Poly (lactic-*co*-glycolic acid) nanospheres allow for high L-asparaginase encapsulation yield and activity

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Abstract

L-Asparaginase (ASNase) is an amidohydrolase used as a chemotherapeutic agent for the treatment of acute lymphoblastic leukemia (ALL). The nanoencapsulation of this enzyme is strategic to avoid its immediate immunogenic effects that lead to a decrease in the enzyme half-life. In this work, ASNase-containing nanoparticles (NPs) were prepared by double emulsification, through an ultrasonic sonicator or an Ultra-Turrax, using two copolymers of 50:50 (w/w) poly (lactic-*co*-glycolic acid) (PLGA) with different ranges of molecular weight (24–38 kDa and 30–60 kDa) and varying the concentration of polyvinyl alcohol (PVA) as a stabilizer (0.5, 1.0, 1.5 and 2.0%) as well as the emulsification time (30 and 60 s). Using 24–38 kDa PLGA and 1.0% PVA, we obtained by cavitation NPs with hydrodynamic diameter of 384 nm, polydispersity index of 0.143 and Zeta potential of −16.4 mV, whose ASNase encapsulation efficiency was as high as 87 ± 2%. The encapsulated enzyme showed an activity 22% higher than that of the free enzyme, and no conformational changes were detected by circular dichroism. The enzyme release from NPs entrapped in dialysis bags (500 kDa molecular weight cut-off) allowed selecting a controlled system able to release about 60% of the enzyme within 14 days, for which the Korsmeyer-Peppas model provided the best correlation ($R^2 = 0.966$).

Keywords: L-Asparaginase; eEnzyme encapsulation; pPoly (lactic-*co*-glycolic acid); dDouble emulsification; cCavitation

1.1 Introduction

The enzyme L-asparaginase (ASNase) is a key chemotherapeutic agent for the treatment of acute lymphoblastic leukemia (ALL) and other hematopoietic malignancies [1]. ASNase is an amidohydrolase belonging to the N-terminal nucleophile family, which requires autocleavage between Gly167 and Thr168 to become catalytically competent. This behavior differentiates it from other similar enzymes, in which the serine residue acts as the primary nucleophile. The enzyme produced by *Escherichia coli* is a homotetramer with a molecular weight of about 142 kDa [2]. Its catalytic action leads to asparagine (Asn) deamidation, resulting in the formation of aspartate (Asp) and ammonia as a by-product [3]. Since leukemic cells are auxotrophic for Asn [4], a reduction in the blood concentration of this amino acid resulting from ASNase action is an effective therapy for ALL, because under these conditions the cell cycle arrests in the G1 phase leading to apoptosis [5].

However, immunogenic reactions and pharmacokinetic limitations are responsible for early clearance of ASNase from blood plasma, i.e. for a short half-life [6]. To reduce these problems, new biotechnological alternatives for

ASNase production by different microbial sources or recombinant forms have been described, as well as the employment of pegylation or immobilization methods through nanoencapsulation [7].

Until now, the first pharmaceutical technological innovation in the nanomedicine field concerning ASNase was the pegylation with the approval of Oncaspar® in 2006, but this therapeutic enzyme has been used since 1994 when *Escherichia coli* ASNase was approved. Over 20 years, some studies have reported ASNase nanoencapsulation into liposomes [8] and polymersomes [9] that, however, have some limitations. The main drawback of liposomes is the chemical instability of lipid vesicles, while that of polymersomes, which are tough vesicles, is the complexity of their self-assembly that may result in low macromolecule encapsulation yields [10]. The encapsulation in nanospheres could be a strategy able to lower clearance of this enzyme through its confinement in a polymeric matrix [11,12].

An example of biodegradable and biocompatible polymer widely accepted for drug delivery systems like nanospheres is poly (lactic-*co*-glycolic acid) (PLGA) [13]. In reason of ~~these~~its suitable characteristics for pharmaceutical applications, PLGA is approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Currently 14 PLGA-based drug products, approved by FDA, are available in the United States market. It is noteworthy in this respect that FDA imposes that, to register new products with nanotechnological application, they must have physical or chemical properties or biological effects that can be attributed to their size, even in a range exceeding the nanometer scale, i.e. above 1 ~~micrometer~~µm (1000 nm).

Dispersion of PLGA nanoparticles within a hydrogel matrix allows for their localization at the site of formulation injection [14], but in ASNase-based therapy of ~~leukaemia~~leukemia, which is a nonsolid cancer of the blood and bone marrow, there is not requirement for enhanced permeation and retention effect; therefore, in this case an effective nanoparticle treatment classically relies on long-circulating nanoparticles [15].

Based on this background, we developed PLGA nanoparticles for the encapsulation of ASNase. Two different methods of producing NPs by double emulsification were studied, namely ultrasonic cavitation and dispersion. Cavitation allowed preparing a promising system of PLGA-containing NPs with a loading capacity of ~~more than~~ ≥80% of ASNase.

~~2.~~2 Materials and methods

~~2.1.~~2.1 Materials

L-Asparaginase was obtained from Changzhou Qianhong Bio-Pharma Co. Ltd. (Changzhou, Jiangsu, China). 50:50 (w/w) poly (lactic-*co*-glycolic acid) (PLGA) with average molecular weight (Mw) of 24–38 kDa and 30–60 kDa, designated as PLGA 1 and PLGA 2, respectively, polyvinyl alcohol (PVA) (Mw of 31–50 kDa), bicinchoninic acid (BCA) assay kit, asparagine and trichloroacetic acid were purchased from Sigma Aldrich® (São Paulo, SP, Brazil), while the Nessler reagent was obtained from Merck Millipore® (Barueri, SP, Brazil). All the other analytical grade reagents were purchased from Synth® (São Paulo, SP, Brazil), while water was purified using a Milli-Q water purification system (MilliPore, Bedford, MA, USA).

~~2.2.~~2.2 Nanoparticles preparation

Table 1 shows the variables analyzed for NPs preparation by the water-in-oil-in-water solvent evaporation technique. Briefly, 250 µl of 0.1 M phosphate buffered saline (PBS), pH 7.4, was emulsified in 2.5 ml of chloroform containing 50 mg of PLGA. This primary water/oil (W_1/O) emulsion was obtained by either a) cavitation on ice bath with a 750-watt ultrasonic probe, model Vibra-Cell VC 750 (Sonics & Materials, Newtown, CT, USA), with the sonication intensity set at an amplitude of 50% of the total power, or b) emulsification with Ultra-Turrax disperser, model T18 basic (IKA, Wilmington, NC, USA), at 4000 rpm. The second double (W_1/O/W_2) emulsion was prepared by addition of the first W_1/O emulsion into 10 ml of 0.5, 1.0, 1.5 or 2.0% aqueous PVA solution for three cycles of 30 or 60 s under the conditions described above. The final preparation was magnetically stirred at 450 rpm for 24 h at room temperature (25 ± 1 °C) until completion of the organic solvent evaporation.

