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Peptide-loaded solid lipid nanoparticles (SLN): influence of production parameters

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Abstract

Solid lipid nanoparticles (SLN) are an alternative to particulate carriers made of biodegradable polyesters. The SLN have been sought as vehicles for drug molecules, and their production often uses physiological lipids or lipid molecules with an history of safe use in human medicine. However, little has been studied regarding the incorporation of peptides into SLN. This report describes the first studies on the incorporation of lysozyme, as a model peptide, in SLN. Previous to nanoparticle preparation, lysozyme was solubilised, until saturation, into the melted lipid phase. Production was carried out by a cold homogenisation process. The entrapment efficiency was dependent on the initial solubility of the peptide in the lipid phase of the final preparation. The influence of formulation parameters (e.g. type of lipid, time of exposure to different temperatures, pressure and the number of homogenisation cycles) on the integrity and activity of the enzyme, was also assessed. The lysozyme molecule remained intact throughout the process without loosing its activity, as shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the rate of lysis of *Micrococcus lysolideikticus*, respectively. This study shows that some proteins are able to endure the harsh procedures of formulation by high pressure homogenisation, making possible the use of SLN as antigen carriers for vaccine delivery. © 1997 Elsevier Science B.V.

Keywords: Solid lipid nanoparticles; Protein antigens; Vaccines; Lysozyme

1. Introduction

Biodegradable particulate carrier systems have been sought as vehicles for protein antigens and an extensive work has been developed in the area

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of vaccine formulation, using various base molecules to make nanoparticles/microparticles, which try to release their payload of antigen in a controlled manner. Nanoparticles/microparticles have also been investigated for their adjuvant properties, with antigens adsorbed onto their surface or entrapped within the polymer matrix. The adjuvanticity of different antigen-containing particulate systems, including liposomes (Allison and Gregoriadis, 1974), immunostimulating complexes (ISCOMs) (Browning et al., 1992) and nanoparticles/microparticles made of polymers, such as polymethylmethacrylate (Stieneker et al., 1991), polyalkylcyanoacrylate (O'Hagan et al., 1989) and polyacrylamide (Edman and Sjöholm, 1982), has also been studied. Furthermore, microspheres and microcapsules made of polylactic acid (PLA) and its copolymer with glycolic acid (PLGA) were used to deliver antigens at relatively predictable times (Alonso et al., 1994). However, most of these polymers present problems associated with the costs and the potentially toxic organic solvents used for microsphere production. Cytotoxic effects produced in vitro by PLA and PLGA particles (Maaßen et al., 1993) and polyalkylcyanoacrylates nanoparticles (Lenaerts et al., 1984) were observed.

An alternative to these systems consists of solid lipid nanoparticles (SLN) prepared either with physiological lipids or lipid molecules with an history of safe use in human medicine, which attract increasing attention as colloidal drug carriers. Under optimised conditions they can be produced to incorporate lipophilic or hydrophilic drugs and seem to fulfil the requirements for an optimum particulate carrier system (Müller et al., 1995).

Although many protein antigens have been microencapsulated into polymer microspheres for immunisation purposes, e.g. tetanus toxoid (Alonso et al., 1994), cholera toxin B subunit (Almeida et al., 1992), diphtheria toxoid (Singh et al., 1991), and staphylococcal enterotoxin B (Eldridge et al., 1991), little has been studied regarding the incorporation of peptide antigens into SLN. To our knowledge, few experiments have been described on the incorporation of recombinant malaria protein antigens into lipospheres, using a technique which involves the cooling of an emulsion prepared with melted lipids (Amselem et al., 1992).

The purpose of this work was to explore the possibility of producing vaccine formulations using SLN. The studies included the incorporation of a model protein molecule into SLN, using the cold dispersion technique, in a high pressure homogeniser (Müller et al., 1995), and evaluated its stability throughout the procedure. The effects of production parameters, as well as the effects of excipients on the integrity of the protein were assessed. The model protein selected was lysozyme (14 000 Da, 129 amino acids) because it is one of the best characterised and most studied of all proteins (Dobson et al., 1994). Moreover, it allows the assessment of changes in the molecule integrity and stability, due to the formulation procedure, through the determination of its specific activity.

2. Materials and methods

2.1. Materials

The hard fats used (Witepsol E 85 and Softisan 142) were obtained from Hüls AG (Witten, Germany). Monosteol (propyleneglycol palmitostearate) and Superpolystate (polyoxyethyleneglycol 300 mono, di-stearate) were provided by Gattefossé (Weil a. R., Germany). Cetyl alcohol was obtained from Caesar und Loretz, Hilden, Germany. Lysozyme (from chicken egg-white), Tween 80 and sodium cholate were purchased from Sigma GmbH (Deisenhofen, Germany). Poloxamers 182 and 188 (ICI, Wilton, UK) were also used. Acrylamide, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethylethylene diamine (ThMED) and the standard molecular weight marker proteins were supplied by Bio-Rad (Munich, Germany). For the determination of lysozyme activity, *Micrococcus lysolydeikticus* was obtained from Sigma. All chemicals and reagents not specified in the text were of analytical grade or equivalent.

