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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Albumin/asparaginase capsules prepared by ultrasound to retain ammonia

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Abstract Asparaginase reduces the levels of asparagine in blood, which is an essential amino acid for the proliferation of lymphoblastic malign cells. Asparaginase converts asparagine into aspartic acid and ammonia. The accumulation of ammonia in the bloodstream leads to hyperammonemia, described as one of the most significant side effects of asparaginase therapy. Therefore, there is a need for asparaginase formulations with the potential to reduce hyperammonemia. We incorporated 2 % of therapeutic enzyme in albumin-based capsules. The presence of asparaginase in the interface of bovine serum albumin (BSA) capsules showed the ability to hydrolyze the asparagine and retain the forming ammonia at the surface of the capsules. The incorporation of Poloxamer 407 in the capsule formulation further increased the ratio aspartic acid/ammonia from 1.92 to 2.46 (and 1.10 from the free enzyme), decreasing the levels of free ammonia. This capacity to retain ammonia can be due to electrostatic interactions and retention of ammonia at the surface of the capsules. The developed BSA/asparaginase capsules did not cause significant cytotoxic effect on mouse leukemic macrophage cell line RAW 264.7. The new BSA/asparaginase capsules could potentially be used in the treatment of acute lymphoblastic leukemia preventing hyperammonemia associated with acute lymphoblastic leukemia (ALL) treatment with asparaginase.

Keywords Acute lymphoblastic leukemia · Asparaginase · Ultrasound · Capsules · Ammonia retention

Introduction

Asparaginase (EC 3.5.1.1) has been used for the treatment of malignant hematopoietic diseases in children since 1970. These diseases include pediatric acute lymphoblastic leukemia (ALL), acute myelocitic leukemia, lymphosarcoma, and non-Hodgkin lymphomas (Muller and Boos 1998; El-Naggar et al. 2014). Asparaginase belongs to a family of homologous amidohydrolases. It catalyzes the hydrolysis of the side chain amide bond of asparagine, resulting in the formation of aspartic acid and ammonia (Kumar and Sobha 2012).

For tumor cells, blood serum asparagine is essential for their growth because they are unable to synthetize their own asparagine. On the contrary, the growth of normal cells is independent on its requirement as this amino acid can be synthesized by their own enzyme asparagine synthetase (Wetzler et al. 2007; El-Naggar et al. 2014). The administration of asparaginase deprives tumor cells of their extracellular source of asparagine. This deprivation leads to tumor cell death by taking advantage of their inability to complete protein synthesis. Some secondary effects of asparaginase therapy include hyperglycemia, pancreatitis, hepatoxicity, hypersensitivity reactions, impairment of central nervous system, hyperammonemia, and occasionally anaphylactic shock (Fu and Sakamoto 2007; Kumar and Sobha 2012; Rizzari et al. 2013).

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Hyperammonemic encephalopathy is a frequently fatal complication in people treated with asparaginase. It is characterized by increased serum ammonia levels in the absence of some liver disease that can cause hepatic failure or any other identifiable cause (Nott et al. 2007; Lyles et al. 2011). Hyperammonemia was defined as ammonia concentrations higher than 50 μmol/L and clinical significant hyperammonemia as ammonia concentrations higher than 100 μmol/L (Heitink-Pollé et al. 2012). Clinical signs of hyperammonemia can be manifested by several symptoms affecting the central nervous system such as headache, lethargy, depression, cerebral edema, coma, and even death if the increase in blood ammonia is not interrupted (Paulides et al. 2013; Nussbaum et al. 2014). To reduce it, it is necessary to reduce exogenous nitrogen intake by protein restriction and minimize gastrointestinal bleeding. Nitrogen excretion can also be enhanced by hemodialysis or ammonia-trapping therapy with sodium benzoate and sodium phenylacetate (Lyles et al. 2011).

Another possible solution to avoid hyperammonemia is the development of capsules with the capacity to maintain asparaginase activity after immobilization while capturing the ammonia resulting from asparaginase activity. Asparaginase can be immobilized on different supports like silk fibroin (Zhang et al. 2011) or chitosan (Bahreini et al. 2014) particles, conjugated with sericin protein microparticles (Zhang et al. 2004), or encapsulated in carboxymethyl konjac glucomannan–chitosan nanocapsules (Wang et al. 2008). The enzyme also can be immobilized into a hydrogel matrix made of polyethylene glycol and bovine serum albumin (BSA) (Jean-François et al. 1997) or entrapped into a hydrogel superparamagnetic particles (Teodor et al. 2009). Polyaniline nanofibers (Ghosh et al. 2012) and fructose polymer levan (Vina et al. 2001) are also used for asparaginase immobilization.

These systems focused on the improvement of asparaginase activity/stability and not on the retention of the forming ammonia. The development of new systems, with the capacity to retain the forming ammonia thus reducing the plasma ammonia concentration resultant from asparaginase activity, is here explored.

