



## Effects of salt addition on the microencapsulation of proteins using W/O/W double emulsion technique

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The influence of co-encapsulation of stabilizing additives together with BSA on microsphere characteristics using the modified water-in-oil-in-water emulsion solvent evaporation (W/O/W) method was investigated. For this purpose, poly(L-lactide) microspheres containing bovine serum albumin (BSA) were prepared. The morphology, porosity, specific surface area, particle size, encapsulation efficiency and kinetics of drug release of protein loaded microspheres were analysed in relation to the influence of co-encapsulated stabilizing additives such as electrolytes. High salt concentrations in the internal ( $W_1$ ) aqueous phase, often necessary to stabilize protein or antigen solutions, led to an increase in particle size, particle size distribution, porosity and specific surface area. Bulk density and encapsulation efficiency decreased. The release profile was characterized by a high initial burst due to the highly porous structure. Addition of salt to the external or continuous water phase ( $W_2$ ), however, stabilized the encapsulation process and, therefore, resulted in improved microsphere characteristics as a dense morphology, a reduced initial burst release, a drastically increased bulk density and encapsulation efficiency. Analysis of the specific surface area (BET) showed that the addition of salt to  $W_2$ , regardless of the salt concentration in the  $W_1$  phase, decreased the surface area of the microspheres approximately 23-fold. Microsphere properties were influenced by salts additions through the osmotic pressure gradients between the two aqueous phases and the water flux during microsphere formation. Release profiles and encapsulation efficiencies correlated well with the porosity and the surface area of microspheres. Furthermore, the influence of a low molecular weight drug and different time-points of salt addition to  $W_2$  on microsphere characteristics were studied by encapsulation of acid orange 63 (AO63), confirming the results obtained with BSA. This study suggests that modification of the external water phase by adding salts is a simple and efficient method to encapsulate stabilized protein solution, with high encapsulation efficiency and good microsphere characteristics.

**Keywords:** W/O/W encapsulation method, microspheres, morphology, process stability, additives, protein release.

### Introduction

During the last two decades, parenteral drug delivery systems based on biodegradable microspheres have extensively been investigated (Okada and Togucki 1995). While the microencapsulation of peptides has led to several commercially

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available products, the encapsulation of proteins still presents considerable difficulties. In contrast to peptides, proteins possess a more complex 3-dimensional structure, necessary for their biological activity, and consequently, present more problems concerning stability (Manning *et al.* 1989).

During microencapsulation, proteins are exposed to organic solvents, heat, extreme shear forces and/or ultrasonication, as well as increasing concentrations of additives, destabilizing proteins (Charman *et al.* 1993, Lu and Park 1995). Furthermore, a decrease in pH is observed in microspheres during release due to degradation products of poly(lactide-co-glycolide), PLG. Therefore, the complexity of their molecular structure often requires stabilization during storage, preparation and rehydration of microspheres to retain their biological activity (Johansen *et al.* 1998, Morlock *et al.* 1997). As a consequence, the encapsulation process of proteins has to be adapted precisely to ensure protein stability. The co-encapsulation of additives such as salts or sugars, which may interact with the aqueous protein (Cleland and Jones 1996), is a promising approach to retain the biological activity.

Among the microencapsulation techniques, the double emulsion or W/O/W method is one of the most frequently used methods for encapsulating water soluble drugs (Ogawa *et al.* 1988). An aqueous solution (internal aqueous phase,  $W_1$ ) of the drug is emulsified into an organic solution containing the polymer. This primary emulsion ( $W_1/O$ ) is then dispersed in a second aqueous phase containing a suitable emulsifier (external/outer water phase,  $W_2$ ), resulting in a transient formation of a double emulsion ( $W_1/O/W_2$ ). Solid microspheres are collected following the complete removal of the volatile organic solvent.

Since the microencapsulation procedure offers a large number of possibilities for modifications, the success of this technology strongly depends on the adaptation of process variables. Formulation parameters affecting the final product have been identified, including polymer composition and concentration, rates of solvent diffusion, drug loading, phase ratio of emulsion system, emulsion stability, homogenization techniques, and temperature (Alex and Bodmeier 1990, Nihant *et al.* 1994, Schugens *et al.* 1994, Crotts and Park 1995, Pistel *et al.* 1999).

While the introduction of sensitive proteins in the form of aqueous solutions is an attractive feature of the W/O/W process, it also creates various problems. Proteins often require buffer salts and other excipients for their stabilization (Manning *et al.* 1989). These additives to the  $W_1$  phase, such as buffer salts, amino acids, carbohydrates and polymers are needed to maintain protein integrity. On the other hand, an increased osmotic pressure results from these additives in the  $W_1$ -phase, which was shown to decrease the encapsulation efficiency (Morlock *et al.* 1997). Diluting the protein solution to reduce the osmotic pressure of the internal phase is not an acceptable alternative because the encapsulation efficiency decreases markedly with the increasing volume of the  $W_1$  phase (Alex and Bodmeier 1990, Crotts and Park 1995). Therefore, protein solutions are frequently desalinated prior to preparation of the  $W_1/O$  emulsion. This procedure can destabilize the protein and usually leads to drug loss by adsorption of protein to membranes and reaction vessels, which is undesirable for expensive proteins.

The present study was undertaken to improve the microencapsulation process for protein or antigen solutions using the W/O/W double emulsion technique, without resorting to desalination procedures. L-Polylactic acid (L-PLA) was chosen as the prototype of very hydrophobic polyester. Moreover, its slow

degradation properties should facilitate the analysis of the pore diffusion process without interference from polymer erosion. Bovine serum albumin was used as a model protein. To demonstrate the influence of salts in the internal  $W_1$  phase different concentrations of NaCl were added. The approach was to stabilize the microencapsulation process by also adding salts to the external  $W_2$  phase to compensate the osmotic gradients. The effect of additives on the W/O/W double emulsion microencapsulation process and its influence on the microsphere characteristics, especially surface morphology, were analysed.

In addition, for further demonstration of the stabilizing effect of salt in the continuous water phase, the low molecular weight water soluble dye, acid orange 63 (AO63), was encapsulated into L-PLA microspheres. In these experiments, the influence of the low molecular weight and the importance of the time-point of salt addition were investigated.

