In vivo half life of nanoencapsulated L-asparaginase

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In the present study, antileukemic enzyme L-asparaginase (ASNase) was encapsulated into poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanocapsules in order to decrease the immunogenicity and toxicity of the enzyme and to increase its *in vivo* half life in mice.

Nanocapsules were prepared by water-in-oil-in-water approach and each phase was changed systematically. By changing the pH of the w_2 phase to the isolelectric point of L-ASNase, the encapsulation efficiency was increased from 23.7% to 28.0%. Also, modification of ASNase with PEG_2 increased the encapsulation efficiency from 23.7% to 27.9% and protected the enzyme against denaturation. Combination of the various optima enabled a substantial increase in the activity (0.074–0.429 U/mg nanocapsule).

The enzyme activity in the blood due to unmodified PHBV nanocapsules dropped to 38% of its initial value 4 h after injection. When the same sample was tested for the enzyme content in the circulation by using the radio-labeled enzyme a much lower enzyme (30% of initial) could be detected after a shorter time (3 h). The PHBV nanocapsules with heparin conjugated on their surface had a longer presence in the circulation than unmodified PHBV nanocapsules. After 6 h, around 50% of the enzyme was still present in the blood. Radioactivity measurements using the same sample showed a sharp decrease in enzyme amount in the circulation in the early stages. However, radioactivity was still detectable at the eighth hour.

No adverse effects and symptoms of anaphylaxis were observed upon injection of encapsulated ASNase-PHBV nanocapsules to mice i.v. through the tail vein.

1. Introduction

Peptides or proteins are attracting more attention as drugs as their functions are better understood and progress is made in biotechnology and bioengineering. The development of DNA-recombinant technology, in particular, has made these compounds available on a larger scale than in the past. However, unprotected therapeutic polypeptides possess a short *in vivo* half life and antigenic property when they are introduced into the body. For this reason effective administration of protein drugs necessitates their protection from the hostile immunological system of the body.

L-asparaginase (L-ASNase; L-asparagine amido hydrolase, E.C.3.5.1.1.) is an antitumor agent effective in human acute leukemia. L-Asparaginase exerts its antitumor activity by depleting the nonessential amino acid L-asparagine [1]. Circulating pools of L-asparagine and L-aspartic acid are present in human blood and the normal human cell can obtain L-asparagine from the circulating blood pool, or L-asparagine may be synthesized by the transamination of L-aspartic acid, with glutamine acting as the amine donor and L-asparagine synthase catalyzing the reaction. Therefore, in the normal cell, L-asparagine is a nonessential amino acid because it can be synthesized from L-aspartic acid and L-glutamine.

Certain malignant cells, such as those in acute lymphocytic leukemia are unable to synthesize L-asparagine due to the lack of L-asparagine synthetase activity. When L-asparagine is depleted by the activity of L-ASNase in plasma, the malignant cells cannot obtain

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L-asparagine from extracellular sources, to maintain protein synthesis. Thus, inhibition of RNA and DNA synthesis occurs, resulting in tumor cell death [2].

The heterologous origin of the enzyme leads to an immune response upon treatment with ASNase. Anaphylactic reactions may be observed in up to 33% of patients [3]. This high rate is related to the fact that L-asparaginase is a polypeptide of bacterial origin that can stimulate production of IgE or other immunoglobulins [4].

Nonallergic toxicities of L-asparaginase generally have been attributed to the direct amino acid-depleting effect of the enzyme which results in inhibition of protein synthesis [5]. Clinically important toxicities include liver dysfunction, pancreatitis, hyperglycemia, coagulopathy, and central nervous system dysfunction. An increasing number of hemorrhagic and thrombotic events were reported by asparaginase treatment [6].

Prolonged reduction in the serum concentration of asparaginase was reported after the administration of ASNase [7]. This would allow enough time to induce *de novo* synthesis of asparagine synthase to such a level that protein synthesis can proceed normally. For this reason, encapsulation of L-ASNase within membraneous structures that enable controlled substrate utilization may help to reduce these toxicities.

Numerous modification and coupling studies have been carried out to improve pharmacodynamic parameters, enzymic efficacy and also to minimize the immunogenic effects observed with the clinical use of foreign proteins such as L-ASNase. These studies include physical encapsulation of L-asparaginase within liposomes [8], red blood cells [9, 10] and biocompatible hydrogels [11] and chemical modification of enzyme with nonimmunogenic polymers [12, 13]. The packing of L-ASNase in synthetic microcapsules has been described but this approach has serious limitations related to the accumulation of undegradable material in the body [14].

A new liposomal formulation of L-ASNase with high encapsulation efficiency (40–98%, for small and large liposomes, respectively) and stability in biological fluids has been indicated [15]. The increase in circulating half life ($t_{1/2}$) observed for small liposomes (28.75 h, 170 nm) as compared with large liposomes (0.11 h, 1249 nm) and with the half life of free asparaginase (1.96 h).

Palmitoyl L-asparaginase has been used for higher encapsulation purposes into liposomes [8]. The activity of palmitoyl L-ASNase was reported as 30% for multilamellar vesicle (MLV) and 51–74% after extrusion procedure. Liposomal encapsulation of palmitoyl L-ASNase as compared with free palmitoyl L-ASNase resulted in a prolongation of half life in blood (from 2.9 h to longer than 23.7 h).

