

3.2.2.2.2. In vitro drug release of lysozyme from in situ formulations based on DMSO

3.2.2.2.2.1. Lysozyme incorporation into PLGA solutions in DMSO

Due to the solubility of lysozyme in DMSO, the protein could be incorporated into the PLGA solution in several ways. Dividing the solvent portion to separately dissolve protein and polymer before combining them was not possible because of the already high viscosity of a 40 % PLGA solution in DMSO (957 +/- 103 mPas at 20°C). In contrast to a previous report (Chin et al., 1994), where the formation of a viscous gel was described upon addition of about 5 % lysozyme to DMSO, the viscosity of the protein solution in DMSO was no issue. Solutions containing 0 %, 3.4 %, 7.1 % and 9.6 % lysozyme in DMSO had viscosities of only 1.5, 2.4, 4.4 and 5.4 mPas at 20°C, respectively. The reason for the discrepancy with the previous report could be due to the used protein material or the dissolution process. Lyophilized lysozyme gelled immediately after coming in contact with DMSO. Without sufficient agitation, the semisolid particles sedimented prior to dissolution, which led to a gel layer at the bottom of the vials. This gel layer impeded further protein dissolution.

Therefore, the following three methods to prepare lysozyme containing biodegradable in-situ forming implant and microparticle systems were investigated. Lysozyme was 1. dispersed in PLGA solution (“dispersed”), 2. mixed with PLGA powder prior to DMSO addition (“co-dissolution”) and 3. lysozyme was dissolved in DMSO prior to PLGA addition (“dissolved”).

High shear mixing was avoided due to potential stability problems. Large agglomerated gel particles of up to 400 μm in size (Figure 89a) were observed when lysozyme was added stepwise to a 40 % PLGA solution under vigorous vortexing. The high viscosity of the polymer solution made it difficult to disperse the rapidly swelling lyophilized protein material.

DMSO addition to a PLGA / lysozyme powder blend prepared by vortexing resulted in gel particles of up to 100 μm (Figure 89b). Although the distribution of lysozyme was improved when compared to the dispersion method, a homogenous mixture was not obtained.

PLGA addition to lysozyme solutions in DMSO resulted in very homogenous in situ implant formulation (Figure 89c). But instead of an expected solution, a dispersion of a liquid phase with droplets of less than 10 μm was obtained after dissolving the polymer in the protein solution. Lysozyme and PLGA were incompatible in DMSO.

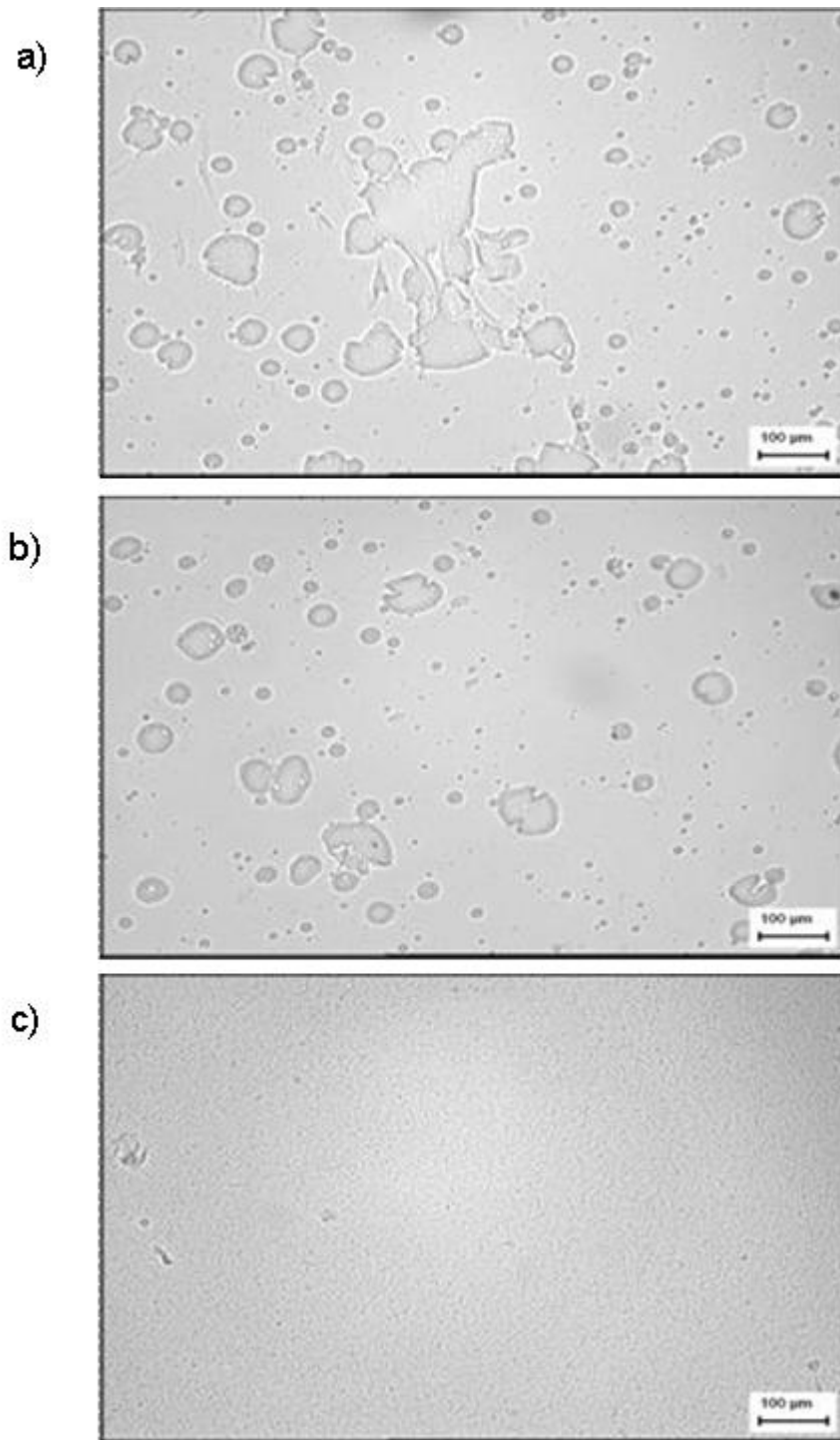


Fig. 89: Microscopic appearance of 2.7 %lysozyme containing 40 % PLGA (RG 502H) solutions depending on preparation technique a) lysozyme to PLGA solution, b) DMSO to PLGA / lysozyme blend and c) PLGA to lysozyme solution

The quality of the protein-containing polymer solutions affected the in vitro drug release of corresponding in-situ microparticle and in-situ implant systems (Figures 90a and b). Increased release rates were obtained with both in-situ formulations, if large protein aggregates were present in the PLGA solution. The effect of the particle size on the initial release from both in-situ systems was attributed to a contribution of a sedimentation process, which was indicated by noticing lysozyme particle accumulation at the bottom of the PLGA solutions upon standing. As previously reported, sedimentation of large drug particles could alter the drug distribution in polymer solutions (Maa and Hsu, 1997). Average diffusion pathways from aggregates to the implant surface could be shortened due to the formation of surface near protein reservoirs, which were released more rapidly.

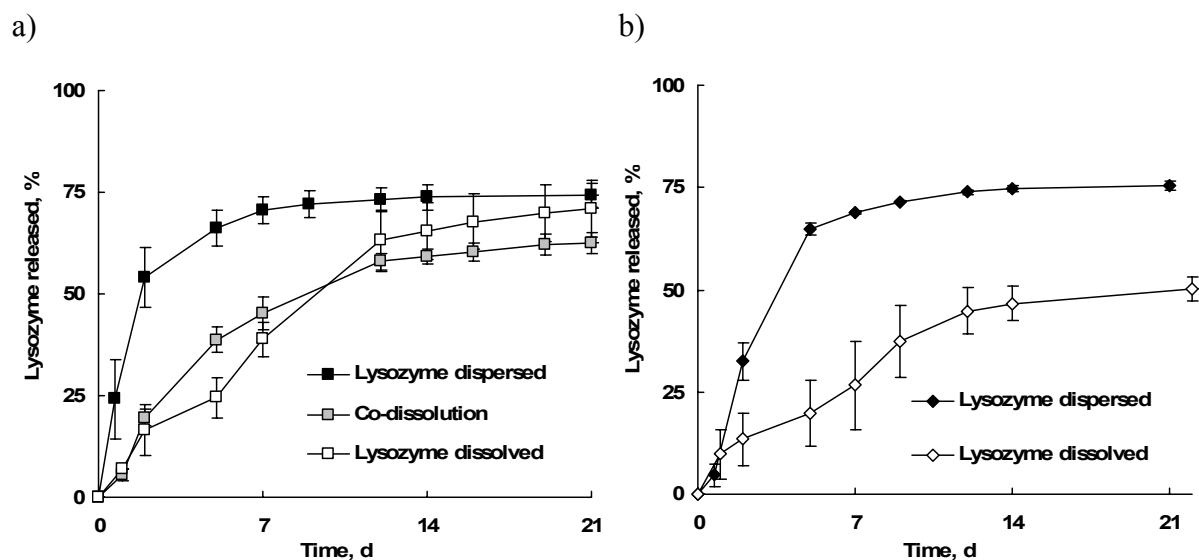


Fig. 90: Lysozyme release dependent on preparation method: a) in-situ microparticle formulations [40 % PLGA (RG 502H) solutions in DMSO containing 4 % lysozyme (relative to polymer) emulsified into sesame oil] and b) corresponding in-situ implants

3.2.2.2.2. Effect of polymer concentration

The rate of lysozyme release from in situ microparticles decreased increasing the PLGA (RG 502H) concentration (Figure 91a), which was in agreement with previous reports (Lambert and Peck, 1995). The initial release of lysozyme from comparable in situ implants was previously

attributed to protein situated in or nearby the polymer-poor phase forming during polymer phase separation in contact with water (Graham et al., 1999). Accordingly, a higher PLGA concentration, which leads to an increased viscosity of the polymer solution, would decelerate the phase separation and thus decrease the release rate. Especially the increase from 30 % to 40 % decreased the initial lysozyme release and changed the release characteristic from a burst-type profile to controlled release over 12 days.

A little inflection in the curve of the in situ microparticles with the 40 % PLGA concentration at about 5 days divided the initial from the second release phase. According to a previous report on the mass loss behavior of conventional microparticles based on the same PLGA grade (Tracy et al., 1999), the release phase between 5 and 12 days could be ascribed to polymer erosion.

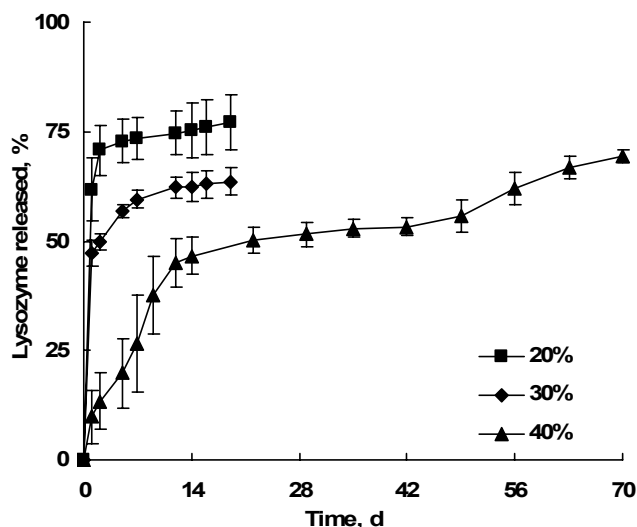


Fig. 91: Lysozyme release from in situ microparticles (RG 502H in DMSO, 4 % drug loading based on polymer emulsified with sesame oil) as a function of the polymer concentration

A similar effect of the polymer concentration on the initial release was seen with in situ implants containing capped PLGA (Figure 92). The initial diffusional drug release phase decreased increasing the concentration of RG 502S from 20 % over 30 % to 40 %. Interestingly the initial phase appeared to be prolonged beyond 2 days for the 30 % concentration, which led to an overlay with the following erosion phase and thus to a more continuous pattern. The reason for the prolonged initial release phase with the 30 % concentration could be due to a balance between

the phase separation / drug release rate in the nascent implant and a suitable porosity of the resulting hardened implant. Thus, a large burst as with 20 % or a phase of limited drug release between 4 and 7 days as with 40 % was not obtained with the 30 % polymer concentration. The recovery of lysozyme after the erosion phase (7-21 d) was between 81 +/- 10 % (40 % RG 502S) and 96 +/- 2 % (30 % RG 502S).

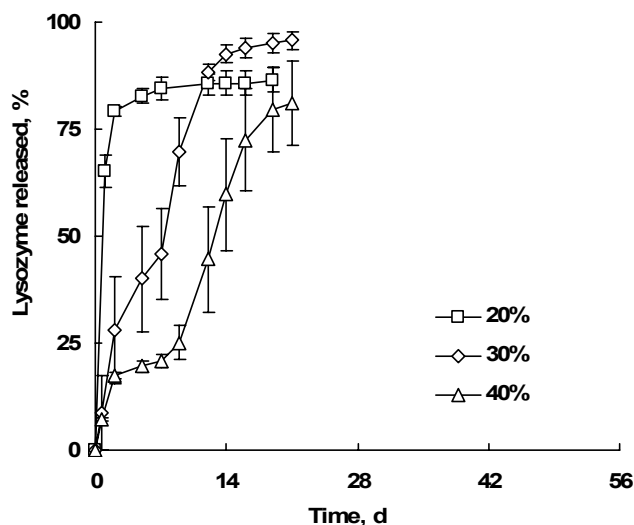


Fig. 92: Lysozyme release from in situ implants (RG 502S in DMSO, 4 % drug loading based on polymer) as a function of the polymer concentration

An increase of polymer concentration from 20 % to 30 % also decreased the initial release of in situ microparticles based on capped PLGA, whereas no further decrease was achieved at 40 % (Figure 93a). The release curves of the formulations based on 30 % and 40 % PLGA solutions were very similar except for the extent of an apparent late release phase at day 42. Interestingly, this phase occurred at the same time-point were it was observed with triacetin-based formulations but with the uncapped polymer type and was attributed to release of drug from the slower degrading shell of the formulations (paragraph 3.2.1.2.2.).

Previously, a concept was introduced, which divided the total drug loading into different compartments in order to facilitate modeling of protein release from PLGA microparticles (Batycky et al., 1997). Accordingly, comparable release pattern (relative expression) independent of the polymer concentration would suggest comparably distributed drug portions between the compartments.

The shape of the release curve obtained with the in situ microparticles with 20 % polymer concentration differed from the higher concentrations. A distinct polymer erosion-controlled release phase between 7 and 21 days was not observed. This could indicate that lysozyme was probably not located in the center of the PLGA matrix, which, based on previous findings (Li et al., 1990b and Tracy et al., 1999), was expected to be responsible for protein release during the first erosion period of the capped PLGA (7 - 21 days). A late release phase, resulting from drug release from the slower degrading external layer of the PLGA matrix, was again indicated.

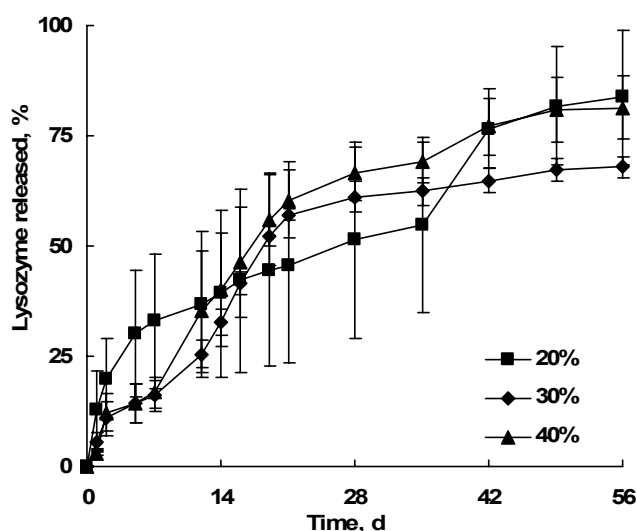


Fig. 93: Lysozyme release from in situ microparticles (RG 502S in DMSO, 4 % drug loading based on polymer emulsified with sesame oil) as a function of the polymer concentration

A release profile without erosion phase between 7 and 21 days, as observed with the 20 % in situ microparticle formulation based on capped PLGA, was also seen with an in situ microparticle emulsion, which was formulated in an alternative way. This formulation had a similar total composition as an in situ microparticle emulsion based on a 40 % PLGA solution. However, the polymer was suspended in the oil phase with which the solution of lysozyme in DMSO was emulsified. The PLGA particles were thereby expected to soak up the protein solution, which should lead to a higher lysozyme concentration at or near the surface of the solvated particles.

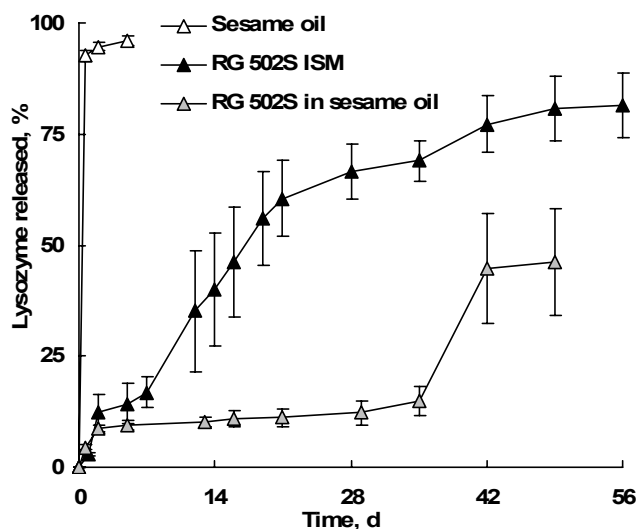


Fig. 94: Lysozyme release from an emulsion of lysozyme dissolved in DMSO and emulsified with sesame oil or from the same emulsion additionally containing capped PLGA suspended in the sesame oil or from the corresponding in situ microparticle emulsion (40 % PLGA solution containing 4 % drug - based on polymer emulsified with sesame oil; phase ratio 1:1, 50 mixing cycles at 1/s)

Interestingly, the release from this system showed a relatively little burst (9 % in 2 days) and a distinct second burst at 42 days (Figure 94). More or less immediate release was obtained, if the lysozyme solution in DMSO was emulsified with sesame oil only. Thus, the decreased burst was related to the presence of polymer and not due to protein instability during the oil-in-oil emulsification. The low burst indicated that lysozyme could be sufficiently incorporated into the shell of the solvated particles or was adsorbed to the surface of the particles during the emulsification step. The non-appearance of a release phase between 7 and 21 days supported protein enrichment in the external region of the polymer solution phase and the absence of protein in the core of a PLGA matrix.

The relatively large late release phase at day 42, which would be attributed to release upon erosion of the slower degrading shell of PLGA matrices (paragraph 3.2.1.2.2.), fitted also well with the assumption of lysozyme enrichment in the external layer of the polymer particles. The onsets of this late phase with formulations based on uncapped (triacetin-based in situ implant) and capped PLGA appeared to be similar, which would not be expected from the apparent degradation rates for both polymers (Tracy et al., 1999) but would be in agreement with the

degradation by random chain scission, where the end-group functionality should be of minor importance. This could indicate that capping of the free carboxylic groups just retards the onset of autocatalytic effects in the core of PLGA matrices, be it due to slower water diffusion into the polymer bulk (hydrophobicity) or due to a contribution of the chain-ends of PLGA on the initiation of autocatalytic effects.

3.2.2.2.3. Comparison of uncapped and capped PLGA

Lysozyme release from in situ microparticles based on 4 % (based on polymer) lysozyme-loaded PLGA solutions in DMSO depended on the PLGA type (Figure 95). The initial release was higher with the uncapped compared to the capped polymer type at 20 % and 30 % polymer concentration, which could be due to a faster phase separation with the more hydrophilic, uncapped polymer. A release characteristic dominated by the initial burst could be avoided with a concentration of 20 % capped polymer, whereas 40 % uncapped polymer were necessary to sufficiently slow down the phase separation and obtain controlled release.

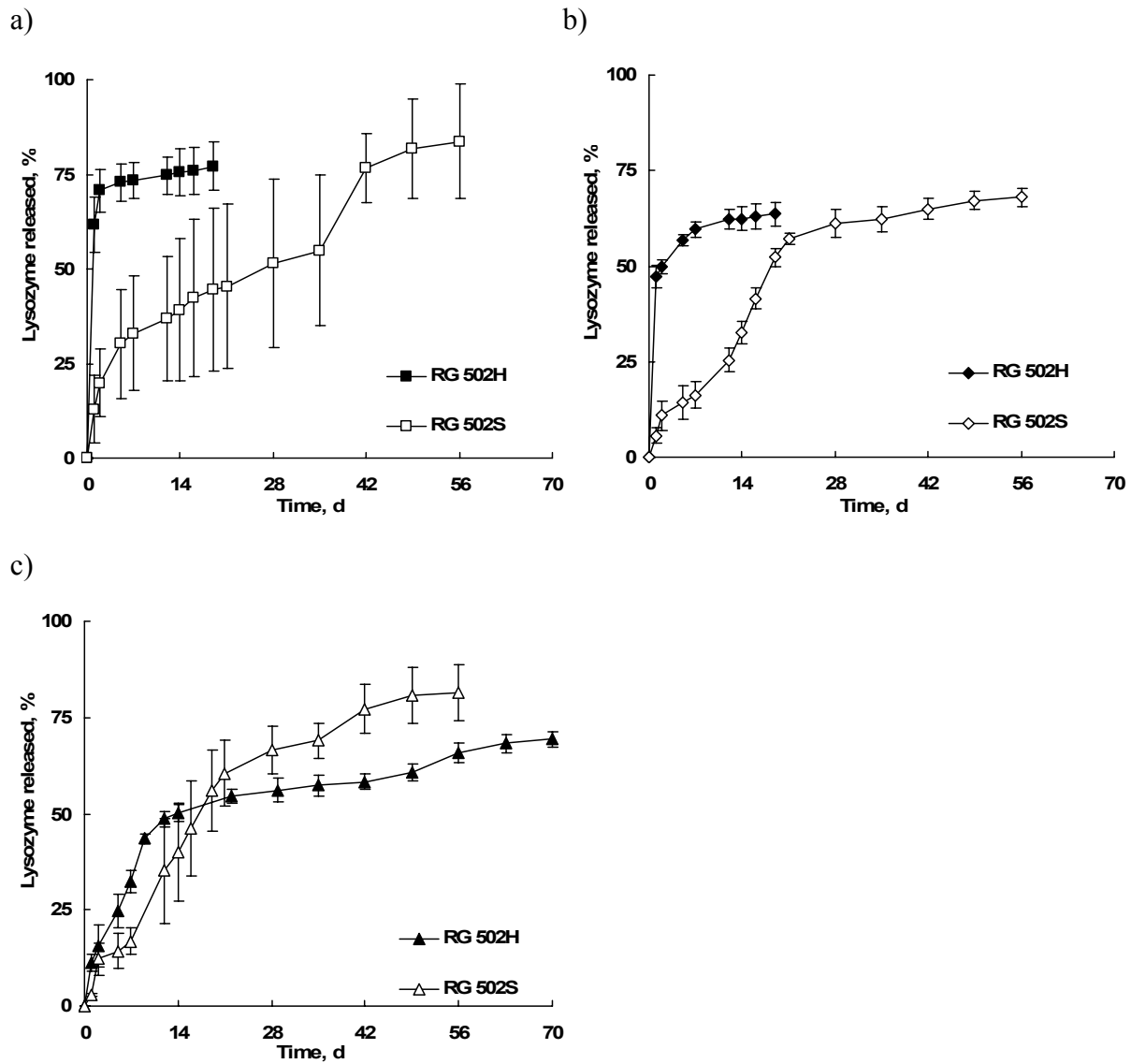


Fig. 95: Lysozyme release from in situ microparticles based on a) 20 %, b) 30 % and c) 40 % PLGA in DMSO (4 % drug loading based on polymer) emulsified with sesame oil as a function of the polymer type (capped PLGA, RG 502S vs. uncapped PLGA, RG 502H)

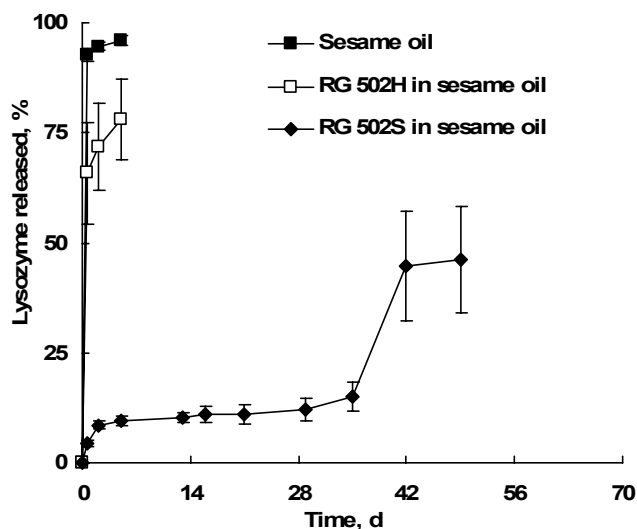


Fig. 96: Lysozyme release from oil in oil emulsions of lysozyme solutions in DMSO with sesame oil with or without suspended PLGA (total composition corresponding to a ISM-emulsion based on a 40 % PLGA solution containing 4 % drug - based on polymer; phase ratio 1:1, 50 mixing cycles at 1/s) as a function of the polymer type

A better control of the initial release with capped PLGA compared to uncapped PLGA was also obtained, when lysozyme dissolved in DMSO was emulsified into the sesame oil containing suspended PLGA particles (similar to an ISM-system based on a 40 % PLGA solution). Only 9 % of the lysozyme loading was released within 2 days from the formulation containing capped particles, whereas 78 % were released with the uncapped polymer (Figure 96). This indicated a better entrapment of lysozyme into the particles upon soaking up the protein solution. The better entrapment could be explained by a slower phase separation of the soaked and solvated particles upon contact with water as suggested for the in situ microparticle formulations. However, a contributing factor with this approach could be a deeper penetration of lysozyme into the capped PLGA particles. A faster dissolution of capped compared to uncapped PLGA was shown previously with NMP (Vivian Voigt, 2005), which has a comparable properties as a PLGA solvent (Hildebrandt solubility parameters: $\delta_{\text{NMP}} = 22.9 \text{ MPa}^{1/2}$ and $\delta_{\text{DMSO}} = 26.6 \text{ MPa}^{1/2}$; values from product brochure of AgsolEx-1, ISP, US and from Terada and Marchessault, 1999).