BSA is widely used in the development of systems for drug delivery because of its abundance, ease of purification, biodegradability, low cost, nontoxicity, and nonimmunogenicity (Elzoghby et al. 2012a). Albumin is used to prepare protein capsules that can be easily functionalized chemically due to the reactive groups on their surfaces like amino, thiol, and carboxylic groups (Loureiro et al. 2014). Due to a high content of charged amino acids (for example, lysine, arginine, aspartate, and glutamate), the albumin nanoparticles allow electrostatic adsorption of positively or negatively charged molecules (Elzoghby et al. 2012b). Functionalized nanoparticles can be applied for targeted drug delivery, improving cellular uptake and alleviating undesired toxic side effects associated with chemotherapeutic treatments (Loureiro et al. 2015b).

The production of albumin-based nanoparticles can be achieved using high-intensity ultrasound, which is a high-energy emulsification method. This method consists in a combination of two acoustic phenomena, emulsification and cavitation, and it is used to produce aqueous suspensions of proteinaceous microcapsules (Silva et al. 2012). It was demonstrated that high shear forces applied to biphasic water/hydrophobic solvent systems can create a microscopic dispersion of the protein solution. In this way, the proteins tend to migrate to the water/solvent interface, stabilizing the microcapsules (Silva et al. 2012; Loureiro et al. 2015b).

In this paper, we report the development of BSA-based capsules by ultrasound to immobilize asparaginase and evaluate their ability to retain the ammonia resulting from asparaginase activity (Fig. 1). The potential of BSA to form stable particles by ultrasound and high-pressure homogenization was already described by Loureiro et al. (2015b) and Silva et al. (2012).

To evaluate the capsules' capacity to retain ammonia, a new HPLC-MS method was developed, avoiding the amino acid derivatization process. The forming aspartic acid was quantified through the new HPLC-MS method and compared with the ammonia quantified by the Nessler method (Stecher et al. 1999; Cappelletti et al. 2008).

The systemic application of BSA/asparaginase microcapsules by intravenous injection is expected to decrease the ammonia level below 50 μmol/L, considered the limit value for hyperammonemia. Normal asparagine levels vary between 40 and 80 μM, and the administration of these capsules should be also capable to obtain a complete asparagine depletion. However, good results were already obtained by an asparagine depletion of 0.2 and 0.5 μM (Tong et al. 2014; Appel et al. 2008).

With the BSA/asparaginase formulations, we intend to develop new therapeutic systems for the treatment of ALL, which will hydrolyze the asparagine to therapeutic levels while reducing the concentration of ammonia and thus avoiding hyperammonemia.

Materials and methods

Materials

Asparaginase was obtained from Changzhou Qianhong Bio-Pharma Co., Ltd. (Jiangsu, China). Asparagine, aspartic acid, and Poloxamer 407 (Pol_{407}) were obtained from Sigma (USA). BSA, Dulbecco's modified Eagle's medium (DMEM), glutamine, tetrazolium bromide (MTT), and Nessler reagent were obtained from Sigma-Aldrich (USA). Dimethyl sulfoxide (DMSO) was obtained from Fisher

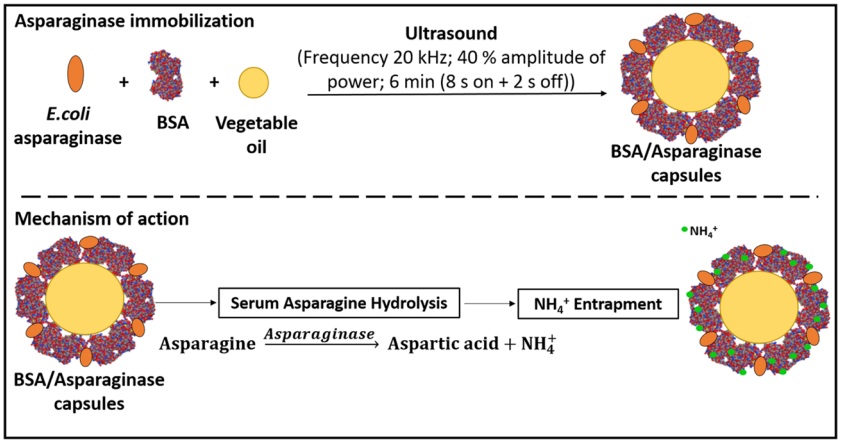

Fig. 1 Scheme of production of BSA/asparaginase capsules and retention of ammonia resultant from asparaginase activity on the capsules' surface

Scientific (USA) and fetal bovine serum (FBS) from Biochrom (Berlin, Germany).

Asparaginase enzymatic assay

The hydrolysis reaction of asparagine was performed with 0.001 and 0.0015 g/L of asparaginase and 100 mM of asparagine, at 37 °C, in ultra-pure water. Ultra-pure water was selected as the reactional medium for this assay because 50 mM Tris buffer affected the quantification of aspartic acid by HPLC-MS. After 240 min of reaction, the enzyme was inactivated in an ice-cold bath and removed from the reaction medium by ultra-centrifugation in Eppendorfs with a membrane cutoff of 5000 Da. After ultra-centrifugation, the membrane was washed with ultra-pure water to prevent amino acid retention in the membrane. The aspartic acid lost during ultra-centrifugation was recovered and quantified. The average retention for the aspartic acid was about 5.2 %. The amount of amino acid entrapped in the filtration membrane was then considered.