2.2. SLN preparation

Lysozyme (1 to 10 mg ml^{-1}) and Poloxamer 182 above its critical micellar concentration were dissolved in distilled water at room temperature, and this solution was added dropwise to 20 g of melted lipids, under vigorous stirring (Ultra-Turrax T25, Janke und Kunkel, Staufen i.Br., Germany), at a temperature not exceeding 50°C to avoid protein degradation. At temperatures higher than its cloud point, Poloxamer 182 promotes the solubilisation in hydrophobic media, allowing the incorporation of lysozyme. This mixture was then transferred to a rotary-evaporator to eliminate the water, resulting in the solubilisation of lysozyme in the lipid phase. The process is then repeated until the protein reaches its saturation level. The volume of aqueous protein solution used was dependent on protein concentration. The resultant lipid phase containing the protein solubilised was then used to produce SLN using high pressure homogenisation (HPH), by the cold dispersion technique previously described (Müller et al., 1995). Briefly, the method consisted of grinding the lysozyme-containing lipid mixture, in liquid nitrogen, with a mortar mill (Retsch, Haan, Germany) yielding a lipid powder. This was dispersed (5% w/w) in a 3% (w/w) aqueous surfactant solution (sodium cholate, Poloxamer 188 or Tween 80), using a high speed stirrer (Ultra-Turrax T25) and homogenised at 25°C with an APV Gaulin Micron LAB 40 homogeniser (Lübeck, Germany) at 1000 bar/3 cycles.

2.3. Particle characterisation

Particle size was determined both by laser diffractometry (MasterSizer X, Malvern Instruments, Malvern, UK) and photon correlation spectroscopy (PCS) (Malvern Zetasizer IV, Malvern Instruments, UK). For the laser diffractometry data, samples were measured in demineralised water and the diameters 50% (D50) and 90% (D90) were used to characterise the SLN dispersions. From PCS measurements a mean diameter of the population was determined and a polydispersity index was obtained as a measure of the width of distribution. Surface charge was assessed through the measurement of zeta potential in a 50 μSi cm^{-1} NaCl solution, using laser Doppler anemometry (Malvern Zetasizer IV).

2.4. Protein recovery from SLN

The amount of protein encapsulated per unit weight of nanoparticles was determined after filtration of the SLN suspension to separate the solid lipids from the aqueous medium. This was carried out using the centrifugation/filtration Centrisart® I system (SM 13269; 100 000 Da) from Sartorius AG (Goettingen, Germany), at 4800 rpm/17 h (centrifuge Haraeus SepatechBiofuge 22R). To prevent losses of lysozyme due to adsorption, the filter membranes and the centrifugation tubes were previously saturated with a solution of the protein and subsequently washed with double-distilled water to remove any traces of non-adsorbed protein. The degree of encapsulation was calculated indirectly by determining the amount of protein remaining in the supernatant after centrifugation/filtration. Lysozyme content was also measured directly in the SLN. Briefly, an appropriate amount of particles was melted at 50°C, and and lysozyme was extracted with 2 × 0.9% aqueous NaCl, at the same temperature. Each extraction was preceeded by vigorous agitation during 30 min. The supernatants from SLN suspensions and aqueous phases from the extractions, were assayed by the bicinchoninic acid protein assay (Smith et al., 1985), performed in microtitre plates as described elsewhere (Almeida, 1993). Results were expressed as mean $\pm$ S.D. ($n = 8$).

2.5. Lysozyme stability studies

The effects of production parameters (temperature, pressure and number of homogenisation cycles), as well as the effects of excipients on lysozyme stability were assessed. The influence of temperature was studied through the assessment of the molecule's integrity before and after it had been submitted to a temperature of 50°C, for periods up to 5 hours. Similarly, the influence of the pressure applied during the homogenisation

Table 1
Amount of lysozyme solubilised in melted lipid mixtures (50°C) previous to SLN preparation

Lipid	Melting point (°C)	Lysozyme solubilised (μg protein/g lipid)	
		Determined (± S.D.; $n = 3$)	Theoretical
Witepsol E85	44.4[a]	50 ± 28	48
Witepsol E85/cetyl alcohol (75:25)	41.4[a]	200 ± 19	236
Softisan 142	45.9[a]	75 ± 40	80
Softisan 142/cetyl alcohol (75:25)	41.6[a]	250 ± 25	277
Monosteol	33–36[b]	515 ± 200	539
Superpolystate	32–36[b]	700 ± 140	712

[a] Determined by differential scanning calorimetry.
[b] From the literature.