The release of in situ implant formulations confirmed that the main difference between both polymers at a concentration of 40 % was the erosion-controlled release phase (Figure 97). The initial, diffusion-controlled release phase could be well distinguished from the erosion-controlled

phase with the capped polymer type. The shape of the erosion-controlled portion of the curve (7-21 days) was sigmoidal. The amount of drug released during this phase was higher with the capped compared to the uncapped PLGA grade.

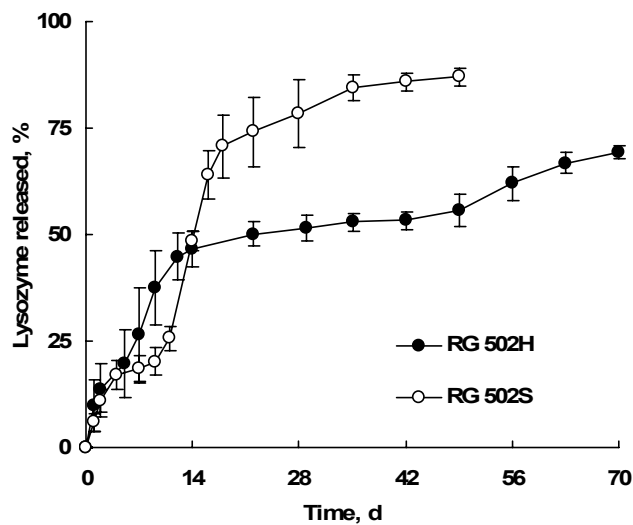


Fig. 97: Lysozyme release from in situ implants based on 40 % PLGA in DMSO (4 % drug loading based on polymer) as a function of the polymer type (capped PLGA, RG 502S vs. uncapped PLGA, RG 502H)

3.2.2.2.4. Drug loading

Lysozyme release from the in situ microparticle formulations with uncapped PLGA was dependent on drug loadings between 2 % and 6 % (Figure 98). Especially the initial release increased with increasing drug loading. A late release phase occurred but was of minor importance.

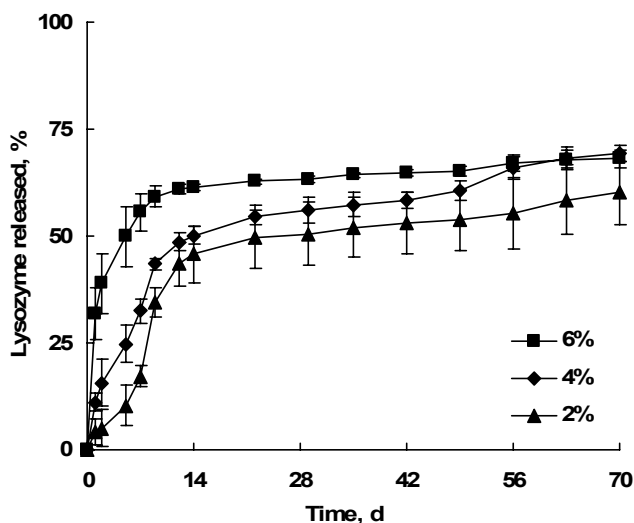


Fig. 98: Lysozyme release from in situ microparticles consisting of 40 % uncapped PLGA (RG 502H) in DMSO emulsified with sesame oil as a function of drug loading (based on polymer)

The release patterns of the corresponding in situ implants showed a similar effect of the lysozyme loading. The profiles could be differentiated into four phases (Figure 99a). The plot of the fraction released against square root of time (Higuchi plot) highlighted the different release rates within the first 14 days. At about 14 days, the profiles ran into a plateau. A late release phase commenced with all drug loadings after about 42 days. The amount of lysozyme released within this release phase appeared to be dependent on the drug loading.

The linearization of the release profiles of the in situ implants with 4 % and 6 % drug loading (based on polymer) was obtained between 0 and 5 days with the square root of time expression (Higuchi plot) of the release data. The behavior indicated a diffusion-controlled initial release from the nascent polymer matrices (Figure 99b), as reported previously (Higuchi, 1961). The release within the initial period was probably due to drug, which was located in the

interconnected polymer-poor phase in the nascent implants, as suggested previously (Graham et al., 1999). Following the idea to divide the total drug payload into different compartments (Batycky et al., 1997), this drug fraction would constitute the first compartment.

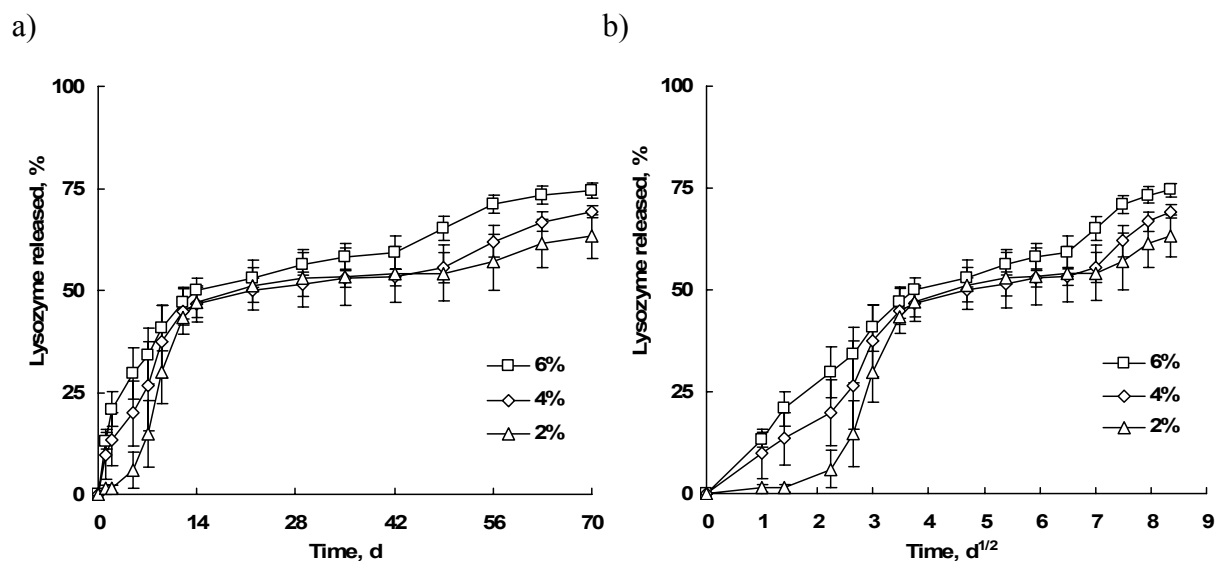


Fig. 99: Lysozyme release from in situ implants consisting of 40 % uncapped PLGA (RG 502H) in DMSO as a function of drug loading (based on polymer) expressed as a) fraction released over incubation time and b) fraction released over the square root of time

The release phase between 5 and 14 days appeared to be sigmoidal in shape, which was most obvious in case of the 2 % drug loading, where no initial release interfered (Figure 99). In agreement with the morphology of the in situ implant (Figure 85) and previously reported data on the mass loss of conventional microparticles (based on the same PLGA grade) during incubation (Tracy et al., 1999), this phase would be attributed to polymer erosion-controlled drug release from the center of the polymer matrices. PLGA is known to degrade faster in the center of a matrix due to autocatalysis (Vert et al., 1997).

Opposing trends were seen, when the drug fractions released within the initial (0-5 days) and the second release phase (5-14 days) were plotted against the drug loading (Figure 100). The portion of lysozyme released within the first 5 days increased, whereas the fraction released between 5 and 14 days decreased with increasing drug loading. However, the fraction of lysozyme released within the both periods (0-14 days) appeared to be independent of the drug loading.

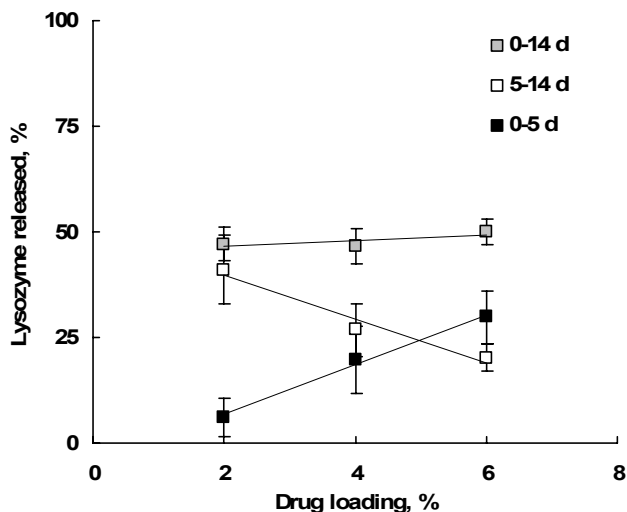


Fig. 100: Relative amounts of lysozyme released from in situ implants consisting of 40 % uncapped PLGA (RG 502H) per time period in dependence of the drug loading

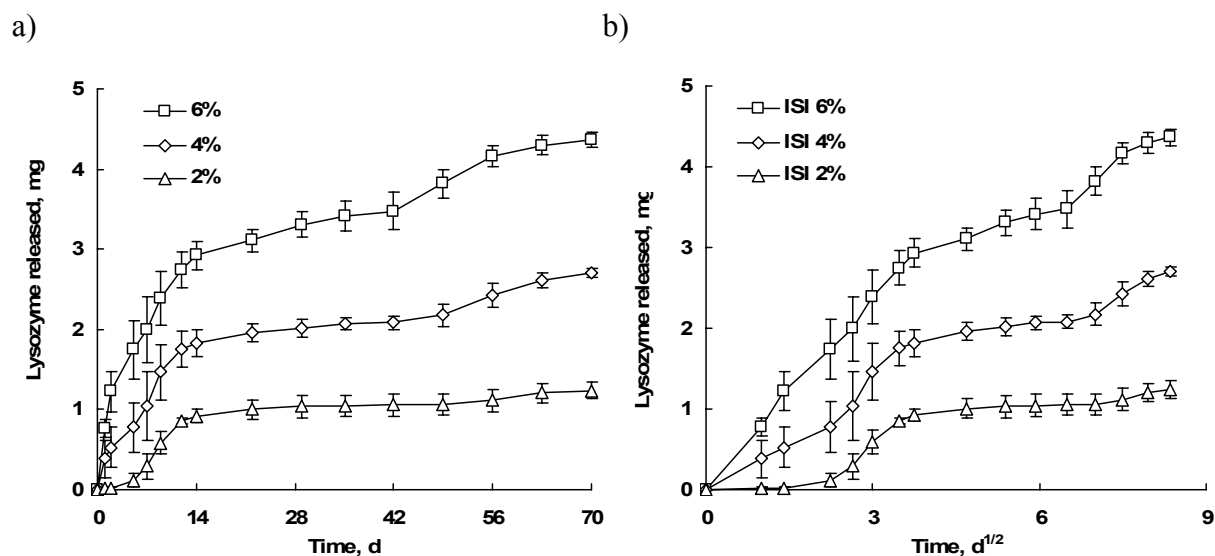


Fig. 101: Lysozyme release from in situ implants consisting of 40 % uncapped PLGA (RG 502H) in DMSO as a function of drug loading (based on polymer) expressed as a) amount (mg) released over incubation time and b) amount (mg) released over the square root of the incubation time

The absolute expression of the release data suggested, that only the initial release period (0-5 days) was affected by an alteration of the drug loading between 2 % and 6 % (Figures 101a and

b). A plot of the amount of lysozyme released within both time intervals against the drug loading visualized this difference (Figure 102). The total mass of lysozyme released within the initial time-period increased with increasing drug loading, whereas the released amount between 5 and 14 days appeared to be independent of the drug loading. A slight overlay of both release phases needs to be considered when interpreting the inclinations.

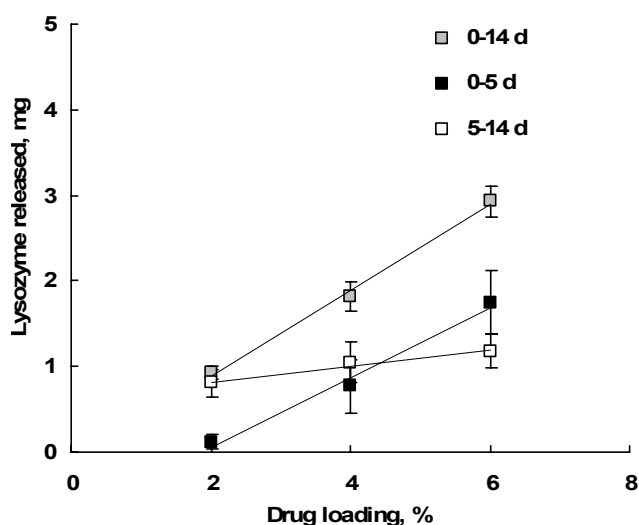


Fig. 102: Absolute amount of lysozyme released from in situ implants consisting of 40 % uncapped PLGA (RG 502H) per time period in dependence of the drug loading

A dependence of the initial release on the loading of lysozyme could be simply attributed to an accelerated phase separation rate due to an increase of hydrophilic additives (here: drug) in the polymer solution (Graham et al., 1999). However, the converging release patterns after 14 days could indicate the presence of a drug loading-independent super-compartment, which divides into the initial diffusion-controlled and the following erosion-controlled drug compartment. The formation of these sub-compartments appeared to be drug loading-dependent probably due to the limited capacity of the erosion compartment for lysozyme. On the basis of a previous suggestion (Batycky et al., 1997), such a scenario could be explained with polymer-poor phase with or without immediate access to the release medium. However, protein stability issues could also be involved considering drug adsorbed to the interface between polymer-poor and polymer-rich phase or to the surface of hardened polymer as the saturable compartment, which would be mobilized by polymer erosion.

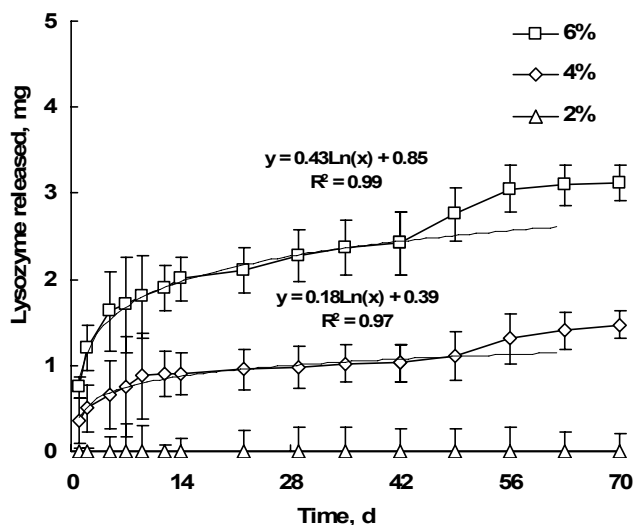


Fig. 103: Lysozyme release from in situ implants consisting of 40 % uncapped PLGA (RG 502H) in DMSO as a function of drug loading (based on polymer) normalized against the release profile of the 2 % drug-containing formulation

Interestingly, the release data obtained after normalization against the profile of the 2 % lysozyme-containing formulation, in order to eliminate the drug loading-independent erosion-phase between 5 and 14 days, apparently represented a logarithmic release kinetic between 0 and 42 days (Figure 103). Such a kinetic has never been reported. However, the kinetic of lysozyme adsorption to contact lenses appeared to possess a logarithmic character after an induction period (Castillo et al., 1985). If dissolution of reversible aggregates would show the same kinetic as aggregation, the unusual kinetic could be explained with an underlying disaggregation-controlled release mechanism of lysozyme. The phase of minimal lysozyme release between 14 and 42 days could therefore be related to the initial release period.

The presence of the release phase beyond 42 days after normalization as well as the deviation from the logarithmic release revealed, that this release phase differed from the other two. As discussed for long-term release data on other formulations based on uncapped PLGA (paragraphs 3.2.1.2.2. and 3.2.2.2.2.2.), the late release phase beyond 42 days could be due to a second erosion-controlled release phase, where drug entrapped into the slower degrading implant shell would be released.

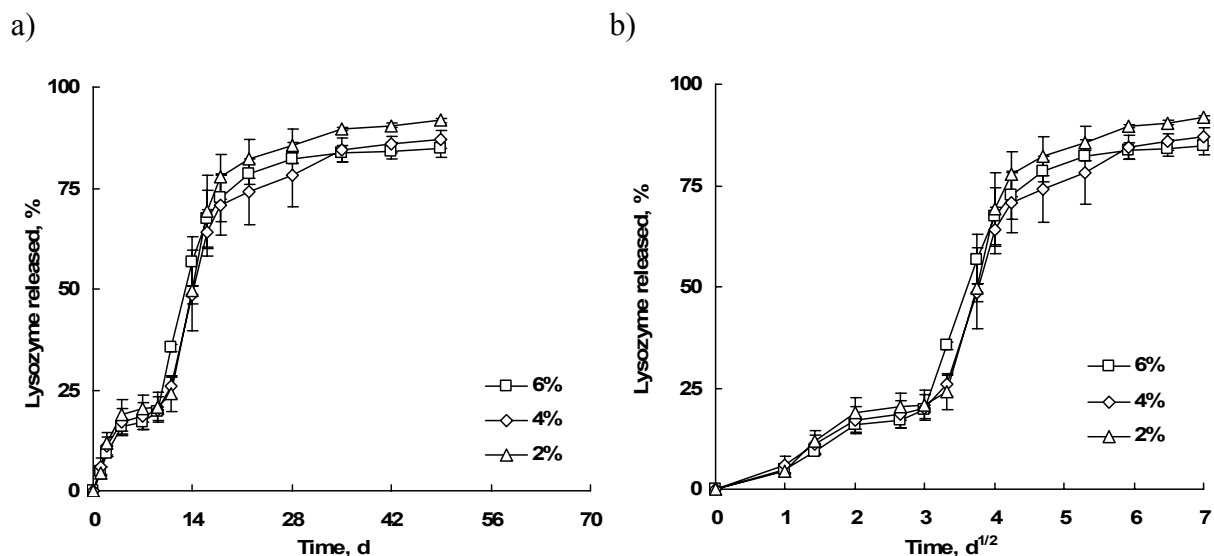


Fig. 104: Lysozyme release from in situ implants consisting of 40 % capped PLGA (RG 502S) in DMSO as a function of drug loading (based on polymer) expressed as a) fraction released over incubation time and b) fraction released over the square root of the incubation time

The release patterns of in-situ forming implants based on capped PLGA were independent of the drug loading in the range of 2 % to 6 % (Figure 104). The drug appeared to be evenly distributed among the diffusion and the erosion compartments.

Compared to the insensitivity of the in situ implants based on capped polymer to changes of the drug loading, the sensitivity with uncapped PLGA could indicate, that positively-charged lysozyme ($pI \sim 11$) preferentially interacted with free carboxylic groups, which could have led to an altered drug distribution. The preferential adsorption of lysozyme to hydrophilic surfaces was in agreement other reports (Voeroes, 2004 and Park et al., 1998).

3.2.2.2.2.5. Comparison of in situ microparticle with in situ implant systems

The release of lysozyme from in situ microparticle formulations based on 40 % uncapped PLGA was more or less similar to the corresponding in situ implants at 2 % and 4 % drug loading (Figures 105a and b). Similarly to triacetin, DMSO would likely be immiscible with sesame oil, since less than 2 % of DMSO could be incorporated into vegetative oils with comparable fatty acid compositions as sesame oil (peanut, soybean and safflower oil). Hence, the phases separated according to their densities. The oil accumulated at the top, whereas the polymer solution formed an implant-like structure on the bottom of the vial (Figure 88). The implant-like structure and the fast polymer precipitation from the solution in DMSO probably impeded effects of sesame oil incorporated in the polymer solution phase on the release, as proposed for the in situ microparticle formulations based on triacetin (paragraph 3.2.1.2.4.). However, the initial burst was increased with the in situ microparticle system with the 6 % drug loading (Figure 105c).