Ammonia quantification by Nessler method

Ammonia quantification was determined by a stopped assay using Nessler's reagent. The ammonia produced in 10 μL of the reaction mixture was determined by the addition of 990 μL Nessler's reagent. The optical density of the solution was read at 436 nm (BioTek®, Synergi MX), and the ammonia concentration was determined on the basis of a standard curve previously obtained with ammonium sulfate as a standard (Stecher et al. 1999; Cappelletti et al. 2008).

Aspartic acid quantification by HPLC-MS without derivatization

Mass spectrometer method Mass spectrometer analysis was performed on Finnigan LXQ mass spectrometer in a positive ionization mode. The aspartic acid solution was prepared in ultra-pure water with 20 % of acetonitrile and then filtered with 0.2-μm filters. High flow source conditions were optimized for aspartic acid with a flow rate of 5 μL/min. Source voltage was 4.95 kV, and source current was 0.60 μA. Sheath gas, aux gas, and sweep gas were 40, 20, and 19.98 arb, respectively. Capillary voltage was 17.77 V, capillary temperature was 274.88 °C, and the tube lens voltage was 25.01 V.

HPLC-MS method A reverse-phase HPLC system linked with mass spectrometer was used to isolate aspartic acid. During methods' optimization, two columns were tested: the Kinetex™ 2.6 μm C18 100 Å, LC Column 100 × 4.6 mm (Phenomenex®) and the Synergi Hydro-RP, 80 Å, 4 μm and 150 × 4.60 mm (Phenomenex®). After optimization, the Synergi Hydro-RP column with ammonium bicarbonate 0.01 M was selected, and the pH adjusted to 6 with formic acid (A) and acetonitrile (B) as elution solvents. A sample volume of 25 μL was injected with a flow rate of 0.3 mL/min for 20 min. The gradient program started with 95 % solvent A during the first 8 min, decreased linearly to 40 % solvent A in the next 7 min, and returned to the initial 95 % during the next 5 min. In consequence, the solvent B started with 5 % during the first 8 min, increased linearly to 60 % solvent A in the next 7 min, and returned to the initial 5 % during the next 5 min.

The peak identification was performed through the MS detector and compared to the elution times obtained when the amino acids were analyzed separately, in the same conditions. For a more correct calculation of the peak area, the "Base Peak" control from Xcalibur program was used. With this control, it is possible to obtain the peak from each amino acid individualized by selecting their mass weight.

Effect of BSA on asparaginase stability

To study the effect of BSA on asparaginase stability, several solutions with different ratios of asparaginase and BSA dissolved in PBS were prepared with a protein final concentration of 10 mg/mL. The solutions were stored at 4 °C, and the

activity was determined over time. Penicillin and streptomycin were added to the BSA/asparaginase solutions to inhibit the growth of microorganisms which could affect enzyme activity and stability. The asparaginase activity was determined weekly for 107 days, on 50 mM Tris pH 8.6, with a 0.001 g/L of asparaginase and 100 mM of asparagine. The reaction mixture was incubated at 37 °C during 4 h, and the activity was determined by the Nessler method.

BSA-based capsules prepared by ultrasounds

The BSA, BSA/asparaginase, and BSA/asparaginase/Pol_{407}capsules were prepared by sonication using Sonics® Vibra-Cell™. For BSA capsules, BSA was dissolved in PBS, pH 7.4, at a final concentration of 10 mg/mL. For BSA/asparaginase capsules, both components were dissolved in PBS, pH 7.4, at a final concentration of 10 mg/mL with an enzyme percentage of 2 % (*w*/*w*). To BSA/asparaginase/Pol_{407} capsules, the Poloxamer 407 was added to the previous BSA/asparaginase solution with a final concentration of 5 mg/mL. The amount of Poloxamer 407 used on the capsule formulation was based on the work of Loureiro et al. (2014). The aqueous phase was sonicated with 5 % (*v*/*v*) of vegetable oil with a frequency of 20 kHz and 40 % amplitude of power. The solution was sonicated during 6 min with cycles of 10 s (8 s of ultrasound pulses on plus 2 s of ultrasound pulses off). After synthesis, the phase with the capsules was collected by centrifugation (1000×*g* during 45 min, at 4 °C) using centricon tubes (molecular weight cutoff of 100 kDa) (Amicon Ultra-15, Millipore) (Loureiro et al. 2014). After separation, the free protein in the aqueous phase was quantified by the Lowry method (Peterson 1977) in order to determine the capsule formation efficiency using the formula:

$$\text{Formation efficiency}\ (\%) = \frac{[\text{Protein}]_{\text{total}} - [\text{Protein}]_{\text{free}}}{[\text{Protein}]_{\text{total}}} \times 100 \quad (1)$$

where $[\text{Protein}]_{\text{total}}$ and $[\text{Protein}]_{\text{free}}$ is the total concentration of protein added in formulation and the free concentration of protein in the aqueous phase solution after centrifugation, respectively.

Capsule characterization

The emulsions were dispersed in ultra-pure water and analyzed at 25 °C for their size distribution, polydispersity index (PDI), and *Z*-potential. The parameters were determined by photon correlation spectroscopy, using dynamic light scattering (Malvern Instruments, Nano-ZS). The values for viscosity and refractive index were 0.8872 cP and 1.330, respectively. Each sample was measured in triplicate, and the results are presented as mean value ± standard deviation.

