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Chemical Functionalization in Bionanoparticles

julho de 2015

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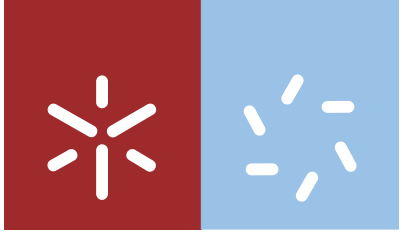

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Chemical Functionalization in Bionanoparticles

Dissertação de Mestrado
Mestrado em Biofísica e Bionanossistemas

Trabalho efetuado sob a orientação do
Professor Doutor Artur Cavaco-Paulo
e coorientação do
Doutor Artur Jorge Araújo Magalhães Ribeiro

julho de 2015

DECLARAÇÃO

Nome: Ana Catarina Malheiro Tinoco

Endereço eletrónico: rina.tinoco@gmail.com Telefone: 915313882/253676538

Bilhete de Identidade/Cartão do Cidadão: 14142648

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Orientador:

Professor Doutor Artur Cavaco-Paulo

Co-orientador:

Doutor Artur Jorge Araújo Magalhães Ribeiro

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Abstract

Asparaginase is an enzyme that has been extensively used for the treatment of acute lymphoblastic leukemia (ALL) and other lymphoproliferative malignancies. This enzyme catalyzes the hydrolysis of asparagine into aspartic acid and ammonia, resulting in leukemic cancer cells death. One of the side-effects of asparaginase therapy is hyperammonemia that is caused by an abrupt elevation of ammonia levels in plasma after asparaginase therapy and can lead to cerebral edema, herniation, coma, and even death.

The main objective of the work was the development of asparaginase immobilized nanoparticles with the capacity to retain at surface the free ammonia, reducing the levels of circulating ammonia and avoiding hyperammonemia.

The BSA particles were produced by two high-energy emulsification methods, ultrasounds and high pressure homogenization. In terms of physical characteristics, the best nanoparticles were the ones prepared by homogenization, since the particles where in the nano-range (lower than 200 nm) and exhibit a lower PDI. Relatively to the activity, all the particles produced by ultrasounds almost lost the asparaginase activity after storage for two months at 4 °C. For this particles, two surfactants were tested, Poloxamer 407 and zein, in order to stabilize the particles. Generally with the surfactants, it was observed an improvement on asparaginase activity during the first month, still the enzyme activity decreased almost to zero after two months. All the particles prepared by homogenization, except the BSA:Asparaginase:Pol$_{407}$ D, also lost the activity after two months at 4 °C. The BSA:Asparaginase:Pol$_{407}$ D were the only particles that stabilize the enzyme and retain 87 % of the initial activity two months after immobilization.

The development of a new HPLC-MS method to quantify the asparagine and aspartic acid together with the ammonia quantification by Nesslerization, confirmed the ability of the particles with asparaginase to retain the ammonia resulting from the asparaginase activity. This capacity to retain ammonia could be used to avoid hyperammonemia during the treatment of ALL.

The cytotoxic effect of the particles prepared by ultrasounds and tested regarding their ability to retain ammonia, was evaluated using the mouse leukemic macrophage cell line RAW 264.7. The particles which displayed more cytotoxicity to the cells were the ones with zein on the formulation; while the BSA:Asparaginase B particles did not show any cytotoxic effect with a cellular viability around 93 % for all tested concentrations. Generally, the cells incubated with the particles had cellular viability always superior to 73 %.

Resumo

A asparaginase é uma enzima que tem sido vastamente utilizada para o tratamento da Leucemia Linfoblástica Aguda (ALL) e outras doenças linfoproliferativas. Esta enzima catalisa a hidrólise da asparagina em ácido aspártico e amónia, resultando na morte das células leucémicas. Um dos efeitos colaterais desta terapia é a hiperamonemia, causada por uma elevação abrupta dos níveis de amónia no plasma sanguíneo, podendo resultar em edemas cerebrais, coma ou até morte.

O principal objetivo deste trabalho foi o desenvolvimento de nanopartículas onde a asparaginase pudesse ser imobilizada e com a capacidade de reter a amónia livre, reduzindo assim os níveis de amónia no plasma e evitando a hiperamonemia.

As partículas de BSA foram produzidas por dois métodos de alta-energia, os ultrassons e a homogeneização de alta-pressão. Em termos de características físicas, as melhores partículas foram as preparadas por homogeneização, uma vez que apresentavam um tamanho inferior a 200 nm e exibiam um baixo PDI. Relativamente à atividade, todas as partículas preparadas por ultrassons apresentavam uma atividade da asparaginase muito baixa ou nula após dois meses a 4 °C. De modo a estabilizar estas partículas, foram testados dois surfactantes, Poloxamer 407 e zeína. Globalmente, a adição de surfactantes melhorou a atividade da asparaginase durante o primeiro mês, dimunuindo quase até zero após dois meses. Todas as nanopartículas preparadas por homogeneização, exceto as BSA:Asparaginase:Pol407 D, também perderam a atividade após dois meses a 4 °C. As nanopartículas BSA:Asparaginase:Pol407 D demostraram ser as únicas partículas capazes de estabilizar a enzima, retendo 87 % da atividade inicial mesmo dois meses após imobilização.

O desenvolvimento do método de HPLC-MS para quantificar simultaneamente a asparagina e ácido aspártico, em conjunto com a quantificação de amónia pelo método de Nessler, confirmaram a capacidade das partículas com asparaginase em reter a amónia resultante da atividade desta enzima. Esta capacidade para reter a amónia livre pode ser utilizada para evitar a hiperamonemia durante o tratamento da ALL.

A citotoxicidade das partículas preparadas por ultrassons, as mesmas testadas relativamente à sua capacidade em reter amónia, foi avaliada com a linha celular de macrófagos de ratinho RAW 264.7. As partículas que demostraram uma maior citotoxicidade foram aquelas que incluíam zeina na sua formulação; as partículas BSA:Asapraginase B não demonstraram qualquer citotoxicidade, apresentando uma viabilidade celular superior a 93 % para todas as concentrações testadas. De um modo geral, as células incubadas com todas as partículas apresentaram uma viabilidade celular superior a 73 %.

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LIST OF ABREVIATIONS

[S]	Initial concentration of the substrate,
ALL	Acute Lymphoblastic Leukemia
BSA	Bovine Serum Albumin
CLEA	Cross-Linked Enzyme Aggregates
DOC	Deoxycholate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
FBS	Fetal Bovine Serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC-MS	High Performance Liquid Chromatography–Mass Spectrometry
K_M	Michaelis-Menten constant
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight mass spectroscopy.
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
PBS	Phosphate-buffered saline
PEG	Polyethylene Glycol
Pol407	Poloxamer 407
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TCA	Trichloroacetic Acid
TEMED	Tetramethylethylenediamine
TFA	Trifloroacetic acid
v	steady-state rate of the enzymatic reaction,
V_{max}	limiting value of v at saturating concentrations of the substrate

Chapter 1: Introduction

1.1. Enzymes Immobilization

By definition, enzymes are protein molecules that have the capacity to catalyze chemical and metabolic reactions important to sustain life, showing a high specificity for those processes. These biological catalysts have the potential to work in mild reactions conditions as neutral pH, atmospheric pressure and near room temperature. However, enzymes can be very expensive duo to the high cost of acquisition and purification, instability of their structures once they are isolated from their natural environment and their sensitivity both for process conditions and trace levels of some substances that can act as inhibitors. Enzymes present some main characteristics like its basic function is to increase the rate of a reaction, they act specifically with only one substrate converting it into products and some enzymes can be regulated from a low activity state to a high activity state and vice-versa (Mateo *et al.*, 2007; Nisha *et al.* , 2012; Krajewska, 2004).