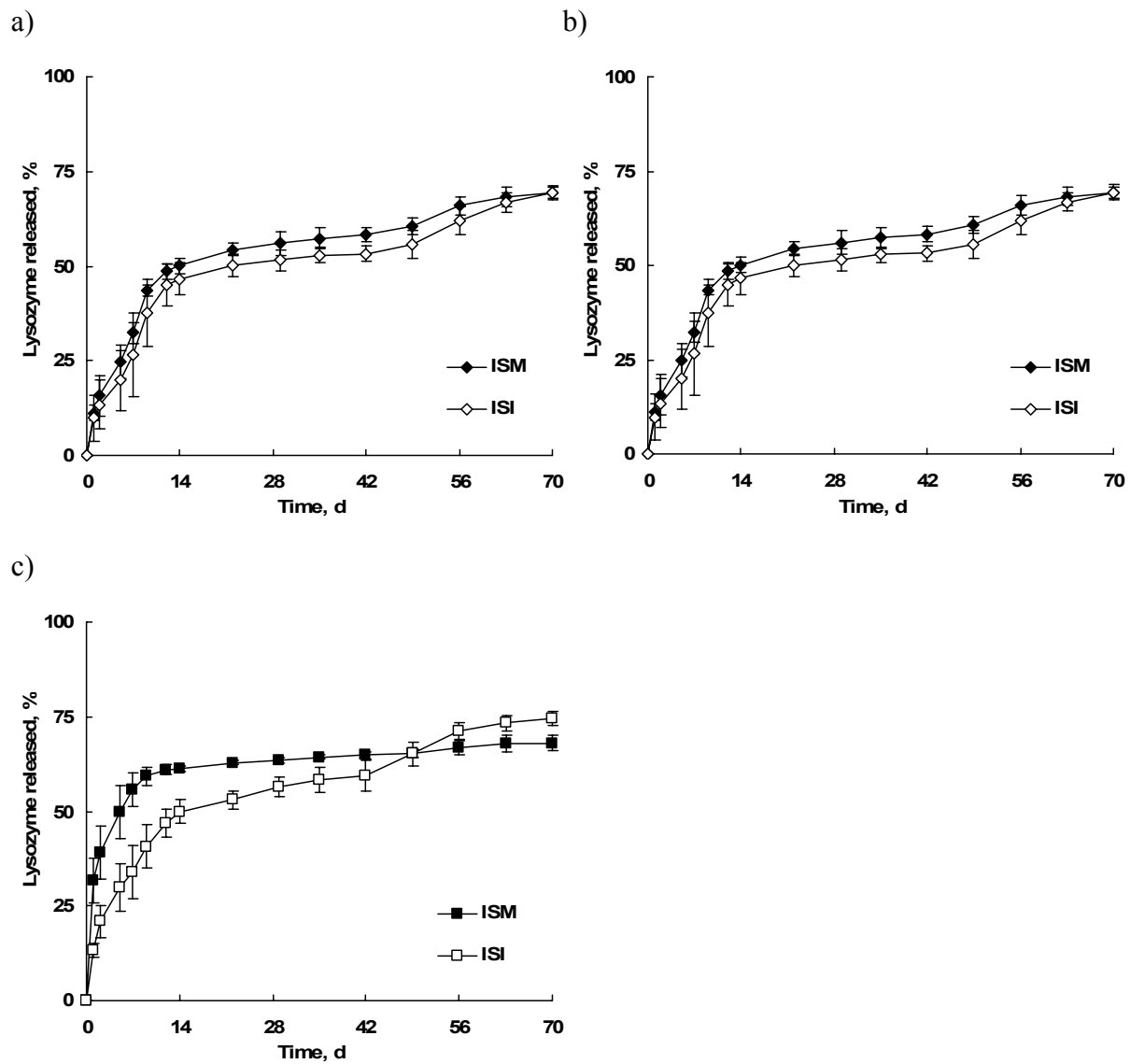


Fig. 105: Lysozyme release from in situ implants (ISI) based on 40 % uncapped PLGA (RG 502H) in DMSO and corresponding in situ microparticle systems (ISM) prepared with sesame oil; a) 2 %, b) 4 % and c) 6 % drug loading (based on polymer)

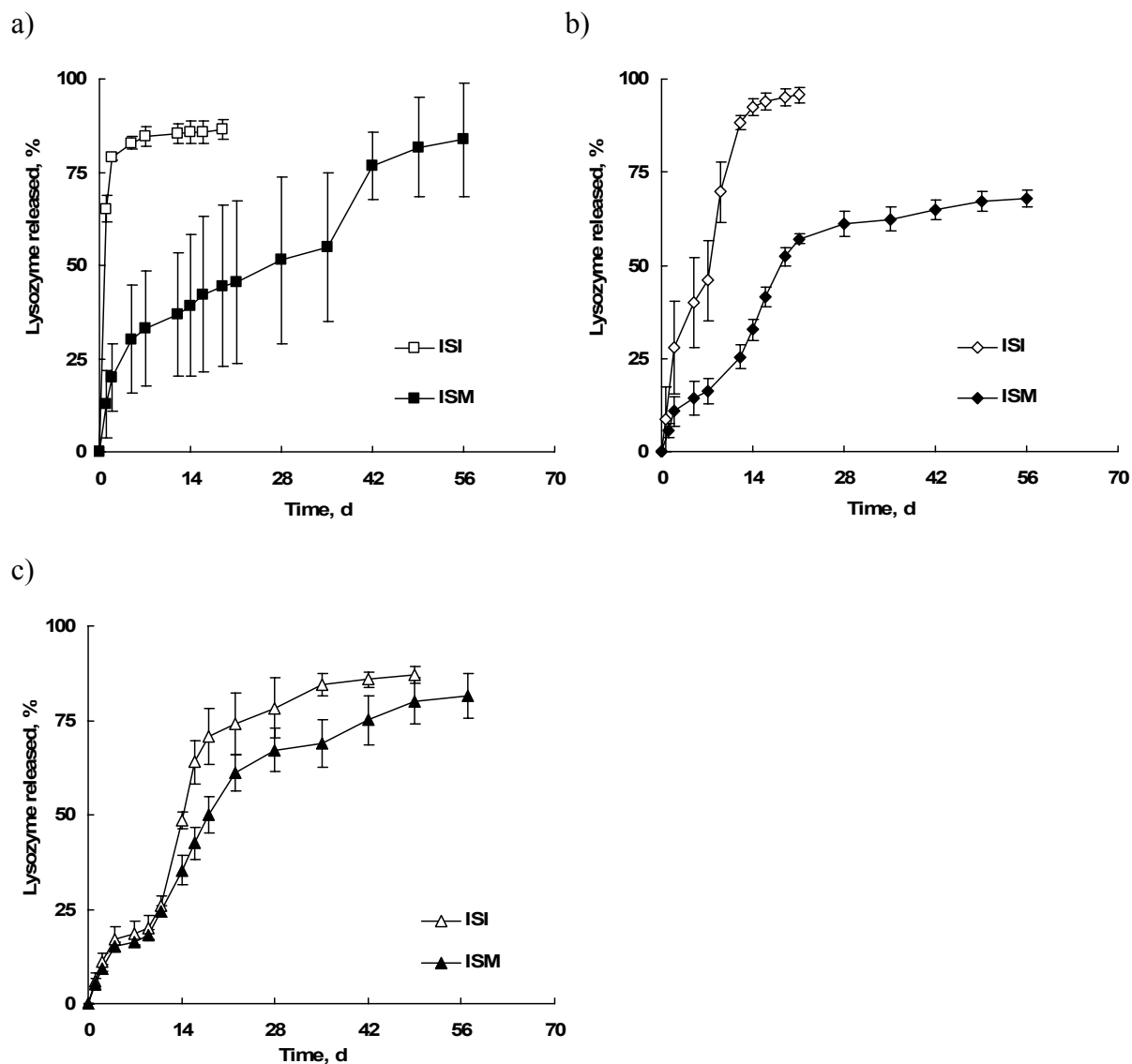


Fig. 106: Lysozyme release from in situ implants (ISI) based on capped PLGA (RG 502S) in DMSO containing 4 % drug (based on polymer) and corresponding in situ microparticle systems (ISM) prepared with sesame oil; a) 20 %, b) 30 % and c) 40 % polymer concentration

In contrast to the very small effect of the drug loading (2-6 %) on the release of the in situ systems based on uncapped polymer, the release profiles differed more pronounced between the in situ systems based on capped polymer when the polymer concentration was varied. The emulsification step with sesame oil decreased the initial release at polymer concentrations 20 % and 30 %, whereas no further decrease was obtained at the 40 % PLGA concentration (Figure

106). The decreased burst was probably due to a slower phase separation after emulsification. A slower phase separation could not be explained with an increase of the polymer concentration due to dissipation of the solvent during emulsification, considering the limited miscibility of DMSO and sesame oil. A possible explanation could be that some sesame oil remained associated with the surface of the polymer solution due to the presence of hydrophobic end-groups (“longer-chain” alkyl alcohol in case of RG 502S) of PLGA at the interface. Such a sesame oil film could decrease the affinity of the polymer solution to the precipitating aqueous medium and therefore decrease the phase separation rate (Brodbeck et al., 1999). That this behavior could be unique for RG 502S was supported through the very high initial releases obtained with in situ microparticle systems based on low concentrations of uncapped PLGA (Figure 95). However, in situ implants with low concentrations of RG 502H were not investigated.

3.2.2.2.2.6. Mathematical modeling of lysozyme release from in situ implants

The interesting release behavior of the 40 % PLGA-containing in situ implant systems based on DMSO as a function (paragraph 3.2.2.2.2.4.) facilitated a quantitative description of the two- or three-phasic release patterns as a function of the drug loading.

Phase 1: Diffusion-controlled release

The release of lysozyme from in situ implants based on 40 % PLGA was attributed to three phases. The first release phase located between 0 and 4-5 days, which was characterized by the polymer phase separation process, could be linearized plotting the cumulative amount released against the square root of time (Figures 101 and 106). According to existing models on matrix diffusion (Siepmann and Peppas, 2001), an empirical description of the release profiles for the first compartment could be

$$M_t/M_\infty^1 = k * t_{rel}^{1/2} \quad (\text{Equation 2}).$$

There, M_t is to the cumulative mass of lysozyme released at time-point t ; M_∞^1 is drug loading of the first compartment; k is a release rate constant and t_{rel} the actual release time. For the capped polymer, the fraction of the total drug loading, which was released from the first compartment,

was independent of the drug loading between 2 % to 6 %. Thus, M_{∞}^1 could be expressed as fraction of the total drug loading (M_{∞}) (Equation 3).

$$M_{\infty}^1 = f * M_{\infty} \quad (\text{Equation 3})$$

The release data indicated a slight lag time (t_{lag}) after immersion of the in situ implants (Figure 106b). The actual release time could be calculated through subtraction of the lag time from the total incubation period (t) (Equation 4).

$$t_{rel} = t - t_{lag} \quad (\text{Equation 4})$$

An empirical description of the release curve could be derived after integration of equations 3 and 4 into equation 2 (Equation 5).

$$M_t/M_{\infty} = \begin{cases} 0 & \text{if } 0 < t < t_{lag} \\ f * k * (t - t_{lag})^{1/2} & \text{if } t_{lag} \leq t \leq t_{end} \end{cases} \quad (\text{Equation 5})$$

The time, where the release phase ended (t_{end}) was 4 days for the formulations containing the capped polymer. The lag time was approximately 0.71 days and the average fraction (f) of the total drug loading, which was released within the initial period from the in situ implants, was 17.16 +/- 2.98 %. The release rate constant (k) was 0.55 d^{-1/2}. The values were averages obtained from the release data of all three drug loadings. The resulting release equation for an estimation of lysozyme release from in situ implants based on 40 % capped PLGA in DMSO in the initial release period would be:

$$M_t/M_\infty = \begin{cases} 0 & ; 0 < t < 0.71 \\ 17.16 \% * 0.55 \text{ d}^{-1/2} * (t - t_{\text{lag}})^{1/2} & ; 0.71 \text{ d} \leq t \leq 4 \text{ d} \end{cases} \quad (\text{Equation 6}).$$

The fit represented the actual release curve in the initial phase (0-4 day) very well but it also revealed the unsuitability to describe the release curve beyond an incubation time of 4 days (Figure 107). The initial time period could represent the interval were the in situ formation (hardening) of the implants occurred (Figure 87).

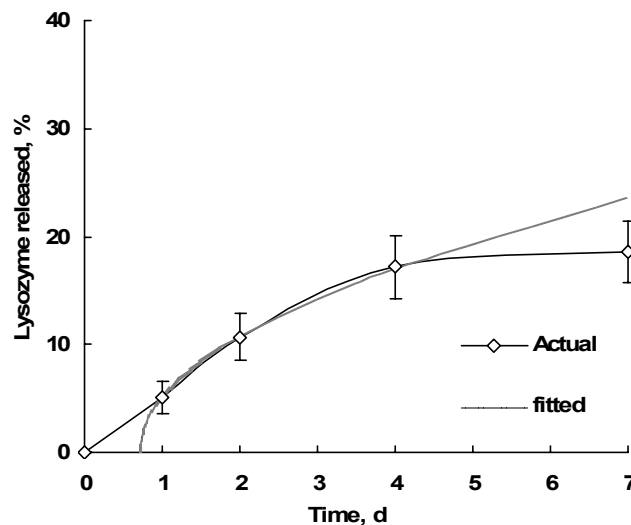


Fig. 107: Actual release and release curve fitted to the in the initial phase (0-4 d) of the actual patterns from in situ implants based on 40 % capped PLGA (RG 502S) in DMSO with lysozyme loadings between 2 and 6 %.

Accordingly, the model for the initial release of the in situ implants based on 40 % uncapped PLGA was formulated. There, the dependence of the fraction (f) released within the first 5 days on the total drug loading had to be addressed. This was solved assuming a linear relation between the compartment and the total drug loading, which was suggested for payloads between 2 % and 6 % (Figure 102). Equation 3 was therefore extended with a term, expressing the increase of drug

release within the initial release period with increasing drug loading (c_0 , %w/w based on polymer) (Equation 7, $R^2 = 0.991$).

$$f = 5.94 * c_0 - 5.24 \quad \text{(Equation 7).}$$

In contrast to the in situ implants with the capped polymer, no lag time was observed with the uncapped polymer. The release rate constant ($k = 0.457 \text{ d}^{1/2}$) was determined from the 4 % and the 6 % drug loading, due to the low release with the 2 % drug containing formulations in the initial period. Thus, the fitting would only include the higher drug loadings. Inserting equation 7 into equation 3 and then into equation 2, the amount released between 0 and 5 days could be expressed with the following equation

$$M_t/M_\infty = (5.94 * c_0 - 5.24) * 0.457 \text{ d}^{1/2} * t^{1/2} \quad \text{(Equation 8).}$$

Equation 8 appeared to reasonably represent the release patterns of the 4 % and the 6 % drug loading within the initial release period (Figure 108). However, the release of the 2 % drug loading was overestimated. The assumption of linear relation between the compartment and the total drug loading would require further refinement, since the onset of the erosion-controlled release phase obviously affected the 5 day time-point at least of the formulation with the 2 % drug loading. Different drug loadings as well as more sampling time-points in the initial release period would be needed therefore.

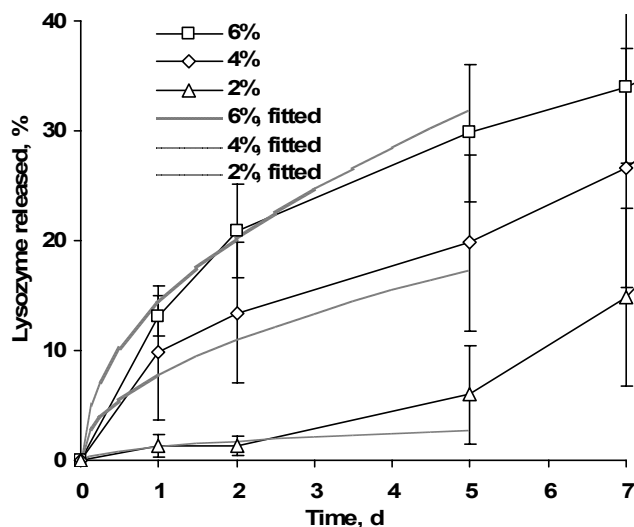


Fig. 108: Actual release and release curve fitted to the in the initial phase (0-5 d) of the actual patterns from in situ implants based on 40 % uncapped PLGA (RG 502H) in DMSO with lysozyme loadings of 2 %, 4 % and 6 %.

Further refinements of the release and extension to a broader range of drug loadings, as well as to the effect of the polymer concentration could be conducted. Investigations should also include considerations with regard to alternative release kinetics (e.g., logarithmic, Figure 103 or exponential, Batycky et al., 1997).

Phase 2: Erosion-controlled release

The erosion of the polymer matrix plays an important role for the release of encapsulated drug, if the diffusional release through the polymer phase is limited, for example due to very low diffusion coefficients. The importance of the erosional release was shown for certain low-molecular-weight drugs as well as for peptides and proteins (Shah et al., 1992 and D'Souza et al., 2005). The kinetic of this release phase is often described as zero-order (e.g., Zolnik et al., 2006). However, other researcher took the more sigmoidal shape of the release curve into account and applied mathematical descriptions, like the Weibull-function (D'Souza et al., 2005) or statistical methods like Monte Carlo simulations (Goepferich, 1997) to approximate the actual release characteristics.

A general model of all occurring release events (burst, induction period and the sigmoidal release curve) was reported previously (Batycky et al., 1997). There, the observed release phases were attributed to different compartments within conventional microparticles. The erosion-controlled release of the used protein was thereby correlated to the degradation behavior of the bulk-eroding PLGA matrix through a link between PLGA-erosion and the porosity of the polymer matrix.

A framework for the modeling of the mass loss behavior of bulk-eroding polymer matrices was developed here, which could facilitate a quantitative description of erosion-controlled release patterns of drugs from PLGA matrices. The basis of the model is the pseudo-first-order kinetic with which the number-average molecular weight (NAMW) of degrading PL(G)A decreases in case of a random chain scission mechanism. The logarithm of the NAMW is thus linearly related to the degradation time. The same would be true for the complete molecular weight distribution, which enables to visualize the degradation process as a time-dependent shift of the molecular weight distribution of the polymer to lower molecular weights (Figure 109), which is typically seen in gel permeation chromatograms following degradation processes (Zhu et al., 1991 and Birnbaum and Brannon-Peppas, 2003). The incubation time could be also expressed as z-axis in a three-dimensional Cartesian coordinate system.

According to Tung (1966), Figure 109 also exemplifies the possibility to approximate the molecular weight distribution of polymers (here: RG 502H with weight-average molecular weight = 10850 g/mol and polydispersity 1.94) with a normal distribution, if the detector response (\sim molar concentration) is plotted against the logarithm of the molecular weight. The fitting of the molecular weight distribution of PLGA at the initial time-point revealed a mean

($\mu(t=0)$) of 9.076 and a standard deviation (σ) of 0.805 ($R^2=0.992$ with amplitude 2.294). Ideally, a change of this standard deviation during degradation would not be expected based on a random chain scission of a homogeneously degrading PLGA matrix.

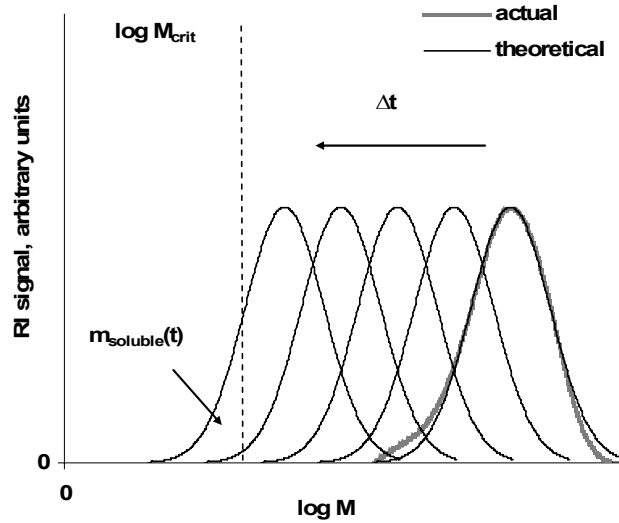


Fig. 109: Schematic representation of the effect of the degradation process on the molecular weight distribution of a PLGA matrix homogeneously degrading according to a random chain scission mechanism

The progressive decrease of the molecular weight leads to the crossing of a critical molecular weight (M_c) boundary, where the oligomers become soluble ($M_c = 1050\text{--}1150$ g/mol; Park, 1994). As depicted in Figure 109, the area under the curve (AUC) below the critical molecular weight would represent the mass of soluble oligomers, which are able to diffuse through the PLGA matrix. Assuming the formation of soluble oligomers as rate-controlling step for their release, the time-dependent formation of a soluble oligomer mass fraction ($f_{sol}(t) = m_{sol}(t) / m_{total}$), can be expressed accordingly (Equation 9).

$$\frac{m_{sol}(t)}{m_{total}} = \int_0^{\ln M_c} \frac{e^{-\frac{(\ln M - \mu(t))^2}{2\sigma^2}}}{\sigma\sqrt{2\pi}} d \ln M \quad (\text{Equation 9})$$

Since the degradation process proceeds according to a pseudo first order kinetics the time-dependent mean of the lognormal distribution ($\mu(t)$) of molecular weights could be expressed as follows

$$\mu(t) = \mu(t=0) - k' * t \quad (\text{Equation 10}),$$

where k' is the first-order degradation constant and t the incubation time. A pseudo-first-order degradation constant (k'_{obs}) of 0.116 d^{-1} was obtained for the linear decrease of the natural logarithm of the NAMW from the analysis of PLGA powder (RG 502H) upon incubation in the release buffer (paragraph 3.1.2.1.). The value was in agreement with previously reported values obtained with a similar PLGA grade (Tracy et al., 1999). Using a critical molecular weight of 1100 g/mol (Park, 1994), the fraction of soluble oligomers generated upon degradation of the PLGA based in situ implants could be determined for defined time-points with MS Excel.

However, the resulting pattern was much slower compared to the release of lysozyme (Figure 110a) and did not correspond to the loss of the whole implant core within 14 days of incubation (Figure 2). The underestimation of the degradation was likely caused by an underestimated degradation rate. The commonly applied degradation methodology focuses on the analysis of the residual polymer mass. This disregards the heterogeneous degradation of PLGA matrices due to autocatalytic effects in the core (Vert et al., 1997). Thus, an “average degradation rate constant” of the PLGA matrix is determined, where the influence of the implant shell increases with increasing incubation time, which results in an underestimation of the implant core and an overestimation of the implant shell degradation. However, a twice higher degradation rate than the observed one resulted in an excellent representation of the release curve of lysozyme with the calculated generation of soluble oligomers in the core of the PLGA matrix (Figure 110b).

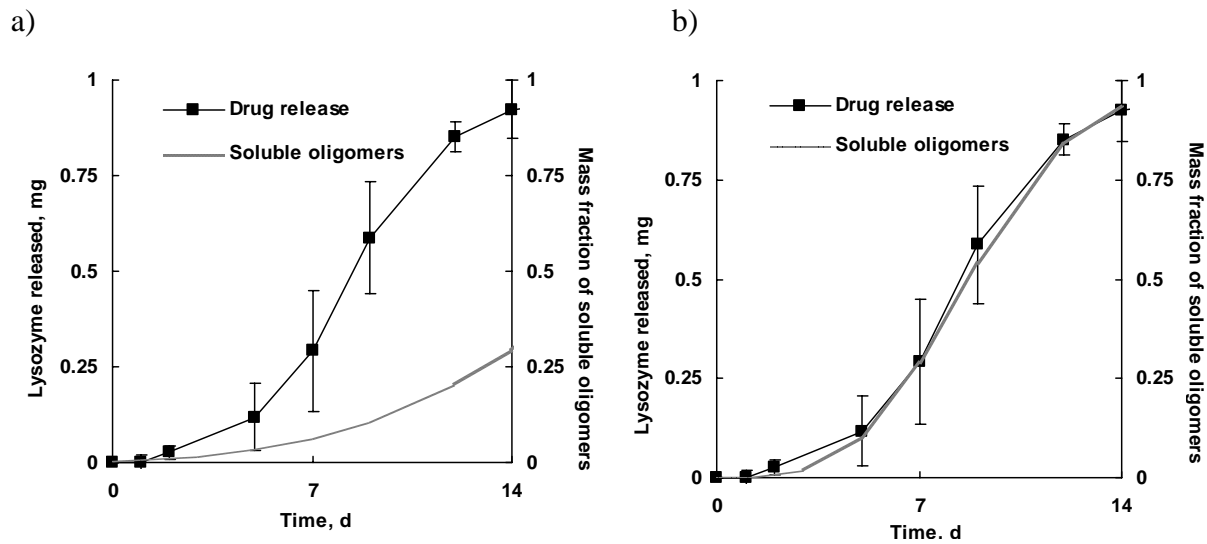


Fig. 110: Comparison of the drug release pattern of the in situ implant based on 40 % RG 502H in DMSO containing 2 % lysozyme with the simulated generation of soluble PLGA oligomers from the implant core during incubation in release medium a) with an experimentally determined degradation rate $k'_{obs} = 0.116 \text{ d}^{-1}$ and b) with an estimated degradation rate for the core of $k'_{est} = 2 * k'_{obs}$

Phase 3: Erosion-controlled release?

The assumption of erosion-controlled lysozyme release from the core and from the shell of the implants was in agreement with the occurrence of a late release phase of lysozyme between days 42 and 70 independent of the drug loading. The biological activity of the material was 61 +/- 8 %. An observation of a late protein release between 70 and 90 days was already reported previously for microparticles based on a higher molecular weight 50:50 PLGA [32]. However, it was attributed to an experimental error. That this release phase has been rarely reported is rational, considering the location far beyond the commonly expected release period for the investigated polymer types (Wei et al., 2004). However, this would have to be considered for drug products for safety reasons. Based on the erosion model, the ratio of both degradation rates could be roughly estimated from the ratio of the midpoints of both erosion phases (56 d / 9.5 d). Thus, the degradation rate for the matrix shell appeared to be six-times slower compared to the matrix core. Although total protein recoveries of 63-74 % were observed, some uncertainties for the

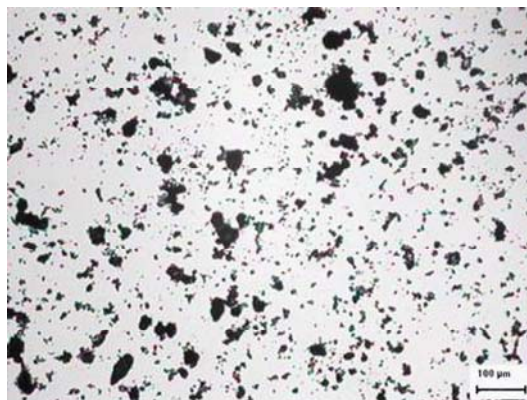
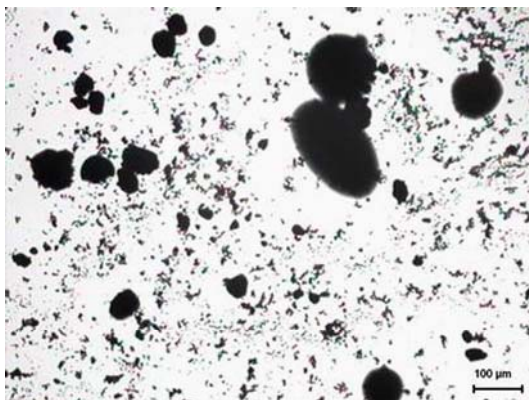
prediction of the drug loading dependent distribution of lysozyme among the compartments of the in situ implants remain.