## Materials

L-Polylactic acid (L-PLA, Mw 40 kDa) was a gift from Novartis Pharma LTD (Switzerland) and poly(vinyl alcohol) (PVA) (88% hydrolysed, Mw 130 kDa, Mowiol<sup>®</sup> 18-88) was obtained from Hoechst AG (Germany). Bovine serum albumin (BSA), acid orange 63 (AO63), sodium dodecyl sulphate (SDS) and the BCA-assay were supplied by Sigma (Germany). All other materials of analytical quality were purchased from Merck (Germany).

## Methods

### *Preparation of microspheres*

BSA and AO63 loaded microspheres were prepared by a modified double emulsion technique ( $W_1/O/W_2$ ) (Ogawa *et al.* 1988). Briefly, a solution of polymer (L-PLA) in dichloromethane (DCM) (1 g/3.77 ml (BSA-loaded microspheres) and 0.4 g/10 ml (AO63 loaded microspheres)) was emulsified with 1 ml of an aqueous drug solution ( $W_1$ ) (50 mg BSA/ml, theoretical drug loading 5% and 40 mg AO63/ml, theoretical drug loading 10%) in a 10 ml polypropylene syringe using a high speed homogenizer ultra turrax (type TP18/10, IKA, Germany) operating at 20 000 rpm for 30 s to form the first inner  $W_1/O$  emulsion. This primary emulsion was immediately injected into 300 ml of an aqueous solution of poly(vinyl alcohol) (0.5% w/v) (external phase,  $W_2$ ) and homogenized for 30 s using an ultra turrax (type T25, IKA, Germany) at 8000 rpm to produce a transient  $W_1/O/W_2$  emulsion. As described below, different salt concentrations were added to the internal and/or external water phase,  $W_1$  or  $W_2$  respectively. The resulting  $W_1/O/W_2$  emulsion was stirred at 200 rpm for 3 h with a propeller mixer to allow solvent extraction and evaporation and microsphere hardening. The microspheres were then isolated by centrifugation at 2500 rpm for 5 min, washed three times with distilled water and freeze-dried. The duration of lyophilization differed in dependence of salt addition to  $W_1$  and  $W_2$  from 15 h (no salt in  $W_1$  and salt in  $W_2$ ) up to 3 days (high salt concentration in  $W_1$  and no salt in  $W_2$ ). The final product was stored at +4°C in a desiccator.

### *Variations of BSA-loaded microspheres*

Three alternative procedures were carried out involving:

- addition of different NaCl concentrations to the internal water phase  $W_1$  (0, 2.5, 5.0, 7.5, 10.0 mg/ml) (group A),
- addition of different NaCl concentrations to the external water phase  $W_2$  (0, 2.5, 5.0, 7.5, 10.0% (m/v)) (group B), and
- addition of NaCl to both internal and external water phase simultaneously. The NaCl concentration in  $W_1$  was fixed at 10 mg/ml, whereas the NaCl concentration in  $W_2$  was varied (0, 2.5, 5, 7.5, and 10% (m/v)) (group C).

### *Variations of A063-loaded microspheres*

In order to evaluate the effect of salt concentration in the external aqueous phase on microsphere preparation, as well as the importance of time point of salt addition, three different alternative procedures were carried out involving:

- addition of different NaCl concentrations to the external water phase (0, 2.5, 5.0, 7.5, 10.0% (m/v)), and
- addition of 10% NaCl to the external water phase before, 2 min after and 30 min after injection of the  $W_1/O$  emulsion to the  $W_2$ -phase.

### *Drug content*

*Determination of BSA microsphere drug content.* Protein content was determined by a DMSO/NaOH/SDS method. Microspheres (10 mg), accurately weighed, were completely dissolved (1 h) in 2 ml DMSO by occasional shaking. Four millilitres of a NaOH/SDS-solution (0.25 N, 0.5% w/v) was added and the mixture was agitated in a rotating bottle apparatus (Rotatherm, Liebig, Germany) at 30 rpm and 37°C for 4 h. The amount of encapsulated BSA was determined using the BCS-assay (Pierce Chemicals, USA). The BCS working solution was prepared by combining 50 parts of BCA stock solution with one part of 4%  $CuSO_4 \cdot 5 H_2O$  just before the protein assay. The samples (50  $\mu$ l) were pipetted into microplate wells (Microwell-plates, Nunc, Germany) and were mixed with 200  $\mu$ l of BCA working solution. This microtiter plate was incubated at 60°C for 45 min, cooled for 15 min to room temperature and the intensity of colour development was then measured at 570 nm with a Microplate Reader (Titertek Plus MS212, ICN, SLT Labinstrumente Deutschland GmbH, Germany). All samples and standards were assayed in triplicate.

The encapsulation efficiency was expressed as the percentage of protein entrapped compared to the initial amount of protein. The theoretical loading was defined as the ratio between the initial amount of protein over the total amount of polymer, while the actual loading was equal to the ratio between the amount of entrapped protein over the total amount of polymer.

*Determination of A063 microsphere drug content.* The content of A063 in the microspheres was determined by alkaline hydrolysis. Briefly, 10 mg of microspheres, accurately weighed, were dispersed in 1.5 ml of a 0.5 N-NaOH. The dispersion was shaken at room temperature for 4 h with an Eppendorf mixer (type 5432, Eppendorf, Germany) and then for 20 h with an orbital shaker apparatus

(Rotatherm, Liebsch, Germany) at 30 rpm and 37°C. Following centrifugation at 5000 rpm for 5 min (type 5415C, Eppendorf, Germany) the AO63 content of the supernatant was determined spectrophotometrically at 426 nm (UV 160, Shimadzu, Japan) against a series of AO63 standards prepared in 0.5 N-NaOH. Each microsphere batch was measured in triplicate.