(500 and 1000 bar) and the number of cycles (1 to 3 cycles), both at room temperature (≈ 25°C) and 50°C, were studied. The stability of lysozyme was also evaluated after melting the lipids in which lysozyme had been incorporated, at 50°C, followed by appropriate extraction with distilled water, as described for the determination of the amount of protein microencapsulated. To assess the influence of the surfactants, lysozyme was dissolved (8 μg ml^{-1}) in water and in aqueous solutions containing either 3% Tween 80, 3% sodium cholate, or 3% Poloxamer 188. These solutions were analysed for lysozyme activity at days 1 and 10 after preparation.

Protein samples from these experiments and also those collected throughout the actual encapsulation procedure were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 14% gel in a Minigel System SF 200 (Hoefer Scientific Instruments, San Francisco, USA), according to the method described by Lugtenberg et al. (1975). Gels were stained for protein using Coomassie Brilliant Blue R-250 (Sigma).

The stability was assessed by measuring in vitro the activity of the enzyme throughout the formulation procedures by the ability of lysozyme to lyse freeze-dried *Micrococcus lysolydeikticus* at a known concentration, at 25°C (Sugar, 1952). This results in a reduction of turbidity that can be measured with a spectrophotometer (Uvikon 710, Kontron Instruments). Three determinations were made per each sample and the results were expressed either as percentage of decrease in optical density (ΔA_{450} nm) of a suspension of bacteria, in activity units g^{-1}, or units ml^{-1} (mean ± S.D.), where applicable. One conventional activity unit of lysozyme represents a decrease of optical density of 0.001 min^{-1} at 25°C and pH 7.0, in a 3-ml suspension (initial optical density = 0.750) of *Micrococcus lysolydeikticus* cells (Portenhauser, 1983).

3. Results

3.1. Solubilisation of lysozyme in melted lipids

Due to their hydrophobic nature, SLN may be more appropriate to incorporate lipophilic drugs which can be easily dissolved in melted lipid mixtures. Hydrophilic proteins are expected to be poorly microencapsulated into SLN, tending to partition in the water phase during the process. The partition is further enhanced by the use of surfactants as emulsion stabilisers. On the other hand, the melting range of the lipid should be around 50°C, to guarantee the stability of lysozyme (Jollès, 1967), limiting the choice of lipid materials to those presenting melting points up to that value.

Despite the use of a solubilisation technique with a surfactant, only small quantities of lysozyme could be incorporated in the lipids (Table 1). Nevertheless, it is evident that the amount solubilised depends on the lipid composition, be-

ing higher for lipids which possess a larger surface-active fraction that facilitates emulsification, e.g. Monosteol and Superpolystate. This is also confirmed by the results obtained with the mixtures of Witepsol E 85/cetyl alcohol (75:25) and Softisan 142/cetyl alcohol (75:25), which incorporated higher amounts of lysozyme, when compared to the hard fats alone.

3.2. Effect of excipients on lysozyme stability

Proteins are characterised by their complex and well defined tertiary and/or quaternary structure which have important implications regarding their biological activity. These molecules usually present problems related to instability and loss of activity during pharmaceutical formulation techniques. The incorporation of polypeptides into hydrophobic media, together with the presence of surfactants, would probably damage these molecules. The effect of lipid excipients on lysozyme integrity was assessed by SDS-PAGE. As shown in Fig. 1, no changes in the pattern of migration of lysozyme extracted from lipids could be detected. The extra band of higher molecular weight, shown in all lanes including the control solution, is due to the presence of an impurity in this 95% pure commercially available hen egg-white lysozyme. No additional bands were observed, suggesting that the integrity of the molecule was maintained after being solubilised and extracted from several lipid mixtures.

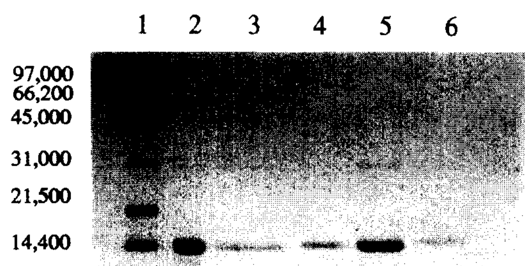

Fig. 1. SDS-PAGE (14% gel) of lysozyme after being extracted from several lipid mixtures. Lanes: (1) molecular weight markers; (2) lysozyme in water (500 μg ml^{-1}); (3) extract from Softisan 142/cetyl alcohol; (4) extract from Witepsol E85/cetyl alcohol; (5) extract from Monosteol; (6) extract from Superpolystate.

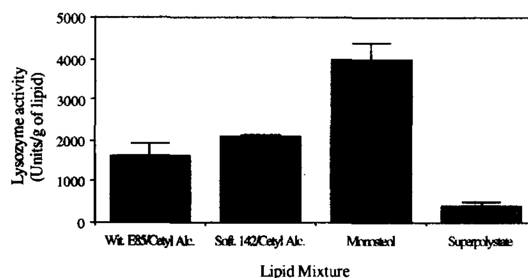

Fig. 2. Activity of lysozyme after extraction from the lipid mixtures used to prepare SLN. Amounts incorporated are shown in Table 1.