3.2.2.2.2.7. Addition of release modifying agents

Parenterally applicable excipients were co-incorporated into in situ implant formulations based on 40 % uncapped PLGA (RG 502H) in DMSO in order to obtain more continuous release profiles and higher drug recoveries after release. Magnesium hydroxide was selected as buffering agent, which was reported to inhibit proteolytic degradation of BSA through weakening of the acidic microclimate developing during PLGA degradation (Zhu and Schwendeman, 2000b). Disodium edetate (Na EDTA), usually applied in protein stabilization as chelating agent, was thought to potentially compete with the carboxylic acid groups of uncapped PLGA for the positively charged lysozyme (pI 11 and 14 kDa) and therefore decrease protein adsorption to the polymer surface. The amphiphilic polyvinyl pyrrolidone (PVP) was reported to inhibit protein adsorption onto surfaces (Robinson and Williams, 2002) and the arginine-rich, positively charged protein salmon protamine sulfate was also added as competitor for lysozyme adsorption. It was recently reported that basic additives, such as arginine derivatives (Shiraki et al., 2004) and polyamines (Kuduo et al., 2003), would not only prevent proteins from aggregation but even decrease heat-induced denaturation, which requires thermodynamic stabilization.

Except for PVP, which was soluble, all other substances were insoluble in DMSO. Macroscopic examination of the raw materials revealed some larger particles in magnesium hydroxide and sodium edetate, whereas protamine sulfate powder consisted of large crystals (Figure 111, left). Ball milling appeared to be able to sufficiently micronize the substances in order to guarantee an efficient incorporation into the in situ implants. Only a few agglomerates remained (Figure 111, right).

Magnesium hydroxide



Protamine sulfate



Sodium edetate



Fig. 111: Macroscopic appearance of the additives as received (left) and after ball milling (right)

Lysozyme release was affected by the addition of the additives. Although magnesium hydroxide was reported to stabilize acid-labile proteins, the co-incorporation of the slightly soluble (~12 mg/l) base changed the release profile markedly and decreased the total recovery of lysozyme (Figure 112). Only a limited amount (15 %) of the drug loading was released within an initial

release phase of 9 days, whereas most of the totally recovered lysozyme (32 % of 47 %) was released between 35 and 77 days. The decreased release within the initial release could be attributed to the increased lysozyme adsorption to PLGA at an increased medium pH (paragraph 3.1.1.2.). The pronounced late release phase (>42 days), which was attributed to erosion-controlled release of lysozyme from the slower degrading implant shell, pointed to a higher drug loading of the compartment. This could be due to an altered drug distribution after the in situ implant formation but could also be an effect of an altered erosion behavior due to a slowed degradation (Zhang et al., 1997).

A slightly lower initial release was obtained with the addition of Na EDTA, which might be due to a decreased solubility of lysozyme in the polymer-poor phase in presence of freely soluble Na EDTA (~100 g/L). The high solubility could also explain the limited duration of this effect. The release profiles of the formulations with and without Na EDTA overlaid beyond an incubation time of 7 days.

The most interesting effects were obtained with protamine sulfate and PVP. The incorporation of PVP increased the amount of lysozyme released in the first erosion phase (5/6 – 14 days) from about 26 % to 36 %, whereas the initial release was unaffected. According to previous investigations (Robinson and Williams, 2002), the effect of PVP might be explained by an inhibited protein adsorption from the polymer-poor phase to the PLGA surface. Preferential partitioning of amphiphilic polymers into the polymer-poor phase during the in situ formation of implants was shown previously with pluronic (DesNoyer and McHugh, 2003). The late release phase was unaffected by PVP addition (about 12-13 % between 49 and 79 days). Although, the total recovery of lysozyme increased markedly, the release of lysozyme was still incomplete. An increased amount of PVP might help to quantitatively inhibit protein losses due to a better surface saturation with PVP.

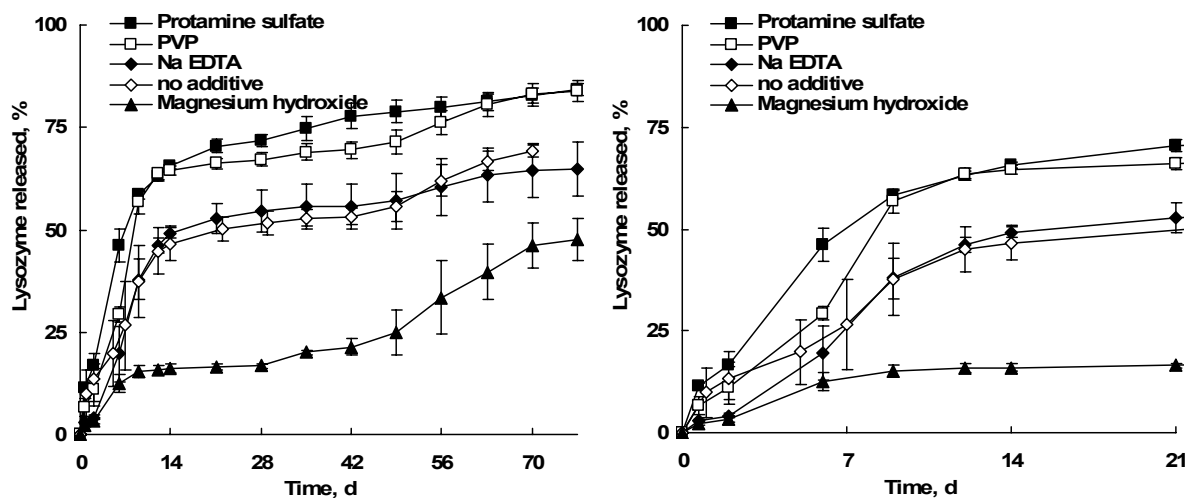


Fig. 112: Lysozyme release from in situ implants consisting of 40 % uncapped PLGA (RG 502H) in DMSO with 4 % drug loading (based on polymer) varying in the added excipient (mass ratio of drug to excipient 2:1) in dependence of the incubation time

The addition of protamine sulfate showed an effect on lysozyme release comparable to PVP. The initial release was more or less unaffected, whereas the lysozyme fraction released during the erosion phase increased markedly. In contrast to PVP, the late release phase disappeared. An interesting feature of protamine sulfate was its detectability using the Coomassie assay. This allowed quantification of protamine sulfate and lysozyme together, whereas lysozyme could be separately measured with HPLC. Compared to lysozyme, the release of the co-incorporated protamine sulfate was slower within the first 21 days and a significant portion appeared during the late release phase, beyond 42 days (Figure 113). The preferential release of lysozyme from the same formulation was thereby in agreement with a lower isoelectric point compared to protamine (pI 11 vs. pI 13.8) and did not correlate with the molecular weight (14 kDa vs. 4 kDa; Hoffmann et al., 1990). This indicated that a competition for electrostatic interactions with negatively charged carboxylate groups of PLGA contributed to the enhanced release of lysozyme. Interestingly, the combination of the release patterns of lysozyme (4 % loading) and protamine sulfate (2 %), which corresponds to a total protein release, matched quite exactly the release patterns of the in situ implants containing 6 % lysozyme, solely. This further supported that protamine sulfate competed with lysozyme for the same target.

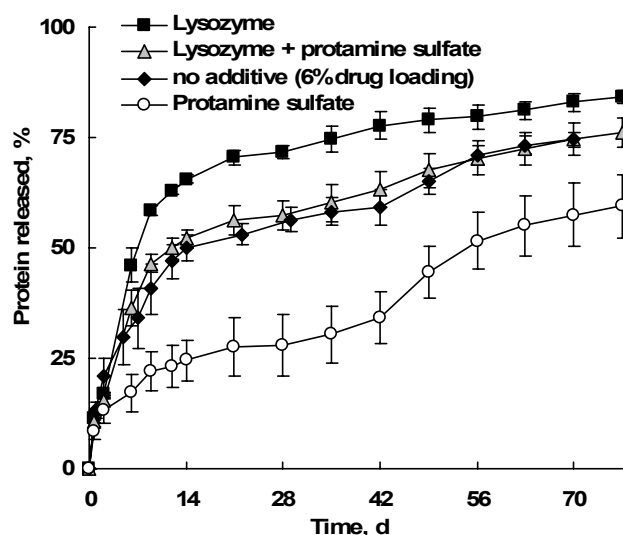


Fig. 113: Lysozyme and protamine sulfate release from in situ implants consisting of 40 % uncapped PLGA (RG 502H) in DMSO with 4 % lysozyme and 2 % protamine sulfate loading (based on polymer) in comparison to the protamine-free formulation loaded with 6 % lysozyme over the incubation time

Although the recovery of lysozyme could be increased by addition of protamine sulfate from 70 % to about 85 %, the release of both proteins was incomplete. Furthermore, to sacrifice one protein for the release of another would not eliminate the risk of the formation of immunogenic aggregates at all. Thus, recently investigated low molecular weight derivatives of protamine sulfate (Chang et al., 2001) might be more stable and exhibit an increased resistance to irrecoverable losses during the release. The advantage of utilizing protein stabilizers acting exclusively by a competitive mechanism (e.g. proton scavenger or surface occupying agents) is the avoidance of direct contacts, which can lead to destabilization inherently (Weert et al., 2004).

3.2.3. Blends of water-miscible and water-immiscible polymer solvents

3.2.3.1. The ternary solvent system DMSO / ethyl acetate / water

The ternary solvent system consisting of DMSO, ethyl acetate and water was selected in order to facilitate adjustments of the solubility of lysozyme during incorporation into the in situ formulations. The pharmaceutically acceptable solvent DMSO was used because of its ability to dissolve both the model protein (lysozyme) and the biodegradable polymer (PLGA). Furthermore, lysozyme, which was denatured upon dissolution in DMSO, could be completely recovered from a non-aqueous solution in DMSO and the protein possessed full biological activity (paragraph 3.1.3.2.3.). The biocompatible, GRAS-listed ethyl acetate (Royals et al., 1999 and Kranz et al., 2001) dissolves the polymer but is a nonsolvent for the protein. Ethyl acetate was applied in order to allow adjustments of the protein solubility. The feasibility of a nonaqueous in situ protein precipitation step during the preparation of in situ systems was investigated in order to homogeneously disperse the protein in the polymer solution. Additionally, the hypothesis was tested, whether lysozyme, precipitation under non-aqueous conditions would lead to storage stable formulations due to kinetic trapping of the protein (Griebenow and Klibanov, 1996).

Additionally to DMSO and ethyl acetate, water was introduced into the solvent system since preliminary investigations suggested markedly decreased dissolution times for lysozyme in DMSO in the presence of small amounts of water (from about 2 h to less than 0.5 h). This would be desirable for formulations, which require reconstitution prior to administration, e.g. where protein and polymer had to be stored separately from the solvent systems due to storage stability issues. Finally, it was expected to gain further insight into the developed protein separation method, which could lead to further optimization of the extraction conditions (e.g., completely omit buffer / ethyl acetate interface when extracting hydrated implants).

3.2.3.1.1. DMSO / ethyl acetate / water blends

Ternary phase diagrams were determined for the solvent system DMSO / ethyl acetate / water with and without 2.7 % lysozyme (equivalent to a 4 % target loading based on PLGA only) or 40 % PLGA. A high PLGA concentration of 40 % was selected, because the initial drug release (burst) is lower at higher polymer concentrations (Lambert and Peck et al., 1995). The

diagrams should identify suitable conditions for lysozyme incorporation into the in-situ drug delivery systems in dissolved or finely dispersed form.

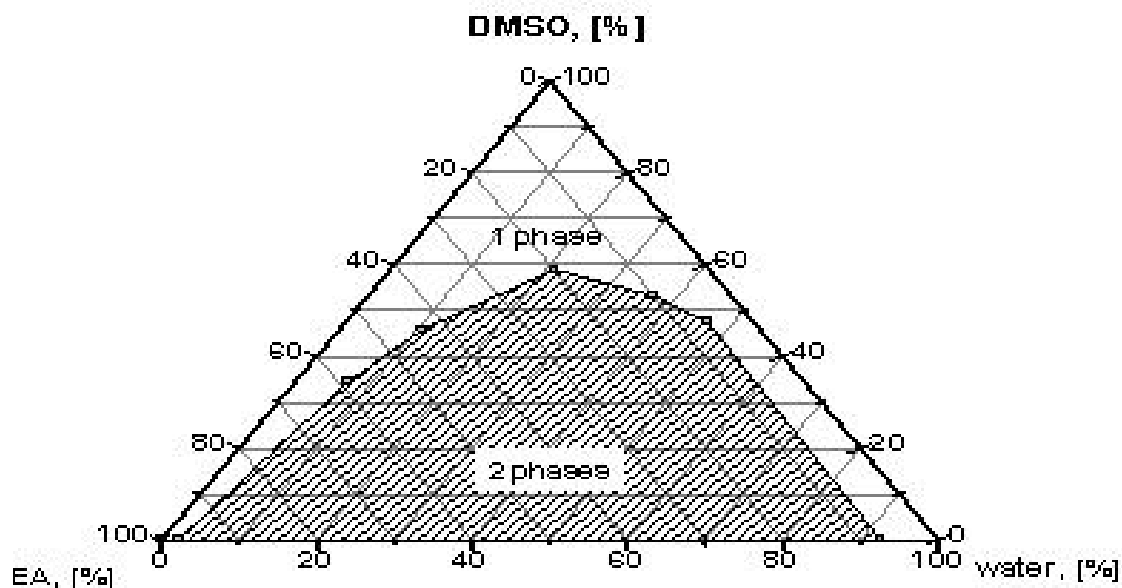


Fig. 114: Phase diagram for DMSO, ethyl acetate (EA) and water mixtures (room temperature)

The phase diagram of the ternary solvent system showed areas of miscibility (1 phase) and immiscibility (2 phases) (Figure 114). Water and ethyl acetate were completely miscible with DMSO, whereas only 8 % ethyl acetate could be added to water and only 2 % water to ethyl acetate before phase separation occurred. Compositions with DMSO contents higher than 60 % were miscible independent of their ethyl acetate to water ratio.

3.2.3.1.2. Lysozyme in DMSO / ethyl acetate / water blends

The two phase region in the phase diagram consisted of two regions, namely the miscibility gap of the ternary solvent mixture, which was unaffected by the low protein concentration, and a region where protein precipitation occurred (Figure 115). The phase boundary for precipitation of lysozyme depended on the water content (0-8.5 %) and was between ethyl acetate concentrations of 29 to 47 %.

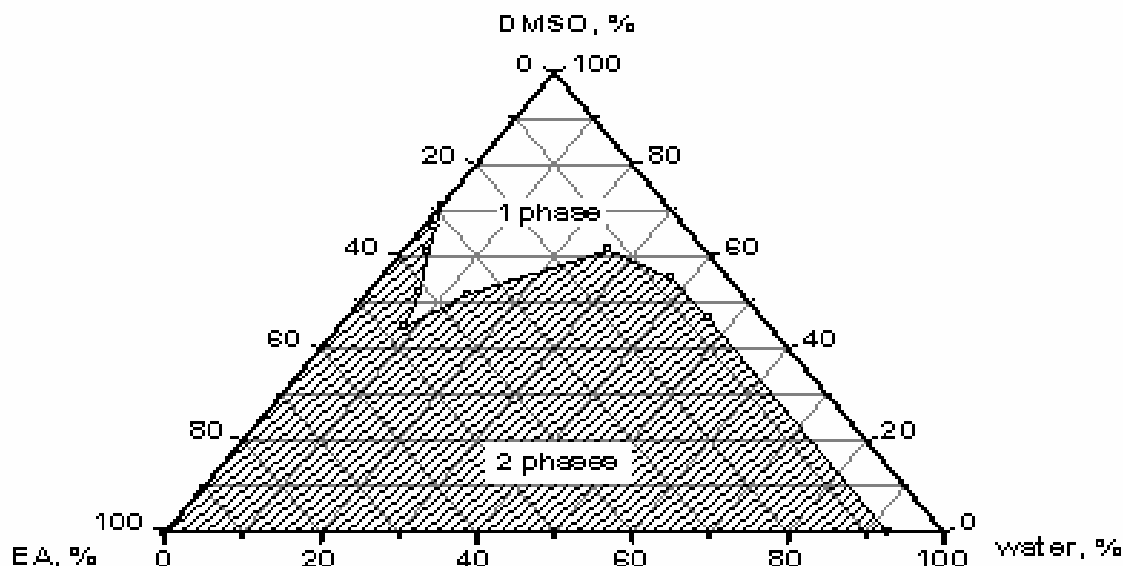


Fig. 115: Phase diagram for DMSO, ethyl acetate (EA) and water mixtures containing 2.7 % lysozyme

A slow appearance of cloudiness accompanied by protein gelation was observed during titration, when the composition approached the boundary for protein precipitation. Both phenomena were in accordance with observations made during lysozyme crystallization close to the liquid-liquid phase boundary in aqueous systems (Galkin and Vekilov, 2000). However, whether cloudiness observed before protein precipitation was caused by crystals or simply by aggregate formation was not further investigated since a precipitate could be obtained without gelation through continuous titration of the protein solutions with the non-solvent ethyl acetate under gentle agitation.

3.2.3.1.3. PLGA in DMSO / ethyl acetate / water blends

A one phase region in the phase diagram of 40 % PLGA (RG 502H) solutions was apparent at low water contents (Figure 116). Dependent on the DMSO / ethyl acetate ratio, between 2 and 9 % water could be incorporated into the polymer solutions before liquid-liquid phase separation occurred. The phase diagram of the corresponding end-capped polymer type, RG 502S, did not differ significantly from RG 502H (Figure 117).

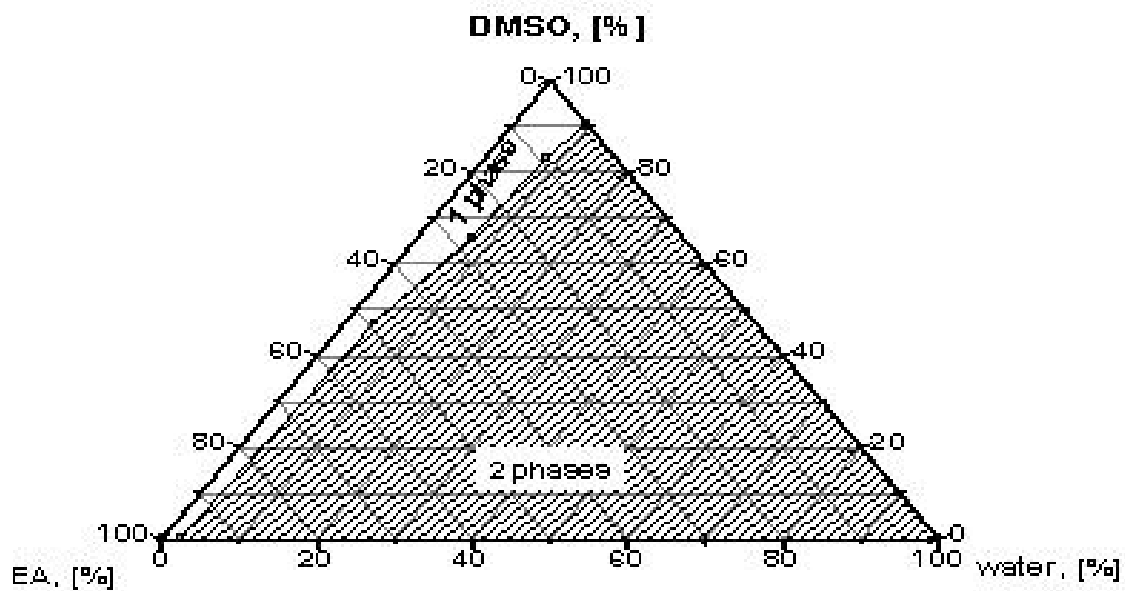


Fig. 116: Phase diagram for 40 % RG 502H in DMSO, ethyl acetate (EA) and water mixtures

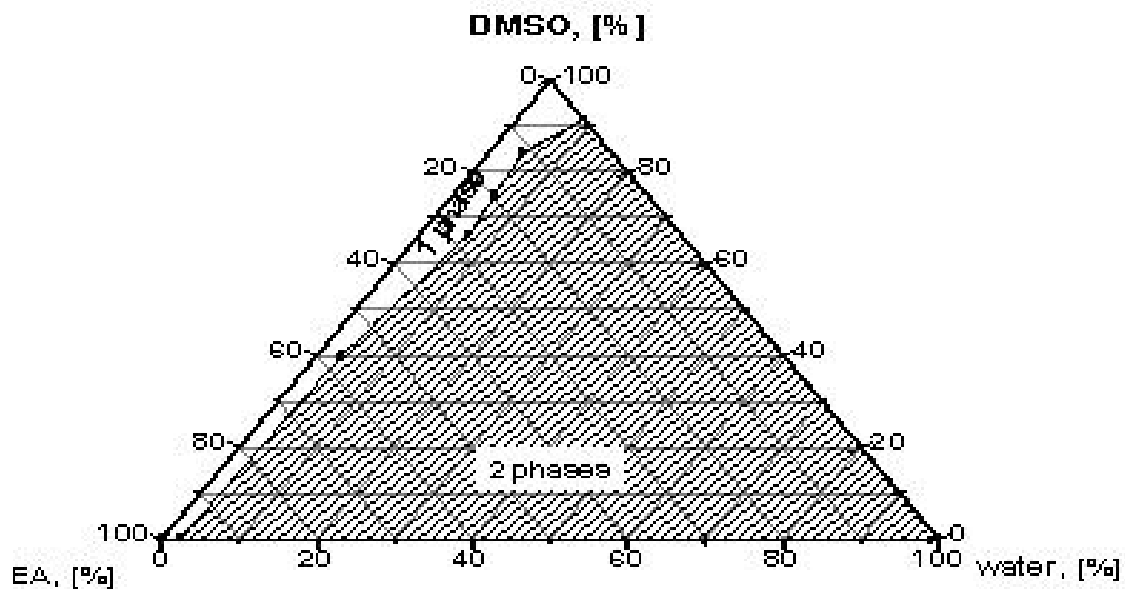


Fig. 117: Phase diagram for 40 % RG 502S in DMSO, ethyl acetate (EA) and water mixtures

The phase diagrams for PLGA and lysozyme revealed the compositions at which the model protein could be incorporated into polymer solutions either in dissolved or suspended state (Figure 118).

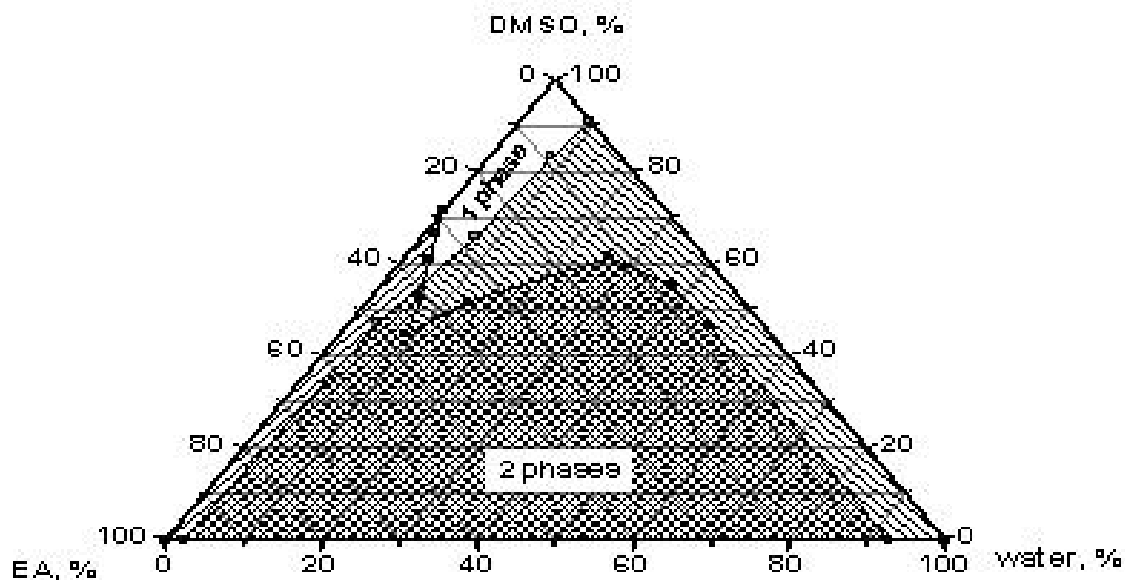


Fig. 118: Comparison of phase diagrams obtained with 2.7 % lysozyme and 40% PLGA (RG 502H) containing blends of DMSO, ethyl acetate (EA) and water

DMSO / ethyl acetate ratios in the range between 71:29 and 60:40 resulted in protein suspensions. These suspensions could turn into protein solutions prior to PLGA coacervation/precipitation upon contact with aqueous media (release medium/ tissue fluid).