~~Table 1.~~Table 1 Variables investigated in the first emulsification to obtain nanoparticles with low polydispersity index using 50:50 (w/w) PLGA (average molecular weight of 24–38 kDa and 30–60 kDa) and PVA (31–50 kDa) as a stabilizer.

alt-text: Table 1

System[a]	PVA concentration (%)	Emulsification technique	Time of 3-cycle homogenization (s)
1	0.5	US C[b]	30
2	0.5	US C[b]	60
3	1.0	US C[b]	30
4	1.0	US C[b]	60

5	1.5	US C[b]	30
6	1.5	US C[b]	60
7	2.0	US C[b]	30
8	2.0	US C[b]	60
9	0.5	U-T D[c]	30
10	0.5	U-T D[c]	60
11	1.0	U-T D[c]	30
12	1.0	U-T D[c]	60
13	1.5	U-T D[c]	30
14	1.5	U-T D[c]	60
15	2.0	U-T D[c]	30
16	2.0	U-T D[c]	60

[a] Each nanoparticle system was prepared using either PLGA 1 or PLGA 2.

[b] Ultrasonic cavitation.

[c] Dispersion with Ultra-Turrax disperser.

~~2.3.~~2.3 Dynamic light scattering analysis

Dynamic light scattering (DLS) analysis of NPs was carried out at 25 °C and a detector angle of 90° in a Zetasizer, model Nano ZS (Malvern Instruments, Worcestershire, UK), equipped with a He-Ne laser ($\lambda = 633$ nm). For this analysis, samples were prepared without filtration (to ensure that large populations were not discarded) and 1:100 diluted in PBS. The polydispersity index (PDI) was obtained from the correlation function by using a cumulative analysis. PDI values greater than ~0.7 were assumed to indicate systems with broad size distribution. Assuming non-interacting particles, the hydrodynamic diameter (D_f) was expressed either as DLS Size Intensity or DLS Size Number, from intensity-weighted or number-weighted distribution, respectively, through the Stokes-Einstein equation:

$$Df = \frac{k_B\, T}{6\pi\eta R_h} \quad \textbf{(1)}$$

where k_B is the Boltzmann constant (1.3806×10^{-23} J/K), T the temperature, η the absolute viscosity of PBS and R_h the NP hydrodynamic radius.

~~2.4.~~2.4 Transmission electron microscopy

For transmission electron microscopy (TEM), 5.0 µl-aliquots of each NP-containing sample were diluted in distilled water, and a drop of the resulting dispersion was placed onto a carbon-coated copper grid. The grid was then observed by a transmission electron microscope, model Tecnai Spirit Biotwin G2, FEI (Hillsboro, Oregon, USA), operating at an accelerating voltage of 200 kV.

~~2.5.~~2.5 Stability of encapsulated enzyme

To evaluate the ASNase secondary structure, Far-UV circular dichroism (CD) analysis was performed on free protein and encapsulated protein recovered from the NPs. Data were collected on a CD Spectrometer, model J-1500 (Jasco, Oklahoma City, OK, USA), in the 180–260 nm range using a cell path length of 10 mm at 25 °C and a protein concentration of 10.0 mg/ml. Spectra were corrected for PBS contribution and expressed as mean residue ellipticity (mdeg) as a function of wavelength (nm).

~~2.6.~~2.6 Enzymatic activity assay

ASNase activity was measured according to the Nessler method, by determining the quantity of ammonia released from Asn hydrolysis catalyzed by this amidohydrolase. The reaction was performed in two steps. Tubes containing 500 µl of 50 mM Tris–HCl (pH 8.8), 450 µl of water, 50 µl of 189 mM Asn and 50 µl of enzyme solution were first incubated at 37 °C for 30 min. After stopping the reaction with 50 µl of 1.5 M trichloroacetic acid (TCA), 100 µl-aliquots from the former step were diluted in 2.15 ml of water, and 250 µl of Nessler's reagent were added. The results were recorded spectrophotometrically at $\lambda = 436$ nm using a SpectraMax Absorbance Microplate Reader (Molecular Devices, São Paulo, SP, Brazil) after 1 min. One unit of ASNase activity was defined as the amount of enzyme required to release 1 µmol of ammonia ~~per minute~~/min at pH 8.6 and 37 °C. To this purpose, we used a standard curve of ammonium sulfate with amount ranging from 0 to 1.2 µmol.

~~2.7.~~2.7 Encapsulation efficiency assay

ASNase-loaded NPs were submitted to three cycles of centrifugation at 3220 *g* for 15 min and resuspended in 5.0 ml of PBS, pH 7.4, to remove any non-encapsulated molecule of the enzyme. The pellet was dissolved in 1.0 ml of 50% dimethyl sulfoxide (DMSO) in PBS (v/v). and the enzyme concentration quantified as total proteins using a BCA Kit. ASNase encapsulation efficiency ($EE_{\%}$) was calculated according to the equation:

$$EE_{\%} = \frac{m_p}{m_{p0}} \bullet 100 \quad \textbf{(2)}$$

where m_{p0} is the initial mass of protein and m_p the mass of protein in the pellet solution. The pellet solution was also subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN II Multiscreen apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and the gel stained with Coomassie blue. The protein molecular weight marker was acquired from Bio-Rad Laboratories (Hercules, CA, USA). Another electrophoretic separation with retention of native properties (Native-SDS-PAGE) was performed to evaluate the enzymatic activity in this pellet solution through zymogram according to the L-aspartyl-β-hydroxamic acid method. According to this method, L-aspartyl-β-hydroxamic acid is hydrolyzed by ASNase, producing aspartic acid, hydroxylamine and red hydroxamic acids complexes with ferric chloride absorbing at 490 nm.

~~2.8.~~2.8 *In vitro* ASNase release

The *in vitro* release of ASNase from the NPs was investigated by dialysis at 37 °C under gentle magnetic stirring at 100 rpm. Briefly, 1.0 ml of protein-encapsulated NPs dispersion in PBS, pH 7.4, was added to a dialysis bag (500 kDa molecular weight cut-off) immersed in 100 ml of PBS (release medium). At selected time intervals, 70 µl of release medium were withdrawn and replaced with an equal volume of fresh medium. The amounts of released proteins as well as proteins remaining in the dialysis bag were determined by the BCA method. The release experiments were carried out in triplicate, and the results expressed as mean values ± standard deviations. The percent release of ASNase at time t was calculated according to the equation:

$$P_t(\%) = \frac{M_t}{M_0} \bullet 100 \quad P_t(\%) = \frac{M_t}{M_0} \times 100 \quad \textbf{(3)}$$

where M_t is the cumulative absolute amount of enzyme released at time t during dialysis and M_0 the absolute amount of enzyme encapsulated within the system at the beginning ($t = 0$).

As extensively reviewed by Ma et al. (2018), enzyme release from nanospheres can follow different kinetics depending on the phenomenon limiting the process and the presence or not of a lag period preceding the release. Because the release of ASNase in this work has occurred since the beginning, we tested only the main release models available in the literature without any effort to take into account such a lag period.