The *Micrococcus lysolydeikticus* assay for specific activity was carried out before and after incorporation into the lipids, as well as in lysozyme solutions containing surfactants. The effect of the solubilisation procedure on enzymatic activity also depends on the composition of the lipid mixture (Fig. 2). Some differences in activity between extracts from Monosteol, Witepsol E85/cetyl alcohol (75:25) and Softisan 142/cetyl alcohol (75:25) may be due to their different lysozyme contents (Table 1). For example, the low activity presented by the protein extracted from Superpolystate seems to be in contradiction with the relatively high amount of enzyme incorporated. Lysozyme is active over a wide range of pH, but Superpolystate originates very acidic aqueous extracts (pH ≤ 2) which, together with the temperature of extraction (50°C), may account for the loss of activity. In spite of showing a normal pattern of migration in SDS-PAGE, this sample reveals a very fainted band, confirming the loss of protein (Fig. 1).

Data in Fig. 3 suggest that immediately after preparation (day 1), the activity of lysozyme dissolved in an aqueous solution containing 3% Tween 80 (90.5 ± 9.5% activity), or Poloxamer 188 (95.2 ± 0.6% activity), is not significantly different from that of the control solution in distilled water (100.0 ± 0.5% activity). However, the same amount of enzyme dissolved in 3% sodium cholate presented only 76.2 ± 4.7% activity when compared to the control, which may result from an interference of the surfactant on the enzymatic activity of lysozyme. Solutions were kept at 4°C and after 10 days showed a loss of activity, which

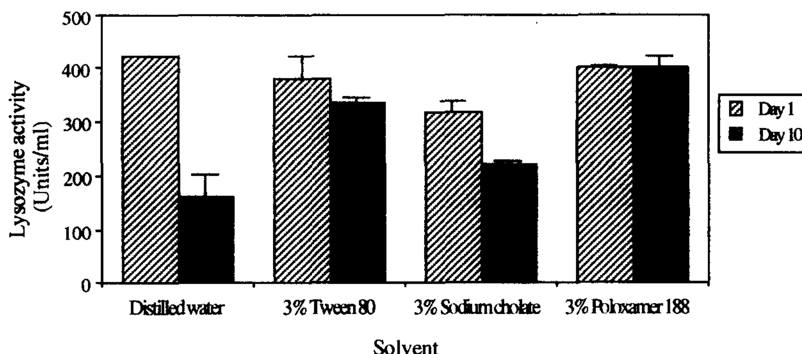

Fig. 3. Activity of lysozyme (8 μg ml^{-1}) in the presence of surfactants (mean ± S.D.; $n = 3$).

was more significant for lysozyme dissolved in distilled water (61.5% lost) than in those containing surfactants (no loss for Poloxamer 188, 12.6% loss for Tween 80, and 31.5% loss for sodium cholate). These results suggest that lysozyme is more stable in aqueous solutions of Poloxamer 188 or Tween 80 than in distilled water or sodium cholate solution. Thus, Poloxamer 188 or Tween 80 were the surfactants chosen to be used in the subsequent formulations of SLN.

3.3. Effect of the homogenisation parameters

The assessment of the effect of homogenisation parameters on the stability of lysozyme revealed that the molecule remains stable throughout harsh conditions such as those involved in HPH. After periods up to 5 h at 50°C, the molecule analysed both by SDS-PAGE and activity assay, remains unchanged when compared to the control stock solution (Figs. 4 and 5a), indicating that the temperatures used in the homogenisation process do not cause any detectable damage to lysozyme. Similarly, homogenisation pressures as high as 1000 bar applied up to 3 cycles at 50°C appear not to cause visible degradation to this protein, since the rate of substrate consumption is not different from that shown by the control solution (Fig. 5b) and no changes in the pattern of migration were detected by SDS-PAGE (Fig. 6).

3.4. Characterisation of lysozyme-containing SLN

As shown in Table 1 for the initial incorporation of lysozyme in the melted lipids, the attempts made to increase lysozyme loading in SLN through the combined use of less hydrophobic lipid mixtures and surfactant resulted in higher amounts of protein incorporated. However, some of the lysozyme-containing lipids did not originate stable SLN formulations. In some cases (Monosteol and Superpolystate) nanoparticles did not form and a transparent liquid was produced instead, due to the dispersion of these self-emulsifying lipids in the aqueous surfactant solution. Thus, the subsequent studies were carried out with Witepsol E85/cetyl alcohol (75:25) and Softisan 142/cetyl alcohol (75:25), which gave better formulations (Table 2).