To avoid adverse reactions and to prolong the enzyme half life, entrapment of asparaginase into erythrocytes has been employed succesfully with monkeys and mice [16, 17]. Enzyme loaded monkey erythrocyte ghosts extended the circulation half life of L-asparaginase to several days. Naqi *et al*. [18] investigated the entrapment of L-ASNase into canine erythrocytes by a single dialysis encapsulation. *In vitro* cell survival studies showed that carrier cells did circulate and that L-ASNase had a half life of 6.5 days. Carrier cells prepared with inulin and (^{14}C) sucrose were stored at 4 °C for two weeks and began to show signs of deterioration after 2 days. Kravtzoff *et al*. [10] evaluated the pharmacokinetic and pharmacodynamics parameters of *E. coli* L-asparaginase loaded in RBCs. The results obtained from 13 patients showed a good correlation between the life spans of RBCs and L-ASNase loaded in RBCs. The elimination of the enzyme was biphasic. A rapid partial clearance was observed during the first 24 h representing 47% of the total injected dose. However, the RBCs circulating after 24 h had a normal *in vivo* life span ($t_{1/2} = 27$ days).

Encapsulation of therapeutic proteins into biodegradable, biocompatible polymeric nanocapsules would be a safe way of delivering enzyme and protein drugs. Very recently Gaspar *et al*. [19] described the development and *in vitro* characterization of PLGA nanospheres containing L-asparaginase. The aim of the work was to determine the adequate conditions for the preparation of L-asparaginase loaded nanoparticles with a narrow size distribution and a high loading of the active enzyme. The nanoparticles made of PLGA with free carboxyl end groups (more hydrophilic) had a high protein loading (4.86% (w/w)) and provided a continuous delivery of the active asparaginase for 20 days. However, the enzyme loading was low (2.65% w/v) and no active enzyme was detected in the release medium after a 14-day *in vitro* incubation period when nanoparticles were made of PLGA with esterified carboxyl end group.

Intravascular delivery of microparticles and nanoparticles has thus far been used in a limited number of studies. After the particles are injected into the blood stream they are rapidly conditioned (or coated) by elements, such as plasma proteins and glycoproteins of the circulation. This process, known as opsonization, is critical in dictating the subsequent fate of the administered particles. Normally for nanoparticles and other foreign materials that are recognized as foreign, the opsonization process renders the injected material easily recognizable by the major defense system of the body, the reticuloendothelial system. The macrophage (Kupffer) cells of the liver and to a lesser extent the macrophages of the spleen and the circulating macrophages play a critical role in removing opsonized particles [20].

It is argued that the modification of the surfaces of the particles intended for intravenous injection by a polymer, to provide a hydrophilic and a steric barrier, would minimize the uptake of the plasma components, opsonization, and recognition phenomena. This concept of disguising particles with hydrophilic surface coating has now been applied to biodegradable particles as well as to liposomes and emulsions [21]. It is possible to disguise particles by making their surfaces both hydrophilic and sterically stable using polymer adsorption. Particles that carry adsorbed or grafted layers of polymers can display excellent stability characteristics because of the unfavorable thermodynamics for the close proximity of the adsorbed or grafted polymer chains.

Coating with polyoxyethylene–polyoxypropylene block copolymers reduces their surface hydrophobicity which may be the reason why such particles are less prone to phagocytosis [22]. Alternatively, the hydrophilic coating layer may reduce or prevent opsonization; a process necessary for recognition of most particles, i.e., latex microspheres, certain classes of liposomes and

pathogenic microorganisms can attract suppressive subtances called dynopsins from plasma or serum [23, 24].

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a very promising biodegradable polymer which has recently been used as a material for the preparation of microcapsules [25, 26], microparticles and nanoparticles [27, 28]. This polymer is entirely natural and produced by microorganisms as well as by genetically modified plants. In the present study, antileukemic enzyme L-asparaginase was encapsulated in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanocapsules in order to diminish immunogenic, toxic property of the enzyme and increase its circulation half life.

Surface modification of nanocapsules was performed to increase the hydrophilicity and prevent easy opsonization by reticuloendothelial system. For that purpose, low molecular weight PHBV was prepared and conjugated to low molecular weight heparin.

In vivo studies were performed to compare and test encapsulated L-asparaginase preparations in mice model. These were compared in terms of pharmacodynamic properties in circulation and in immunogenicity of the preparations.

2. Materials and methods

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), (PHBV, containing 8% 3-hydroxyvalerate, MW 297 000, determined by viscometric measurement) was obtained from Aldrich Chem. Co. (USA). Polyvinylalcohol (PVA) (molecular weight 30 000–70 000 Da), sodium borohydride ($NaBH_4$), polyethylene glycol (PEG, MW 5000), L-asparaginase (*E. Coli*, EC 3.5.1.1, 155 units/mg protein, in 50% glycerol), malic dehydrogenase, (Porcine Heart, 600 units/mg) and glutamic oxaloacetate dehydrogenase (GOT, EC 2.6.1.1, 309 units/mg protein), cyanuric chloride, Sepharose CL 4-B, low molecular weight heparin, goat anti-mouse (whole molecule) alkaline phosphatase conjugate, and p-nitrophenylphosphate were obtained from Sigma Chemical Co. (St. Louis, USA).