Recently, numerous efforts have been devoted to the development of insoluble immobilize enzymes that present many advantages for a variety of applications, comparing with their soluble counterparts. For example, as they can be easily reused multiple times for the same reaction with longer half-lives and less degradation, immobilized enzymes can reduce the productions costs. They can also be used as stable and reusable devices for analytical and medical applications, as selective adsorbents for purification of molecules, and as effective micro-devices for controlled release of proteins drugs. Thereby, the immobilization techniques provided a straightforward method of controlling reaction rate as well as reaction start and stop time (Spahn and Minteer, 2008; Cao, 2006; Ansari and Husain, 2012; Sheldon *et al.*, 2005).

Enzyme immobilization is the process of confining the enzyme molecules to a phase different from the one that substrates and products are included, generally using inert polymers and inorganic materials as carrier matrices (Datta *et al.*, 2012). To a successful approach, these matrices have to present some important features as inertness, physical strength, stability and ability to increase enzyme specificity/activity. They also have to reduce product's inhibition, nonspecific adsorption and microbial contamination (Datta *et al.*, 2012). If properly designed, immobilization can be a powerful tool to improve almost all enzyme properties like stability, activity, specificity, selectivity, reduction of reaction products' inhibition and protection against autolysis (Mateo *et al.*, 2007; Ansari and Husain, 2012).

In this way, immobilized enzymes may also exhibit much better functional properties than the corresponding soluble enzymes by very simple immobilization protocols (Mateo *et al*, 2007).

1.1.1. Enzymes Immobilization Techniques

Basically, three traditional methods of enzyme immobilization can be distinguished: cross-linking, binding to a support (carrier) and entrapment or encapsulation (Sheldon, 2007). Although the basic methods of enzyme immobilization can be categorized only into a few different methods, hundreds of variations have been developed that take in consideration combinations of these original methods, which can facilitate the design of robust immobilized enzymes for a variety of applications (Cao, 2006; Sheldon *et al.*, 2005). A disadvantage of the immobilization methods that take in account the formation of an enzyme-carrier conjugate is a decrease in enzymes catalytic activity, resulting from the introduction of a large proportion of non-catalytic mass (Sheldon *et al.*, 2005).

The stability of an immobilized enzyme can be influenced by many factors as the chemical and physical structure of the carrier, the conditions under which the enzyme is immobilized, the microenvironment in which the enzyme molecule is located, the properties of its interaction with the carrier, the binding position and the number of the bonds between the enzyme and the carrier (Spahn and Minteer, 2008). Moreover, the activity of the immobilized enzymes can be enhanced by the microenvironment effect, conformational change of the enzyme and flexibility of that conformational change, molecular orientation and binding mode (Cao, 2006; Krajewska, 2004).

1.1.1.1. Covalent Enzyme Immobilization

In general, covalent bonding of an enzyme to a carrier is based on a chemical reaction between the active amino groups located on the enzyme surface and a functional group that are attached to the carrier surface, or vice-versa. This type of immobilization, with a covalent bond between the enzyme and the carrier, provide the strongest linkages of all the immobilization methods, minimizing the leakage of enzyme from the matrix (Cao, 2006; Divya *et al.*, 1998; Nisha *et al.*, 2012).

The performance of the enzyme-carrier conjugate can be affected by the physical and chemical nature of the carrier, the conformation of the enzyme when immobilized, enzyme orientation and the number of bonds formed between the enzyme and the carrier. These properties can cause the freezing of the enzyme conformation due to the multipoint attachment and due to the fact that this kind of bond is irreversible (Cao, 2006).

Multipoint covalent attachment of enzymes on highly activated pre-existing supports via short spacer arms and involved many residues of the enzymes surface promotes a rigidification of the structure

of the immobilized enzyme (Figure 1). This rigidification can increase enzyme stability since it should reduce any conformational change involved in enzyme inactivation by heat, organic solvent and extreme pH (Mateo *et al.*, 2002). A support suitable for protein multipoint immobilization should present a high superficial density of reactive groups that interact with groups frequently placed in the enzyme surface, so it is possible to achieve a strong multipoint covalent attachment. It also needs stable reactive groups that permit long enzyme-support reaction periods and a final inert surface after immobilization, by destroying or blocking the remaining reactive groups in the support without affecting the enzyme (Mateo *et al.*, 2000; Mateo *et al*, 2007; Cowan and Wood, 1995).

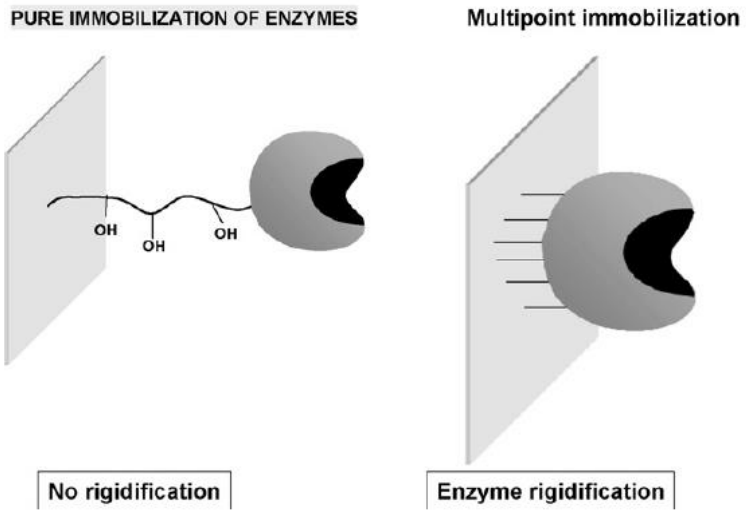

Figure 1 – Effect of multipoint immobilization on enzymes stability via short spacer arms (adapted from Mateo *et al.*, 2007).

1.1.1.2. Enzyme Immobilization based on adsorption

The adsorption method (Figure 2) involves the enzyme being physically adsorbed on the surface of a carrier matrix, often a polymer matrix (Spahn and Minteer, 2008). The enzyme immobilization via non-covalent bonding can be divided into hydrophobic adsorption between regions of the enzyme and the carrier; electrostatic adsorption that is based on the charge-charge interaction among the carrier and the enzymes; and non-specific physical adsorption which include Van der Waals forces, hydrogen bonds and hydrophilic interaction (Sheldon, 2007; Nisha *et al.*, 2012).

Adsorption is one of the simplest methods of physical immobilization of enzymes with the added advantage of being inexpensive and mild to the enzyme. Others advantages of this technique is its

reversibility, which enables not only the purification of proteins but also the reuse of the carriers, and the possible high retention of activity since there is no chemical modification in the enzyme (Diaz and Balkus, 1996). However, the immobilized enzymes prepared by adsorption tend to leak from the carriers, owing to the relatively weak interaction between the enzyme and the carrier, which can be destroyed by desorption forces such as high ionic strength and pH (Cao, 2006; Pierre *et al.*, 2006).

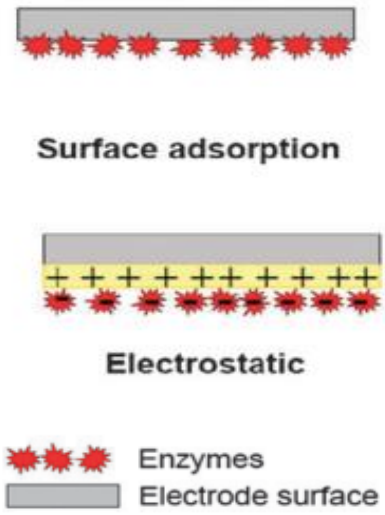

Figure 2 – Enzyme immobilization by surface adsorption and electrostatic interaction (adapted from Ronkainen, 2010).