Among them, the first-order model can be described by the equation (Ma et al. 2018)(It is the same reference as for Query 8 (see four lines before). Therefore, the same reference number should be used.):

$$\frac{M_t}{M_\infty} = 1 - e^{-k_1 t} \quad \textbf{(4)}$$

where M_∞ is the absolute cumulative amount of enzyme released at infinite time that should be equal to M_0 in Eq. (3), M_t/M_∞ the fraction of enzyme released at time t (h), and k_1 the first-order kinetic constant (h^{-1}) [16]. This kinetic constant was estimated as the slope of the straight line obtained plotting the $\log_{10}$ of the Percent Cumulative Retention of the enzyme versus time.

The Korsmeyer-Peppas model is expressed by the equation (Korsmeyer et al. 1983):

$$\frac{M_t}{M_\infty} = k_K \, t^n \quad \textbf{(5)}$$

where n is the release exponent pointing out the mechanism of enzyme release and k_K a constant incorporating structural and geometric characteristics of the enzyme dosage form. These parameters were estimated as the slope and the intercept on the ordinate axis, respectively, of the straight line obtained plotting, in a log-$\log_{10}$ plot, the Percent Cumulative Release of the enzyme versus time.

Almost all the remaining release models can essentially be thought of as special cases of the last one. So, when $n = 1$, the system shows a non-Fickian release with characteristic zero-order kinetics, and Eq~~uation~~ (5) simplifies

to:

$$\frac{M_t}{M_\infty} = k_0\, t \tag{6}$$

where k_0 is the zero-order kinetic constant, which was estimated as the slope of the straight line obtained by simply plotting the Percent Cumulative Release of the enzyme versus t.

When $n = 0.5$, the release takes place by Fickian diffusion with negligible relaxation coefficient, and Equation (5) simplifies to the so-called Higuchi model (Higuchi 1961; Haidar et al. 2008):

$$\frac{M_t}{M_\infty} = k_{\mathrm{H}}\sqrt{t} \tag{7}$$

where k_{H} is the Higuchi constant that depends on the diffusion coefficient, the enzyme solubility in the dissolution medium, the porosity, and the enzyme concentration in the NP. This kinetic constant was estimated as the slope of the straight line obtained by plotting the Percent Cumulative Release of the enzyme versus the square root of time.

The Origin Pro-8 software (Origin Lab Corporation, Wellesley Hills, MA, USA) was used to perform the linear regression considering the determination coefficient (R^2), while the Akaike Information Criterion (AIC) was determined according to Eltayeb et al. [17].

2.9.2.9 Haemolysis assay

The haemolysis assay was performed for NPs with or without ASNase. NPs were previously lyophilized at −50 °C on a freeze drier, model L101 (Liotop, São Carlos, SP, Brazil). After lyophilization NPs were kept at −80 °C in an ultrafreezer, model MDF-U72 V (Sanyo, Osaka, Japan), and then resuspended in 0.9% NaCl salt solution, pH = 7.4, for the haemolysis assay. The systems were prepared mixing 1.0 mL ml of the sample and 50 µL l of sheep red blood cells, stirring for 1 h at 37 °C, and then centrifuging at 1788 *g* for 3 min. Haemoglobin release upon haemolysis was read in a UV–Vis spectrophotometer (SpectraMax Absorbance Microplate Reader, Molecular Devices, São Paulo, SP, Brazil) at 540 nm. The absorbance of each sample (A_s) was compared with those of saline solution taken as negative control (A_b) and water as positive control (A_w). The haemolytic effect was expressed as % haemolysis by the equation:

$$\%\text{haemolysis} = \frac{[(A_s) - (A_b)]}{[(A_w) - (A_b)]} \times 100 \tag{8}$$

2.10.2.10 2.9. Statistical analysis

Results are given as means of triplicates ($n = 3$) and standard deviation (SD), while a p value < 0.05 was considered to be indicative of statistical significance. Statistical comparisons were performed by one-way Analysis of Variance (ANOVA), and the Tukey's post hoc test was used to compare all the results among them by means of the Origin Pro-8 software (Origin Lab Corporation, Wellesley Hills, MA, USA).

3.3 Results

3.1.3.1 Screening of a system for ASNase encapsulation

Size distribution by intensity of NPs prepared using either PLGA 1 (30–60 kDa) or PLGA 2 (24–38 kDa) (Table 2) indicated that some of the systems (system 12 with PLGA 1; systems 14 and 16 with either PLGA 1 or PLGA 2) had a hydrodynamic diameter in the micrometric range (>1000 nm) with main population composed of micro- rather than nanoparticles.

Table 2.Table 2 Hydrodynamic diameters by intensity (HD_I) and by number (HD_N) of nanoparticles prepared using either PLGA 1 (30–60 K kDa) or PLGA 2 (24–38 kDa).

alt-text: Table 2

System[a]	PLGA 1		PLGA 2	
	HD_I (nm)	HD_N (nm)	HD_I (nm)	HD_N (nm)
1	n.d.[b]	n.d.	n.d.	n.d.
2	n.d.	n.d.	n.d.	n.d.
3	n.d.	n.d.	n.d.	n.d.
4	400 (100%)[c]	414 (100%)	389 (100%)	410 (100%)

5	808 (100%)	627 (100%)	n.d.	n.d.
6	615 (99.1%),	131 (20.1%),	485 (99.5%),	548 (92%),
	147 (0.6%), 5288 (0.3%)	486 (79.9%)	4831 (0.4%)	5078 (8.0%)
7	697 (99.2%), 5513 (0.8%)	619 (100%)	n.d.	n.d.
8	608 (100%)	510 (100%)	593 (99.2%), 5262 (0.8%)	690 (85.9%), 154 (1.1%)
9	n.d.	n.d.	n.d.	n.d.
10	n.d.	n.d.	n.d.	n.d.
11	n.d.	n.d.	n.d.	n.d.
12	1023 (89.5%),	1173 (46%),	678 (100%)	648 (100%)
	5105 (8.4%), 277 (2.1%)	5390 (53.6%), 276 (0.4%)		
13	n.d.	n.d.	n.d.	n.d.
14	1234 (78.3%), 5330 (19.1%),	1432 (30.5%), 5446 (45.6%),	670 (72.7%), 1262 (27.3%)	646 (71.3%), 174 (28.7%)
	324 (2.6%)	350.8 (0.7%)		
15	n.d.	n.d.	n.d.	n.d.
16	1350 (80.6%),	1251 (53.7%),	562 (100%)	536 (100%)
	5124 (14.7%), 345 (4.8%)	5326 (45.6%), 351 (0.7%)		

[a] All nanoparticles were prepared under the conditions listed in Table 1 using either PLGA 1 or PLGA 2.