Nessler's Reagent was purchased from Aldrich Chemical Company Inc. (Milwaukee, USA). Tritiated ^3H-$NaBH_4$ was obtained from Amersham Pharmacia Biotech (England). Toluidine blue was obtained from Merck (Germany). Balb/C mice were obtained from the animal house of Gulhane Military Academy of Medicine (Ankara, Turkey). All the other chemicals used were of analytical grade.

2.1. Preparation of PEG_2 and PEG'ylated ASNase

Activated PEG, 2-4 bis(o-methoxy-polyethylene glycol)-6-chloro-s-triazine) (PEG_2), was prepared from monomethoxypolyethylene glycol according to the method of Ona *et al*. [29]. Monomethoxypolyethylene glycol (110 g, MW 5000) was dissolved in 500 ml of anhydrous benzene. The solution was refluxed in the presence of granular Molecular Sieves 4A for 6 h to remove water. After cooling, zinc oxide (50 g) and cyanuric chloride (1.85 g) were added, and the mixture was refluxed for 53 h. The resulting mixture was diluted with 500 ml of benzene and filtered. The product (PEG_2) was precipitated by addition of petroleum ether (twice the volume of the solution) followed by drying under vacuum to obtain 108 g of activated PEG.

Active PEG (PEG_2) (10 mg, MW 10 000) was mixed with L-asparaginase (0.1 ml, 6 mg/ml in 50% glycerol, pH 6.5). The enzyme-PEG_2 solution was incubated for 1 h at room temperature and then used as ''water phase one'' (w_1) in nanocapsule preparation.

2.2. Radiolabeling of enzyme

Native asparaginase was tritiated using a modification of the method of Fernandes and Gregoriadis [30]. Asparaginase (1.2 mg) was mixed with K_2HPO_4 (2 ml, 0.75 M, pH 9) and cooled in an ice-water bath. $NaBH_3$ (10 μmol) was supplemented with tracer [^3H]-$NaBH_4$ (1mCi), mixed with formaldehyde solution (30 μmol, 2.5 ml) and the mixture was stirred for 2 h at 4 °C and then dialyzed thrice against PBS (2 L) at 4 °C for 48 h. The labeled enzyme was precipitated by addition of ammonium sulfate and incubated for 2 h at 4 °C. Precipitated enzyme was collected by centrifugation with a clinical centrifuge (IEC/Division, USA) at 5000 rpm and redissolved in 0.4 ml PBS.

2.3. Low molecular weight PHBV preparation by treatment with $NaBH_4$

Low molecular weight PHBV was prepared as described by Baran [31]. PHBV powder (300 mg) was dissolved in chloroform (9 ml). $NaBH_4$ (3 mg) was dissolved in methanol (1 ml) and mixed with PHBV solution. The solution was continuously stirred on a magnetic stirrer and precipitated in cold methanol at predetermined time points to obtain PHBV with different molecular weights. Afterwards, the precipitate was filtered and dried under vacuum at room temperature.

2.4. Encapsulation of L-asparaginase in PHBV nanocapsules

Nanocapsules were prepared by the water-in-oil-in-water approach by solvent evaporation, applying a modified version of the procedure for poly(lactic-co-glycolic acid) nanospheres described by Blanko and Alonso [32]. An aqueous solution of protein (0.1 ml, 0.6 mg L-asparaginase) was emulsified in dichloromethane (0.6 ml) containing PHBV (30 mg) by probe sonication for 15 s (ultrasonic homogenizer, 4710 series, Cole-Parmer Instruments, Chicago, USA) at an output of 24 W. The first emulsion (w/o) was added into aqueous solution of PVA (2 ml, 1% w/v) to form the second emulsion [(w_1/o)/w_2)]. The double emulsion was then added into PVA (50 ml, 0.3%) in a flask and the solvent was evaporated at 4 °C by mixing overnight with a magnetic stirrer. Nanocapsules were precipitated by centrifugation at 15 000 g for 25 min and washed with Tris–HCl buffer (pH 7.4, 10 mM). The nanocapsules were suspended in Tris–HCl buffer (4 ml, pH 7.4, 10 mM). In the study this procedure was referred to as the ''base preparation''.

2.5. Optimization of encapsulation conditions of L-asparaginase

2.5.1. *Effect of modification of L-asparaginase with* PEG_2

L-asparaginase modified with active PEG prepared in Section 2.1 was used in microencapsulation as an alternative to unmodified ASNase.

2.5.2. *Influence of use of isoelectric point as the medium pH*

The effect of the use of isolectric point pH was determined by adjusting the pH of w_2 phase to the isoelectric point of L-asparaginase (pI: 4.9) with 0.1 M phosphate buffer prior to emulsification.