1.1.1.3. Immobilization by Enzyme Entrapment

Enzyme entrapment refers to the confinement of the enzymes in a synthetic or natural polymeric networks formed by chemical or physical means, such as cross-linking or gelation, respectively (Figure 3). It can be achieved by gel or fiber entrapping and microencapsulation and these networks allow the retention of the enzyme inside the network but, simultaneously, the free movement of the substrates and the products, since it is a permeable membrane for those compounds (Cao, 2006; Nisha *et al.*, 2012).

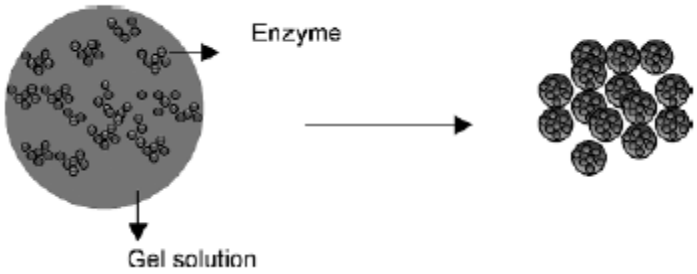

Figure 3 – Enzyme entrapment on a gel solution (adapted from Cao, 2006).

1.1.1.4. Immobilization by Enzyme Encapsulation

Immobilization of enzymes by encapsulation is the method that incorporates the enzyme molecules within spherical semipermeable membranes, as liposomes, polymeric particles and microemulsion droplets. These membranes have a selective controlled permeability, since the enzyme molecules are physically confined to the interior of the membrane but the substrates and products are able to diffuse freely across the membrane if the their pore size is bigger than the size of both compounds (Figure 4). There are many factors that have the capacity to affect the activity of the encapsulate enzymes, for example the thickness of the membrane, the pore size, the processes used to form this conjugate and the properties of the enzyme (Cao, 2006; Nisha *et al.*, 2012; Betancor and Luckarift, 2008).

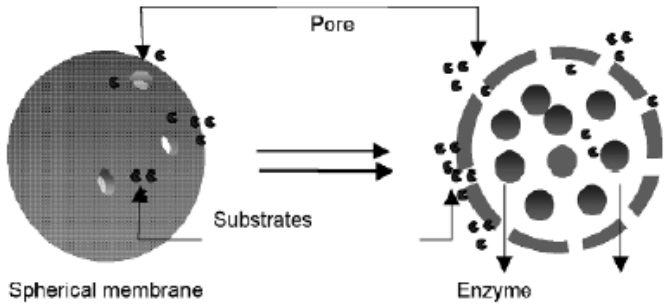

Figure 4 – Enzyme encapsulation and free movement of substrates and products across the membrane pores (adapted from Cao, 2006).

1.1.1.5. Cross-linking Enzyme Immobilization

Cross-linking enzyme immobilization is a carrier-free method and results in the formation of aggregates connected by intermolecular linkages, being these aggregates composed by a covalent linkage between the enzymes molecules and bi-functional reagents with low molar mass. These bi-functional reagents has the ability to react rapidly with some groups located at the surface of the enzyme, as carboxyl and amino groups (Fernandes *et al.*, 2010; Sheldon, 2007).

Generally, the precipitation of proteins as solid aggregates is possible by the simple addition of some compounds to the aqueous solutions of proteins, as salts, organic solvents or non-ionic polymers. These solid aggregates of proteins or enzymes are stabilized by non-covalent bonding, without perturbation of their tertiary structure that is, without denaturation. After the precipitation, it is possible to

remove the aggregates from the solution where the enzymes precipitate and dissolve them in a new aqueous solution, purifying the enzymes. Considering this precipitation process, the pre-organized structure and catalytic activity of the aggregates can be maintained by cross-linking of these solid aggregates, making them permanently insoluble. These technique leads to the development of a new family of immobilized enzymes called CLEA: cross-linked enzyme aggregates (Figure 5) (Sheldon, 2007; Mateo *et al.,* 2007; Cao *et al.*, 2003).

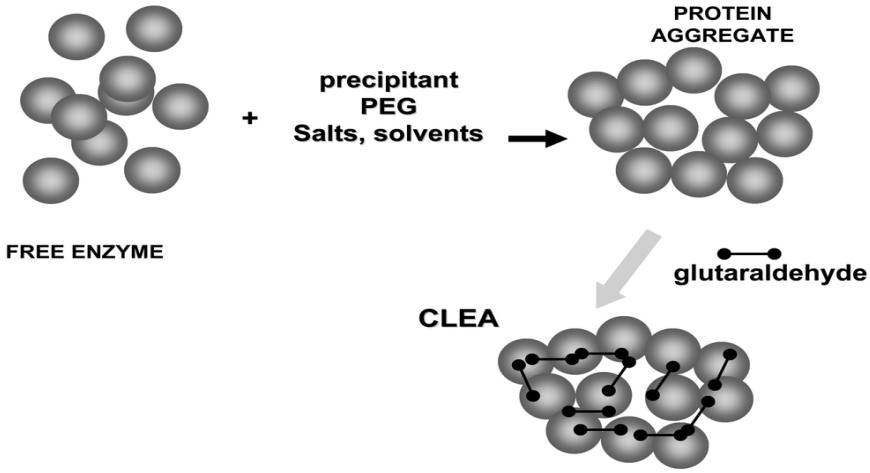

Figure 5 – Formation of a solid aggregate of enzymes by cross-linking immobilization (adapted from Barbosa *et al.,* 2014).

Sometimes, cross-linking between the subunits of an enzyme also occurs, playing a critical role in multimeric proteins stabilization, as it prevents protein dissociation and helping on the reassociation of its subunits (Cao *et al.,* 2000). There is another family of immobilized enzymes called CLECs (cross-linked enzyme crystals) which are significantly more stable to denaturation by heat, organic solvents and proteolysis than the corresponding soluble enzyme, allowing reactions at higher temperatures and in aqueous organic solvent mixtures. Comparing to immobilized or soluble enzymes, CLECs also have a higher activity per unit volume. Their operational stability, controllable particle size and ease of recycling, coupled with their high catalyst activities, made them ideally suited for industrial bio-transformations. Despite these attractive features, a disadvantage of CLECs is the need to crystallize the enzyme, an arduous procedure which require the enzyme in a high purity stage (Sheldon, 2007; Roy and Abraham, 2004; Govardhan, 1999).

1.2. Protein PEGylation

PEGylation refers to the modification of a peptide, protein or non-peptide molecule by the covalent attachment of one or more polyethylene glycol (PEG) chains. The interest for this procedure has been rising since it allows the enhancement of the therapeutic and biotechnological potential of the compounds attached to PEG. When the PEG chain is properly linked to the protein, it can modify several of its characteristics while the enzymatic activity and the receptor recognition are maintained (Veronese, 2001; Veronese and Pasut, 2005).

1.2.1. PEG characteristics

PEG is synthesized by ring opening polymerization of ethylene oxide using methanol or water as initiator, to yield methoxy–PEG (mPEG–OH) or diol-PEG (HO–PEG–OH), respectively (Pasut and Veronese, 2007). Many studies report that the conjugated proteins have unchanged secondary and tertiary structures, although is normal to verify a reduction in enzyme activity after PEGylation due to the steric interference of polymer chains during the biological processes (Pasut and Veronese, 2012).

PEG has some key properties as great flexibility due to the absence of bulky substituents along the chain as well as the high hydration of the polymeric backbone. PEG also have high solubility in water and in many organic solvents, did not present toxicity to the cells and is approved by FDA for human use. When conjugated with proteins, PEG improves the conjugates bioavailability and their plasma half-life owing to the increased hydrodynamic volume that reduces the kidney clearance; it protects the proteins against degrading enzymes and prevents or reduce protein aggregation, immunogenicity and antigenicity (Harris and Chess, 2003; Pasut and Veronese, 2012). The reduction of immunogenicity is attributed to the shielding effect of polymeric chains around the protein since it prevents proteins interaction with degrading enzymes or antibodies (Pasut and Veronese, 2007; Pasut *et al.,* 2004). Generally, the factors that affect the properties of the conjugate are structure, molecular weight, number of PEG chains attached to the polypeptide, the location of PEG linkages on the protein and the method used to make the PEG-polypeptide conjugation (Roberts *et al.*, 2002).