#### *Particle size and particle size distribution*

Particle size and particle size distribution were analysed, dispersing 10 mg of the samples in an aqueous solution of Tween<sup>®</sup> 20 (0.1% w/v). The measurements were carried out by laser light scattering using a Malvern Mastersizer X (Malvern Instruments, UK). The 300 mm lens utilized for BSA microspheres covered a particle size range of 1.2–600 µm and the lens 100 mm lens covering a particle size range of 0.5–180 µm, was used for AO63 microspheres. The calculations of the particle sizes was carried out using the standard modulus of the Malvern software according to the theory of Mie. The weighted average of the volume distribution  $D[4.3]$  was used to describe particle size. This parameter is defined as follows:  $D[4.3] = \sum nd^4 / \sum nd^3$  ( $n$  = number of particles in each area of particle sizes,  $d$  = medium particle diameter in the area of particle sizes). Each sample was measured in triplicate.

#### *Release of BSA and AO63 from microspheres under in vitro conditions*

Eighty milligrams of the BSA and 40 mg of the AO63 microspheres were suspended in 4 ml of PBS-buffer pH 7.4 (8 g NaCl, 0.2 g KCl, 0.24 g KH<sub>2</sub>PO<sub>4</sub>, 1.81 g Na<sub>2</sub>HPO<sub>4</sub>\*2 H<sub>2</sub>O, 0.5 g NaN<sub>3</sub>, 0.1 g Tween<sup>®</sup> 20 ad 1000 ml distilled water). The samples were agitated in a rotating bottle apparatus (Rotatherm, Liebsch, Germany) at 30 rpm at 37°C. At defined time intervals, the buffer was completely withdrawn after centrifugation and replaced by 4 ml of fresh buffer. The amount of drug released was determined spectrophotometrically at 278 nm (BSA) and 450 nm (AO63) (UV 160, Shimadzu, Japan) against a BSA or AO63 standard curve. Each microsphere batch was studied in triplicate.

#### *Surface morphology and internal morphology*

Cross-sections of the microspheres were obtained by embedding them into a tissue freezing medium (Leica Instruments, Germany) and cutting them using a microtome (Frigocut Mod. 2700, Reichert-Jung, Germany). Microspheres were dried *in vacuo* and subsequently sputter-coated with a gold layer at 25 mA in argon atmosphere at 0.3 hPa for 2 min (Edwards/Kneise Sputter Coater S150, Edwards, Germany). The coating procedure was repeated three times. The external and internal morphology of microspheres were analysed by scanning electron microscopy (SEM) (Hitachi S510, Hitachi Denshi GmbH, Germany) *in vacuo* (0.001 mbar) and at a voltage of 25 kV.

#### *Bulk density*

A known weight of microspheres (0.5–1 g) was transferred to a 2 ml graduated syringe and the initial volume recorded. The syringe was then tapped 1250 times. Tapped bulk density (BD) of the BSA microspheres was determined by measuring

the volume occupied after 1250 tappings. BD was expressed as g/ml using the following equation:  $BD = \text{weight of microspheres} / \text{final volume after tapping}$ .

### Specific surface area (nitrogen sorption analysis)

Specific surface area (SSA) of BSA microspheres was determined by the Brunauer-Emmett-Teller method (BET) (Brunauer *et al.* 1938). The BET method is based on the adsorption and desorption of nitrogen or krypton gas at the surface and within the pores of the microspheres.

After flooding the vacuum chamber with dry nitrogen gas, microspheres were transferred to a sample cell, accurately weighed, then gassed out at 25°C for at least 1 h immediately prior to analysis. The specific surface area was performed using a Gemini 2375 (V3.02). Sorption measurements were performed using ultra pure nitrogen as the adsorbate and liquid nitrogen as a coolant. Surface area was calculated by the BET method using five adsorption points in the  $P/P_0$  range of 0.05–0.3.

## Results and discussion

BSA was encapsulated in L-PLA microspheres and the presence of stabilizing agents was mimicked by separately adding NaCl to the internal water phase,  $W_1$ , in different concentrations (0–10 mg/ml), as shown in table 1. These salt concentrations represent common concentrations used in buffers for stabilizing proteins. The preparation process for the microsphere batch with the highest salt concentration in the inner water phase (batch A5) was then stabilized with salt in the external phase (0–10% w/v), as the microspheres showed poor characteristics or

Table 1. Characteristics of bovine serum albumin loaded microspheres.

Variation	MP batch	NaCl addition		Encaps. eff <sup>a</sup> (%)	Placebo MP		
		$W_1$ (mg/ml)	$W_2$ (%)		SSA <sup>b</sup> (m <sup>2</sup> /g)	Density (g/ml)	Bulk density (g/ml)
Addition of NaCl to the inner water phase ( $W_1$ )	A-1	0	0	71.2 ± 2.55	1.402	1.372	0.306
	A-2	2.5	0	48.1 ± 2.79			
	A-3	5.0	0	47.4 ± 2.12			
	A-4	7.5	0	46.5 ± 3.35			
	A-5	10.0	0	38.9 ± 1.13			
Addition of NaCl to both, inner ( $W_1$ ) and outer ( $W_2$ ) water phase simultaneously	B-1	10.0	2.5	75.5 ± 4.99	0.719	1.396	0.478
	B-2	10.0	5.0	72.4 ± 4.24			
	B-3	10.0	7.5	74.8 ± 5.71			
	B-4	10.0	10.0	72.6 ± 0.81			
Addition of NaCl to the outer water phase ( $W_2$ )	C-1	0	2.5	72.0 ± 1.10	0.780	1.380	0.523
	C-2	0	5.0	73.4 ± 3.58			
	C-3	0	7.5	74.0 ± 3.25			
	C-4	0	10.0	75.8 ± 2.74			

<sup>a</sup> Theoretical drug loading 5% w/w, determined with the DMSO/SDS/NaOH method.

<sup>b</sup> Specific surface area.

even became aggregated. For a complete comparison of the results, batches of microspheres with only salt in the external phase were additionally produced.

### *Characterization of microspheres*

Protein loaded microspheres prepared without salt, either in the inner water phase or in the outer water phase, showed a smooth surface with few pores and a spherical geometry, without aggregation phenomena, as confirmed by SEM analysis (figure 1(a)). The addition of salt to the internal water phase, however, had a significant effect on the external and internal structure of the microspheres, as clearly evident from the SEM photographs (figure 1(b) + (c)). A higher salt concentration in the inner water phase led to an increasingly porous structure with a significantly irregular, spongy shape. At salt concentrations of 10 mg/ml in  $W_1$ , microspheres lost their spherical shape, due to the very large pores, which in some cases led even to fusion and aggregation between the microspheres. By adding NaCl to the continuous, outer water phase, it was possible to reverse the phenomenon of increasingly porous structure. It was interesting to note that a small addition of NaCl of as little as 2.5% to the external water phase already reduced pore formation (figure 1(d)) and it should also be noted that microspheres prepared exclusively with salt in the outer phase displayed a smooth surface without pores and a spherical and absolutely dense and compact shape.