2.5.3. *Effect of PHBV molecular weight*

Low molecular weight PHBV (30 mg, average MW 23 000 Da) prepared by $NaBH_4$ treatment was used in the preparation of oil phase in the nanocapsule preparation.

2.5.4. *Combination of optimum preparation conditions for ASNase*

The modifications of the phases which yielded optimum results were combined in one final preparation. The composition of the optimum batch was as follows: w_1 phase; PEG_2 (10 mg), oil phase (O): low molecular weight PHBV (Av. MW 23 000 Da), and w_2 phase: 4% PVA and isoelectric point adjustment.

2.6. Microscopy

A nanocapsule suspension in Tris–HCl buffer (pH 7.4) was carefully placed onto aluminum stubs and dried at room temperature. Dried capsules were coated with an ultrathin layer of gold under vacuum. Scanning electron micrographs of PHBV nanocapsules were obtained by using a Cambridge Stereoscan S4-10 scanning electron microscope.

2.7. Measurement of ASNase activity

2.7.1. *Nessler's reaction*

In vitro L-asparaginase activity was measured by Nessler's method. The assay procedure is based on direct Nesslerization of ammonia [2]. Enzyme solution (10–100 µl) was added to Tris–HCl (pH 8.5, 50 mM) in a final volume of 1.5 ml. The reaction was started with addition of L-asparagine solution (0.5 ml, 10 mM, in 50 mM Tris–HCl, pH 8.5) and allowed to proceed for 5–60 min at 37 °C. The incubation was stopped with trichloroacetic acid (0.5 ml, 15%) and the volume was adjusted to 4.5 ml with distilled water. Nessler's reagent (0.5 ml) was added, and the mixture was allowed to stand at room temperature for 15 min, and then the absorbance at 500 nm was measured with UV/Vis spectrophotometer.

The extinction coefficient of NH_4Cl (used as a standard for ammonia) was found to be $0.1383\ \mu mol^{-1}\ cm^{-1}$. L-asparaginase specific activity (Unit) was calculated as: $(\Delta A_{500}/min)/(0.1383)/mg$.

2.7.2. *Specific activity of L-asparaginase* in vitro *and in mouse serum*

To measure L-asparaginase activity in the serum of mice, coupled enzyme assay was used instead of Nessler's method due to the higher sensitivity [33]. The enzyme mixture was prepared by mixing glycerol (100 ml, 87%), Tris–HCl buffer (pH 8.45, 50 ml, 0.5 M), NADH (50 mg), α-ketoglutaric acid (50 mg), GOT (180 U), MDH (110 U) and the volume was adjusted to 500 ml with distilled water. Asparaginase containing plasma (50 µl) was added into this enzyme-coupled activity assay mixture (2.25 ml) and incubated for 15 min at 37 °C. Then, 250 µl 10 mM L-asparagine in Tris–HCl buffer (pH 8.5) was added and the decrease in the absorbance of NADH was recorded at 340 nm using a UV/Vis spectrophotometer.

2.8. Determination of encapsulation efficiency of L-asparaginase

The amount of L-asparaginase encapsulated in nanocapsules was determined by dissolving dry nanocapsules (5 mg) in chloroform (2 ml) and extracting repeatedly with distilled water (2 ml). Then, Bradford method [34] was used to quantify the protein in the aqueous extracts. The final concentrations of the reagents, after being added to protein solutions (0.5 ml), were: Coomassie Brilliant Blue G-250 (0.01% (w/v)), ethanol (4.7%), and phosphoric acid (8.5%). The color intensity was measured spectrophotometrically at 595 nm. The encapsulation efficiency (E.E. %) of the enzyme in nanocapsules was calculated as the ratio of the amount of protein in the nanocapsules and the input protein, multiplied by 100.

2.9. Heparin conjugation to low Mw PHBV

Hydroxyl groups of low Mw PHBV was activated by cyanuric chloride as described in Section 2.1. for PEG activation. Low MW PHBV (500 mg, MW 23 000 Da) was dissolved in chloroform. The solution was mixed for 6 h with granular molecular sieve to remove any traces of water. Cyanuric chloride (0.0185 g) and zinc oxide (0.5 g) were added and the solution was mixed on a magnetic stirrer for 53 h at room temperature. After filtering the suspension, activated PHBV in the filtered solution was precipitated in cold methanol and dried under vacuum.

Low molecular weight heparin (20 mg) was dissolved in borate buffer (1 ml, 0.1 M, pH 10). Activated PHBV (100 mg) was dissolved in DMSO/chloroform (5 ml, 1 : 1 volume ratio), and heparin was added into the solution. The polymer-heparin solution was mixed for 48 h at room temperature. The PHBV-heparin was dialyzed extensively against distilled water and the precipitated polymer was freeze-dried.

