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Stabilisation and determination of the biological activity of L-asparaginase in poly(D,L-lactide-co-glycolide) nanospheres

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Abstract

The preservation of biological activity of protein drugs in formulations is still a major challenge for successful drug delivery. The enzyme L-asparaginase, which exhibits a short in vivo half-life and is only active against leukaemia in its tetrameric form, was encapsulated in poly(D,L-lactide-co-glycolide) nanospheres by the (w/o)/w-emulsion solvent evaporation technique in presence of various potential stabilisers. Elucidation of the preparation steps revealed that the enzyme is denaturated at the aqueous/organic interface and by sonication. The preparation of L-asparaginase nanospheres with trehalose, PEG 400, and glycerol as components of the inner aqueous phase yielded colloidal formulations with increased biological activity as determined by an improved protocol for quantification of the active enzyme encapsulated. After lyophilisation the enzyme activity and particle size distribution were retained only by use of Pluronic F68 as a lyoprotectant. Despite the unaltered particle size and improved biological activity, the release profile of the enzyme was strongly altered by coencapsulation of the stabilisers resulting in increased first bursts. In consequence, the biological activity of L-asparaginase during preparation and storage can be improved by combining appropriate additives but concurrently the release profile is influenced.

Keywords: L-Asparaginase; Lyoprotectant; Nanospheres; PLGA; Stabilisation; Surfactant

1. Introduction

Due to the delicate molecular architecture of proteins, the development of delivery systems for protein drugs is still a challenge in pharmaceutical technology. Proteins are susceptible to proteolytic degradation and poorly absorbable due to their hydrophilicity and high molecular weight. As it is now generally accepted that colloidal carriers can cross cellular barriers probably leading to higher availability, nanospheres might be useful for protein delivery. Although encapsulation shields the protein from proteolytic attack and can prolong the release, instability of the entrapped protein due to unfavorable microenvironment within the nanospheres can affect the integrity of the protein. At the best the biological activity is lost, at the worst immunogenic or toxic degradation products are formed. Thus, stabilisation of protein drugs is a major issue for successful therapy.

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L-Asparaginase is widely used for the treatment of haematopoetic diseases such as acute lymphoblastic leukaemia. The enzyme is able to destroy asparagine-dependent tumors by degrading L-asparagine in circulation and famishing malignant cells. The major limiting factor in clinical use is the short half-life in vivo ($t_{1/2}$ = 1.2 days), which necessitates multiple injections via the parenteral route (Müller and Boos, 1998). Apart from hypersensitivity, which ranges from mild allergic reactions to anaphylaxis, also immunogenicity of the enzyme was reported. Due to formation of neutralizing antibodies the half-life of circulating L-asparaginase can be shortened to 2.5 h. By covalent conjugation of PEG to L-asparaginase the half-life was increased to 15 days and immunogenicity was reduced (Ho et al., 1986).

In order to prolong the half-life and to stabilise the enzyme, L-asparaginase was incorporated in liposomes (Cruz et al., 1993) and nanospheres made from poly(D,L-lactide-co-glycolide) (PLGA) (Gaspar et al., 1998). Due to their biocompatibility and biodegradability these copolymers were approved by the FDA for use in humans. As reported by Gaspar et al., L-asparaginase-loaded nanoparticles made from PLGA with free carboxyl-end groups as referred to the "H-type of PLGA" had a high protein loading and provided a continuous delivery of the enzyme for 20 days. When the carboxyl-end groups of PLGA were esterified, the protein loading was lower and no active enzyme was released.

The aim of this work was to identify additives to further improve the biological activity of L-asparaginase incorporated in PLGA nanospheres. As only the tetrameric form of L-asparaginase possesses enzymatic activity, preservation of biological activity during preparation, storage, and release were investigated. To identify sources of inactivation, the preparation process was examined step by step and the influence of different stabilisers on particle size as well as biological activity of L-asparaginase was estimated. As a prerequisite for quantification of L-asparaginase entrapped, a protocol for determination of biological active L-asparaginase after preparation of nanospheres was established. Moreover, the suitability of the stabilisers for lyoprotection and their influence on the in vitro release profile using an optimised test medium were investigated.

2. Materials and methods

2.1. Materials

L-Asparaginase (EC 3.5.1.1) from *Escherichia coli* was purchased from Sigma (Vienna, Austria) as an aqueous solution with glycerol or as a lyophilised powder. PEG-asparaginase from *E. coli* was obtained from Sigma (Vienna, Austria). The type of poly(D,L-lactic-co-glycolic acid) used was Resomer® RG503H (lactide/glycolide ratio 50:50, inherent viscosity 0.32–0.44 dl/g, acid number >3 mg KOH/g) and obtained from Boehringer Ingelheim (Ingelheim, Germany). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Preparation of L-asparaginase-loaded nanospheres

L-Asparaginase-loaded nanospheres were prepared by conveniently adjusting the water-in-oil-in-water solvent evaporation technique (Ertl et al., 1999). In brief, 200 μl of an aqueous solution of L-asparaginase containing 25% glycerol at the most or other additives (Table 3) was emulsified in 1 ml ethyl acetate containing 200 mg PLGA by sonication (sonifier: Bandelin electronic UW 70/HD 70, tip: MS 72/D, Berlin, Germany) for 30 s in an ice bath. Addition of 3 ml of an aqueous solution of Pluronic F68 (5%) to the w/o emulsion and sonification for 20 s under cooling with ice yielded the (w/o)/w emulsion. Then, the double emulsion was diluted into 50 ml of 1% Pluronic F68 in aqueous 2-propanol (2%) and the suspension was maintained under magnetic stirring for 1 h at 600 rpm followed by evaporation of residual ethyl acetate under vacuum. The nanoparticles were collected by centrifugation (8000 rpm, 10 min, 4 °C) and resuspended in distilled water. The washing step was repeated twice. Prior to storage at −20 °C the loaded nanoparticles were filtered (1 μm pore size) to exclude few aggregates and resuspended in an aqueous solution of Pluronic F68 (1%).