Scanning electron micrographs of AO63 loaded microspheres show that the encapsulation without modifying the preparation method led to extremely porous microspheres (figure 2(a)). Again, the addition of salt to the external phase led to microspheres with a smooth, non-porous surface and a spherical shape (figure 2(b)).

Under the influence of an osmotic gradient, the organic phase of a W/O/W emulsion acts as a semipermeable membrane allowing the passage of water across the organic phase. This leads either to swelling or shrinkage of the internal droplets, depending on the direction of the osmotic gradient (Florence and Whitehill 1982).

In the case of group A microspheres the high salt concentration in  $W_1$  forces water to pass along the osmotic pressure gradient, from the external to the internal phase, resulting in swelling of the internal droplets and of the embryonic microspheres. The higher phase volume ratio leads to a destabilization of the W/O/W emulsion. Occasionally, a rupture of the polymeric layer among group A microspheres, due to the extreme enlargement of the internal water phase, was observed, thus resulting in a release of the inner phase into the external water phase and a loss of drug during encapsulation. Therefore, a breakdown of the double emulsion is possible at very high salt concentrations caused by the coalescence of the internal water droplets. It is then impossible to form microspheres. The removal of solvent coacervates the polymer, thereby immobilizing and encapsulating the inner aqueous phase within a spherical polymeric matrix. However, the influx of water from the external to the internal water phase occurs faster than the coacervation, as the initial droplet size increases as the percentage of dispersed aqueous water phase increases. It was thought that the porous structure of microspheres of formulations A resulted from the inclusion of primary emulsion droplets within the polymer layer. It is conceivable that a pore would develop at the site of an aqueous droplet upon solidification of the polymer matrix (Crotts and Park 1995). In other words,

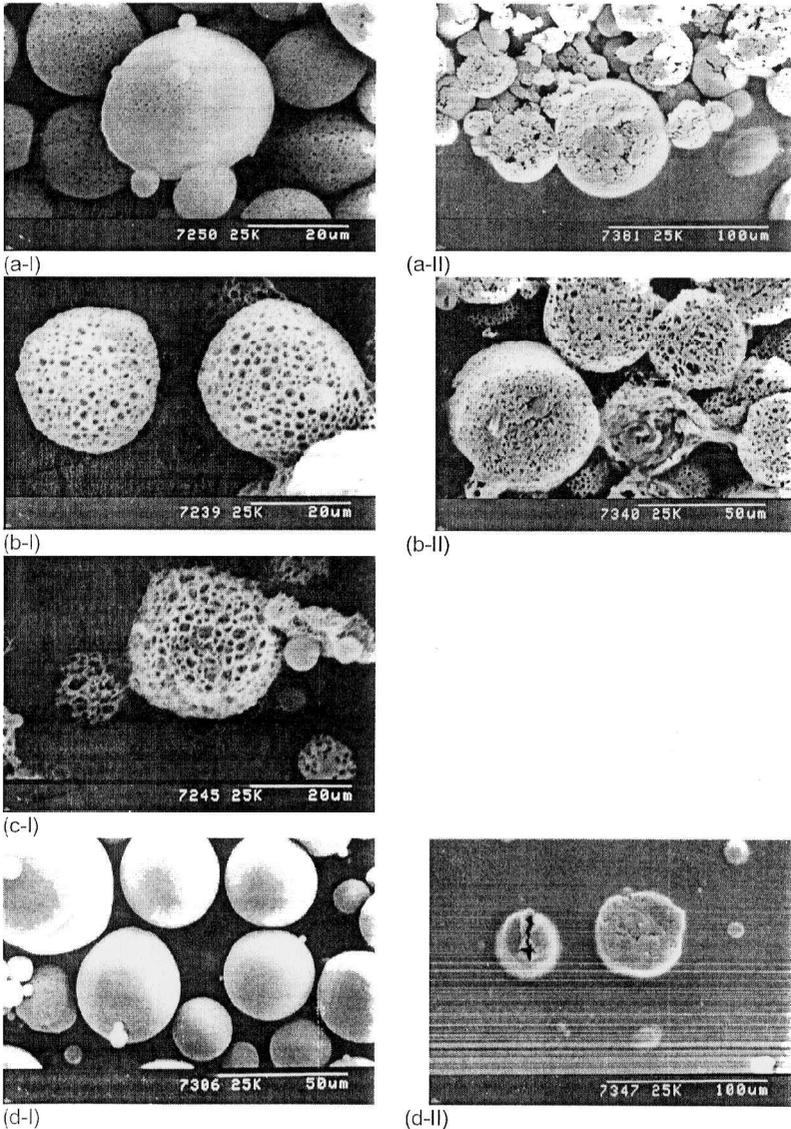


Figure 1. Surface morphology (I) and cross sections (II) of BSA loaded microspheres prepared with different salt concentrations in the inner water phase [(a) 0 mg/ml, (b) 5 mg/ml, (c) 10 mg/ml] and microspheres prepared with 10 mg/ml NaCl in the inner water phase and additionally 2.5% NaCl in the outer water phase (d) (C-II: No cross sections were obtained).

the structure of the W/O/W emulsion at the time of coacervation defines size and morphology of the final microspheres. During further processing, water still remained in the microspheres, thus causing the large volume and the extremely porous structure of the microspheres, which persisted even after drying. In the solidified state, the polymer chains were no longer flexible enough to rebuild a dense, compact microsphere matrix in the case of batches A2–A5. This theory was supported by the observation that, immediately after preparation, microspheres of