3.2.3.1.4. Lysozyme incorporation into PLGA solutions

The phase diagrams of lysozyme and PLGA facilitated the selection of solvent conditions, which were expected to allow incorporation of lysozyme in either dissolved or dispersed state. According to the results on the DMSO only systems (paragraph 3.2.2.2.1.) lysozyme was dissolved in DMSO or DMSO / water prior to the addition of ethyl acetate and polymer. A splitting of the solvents into two portions in order to separately dissolve protein and polymer

before combining them, was not possible because of the already high viscosity of 40 % PLGA solutions (DMSO: 957 +/- 103 mPas, ethyl acetate: 20,121 +/- 546 mPas).

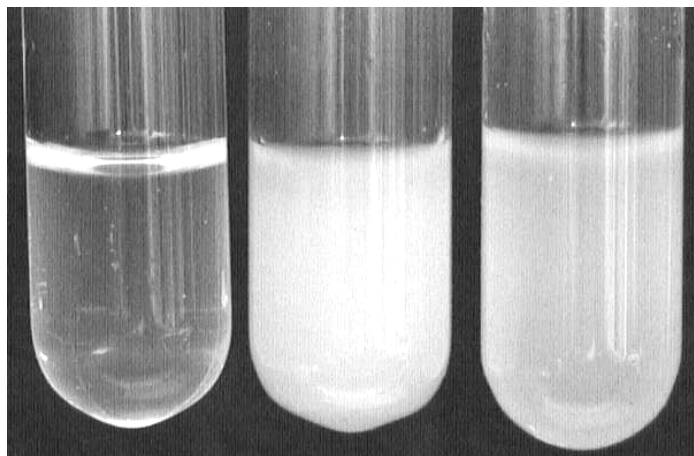


Fig. 119: Appearance of 4 % lysozyme (based on polymer) containing 30 % PLGA (RG 502H) solutions differing in solvent ratios DMSO / ethyl acetate / water (from left to right: 100/0/0, 75/25/0 and 70.5/23.5/6)

A dependence of the turbidity of lysozyme-containing PLGA solutions on the solvent ratio DMSO / ethyl acetate / water was clearly seen, when 30 % PLGA solutions were investigated (Figure 119). A pronounced turbidity hampered the optical differentiation of the solvent ratios in 40 % PLGA solutions even though the formulation was based on DMSO only. The protein precipitated upon addition of the nonsolvent ethyl acetate (DMSO / ethyl acetate / water 75/25/0). The turbidity decreased upon addition of water (DMSO / ethyl acetate / water 70.5/23.5/6) at the same DMSO / ethyl acetate ratio, which indicated that the precipitate consisted of the protein and not the polymer. These compositions were selected from the one phase regions of the individual protein and polymer phase diagrams (Figure 119). The apparent turbidity of the combined protein / PLGA system indicated a shift of the phase boundary for the protein in concentrated PLGA solutions. A quantification of the dissolved protein fraction in the supernatant of the dispersions confirmed this. The fraction of dissolved lysozyme in 1.6% protein-containing formulations was reduced to 0.242 +/- 0.019 %, 0.151 +/- 0.075 % and 0.002 +/- 0.001 % for DMSO / EA / water ratios 100/0/0, 70.5/23.5/6 and 75/25/0, respectively.

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

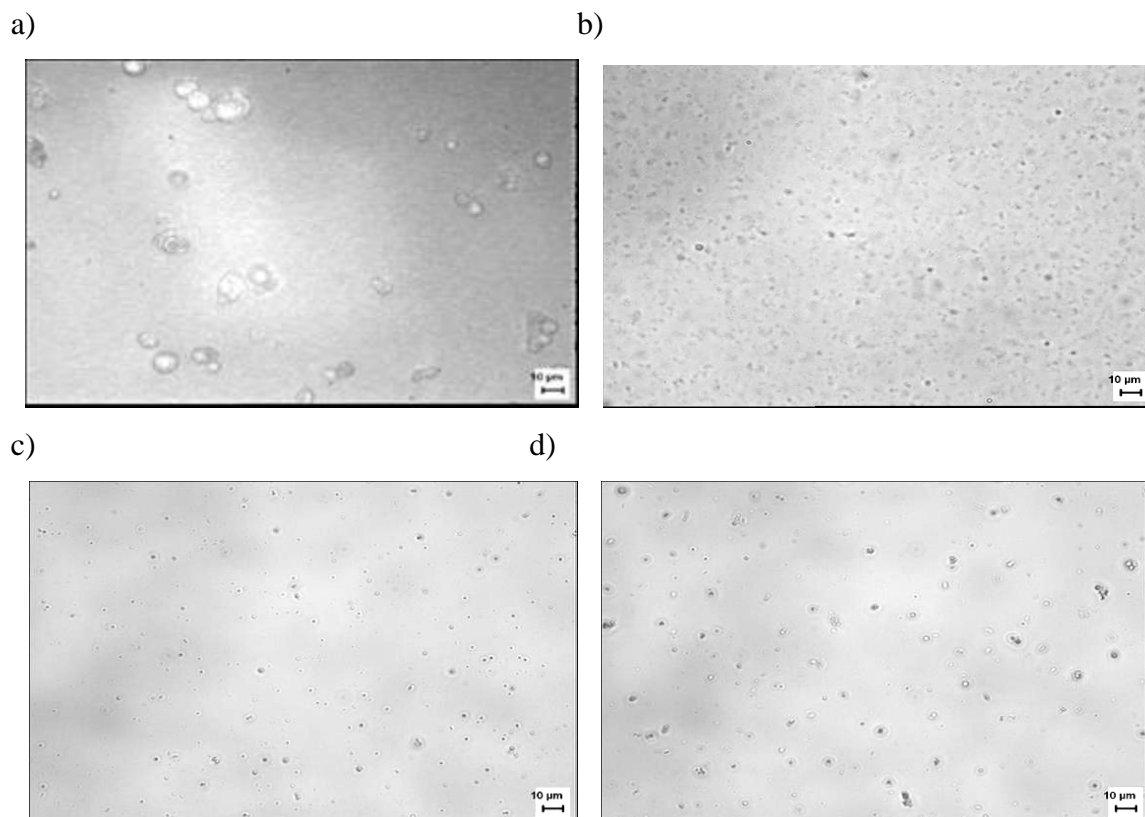


Fig. 120: Microscopic pictures of a) lyophilized (as received) lysozyme dispersed in sesame oil and precipitated lysozyme in 30 % PLGA solutions in DMSO / ethyl acetate / water ratios b) 100/0/0, c) 75/25/0 and d) 70.5/23.5/6 (magnified 400:1)

3.2.3.1.4.1. Particle size of precipitated lysozyme

In order to evaluate the particle size of precipitated protein, 4 % lysozyme (based on polymer)-containing 30 % PLGA solutions in DMSO / ethyl acetate / water (100/0/0, 75/25/0 and 70.5/23.5/6) were compared to a dispersion of lyophilized lysozyme (as received) in the protein nonsolvent sesame oil. The particle size of lyophilized lysozyme dispersed in sesame oil was

around 11.2 (5.6) μm (measured with light microscopy, Figure 120 a). This was larger than the upper limit of detection (3 μm) for photon correlation spectroscopy (PCS). The size of lysozyme particles formed in PLGA solutions in DMSO could not be determined with PCS as well. This was probably due to comparable refractive indices of dispersed and continuous phases after liquid-liquid phase separation, since microscopic pictures of the protein particles (Figure 120 b) suggested a mean size, which was comparable to the size of precipitated lysozyme particles in polymer solutions in DMSO/ ethyl acetate/ water (75/25/0 and 70.5/23.5/6) (Figure 120 c and d). The average diameters of these two DMSO/ ethyl acetate/ water systems obtained from three PCS measurements were 2.2 (0.2) and 2.2 (0.3) μm (PCS measurement), respectively. Although broad size distributions were indicated by high polydispersity indices of 1.0, large particles comparable to the dispersed raw material (~ 11 μm) were not observed upon microscopic evaluation.

3.2.3.1.4.1. Effect of the DMSO / ethyl acetate / water ratio on lysozyme release

Lysozyme release from in-situ microparticles was only slightly affected by the investigated DMSO / ethyl acetate / water ratios (Figure 121a), although the addition of polymer nonsolvents (water) and solvents with limited water affinity (ethyl acetate) were expected to alter the phase separation of the polymer and thus the release (Brodbeck et al., 1999 and Graham et al., 1999). Ethyl acetate could be partially removed from the polymer solution phase during emulsification into sesame oil. Ethyl acetate is completely miscible with sesame oil. The residual amounts of ethyl acetate as well the incorporated quantity of water were probably insufficient to alter the release of lysozyme from in situ microparticle formulation. On the other hand, the superimposing release pattern from the three formulations showed that an in-situ precipitation could lead to more homogenous lysozyme dispersions in PLGA solutions and thus lower initial releases compared with conventional dispersing techniques (paragraph 3.2.2.2.2.1.).

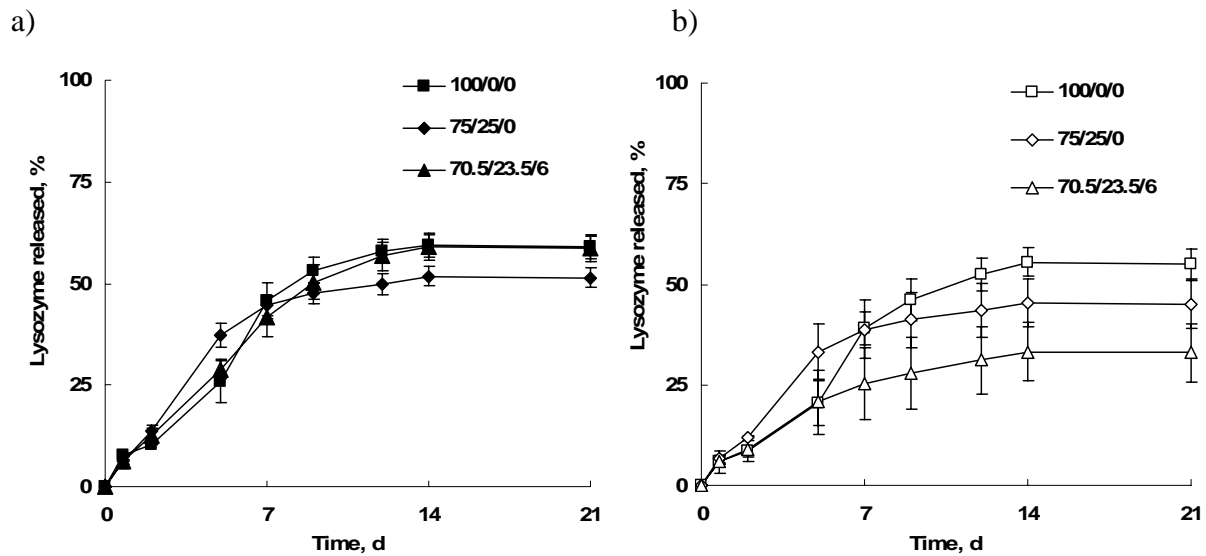


Fig. 121: Lysozyme release as a function of the solvent composition in the polymer solution: a) in-situ microparticle formulations [40 % PLGA (RG 502H) solutions in DMSO / ethyl acetate / water containing 4 % (relative to polymer) lysozyme] and b) corresponding in-situ implants

However, differences between the release patterns of the corresponding in-situ implants were more pronounced (Figure 121b). Especially the DMSO / ethyl acetate / water 70.5/23.5/6 implant formulation showed a different behavior compared to the other two solvent ratios (Figure 122). Lysozyme release from the in situ microparticles was more complete compared to the in situ implant. The release from the in situ implants during the polymer erosion-controlled phase between 7 and 14 days was much lower compared to the in situ microparticles.

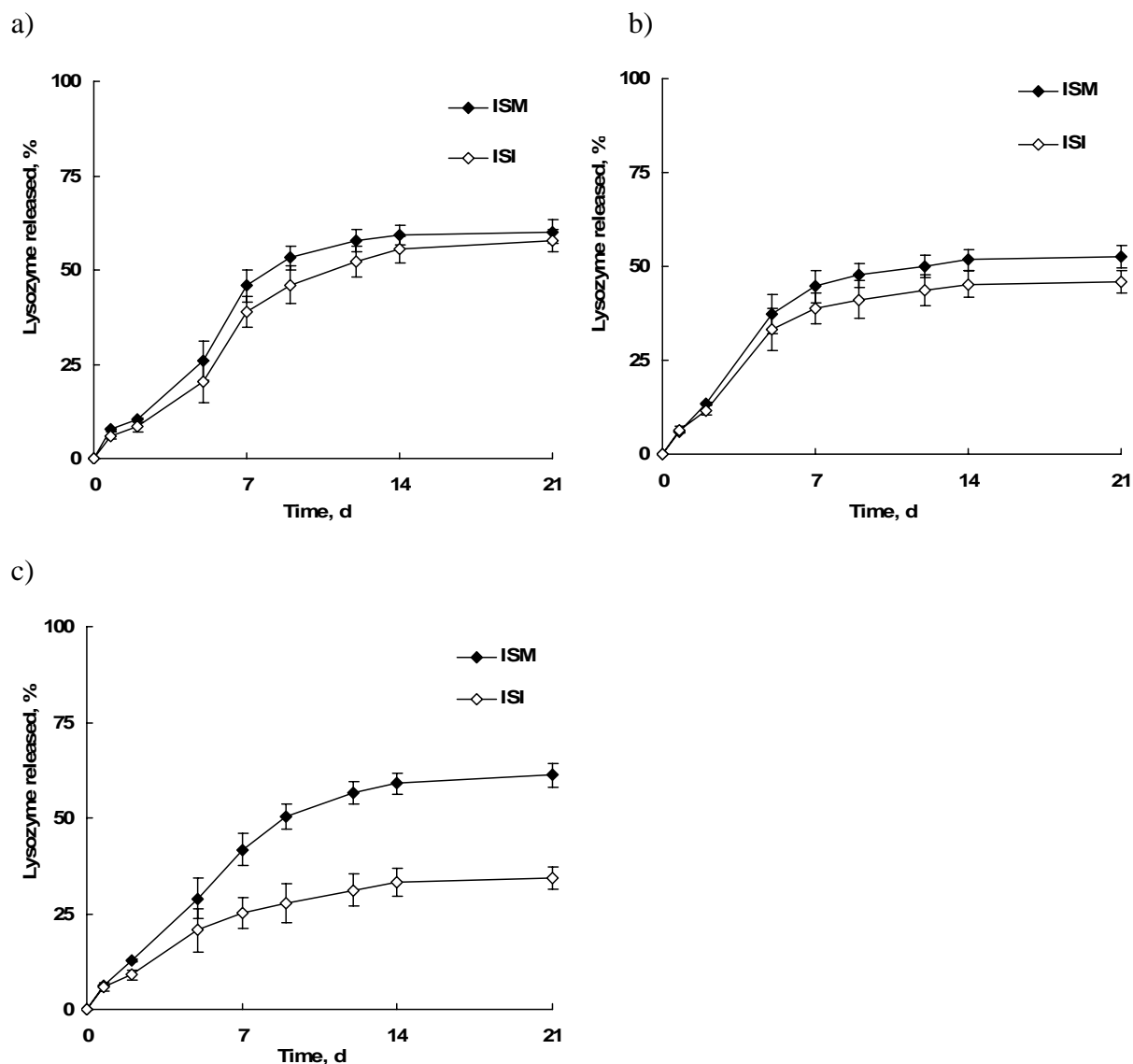


Fig. 122: Lysozyme release from in situ microparticles and corresponding implants [40 % PLGA (RG 502H) solutions in blends of DMSO / ethyl acetate / water containing 4 % (relative to polymer) lysozyme] as a function of the solvent composition in the polymer solution: a) 100/0/0 b) 75/25/0 and c) 70.5/23.5/6

Similar to the in situ formulations based on the DMSO / ethyl acetate / water 70.5/23.5/6, the initial release was higher with the in situ microparticles than with the in situ implant at a solvent ratio of 70/20/10 ratio (Figure 123a), which corresponds to a composition outside the 1 phase region of the phase diagrams (Figure 118). Thus, the polymer solution was already separated into a polymer-rich (dark) and polymer-poor phase during preparation of the in situ systems (Figure

123b). Only a small burst was obtained with the in situ implant although a polymer-poor phase was already present in the formulation prior to injection into the release medium. The release was performed in horizontal test tube position, which impeded a more quantitative comparison with the other solvent ratios (Figure 122).

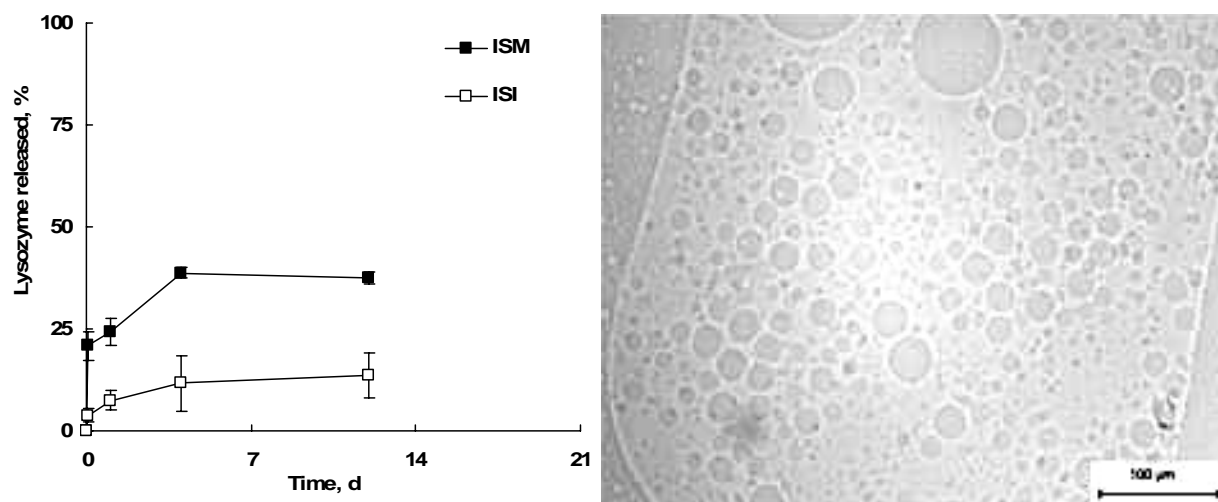


Fig. 123: a) Lysozyme release from in situ microparticles and corresponding implants based on 40 % PLGA (RG 502H) solutions in a 70/20/10 blend of DMSO / ethyl acetate / water containing 4 % (relative to polymer) lysozyme; b) microscopic picture of the (phase separated) in situ implant formulation

3.2.3.1.4.2. Effect of the lysozyme supplier on drug release

A change of the lysozyme supplier from Roth (standard) to Sigma changed the release behavior of the in situ formulations, especially the in situ implant based on DMSO / ethyl acetate / water ratio 75/25/0 (Figure 124b). The release characteristics matched quite exactly the patterns obtained with the 70.5/23.5/6 blend, whereas before no considerable difference between the in situ formulations for this solvent ratio was observed. The in situ microparticle systems were again more robust to changes of the solvent ratio (Figure 124a).

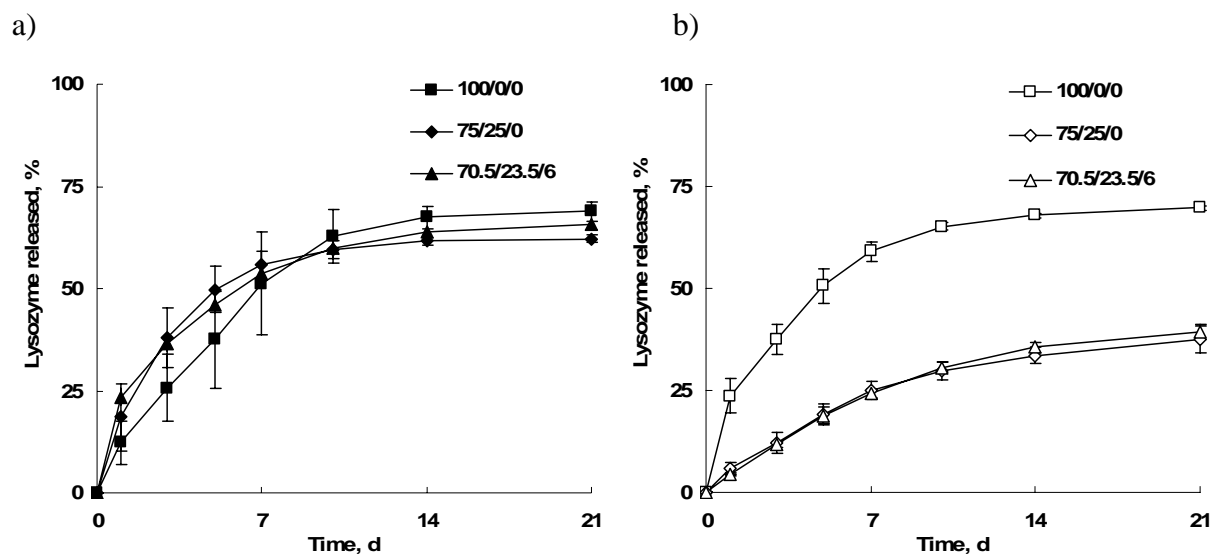


Fig. 124: Lysozyme (Sigma) release as a function of the solvent composition in the polymer solution: a) in-situ microparticle formulations [40 % RG 502H solutions in blends of DMSO / ethyl acetate / water containing 4 % (relative to polymer) lysozyme] and b) corresponding in-situ implants

The direct comparison of the supplier confirmed that the most obvious difference between the lysozyme qualities occurred with the solvent ratio 75/25/0 (Figure 125). However, the release differed also slightly for the other solvent ratios.

The altered release behavior of lysozyme as function of the supplier was unexpected. A difference between both materials was noticed when they were dissolved in DMSO initially, in order to prepare the in situ formulations. Lysozyme from Roth dissolved within 90 minutes, whereas it took about 150 minutes to dissolve the protein supplied from Sigma. It was previously suggested, that the solubility of proteins in organic solvents depends on the pH at which a protein was lyophilized (Chin et al., 1994 and Bromberg and Klivanov, 1995). The higher the difference between this pH and the isoelectric point (pI) of proteins (higher net charge), the higher was the solubility in organic solvents. Indeed, the measurement of the pH of aqueous solutions (3.1 +/- 0.1 % m/v) of both lysozyme materials pointed to a difference in the solution pH from which the protein was lyophilized. The pH of the Roth (pH 3.2) was lower than the pH of the Sigma (pH 3.8) lysozyme solution, which should correlate with the pH prior to lyophilization. However, it was not clear how an alteration of the lysozyme solubility could affect the release behavior in the

way observed. Follow up studies should include stability considerations, since alterations of the protein net charge could have affected the stability of the protein (aggregation behavior in the organic solvent blends or the adsorption behavior to PLGA) and thus the drug release.