The mean diameter and particle size distribution were examined by laser diffraction (Shimadzu Laser Diffraction Type Particle Size Analyzer SALD-1100). The nanospheres were suspended in water containing 0.2% Tween 20 as a wetting agent to minimize agglomeration, sonicated for 1 min and analyzed under

continuous stirring in duplicate. Sizes were expressed as volume diameters.

2.3. Quantification of active L-asparaginase in nanospheres

In an effort to determine the biological activity of the enzyme after preparation of the nanospheres different methods for improved extraction were investigated. Apart from lysis of the nanoparticles in organic solvents and alkaline solution, the nanoparticles were mechanically disintegrated to facilitate the release of the enzyme in its biologically active form.

Mechanical pulping of the nanospheres was performed by grinding a frozen suspension of 53 mg L-asparaginase-loaded nanoparticles with 100 mg chromium trioxide particles (median 260 nm) under liquid nitrogen with mortar and pestle for 15 min. After evaporation of the nitrogen, the enzyme was extracted from the suspension with 4 ml 66 mM phosphate buffer pH 7.4 containing 1% Pluronic F68 by end-over-end rotation for 15 min. The disrupted nanoparticles and the chromium particles were collected by centrifugation and the extraction step was repeated twice. The protein content and the enzymatic activity of the supernatants were determined as follows (see Section 2.4).

To disintegrate the enzyme-loaded nanospheres by high speed homogenisation, 53 mg L-asparaginase-loaded nanoparticles were suspended in 4 ml distilled water and homogenized with an Ultra-Turrax (Ultra-Turrax T8, 100 W, IKA Labortechnik, Germany, setting 5) for 10 min in an ice bath. After rinsing the tip with 1.5 ml distilled water, the homogenized suspension was extracted by end-over-end rotation as described above.

2.4. Determination of the L-asparaginase content of nanospheres

After disintegration or dissolution, the payload of the nanoparticles was characterized by two different methods to gain information about the total protein content as well as the activity of the enzyme entrapped.

The protein content was estimated by the micro BCA^{TM} protein-assay (Pierce, Bonn, Germany) relying on reduction of Cu^{2+} by proteins in alkaline solution. The violet coloured bicinchonic acid–Cu^{+} complex is quantified spectrophotometrically at 562 nm. In practice, 450 μl protein sample and 450 μl reagent were incubated for 1 h at 60 °C under protection from light. After chilling to room temperature for 20 min, the absorption was read at 562 nm. The corresponding solvent without protein was used as a blank. No interference with the PLGA and the stabilisers was observed.

The biological activity of L-asparaginase was determined by the method described by Jayaram (Jayaram et al., 1974). It relies on a cascade of enzymatic reactions initiated by conversion of L-asparagine to L-aspartic acid by L-asparaginase. Then, L-aspartic acid is converted to oxaloacetate by aid of L-glutamic oxaloacetate transaminase and alpha-ketoglutarate. Finally, the oxaloacetate is reduced to malic acid by L-malic dehydrogenase with concurrent oxidation of NADH to NAD. Thus, the enzymatic activity of one unit L-asparaginase corresponds to formation of 1 mmol NAD per min at 37 °C. In practice, the reagent was prepared from 100 ml anhydrous glycerol, 50 ml 0.5 M Tris-buffer pH 8.45, 50 mg alpha-ketoglutarate-sodium, 180 U L-glutamic oxaloacetate transaminase, 110 U L-malic dehydrogenase, and 50 mg NADH adjusted to 500 ml with distilled water. Immediately prior to use, 10 mM L-asparagine in 50 mM Tris/HCl-buffer pH 8.45 are added to the solution at a ratio of 9:1 (v/v). After incubation of 2 ml reagent and 0.5 ml L-asparaginase containing sample for exactly 1 h at 37 °C, the decrease in absorption at 340 nm was determined. The detection limit was 0.001 U L-asparaginase per ml.

All determinations were performed in triplicate and repeated at least twice.

2.5. In vitro release studies

After resuspending the nanospheres in 50 mM Tris-buffer pH 7.4 containing 1% Pluronic F68 as a stabiliser and 0.01% benzalkonium chloride as a preservative aliquots of 1.5 ml suspension containing 2–4 mg nanoparticles were transferred into Eppendorf cups. The tightly sealed samples were incubated at 37 °C in a water bath under end-over-end rotation at 40 rpm and protection from light. From day 3, every other day the release medium was adjusted to pH 7.0–7.5 by addition of 5N NaOH. At predetermined intervals, three samples were collected

and centrifuged (10 min, 8000 rpm, 4 °C). The supernatant was assayed for enzymatic activity and protein content as described above. Occasionally, the protein content of the pellet was determined after dissolving the polymeric matrix in 0.1N NaOH.