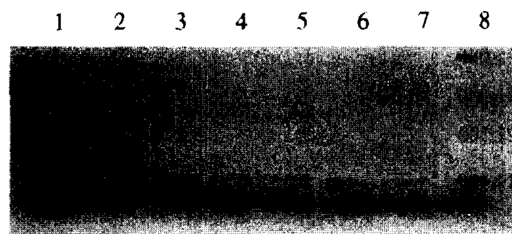

Fig. 4. SDS-PAGE (14% gel) of 500 μg ml^{-1} lysozyme in distilled water at 50°C. After preparation, solution was incubated and samples collected at different times. Lanes: (1) molecular weight markers; (2) control at room temperature; (3) 30 min; (4) 1 h; (5) 2 h; (6) 3 h; (7) 4 h and (8) 5 h.

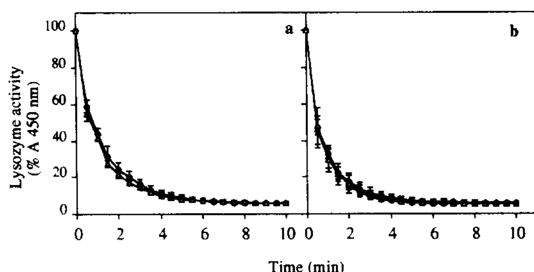

Fig. 5. (a) Activity of 500 μg ml^{-1} lysozyme in distilled water at 50°C; (●) control at room temperature; (Δ) 5 h (mean ± S.D.; $n = 3$). (b) Activity of 500 μg ml^{-1} lysozyme in distilled water under HPH conditions; (○) initial solution; (◆) 1000 bar/3 cycles, room temperature; (Δ) 1000 bar/3 cycles, 50°C (mean ± S.D.; $n = 3$).

The protein entrapment into the particles, as a phenomenon, was demonstrated by the lytic activity in aqueous extracts (Fig. 7) and the presence of lysozyme bands in SDS-PAGE (Fig. 8). The observations made in two different SLN formulations through two different analytical methods, imply that this is not an isolated phenomenon and at least part of the protein remains entrapped in the lipid during nanoparticle formation. In any case, loading efficiencies were low, due to both the low initial amounts of protein solubilised in the lipid mixture (Table 2) and the partitioning of enzyme into the aqueous phase. In relation to the initial amount of lysozyme we found in the aqueous phase of SLN dispersions 43.2 ± 11.4% (w/w) of protein for Softisan 142/cetyl alcohol (75:25) and 59.2 ± 2.9% (wiw) for Witepsol E85/cetyl alcohol (75:25), approximately ($n = 3$). This was also confirmed by the activity measurements showed in Fig. 7.

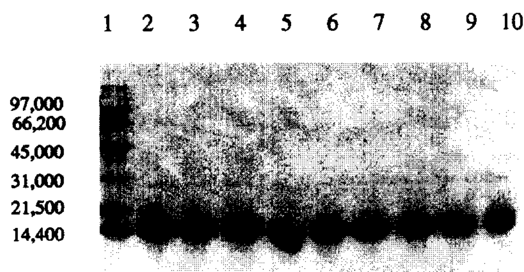

Fig. 6. SDS-PAGE (14% gel) of 500 μg ml^{-1} lysozyme in distilled water. After preparation, solution was submitted to different HPH treatments. Lanes: (1) molecular weight markers; (2) initial solution; (3) 500 bar/1 cycles/25°C; (4) 500 bar/3 cycles/25°C; (5) 1000 bar/1 cycles/25°C; (6) 1000 bar/3 cycles/25°C; (7) 500 bar/1 cycles/50°C; (8) 500 bar/3 cycles/50°C; (9) 1000 bar/1 cycles/50°C; (10) 1000 bar/3 cycles/50°C.

On the other hand, the SLN prepared with both lipid mixtures presented broad particle size distribution (Table 2). Laser diffractometry measurements showed nanoparticle populations that reach the micrometre range for both Softisan 142/cetyl alcohol (75:25) and Witepsol E85/cetyl alcohol (75:25).

As a final proof that lysozyme molecule could resist the different steps of SLN production by HPH, protein electrophoresis and activity determinations were performed after appropriate extraction, in both the aqueous and lipidic components of formulations. Once again, lysozyme showed no changes in its pattern of mobility and no differences from the control solution could be detected by SDS-PAGE (Fig. 8). Even the samples from SLN formulations produced by the hot dispersion (Müller et al., 1995) show no alterations when compared with the control on lane 2. These were produced by HPH of the melted lipids at 50°C/1000 bar/3 cycles (data not shown).

Finally, the findings obtained from activity measurements show no loss of enzyme activity (Fig. 7). Thus, under the experimental conditions it appears that the usual homogenisation parameters for SLN preparation do not affect lysozyme. However, an increase in activity was consistently observed for the Witepsol E85/cetyl alcohol (75:25) formulation, suggesting an apparent activation of the enzyme. Further investigation is being performed to analyse fully this observation.