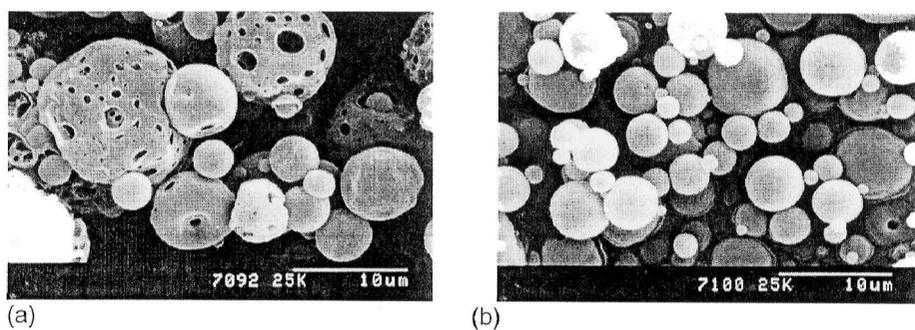


Figure 2. Scanning electron micrographs of acid orange 63 loaded microspheres prepared without (a), and with 10% (b), NaCl in the outer water phase.

group A (high salt concentration in  $W_1$ ) exhibited an extremely large volume (20-fold higher for A5 compared to batch C microsphere). The large amount of incorporated water led additionally to a prolonged lyophilization of 3 days. In contrast, microspheres of group B and C were very compact and dense, needing less than 15 h of lyophilization.

On the other hand, high salt concentrations in the continuous external water phase, regardless of the salt concentration in  $W_1$ , caused internal water droplets to remain small and, consequently, the porosity was reduced.

The large volume caused by the spongy and irregular network-like structure of microspheres prepared with high salt concentration in  $W_1$  (A2–A5) was quantified by determining the bulk density (table 1). Increasing the salt concentration in the inner water phase led to a decrease in the bulk density. Furthermore, microspheres prepared with salt in the external phase presented free-flowing microspheres because of the dense and smooth surface. In contrast, microspheres with a rough and porous surface (A3–A5) were not able to flow because of the high friction of the surface.

The densities of the placebo microspheres confirmed values found for L-PLA. However, the specific surface area (SSA) of A5 (10 mg/ml NaCl in  $W_1$ , 0% NaCl in  $W_2$ ) was found to be  $18.14 \text{ m}^2/\text{g}$ . This high value was indicative for highly porous particles. SSA of particles completely devoid of pores (salt addition to  $W_2$ ) and having a density of  $1.38 \text{ g/ml}$  is  $0.78 \text{ m}^2/\text{g}$ . Therefore, the high value obtained (23 times that of a non-porous microsphere) confirms the very porous feature.

The study of parameters influencing the morphology of microspheres has been the subject of numerous papers. Several factors such as polymer molecular weight, co-solvent concentration, peptide concentration, volume of the inner water phase, effect of solvent removal technique, shear force and emulsion stability and pH in the external phase, were found to influence the matrix porosity and, hence, the surface area of the microspheres (Schugens *et al.* 1994, Crotts and Park 1995, Sah *et al.* 1995b, Leo *et al.* 1998).

Apart from SEM and SSA data, the determination of the encapsulation efficiency as a function of salt concentrations also confirms the important influence of additives in the inner and/or outer water phase. As previously reported, the encapsulation efficiency of the drug into microspheres increased with addition of sodium chloride to the external aqueous poly(vinyl alcohol) solution (Uchida *et al.* 1995, 1996, Esposito *et al.* 1997, Takada *et al.* 1997).

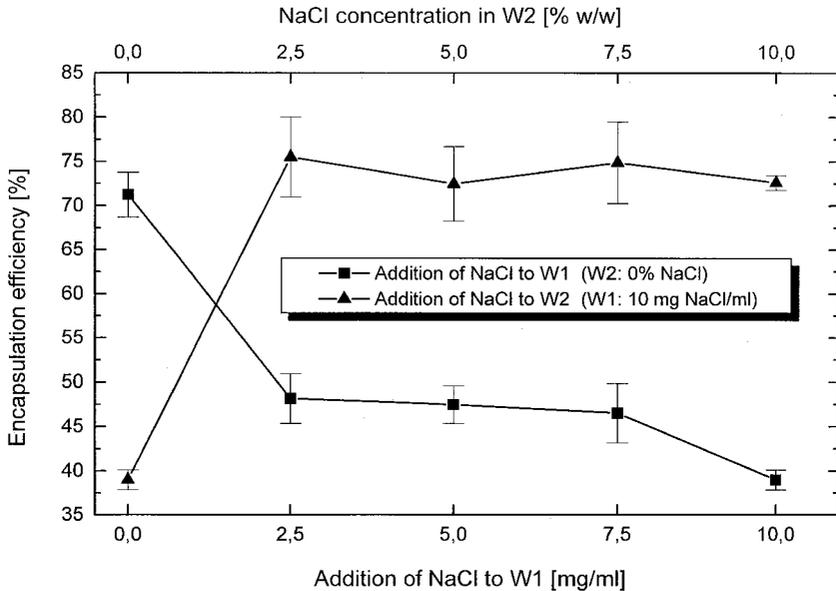


Figure 3. The influence of NaCl addition to the inner and/or outer water phase on the encapsulation efficiency of BSA. (■): Addition of different NaCl concentrations to the inner ( $W_1$ ) water phase (0, 2.5, 5.0, 7.5 and 10.0 mg/ml) (batches A1–A5). (▲): Addition of NaCl to both, inner ( $W_1$ ) and outer ( $W_2$ ) water phase simultaneously. The NaCl concentration of  $W_1$  was fixed at 10 mg/ml, whereas the NaCl concentration in  $W_2$  was varied (0, 2.5, 5.0, 7.5 and 10.0% m/v) (batches A5 and B1–B4). The drug content was determined with the DMSO/NaOH/SDS method.

Table 2. Characteristics of acid orange 63 loaded microspheres.