3. Results

3.1. L-Asparaginase stability during nanoparticle preparation

The double emulsion technique for preparation of PLGA nanospheres comprises various steps critical for biological activity of proteins. First, during formation of the w/o emulsion, the protein can be affected by the water/organic solvent interface. Interfacial adsorption can be followed by unfolding which results in reduced biological activity. Upon emulsification of an aqueous solution of L-asparaginase in ethyl acetate at a volume ratio of 1:2 (aqueous/organic phase) there was a substantial decrease in enzyme activity with dilution of the protein solution (Table 1). As the loss of activity was strongly dependent on the concentration of the enzyme and the interfacial area provoked by emulsification was comparable, the absolute amount of enzyme denaturated seems to be rather constant at all enzyme dilutions investigated. Thus, the loss in enzyme activity was in diluted solutions quite higher than in more concentrated ones. In comparison to the enzyme activity, the protein content of the aqueous phase was not affected by exposition to the organic solvent. Consequently, the contact with lipophilic interfaces provoked denaturation, but insoluble aggregates were not formed.

Another source of inactivation during preparation of nanospheres are shear forces and cavitation stress as necessary for formation of nanodroplets. Despite cooling, ultrasonication provokes cavitations with local extremes of temperature and radical formation which adversely affect proteins (Suslick et al., 1986; Reisz and Kondo, 1992). Although reduction of exposure from 30 to 15 s increased the L-asparaginase activity by 25.7 ± 1.3%, insufficient emulsification resulted in immediate coalescence of the droplets. Thus, ultrasonication for 30 s at the minimum was required. When increasing concentrations of L-asparaginase in aqueous solution were emulsified with ethyl acetate by ultrasound, the loss of enzymatic activity decreased concurrently (Table 2). Whereas the loss of enzyme activity was 12.4 ± 4.2% in case of manual emulsification (Table 1), the enzyme activity decreased by 63.1 ± 1.2% upon emulsification with ultrasound at 166.5 μg L-asparaginase (Table 2). Thus, an additional amount of 50.7% L-asparaginase were subject to inactivation due to enlargement of organic/water interfaces and cavitation stress provoked by ultrasonication.

Due to the hydrophobicity of the PLGA, unfolding and adsorption of proteins to the matrix by hydrophobic interactions is another factor detrimental for biological activity. After dispersing 2 mg PLGA as supplied in an aqueous solution of L-asparaginase, the enzyme activity decreased by an negligible extent of 2.4 ± 5.6%. According to this preliminary result,

Table 1
Influence of ethyl acetate on the enzymatic activity of L-asparaginase in aqueous solution

	L-Asparaginase in aqueous phase prior to shakeing (μg/ml)	Protein content (%)	Enzyme activity (%)
Without ethyl acetate	110.00	100.0 ± 2.0	100.0 ± 5.1
With ethyl acetate	220.00	98.1 ± 3.1	97.4 ± 5.2
	166.50	102.0 ± 2.9	87.6 ± 4.2
	110.00	107.0 ± 0.6	76.5 ± 4.6
	55.00	104.2 ± 1.8	69.1 ± 4.1
	27.50	101.8 ± 4.1	60.6 ± 0.1
	13.75	97.3 ± 3.8	50.0 ± 0.0
	6.88	99.3 ± 5.7	21.2 ± 4.4
	3.44	98.6 ± 4.2	9.2 ± 1.7

Serial dilutions of L-asparaginase were manually shaken with two volumes of ethyl acetate. After evaporation of residual organic solvent under reduced pressure the aqueous phase was assayed for protein content and enzyme activity. The protein content and the enzyme activity of the corresponding non-treated aqueous solution corresponds to 100%.

Table 2
Influence of emulsification with ethyl acetate by sonication on the activity of L-asparaginase

L-Asparaginase in first aqueous phase (μg/0.2 ml)	Enzyme activity prior emulsification (U)	Enzyme activity after emulsification (U)	Enzyme activity after emulsification (%)
1262.8	195.7 ± 0.6	159.3 ± 1.6	81.4
291.3	45.1 ± 3.9	29.9 ± 0.4	66.3
166.5	25.8 ± 0.2	9.5 ± 0.2	36.9

The double emulsion was prepared according to the standard procedure without addition of PLGA followed by addition of 0.4 ml aqueous Pluronic F68 solution and centrifugation. After evaporation of residual organic solvent under reduced pressure the aqueous phase was assayed for enzyme activity.

the stability of the enzyme is not affected by the matrix. But it should be considered that an organic solution of the polymer is used for preparation of the nanoparticles.

3.2. Stabilisation of L-asparaginase in aqueous solution

As ultrasonication and organic solvents are essential for preparation of nanospheres, the stabilising effect of different additives and pH on the activity of L-asparaginase in aqueous solution was investigated (Table 3). The L-asparaginase exhibited high stability in aqueous solution for 24 h at pH 8.0. Although the optimum activity was observed at pH 7–9 (Stecher et al., 1999), upon incubation in phosphate-buffer pH 7.4 the enzyme activity decreased by nearly 70% and in Tris-buffer pH 8.45 by about 30%. In contrast to glycerol, PEG 400, and carboxymethylcellulose, the addition of detergents such as sodium cholate or Pluronic F68 strongly improved the stability of the enzyme. Among different carbohydrates and polyoles including dextran and PEG, which are known to stabilise enzymes by hydration, only 10% trehalose was found to stabilise the enzyme. Additionally, in presence of proteins such as serum albumin or gelatine the enzymatic stability of L-asparaginase was also maintained.