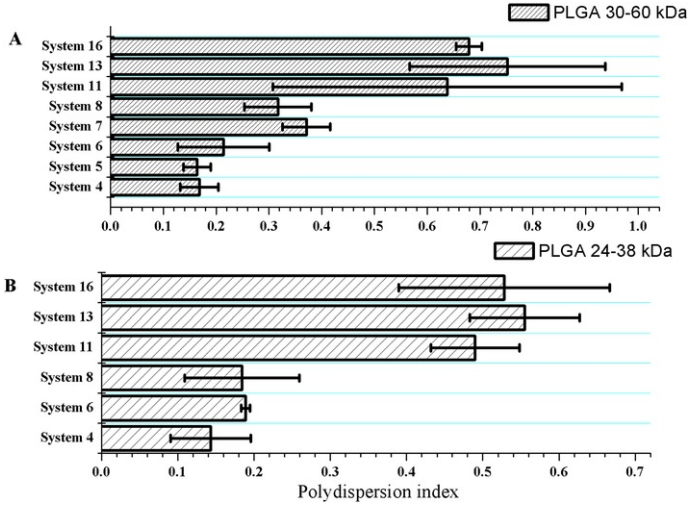
[b] n.d. = not determined because of phase separation.

[c] Percentages do refer to size distribution of heterogeneous systems.

Regarding the emulsification time, systems prepared using either PLGA 1 or PLGA 2 as copolymers for a homogenization time of 30 s showed overall phase separation soon after preparation. The only exceptions were systems 5 and 7 prepared with PLGA 1 that emulsified even by ultrasonic cavitation, likely because the simultaneous use of PVA at high levels (1.5 and 2.0%) and cavitation prevented the immediate phase separation. Systems made with PLGA 1 using Ultra-Turrax disperser for 60 s resulted in large NPs with size in the micrometric range (1000–5000 nm), while cavitation for the same time could ensure narrower nanoparticles. On the other hand, using PLGA 2 only the longer time of ultrasonic cavitation allowed obtaining NPs with acceptably small hydrodynamic diameters.

PVA concentration did not interfere in the hydrodynamic diameter of NPs prepared by cavitation with PLGA 1, whereas the size of those prepared by Ultra-Turrax disperser increased with the increase in PVA concentration. At the highest PVA concentration (2.0%), PLGA 2 resulted in NPs with hydrodynamic diameter about 100 nm larger than those prepared with lowest PVA concentrations, which, however, did not imply any change in their nanometric scale. These results taken together suggest that the use of cavitation for 60 s and 1.0% PVA are necessary requirements to produce stable NPs with both polymers. However, a monomodal size distribution profile was observed for NPs prepared with PLGA 2 rather than with PLGA 1, by both intensity and number. In general, PLGA 2 resulted in particles in the nanometric scale with smaller ~~in~~size compared with PLGA 1, while some of the particles obtained with the latter polymer exhibited hydrodynamic diameters even in the micrometric scale.

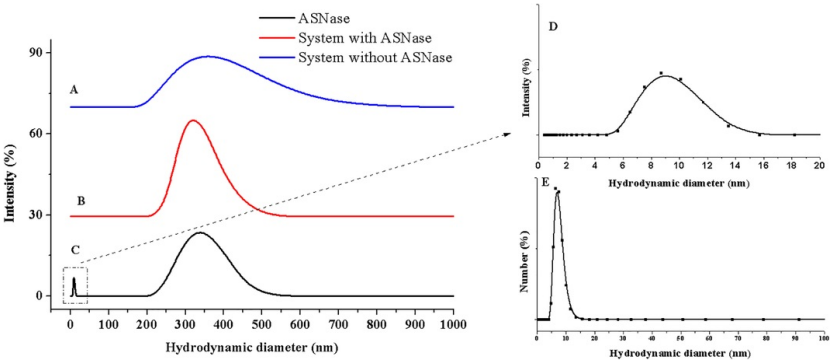
Fig~~ure~~. 1 shows the values of the polydispersity index (PDI) for systems obtained with both copolymers that did not exhibit any phase separation. The analysis of variance indicated a statistically significant difference between PDI values obtained with each copolymer ($p < 0.05$). Both PLGA 1 and PLGA 2 under the above conditions (cavitation for the longer emulsification time using 1.0% PVA) ensured the best size distribution of nanoparticles with the lowest PDI values.



~~Figure 1.~~**Fig. 1** Polydispersity index of nanoparticles prepared: A) with PLGA 1 (30–60 kDa) and B) with PLGA 2 (24–38 kDa).

alt-text: Fig. 1

The system with the smallest particle size and the lowest PDI, i.e., the system 4 prepared with PLGA 2, was chosen to encapsulate ASNase. One can see in Figure 2, curve A, the dynamic light scattering profile of NPs without enzyme, which indicates a hydrodynamic diameter of 384 ± 14 nm and low polydispersity index (PDI = 0.14 ± 0.03). Although the incorporation of the enzyme did not significantly alter the size of NPs (curve B), which showed a hydrodynamic diameter of 332 ± 18 nm, it significantly increased the degree of polydispersity (PDI = 0.92 ± 0.05). Curve C, which refers to the solution of ASNase in PBS, pH 7.4, points out an initial peak (magnified in inset D) referring to the non-aggregated enzyme with hydrodynamic diameter of 9.1 ± 0.0 nm, and another peak that may be ascribed to aggregates. The values of Zeta potential of NPs without and with enzyme were negative and statistically coincident (–16.9 ± 0.1 mV and –16.7 ± 0.2 mV, respectively), thus suggesting that the enzyme was mainly confined within them.



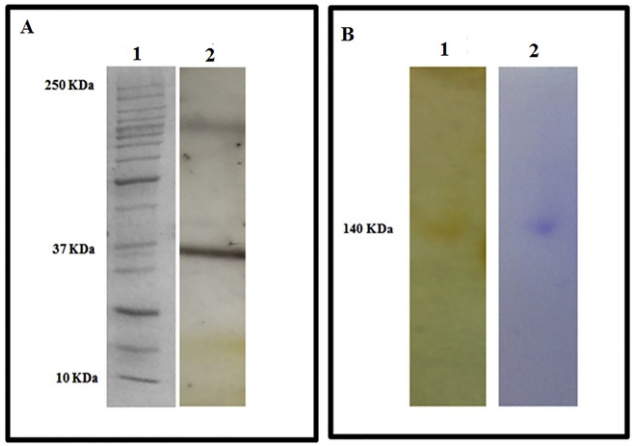
~~Figure 2.~~**Fig. 2** Dynamic light scattering profiles of nanoparticles and ASNase by intensity: A) nanoparticle without enzyme, B) nanoparticle with enzyme, C) ASNase solution in PBS, pH 7.4. D) Inset showing magnification of the former peak of ASNase solution in its form without aggregates. E) Profile by number of ASNase in PBS, pH 7.4.

alt-text: Fig. 2

~~3.2.~~3.2 Encapsulated ASNase stability

The presence of encapsulated enzyme within the NPs was confirmed by the presence of a band at 37 kDa, corresponding to the enzyme monomer, in the SDS-PAGE of the solution resulting from the dissolution of ASNase-containing NPs in DMSO/PBS (Fig. 3A). The absence of any other evident band in this electrophoretic profile is a proof of the purity of encapsulated ASNase and confirms the effectiveness of the proposed protocol for NP preparation.