1.3. Asparaginase

In 1953, Kidd noted the remarkable observation that guinea pig serum had antileukemia activity in mice. But, just in 1961, Broome attributed the cause of this antileukemia activity to depletion of asparagine by the enzyme asparaginase (Rytting, 2012). Since 1970, asparaginase has been used for the treatment of malignant hematopoietic diseases in children as a single agent or in combination with other chemotherapy agents. Those diseases include, principally, pediatric acute lymphoblastic leukemia (ALL), acute myelocitic leukemia, lymphosarcoma treatment and non-Hodgkin lymphomas (Muller and Boos, 1998; El-Naggar *et al.*, 2014).

Although the successful treatment in the last years, a significant percentage of the ALL patients (10-25 %) relapse early, normally during the 3 years after the induction phase. In order to decrease this relapse, pharmacological changes have been made to try to improve the treatment of relapsed patients, however these changes have not been very successful basically due to acquired drug resistance (Avramis and Panosyan, 2005).

Asparaginase (EC 3.5.1.1) belongs to a group of homologous amidohydrolases family, characterized by catalyzing the hydrolysis reaction of the sidechain amide bond of asparagine, resulting in the formation of aspartate and ammonia (Figure 6) (Kumar and Sobha, 2012). The asparaginase reaction can be assayed by measuring the release of aspartate in a glutamate-oxaloacetate amidotransferase/malate dehydrogenase coupled enzymatic test and the release of ammonia in a simple Nessler test or by the disappearance of asparagine (Michalska and Jaskolski, 2006).

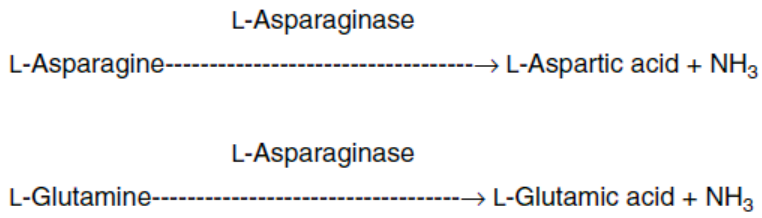

Figure 6 – Reaction of degradation of asparagine and glutamine, catalyzed by asparaginase (adapted from Fu and Sakamoto, 2007).

Heterologous enzymes are typically not suitable for human therapy, especially when a repeated or prolonged administration is required, once they are recognized as non-human by the human body and can result in adverse immunological reactions. Additionally, it is important that the therapeutic enzymes for cancer treatment present some pharmacological properties like: (i) must be highly specific to the

substrate in order to avoid any undesired side effects, (ii) should exhibit a stability and high catalytic activity under physiological conditions to afford a reasonable dosing regimen; and (iii) must be amenable to formulation at the administration dose without adverse effects such as aggregate formation or inactivation (Cantor *et al.*, 2012).

1.3.1. Preparations of Asparaginase available for therapy

The asparaginase is currently available in three different formulations: two are derived from *Escherichia coli* (native asparaginase and asparaginase conjugated with PEG) and one from *Erwinia chrysanthemi*. All three formulations share the same mechanism of action but have different pharmacokinetic properties (Table I), which do not make them easily switchable (Cantor *et al.*, 2012; Rizzari *et al.*, 2013).

Table I - Chemical and pharmacological properties of different asparaginase preparations

Source	Molecular weight (kDa)	K_M (M) ASN	K_M (M) GLN	Isoeletric point (pH)	Apparent $t\frac{1}{2}$ (days)
E. chrysanthemi	138	12×10^{-6}	1.10×10^{-3}	8.7	0.65±0.13
E. coli	141	10×10^{-6}	6.25×10^{-3}	5.0	1.24±0.17
PEG-*E. coli*	145	10×10^{-6}	--	5.0	5.73±3.24

Asparagine (ASN); Glutamine (GLN)
Data according to Muller and Boos, 1998 and Asselin *et al.*, 1993

Asselin *et al* (1993) found that the serum half-life of *E. coli* asparaginase activity was 1.24±0.17 days, approximately the double of the half-life of *E. chrysanthemi* activity (0.65±0.13 day). They also reported that the serum half-life of *E. coli* asparaginase activity increased to 5.73±3.24 days when the enzyme was conjugated with PEG. There are also differences on the time required to the serum asparaginase return to normal concentrations, being necessary 4 days for *E. chrysanthemi* asparaginase versus 11 days for *E. coli* asparaginase (Duval, 2002; Muller and Boos, 1998).

The antileukemic activity of asparaginase can be influenced by many factors. Some of them are biochemical factors like the rate of hydrolysis and the K_M of the enzyme for asparagine and glutamine, and others are pharmacological factors as serum clearance of the enzyme and tumor cell resistance to asparaginase. The antileukemic activity is also affected by the host immunological effects of anti-

asparaginase antibody formation, the contributions from the nutrient intake and the augmented asparagine 'input' from the *de novo* biosynthesis of asparagine by the liver (Avramis and Panosyan, 2005).

1.3.2. Asparaginase Types and Structure

Generally, bacteria produce two distinct asparaginases: type I and type II. Type I asparaginase is cytoplasmic, display high K_M values relatively to asparagine and show also glutaminase activity. Type II asparaginase is periplasmic, exhibits low K_M values against asparagine owing to the high affinity to this molecule and have low activity towards glutamine. Additionally, *E. coli* synthesizes a third type of asparaginase, asparaginase type III or EcAIII, that is classified as a plant-type asparaginase once this protein has a 70% amino acid sequence similarity to the asparaginase from plants, and belong to the Ntn-hydrolases family. Because type I has a much lower affinity for asparagine, only type II asparaginase has the anti-tumor properties and is used for the treatment of malignant hematopoietic diseases (Cappelletti *et al.*, 2008; Kumar and Sobha, 2012; Michalska and Jaskolski, 2006). Although both *E. coli* and *E. chrysanthemi* asparaginase II have glutaminase activities, it only represent 3-9% of the total asparaginase activity once the K_M value for glutamine is 100 times higher than that for asparagine (Aslanian and Kilberg, 2001).

The functional form of *E. coli* asparaginase II exists as a homotretramer with a molecular mass between 133 and 141 kDa and each monomer composed by 300-350 amino acids (326 according to Protein Data Bank) (Cappelletti *et al.*, 2008; Kozak *et al.*, 2002). This enzyme has a nearly ideal 222 symmetry and is composed of four identical subunits bound principally by non-covalent forces (Figure 7), where the subunit A is connected to the subunit C (dimer A/C) and the subunit B is connected to the subunit D (dimer B/D). The asparaginase tetramer has four active sites and can be classified as a dimer of dimers because the dimer A/C is linked to the dimer B/D and each interface of the intimate dimer has two active sites (Kozak *et al.*, 2002; Muller and Boos, 1998; Michalska and Jaskolski, 2006). Although the dimers contains all the functional groups and the structural elements to create a complete active site environment, the enzyme is only active and functional when the tetramer is complete by the approximation of the dimer A/C and the dimer B/D (Khushoo *et al.,* 2004; Fu and Sakamoto, 2007).