The reaction between toluidine blue and heparin was performed essentially as described by Smith *et al*. [35] but with some modifications which allowed direct determination of immobilized heparin content. A Heparin-PHBV sample (3 mg) or nonconjugated PHBV (3 mg, as a control) was dissolved in dichloromethane (1 ml) in a test tube. Toluidine blue solution (2.5 ml,

0.005%, 0.01 N HCl containing 0.2% NaCl) was pipetted into the test tube. The mixture was vortexed vigorously for 5 min. It was then diluted with NaCl (0.2%) to a total volume of 5 ml and agitated by a vortex mixer for 30 s. Hexane (5 ml) was then added to each tube and the tubes were shaken vigorously for another 30 s to separate the heparin-dye complex formed. Aqueous solution (0.5 ml) was mixed with ethanol (2.5 ml) and the absorbance was measured at 621 nm by using UV-visible spectrophotometer. Varying amounts of the standard heparin in saline solution (2.5 ml, 0.2%) (which would result in a standard curve with a concentration range of approximately 10–70 μg of heparin) were added to the dye solution (2.5 ml) and treated as polymer samples.

2.10. Determination of *in vivo* half life of L-ASNase preparations

Studies to measure the residence of modified and unmodified asparaginase were undertaken using female Balb/C mice. The animals were housed in the animal care facility (METU), air-conditioned, with a 12 h light/12 h dark cycle. The nanocapsule preparation was as described in Section 2.4 except that Tween 80 in PBS (0.4%) was used instead of Tris–HCl to wash and resuspend nanocapsules because of the toxicity of the latter compound. After washing and centrifuging nanocapsules, the pellet was dissolved in Tween 80-PBS (2 ml) medium and filtered through 5 μm filter in order to prevent vein occlusion during *in vivo* testing by aggregates and large particles (the capillary is 9 μm in diameter).

Mice were maintained in a warm chamber to dilate their lateral tail veins and then the filtered suspension (0.5 ml, 0.37 U and 0.58 U for unmodified and heparin conjugated nanocapsules, respectively) was injected. Blood samples were collected from the opposite lateral vein at predetermined time periods into plastic microtubes, containing heparin (10 μl, 5 mg/ml). Whole blood samples were frozen at $-70\,^{\circ}C$ and thawed at room temperature in order to rupture the nanocapsule wall and to release the enzyme into the medium. Thawed blood samples were then tested for their asparaginase activities by coupled enzyme assay method. Turbidity from serum proteins was eliminated by preincubating blood sample in enzyme mixture without adding the substrate solution for 15 min at 37 °C. After incubation, substrate solution was added and the reaction for asparaginase activity measurement was started as described in coupled enzyme assay (Section 2.7 b).

For radiolabeled enzyme encapsulated nanocapsules, 50 μl of heparinized whole blood was mixed with H_2O_2 (100 μl) to bleach the haemoglobin and mixed with 20 ml scintillation cocktail for the detection of their beta emission.

2.11. Antigenicity testing for L-ASNase preparations

For toxicity studies an adaptation of Jorge *et al*. [8] was used. Four Balb/C mice were sensitized for encapsulated ASNase and three for unmodified ASNase with subcutaneous injections of 100 μg of each of free or encapsulated ASNase on days 0, 10, and 20. On day 30, animals were challenged intravenously (in the tail vein) with the same dose (100 μg asparaginase) of free or encapsulated enzyme. Deaths or symptoms of hypersensitivity were recorded. For ELISA test blood samples were collected from mice tail three days after the last injection.

2.12. ELISA test for anti asparaginase Ig in serum

ELISA test was performed according to method by Koerholz *et al*. [36]. L-asparaginase preparations (1 mg/ml) were collected on 96 well ELISA plate by overnight incubation in carbonate buffer (pH 9.8) at 4 °C. The plates were then washed thrice using PBS-Tween 20 (0.005%, pH 7.4). Sera were diluted 1 : 20, 1 : 40, and 1 : 60 in PBS-BSA (1%)-EDTA (5% v/v of a 0.2 M solution). They were added to plates in duplicates and incubated for 2 h at 37 °C in order to allow specific antibodies to bind to the solid phase L-ASNase. Excess material was removed by washing the plates thrice with PBS-Tween. To quantitate specific antibodies bound to the solid-phase L-ASNase, a goat anti-mouse IgG antibody conjugated to alkaline phosphatase was added to each well in an appropriate dilution in PBS-Tween and incubated at room temperature. Unbound conjugate was removed by three washings with PBS-Tween and bound conjugate was quantified by adding substrate p-nitrophenylphosphate (1 mg/ml) diluted in diethanolamine buffer (pH 9.6). After 30 min at room temperature the optical density was measured at 405 nm using an automated ELISA reader (Molecular Devices, Kinetic Microplate Reader, USA). Sera which were never exposed to L-ASNase served as controls.

3. Results and Discussion

3.1. Effect of modification of enzyme by conjugation with PEG

Modification of L-asparaginase with polyethylene glycol yielded a higher encapsulation efficiency and specific activity in comparison to unmodified enzyme. This increase was from 23.7% to 27.9% for encapsulation efficiency (Table I) and from 0.074 to 0.276 U/mg polymer or 47 U/mg protein to 104 U/mg protein for specific activity (Fig. 1(a) and (b)). During the encapsulation process it is expected that PEG_2 increases asparaginase hydrophobicity and partitioning of the enzyme with oil phase and also partly protects it from damages incurred during sonication and from the denaturating effect of the solvent dichloromethane.