3.3. Methods for estimation of encapsulated active L-asparaginase

Up to now the biological activity of the entrapped enzyme can only be estimated from the release profile, disregarding probable loss of activity caused by storage of the particles or by an inappropriate release medium. To assess the quality of the nanospheres immediately after preparation, different methods for

Table 3
Influence of pH and additives on activity of L-asparaginase

Medium	Enzyme activity (%)	Medium	Enzyme activity (%)
Water pH 5.6	72.1 ± 5.3	1% aqueous dextran 4000	39.7 ± 3.6
Water pH 8.0	93.9 ± 1.9	10% aqueous dextran 4000	24.3 ± 0.9
Phosphate-buffer pH 7.4	31.5 ± 4.6	0.5% aqueous gelatine	97.4 ± 0.3
0.5 M Tris-buffer pH 8.45	67.3 ± 10.4	1% aqueous gelatine	98.3 ± 1.2
0.05 M Tris-buffer pH 8.45	60.4 ± 2.7	0.1% aqueous BSA	105.7 ± 1.6
0.17% aqueous glycerol	67.2 ± 2.7	1% aqueous BSA	102.4 ± 0.2
1% aqueous glycerol	48.9 ± 0.8	1% aqueous glucose	23.2 ± 0.3
20% aqueous glycerol	20.9 ± 0.2	1% aqueous lactose	21.6 ± 1.6
1% aqueous Na-cholate	96.5 ± 3.3	1% aqueous sucrose	38.3 ± 4.1
1% aqueous Pluronic F68	100.5 ± 1.7	10% aqueous sucrose	61.0 ± 1.8
1% aqueous PEG 400	70.1 ± 1.2	1% aqueous trehalose	57.4 ± 2.0
10% aqueous PEG 400	46.4 ± 0.9	10% aqueous trehalose	106.3 ± 2.7
20% aqueous PEG 400	35.9 ± 0.6	20% aqueous trehalose	87.7 ± 2.2
1% aqueous CMC	81.1 ± 6.4		

After incubation of L-asparaginase (0.2 μg/ml) in the corresponding medium for 24 h at room temperature the enzyme activity was determined and related to the activity immediately determined after preparation of the solution (100%). CMC, carboxymethylcellulose.

destruction of the polymeric matrix followed by activity testing of the enzyme released were investigated.

As currently described in the literature (Sharif and O'Hagan, 1995), L-asparaginase-loaded nanospheres were dissolved in ethyl acetate followed by repeated extraction with Tris-buffer but no activity of the enzyme was detected. Similarly, dissolution of the nanoparticles in an emulsion of ethyl acetate/Tris-buffer and subsequent extraction of the broken emulsion yielded only 3% of the activity of encapsulated L-asparaginase.

Alternatively, mechanical destruction of the polymeric matrix at low temperature was supposed to maintain biological activity. After the suspension of the nanoparticles was frozen with liquid nitrogen and grounded with chromium trioxide, the subsequent extraction yielded 23% of the enzyme encapsulated as determined by the protein assay. But the extracted enzyme exhibited only 66% biological activity (Fig. 1). When the nanospheres were disintegrated by high speed homogenisation about 30% of the total content of L-asparaginase with 67% enzymatic activity were recovered. It is likely that the discrepancy between the protein content and its biological activity is due to mechanical stress by shear forces, which results in denaturation of L-asparaginase. This hypothesis is supported by the observation that about 16.5 ± 1.0% of total enzyme activity was localized at the surface of the nanoparticles (data not shown).

In comparison, release of the encapsulated enzyme by end-over-end rotation for 1 h at room temperature in 66 mM phosphate-buffer pH 7.4 yielded 24% L-asparaginase with 98% enzymatic activity. Thus, the surface-associated enzyme and about 8% of the enzyme entrapped within the polymeric matrix were recovered by this method. In order to increase the amount of encapsulated enzyme available for quantification, the polymeric matrix was hydrolyzed at elevated temperature at high ionic strength of the buffer. Upon incubation at 50 or 60 °C in 0.5 M Tris-buffer pH 8.45 containing 1% Pluronic F68 for stabilisation, melting and coalescence of the nanoparticles as well as excessive loss of enzymatic activity were observed. In contrast, upon incubation at 42 °C for 20 h, the nanoparticles were dissolved and 19.5% active enzyme were detected. In comparison, free L-asparaginase lost 31.1 ± 0.9% of its biological activity under these conditions. Due to interference of Tris-buffer with the protein assay, the total amount of enzyme released could only be calculated and was 27.9 ± 1.1%. In comparison to end-over-end rotation for 1 h, the additional release of entrapped enzyme amounted to only 4%. Among the different methods investigated, release