Cellular viability assay

Cell culture maintenance RAW 264.7 (ATCC® TIB-71™) cell line (mouse leukemic macrophage cell line) was cultured in DMEM media, supplemented with 10 % FBS, 1 % glutamine (2 mM), 1 % sodium pyruvate (1 mM), 10 mM HEPES, and 1 % (*v*/*v*) penicillin/streptomycin solution. Cells were maintained in 75-cm^2 tissue culture flasks at 37 °C in a humidified atmosphere with 5 % CO_2. The cell culture medium was renewed twice a week. Subculture was performed when confluence reached values near 80–90 %. For subcultures and plating, the adherent cells were detached with a scrapper in 5 mL of fresh culture media. The cell suspension was centrifuged for 5 min at 160×*g*. The supernatant was discarded, and the fresh medium was added to obtain a new cell suspension. The cell suspension was loaded in a *Neubaeur* chamber, and the concentration of cells present in the suspension was estimated.

Cell viability assessed by MTT assay Cells were seeded at a density of 25,000 cells/well on a 96-well tissue culture plate the day before the experiments. RAW cells were exposed to four concentrations (25, 50, 100, and 200 μg/mL) of capsules with asparaginase and to two concentrations of free asparaginase (2 and 4 μg/mL). The two concentrations of free enzyme correspond to the amount of enzyme present in the highest concentrations of capsules tested. Cells incubated with DMSO (30 % of the total volume) and cells without the addition of capsules/compounds were used as positive and negative controls of cytotoxicity, respectively. Raw cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2. At the end of 24 h of contact, cell metabolic activity was assessed by the MTT viability assay (Ribeiro et al. 2013). After incubation, the medium with the capsules and enzyme was removed and 110 μL of medium with MTT (5 mg/mL) was added to each well, and cells were further incubated at 37 °C for 2 h. The MTT solution was carefully decanted, and formazan crystals were dissolved in 110 μL of a DMSO/EtOH (1:1 (*v*/*v*)) mixture. Color was measured with 96-well plate reader at 570 nm in a microplate reader SpectraMax Plus (Molecular Devices).

Statistical analysis

Data are presented as average standard deviation (SD), $n = 3$. Statistical comparisons were performed by one-way ANOVA with GraphPad Prism 5.0 software (La Jolla, CA, USA). Tukey's post hoc test was used to compare all the results between them, and a Dunnet's test was used to compare the results with a specific control. A p value of <0.05 was considered to be statistically significant.

Results

BSA effect on asparaginase

Several BSA/asparaginase solutions were prepared to determine the ratio which conferred higher enzyme stability along time. Enzyme activity was measured weekly for 107 days by ammonia quantification using the Nessler method (Stecher et al. 1999; Cappelletti et al. 2008). The amount of ammonia produced by the enzyme was determined by comparing the absorbance of samples at 436 nm with the absorbance of a calibration curve prepared with ammonium sulfate (Eq. 2).

$$\text{Absorbance} = 0.03046[\text{ammonia}]\text{mM} + 0.03719;\quad R^2 = 0.9976 \tag{2}$$

Asparaginase half-life times were determined in some BSA/asparaginase samples. For other samples, the half-life time was estimated at the end of storage as the final enzyme activity was higher than half of the initial activity (Table 1).

As illustrated in Table 1, there was an increase in the half-life time of asparaginase with increasing percentage of BSA in solution. Comparing all the tested BSA/asparaginase ratios, the ratio which stabilized the enzyme for a longer period was the 98 % BSA/2 % asparaginase with an estimated half-life of 148.95 days. This proportion was chosen for the preparation of BSA/asparaginase capsules by ultrasound.

BSA/asparaginase capsules prepared by ultrasound

Three different types of BSA capsules were prepared by sonication: BSA, BSA/asparaginase and BSA/asparaginase/Pol_{407}. Capsule formation efficiencies were 95.6, 93.9, and 89.8 % for BSA/asparaginase and BSA/asparaginase/Pol_{407}, respectively.

The ability of capsules to hydrolyze asparagine was determined by the Nessler method 1 day after synthesis and compared to the activity of free asparaginase dissolved in PBS (Fig. 2). BSA capsules were also tested for the ability to hydrolyze asparagine although they did not show any enzymatic activity (data not shown).

There was an increase on asparaginase activity after immobilization for both tested capsules, with a significant increase of 29.73 % for the BSA/asparaginase capsules relatively to the free asparaginase (p value ≤0.01). The capsules with Poloxamer 407 showed lower asparaginase activity comparatively to the capsules without Poloxamer 407.

The capsules' Z-average and PDI were assessed by photon correlation spectroscopy, whereas laser Doppler anemometry was employed for Z-potential. Capsules' physical stability was evaluated over 2 months, by weekly measurements of capsules' Z-average, PDI, and Z-potential, as represented on Fig. 3 for BSA/asparaginase and BSA/asparaginase/Pol_{407}. For BSA capsules, their characteristics at day 1 were represented on Table 2, as they were stable over time.