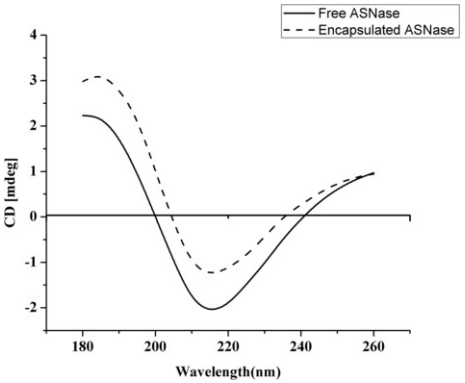
The non-denaturing gel zymogram of Fig. 3B shows a band at 140 kDa that qualitatively confirmed the encapsulated ASNase activity as well as the molecular weight of the homotetrameric enzyme reported by Sanches et al. [2].



~~Figure 3.~~Fig. 3 A) SDS-PAGE profile under denaturing conditions of ASNase-containing NPs dissolved in DMSO/PBS. B) Non-denaturing gel zymogram qualitatively confirming the activity of encapsulated ASNase.

alt-text: Fig. 3

The circular dichroism spectra of free and encapsulated enzyme illustrated in Fig. 4 indicates preservation of the secondary structure of a homotetramer belonging to α/β class of proteins like a α-helical structure even after the encapsulation process, thus confirming the stability of encapsulated ASNase.

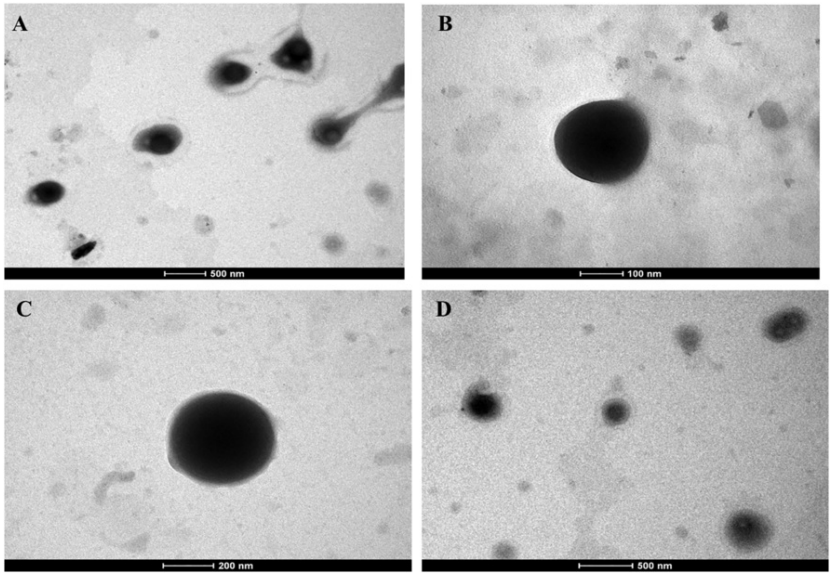


~~Figure 4.~~Fig. 4 Circular dichroism spectra of free and encapsulated ASNase.

alt-text: Fig. 4

~~3.3.~~3.3 Transmission electron microscopy

As shown in Fig. 5, individual, dispersed nanosphere-like particles with no evidence of aggregation were observed. The mean diameter of particles measured using the Image J program was 390 ± 88 nm.



~~Figure 5.~~Fig. 5 Transmission electron microscopy images of nanoparticles prepared with PLGA 2 (24–38 kDa). Accelerating voltage = 200 kV. In micrographs A and D it is possible to notice fields with several nanospheres. Micrograph A of five nanospheres allowed measuring the mean diameter (390 nm) and standard deviation (SD = 88 nm) by the Image J program. In micrographs B and C it is possible to identify individual nanostructures with a difference in size of around 100 nm.

alt-text: Fig. 5

~~3.4.~~3.4 ASNase activity

As can be seen in Table 3, the values of both volumetric and specific activities of encapsulated ASNase were significantly higher than those of the free enzyme, which suggests enzyme concentration induced by the encapsulation process.

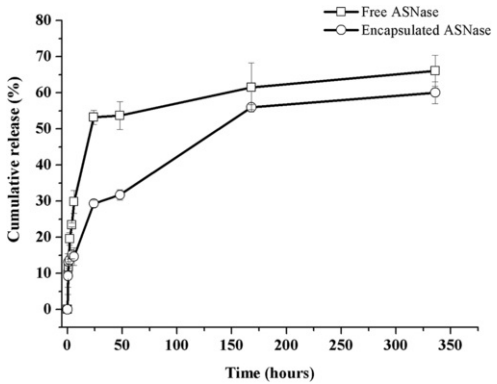
~~Table 3.~~Table 3 Values of volumetric and specific activities of both free and encapsulated ASNase. Results are expressed as means of three replicates ($n = 3$) ± standard deviations.

alt-text: Table 3

Activities	Free ASNase	Encapsulated ASNase
Volumetric activity (U/ml)	1.5 ± 0.1	1.9 ± 0.1
Specific activity (U/mg)	213 ± 5	265 ± 6

~~3.5.~~3.5 *In vitro* ASNase release

Fig. 6 shows a comparison between the profiles of *in vitro* release from dialysis bags of the free enzyme and the enzyme contained in PLGA nanoparticles. As expected, the encapsulated enzyme release was slower; in fact, about 56 and 60% of ASNase was released from NPs within 7 and 14 days, respectively, while ~~more than~~ ≥61 and 66% releases were observed for free enzyme after the same time intervals.



~~Figure 6.~~Fig. 6 Cumulative release of free and encapsulated ASNase from dialysis bags.

alt-text: Fig. 6

The experimental data of the cumulative release of ASNase from NPs vs. time were then processed using the zero-order (Eq. (6)), first-order (Eq. (4)), Higuchi (Eq. (7)) and Korsmeyer-Peppas (Eq. (5)) kinetic models. Only the Korsmeyer-Peppas model was able to satisfactorily fit the experimental data with a correlation coefficient (R^2) of 0.966 (Table 4) and a value of the release exponent < 0.5 ($n = 0.291$) (Fig. 7), while all the other models showed poorer or unsatisfactory correlation. As expected, the Korsmeyer-Peppas model also showed the lowest value of the AIC (--50.72) (Table 4), confirming its best fitting compared with the others.

~~Table 4.~~Table 4 Model parameters for L-asparaginase release from nanoparticles.

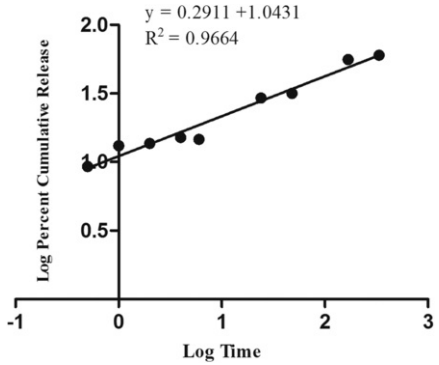
alt-text: Table 4

Model	R^{2a}	R_{adj}^{2b}	AIC[c]
Zero-order	0.845	0.812	46.63
First-order	0.860	0.841	--43.49
Higuchi	0.961	0.956	33.55
Korsmeyer-Peppas	0.966	0.961	--50.72

[a] Determination coefficient.