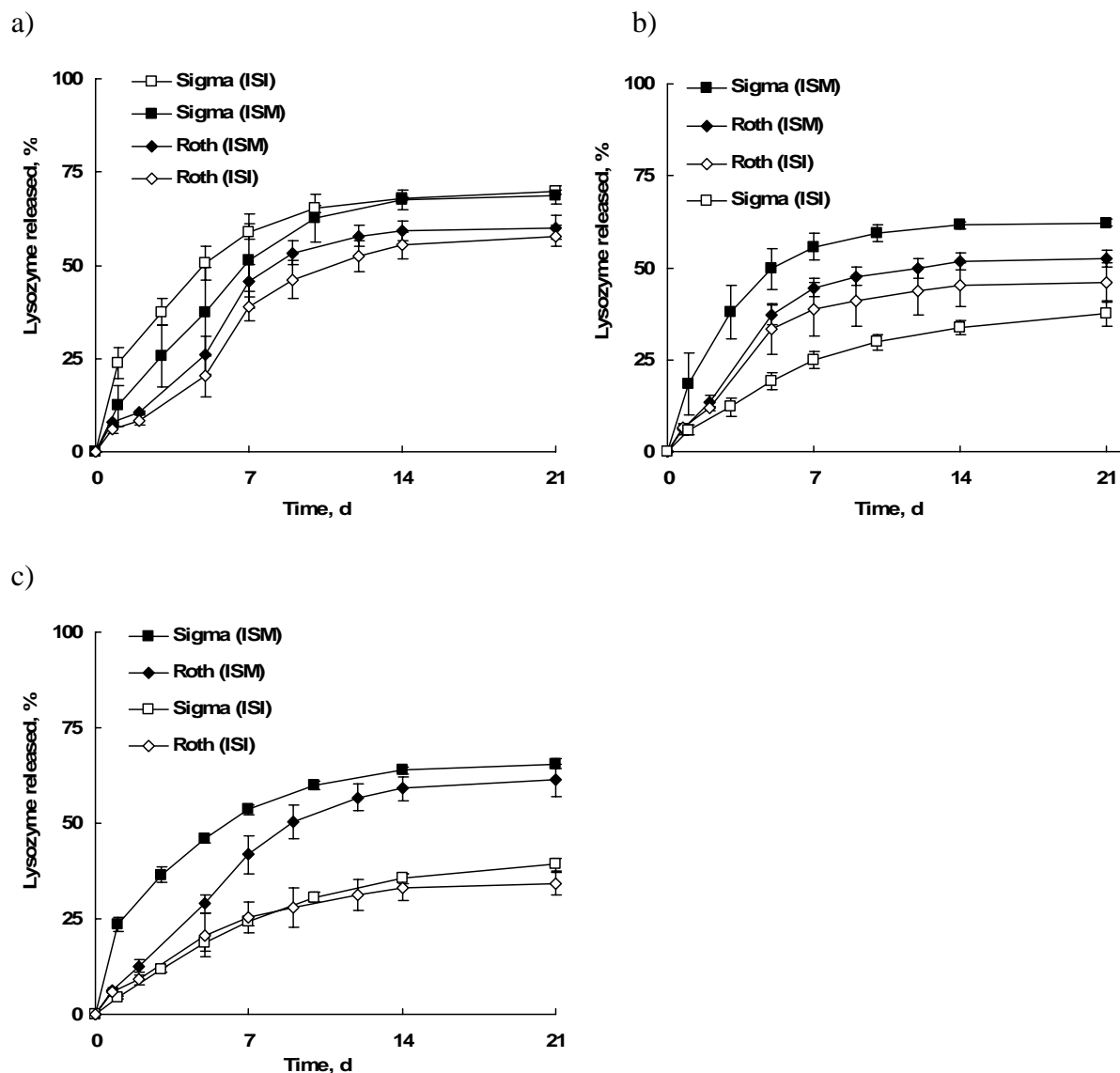


Fig. 125: Lysozyme release from in situ microparticles and corresponding implants [40 % PLGA (RG 502H) solutions in blends of DMSO / ethyl acetate / water containing 4 % (relative to polymer) lysozyme] as a function of the lysozyme supplier and solvent ratios: a) 100/0/0 b) 75/25/0 and c) 70.5/23.5/6

3.2.3.2. The ternary solvent system DMSO / triacetin / water

The protein non-solvent / polymer solvent triacetin was investigated as alternative to ethyl acetate as precipitant for lysozyme. Its advantage over ethyl acetate as formulation ingredient was the lower volatility (0.00248 mm Hg at 25°C vs. 76 mm Hg at 20°C). Thus, the risk of changes of the solvent compositions due to evaporative losses during long-term storage of the in situ formulations was decreased.

Ternary phase diagrams were determined similar to the DMSO / ethyl acetate / water system. The phase diagram of the ternary solvent system was comparable to the system with ethyl acetate (Figure 126 vs. 114). The phase boundary in total appeared to be slightly shifted to the right. Water and triacetin were completely miscible with DMSO, whereas only 6 % triacetin could be added to water and only 3 % water to triacetin before phase separation occurred. As with ethyl acetate, compositions with DMSO contents higher than 60 % were miscible independent of their triacetin to water ratio.

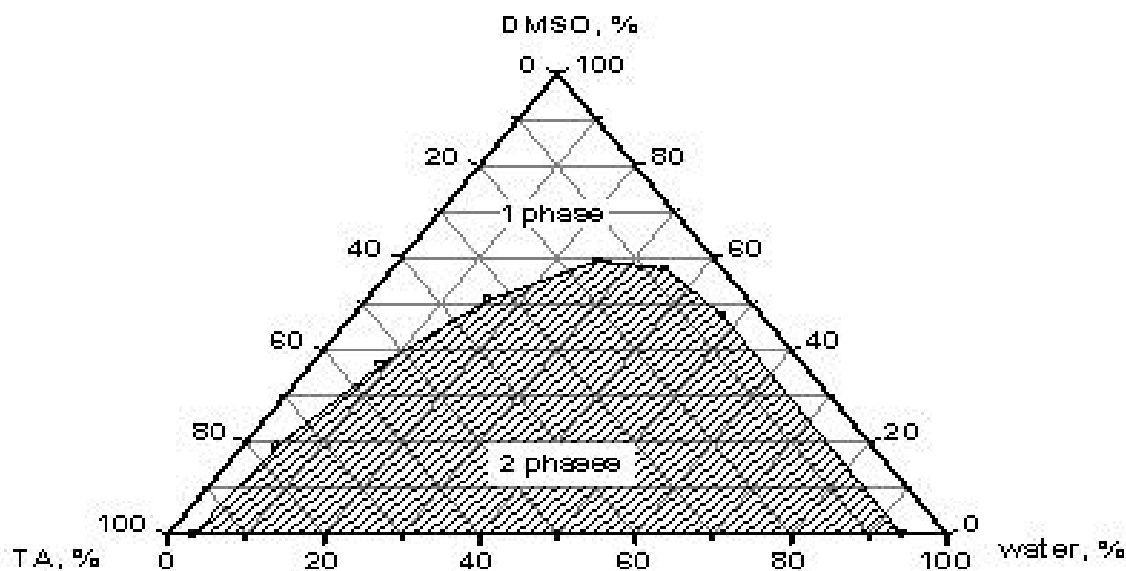


Fig. 126: Phase diagram for DMSO, triacetin (TA) and water mixtures (room temperature)

3.2.3.2.1. Lysozyme stability in DMSO / triacetin / water blends

The phase diagram of the ternary solvent system containing lysozyme was also close to the system with ethyl acetate (Figure 127 vs. 115). The solid – liquid phase boundary, where precipitation of lysozyme occurred, was located between triacetin concentrations of 29 to 46 % depended on the water content (0-8.6 %). Thus, the results were very similar compared with the ternary solvent system with ethyl acetate.

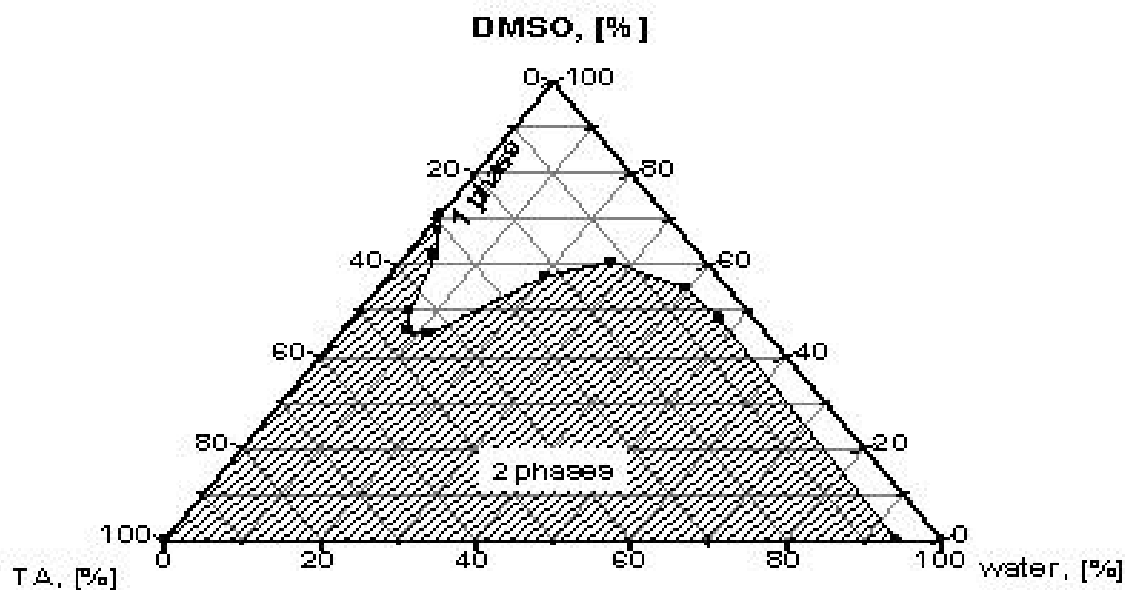


Fig. 127: Phase diagram for DMSO, triacetin and water mixtures containing 2.7 % lysozyme

When the lysozyme dispersions in the DMSO / triacetin 50/50 blend and in triacetin were filtrated and extracted using the developed protein separation method, residual protein solubilities of 0.036 +/- 0.001 mg/g and 0.004 +/- 0.003 mg/g were measured, respectively. This corresponds to mass ratios of dissolved to undissolved protein of 750/1 for the 50/50 blend and 6750/1 for triacetin.

The DMSO / triacetin ratios 100/0, 50/50 and 0/100 were selected to test the long-term stability of lysozyme in dependence of the physical state and the effect of applying a non-aqueous precipitation step.

In “DMSO / triacetin” samples, lysozyme was first dissolved in DMSO before precipitation was induced through addition of triacetin. In case of the “triacetin / DMSO” sample, the protein (as received) was dispersed in triacetin and then DMSO was added to exclude the non-aqueous precipitation step.

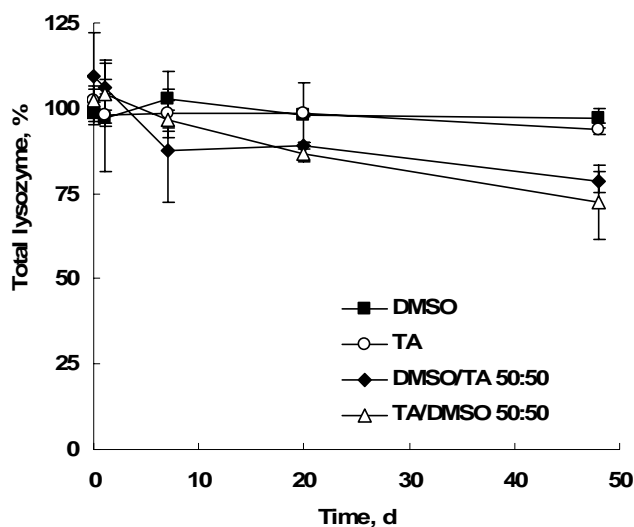


Fig. 128: Recovered mass (“total recovery”) of lysozyme separated from 2.7 % protein-containing DMSO / triacetin (TA) blends relative to the theoretical value as function of storage time at ambient temperature in a desiccator

Lysozyme stored 48 days in the DMSO / triacetin systems at room temperature was completely recoverable from the DMSO solution as well as from the dispersion in triacetin. A comparable loss of recoverable material was noticed in the 50/50 blends (Figure 128) and suggested that the stability was independent of the precipitation step involved in the preparation of the DMSO / triacetin sample. A small shoulder in the HPLC-chromatograms of all DMSO samples (>14.2 min retention time) was found for both systems at 48 days but not for the neat triacetin.

A solution of lysozyme in DMSO was also incubated at 4°C and at 40°C. The total lysozyme recovery decreased to 83.9 +/- 0.5 % in the 40°C sample during 48 days storage (Figure 129), which was accompanied by the development of a shoulder behind the native peak in the HPLC chromatograms.

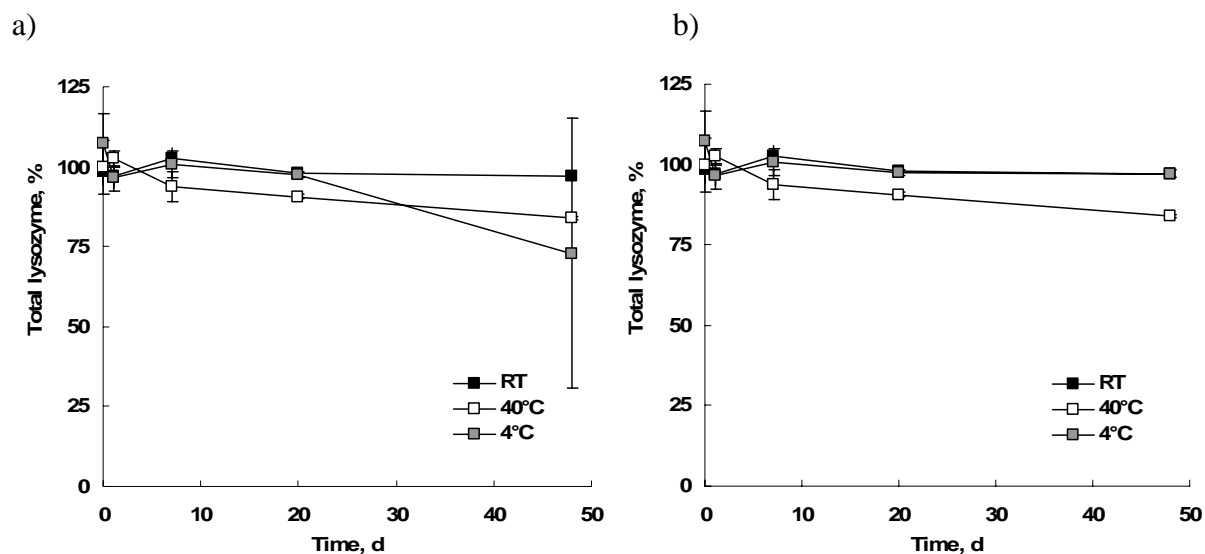


Fig. 129: Recovered mass (“total recovery”) of lysozyme separated from 2.7 % protein solutions in DMSO relative to the theoretical value vs. storage time at ambient, 4°C or at elevated temperature (40°C) in a desiccator; a) all samples n=3 and b) exclusion of one 4°C sample, n=2

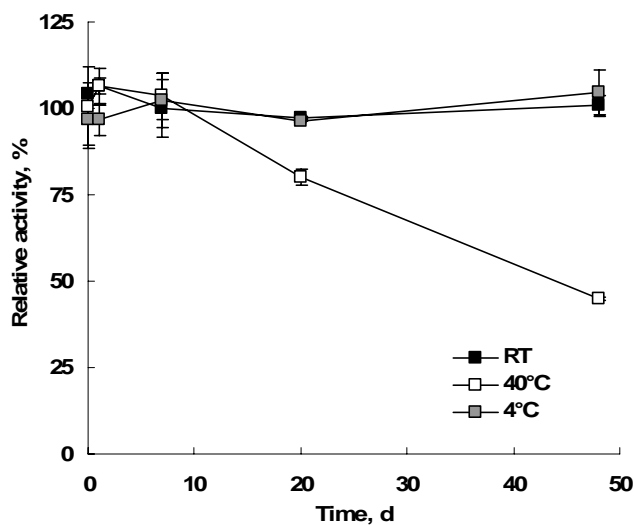


Fig. 130: Relative activity of lysozyme (active / total recovery, [%]) recoverable upon extraction of 2.7 % lysozyme solutions in DMSO in dependence of storage at low (4°C), ambient (RT) or elevated temperature (40°C) in a desiccator as a function of incubation time

Furthermore, additional bands occurred in the electrophoresis (Figure 132, lane 3), which pointed to aggregates as the reason for the peak shoulder in the HPLC-chromatograms. Quantification under exclusion of the area under this shoulder (>14.2 min retention time) resulted in total lysozyme recoveries of 84.7 ± 0.6 % after 20 days and 68 ± 0.3 after 48 days incubation. Allowing for a certain inaccuracy due to the difficult differentiation of the peak areas, these total recoveries were in good agreement with the relative activities (Figure 130), which would suggest that inactive but still water-soluble aggregates were formed in the 40°C sample during incubation.

The total recovery at 4°C appeared to be decreased upon analysis of all three samples at 48 days storage (Figure 129a). But only one of the three samples was affected (24 % of the expected concentration), whereas lysozyme could be almost completely recovered from the other two specimen (95 and 96 %). Exclusion of the peculiar sample would mean that lysozyme would be stable in DMSO at 4°C (Figure 129b), where DMSO would be frozen (melting point: 18°C). However, no justification for excluding this value was found. Despite the question of the total recovery, the activity relative to the total recovery was unchanged at 4°C (Figure 130). Similarly, only fully active lysozyme was recovered from all samples stored at ambient condition (Figure 129).

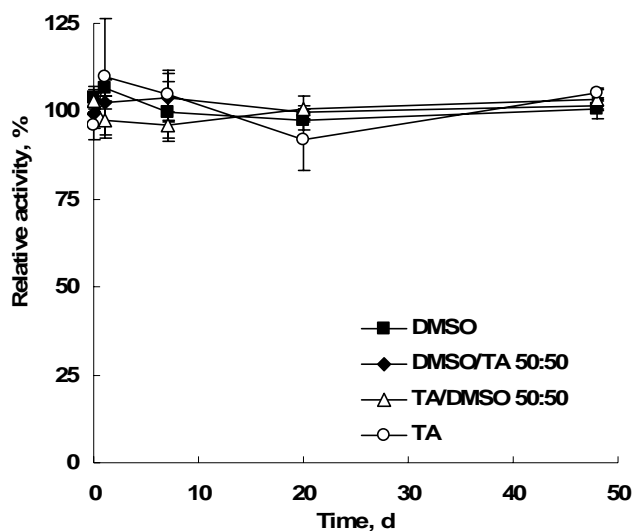


Fig. 131: Relative activity of lysozyme (active / total recovery, [%]) recoverable upon extraction of 2.7 % lysozyme containing DMSO/ triacetin (TA) mixtures vs. storage time at ambient temperature in a desiccator

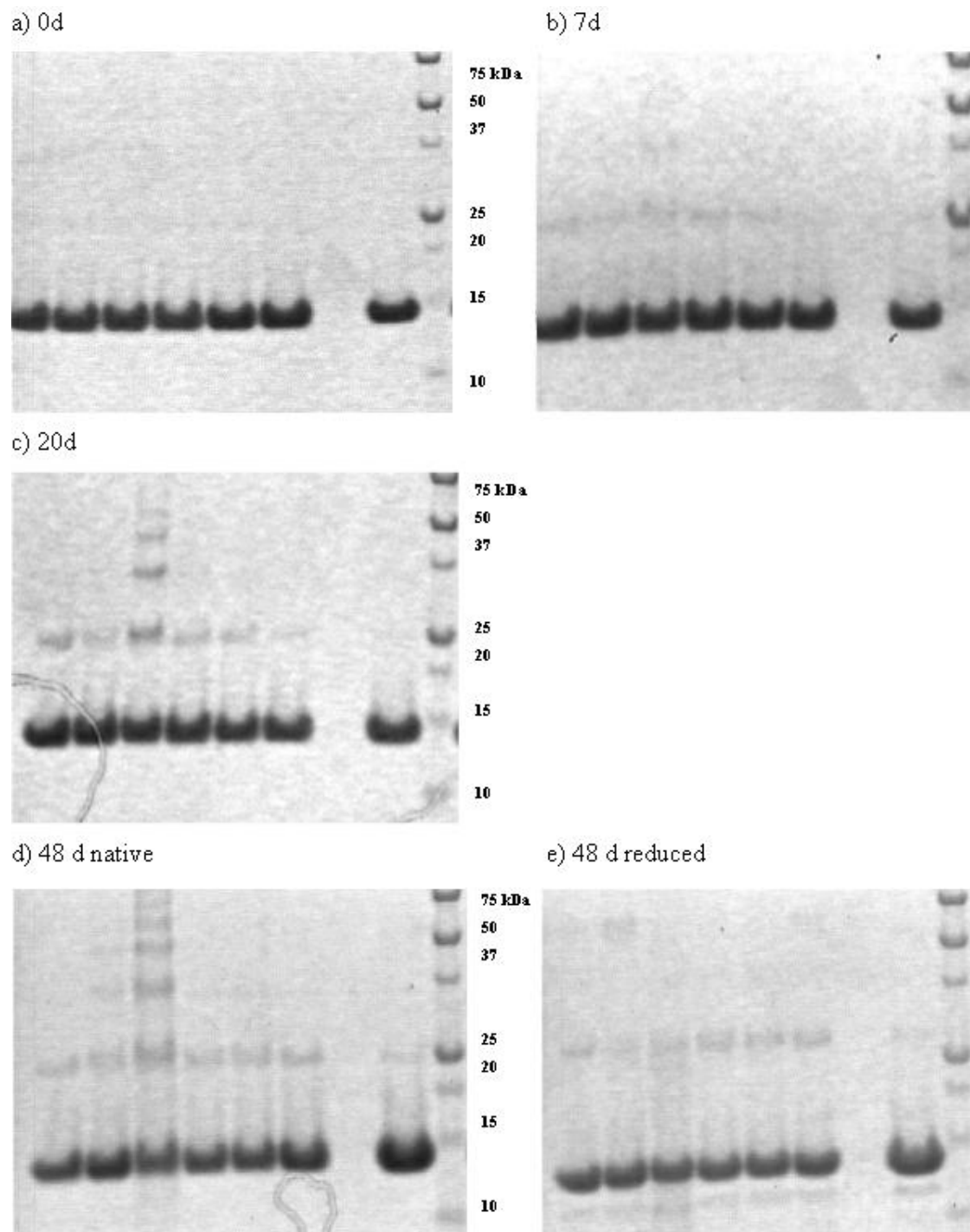


Fig. 132: Native PAGE of lysozyme recovered from DMSO/ triacetin (TA) solvent blends after a) 0 days, b) 7 days, c) 20 days and d) 48 days as well as e) reducing PAGE after 48 d storage; Lanes: 1) DMSO/4°C, 2) DMSO, 3) DMSO/40°C, 4) DMSO/ TA 50/50, 5) TA/ DMSO 50/50, 6) TA, 7) blank, 8) freshly prepared lysozyme standards (lysozyme in acetate buffer pH 5) and 9) marker

Native gel electrophoresis revealed only little dimer formation in the lysozyme dispersions in the 50/50 blends (lanes 4 and 5) and even less in triacetin (lane 6) in dependence of incubation time (Figures 132a-d). However, additional bands at multiples of the molecular weights of the native lysozyme band ($n \times 14$ kDa) were observed for the DMSO solution of lysozyme stored at room temperature (lane 1), although the total protein recovery of that sample was not affected during storage, which indicated formation of reversible aggregates.

More pronounced aggregate formation was seen with the lysozyme solution stored at 40°C (lane 3). The multimer bands in the stressed samples disappeared when gel electrophoresis was conducted under reducing conditions (Figure 132e), which suggested the formation of covalently bound (disulfide-crosslinked) aggregates in the stressed sample during 48 days storage. The slow lysozyme degradation in DMSO at ambient temperature did not allow the conclusion, whether changes observed for the stressed lysozyme solution would be representative for longer-term changes at room temperature.

Interestingly, despite the decrease of the total recovery in the 50/50 blends, electrophoresis did not exhibit multimer bands in the samples. This could indicate that the decrease of total lysozyme recovery in the blends could be due to caking of sedimented larger protein particles instead of irreversible protein aggregation as in case of the stressed DMSO samples. As observed during dissolution of lyophilized lysozyme in DMSO and in accordance with previous observations (Chang et al., 1991), sedimentation of partially solvated protein particles could lead to the formation of an apparently insoluble gel layer if not properly agitated. Thus, an effect of inhomogeneous drug distribution on the total lysozyme recovery could not be excluded.

3.2.3.2.2. PLGA in DMSO / triacetin / water blends

The phase diagrams of the polymer solutions (40 % RG 502H and RG 502S) in triacetin-containing blends were very similar to the ethyl acetate systems. A one phase region occurred at low water contents (Figures 133 and 134). The boundary of liquid-liquid phase separation varied with the DMSO / triacetin ratio between 2 and 9 % water contents.