4. Discussion

There is an urgent need for pharmaceutically acceptable vaccine delivery systems and adjuvants for antigens. Publications have emphasised the adjuvant properties of colloidal carriers when administered by parenteral and non-parenteral routes and a great effort has been put into the development of these antigen carriers (Eldridge et

Table 2
Composition and particle size of lysozyme-containing SLN

Lipid matrix	Lysozyme content in the formulation (μg protein/g lipid)	Particle size (μm ± S.D., laser diffractometry)	Particle size PCS (nm ± S.D.)	Zeta potential (mV ± S.D.)
S 142/CA (75:25)	≈225	$D50 = 0.60 \pm 0.00$ $D90 = 1.86 \pm 0.02$	644 ± 12.9	−11.0 ± 0.2
W E85/CA (75:25)	≈300	$D50 = 0.58 \pm 0.00$ $D90 = 0.93 \pm 0.01$	549 ± 7.0	−9.8 ± 0.7

W E85, Witepsol E85; S 142, Softisan 142; CA, cetyl alcohol.

al., 1991; Singh et al., 1991; Almeida et al., 1992; Amselem et al., 1992; Alonso et al., 1994). The advantages of SLN as a potential vaccine delivery system are their safety and biocompatibility. In contrast with the usual methods for nanoparticle production, SLN preparations do not involve the use of toxic solvents.

The contact between lysozyme and the most SLN excipients appears not to damage the protein molecule or it does not act on the activity. The SDS-PAGE showed that lysozyme maintains its integrity (Fig. 1), but such observation does not necessarily indicate that the enzyme molecule retains its conformation during all the process. For this reason the *Micrococcus lysolydeikticus* activity assay was carried out, revealing that the enzymatic activity was not affected (Fig. 2). It is established that the compact structure of proteins is determined by its interactions with the environment. If a protein is transferred from an aqueous to an non-aqueous medium, such as those used in the SLN formulations, internal hydrophobic interactions cease to exist and changes in structure may occur (Norde, 1984). Lysozyme structure will probably change after solubilisation in lipid mixtures. Since the analytical procedures include an extraction of lysozyme into an aqueous solution, the molecule may refold and recover its native conformation, which can occur within seconds (Dobson et al., 1994). Similarly, it was reported that incorporation of lysozyme into, and release from, hydrophobic ointment-like poly(ortho esters) does not lead to loss of activity (Wüthrich et

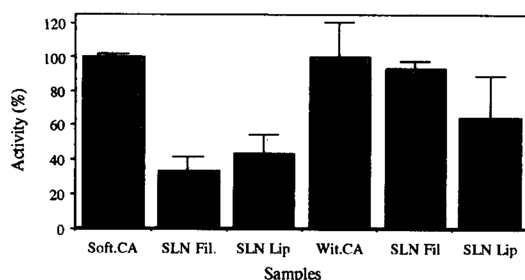

Fig. 7. Activity of lysozyme extracted from SLN formulations, prepared by the cold homogenisation technique, compared to the activity of the enzyme in the initial lipid mixture (mean ± S.D.; $n = 3$). Abbreviations: Soft/CA, Softisan 142/cetyl alcohol; Wit/CA, Witepsol E85/cetyl alcohol; SLN fil., aqueous filtrate from SLN preparations; SLN lip., lipid phase from SLN preparations.

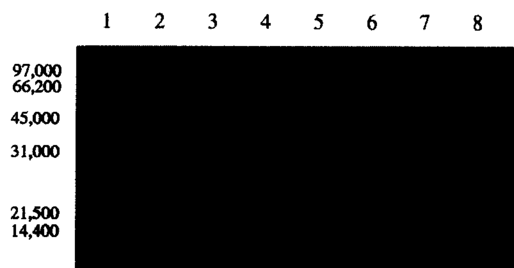

Fig. 8. SDS-PAGE (14% gel) of lysozyme present in SLN formulations. Lanes: (1) molecular weight markers; (2) lysozyme in water (500 μg ml^{-1}); (3) extract from Softisan 142/cetyl alcohol; (4) extract from Softisan 142/cetyl alcohol SLN produced by cold HPH; (5) extract from Softisan 142/cetyl alcohol SLN produced by hot HPH; (6) extract from Witepsol E85/cetyl alcohol; (7) extract from Witepsol E85/cetyl alcohol SLN produced by cold HPH; (8) extract from Witepsol E85/cetyl alcohol SLN produced by hot HPH.

al., 1992). As the stability of protein molecules is likely to be affected by hydrophobic media, such as the lipids and the surfactants used, the effect of this exposure is probably both antigen- and medium-dependent, and a range of combinations may need to be assessed for their suitability for each individual antigen.

Therefore, depending on the antigen to be incorporated, exposure to the usual SLN excipients is not necessarily a detrimental step during vaccine formulation.