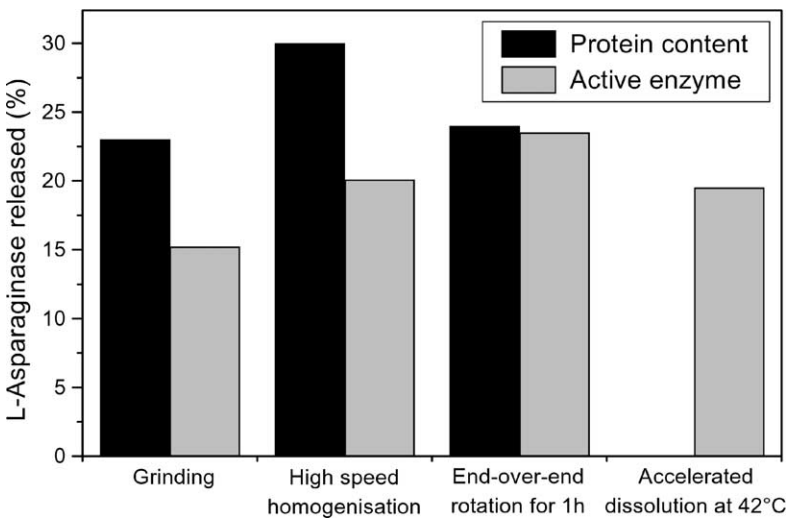

Fig. 1. Comparison of protein content and total biological activity of L-asparaginase released from PLGA nanospheres after pulping by different methods.

Table 4
Characteristics of L-asparaginase-loaded nanospheres prepared in presence of different stabilisers

Inner aqueous phase	Median (nm)	IQR (nm)	NRY (%)	Final loading (%)	EE (%)
ASN in water	420	165	55.2 ± 1.2	0.39	73.6
ASN in 1% aqueous Pluronic F68	380	150	47.5 ± 0.7	0.36	58.2
ASN in 0.1% aqueous HSA	360	155	60.4 ± 1.3	0.29	58.6
ASN in 10% aqueous PEG 400	410	155	61.9 ± 3.1	0.13	26.4
ASN in 10% aqueous trehalose	350	145	57.2 ± 0.9	0.41	79.2
ASN in 0.5 M Tris-buffer pH 8.45	340	150	46.9 ± 1.0	0.45	71.1
Glycerol stabilised ASN	370	155	58.4 ± 2.0	0.39	76.9
PEG-ASN	370	150	57.8 ± 2.0	0.23	45.4

IQR: mean interquartile range ($(D_{75} - D_{25})/2$); NRY: nanosphere recovery yield; EE: encapsulation efficiency; ASN: L-asparaginase; HSA: human serum albumin.

by end-over-end rotation or accelerated hydrolysis of the PLGA at 42 °C seems to be appropriate for determination of the biological activity of encapsulated L-asparaginase.

3.4. Stabilisation of L-asparaginase in nanoparticles

In view of the results of the stability tests of L-asparaginase in aqueous solution and determination of entrapped enzyme, L-asparaginase-loaded nanoparticles were prepared in presence of different stabilisers. As PEG 400 was reported to reduce adsorption of proteins to polymers and to promote sufficient release of proteins entrapped, it was also included as a stabiliser (Péan et al., 1999).

Independent from the type of additive, all the nanoparticle preparations exhibited monomodal size distribution and the median was 374 ± 26 nm (Table 4). Additionally, monodisperse formulations were obtained according to an interquartile range of 153 ± 6 nm. Whereas these parameters nearly remained unaffected by filtration, the yield was reduced. Apart from particle loss by filtration, the presence of Pluronic F68 and Tris in both aqueous phases resulted in yields lower than 50%. As compared to nanoparticles prepared from aqueous solutions of L-asparaginase, the encapsulation efficiency was rather low in presence of PEG 400. A similar effect was observed upon use of PEGylated L-asparaginase. But Pluronic F68, human serum albumin, trehalose, and Tris-buffer as well as glycerol stabilised the enzyme and yielded nanospheres with comparable encapsulation efficiency.

Besides yield and encapsulation efficiency the activity of the enzyme incorporated is another important issue in preparation of nanospheres. In presence of human serum albumin as a stabiliser the total enzyme content could not be determined due to interference of the protein assay (Table 5). As the enzyme activity detected was about half of stabiliser-free nanoparticles, human serum albumin is not recommended for stabilisation. When Tris-buffer was a component of the inner and outer aqueous phase, obviously the entrapped enzyme was denaturated by high amounts of buffer-substances. Although the activity could not be determined, presence of the enzyme was confirmed qualitatively by SDS–PAGE of these nanospheres after dissolution in NaOH and neutralization. As the blotted protein was detectable only after colloidal gold stain, rather low amounts of native enzyme were present (data not shown). Additionally, as compared to L-asparaginase-loaded nanoparticles without stabiliser, in presence of Pluronic F68 the activity of the enzyme decreased to about a third. Though the activity of encapsulated PEGylated L-asparaginase as determined by end-over-end rotation was comparable to that of L-asparaginase in nanoparticles without additive, the accelerated hydrolysis showed an five-fold increase. This discrepancy might be due to stabilising salts in PEGylated L-asparaginase which generate more dense nanoparticles. In turn, this results in low release rates of the incorporated enzyme. In contrast, presence of trehalose, PEG 400, and glycerol during manufacturing yielded nanoparticles with an L-asparaginase activity being two, three, and four times higher than that of additive-free nanoparticles.