[b] Adjusted determination coefficient.

[c] Akaike Information Criterion.



~~Figure 7.~~Fig. 7 Bi-log plot of percent cumulative release of ASNase from nanoparticles versus time, expressed in h.

alt-text: Fig. 7

~~3.6.~~3.6 Haemocompatibility of nanoparticles by haemolysis assay

As expected, no haemolytic effect was observed for PLGA nanoparticles with or without ASNase (Supplementary material, Table S1).

~~4.~~4 Discussion

The main parameters influencing formation and stability of PLGA NPs have been extensively studied [18] ~~(Vandervoort and Ludwig 2002; Dillen et al., 2004; Carraro et al. 2014; Akl et al. 2016)~~. However, when the aim is to prepare NPs for protein encapsulation, other insights are needed, since the preparation conditions can lead to protein instability.

NPs size distribution by DLS was evaluated in this study under three aspects: the distribution by intensity, the distribution by number and the polydispersity index. Comparison of distributions by intensity and by number is fundamental for choosing between one NP~~s~~ system or another, since it allows identifying possible peaks due to sample impurity, which are visible in the distribution by intensity but do not appear in that by number (Dionzou et al., 2016). In the case of NPs investigated in the present study, some of the peaks in the micrometric scale were apparent even when analyzed by number, indicating that they were not related to dusts, but to the system itself. In other words, some conditions resulted in micrometric particle aggregation, what is not adequate for ASNase intravenous administration.

Phase separation occurred in systems prepared with PLGA 1 with a homogenization time of 30 s, except for systems 5 and 7 prepared with the highest PVA concentration. This can be explained by the simultaneous effects of cavitation and PVA that reduced the surface tension and stabilized the phases during the emulsification process (Lawrence and Rees 2000). According to Sharma et al. (2016), the concentration of stabilizer used to prepare NPs is of fundamental importance, because in suitable concentration it can avoid the system coalescence. In fact, the same authors observed that low PVA concentrations did not allow the formation of a stable emulsion and that phase separation occurred after a few hours of the emulsification process, leading to the formation of polymer aggregates.

The concentration of stabilizer plays a significant role in the protection of droplets, because it can avoid the coalescence of globules in the double emulsion–solvent evaporation technique [18]. As a confirmation of this, the lowest PVA concentration (0.5%) caused phase separation with both copolymers even in systems prepared with stirring time of 60 s, regardless of the technique used; thus, higher PVA concentrations are necessary to allow stabilization of the system.

In addition to the PVA concentration, the homogenization time was shown to be another important factor to be controlled in the preparation of nanoparticles for protein encapsulation, in relation either to the stress caused by agitation or sonication (Maruyama et al., 2015) or to the large interface between aqueous and organic phases, while shear stress is primarily responsible for protein instability [19]. In this study, a homogenization time of 60 s was the one that led to the greatest number of systems with no phase separation, because kinetic energy is a fundamental step for NPs elaboration and influences the NPs shape and size distribution [18].

In general, a concentration of PVA higher than 0.5% and an emulsification time of 60 s resulted in stable systems either with PLGA 1 or PLGA 2. However, PDI was lower for systems prepared by ultrasonic cavitation compared with Ultra-Turrax dispersion. Ultrasonic cavitation proved to be more advantageous because it allowed for immediate emulsification of phases as well as reduction in the size and polydispersity of NPs made either with PLGA 1 or with PLGA 2. This can be explained by the fact that during cavitation sequential formation (increase and decrease) of vapor bubbles takes place in the liquid, whose collapse or implosion generates high local temperatures and pressures in a very short time, thereby in turn generating very high shear stress (Gilca et al. 2015). These results are in accordance with those of Hashtjin and Abbasi [20], who observed that cavitation provides smaller droplet size, lower NPs polydispersity and greater stability of final product compared with Ultra-Turrax homogenization.

Besides double emulsion, other methods could be used to produce PLGA drug delivery systems. Core/shell microcapsules were successfully prepared by co-axial electrohydrodynamic atomization, in which needles are infused with drug and polymer solutions, a high voltage is applied to generate charges, a jet is formed that breaks into smaller droplets, droplets undergo further reduction in size due to solvent evaporation and, finally, particles with defined size and shape are produced [21]. Advantages of this method are the precise control of particle size and distribution, high reproducibility and suitability for both hydrophobic and hydrophilic drugs. It offered yields ranging between 50 and 70~~.~~% in the encapsulation of drugs with different water solubility such as paracetamol and indomethacin [22].

As can be seen in Table 2, the hydrodynamic diameter of NPs was larger using PLGA 1 rather than PLGA 2. In addition, no particles in the micrometer size range with ~~more than~~ ≥90% of distribution by intensity were observed using the latter copolymer. These results confirm that the size of PLGA NPs increases proportionally with the molecular weight of this polymer (Mittal et al. 2007).

In some cases, the key factor in NPs efficacy is their ability to selectively target the tissue of interest whil~~st~~e avoiding potential off-target effects on other tissues. To this purpose, Fullstone et al. (2015) demonstrated the possibility to increase NPs specificity for tumors by testing the ability of NPs with different size to cross fenestrations with pore size corresponding either to normal vessels (60 nm) or to those associated with tumors (240 nm). Herein,

we selected the system 4, prepared with PLGA 2 to encapsulate ASNase considering both the size and PDI of the NPs. This choice was based on the fact that the treatment of non-solid cancers such as ~~leukaemia~~leukemia needs longer circulation times, just like those expected with this nanoscale-based strategy. In addition, to avoid clearance in kidneys, nanostructures with size larger than 8 nm are desirable [23]. So, in accordance with FDA guidance, a nanotechnological approach was employed for this protein drug delivery.

The particle size of system 4 after incorporation of ASNase (332 ± 18 nm) shows that enzyme encapsulation did not alter NPs size, although an increase in PDI was observed up to 0.92 ± 0.05. Such an increase can be explained by the residual presence of enzyme aggregates, already detected by DLS analysis of the free enzyme. Observing the ASNase size distribution by number, one can see that these aggregates represent a small fraction of the total sample, nonetheless the distribution by intensity indicates that even a few large particles may have been enough to generate a light scattering signal [24,25].

The NPs Zeta potential was negative either with (~~-~~16.9 ± 0.1 mV) or without (~~-~~16.7 ± 0.2 mV) encapsulated enzyme, and its values agree with those reported in other studies for PLGA NPs (Panyam ~~et al.~~and Labhasetwar 200~~2~~3). For instance, Rodriguez-Nogales et al. [26] reported a practically coincident Zeta potential for PLGA NPs (~~-~~16 ± 2 mV), which was ascribed to the acid functional groups of the copolymer in NPs polymer matrix.

The presence of the enzyme in the system was qualitatively proven by electrophoresis (Fig. 3~~Fig. 4~~, panel A), and its activity shown in the ASNase zymogram obtained after NPs dissolution with 50% DMSO (~~Fig. 4~~Fig. 3, panel B). The occurrence of an active ASNase band indicates that active ASNase was encapsulated even under conditions capable of altering the enzyme structure such as sonication and organic solvent exposure (Fonte et al. 2015).