MP-batch	Salt type	Salt concentration in $W_2$ (%)	Timepoint of salt addition (min)	Yield (%)	Encaps. eff <sup>a</sup> (%)	Size $D$ (4.3) ( $\mu\text{m}$ )
AO-1	NaCl	0	0	74.2	0.0 $\pm$ 0.00	6.54
AO-2	NaCl	2.5	0	80.4	23.8 $\pm$ 0.56	5.99
AO-3	NaCl	5.0	0	80.3	77.2 $\pm$ 0.77	5.67
AO-4	NaCl	7.5	0	81.4	80.5 $\pm$ 1.58	4.88
AO-5	NaCl	10.0	0	82.2	87.8 $\pm$ 2.46	4.54
AO-6	NaCl	10.0	3	69.9	12.4 $\pm$ 0.08	5.12
AO-7	NaCl	10.0	15	70.4	0.4 $\pm$ 0.03	6.31

<sup>a</sup>Theoretical drug loading 10% w/w, determined with the alkaline hydrolysis method.

In this study, the entrapment of BSA in microspheres was drastically reduced from 72% to 38% when the NaCl concentration was increased from 0 mg/ml to 10 mg/ml in the inner water phase. The addition of salt to the outer water phase, simultaneously to high salt concentration in  $W_1$ , again increased encapsulation efficiency, as shown in figure 3.

Table 2 summarizes the encapsulation efficiencies of AO63 loaded microspheres in relation to NaCl concentration in  $W_2$  and the time-point of salt addition. Microspheres with very low encapsulation efficiency  $<2\%$  were obtained when

particles were prepared without salt in  $W_2$ , whereas the addition of 5% NaCl already led to a significant increase of the encapsulation efficiency of up to 80%. Furthermore, the salt concentration of the external phase was changed at different time intervals in relation to the second emulsification step, from 3–15 min after preparation of the W/O/W emulsion. As can be seen from table 2, the addition of salt after 15 min to the external  $W_2$  phase did not affect encapsulation efficiencies. Microspheres were similar to those prepared without salt in  $W_2$ . An increase in encapsulation efficiency and a reduction of surface pores were observed when adding NaCl 3 min after the second emulsification step was carried out. However, differences to microspheres prepared with salt in  $W_2$  from the beginning of the second emulsification were still noticeable.

An important prerequisite for the encapsulation of a drug is the dispersion of the aqueous phase into small droplets (Alex and Bodmeier 1990). The organic (middle) phase separates the internal water droplets from the external aqueous phase, thus acting as a diffusional barrier and preventing the diffusion of the protein into the outer phase. Nevertheless, an exchange between the two aqueous phases (external and internal) might occur as a consequence of protein solubility in the outer phase and of the instability of the W/O/W emulsion, caused by higher osmotic pressure in the internal water droplets. The influx of water into the internal aqueous phase increased the volume of  $W_1$  and the thickness of the oil layer around the internal droplets decreased. If the osmotic pressure difference across the organic phase, O, is extreme, then the passage of water is so rapid that an almost immediate rupture of the O phase with expulsion of the internal droplets occurs. However, the migration of inner water droplets and subsequent fusion with the outer phase is only possible during the initial stages of the second emulsification process, prior to the formation of the viscous boundary. The importance of the time dependent addition of salt was demonstrated for AO63 loaded microspheres. The addition, 15 min after the second emulsification step, led to microspheres comparable to those prepared without salt, whereas injecting the  $W_1/O$  emulsion into the  $W_2$  phase containing salt improved encapsulation efficiency. It was demonstrated that diffusion and drug loss across the droplet interface occurred mainly during the first minutes after the second emulsification step. Polymer precipitation to a dense matrix on the outer surface hindered further drug diffusion to the outer phase.

Similar observations were made in studies where the volume of the internal phase (Alex and Bodmeier 1990) and the total drug content were varied (Uchida *et al.* 1996). An increase in  $W_1$  volume leads to more porous microspheres and a decrease in microencapsulation efficiency. As well as salts, higher theoretical drug loading from low molecular weight substances also increases the osmotic pressure differences between the internal and external aqueous phase resulting in a more intensive mixing of the internal and external phase. Encapsulation efficiency decreases as theoretical drug load increases. Due to the colligative character of the osmotic pressure, this effect is especially enhanced for low molecular drugs as seen in the case of AO63, whereas the influence of proteins is negligible in this case due to their high molecular weight. However, labile proteins are often encapsulated together with stabilizing agents such as buffer salts or carbohydrates (Cleland and Jones 1996, Morlock *et al.* 1997). Since the stabilizers contribute to the osmotic pressure because of their low molecule weight, microspheres characteristics are also influenced. The loss of AO63 was enhanced by the low polymer

concentration (2.5% w/v) used for the preparation of AO63 microspheres, since the low kinematic viscosity of the L-PLA solution allows migration of the aqueous drug droplets out of the organic phase during formation of the second emulsion. In general, higher viscosity of the organic phase O stabilizes the film around the internal water droplets, therefore reducing possible coalescence of the internal phase and reducing the mixing with the external phase.

**The low encapsulation efficiency could also be a result of the quicker hardening of the microspheres.** In the case of batches A3–A5, **the increase in  $W_1$  due to its high salt concentration may open the possibility for solvent diffusion into two directions:** On one hand, solvent diffusion into the internal water phase, and on the other hand, diffusion into the external phase—the more important direction. Although the total water volume remains the same, **the porous polymeric matrix can now be created from outside to inside and,** to a minor extent, from inside to outside. It is obvious that **interconnecting channels might, therefore, be created more rapidly and drug loss enhanced.** A reduced increase of  $W_1$  volume was achieved by increasing the osmotic pressure in the external phase through addition of various amounts of salt. Significant improvement of encapsulation efficiencies and a drastic reduction in microsphere porosity were the result.

#### *In vitro release studies*

Apart from the morphological shape of microspheres, the *in vitro* release is the most important parameter in the investigation of a parenteral depot system. In general, BSA release occurs in three phases: a first, rapid release (burst release) followed by a second phase (lag-phase), characterized by a slow release rate of BSA related to a protein diffusion into the receptor medium, and a third phase with a rapid release resulting from polymer erosion (Bodmer *et al.* 1992). Phase one and in some cases phase two can be influenced by the preparation method, whereas the erosion phase is dominated by the polymer. BSA release studies were conducted up to 20 days, since the main interest was in the influence of the microencapsulation technique on the pore diffusion characteristics, relevant in phases one and two of the release process.