Table 5
Activity of L-asparaginase after encapsulation in presence of different stabilisers

Inner aqueous phase	Activity of ASN encapsulated			
	End-over-end extraction		Alkaline hydrolysis at 42 °C	
	%	μg	%	μg
ASN in water	10.9 ± 0.4	0.94 ± 0.03	8.3 ± 0.1	0.72 ± 0.1
ASN in 1% aqueous Pluronic F68	3.4 ± 0.2	0.24 ± 0.01	0.058 ± 0.057	0.004 ± 0.004
ASN in 0.1% aqueous HSA	n.d.	0.51 ± 0.05	n.d.	0.52 ± 0.04
ASN in 10% aqueous PEG 400	34.6 ± 2.6	1.09 ± 0.08	28.5 ± 1.1	0.89 ± 0.03
ASN in 10% aqueous trehalose	20.4 ± 0.5	1.90 ± 0.05	15.1 ± 0.3	1.41 ± 0.03
ASN in 0.5M Tris-buffer pH 8.45	u.d.	u.d.	u.d.	u.d.
Glycerol stabilised ASN	45.8 ± 0.4	4.14 ± 0.04	19.5 ± 0.1	1.77 ± 0.01
PEG-ASN	9.2 ± 0.3	0.51 ± 0.02	47.4 ± 0.5	2.63 ± 0.03

ASN: L-asparaginase; HSA: human serum albumin; n.d.: not determined; u.d.: under detection limit.

3.5. *Influence of lyophilisation on particle size and* L-*asparaginase activity*

Due to release of their payload and erosion in aqueous media, the nanoparticles must be frozen or better lyophilised prior to storage. As lyophilisation of L-asparaginase-loaded nanoparticles resulted in formation of large aggregates in the micrometer range, which could not be dissociated by moderate ultrasonication, the effect of different lyoprotecting agents at concentrations currently used was investigated (Table 6). Upon addition of glycerol, trehalose, Tris-buffer, and skim milk powder the volume diameter of 90% (D_{90}) of the L-asparaginase-loaded particles increased from 13 up to 23-fold by lyophilisation. When human serum albumin was present during lyophilisation, a moderate, three-fold increase in particle size was observed. Solely Pluronic F68 really acted as a lyoprotectant since the particle size was maintained despite lyophilisation. Not only aggregation of the particles but also loss of biological activity can derive from lyophilisation. As compared to the L-asparaginase activity of the nanospheres prior to lyophilisation, without any lyoprotective agent the L-asparaginase activity decreased to 24%. In presence of glycerol, Tris-buffer or trehalose the enzyme activity decreased to 3, 32 and 83%, respectively. Although the enzymatic activity was fully retained after lyophilisation in presence of skim milk powder and human serum albumin, the aggregation of the nanoparticles was not prevented by these agents. Thus, only the addition of 1% Pluronic F68 is well suited to prevent loss of enzyme activity as well as nanoparticle aggregation during lyophilisation of L-asparaginase-loaded nanoparticles.

Table 6
Influence of lyoprotectants on particle size and enzyme activity of L-asparaginase-loaded nanoparticles

ASN-loaded nanospheres	Lyoprotectant	D_{50} (nm)	D_{90} (nm)	Activity of ASN encapsulated (%)
After preparation		360	790	–
Prior to lyophilisation		370	1070	18.3
After lyophilisation	Without lyoprotectant	13010	28590	4.5
	0.5% glycerol	8310	22900	0.5
	0.1% HSA	640	3540	21.8
	1% Pluronic F68	400	1130	21.6
	1% trehalose	4680	12850	15.2
	0.05 M Tris-buffer	8470	19570	5.9
	0.1% skim milk powder	7420	19200	22.8

The L-asparaginase (ASN)-loaded nanoparticles were resuspended in the corresponding media, frozen at −80 °C and lyophilised. The lyophilisates were resuspended in water by short pivoting in an ultrasonic bath at low energy. HSA: human serum albumin.

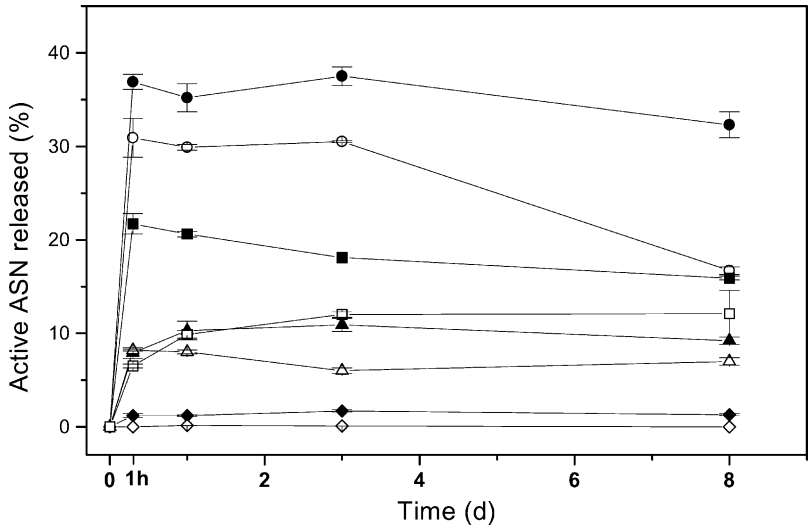

Fig. 2. Release profiles of L-asparaginase (ASN)-loaded nanospheres prepared with different additives. (●) ASN/glycerol; (○) ASN/PEG 400; (■) ASN/trehalose; (□) PEGylated ASN; (▲) ASN/water; (△) ASN/human serum albumin; (◆) ASN/Pluronic F68; (◇) ASN/Tris-buffer.