Correspondingly, HPH conditions (i.e. high pressures and temperature) did not affect the whole structure of lysozyme, as determined by the methods herein employed (Figs. 4–6). Previous studies performed with bovine serum albumin have shown that the condtions usually used during SLN preparation by HPH strongly affect its structure (Weyhers, 1995). Moreover, it has been confirmed that high pressure enhances structural changes in lysozyme (reviewed by Chen (1992)). Therefore, the present results may appear surprising given the extreme environmental conditions to which lysozyme was submitted. The same author (Chen, 1992) state that pressure-induced denaturation is time-dependent and proteins are unlike to be denatured by high pressure since the exposure time is short and the pressure used moderate (1000 bar). Another explanation is that lysozyme is a protein with a high structural stability. Proteins which structure is stabilised by a large Gibbs energy (e.g. lysozyme) are described to behave like 'hard' particles (Norde and Favier, 1992) for their stronger internal coherence, which may contribute for the fast refolding of the molecule after being extracted into an aqueous phase (Norde, 1984; Dobson et al., 1994). In addition, albumin is a much more complex protein and has a low Gibbs energy. It is classified as a 'soft' protein (Norde and Favier, 1992) being more likely to be severely damaged and irreversibly denatured, as shown by Weyhers (1995). Besides, our results confirm the observations of Trenktrog and Müller (1995), who reported that human insulin retains its stability after formulation in an w/o emulsion using a HPH procedure.

The production of SLN was carried out by cold homogenisation technique using a high pressure homogeniser, which is a technique of an easy scaling-up that makes possible the production on industrial scale. Not surprising, the method produced protein-containing formulations with low encapsulation efficiencies, due to the fact that SLN are colloidal drug carriers based on hydrophobic matrixes. This may be further enhanced by the fact that particles produced with low melting point lipids, such as Witepsol E85 and Softisan 142, are amorphous at room temperature (solid or lipid) or only partially recrystallised, which can contribute to the leakage of drug into the aqueous medium (Sieckmann and Westesen, 1992). The recrystallisation behaviour of colloidal dispersions depends on the lipid concentration, presence of surfactants, drugs and the high surface-to-volume ratio of the SLN (Sieckmann and Westesen, 1992, 1994). Müller et al. (1995) reported that low lipid concentration (e.g. 2.5% w/w) seem to disturb the formation of crystals. In this work, melted lipids were solidified in liquid nitrogen for processing by the cold dispersion technique which guarantee that all lipid is in the solid state. This was also reassured by differential scanning calorimetry measurements of the produced SLN (results not shown). Nevertheless, a strong tendency of lysozyme to partition into the aqueous phase is confirmed by the activity determinations which revealed high proportions of enzyme in the SLN supernatant when compared to the lipidic phase (Fig. 7).

However, the aim of this study was to assess the possibility of producing SLN vaccines containing protein antigens. Consequently, the need for a high encapsulation efficiency of antigens is arguable. From a purely scientific point of view it is known that a few micrograms of some antigens can make a protective dose, particularly when administered together with a potent adjuvant. In spite of this, given the cost of some protein antigens the economic factor must also be taken into consideration. Therefore, the waste of antigen created by a low encapsulation efficiency must be avoided and the need for a good encapsulation efficiency of water-soluble molecules may lead to the development of further improved SLN formulations.

These findings indicate that SLN are a potential carrier system for the delivery of protein antigen vaccines, simultaneously avoiding the use of organic solvents. The entrapment efficiency will depend on the lipid mixture employed as well as the conditions of homogenisation. Probably, other protein antigens will behave differently from lysozyme. The above mentioned experiments reported in the literature confirm that every protein must be considered as a special case. Nevertheless, this study has shown that some proteins are able to endure the harsh procedures of formulation by this method, making possible vaccine formulation using SLN. On the other hand, the excipients chosen make the preparations studied impossible to be used for parenteral routes. Thus, more suitable formulations for parenteral administration will have to be investigated, which does not preclude the potential use of SLN as antigen carriers for oral and nasal immunisation.

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References

Allison, A.C. and Gregoriadis, G., Liposomes as immunological adjuvants. *Nature*, 252 (1974) 252.

Almeida, A.J., *Particulate Carriers as Immunological Adjuvants*, Ph.D. Thesis, The University of Aston in Birmingham, UK, 1993.

Almeida, A.J., Alpar, H.O., Williamson, D. and Brown, M.R.W., Poly(lactic acid) microspheres as immunological adjuvants for orally delivered cholera toxin B subunit. *Biochem. Soc. Trans.*, 20 (1992) 316S.

Alonso, M.J., Gupta, R.K., Min, C., Siber, G.R. and Langer, R., Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine*, 12 (1994) 299–306.

Amselem, S., Domb, A.J. and Alving, C.R., Lipospheres as a vaccine carrier system: effects of size, charge, and phospholipid composition. *Vaccine Res.*, 1 (1992) 383–395.