Generally, all BSA/asparaginase capsules were stable over time of storage, in respect of size and PDI, with only a significant difference on BSA/asparaginase capsules' PDI at day 8 (Fig. 3b, d).

Regarding Z-potential, there was a variation for all the formulations during storage. BSA/asparaginase/Pol_{407} capsules exhibited a slight increase on the net negative charge of the capsules with storage (Fig. 3c). For BSA/asparaginase capsules, it was observed a decrease on the net negative charge with the storage time, from −49.10 ± 1.014 to −46.2 ± 0.416 mV (Fig. 3a).

As observed on Table 2, the BSA/asparaginase capsules presented a more negative surface charge when compared with the BSA capsules due to the addition of asparaginase to the formulation. The addition of Poloxamer to the BSA/asparaginase formulation affected both capsules' Z-potential and polydispersity.

Table 1 Asparaginase half-life times of BSA/asparaginase solutions prepared on PBS

Solution	BSA/asparaginase ratio (%)	Initial activity of asparaginase (μmol ammonia/mg asparaginase)[a]	Asparaginase half-life time (days)
A	0/100	31,950 ± 355	46.70
B	20/80	34,000 ± 220	72.08
C	40/60	43,500 ± 1200	89.18
D	60/40	35,600 ± 700	105.13
E	80/20	48,750 ± 750	120.75[b]
F	98/2	46,150 ± 800	148.95[b]
G	100/0	–	–

[a] Enzyme activity was determined at the end of 240 min of incubation, at 37 °C, with 0.001 g/L of asparaginase and 100 mM asparagine. The absorbances of the solutions after Nessler method were measured at 436 nm and compared with the absorbance of a calibration curve (Absorbance = 0.03046 [ammonia]mM + 0.03719)

[b] Estimated half-time of asparaginase

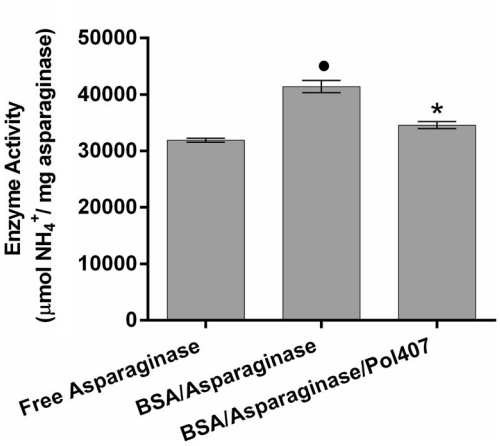

Fig. 2 Activity of asparaginase immobilized on BSA and BSA/Pol_{407} capsules prepared by ultrasounds and free asparaginase on PBS. Activity was determined 1 day after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase and 100 mM of asparagine for 240 min, at 37 °C, in ultra-pure water. Absorbance of the solutions after Nessler method was measured at 436 nm and compared with the absorbance of a calibration curve (Absorbance = 0.03046 [ammonia]mM + 0.03719). The data represents the mean ± SD from three independent experiments. Data were analyzed by one way-ANOVA: p value ≤0.05 (asterisk), p value ≤0.01 (*circle*), compared to the results obtained for free asparaginase

Optimization of a HPLC-MS method for aspartic acid quantification

In order to quantify the aspartic acid by HPLC-MS without derivatization, it was necessary to develop and optimize a new method. As the Tris buffer affected the quantification of aspartic acid by HPLC-MS, ultra-pure water was selected as a reactional medium for asparaginase. When an aspartic acid solution prepared in 50 mM Tris buffer was analyzed, no peaks were detected both in the positive and negative ionization mode.

As both asparagine and aspartic acid are present on the reaction mixture, it was essential to clearly separate the respective peaks to obtain a correct quantification. An aqueous mixture of asparagine and aspartic was analyzed by HPLC-MS in order to observe the pattern of peaks corresponding to each amino acid (data not shown).

A single peak corresponding to the simultaneous elution of asparagine and aspartic acid was initially observed. The overlapping of the peaks does not allow precise aspartic acid quantification. To overcome this drawback, the quantification method was optimized in order to separate the peaks of both amino acids. For the analysis, two different columns were tested: C18 and Hydro-C18 columns. Hydro-C18 columns achieved the best separation of the peaks, probably due to their polar endcapped C18 phase that provides extreme retention of hydrophobic compounds and shows slight polar selectivity.

The mobile phase was also optimized. Water with 0.1 % (*v*/*v*) formic acid and acetonitrile were initially used as eluent; however, this mobile phase was not suitable for the analysis because it did not allow the separation of asparagine and aspartic acid (Fig. S1a).