3.6. Release profile of active L-asparaginase from nanoparticles prepared with stabilisers

As the enzymatic activity decreased to 31.5 ± 4.6% after incubation of free L-asparaginase in 66 mM phosphate-buffer pH 7.4 for 24 h (Table 3), 50 mM Tris-buffer pH 7.4 containing 1% Pluronic F68 as a stabiliser and 0.01% benzalkonium chloride as a preservative was chosen for the release studies. According to preliminary assays, the mass loss of comparable amounts of PLGA nanospheres was 10.2 ± 2.9% within 3 days of incubation. This rather low amount of soluble oligomeric degradation products provoked no considerable pH drop in the release medium. But at day 8, the mass loss of the nanospheres was 33.1 ± 1.7% which acidified the release medium. Consequently, the pH was maintained at neutrality to prevent loss of biological activity of L-asparaginase due to an inappropriate release medium.

The release profile of L-asparaginase from PLGA nanospheres with similar size distributions but different stabilisers showed that the initial burst effect strongly depends on the type of additive incorporated (Fig. 2). As the first burst effect is attributed to the immediate dissolution of L-asparaginase located near the surface of the nanospheres and the nanospheres exhibited similar surface areas, there is an additional influence of the shape and the porosity of nanospheres on the extent of the first burst. Consequently, the surface characteristics of the nanospheres were altered by the additives. Moreover, the additives can promote or hinder the initial release by interference with protein adsorption to the polymeric matrix. As the mass loss of glycerol containing nanospheres was only 3.0 ± 2.2% after 1 day, the presence of the wetting agent promoted the initial release of active enzyme amounting to 36.9 ± 0.8%. During the second phase of release, which corresponds to the release of entrapped enzyme, the activity of the enzyme slightly decreases. A similar vigorous first burst of 30.9 ± 2.1% was observed upon incorporation of PEG 400. This additive was reported to minimize adsorption of proteins to PLGA, but the initial mass loss of 7.0 ± 1.3% also indicates for an additional effect on polymer solubility (Diwan and Park, 2001). In contrast to stabilisation with glycerol, the amount of active enzyme released decreases to 53% at day 8. When trehalose was used to stabilise the activity of L-asparaginase in nanoparticles, 21.7 ± 1.1% biologically active L-asparaginase were released during the initial burst. Subsequently, the amount of active enzyme decreases, which points to inactivation of the released enzyme by time. According to the literature, trehalose is converted into reducing carbohydrates

which might lead to chemical modification of released L-asparaginase (Sánchez et al., 1999). Irrespective of low release rates, which might be due to low mass loss even after 8 days of incubation (19.8 ± 4.1%), only the release profile of nanoparticles containing PEGylated L-asparaginase shows release of continuously increasing amounts of active L-asparaginase. Due to chemical stabilisation of the protein by PEGylation, the enzyme seems to retain activity even after prolonged release. Although the initial release profile of L-asparaginase encapsulated without additives is similar to that of PEGylated L-asparaginase, the unmodified enzyme lost its activity by time. The human serum albumin-containing formulation releases 8.2 ± 0.1% L-asparaginase within 1 h but the enzyme activity in the supernatant decreases with prolonged release. Thus, stabilisation of L-asparaginase by human serum albumin induced reduction of enzyme adsorption was not observed. In accordance with the results of the assays for determination of biological activity of L-asparaginase from nanospheres (Table 5), encapsulation of L-asparaginase in presence of Pluronic F68 and Tris-buffer results in decreased stability of the enzyme released.

4. Discussion

The development of protein pharmaceuticals requires methods for preparation, storage, and application which guarantee structural stability and biological activity of the protein drug. Thus, the procedures involved in formulation have to be screened step-by-step to identify the sources of inactivation.

The preparation of nanoparticles by the double emulsion technique necessitates volatile organic solvents. Although ethyl acetate induces less emulsification-induced denaturation of proteins than methylene chloride (Cleland and Jones, 1996), the loss of L-asparaginase activity was observed to be high particularly at low protein concentrations. These findings support the hypothesis that the loss of protein at the interface between organic and aqueous solvent is constant and part of the protein exerts a self-protecting effect (Putney and Burke, 1998). But even the remaining native protein in the aqueous phase can be compromised as well, since ethyl acetate is soluble in water to 9%. Additionally, the emulsification process requires high input of energy which in turn causes shear and cavitation stress adversely affecting the integrity of L-asparaginase. Although the effects of organic solvent and sonication were not observed at high concentrations of L-asparaginase (Gaspar et al., 1998), even low amounts of denaturated and aggregated protein deriving from the formulation process can provoke immunogenic and toxic side effects.

These findings underline the need for stabilisation of L-asparaginase activity during preparation. Upon stability testing of aqueous solutions of L-asparaginase in presence of different excipients, only serum albumin, trehalose, and Pluronic F68 were found to stabilise the activity of the enzyme. It should be kept in mind, however, that the enzyme lost considerable amounts of activity even in aqueous solution at physiological pH which corresponds to the pH-optimum of L-asparaginase.