Covalent modification of functional groups located on the surface of proteins with amphiphilic polymers like PEG is a useful method to decrease the hydrophilicity of water-soluble proteins. When they are more hydrophobic, their affinity towards the hydrophobic media, the oil phase, is higher [37]. This decreases the leakage into aqueous phase and increases the tendency to accumulate in the oil phase, increasing the encapsulation efficiency. In addition, PEG_2 modification is expected to protect the enzymes from denaturation during ultrasonication of enzyme solution in the preparation of nanocapsules.

TABLE I Experimental conditions and modifications for the optimization of L-asparaginase entrapment into PHBV nanocapsules

Phase	Sample number	1	2	3	4	5	6
	Sample legend	Base	4% PVA	Low MW PHBV[b]	Isoelectric	PEG_2	Isoelect[a]/ PEG_2/ 4% PVA/ Low MW PHBV[b]
	Ingredients						
w_1	Enzyme (asparaginase) 0.6 mg	+	+	+	+	+	+
	PEG_2 (active PEG) 10 mg	—	—	—	—	+	+
O	Dichloromethane 0.6 ml	+	+	+	+	+	+
	Low MW PHBV 30 mg	—	—	+	—	—	+
	PHBV 30 mg	+	+	—	+	+	—
w_2	PVA 2 ml	+1%	+4%	+1%	+1%	+1%	+4%
	Isoelectric pH	—	—	—	+	—	+
Encapsulation efficiency (%)		23.7	29.9	—	28.0	27.9	—

a = Isoelectric pH; b = Low molecular weight PHBV.

3.2 Effect of isoelectric point

Upon setting the pH of the second water phase as the isolelectric point of L-asparaginase the encapsulation of enzyme was increased from 23.7% to 28.0% (Table I). At the isoelectric point the net charge of a protein is zero and this is its least soluble form in an aqueous medium. Under such conditions protein leakage from w_1 phase into w_2 phase is expected to be very low and that it would enable encapsulation of higher amounts of protein.

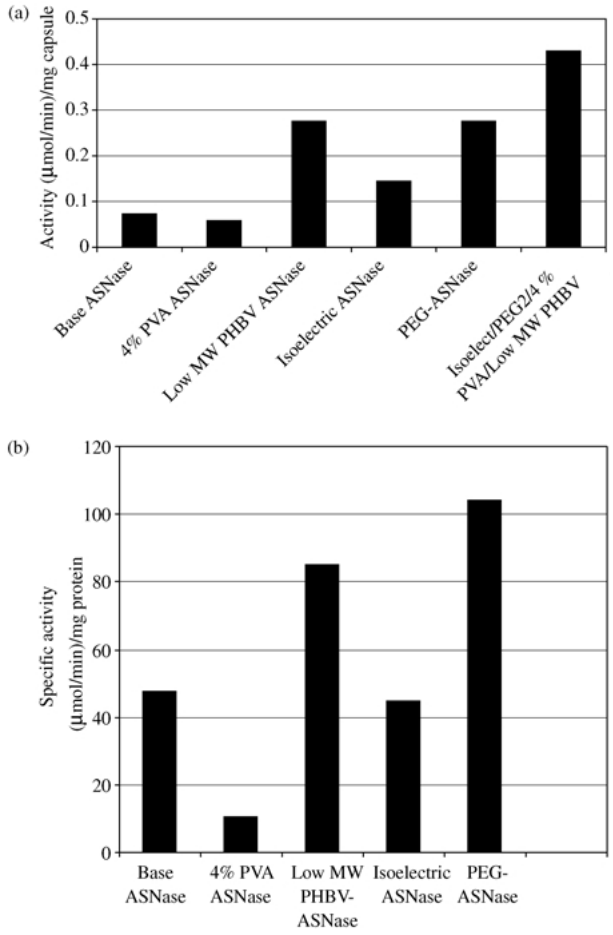

Figure 1 Influence of preparation conditions on specific activities of L-asparaginase encapsulated in PHBV nanocapsules: (a) Activity (µmol/min)/mg dry capsule, (b) specific activity (µmol/min)/mg protein in capsule.

3.3. Effect of molecular weight of PHBV

The use of low MW PHBV (23 000 Da) in nanocapsule preparation, substantially increased the encapsulated ASNase activity from 0.074 to 0.276 U/mg capsule (ca. 4-folds) (Fig. 1(a)) and the specific activity from 47 to 85 U/mg protein (ca. twice). In our earlier studies with catalase [31], although the use of low MW PHBV had not increased the encapsulation efficiency, the activity (U/mg capsule), however, was increased four times compared to use of high MW PHBV (297 000 Da).

3.4. Combination of optimum preparation conditions for ASNase

Combination of optimum encapsulation conditions, as the use of 4% PVA, isoelectric point in w_2, low molecular weight PHBV (modified with 2 h treatment with $NaBH_4$), and chemical modification (PEGylation) of L-ASNase prior to encapsulation enabled a large increase in the activity (from 0.074 to 0.429 U/mg capsule (Fig. 1(a)) in comparison to the base. The activity increase is most probably due to the combination of higher permeability of the nanocapsule wall caused by low MW PHBV and higher stability of enzyme by PEG_2 conjugation.