Although all microsphere formulations consisted of the same polymer composition, release patterns of BSA varied with respect to the initial drug release pattern, also designated as drug burst (figures 4 and 5). It was found that salt had a crucial effect on the initial burst, defined as release within the first 6 h. A salt concentration of 10 mg/ml in the inner water phase led to formation of microspheres that released 43% of BSA when suspended in PBS at 37°C for 6 h, whereas only 6% of BSA was released when salt (2.5%) was additionally added to the outer water phase during preparation, regardless of the high salt concentration in  $W_1$  (figure 6).

The release of AO63 loaded microspheres showed a clearly reduced initial burst and a following continuous release for those microspheres prepared with salt in  $W_2$ . In contrast, batch AO-2 was characterized by a high initial burst, followed only by a slow release (figure 7).

The different release patterns can be explained with the external, as well as with the internal morphology of the microspheres obtained at different salt concentration in  $W_1$  and  $W_2$  (figure 1). One of the factors affecting the hydration of hydrophobic polymers as L-PLA is the matrix structure and, hence, it

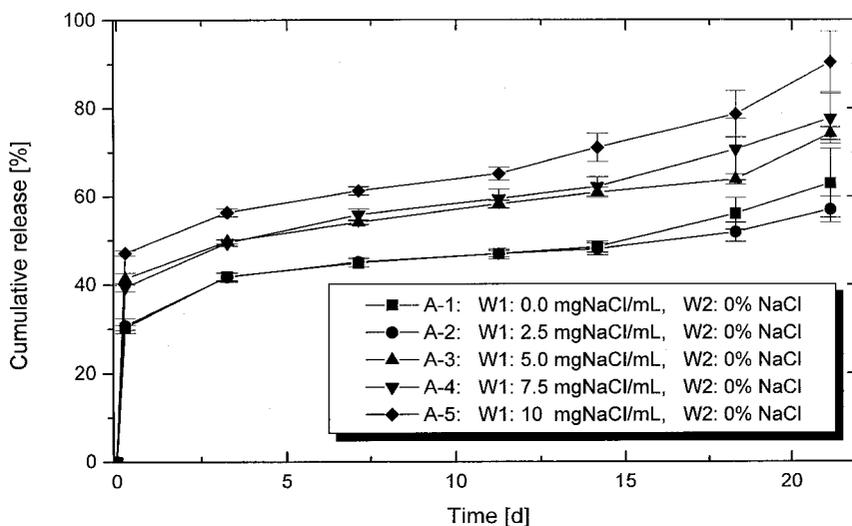


Figure 4. The influence of different NaCl concentrations in the inner water phase (0, 2.5, 5.0, 7.5, 10 mg/ml) on BSA *in vitro* release. ( $W_1$  = inner water phase,  $W_2$  = outer water phase.)

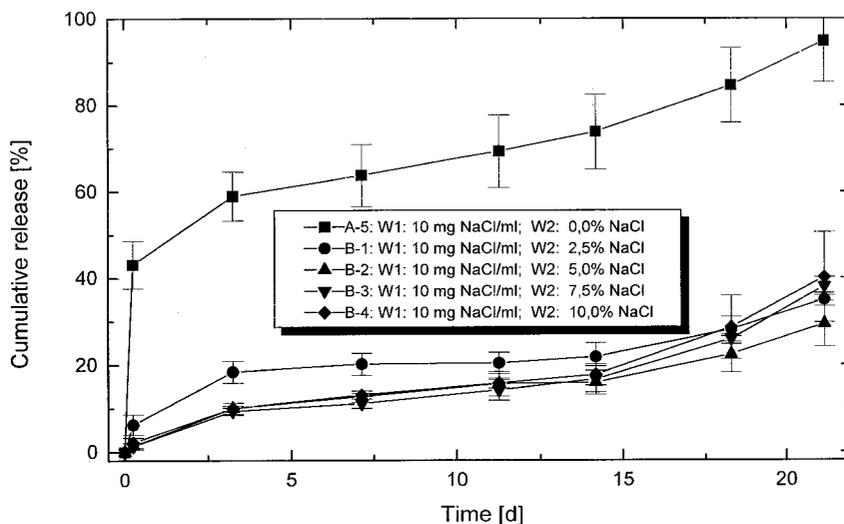


Figure 5. The influence of NaCl addition to both inner and outer water phase simultaneously on BSA *in vitro* release. The NaCl concentration of  $W_1$  was fixed at 10.0 mg/ml, whereas the NaCl concentration in  $W_2$  was varied (0, 2.5, 5.0, 7.5, 10.0% m/v). ( $W_1$  = inner water phase,  $W_2$  = outer water phase.)

influences both diffusion- and erosion-controlled release. In general, proteins are released from microspheres as a result of diffusion through aqueous pores or erosion of the polymeric matrix. The microstructure of microspheres prepared using high salt concentrations in the outer water phase was very dense and compact, which limits the permeation of BSA across the microsphere membrane prior to a significant degradation of the polymer.

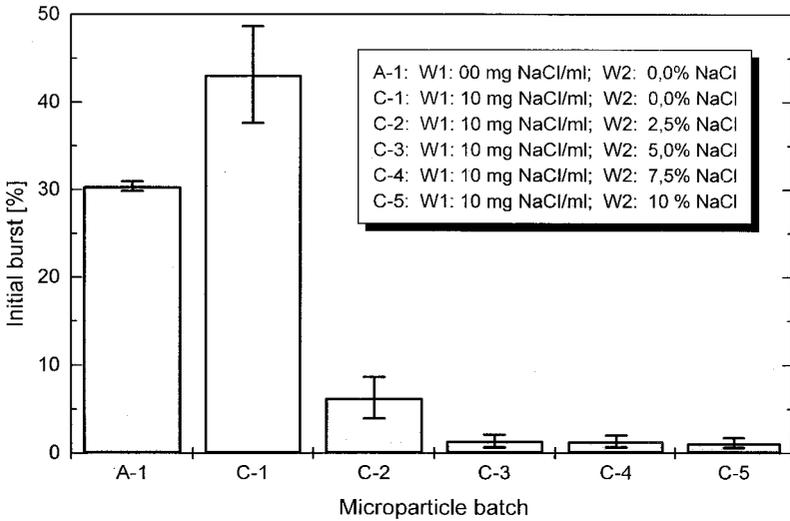


Figure 6. The influence of different NaCl concentrations in the external water phase on the initial burst effect. Except batch A1, all batches were prepared with a high NaCl concentration (10.0 mg/ml) in the inner water phase. The initial burst is defined as the percentage of protein released within the first 6 h ( $W_1$  = inner water phase,  $W_2$  = outer water phase).