Another complicating factor for the characterisation of the product is the quantification of the protein encapsulated. Currently, circular dichroism and FTIR are most frequently used to qualitatively assess protein integrity in the solid state without destruction of the formulation (Yang et al., 1999; Fu et al., 1999; Van de Weert et al., 2000) but quantitative determination of intact protein requires preceding extraction of the protein. As aggregates and artefacts are formed by the extraction methods currently used (Sharif and O'Hagan, 1995), mechanical destruction of the matrix followed by extraction with buffer containing stabilising excipients was investigated. But exposition to shear forces and insufficient pulping of the nanospheres resulted in poor recovery of active enzyme. Despite insufficient extraction, end-over-end rotation for 1 h and accelerated hydrolysis of the polymer at 42 °C were found to be appropriate for estimation of active L-asparaginase co-incorporated with excipients.

The protective effect of serum albumin preferably from humans was attributed to its competition with the protein drug at the water/organic solvent interface. Additionally, some surface active properties of the serum albumin might have contributed to minimisation of the protein fraction denaturated (Morlock et al., 1997). Especially during release the serum albumin could counteract the local pH drop in PLGA microspheres by its buffering effect and reduced incomplete release due to competitive adsorption to the hydrophobic matrix (Johansen et al., 1998). Any

stabilising effect of serum albumin on L-asparaginase activity during encapsulation could not be addressed and moderate release was observed. As already reported, the latter effect might be attributed to high encapsulation efficiency of serum albumin (Meinel et al., 2001). But serum albumin acted as an excellent lyoprotectant as neither particle size distribution nor considerable loss of enzyme activity was observed.

During microencapsulation trehalose was reported to protect proteins against organic solvents by hydration and thus preventing the contact of the protein drug with the organic solvent (Sah, 1999). In accordance, presence of trehalose resulted in nanospheres with retained biological activity of L-asparaginase. During lyophilisation this carbohydrate acts as a water substitute in the solid state forming hydrogen bonds with the protein after removal of water. When a combination of molecular modelling and FTIR was used for prediction of the required level of trehalose for lyoprotection, one molecule of trehalose was found to be necessary for protection of one highly polar residue at the surface of L-asparaginase (Ward et al., 1999). In practice, the activity of the enzyme was almost maintained during lyophilisation but after resuspending the lyophilisate even by aid of sonication a high number of aggregated nanoparticles was observed. During release a high first burst was observed which was most likely due to immediate dissolution of the carbohydrate resulting in pore formation. The pores facilitated immediate release of the L-asparaginase encapsulated with low loss of activity in the release medium.

When Pluronic F68 was used as an additive, protein aggregation and protein adsorption to hydrophobic surfaces were reduced (Blanco and Alonso, 1997). Although aggregation of tetanus toxoid in microspheres was prevented (Alsonso et al., 1994), coencapsulation of Pluronic F68 led to obvious loss of L-asparaginase activity in nanoparticles. Consequently, very low amounts of active L-asparaginase were released. In contrast, Pluronic F68 proved as an excellent additive for maintainance of L-asparaginase activity in aqueous solution and was used as an additive of the release medium. Additionally, Pluronic F68 was the best lyoprotectant under investigation since enzyme activity as well as nanoparticle size distribution was maintained after resuspending the lyophilisate.

Although PEG 400 exerts no stabilising effect on L-asparaginase in solution, this interface active excipient strongly improved the stability of the enzyme during nanoparticle preparation probably by shielding the enzyme from the water/organic solvent interfaces. The release profile of L-asparaginase from PEG 400 containing nanoparticles exhibited a vigorous first burst probably due to inhibition of protein-polymer adsorption and improved polymer solubility (Diwan and Park, 2001). The loss of activity 3 days after incubation was attributed to instability of L-asparaginase in the release medium.

In order to guarantee the pH-optimum for L-asparaginase upon encapsulation and to delay the pH drop upon release by formation of acidic degradation products of the PLGA, Tris was co-incorporated in the nanospheres. In contrast to basic inorganic salts such as magnesium hydroxide (Zhu et al., 2000), calcium carbonate (Johansen et al., 1998) or sodium bicarbonate (Shao and Bailey, 1999), the buffering capacity of Tris was not sufficient to observe any beneficial effect in the release profile.

At the first sight glycerol highly supported the stability of L-asparaginase encapsulated in PLGA nanospheres, but in presence of this additive a vigorous first burst effect resulted from high water solubility of the additive. Even after prolonged release the enzyme activity was maintained. However, glycerol adversely affects enzyme activity and particle size distribution during lyophilisation.

All in all it is suggested that a combination of additives may improve stability of native L-asparaginase in PLGA nanospheres e.g. the use of PEG 400 or glycerol to maintain enzyme integrity during preparation of the nanospheres and Pluronic F68 as a lyoprotectant. Another opportunity is the preparation of protein-loaded nanospheres by use of chemically stabilised protein drugs such as PEGylated enzymes. Nevertheless, upon storage of the lyophilised formulations the addition of lyoprotectants is necessary. Although the in vivo situation is likely to be quite different, adjustment of the release medium to the pH-optimum of the enzyme and presence of additives to minimise loss of biological activity is most important for comparison of beneficial effects of additives. Another major problem encountered with protein instability is the identification of degradation products. In case of L-asparaginase, the pathways leading to loss of activity were identified to start with dissociation of the tetramer to the monomers. After releasing

low molecular fragments, the shortened monomers form aggregates with higher molecular weight than the native tetramer (Jameel et al., 1997). To date it is not known whether the degradation products are simply inactive or also exhibit immunogenicity or toxicity. Thus, full preservation of biological activity by addition of stabilisers is desirable for safe therapy with protein-loaded nanospheres which requires further work in future.

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