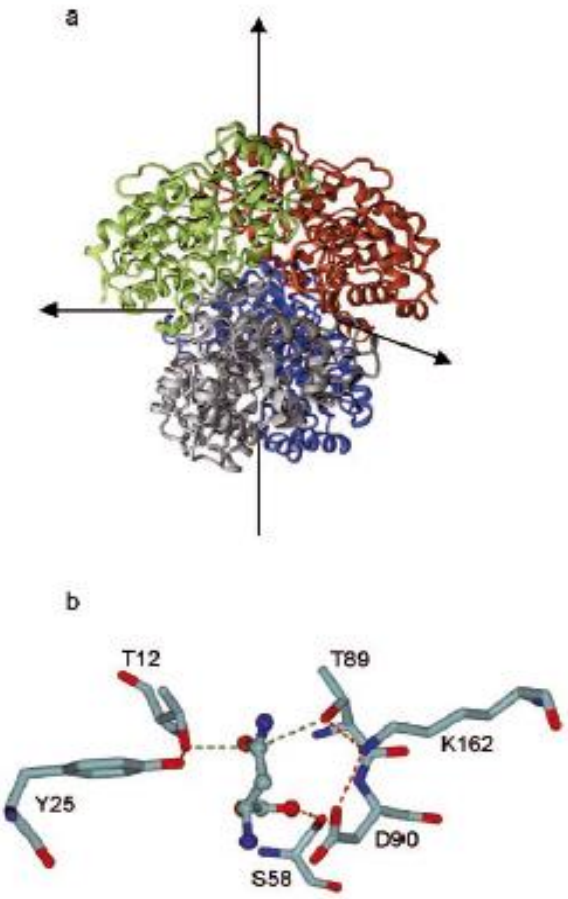

Figure 7 – Structural features of bacterial-type asparaginases. a) the quaternary structure of the periplasmatic *E. coli* asparaginase II. The green (subunit A) and red (subunit C) or gray (subunit B) and blue (subunit D) monomers form the intimate dimer. The arrows correspond to the two-fold axes defining the 222 symmetry of this homotetramer; b) the active site of *E. coli* asparaginase II with an asparagine substrate molecule modeled according to the complex enzyme-substrate. The dotted red lines represent hydrogen bonds while the green ones symbolize a potential line of attack of the active-site Thr residues on the substrate's amide C atom (adapted from Michalska and Jaskolski, 2006).

1.3.3. Acute Lymphoblastic Leukemia

Leukemia is a malignant cancer of the blood and bone marrow, is a cancer of white blood cells, the cells that normally fight infections. This disease is characterized by an uncontrolled increase and excessive multiplications of malignant and immature lymphoblast in bone marrow, which alters the normal blood cells function and, in many instances, can lead to death (Kwan *et al.*, 2009; Jain *et al.*, 2012). Acute lymphoblastic leukemia (ALL) is seen in both children and adults, but is more common

between the ages of 2 and 5 years. The origin of ALL is considered to be multi-factorial, including endogenous or exogenous exposures, genetic predisposition, and fortuitous (Hiroto *et al.*, 2013). There are different types of treatment to ALL and they include steroids, chemotherapy, radiation therapy and intensive combined treatments including steam cell or bone marrow transplants. Despite the variety of drugs available today, their efficacy in treatment of cancers is doubtful and the side effects caused by these chemotherapeutics agents are really aggressive, which can include infertility, secondary neoplasm, nausea and immunosuppression (Jain *et al.*, 2012). Among the antitumor drugs described previously, there is another chemotherapeutic agent in pediatric oncotherapy especially for ALL that is the bacterial enzyme asparaginase. This enzyme has been employed as the most effective chemotherapeutic agent in pediatric ALL and improved the survival rate of pediatric ALL to approximately 90 % in recent trials (El-Naggar *et al.*, 2014).

1.3.4. Asparaginase Mechanism of Action

Asparagine is an amino acid required for the synthesis of protein molecules and survival of the cells. This amino acid can be synthesized within the healthy cell by an enzyme called asparagine synthetase, so it is not considered an essential amino acid in normal cells. This enzyme catalysis the conversion of aspartic acid and glutamine into asparagine and glutamic acid (Figure 8), where glutamine is the donor of amino group and aspartate is transaminated to asparagine in an ATP-dependent reaction. On the other hand, asparagine can also be absorbed from the diet, as soon as it is retained into the body and made available to the cells (Cantor *et al.*, 2012; El-Naggar *et al.*, 2014; Fu and Sakamoto, 2007).

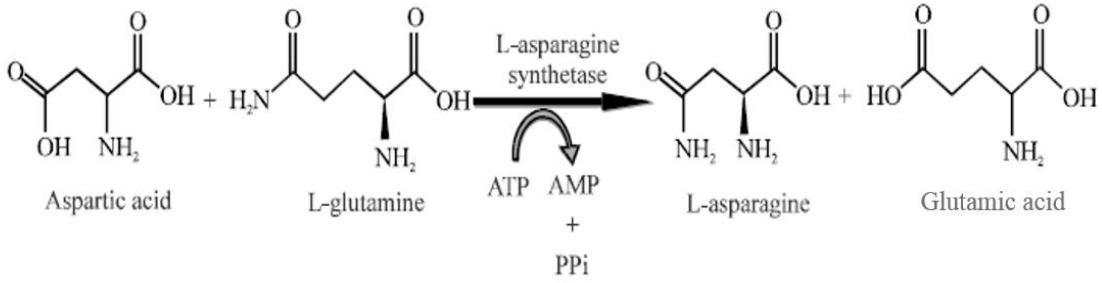

Figure 8 – Biosynthesis of asparagine by asparagine synthetase (adapted from *El-Naggar et al.*, 2014).

However, the availability of asparaginase is different on tumor cells. These cells cannot synthesize sufficient asparagine due to the absence or very low expression of asparagine synthetase and due to the

inability of tumor cells to increase asparagine synthetase activity after asparaginase administration. Thus, the malignant cells are dependent on serum levels of asparagine for their proliferation and survival. In order to keep their rapid malignant proliferation, tumor cells use both extracellular asparagine and intracellular synthesized asparagine, which is limited to satisfy their large asparagine demand (Savitri *et al.*, 2003; Kumar and Sobha, 2012; Wang *et al.*, 2003).

Therefore, asparagine is an essential amino acid for the growth of tumor cells, whereas the growth of normal cells is independent on its requirement since this amino acid can be synthesized by their own enzyme asparagine synthetase, in sufficient quantities for their metabolic needs (El-Naggar *et al.*, 2014; Wetzler *et al.*, 2007). In this way, administration of asparaginase deprives tumor cells of their extracellular source of asparagine which leads to tumor cells death. The death of tumor cells happens because they are dependent of blood serum asparaginase and without it, cells are unable to complete protein synthesis (Figure 9) (Cantor *et al.*, 2012).

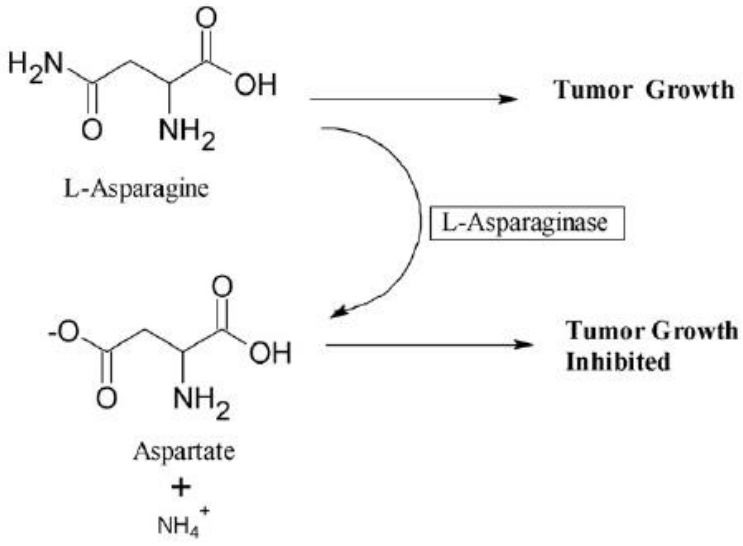

Figure 9 – Schematic representation of asparagine mechanism of action (adapted from Narta *et al.*, 2007).