The circular dichroism spectra of ASNase illustrated in Fig. 4 suggest that there were no conformational changes in the encapsulated enzyme. The negative peaks at 208 and 220 nm corresponding to the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of the α-helix peptide bond were in fact preserved, while the slight decrease in intensity was the likely result of the different enzyme concentrations before and after encapsulation, taking in mind that the enzyme was not fully encapsulated. These results also suggest that the presence of PVA and PLGA in the ASNase-containing medium during the emulsification may have protected it against the stress conditions of the process.

The morphology of NPs was observed by transmission electron microscopy and their size measured with the help of the Image J program. TEM-based average size was only slightly greater (390 ± 88 nm) than that measured by the DLS method. It is worth emphasizing the similarity of these NPs with other PLGA NPs prepared according to other conventional elaboration methods [26–28].

Encapsulation of proteins in nanostructures such as nanoemulsions, liposomes, polymersomes, single-protein nanocapsules and hydrogel nanoparticles has been a challenge, especially to allow for protein stabilization [29]. In this study, the encapsulation of ASNase in the system with the best characteristics from the size and polydispersity viewpoints resulted in an encapsulation efficiency (EE) of 87 ± 2%. In a study with 50:50 (w/w) PLGA NPs made by double emulsification for ASNase encapsulation, Wolf et al. [12] produced nanospheres with EE% between 26 and 70% depending on the aqueous phase used (pluronic, trehalose, glycerol, water, Tris buffer). In general this parameter varied, in the case of ASNase encapsulated in nanostructures, in the range of 30 to 80% [30,31].

It has been reported that the encapsulation of bioactive compounds in sub-microscopic structures like nanoparticles is able to promote their stabilization and protection against alterations and agents impairing their function [29]. ~~(Nagai 2005; Tabata 2006).~~ When applied to enzymes, it has additional advantages such as the possibility of recovering them, increasing their selectivity and protecting them against degradation, thus keeping their active form while circulating in the body (Cellesi and Tirelli 2006). If free ASNase has some tendency to aggregate, the greater activity of the encapsulated enzyme compared to the free enzyme was probably the result of its immobilization within the porous polymer structure, which prevented its aggregation (Rodrigues et al. 2012) and, consequently, improved the exposure of its catalytic site (Mateo et al. 2007).

The release of ASNase from the nanospheres was slow, since about 30% of the enzyme was released within 48 h (Fig~~ure~~. 6). Xie et al. (2008) observed slower release (16~~-~~28% within 144 h) of BSA and lysozyme from PLGA microparticles likely due to polymer degradation. According to Mundargi et al. (2008), PLGA copolymers have been widely used for drug encapsulation not only because of their biocompatibility, but also because their degradation rate and mechanical properties can be modulated according to the poly lactic acid (PLA) to poly glycolic acid (PGA) ratio. These copolymers are broken down into PLA and PGA, which are easily eliminated in the form of CO_2 and water. The rate at which degradation occurs is of fundamental importance for determining the release profile of a bioactive compound and depends on the degree of crystallinity, hydrophilicity and molar mass (Panyam ~~et al.~~and Labhasetwar 200~~2~~3). In a study devoted to ASNase transport, Gaspar et al. [11] observed, after 14 days, a 46% *in vitro* release of the enzyme from NPs prepared from three 50:50 (w/w) PLGA copolymers with different molecular weights (12 kDa and 34 kDa with and without carboxyl-end groups in the polymer chain).

The Korsmeyer-Peppas model was the only one that (Eq. (5)) provided a satisfactory correlation ($R^2 = 0.966$) (Fig. 7). The value of the release exponent estimated by linear regression using Eq. (5) was <~~-~~0.5 ($n = 0.291$), which indicates a pseudo-Fickian diffusion behavior where sorption curves resemble Fickian curves, but the approach to final equilibrium is very slow [32]. According to Albisa et al. [33], pseudo-Fickian behavior predominates when additional effects of swelling, erosion, degradation, stresses, structural changes and relaxation of the material are present.

This result can be explained by the porosity of nanospheres, which may have enabled the diffusion process allowing water to penetrate into the system forcing the protein to diffuse out to the dissolution medium [34]. However, as the ASNase molecules are not sufficiently small to pass through the polymer meshes, the observed initial release may have been the result of partial protein adsorption onto the surface of nanospheres rather than of its encapsulation within the polymeric mesh [35]. This hypothesis agrees with the results of Van Dijkhuizen-Radersma et al. [36], who observed that the BSA hydrodynamic diameter was too large to pass the initial meshes of the hydrogel

matrix; even though no initial release was observed, when the matrix degraded after a certain time, the matrix molecular weight decreased to such an extent that its mesh size became sufficiently large to allow BSA diffusion and further matrix degradation increased the release rate. In other words, ASNase may have been released initially mainly by simple desorption and then by actual degradation of the matrix.

Because ASNase is clinically administered into the bloodstream, a haemolysis assay was performed since some materials can harmfully disturb red blood cells and destroy their cell membrane triggering the release of haemoglobin (Horakova et al., 2018). No haemolytic activity of PLGA nanoparticles with or without ASNase was observed irrespective of their loading (Supplementary material, Table S1), thereby confirming the biocompatibility of the selected polymer.

5.5 Conclusions

In this work, a rational route allowed elaborating L-asparaginase-PLGA nanoparticles varying the stabilizer concentration, the emulsification technique and the time of the homogenization cycle. Although the optimal ranges of these variables were already reported in the scientific literature, it was necessary to consider all possibilities to find the least stressful conditions for the enzyme, i.e. to reach a balance between the conditions for nanoparticles elaboration and those for stabilizing L-asparaginase. We obtained nanoparticles wherein the encapsulated enzyme was highly active. The profile of enzyme release pointed out the proposed system as a potential one to increase the half-life of encapsulated L-asparaginase compared with the free enzyme.

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Uncited reference

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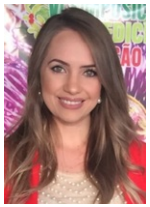
[36] ~~R~~R. Van Dijkhuizen-Radersma, ~~S~~S. Métairie, ~~JR~~J.R. Roosma, ~~K~~K. De Groot and ~~JM~~J.M. Bezemer, Controlled release of proteins from degradable poly(ether-ester) multiblock copolymers, ~~*J Control Release*~~*J. Control. Release* **101**, 2005, 175–186, https://doi.org/10.1016/j.jconrel.2004.08.014.