The mobile phase was replaced by ammonium bicarbonate, pH 6.0 adjusted with formic acid. The eluent and the pH were chosen based on the pKa of the two amino acids. At pH 6.0,

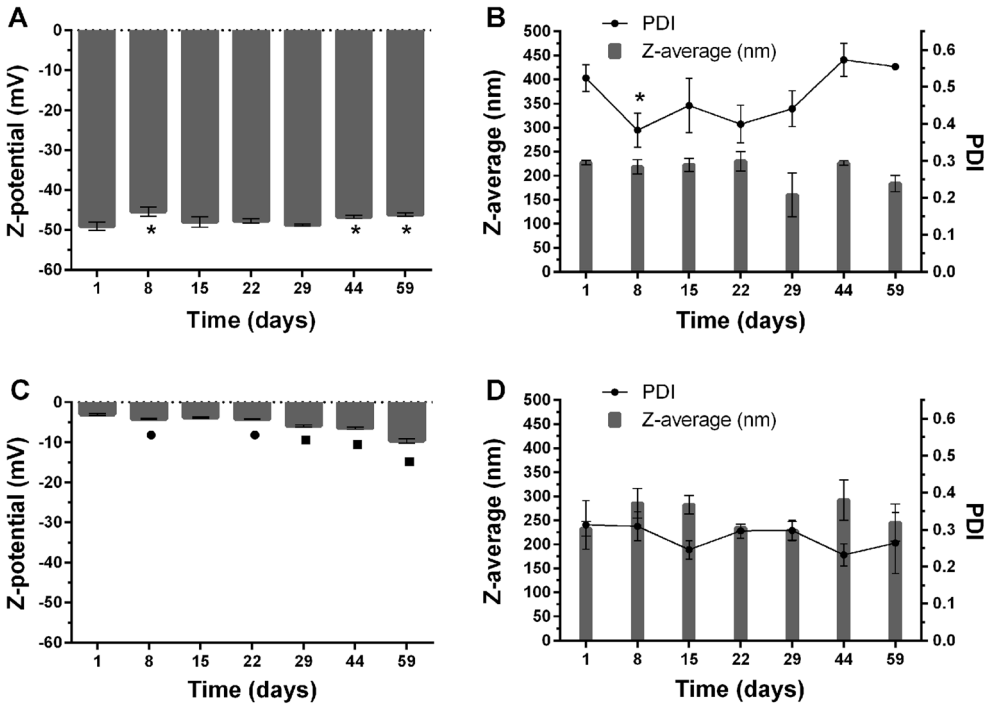

Fig. 3 Characterization of BSA/asparaginase capsules (**a**, **b**) and BSA/asparaginase/Pol_{407} capsules (**c**, **d**), during storage at 4 °C. **a**, **c** *Z*-potential; **b**, **d** *Z*-average and PDI. The data represents the mean ± SD from three independent experiments. Data were analyzed by one way-ANOVA: p value ≤0.05 (*asterisk*), p value ≤0.01 (*circle*), p value ≤0.001 (*diamond*), p value ≤0.0001 (*square*), compared to the results obtained at day 1

Table 2 Physical properties of BSA-based capsules 1 day after synthesis

BSA-based capsules[a]	*Z*-average (nm)	PDI	*Z*-potential (mV)
BSA	183.47 ± 23.38	0.4297 ± 0.038	−28.8 ± 0.9
BSA/asparaginase	227.30 ± 4.60	0.52 ± 0.036	−49.10 ± 1.01
BSA/asparaginase/Pol_{407}	232.67 ± 15.15	0.31 ± 0.066	−3.03 ± 0.26

[a] BSA-based capsules stored at 4 °C

asparagine and aspartic acid have different electrical charge which helped the separation of the two peaks during the chromatography. To increase the peaks' resolution, the analysis was performed in high flow conditions, with a flow rate of 0.3 mL/min.

After column, flow rate, and elution gradient optimization, the chromatographic analysis was performed using the column Hydro-C18, a flow rate of 0.3 mL/min, and ammonium bicarbonate (A) and acetonitrile (B) as eluents. The first peak with a retention time of 4.13 min corresponded to aspartic acid and the second one, with a retention time of 4.60 min, to asparagine (Fig. S1b).

Using this approach, it was possible to identify and separate both asparagine and aspartic acid amino acids without derivatization, when mixed in the same aqueous solution.

BSA/asparaginase capsules' capacity to retain ammonia

In order to study the ability of capsules with asparaginase to retain ammonia, the products of asparagine hydrolysis, aspartic acid, and ammonia were simultaneously quantified after asparaginase activity and compared. The asparaginase concentration used in the capsules was 0.001 g/L, while for the free asparaginase, a concentration of 0.0015 g/L was used. For this concentration of free enzyme, the quantified aspartic acid and ammonia were within the range of values determined for the capsules. The BSA capsules were not tested regarding their ability to retain ammonia since they are not, by themselves, able to hydrolyze asparagine.

The ammonia was quantified by the Nessler method, while the aspartic acid was quantified against an aspartic acid standard curve (Eq. 3) using the new, optimized HPLC-MS method (Table 3).

$$\begin{aligned} &\text{Peak area} = 583946[\text{aspartic acid}]\text{mM} \\ &R^2 = 0.9625 \end{aligned} \quad (3)$$

The main objective of this work was the development of capsules with the ability to hydrolyze asparagine while retaining the forming ammonia. The ammonia retention on the capsules' interface results from the electrostatic interaction between the positively charged ammonia and the superficial negatively charged groups of the capsules.

The ratio between the two products is related with the ability of BSA/asparaginase capsules to retain ammonia. For aspartic acid/ammonia ratios higher than 1, the capsule had the capacity to retain the ammonia resulting from the asparaginase activity.