Some mechanisms of clinical relevance responsible for the tumor cells death are the arrestment of cell cycle in G1 phase, the induction of apoptosis and the cytotoxicity caused by the depletion of asparagine. There are a lot of studies on the mechanisms of action and the occurrence of resistance phenomena to the treatment with asparaginase. This studies demonstrate that a treatment response may only be expected if the malignant cells are unable to increase their expression of asparagine synthetase to an extent that can provide enough asparagine for the cell survival. If the tumor cell respond to the

treatment with an increase on the synthesis of asparagine, it will be observed a decrease in sensitivity of this cells to asparaginase (Kumar and Sobha, 2012; Muller and Boos, 1998).

1.3.5. Toxicity of Asparaginase to Normal Cells

Despite the distinctive mechanism of action of asparaginase, which shows relative selectivity against the malignant cells and not against the normal cells, some patients experience some negative side effects during asparaginase therapy (El-Naggar *et al.*, 2014).

The immunogenicity of exogenous proteins rises with increasing molecular weight and higher complexity of the structure. So proteins like asparaginase are highly immunogenic, since they are composed by a large number of subunits and they have complex quaternary structure. Clinical experience has shown that the application of the asparaginase leads to the formation of specific antibodies to this molecule in many patients (Cantor *et al.*, 2012; Fu and Sakamoto, 2007; Muller and Boos, 1998). In this way, the efficiency of asparaginase has been compromised by a high rate of immunological reactions that can include enzyme inactivation without any clinical manifestation as well as anaphylactic shock; rapid clearance of the enzyme from the blood stream; shortening the enzyme half-life and development of anti-asparaginase antibodies, that neutralize asparaginase activity (El-Naggar *et al.*, 2014; Fu and Sakamoto, 2007). Patients with anti-asparaginase specific IgG antibodies do not have sufficient active enzyme for the depletion of asparagine until a therapeutic effect is verified and, in some cases, asparaginase activity may be undetectable. It is clear that there will be a loss of the therapeutic effect if the enzyme is inactivated and levels of asparagine are inadequate (Rizzari *et al.*, 2013; Wang *et al.*, 2003).

In case of toxicities arising from inhibited proteins, normal tissues with high rates of protein synthesis like pancreas, liver and coagulation systems are the most affected by this therapy, resulting those effects from an impairment of the protein synthesis (El-Naggar *et al.*, 2014; Muller and Boos, 1998). The changes of the coagulation system related to asparaginase manifest themselves in a decrease of several coagulation factors and coagulation inhibiting proteins. The changes affecting the proteins of the coagulation system have a significant clinical impact since they can induce bleeding as well as enhance the probability of thromboembolic events. The occurrence of thrombotic complications in patients who were carry out the treatment with asparaginase varies from less than 10 % to greater than 30 %, with the incidence of thrombus formation increased with the increasing of the age population (Cantor *et al.*, 2012). Other toxic effects related to protein inhibition include abnormalities on lipid metabolism and on

hemostasis, hypoalbuminemia, hyperglycemia due to a decrease in serum insulin and a decrease in lipoproteins, pancreatitis, and hepatoxicity (Fu and Sakamoto, 2007; Kumar and Sobha, 2012).

Additionally, the secondary effects may be associated with life-threatening complications when the central nervous system is involved (Muller and Boos, 1998). An impairment of central nervous system functions is demonstrated as convulsions, irritability, tension, depression, confusion, visions, disorientation, somnolence or even coma and this symptoms are principally due to hyperammonemia (Fu and Sakamoto, 2007; Muller and Boos, 1998).

Hypersensitivity reactions to asparaginase preparations are not rare and the risk to have a severe allergic reaction depend on prior exposures to the enzyme and concomitant medications, for example with corticosteroids. The signs and symptoms of clinical hypersensitivity include rash, urticaria, laryngeal edema, hypotension, bronchospasm and occasionally anaphylactic shock (Cantor *et al.*, 2012; Rizzari *et al.*, 2013). This allergic reactions become more prominent and occur in approximately 5–10 % of patients after repeat exposures to asparaginase and while patients are not receiving corticosteroids. Once patients demonstrate signs of clinical hypersensitivity to this enzyme, they could not continue the same treatment. Although, there are available asparaginases from different bacterial strains that present only limited cross reactivity, which make possible the treatment continuation by selecting an enzyme from a different biological source. The continuation of the treatment also can be possible by using the enzyme linked to PEG (Muller and Boos, 1998; Rizzari *et al.*, 2013).

PEG-asparaginase show some advantages comparatively to asparaginase. Although PEG-asparaginase is less immunogenic than the enzyme alone, it still causes severe allergic reactions in patients who get repeated doses of PEG-asparaginase during treatment. However, patients who have allergic reactions to asparaginase or PEG-asparaginase may continue the treatment with the asparaginase from *E. chrysanthemi*, since antibodies to PEG-asparaginase and asparaginase from *E.coli* do not cross-react with *E. chrysanthemi* asparaginase (Cantor *et al.*, 2012; El-Naggar *et al.*, 2014).

Anti-asparaginase antibodies are the main cause of resistance to the treatment with asparaginase. Asparaginase resistance can be symptomatic when the patients exhibit signs of clinical hypersensitivity, or asymptomatic without any signs. Asymptomatic resistance, also called silent inactivation, is more dangerous than clinical hypersensitivity, as there are no clinical signs of allergy to alert against asparaginase resistance. *In vitro* studies have demonstrated an adaptive mechanism by human leukemia cells that are resistant to asparaginase treatment, where they increase their expression of asparagine synthetase in order to survive against the therapy (Aslanian and Kilberg, 2001; Rizzari *et al.*, 2013).

1.3.5.1. Hyperammonemia

Since 1986, it is known that the treatment with asparaginase can result in hyperammonemia encephalopathy in acute lymphoblastic leukemia, as this enzyme catalysis the hydrolysis of asparagine into aspartate and ammonia (Jörck *et al.*, 2011; Nott *et al.*, 2007; Paulides *et al.*, 2013). This effect is more pronounce when PEG-asparaginase is used in the treatment, because his prolonged half-life does not allow to the ammonia concentration to return to normal values before the next dose is administered (Heitink-Pollé *et al.*, 2013). It is assumed that the increase of ammonia directly corresponds with the activity of asparaginase because ammonia is one of the end products of the biochemical reaction catalyzed by this enzyme (Jörck *et al.*, 2011). The increase of plasma ammonia concentrations during asparaginase therapy may correlate to the effectiveness of asparaginase therapy. So hyperammonemia may be used as a parameter to monitor the enzyme activity of asparaginase, and a lack of ammonia elevation may reflect relative inactivity of this enzyme (Jörck *et al.*, 2011; Nott *et al.*, 2007).

Hyperammonemic encephalopathy is an uncommon but frequently fatal complication following intensive chemotherapy 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 (Lyles *et al.*, 2011; Nott *et al.*, 2007). Hyperammonemia was defined as ammonia concentrations higher than 50 μmol/L and clinical significant hyperammonemia as ammonia concentrations higher than 100 μmol/L (Chen *et al.*, 2010; Heitink-Pollé et al., 2013). Clinical signs of hyperammonemia can be manifested by several of central nervous system symptoms and appearance of these findings is generally proportional to free ammonia concentration. This symptoms include headache, anorexia, irritability, lethargy, dizziness, vomiting, somnolence, disorientation, cerebral edema, coma, and even death if the increase in blood ammonia is not interrupted (Cohn and Roth, 2004; Heitink-Pollé *et al.*, 2013; Nussbaum *et al.*, 2014; Paulides *et al.*, 2013).