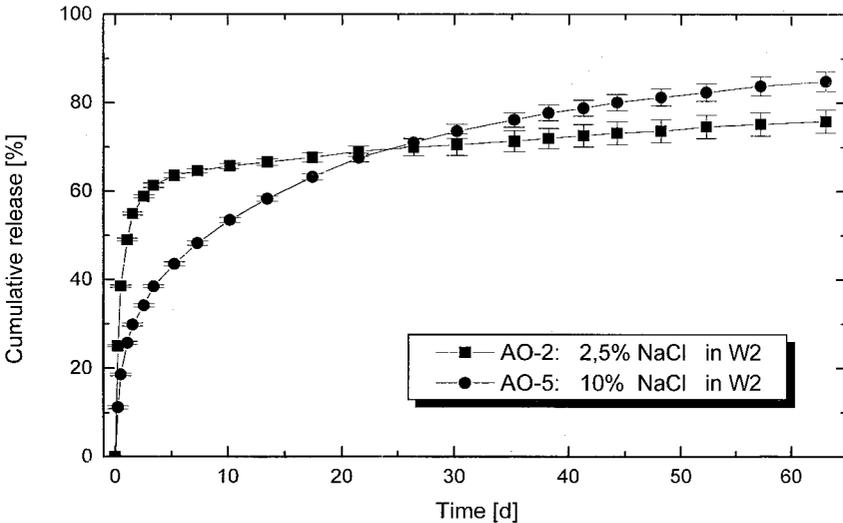


Figure 7. Release profile of acid orange 63 loaded microspheres in dependence of salt concentration in the external water phase ( $W_2$ ).

The drug encapsulated or distributed homogeneously throughout the polymer matrix. On the contrary, high salt concentrations in  $W_1$  and no salt in  $W_2$  produced microspheres with a large porosity that allows an easy hydration of the polymer and the permeation of BSA through microspheres from the beginning of the release study. Furthermore, remaining salt in the microspheres from preparation also increases water influx into microspheres. In this case, the accessibility of

the drug is not dependent upon the physical or chemical erosion of the polymer for release, but on dissolution of the drug within the fluid filled pores and diffusion through the pores. The porosity and, thus, the specific surface area influence the influx of water into the pores and the amount of protein accessible per unit of surface area available. Therefore, microsphere permeability to the large macromolecule BSA appears to be dependent upon the pore size and the salt concentration used to prepare the double emulsion. The microstructure of the microspheres affected primarily the initial stages of the initial drug release phase. Similar results, also relating porosity to the initial burst effect, were previously reported (Cohen *et al.* 1991, Sah *et al.* 1994). They studied the release of BSA from biodegradable microcapsule formulations prepared from various polymer compositions and ratio to protein. An increase in the amount of polymer was found to influence the BSA release profiles because it gave rise to microspheres with a dense, less porous polymeric phase, thereby inhibiting the burst effect. The high burst effect may be the result of interconnecting channels to the surface formed during the preparation process (Crotts and Park 1995). The addition of salt to  $W_2$ , and, thus, the production of a dense and non-porous surface reduced or even eliminated the burst effect.

Furthermore, interfacial effects are possible as ionic interactions between protein, polymer and salt. Heya *et al.* (1991) compared the encapsulation of TRH free base and TRH tartrate. It was demonstrated that ionic interactions between TRH, a basic drug, and PLGA led to more rigid microspheres, thereby eliminating the large initial burst effect. In contrast, a slight reduction of the entrapment ratio and a large increase in burst (up to 100%) were observed when encapsulating TRH tartrate or acids. This was related to the inhibition of ionic interactions.

Another explanation for the high burst effect is that BSA, along with water, appears to migrate to and concentrate near or on the microsphere surface when vacuum dried (Sah *et al.* 1995a). This heterogeneous distribution is enhanced for microspheres prepared with salt in  $W_1$ , as these microspheres were extremely swollen after preparation. In the case of AO63, the fast release was also enhanced by the low molecular weight of the drug and the high water solubility that yielded a high osmotic pressure inside the microspheres after water penetration.

## Conclusion

Additives to the protein solution such as salts led to a drastic destabilization of the W/O/W double emulsion process and, hence, microsphere characteristics were strongly influenced by the change of salt concentration in the inner water phase. Since the salt concentration in the internal water phase  $W_1$  increased porosity and the specific surface area, bulk density and encapsulation efficiency decreased. The release profile was characterized by a high initial burst.

The release profile of BSA encapsulated into L-PLA microspheres using the W/O/W process was consequently attributed to the osmotic pressure inside the particles. An increase in ionic strength of the  $W_2$  phase prevented the migration of the protein from the internal phase into the aqueous, continuous phase during microencapsulation and suppressed the formation of very large pores, leading to a significant drug loss during preparation.

The structure of the microspheres clearly affected the drug release properties, since the protein was released faster from more porous microspheres. The current study demonstrated that, regardless of the salt concentration in  $W_1$ , the addition of salts to the external water phase  $W_2$  changed microsphere morphology to a dense, pore-less structure, characterized by a high bulk density, a distinct increase in encapsulation efficiency and a reduced initial burst.

In summary, this study provides evidence that, although the lifetime of the liquid second emulsion is extremely short, the stability of the secondary emulsion is crucial, because it controls the efficacy of the internal aqueous phase entrapment within the oil layer. The modification of the external water phase through the addition of salts, presented an attractive and simple way to improve and facilitate the encapsulation of stabilized protein solutions without changing or pretreating the protein solution.

Furthermore, higher theoretical drug loadings especially for low molecular weight drugs are achievable by the addition of salt. Even unfavourable preparation conditions as low kinematic viscosity of the polymer solution, large  $W_1/O$ -ratio, high theoretical drug loading and low molecular weight of the drug can be partly overcome.

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