Generally, for all the BSA/asparaginase capsules, a higher aspartic acid concentration relative to the ammonia concentration was observed, with BSA/asparaginase/Pol_{407} capsules showing the highest aspartic acid/ammonia ratio of 2.46 (Table 3).

The free asparaginase also retains some ammonia, although it was not significant when compared with the capsules.

Evaluation of BSA/asparaginase capsules' cytotoxicity by MTT assay

To assess the potential cytotoxic effect of the BSA-based capsules, mouse leukemic macrophage RAW 264.7 cells, which are positive for expression of asparagine synthetase (Suzuki et al. 2002), were used as a model of general cytotoxicity. The cells were exposed to four concentrations of capsules (25, 50, 100, and 200 μg/mL) and two concentrations of asparaginase (2 and 4 μg/mL). The concentrations of asparaginase correspond to the amount of enzyme present in the two highest concentrations of capsules. After 24 h of incubation, the metabolic activity of the cells was evaluated with the MTT assay (Fig. 4).

Analyzing Fig. 4, the BSA/asparaginase capsules did not show any significant toxic effect for any of the tested concentration. These capsules presented cell viability higher than 93.2 ± 5.31 %. When Poloxamer 407 was added to this formulation, the capsules' toxic effect increased for all the concentrations. For BSA capsules without asparaginase, only the highest concentration displayed significant levels of cytotoxicity, with a viability lower than 80 %. However, the incorporation of asparaginase and Poloxamer 407 on the interface of BSA capsules decreased the BSA toxic effect observed for the concentration of 200 μg/mL BSA capsules. The free asparaginase also presented some cytotoxicity for both tested concentrations.

Table 3 Study of the capacity of BSA/asparaginase capsules to retain ammonia

Samples	*Z*-potential (mV)	Aspartic acid[a] (mM)	Ammonia[b] (mM)	Ratio (aspartic acid/ammonia)
BSA/asparaginase	−43.90 ± 0.79	52.52 ± 1.95	27.35 ± 0.43	1.92
BSA/asparaginase/Pol_{407}	−1.75 ± 0.43	37.99 ± 0.96	15.61 ± 0.17	2.46
Asparaginase (0.0015 g/L)	−9.42 ± 2.83	44.91 ± 3.91	40.91 ± 4.75	1.10

[a] Aspartic acid quantified by HPLC-MS comparing the peak area with the calibration curve: Peak area = 583,946 [aspartic acid]mM

[b] Ammonia quantified by Nessler method considering the calibration curve: Absorbance = 0.03046 [ammonia]mM + 0.03719

Discussion

In order to determine the BSA/asparaginase ratio that conferred higher stability to the enzyme when immobilized, asparaginase half-life times of the solutions were determined. The proportion that most stabilized the enzyme was 98 % BSA/2 % asparaginase, and this ratio was chosen for the preparation of BSA/asparaginase capsules. This stabilizing effect of high concentrations of BSA was already reported for other proteins like acetylcholinesterase, ovalbumin, and lysozyme (Sah 1999; Nasseau et al. 2001).

The ability of BSA/asparaginase capsules prepared by ultrasounds to hydrolyze asparagine into aspartic acid and ammonia was determined. The capsules with Poloxamer 407 showed lower asparaginase activity comparatively to the capsules without Poloxamer 407. This could be due to the steric effect of the Poloxamer 31 that could hinder the asparaginase and thus reduce the amount of substrate available near the enzymes' catalytical center.

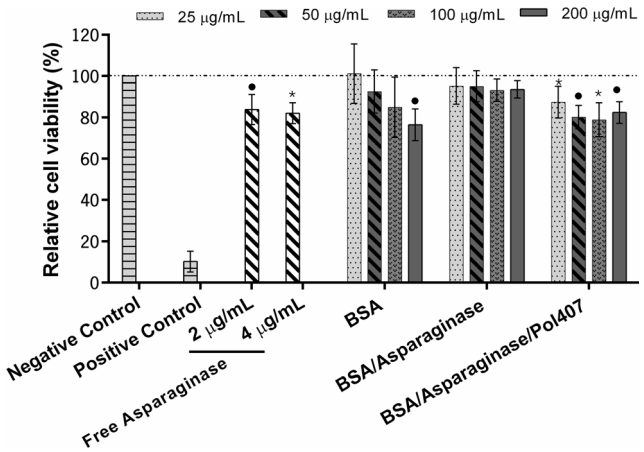

Fig. 4 Relative viability of RAW 264.7 (macrophage cell line from leukemic mouse) evaluated with the MTT assay, after 24-h incubation in medium containing BSA, BSA/asparaginase, and BSA/asparaginase/Pol_{407} capsules and two concentrations of free asparaginase. The free asparaginase concentrations correspond to the amount of enzyme present in the highest capsules' concentrations. Cells incubated with culture medium were used as negative control and cells incubated with 30 % DMSO as positive control of cytotoxicity. Data were determined in relation to the life control. Results are the mean ± SD of triplicate of three independent experiments. Statistical significant differences from the control are indicated as follows: p value ≤0.05 (*asterisk*), p value ≤0.01 (*circle*)

The BSA-based capsules were characterized regarding size, polydispersity, and net charge. The more negative *Z*-potential of BSA/asparaginase/Pol_{407} capsules after 2 months could be due to a rearrangement of Poloxamer 407, leading to desorption of some surfactant molecules from the capsules' surface. This slight variation on the *Z*-potential after storage, from −49 to −45 mv, was not significant to consider a destabilization of the particles. The desorption of Poloxamer 407 will expose more asparaginase and BSA molecules on the capsules' surface, making the *Z*-potential more negative.