During asparaginase treatment, hyperammonemia is probably caused by a combination of hepatotoxic effects of chemotherapeutics, apoptosis of cancer cells resulting in protein catabolism, and additional ammonia release due to application of asparaginase (Cohn and Roth, 2004; Jörck *et al.*, 2011). Patients presenting with acute hyperammonemia are at risk for treatment delay, cerebral edema, herniation, coma, and even death (Nussbaum *et al.*, 2014).

1.4. Protein as a Surfactant Molecule

Almost emulsion-based products in pharmaceutical industries are manufactured by an important process of protein adsorption at oil-water interfaces. Studies over the past years have shown that the properties of proteins adsorbed at oil-water interfaces play a crucial role on the physicochemical properties of emulsions, their characteristics and their stability (Zhai *et al*, 2013).

An emulsion is a heterogeneous, metastable system, comprising a mixture of two liquids that are immiscible, where one of the liquid is dispersed in the other in the form of droplets of colloidal size. It is common to call the liquid in the droplets the dispersed, internal, or discontinuous phase and the surrounding phase the external or continuous phase. The emulsion is stable when the oil-water interface is covered with amphiphilic molecules that are supposed to ensure the kinetic barrier, which prevents the decomposition of the emulsion into separated water and oil (Dimitrova *et al.*, 2004; Hui, 2006).

Proteins are amphiphilic molecules that exhibits a double affinity since they simultaneously exhibit some polar and apolar properties. The polar group of the amphiphilic molecule is constituted by heteroatoms such as oxygen, sulfur, phosphate or azote, and exhibits a strong affinity for polar solvents, particularly water. The apolar group is normally a hydrocarbon chain and exhibit a strong affinity for apolar solvents. The polar portion is called hydrophile or lipophobe while the apolar part is called hydrophobe or lipophile (Salager, 2002).

Many proteins have the ability to promote the formation, increase the stability and produce desirable physicochemical properties in oil-water emulsions. When two immiscible liquids interact, there is a development of "interfacial tension" on the region of contact between the two liquids and, as the area of contact increases, the stability of the emulsion decreases (Hui, 2006; McClements, 2004). To stabilize emulsions is essential to add surfactants or proteins that act like surfactants, in order to decrease the interfacial tension between lipids and water. When the surfactants are added to a system constituted by oil and water, the surfactants rapidly migrate to the interfaces between the two immiscible liquids and orientate the apolar region to the lipid phase and the polar region to the aqueous phase. The surface of the internal phase starts to be covered with the surfactants molecules that are responsible for the phase separation and for the system stabilization against re-coalescence and flocculation (Patino *et al.*, 2003; Zhai *et al.*, 2013; Hui, 2006). Once the droplet is coated by thousands of surfactants molecules, the interfacial tension between the two phases is markedly reduced, which can make the emulsion stable for long periods of time. The droplets storage stability against flocculation and coalescence is also affected by the ability of proteins to generate repulsive interactions (steric and electrostatic) between oil droplets

and to form a protective membrane around the droplets that is resistant to rupture (Hui, 2006; McClements, 2004).

In an aqueous phase, a protein is surrounded in every sides by water molecules, however, at an oil–water interface, it is surrounded by oil molecules on one side and water molecules on the other side. Normally, proteins keep a strongly packed structure, although, when adsorbed to an oil–water interface, the proteins are submitted to changes in its conformation and interactions due to an alteration in its molecular environment. They lose the secondary and higher structure in order to maximize the number of favorable interactions and minimize the number of unfavorable interactions in their new environment. This conformation state, where the configurational free energy of proteins is minimized, makes the structure of adsorbed molecules very different from their native structure in solution. The unfolding exposes the various segments of adsorbed species and facilitates the lateral interaction between two or more adsorbed proteins. After unfolding and orientation to the correct phase, proteins organize in multiple layers on the oil-water interface due to hydrogen, hydrophobic and intermolecular ionic bonding between unfolded protein strands (McClements, 2004; Dimitrova *et al.*, 2004; Zhai *et al.*, 2013; Hui, 2006)(Hui, 2006). The time necessary for these conformational changes to happen its determined by the molecular flexibility and packing of the adsorbed molecules. Moderately flexible proteins such as casein undergo relatively rapid conformational alterations, while more rigid globular proteins like bovine serum albumin (BSA) needs more time (McClements, 2004).

Emulsions formed by proteins are more stable than those formed by low molecular weight surfactants because proteins are the only that, in addition to lowering interfacial tension, can form a membrane-like viscoelastic film around oil droplets via non-covalent interactions and disulfide cross-linking (Damodaran and Razumovsky, 1998).

Surfactants are surface-active molecules that generally compete with proteins for the available droplet surface and thus can displace some or all of the protein from the interface. When a surfactant is bound to a protein, it may stabilize or destabilize the protein structure and it may also promote or prevent protein aggregation, depending on surfactant type, concentration and environmental conditions. In this way, the molecular characteristics of the proteins may be altered, which may change the capacity of proteins to adsorb at the droplet surface and to stabilize emulsions. So it is important to improve the understanding about the nature and origin of protein–surfactant interactions and their influence on the capacity of protein to act as surfactants (McClements, 2004).

1.4.1. Zein as a surfactant

Zein is the main seed storage group of proteins in maize seeds. It is synthesized by polyribosomes bounded to membranes and transported into the lumen of the endoplasmic reticulum on developing endosperm. There, they self-associate due to their hydrophobic properties to form protein bodies, which are stably retained in membrane vesicles (Argos *et al.*, 1982; Lending *et al.*, 1988; Deo *et al.*, 2003). Zein is classified as a prolamin because this group of proteins is rich in proline and glutamine and they are only soluble in strong alcohol solutions. Zein proteins constitute 44–79% of the endosperm protein, depending on the corn variety and separation method used (Lawton, 2002; Matsushima *et al.*, 1997).

Biologically, zein is a combination of proteins varying in solubility and molecular size (Lawton, 2002). They are essentially insoluble in water even at low concentrations of salt and need a high percentage of ethanol in aqueous solutions to maintain the folded conformation. The solubility and chemical reactivity of zein are dependent on the presence of certain functional groups as amides, amines, phenols, hydroxyls and carboxylates. The zein insolubility in water is due to the presence of hydrocarbon groups in zein's side chains. However, zein is soluble in mixtures of water with aliphatic alcohols like ethanol and isopropanol, and also in other organic solvents containing polar groups as carbonyls, hydroxyls and amines (Corradini *et al.*, 2014**;** Deo *et al.*, 2003). According to their solubility in alcoholic solutions in the presence or absence of reducing agents like [3-mercaptoethanol], zein proteins can be divided into four distinct types: α, β, γ, δ.

The α-zeins account for more than 70 % of the total prolamins in maize and are composed by two groups of 19 kDa (Z19) and 22 kDa (Z22) size classes (Matsushima *et al.*, 1997; Lawton, 2002). The amino acid composition analyses of α-zeins show a large amount of hydrophobic residues such as proline, leucine, phenylalanine and alanine, so this group of proteins display highly hydrophobic properties (Matsushima *et al.*, 1997).

Many physico-chemical studies have been made to investigate the structure of zein proteins in solution. Circular dichroism and optical rotatory dispersion experiments of all the zein species on a solution of 70 % methanol determined a α-helical content of 50–60 % and turn or random coil configurations comprising the remaining structure (Argos *et al.*, 1982; Matsushima *et al.,* 1997). The helical wheel model for the zein proteins is composed by nine homologous repeating units, organized in an adjacent and antiparallel form, where helices are collected within a distorted cylinder of oval cross-section with the helical and cylindrical axes lined up (Figure 10a)). The edges of the helical cylinder could be occupied by polar glutamine residues which largely comprise the turn spans between helices. Polar

and hydrophobic residues appropriately distributed along the helical surfaces allow intra- and intermolecular hydrogen bonds and Van der Waals interactions among neighboring helices. This allows the aggregation of rod-shaped zein molecules in molecular planes which would then stack through glutamine interactions at the cylindrical caps (Figure 10b)) (Argos *et al.*, 1982; Shukla and Cheryan, 2001; Liu *et al.*, 2005; Corradini *et al.*, 2014).