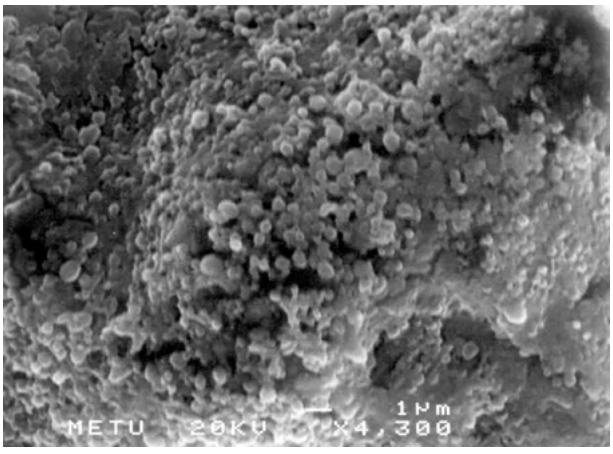

Figure 2 Scanning electron micrograph of High MW PHBV-ASNase nanocapsules.

3.5. Microscopy

Use of low molecular weight PHBV was observed to lead to a decrease in nanocapsule size, from around 750 to about 200 nm (Fig. 2). This is probably because dispersion of oil phase is easier with the low viscosity polymer (a result of chain size reduction), and this would result in smaller droplets in the first emulsification, which in turn reduces the size of the nanocapsules [18].

3.6. Surface modification by heparin conjugation to low MW PHBV

Low MW PHBV was conjugated with heparin. Since low MW PHBV yielded a methoxyester derivative of PHBV [31], with only the terminal hydroxyl group being available for cyanuric chloride activation as was the case in methoxypolyethylene glycol activation by cyanuric chloride. Low molecular weight heparin was successfully conjugated to low MW PHBV. Toluidine blue assay of the heparin-PHBV conjugate showed that 19.23 µg heparin was conjugated to 1 mg polymer. This corresponds to 11.42% (mol/mol) conjugation efficiency or 1.91% (w/w).

3.7. Determination of *in vivo* half life of L-ASNase preparations: Unmodified, PHBV nanocapsules

Enzyme activity (after freeze-thaw to rupture nanocapsule wall and to increase enzyme activity in blood) and radioactivity were used to evaluate the pharmacokinetics of unmodified nanocapsules in blood. The enzyme activity in the blood dropped to 38% after 4 h (Fig. 3). The radioactive measurement showed a much faster drop (Fig. 4). After 3 h 30% of the radioactivity remained in the circulation.

In a recent study by Li *et al*. [38] the half life of PLGA nanoparticles containing BSA was found to be 13.6 min. The PEG conjugated nanoparticles, on the other hand, had exhibited a markedly longer blood clearance (circulatory half life is 4.5 h). Compared to that, PHBV nanocapsules prepared in this study have a remarkably longer residence time in the circulation. Dunn *et al*. [39] studied the influence of surface coating of PLGA nanoparticles by polypropylene oxide–polyethylene oxide block copolymers, poloxamer and polyoxamine,

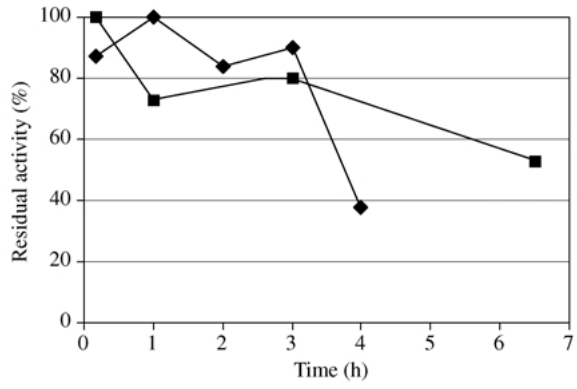

Figure 3 Residual enzyme activity in blood: ASNase encapsulated in unmodified PHBV nanocapsules (-◆-), and ASNase encapsulated in heparin-PHBV nanocapsules(-■-).

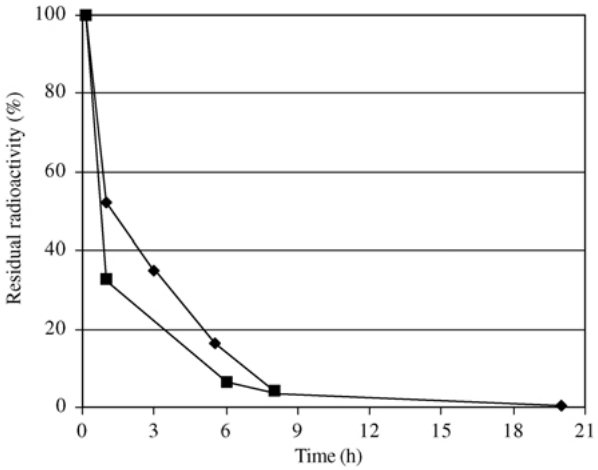

Figure 4 Residual radioactivity in blood: ASNase encapsulated in unmodified PHBV nanocapsules (-◆-), and ASNase encapsulated in heparin-PHBV nanocapsules (-■-).

on their circulation half life. Three hours post injection, 39% and 28% of the administered dose of poloxamer and polyamine coated PLGA nanospheres, respectively, remained in the blood.