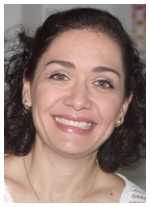
~~**[37]** H~~H. ~~Ashrafi, M~~M. ~~Amini, S~~S. ~~Mohammadi-Samani, Y~~Y. ~~Ghasemi, A~~A. ~~Azadi, MR~~M.R. ~~Tabandeh, E~~E. ~~Kamali-Sarvestani and S~~S. ~~Daneshamouz, Nanostructure L-asparaginase fatty acid bioconjugate: Ssynthesis, preformulation study and biological assessment, *Int J Biol Macromol* *Int. J. Biol. Macromol.* **62**, 2013, 180–187, https://doi.org/10.1016/j.ijbiomac.2013.08.028.~~



Anna Emmanuela Medeiros de Brito has a Master's degree in Pharmaceutical Sciences from the State University of Paraíba (2017) and a degree in Pharmacy from the Federal University of Campina Grande (2012). In the master's degree she executed research related to nanotechnology and the encapsulation of proteins. Currently works with synthesis of drugs focused on nanotechnology.

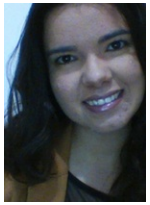
Adalberto Pessoa Jr is a Professor and the Vice-Dean of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil. He has a B.Sc. in Food Engineering (1984) and a Ph.D. in Biochemical and Pharmaceutical Technology (1995). His research interests are on fermentation processes and biomolecule purification, especially aqueous two phase liquid–liquid extraction using polymers and surfactants, fractional precipitation with salts and solvents, cross flow filtration and chromatographic purification. He is also interested in protein drug formulation. Prof. Pessoa Jr. is also an Associate Editor of the Brazilian Journal of Microbiology.

Attilio Converti is Full Professor of Chemistry and Biotechnology of Fermentations at the Polytechnic School of Genoa University and belongs to the Department of Civil, Chemical and Environmental Engineering, where coordinates the Branch of Chemical, Material and Process Engineering of the homonymous PhD program. He is a specialist in different areas of Biotechnology, including applied and environmental microbiology, fermentations, biomaterials, enzymology, biological kinetics and thermodynamics, active principle characterization and encapsulation, and preparation of functional foods. He is authors or co-author of ~~more than~~ ≥300 papers published in international journals and of 4 patents.



Carlota de Oliveira Rangel-Yagui has a B.Sc. in Pharmacy-Biochemistry (1997) and a Ph.D. in Biochemical and Pharmaceutical Technology (2003) from São Paulo University (USP, Brazil), with an 18 months course spent at M.I.T. Her Ph.D. thesis focused on protein purification by micellar systems. She also worked with micellar drug solubilization during her postdoctoral research. Since 2006 she is professor at the USP Faculty o Pharmaceutical Sciences and has been working on biomolecule purification and amphiphilic molecule applications in pharmacy. Her research group is interested on self-aggregated nanostructures for protein drug delivery. She is an Associate Editor of Brazilian Journal of Pharmaceutical Sciences.

José Alexsandro da Silva was Full Professor of Pharmaceutical Technology and Biopharmacy at Pharmacy Department in the State University of Paraíba. He is a specialist in different areas of Pharmaceutical Sciences, including nanoencapsulation of active principle, characterization of nanocarriers. Currently his researches have encompassed fermentations technology. He is authors or co-author of ~~more than~~ ≥30 papers published in international journals and of 2 patents.



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E-Extra

As expected, no haemolytic effect was observed for PLGA nanoparticles with or without ASNase (Supplementary material, Table S1).

Because ASNase is clinically administered into the bloodstream, a haemolysis assay was performed since some materials can harmfully disturb red blood cells and destroy their cell membrane triggering the release of haemoglobin (Horakova et al., 2018). No haemolytic activity of PLGA nanoparticles with or without ASNase was observed irrespective of their loading (Supplementary material, Table S1), thereby confirming the biocompatibility of the selected polymer.

E-component

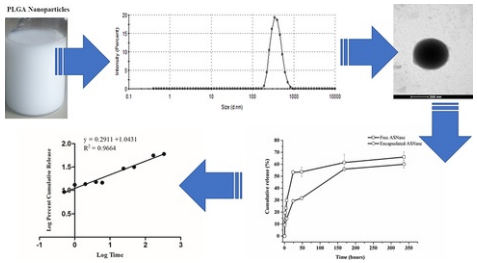
The following are the supplementary data related to this article.

Multimedia Component 1

~~Table S1.~~**Table S1** Results of the haemolysis assay performed in triplicate to check the nanoparticles biocompatibility.

alt-text: Table S1

Graphical abstract



alt-text: Unlabelled Image

Highlights

- L-Asparaginase is encapsulated in 24–38 kDa poly(lactic-*co*-glycolic acid) nanospheres.
- The encapsulated enzyme shows an activity 22% higher than that of the free enzyme.

- A controlled system is able to release about 60% of the enzyme within 14 days.
- The Korsmeyer-Peppas model can satisfactorily describe the enzyme release.

Queries and Answers

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Query:

Citation "Higuchi 1961" has not been found in the reference list. Please supply full details for this reference.

Answer: Full details for this reference are: T. Higuchi, Rate of release of medicaments from ointment bases containing drugs in suspension, *J. Pharm. Sci.* **50**, 1961, 874-875, https://doi.org/10.1002/jps.2600501018.

Query:

Citation "Haidar et al. 2008" has not been found in the reference list. Please supply full details for this reference.

Answer: Full details for this reference are: Z.S. Haidar, R.C. Hamdy and M. Tabrizian, Protein release kinetics for core-shell hybrid nanoparticles based on the layer-by-layer assembly of alginate and chitosan on liposomes, *Biomaterials* **29**, 2008, 1207-1215, https://doi.org/10.1016/j.biomaterials.2007.11.012.

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Citation "Gilca et al. 2015" has not been found in the reference list. Please supply full details for this reference.

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Answer: This citation should be "Panyam and Labhasetwar 2003" rather than "Panyam et al. 2002" Full details for this reference are: J. Panyam and V. Labhasetwar, Biodegradable nanoparticles for drug and gene delivery to cells and tissue, *Adv. Drug Deliv. Rev*. **55**, 2003, 329–347, https://doi.org/10.1016/S0169-409X(02)00228-4.

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No panels A and B were found in Fig. 4. Please check, and amend as necessary.

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Citation "Cellesi and Tirelli 2006" has not been found in the reference list. Please supply full details for this reference.

Answer: Full details for this reference are: F. Cellesi and N. Tirelli, Sol–gel synthesis at neutral pH in W/O microemulsion : a method for enzyme nanoencapsulation in silica gel nanoparticles, *Colloids Surf. A: Physicochem. Eng. Asp.* **288**, 2006, 52–61, https://doi.org/10.1016/j.colsurfa.2006.05.008.

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Citation "Rodrigues et al. 2012" has not been found in the reference list. Please supply full details for this reference.

Answer: Full details for this reference are: R.C. Rodrigues, C. Ortiz, A. Berenguer-Murcia, R. Torres and R. Fernandez-Lafuente, Modifying enzyme activity and selectivity by immobilization, *Chem. Soc. Rev*. **42**, 2013, 6290–6307, doi 10.1039/C2CS35231A.

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Citation "Mateo et al. 2007" has not been found in the reference list. Please supply full details for this reference.

Answer: Full details for this reference are: C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan and R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, *Enzyme Microb. Technol*. **40**, 2007, 1451–1463, https://doi.org/10.1016/j.enzmictec.2007.01.018.

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