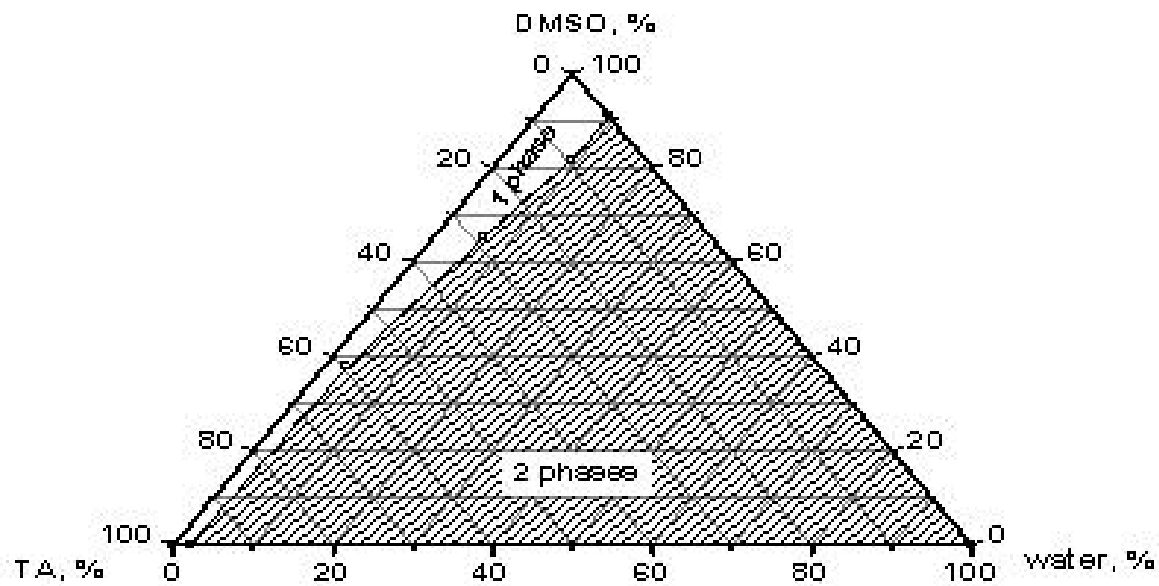


Fig. 133: Phase diagram for 40 % RG 502H in mixtures of DMSO, triacetin (TA) and water

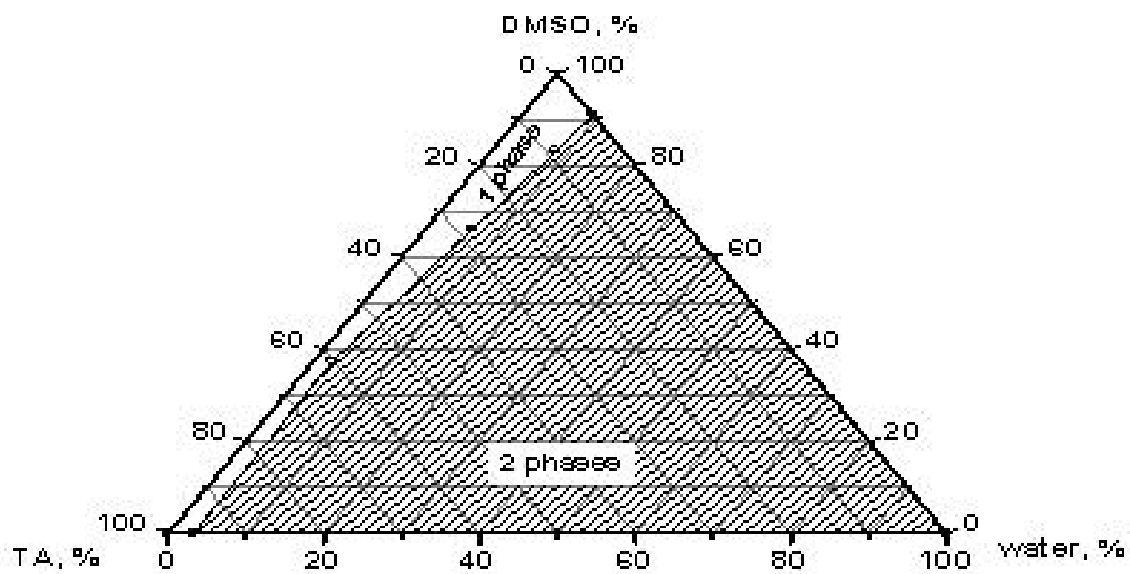


Fig. 134: Phase diagram for 40 % RG 502S in mixtures of DMSO, triacetin (TA) and water

The phase diagrams for PLGA and lysozyme revealed compositions at which the model protein could be incorporated into polymer solutions either in dissolved or dispersed state (Figure 135). DMSO / EA ratios in the range between 71:29 and 60:40 resulted in protein suspensions. These suspensions could turn into protein solutions prior to PLGA coacervation / precipitation upon contact with aqueous media (release medium/ tissue fluid).

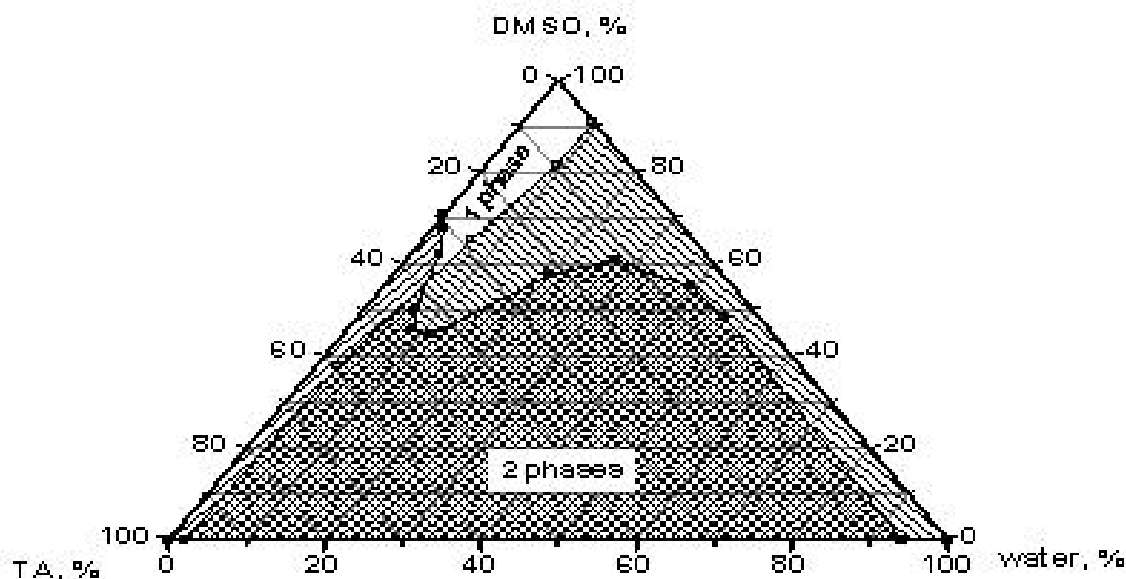


Fig. 135: Comparison of phase diagrams obtained with 2.7 % lysozyme and 40 % PLGA (RG 502H) containing blends of DMSO, triacetin (TA) and water

3.2.3.2.3. Lysozyme stability in PLGA solutions in DMSO / triacetin blends

Lysozyme stability was investigated in 30 % PLGA solutions due to the high viscosity of 40 % PLGA solutions in triacetin. The solubilities of lysozyme in solutions of 30 % RG 502H in DMSO / triacetin (100/0, 50/50, 0/100) and in the solution of 30 % RG 502S in DMSO were 2.424 \pm 0.192 mg/g, 0.009 \pm 0.004 mg/g, 0.005 \pm 0.001 mg/g and 0.122 \pm 0.018 mg/g, respectively. The preparations could be differentiated regarding the state of lysozyme in the polymer solutions. Dispersed lysozyme was present in triacetin and as in-situ precipitated protein in DMSO / triacetin. In contrast to the phase diagrams, lysozyme was not completely soluble in

DMSO solutions of 30 % PLGA, as discussed in previous paragraphs (paragraphs 3.2.2.2.1. and 3.2.3.1.4.).

The polymer types (uncapped vs. capped) were investigated for a potential effect on the protein stability, since proteins could interact with polymers in DMSO solutions (Chang et al., 1991). PLGA solutions in DMSO were used since potential differences in electrostatic interactions are most probable in the solvent with the highest dielectric constant. Thus, the effect of solvent composition and the PLGA-type on the co-incorporated protein was investigated.

Polyacrylamide gel electrophoresis (PAGE) was only used to characterize the recovered material after 98 d, since a time-dependent aggregation was expected from preliminary investigations. Multimer bands could be detected for some of the extracted samples (Figure 136). Especially the DMSO-samples showed pronounced aggregation, whereas only weak multimer bands were seen with the DMSO / triacetin 50:50 blend. The triacetin sample revealed only a single band of monomeric lysozyme. Multimer bands occurring under native and reducing conditions revealed the formation of relatively stable but not necessarily covalent aggregates during storage.

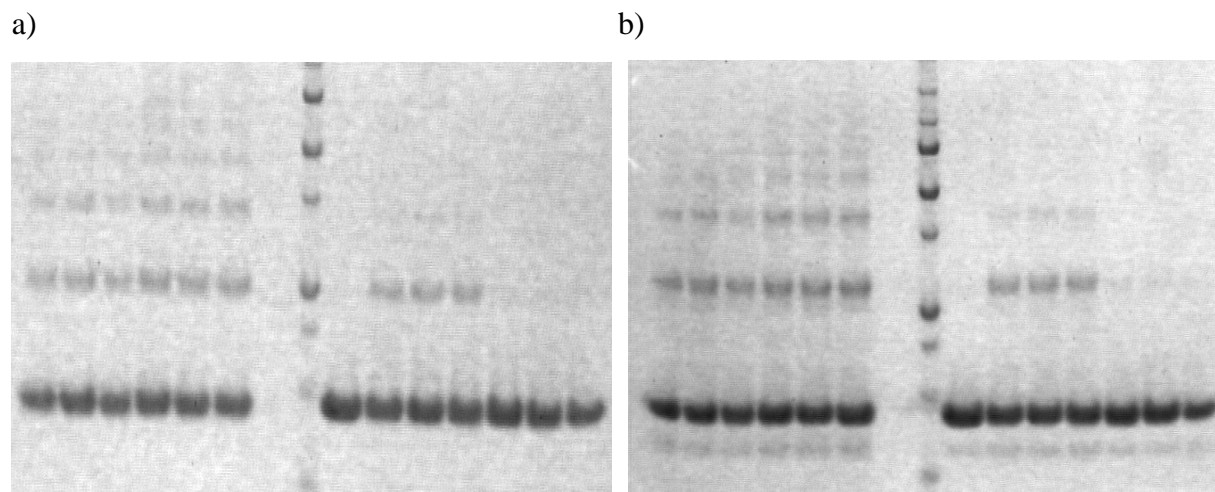


Fig. 136: PAGE of lysozyme recovered from in-situ implant formulations (30 % PLGA solutions containing 4 % lysozyme, based on polymer) after 66 days of incubation a) native and b) reducing conditions (lanes: 1-3. DMSO_502H, 4-6. DMSO_502S, 7. blank, 8. marker, 9. freshly prepared lysozyme standards (lysozyme in acetate buffer pH 5), 10-12. DMSO / triacetin 50/50_RG 502H and 13-15. triacetin_RG 502H)

Total (HPLC) and active (enzyme assay) recovery of lysozyme decreased during 98 d storage of the DMSO samples, whereas DMSO / triacetin 50:50 and triacetin did not show a decrease with storage time (Figures 137 and 138b). A slight drop seen with the latter samples was again likely attributable to sample inhomogeneities due to sedimentation, which was suggested by a gradual recovery increase at later sample time points. Among the DMSO samples, the protein loss was higher in solutions of uncapped PLGA, which could point to a contribution of free carboxylic acid groups to the instability. However, the solubility of lysozyme was higher in solutions of uncapped PLGA in DMSO.

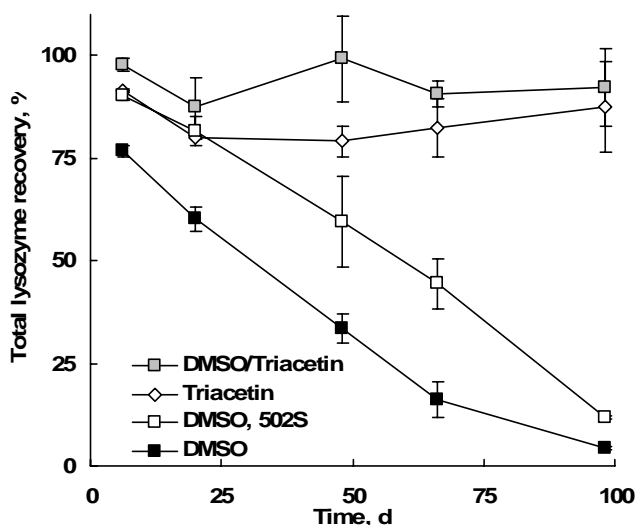


Fig. 137: Recovered mass of lysozyme separated from 4 % (based on polymer) protein containing in-situ implant formulations based on 30 % RG 502H (RG 502S) in DMSO / triacetin blends as a function of storage time at ambient temperature in a desiccator

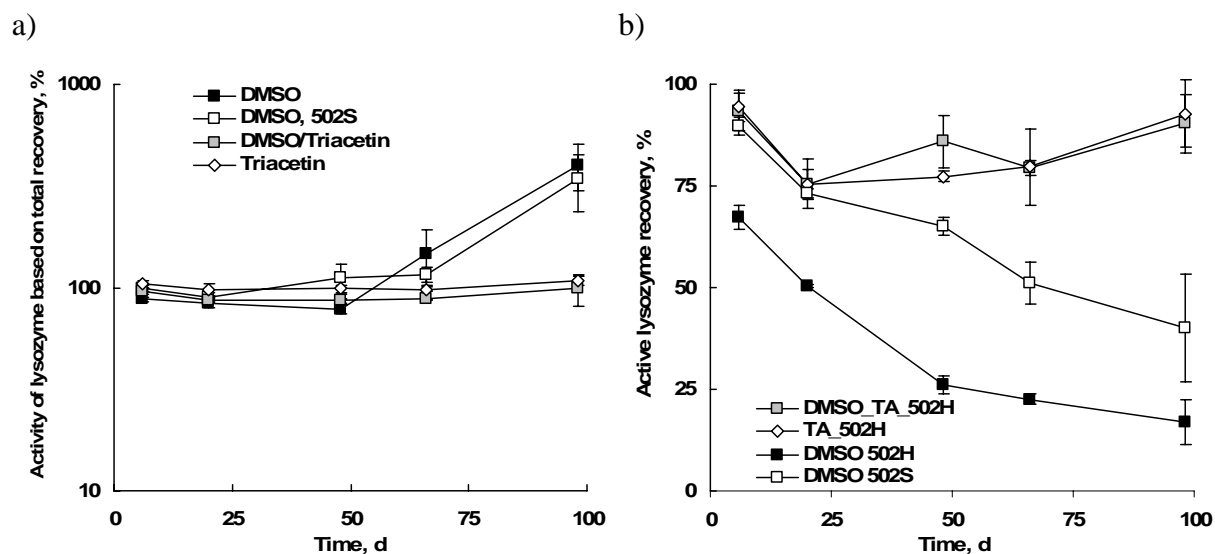


Fig. 138: a) Relative activity (active / total recovery, [%]) and b) active recovery (mass of active lysozyme recovered based on theoretical value) of lysozyme separated from protein containing in-situ implant formulations (30 % PLGA in DMSO / triacetin (TA) blends containing 4 % protein based on PLGA) as a function of storage time at ambient temperature in a desiccator

Tab. 18: Lysozyme total and active recovery (based on theoretical value) and resulting relative activity (active / total recovery, [%]) from the in situ implant formulation based on 30 % RG 502H in DMSO as a function of incubation time in acetate buffer (pH 5) after separation

Storage + incubation time	HPLC %	Active recovery %	Relative activity %
98 d	4.2 (0.4)	16.9 (5.6)	402.8 (101.7)
98 d + 1 d	47.9 (6.8)	25.7 (3.1)	53.9 (5.1)
98 d + 4 d	44.6 (4.6)	28.2 (3.7)	63.1 (4.3)
98 d + 134 d	78.5 (13.9)	55.7 (7.3)	71.7 (3.5)

Electrophoresis suggested that the reduction of the recoverable lysozyme amount during storage was due to aggregation. The recovered material from the DMSO / triacetin blend and triacetin showed more or less full activity throughout the stability study.

Relative activities (active recovery / total recovery [%]) above 100 % were obtained for DMSO samples after 48 d, which indicated an underestimation of the total recovery or an overestimation of the active recovery (Figure 138a). Repetitions of both lysozyme assays after one day resulted in a significantly increased total recovery from a 98 d stored DMSO sample whereas the active recovery was more or less unchanged (Table 18).

Therefore, a time-dependent recovery could be concluded and relative activities above 100 % were obtained since enzymatic activity of lysozyme was always determined after assaying the total recovery. Reanalysis of the aqueous medium obtained from extraction of the in situ implant formulation based on 30 % RG 502H in DMSO after four days indicated the increase to occur primarily within the first day after extraction. A retarded re-dissolution of extracted material in the aqueous medium was concluded. Since only DMSO samples were affected, this effect could be attributed to slower dissolution of extracted aggregated lysozyme. A final analysis of the extracted samples after 134 d revealed a further increase of the total and active recovery as well as an increase of the relative activity. Compared to a retarded dissolution of aggregates after extraction, the long term increase of total and active recovery was likely due to a disaggregation process occurring in the aqueous medium. The increase of the relative activity between 4 to 134 d incubation would be in agreement with a lower activity of lysozyme aggregates compared to the monomeric protein described previously (Snell et al., 2001). The kinetic of the redissolution process appeared to be of a logarithmic character and thus might be linked to the observations reported above (paragraph 3.2.2.2.2.5.).

The drug release of in situ implants was slightly affected by storage at ambient conditions (desiccator) for 4 weeks (Figure 139). The release was faster than with the freshly prepared sample, which could be due to an earlier onset of the erosion phase due to polymer degradation during storage (Dong et al. 2006) or due to an effect of the lysozyme aggregation on the release (similar to paragraph 3.2.2.2.2.1.).

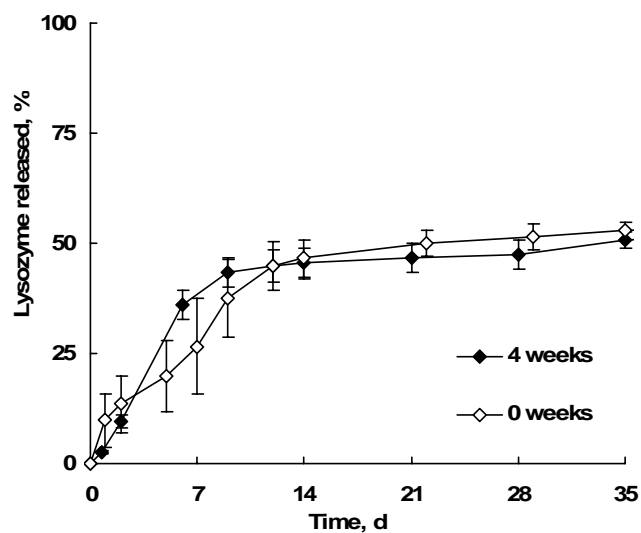


Fig. 139: Lysozyme release from in situ implants based on 40 % PLGA (RG 502H) in DMSO containing 4 % (relative to polymer) lysozyme as a function of storage time at ambient temperature in a desiccator (33 mM acetate buffer pH 5, vertical test tube position)

3.3. External phase composition of ISM

Beside the important effects of the polymer solution phase composition on lysozyme release, also the external phase of the in situ microparticle emulsions affected the release of the protein (paragraphs 3.2.1.2.4., 3.2.3.1.4.1. and 3.2.3.1.4.1.). Furthermore, it was indicated that the non-aqueous emulsions had a limited stability, which could influence the injectability of the formulations. Thus, effects of the external phase composition on lysozyme release and the stability of the emulsion were investigated.

3.3.1. Oil-in-oil vs. water-in-oil in situ microparticles

In situ microparticle emulsions can be formulated as oil-in-oil or alternatively as oil-in-water emulsions. In order to form an emulsion the miscibility of two phases has to be limited. In order to directly compare both in situ microparticle emulsions, triacetin was selected as PLGA solvent, since it possessed a limited miscibility with water as well as with sesame oil (Table 11, paragraph 3.1.3.2.2.).

As described in a previous paragraph (paragraph 3.2.1.2.4.), the incorporation of sesame oil into triacetin-based in situ implants accelerated the release of lysozyme, which could be attributed to a density-driven separation mechanism. The release of lysozyme from the in situ microparticle system with an aqueous phase (0.1 % Lutrol F 68), was even faster compared to the oil-in-oil system with sesame oil (Figure 140). However, the release was not controlled but was mainly characterized by the large burst. Instead of a satisfying encapsulation, a considerable portion of the lysozyme particles (unmicronized) probably dissolved in contact with the aqueous phase during emulsification. The hydrophilic protein is rapidly in contact with water.

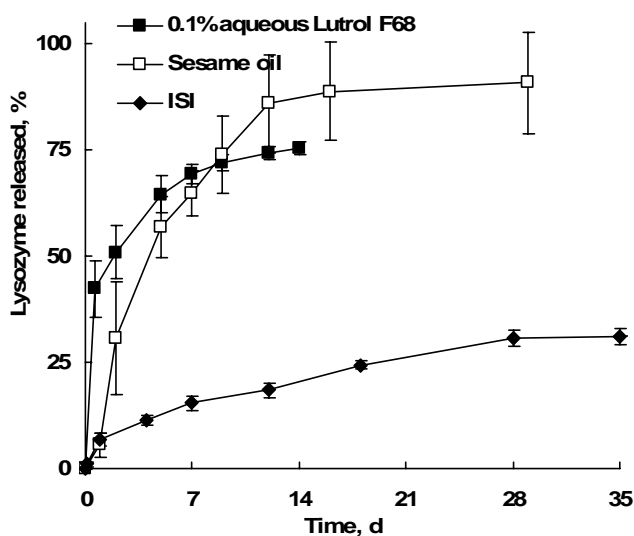


Fig. 140: Lysozyme release from in situ microparticles based on 30 % PLGA (RG 502H) in triacetin containing 4 % lysozyme (relative to polymer) as a function of the type / presence of the external phase

3.3.2. *Internal to external phase ratio*

The internal to external phase ratio was previously shown to affect the release of a small peptide from in situ microparticle formulations (Kranz and Bodmeier, 2007). In contrast to the previous data, ratios above 50:50 were investigated in order to maintain high drug loadings in the in situ formulations, which avoid potential compliance issues due to an increased injection volume (constant drug loading of the internal phase).

As indicated above (paragraph 3.2.1.2.4.), the acceleration of lysozyme release from in situ implant formulations based on triacetin was already achieved by an incorporation of 10 % sesame oil. The release was robust to changes of the phase ratios between 50:50 and 90:10 (Figure 141). However, the triacetin systems were sensitive to changes the internal to external phase ratio between 90:10 and 100:0. According to the previous discussion, this could be understood considering a maximum amount of sesame oil, which can be incorporated in to the polymer solution in triacetin, whereas the excess of sesame oil and the coalesced polymer solution would separate from the surrounding oil phase due to its high density.

A change of the internal to external phase ratio between 67:33 and 33:67 did not affect the initial release of lysozyme from in situ microparticles based on 30 % PLGA solutions in DMSO (Figure 142).

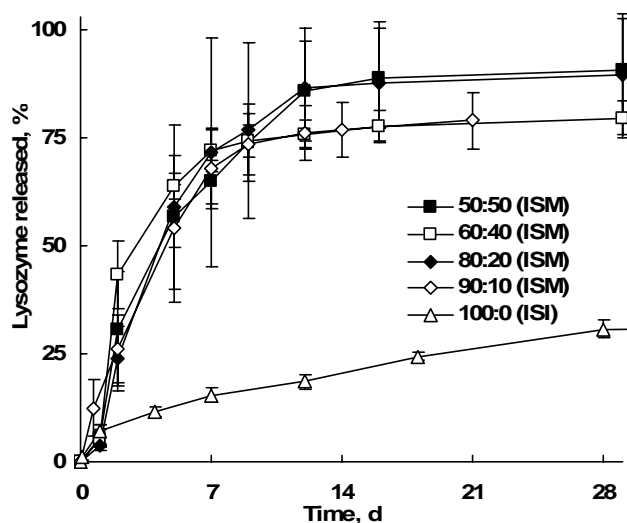


Fig. 141: Lysozyme release from in situ formulations based on 30 % PLGA (RG 502H) in triacetin as a function of the internal (in situ implant, ISI) to external (sesame oil) phase ratio

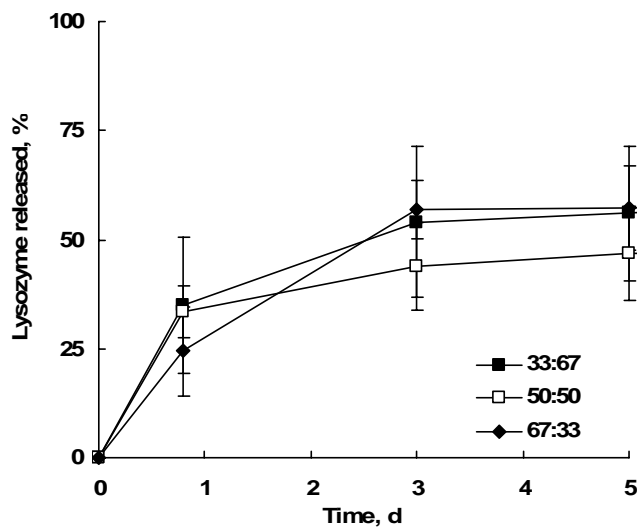


Fig. 142: Lysozyme release from in situ formulations based on 30 % PLGA (RG 502H) in DMSO as a function of the internal to external (sesame oil containing 10 % benzyl benzoate) phase ratio

3.3.3. Mixing rate during emulsification

An increase or decrease of the rate, at which the drug-containing polymer solution and the external sesame oil-based phase were usually blended with the two-syringe system (1/s for 50 mixing cycles), by a factor of two did not affect the initial release of lysozyme from the resulting in situ microparticle systems (Figure 143). Similar to the robustness of the formulations to slight changes of the phase ratio, the robustness to the mixing rate was rational considering a rapid breaking of the emulsions according to their densities.