To escape reticuloendothelial recognition the particles need to be uncharged and hydrophilic to avoid opsonization. Some surface active amphiphilic compounds like block copolymers polyethylene oxide–polypropylene oxide (Poloxamer) and polyoxyethylene sorbitan monooleate (Tween 80) adsorb onto hydrophobic surfaces by their hydrophobic portions, polypropylene and monooleate for poloxamer and Tween 80, respectively [40]. Similarly, PHBV nanocapsules suspended in a Tween 80 solution, can be expected to have Tween 80 molecules bound on them via the monooleate while the polyethylene chains extending outside. This must be the mechanism through which Tween 80 helped extend the circulation duration.

3.8. Heparin coated PHBV nanocapsules

Heparin conjugated PHBV nanocapsules showed a longer circulation compared to unmodified PHBV nanocapsules. After 6 h around 50% of the enzyme activity was present in the blood (Fig. 3). Radioactivity measurements, however, showed a sharp decrease much earlier (Fig. 4). Radioactivity was still detectable at 8 h, indicating that heparin-PHBV nanocapsules have longer circulation time than the unmodified PHBV nanocapsules. Since the heparin molecule is a hydrophilic molecule, hydrophilic chains probably extend outwards into the aqueous phase during nanocapsule preparation forming a hydrophilic layer. A similar mechanism was demonstrated by PEG coated PLGA nanoparticles, where the hydrophilic PEG molecules extended outside, into the aqueous phase, and formed a highly hydrated steric barrier [22].

3.9. Tests for antigenicity of ASNase preparations

In order to test the antigenicity of free ASNase and ASNase encapsulated in PHBV nanocapsules they were

introduced to mice by three subcutaneous and one intravenous injection (challenge). Subcutaneous injections were for the sensitization of the animals before the intravenous injection. Upon injection ASNase encapsulated in PHBV nanocapsules did not show any adverse effects or symptoms of anaphylaxis. However, when the native ASNase was injected two of the three mice showed typical anaphylaxis and died within 30 min of injection. These results clearly indicated that modification or encapsulation of ASNase prevented the development of hypersensitivity. In a similar study by Kravtzoff *et al*. [10], the immunogenicity of the asparaginase remained unmodified by encapsulation of enzyme within red blood cells (RBC) when injected to 13 patients. The formation of IgG or IgM anti-L-asparaginase antibodies was not suppressed and antibody titres were similar to those observed after intravenous L-asparaginase administration (control patients). However, the circulating antibodies did not elicit the adverse reactions. This may be explained by the fact that the internalized enzyme was protected by the RBC membrane and was no longer accessible to circulating antibodies.

3.10. ELISA test for the presence of antiasparaginase Ig in serum

ELISA test of sensitized mice sera yielded high antiasparaginase antibodies for unmodified ASNase and encapsulated ASNase. High antibody levels obtained with nanocapsules must be caused by the release of the enzyme from capsules. As the enzyme is released at the subcutaneous site, macrophages and granulocytes recognize the enzyme and antibodies directed against epitopes of the enzyme. Nevertheless, encapsulation of asparaginase into PHBV nanocapsules have prevented the development of lethal hypersensitivity reactions.

4. Conclusions

The aim of this study was to increase the half life and prevent the toxicity of L-asparaginase in blood by encapsulating in poly(hydroxybutyrate-co-hydroxyvalerate) nanocapsules. The double emulsion method, water-in-oil-in-water solvent evaporation method, was used to encapsulate L-asparaginase. In order to increase encapsulation efficiency and to find optimal conditions for high enzyme activity, each phase of the double emulsion was changed systematically. Modifications, such as the use of isoelectric point and high PVA concentration (4%) in w_2 phase, and the use of active PEG_2 in w_1 phase increased both the encapsulation of L-asparaginase and specific activity. PEG_2 modification protected ASNase from denaturation during encapsulation process. The use of low molecular weight PHBV polymer yielded high enzyme activities (about four times) compared to the base composition probably because of increased permeability of nanocapsule wall. Circulation time of unmodified PHBV nanocapsules was comparatively shorter. The enzyme activity in the serum dropped to 38% after 4 h. The radioactivity measurements revealed a sharper drop. After 3 h, 30% of the radioactivity remained in the circulation.

Heparin conjugated PHBV nanocapsules showed a longer circulation compared to unmodified PHBV nanocapsules. After 6 h, around 50% of the enzyme activity was still present in the blood. Radioactivity measurements, however, showed a sharp decrease at early stages. Radioactivity was still detectable at the eighth hour.

No adverse effects and symptom of anaphylaxis were observed upon injection of encapsulated ASNase in PHBV nanocapsules. Unmodified ASNase, however, resulted in hypersensitivity reactions in two of three mice. These results indicate that encapsulation into PHBV nanocapsules prevented hypersensitivity reactions against L-asparaginase and made these nanocapsules a potential tool for testing towards cancer therapy *in vitro* and *in vivo*.

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