Browning, M., Reid, G., Osborne, R. and Jarrett, O., Incorporation of soluble antigens into ISCOMs: HW gp120 ISCOMs induce virus neutralising antibodies. *Vaccine*, 10 (1992) 585–590.

Chen, T., Formulation concerns of protein drugs. *Drug Dev. Ind. Pharm.*, 18 (1992) 1311–1354.

Dobson, C.M., Evans, P.A. and Radford, S.E., Understanding how proteins fold: the lysozyme story so far. *TIBS*, 19 (1994) 31–37.

Edman, P. and Sjöholm, I., Acrylic microspheres in vivo. V. Immunological properties of immobilized asparaginase in microparticles. *J. Pharm. Sci.*, 71 (1982) 576–580.

Eldridge, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.R. and Gilley, R.M., Biodegradable and biocompatible poly(D,L-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. *Infect. Immun.*, 59 (1991) 2978–2986.

Jollès, P., Relationship between chemical structure and biological activity of hen egg white lysozyme and lysozymes of different species. *Proc. R. Soc. Ser. B*, 167 (1967) 350–364.

Lenaerts, V., Couvreur, P., Christiaens-Leyh, D., Joiris, E., Roland, M., Rollman, E. and Speiser, P., Degradation of poly(isobutylcyanoacrylate) nanoparticles. *Biomaterials*, 5 (1984) 65–68.

Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P. and Van Alphen, L., Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. *FEBS Lett.*, 58 (1975) 254–258.

Maaßen, S., Schwarz, C., Mehnert, W., Lucks, J.S., Yunis-Specht, F., Müller, B.W. and Müller, R.H., Comparison of cytotoxicity between polyester nanoparticles and solid lipid nanoparticles. *Proc. Int. Symp. Control. Release Bioact. Mater.*, 20 (1993) 490–491.

Müller, R.H., Mehnert, W., Lucks, J.S., Schwarz, C., zur Mühlen, A., Weyhers, H., Freitas, C. and Rühl, D., Solid lipid nanoparticles (SLN)—an alternative colloidal carrier system for controlled drug delivery. *Eur. J. Pharm. Biopharm.*, 41 (1995) 62–69.

Norde, W., Physicochemical aspects of the behaviour of biological components at solid/liquid interfaces. In Davis, S.S., Illum, L., McVie, J.G. and Tomlinson, E. (Eds), *Microspheres and Drug Therapy. Pharmaceutical, Immunological and Medical Aspects*, Elsevier, Amsterdam, 1984, pp. 39–59.

Norde, W. and Favier, J.P., Structure of adsorbed and desorbed proteins. *Colloids Surfaces*, 64 (1992) 87–93.

O'Hagan, D.T., Palm, KJ. and Davis, S.S., Poly(butyl-2-cyanoacrylate) particles as adjuvants for oral immunisation. *Vaccine*, 7 (1989) 213–216.

Portenhauser, R., Standards and reference materials. In Bergmeyer, H.V. (Ed), *Methods in Enzymatic Analysis*, Vol. 11, Verlag-Chemie, Basel, 1983, pp. 393–440.

Sieckmann, B. and Westesen, K., Submicron-sized parenteral carrier systems based on lipids. *Pharm. Pharmacol. Lett.*, 1 (1992) 123–126.

Sieckmann, B. and Westesen, K., Thermoanalysis of the recrystallization process of melt-homogenized glyceride nanoparticles. *Colloids Surfaces B: Biointerfaces*, 3 (1994) 159–175.

Singh, M., Singh, A. and Talwar, G.P., Controlled delivery of diphtheria toxoid using biodegradable poly(D,L-lactide) microparticles. *Pharm. Res.*, 8 (1991) 958–961.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.T., Goeke, N.M., Olson B.J. and Klenk, D.C., Measurement of proteins using bicinchoninic acid. *Anal Biochem.*, 150 (1985) 76–85.

Stieneker, F., Kreuter, J. and Löwer, J., High antibody titres in mice with polymethylmethacrylate nanoparticles as adjuvant for HIV vaccines. *AIDS*, 5 (1991) 431–435.

Sugar, D., The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochim. Biophys. Acta*, 8 (1952) 302–309.

Trenktrog, T. and Müller, B.W., Production and characterization of a peptide containing w/o emulsion. *Int. J. Pharm.*, 123 (1995) 199–208.

Weyhers, H., *Feste Lipid Nanopartikel (SLN) für die Gewebsspezifische Arneistoffapplikation*, Ph.D. Thesis, Freie Universitat Berlin, Germany, 1995.

Wüthrich, P., Ng, S.Y., Fritzinger, B.K., Roskos, K.V. and Heller, J., Pulsatile release of lysozyme from ointment-like poly(ortho esters). *J. Control. Release*, 21 (1992) 191–200.