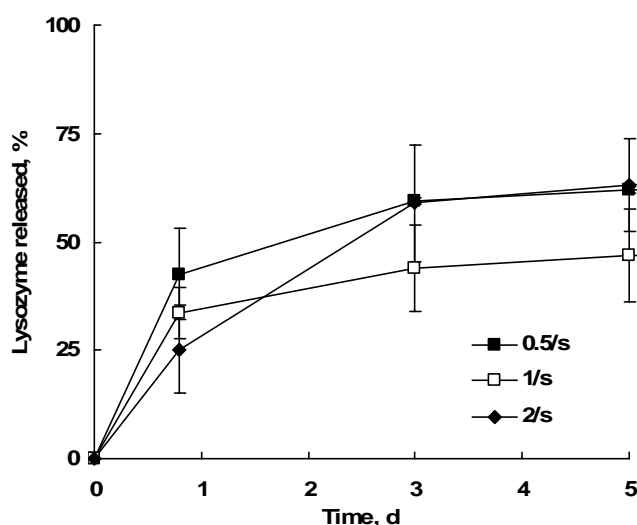


Fig. 143: Lysozyme release from in situ formulations based on 30 % PLGA (RG 502H) in DMSO as a function of the rate of mixing (50 cycles) of the polymer solution and sesame oil containing 10 % benzyl benzoate

It could be concluded, that the investigated in situ microparticle formulations were robust to slight alterations of the internal to external phase ratio as well as to changes of the mixing rate.

3.3.4. Injectability

The injectability of triacetin-based in situ formulations was tested using 1 ml syringes equipped with 20G hypodermic needles. The loaded volumes were about 0.2 ml for the in situ implants, whereas 0.4 ml of the in situ microparticle emulsions were filled into syringes in order to compare similar doses. Both injection volumes were below 1 ml, which was referred to as maximum volume for i.m. administration to the deltoid site (Rodger and King, 2000). The

applied injection speed of 0.5 mm/s (120 s/ml) was slower than recommended for aqueous systems (e.g., 10 s/ml, Mitchell and Whitney, 2001). However, an increase of the injection speed would increase the force required to eject the in situ formulations (linear relation). Since this force also depends on the viscosity of the liquid (Chien et al., 1981), the risk of injection problems with the in situ systems based on the highly viscous PLGA solutions in triacetin would increase. A detachment of the needle from the syringe body was observed, for example, at peak injection forces of about 100 N. The formulations were ejections into air.

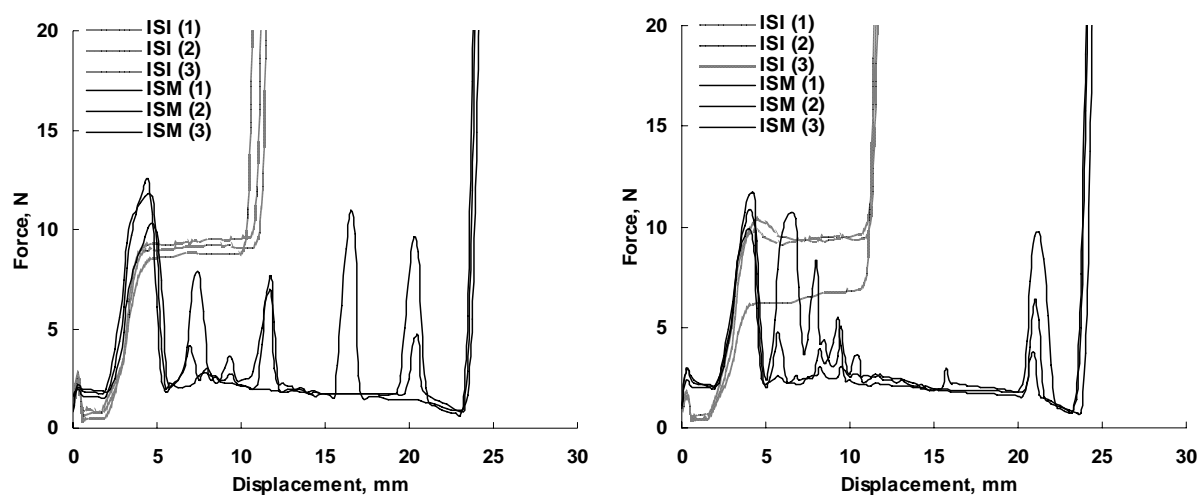


Fig. 144: Required force to eject in situ implants (0.25 g of 30 % PLGA in triacetin) or the corresponding freshly prepared in situ microparticle formulations with sesame oil (0.5 g) from 1 ml syringes equipped with 20G x 1.5 inch hypodermic needles (plunger speed: 0.5 mm/s) over plunger displacement ($n=3$); a) RG 502H and b) RG 502S

The plunger position where the syringes were completely emptied (injection forces >20 N) were about twofold higher with the in situ microparticle emulsions than with the in situ implant formulations in force-plunger displacement-curves (Figure 144). This reflected the different volumes of the in situ formulations loaded to the syringes.

The profiles were in agreement with the limited stability of the in situ microparticle emulsion, indicated previously (paragraphs 3.2.1.1., 3.2.1.2.4. for TA- and 3.2.2.2.1. for DMSO-based in situ microparticle emulsions). Peaks occurring in the force-displacement-plots during ejection were due to coalesced polymer solution droplets (lumps), which were present in the freshly

prepared samples. Oppositely to the in situ microparticle emulsions, the profiles obtained with the homogenous in situ implants were smooth and characterized by a plateau at the maximum injection force. Although the average force during ejection was lower with in situ microparticle emulsions (RG 502H: 3.1 +/- 2.7 N; RG 502S: 3.1 +/- 2.3 N) compared to in situ implants (RG 502H: 6.7 +/- 3.5 N; RG 502S: 6.4 +/- 3.5 N), the maximum forces, which need to be overcome during the injection, were comparable or even higher with the in situ microparticle formulations. Higher values could thereby be rationalized by the fact that triacetin was partially miscible with sesame oil. Thus, some polymer solvent might have partitioned into the oil phase, leaving more concentrated and hence more viscous polymer solution droplets behind.

3.3.5. Stability of oil-in-oil emulsions

The results on the injectability of the in situ microparticle emulsions suggested that the polymer solution droplets need to have a suitable size to pass the syringe needle. Thus, peak forces during injection could be avoided, what potentially leads to an improved injectability compared to the in situ implants. Therefore, the stabilities of oil-in-oil in situ microparticle emulsions were investigated and the effectiveness of emulsifying agents evaluated.

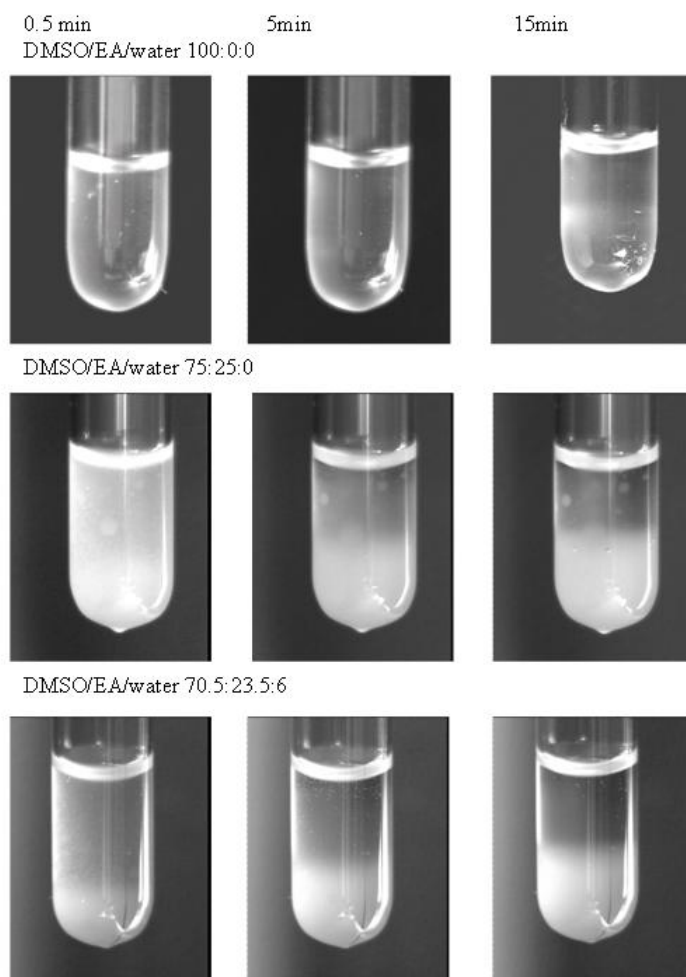


Fig. 145: Drug-free in situ microparticle emulsions of 30 % RG 502H in DMSO / ethyl acetate (EA) / water blends 0.5, 5 and 15 min after emulsification with sesame oil (total volume: 0.5 ml, phase ratio 1:1, 50 mixing cycles at 1/s, emulsions ejected from syringe without needle)

Phase separation with stabilizer-free in situ microparticle emulsions consisting of 30 % RG 502H in DMSO and sesame oil occurred rapidly (Figure 145). Already 0.5 minutes after emulsification two separated areas could be identified with DMSO / ethyl acetate / water ratios 75:25:0 and 70.5/23.5/6. According to a mass ratio of the polymer solution and the sesame oil phase of 1:1, the phase boundary formed at about half of the filling height of the test tubes. The clearer upper part of the broken emulsions represented the oil-rich phase, whereas the turbid mass on the bottom of the vials consisted of the denser polymer solution mainly. Comparable optical properties of DMSO and sesame oil hampered a visual differentiation between the polymer solution based on DMSO only and the oil before 15 min, where a phase boundary could be observed. The formation of this boundary between the polymer solution phase and the oil phase indicated an almost completed phase separation although microscopic pictures showed residual dispersed material in the DMSO only system even beyond a standing time of 15 minutes (Figure 146).

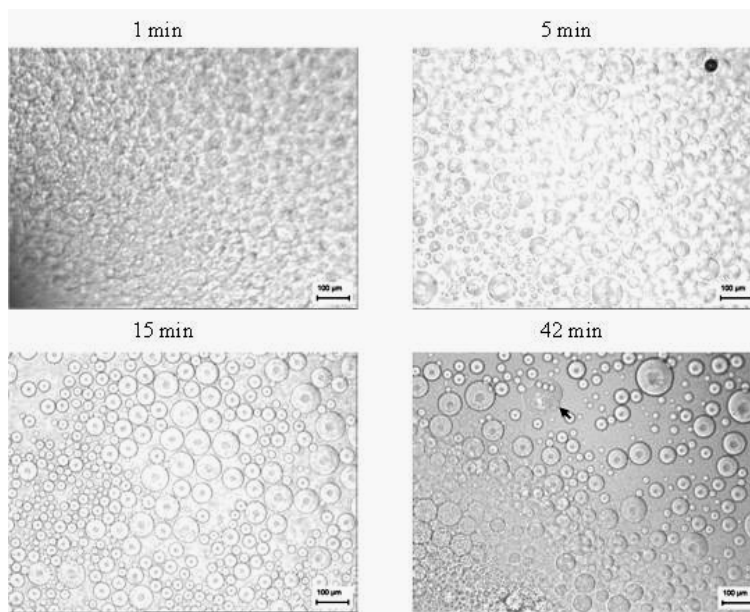


Fig. 146: Microscopic pictures of drug-free in situ microparticle emulsions of 30 % RG 502H in DMSO after emulsification with sesame oil (total volume: 0.5 ml, phase ratio 1:1, 50 mixing cycles at 1/s, emulsions ejected from syringe without needle)

The vast majority of research on the stability of emulsions has been done on aqueous systems. Only a limited number of studies dealt with non-aqueous systems (Hamdan et al., 1995, Cameron and Sherrington, 1996, Imhof and Pine, 1997).

The main focus in the selection of the stabilizing agents was not put on the hydrophilic-lipophilic balance value of the compounds since stabilization of non-aqueous emulsions was reported to be independent of the polarity of the stabilizer (Imhof and Pine, 1997). Bulkier amphiphilic molecules, such as triblock-copolymers of polyethylene oxide (PEO) and polypropylene oxide (PPO), appeared to protect droplets from coalescence. However, the PEO-PPO-PEO triblock-copolymer poloxamer 407 (Lutrol F127) was not soluble in sesame oil and the dispersion in the oil did not stabilize an oil-in-oil emulsion based on DMSO only.

The selected compounds included the nonionic nonpolar emulsifier Span 80 (HLB 4.3) and the soy phosphatidyl cholines Lipoid S45 and S75, which are recommended by the supplier (Lipoid GmbH, Germany) as stabilizer for parenteral suspensions (both) and emulsions (S75). The substances were dissolved in sesame oil to obtain a concentration of 1 % (based on sesame oil).

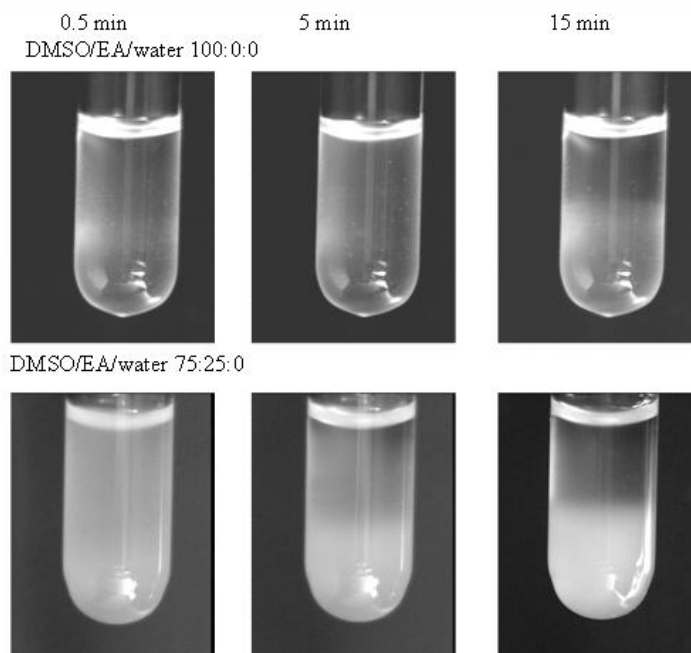


Fig. 147: Drug-free in situ microparticle emulsions of 30 % RG 502H in DMSO / ethyl acetate (EA) / water blends 0.5, 5 and 15 min after emulsification with sesame oil containing 1 % Span 80 (total volume: 0.5 ml, phase ratio 1:1, 50 mixing cycles at 1/s, emulsions ejected from syringe without needle)

A rapid phase separation was observed with the Span 80-containing in situ microparticle emulsions based on 30 % RG 502H in DMSO / ethyl acetate / water 100:0:0 or 75:25:0 (Figure 147). Again, a distinct phase boundary between the oil-rich and the polymer-rich phase was formed within 15 min. Thus, the addition of 1 % Span 80 to sesame oil did not improve the stability of the in situ microparticle emulsions (Figure 147 vs. 145).

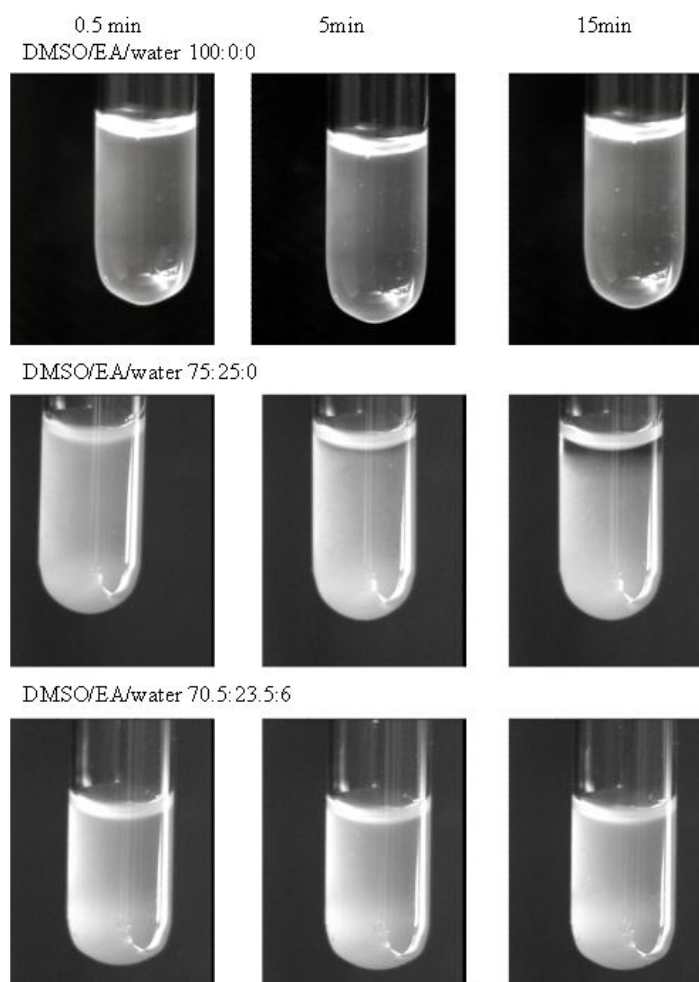


Fig. 148: Drug-free in situ microparticle emulsions of 30 % RG 502H in DMSO / ethyl acetate (EA) / water blends 0.5, 5 and 15 min after emulsification with sesame oil containing 1 % Lipoid S45 (total volume: 0.5 ml, phase ratio 1:1, 50 mixing cycles at 1/s, emulsions ejected from syringe without needle)

In contrast to Span 80, incorporation of the phosphatidyl cholines Lipoid S45 and S75 appeared to result in a stabilization of the in situ microparticle emulsions (Figures 148 and 149). The

formation of separated phases was retarded with Lipoid S45-containing sesame oil in emulsions with the more polar internal phases based on DMSO / ethyl acetate / water 100:0:0 and 70.5:23.5:6 (Figure 148). A clear oil-rich phase on top of the emulsions based on DMSO / ethyl acetate / water ratio 75:25:0 was observed after 15 minutes. Lipoid S75 appeared to be slightly less effective compared to Lipoid S45. A phase boundary was indicated with the polymer solution based on DMSO only (Figure 149).

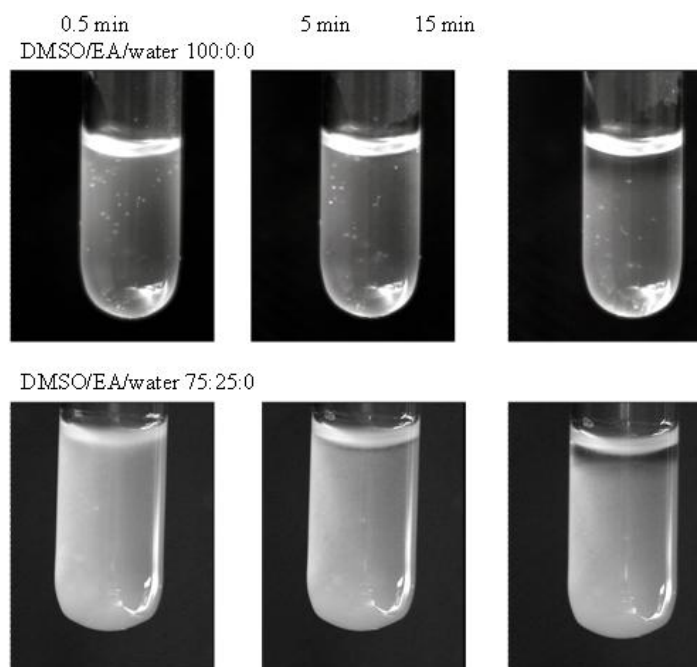


Fig. 149: Drug-free in situ microparticle emulsions of 30 % RG 502H in DMSO / ethyl acetate (EA) / water blends 0.5, 5 and 15 min after emulsification with sesame oil containing 1 % Lipoid S75 (total volume: 0.5 ml, phase ratio 1:1, 50 mixing cycles at 1/s, emulsions ejected from syringe without needle)

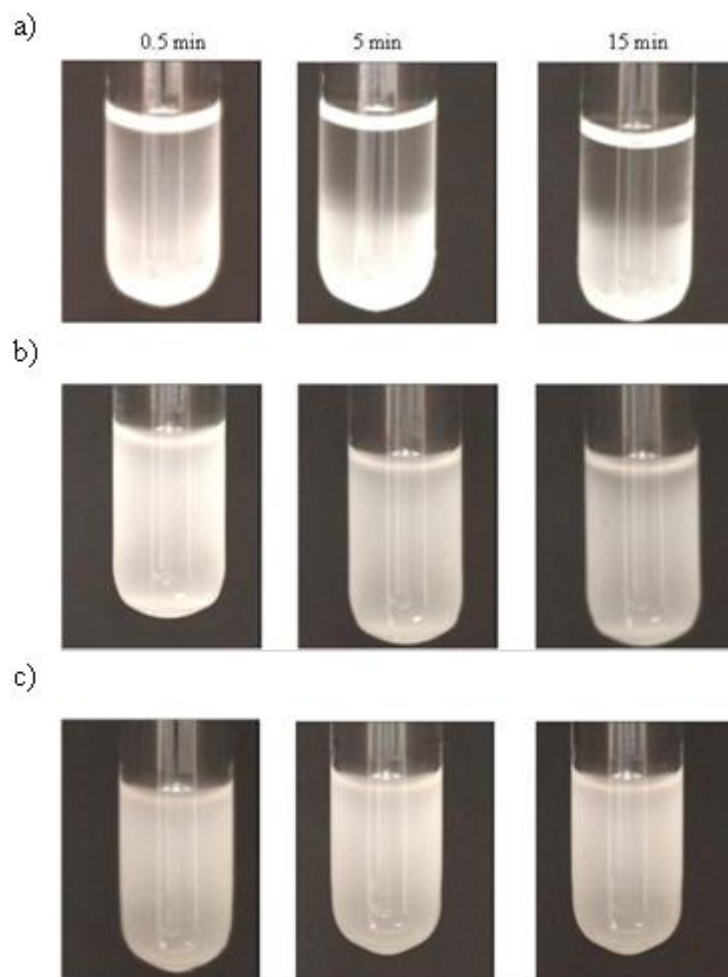


Fig. 150: Drug-free in situ microparticle emulsions of 30 % RG 502H in DMSO / ethyl acetate (EA) / water 70.5:23.5:6 as a function of time after emulsification with medium chain triglycerides containing a) no, b) 1 % and c) 5 % Lipoid S45 (total volume: 0.5 ml, phase ratio 1:1, 50 mixing cycles at 1/s, emulsions ejected from syringe without needle)

A stabilizing effect of Lipoid S45 could be also shown for emulsions of DMSO-based PLGA solutions with medium chain triglycerides (MCT), as alternative oil-phase. Although even 5 % of Lipoid S45 could be dissolved in MCT, an addition of 1 % was already enough to obtain a marked stabilization of an emulsion based on 30 % RG 502H in DMSO / ethyl acetate / water 70.5:23.5:6 (Figure 150).

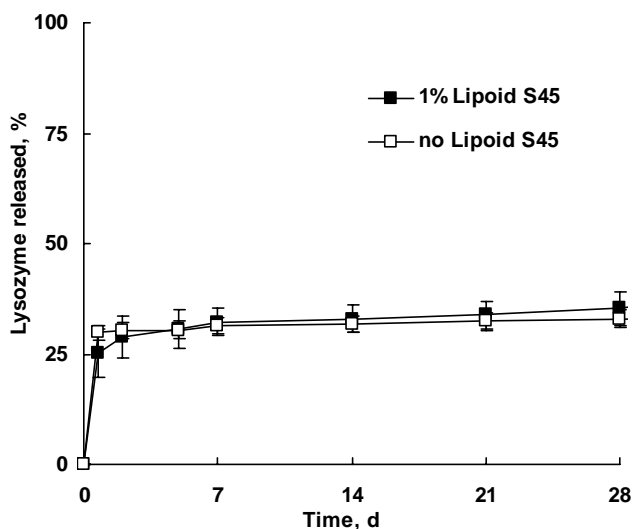


Fig. 151: Lysozyme release from in situ formulations based on 30 % PLGA (RG 502H) in DMSO / ethyl acetate / water 75:25:0 (4 % drug loading) as a function of Lipoid S45 addition to sesame oil (horizontal test tube position)

Results on the effect of surfactant addition on the release pattern obtained under unoptimized release conditions (horizontal test tube position) could not reveal a change upon addition of 1 % Lipoid S45 to the sesame oil phase (Figure 151). However, whether an improved injectability compared to the corresponding in situ implants systems could be obtained without a negative effect on the release characteristics needs to be confirmed with more relevant formulations under optimized release conditions.