As observed on Table 2, the BSA/asparaginase capsules presented a more negative surface charge when compared with the BSA capsules due to the addition of asparaginase to the formulation. Asparaginase has an isoelectric point of 4.9 (Ehrman et al. 1971) and is deprotonated at pH 7.4, acquiring a net negative charge. The more negative *Z*-potential could be justified by the addition of asparaginase to the formulation and its localization at the surface of the capsules.

The addition of Poloxamer to the BSA/asparaginase formulation affected both capsules' *Z*-potential and polydispersity, resulting in a more homogeneous population. The less negative net charge results from the coating of the BSA/asparaginase capsules by the nonionic surfactant, which shields' the surface charge.

In order to prove the capsules' capacity to retain ammonia, an HPLC-MS method for the quantification of asparagine and aspartic acid was optimized. The best results were obtained using Hydro-C18 column and, as a mobile phase, acetonitrile and ammonium bicarbonate, pH 6.0 adjusted with formic acid. Hydro-C18 columns achieved the best separation of the peaks, probably due to their polar endcapped C18 phase that provides extreme retention of hydrophobic compounds and shows slight polar selectivity. The ammonium bicarbonate and the pH were chosen based on the pKa of the two amino acids. At pH 6.0, asparagine and aspartic acid have different electrical charge which helped to separate the two components during the chromatography (Fig. S1).

The main goal of the present work was the development of formulation with the ability to hydrolyze asparagine while

retaining the forming ammonia. To evaluate the ability of the BSA/asparaginase-based capsules, the amount of forming ammonia and aspartic was quantified and compared.

Ammonia retention on the capsules' surface results from the electrostatic interaction between the positively charged ammonia and the superficial negatively charged groups of the capsules. If the capsules' ability to retain ammonia was only related to electrostatic interactions, more negative formulations such as BSA/asparaginase will retain ammonia more efficiently. Nevertheless, the formulation that retained more ammonia was the BSA/asparaginase/Pol_{407} capsule.

The free asparaginase also retains some ammonia, although it was not significant when compared with the amount of ammonia retained by the capsules. The capacity of free asparaginase to retain ammonia is related to the protonation state of asparaginase. At pH 7.4, asparaginase is deprotonated (Baran et al. 2002), and thus, it has some capacity to retain the positively charged ammonia.

The simultaneous quantification of aspartic acid and ammonia confirmed the capsules' interfacial capacity to retain the forming ammonia. However, it seems that the BSA/asparaginase capsules' ability to retain ammonia was not only due to electrostatic interaction between the negative charges on the capsules' interface and the ammonia but could be also related with the entrapment of ammonia in the surface of the capsules.

To determine the cytotoxic effect of the BSA-based capsules, RAW 264.7 cell line was used as a model. The RAW 264.7 is a monocyte macrophage cell line obtained from murine leukemia. This cell line was chosen because it is a cell from the immune system and has the capacity to synthesize asparagine as they express the asparagine synthetase enzyme (Suzuki et al. 2002). Any signs of cytotoxicity will be due to the composition of the capsules and not to the anti-leukemic activity of asparaginase.

Despite that the RAW 264.7 cell line is able to produce its asparagine, the free asparaginase had some cytotoxicity. Besides the hydrolytic activity over asparagine, asparaginase also presents some glutaminase activity which could affect cell viability at some extent.

Although some marginal cytotoxicity, though not relevant, was noted, studies indicate that BSA/Pol_{407} particles are safe to use even in vivo with animals not developing any adverse reaction or losing weight or compromising survival (Loureiro et al. 2015a).

Generally, the developed BSA/asparaginase capsules did not induce significant cytotoxicity in the tested cell line.

In the present work, a new HPLC-MS method for the quantification of aspartic acid without a derivatization step was successfully developed. Higher protective effect of BSA for asparaginase was achieved in a proportion of 98 % BSA/2 % asparaginase. This proportion was selected for the capsules' preparation by ultrasounds.

The new asparaginase immobilization systems were stable over time and showed a higher capacity to retain ammonia than the free asparaginase. This capacity seems not only related with the capsules' net charge but also with an entrapment of ammonia in the capsules' interface. Moreover, all the developed BSA-based capsules did not display significant cytotoxicity to RAW 264.7 cell line. In the future, the capsules' anti-leukemic potential combined with their ability to retain ammonia will be evaluated.

The interfacial capacity of the BSA-asparaginase-based capsules, here presented, to retain ammonia, is of particular interest. The developed systems have the potential to be used as new therapeutic formulations for the treatment of ALL as they maintain the asparaginase activity while retaining ammonia and thus avoiding hyperammonemia.

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Compliance with ethical standards

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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