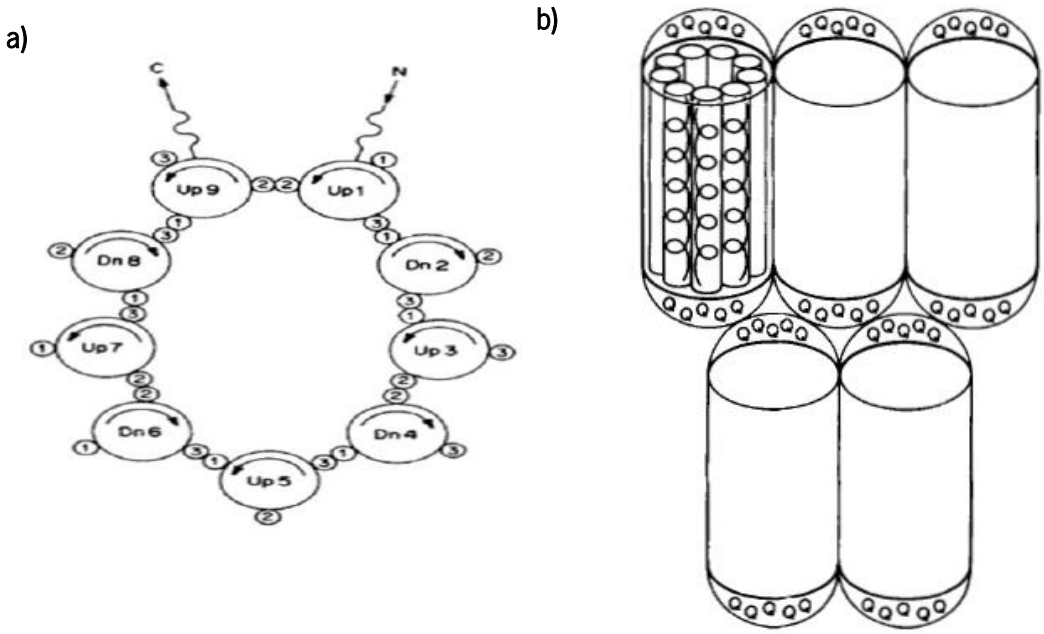

Figure 10 – a) A possible nine-helical zein protein structural model for α-zeins b) a possible model for arrangement of zein proteins within a plane as well for the stacking of molecular planes (adapted from Argos *et al.*, 1982).

On other hand, the analysis of alcohol solutions of zein using small angle X-ray suggested that α-zein adopts a structural model of an elongated rectangular prism shape with the length of 13 nm. This model suggests that the antiparallel helices of the tandem repeat units, each of one formed by a single α-helix joined by loops rich in glutamine, linearly stack in the direction perpendicular to the helical axis (Figure 11). The sides of the cylinder correspond to the surfaces of hydrophobic helices, while the upper and lower surfaces are covered by glutamine bridges, which are hydrophilic (Corradini *et al.*, 2014).

The capacity of zein and its resins to form coatings with properties likes resistance, brightness, smoothness, hydrophobic grease-proof and resistant to microbial attack led to an increase in the study of this proteins as well as an increase in zein commercial interest. Plasticized-zein, zein blended with other moieties, film-forming ability of zein and zein-containing materials have good mechanical properties, very important characteristics to the different applications of this protein (Corradini *et al.*, 2014). Potential

applications of zein include use as biodegradable plastics, fibers, adhesives, coating agents, ceramics, inks, cosmetics, textiles and chewing gum (Shukla and Cheryan, 2001). Zein had also been used in the preparation of microspheres to delay release of drugs until they reach to the intestine, for the protection of the medicines from stomach acid and to provide a mechanism capable to release constantly the drug in the stream (Dong *et al.*, 2004).

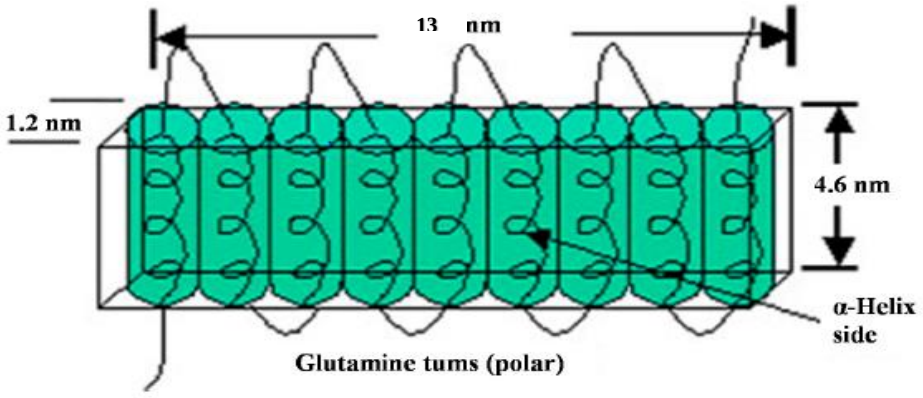

Figure 11 – a) A possible structural model for α-zeins (Z22), where the helical segments are aligned to form a 13 nm long asymmetric cylindrical structure. The sides of the cylinder correspond to the surfaces of hydrophobic helices, while the upper and lower surfaces are connected by glutamine bridges, which are hydrophilic (adapted from (Corradini *et al.*, 2014)).

Chapter 2: Motivation and Objectives

2. Motivation and Objectives

Asparaginase is an enzyme that has been extensively used for the treatment of acute lymphoblastic leukemia and other lymphoproliferative malignancies, for more than 40 years. This enzyme is an amidohydrolase that catalyzes the hydrolysis of asparagine into aspartic acid and ammonia, which leads to the death of leukemic cancer cells that are unable to synthetize the non-essential amino acid asparagine. Without extracellular asparagine, the cancer cells are unable to complete the protein synthesis, culminating with tumor cell death. The major adverse effect of asparaginase therapy, is immunological and allergic reactions, pancreatitis, liver cytolysis, cerebral hemorrhage, thrombosis and hyperammonemia. Hyperammonemia is caused by an abrupt elevation of ammonia levels in plasma after asparaginase therapy and can lead to treatment delay, cerebral edema, herniation, coma, and even death.

The main objective of the work was the development of asparaginase immobilized nanoparticles with the capacity to retain at surface the free ammonia. Thus reducing the levels of circulating ammonia and avoiding hyperammonemia.

To achieve this goal, initially it was necessary to study the behavior of asparaginase at different concentrations of substrate and enzyme, determining the kinetic properties of V_{max} and $K_{M.}$ Two different temperatures were studied 37 °C (normal body temperature) and 40 °C (typical of acute inflammatory states). This assay had the objective to determine if an increase on body temperature will lead to an alteration of the asparaginase activity during treatment.

Relatively to the synthesis of nanoparticles, the aim of this thesis was the elaboration of a nanoparticle formulation with the capacity to preserve the asparaginase activity for a long period of time and to retain the ammonia, resulting from the enzyme activity, at the surface of the nanoparticles. That formulation should be stable in terms of size, polydispersity index and Z-potential during the storage, at 4 °C, and should be non-toxic for cells.

Another goal was the development and optimization of a method by HPLC-MS, avoiding the amino acid derivatization process, in order to quantify simultaneously the asparagine and aspartic acid. Using this approach was possible to compare the concentrations of both products (aspartic acid and ammonia), produced by the asparaginase immobilized on nanoparticles, and determine if the developed nanoparticles were good systems to retain the ammonia at the nanoparticles surface by electrostatic interactions.

Chapter 3: Materials and Methods

Materials

Table II – Reagents used in the work.

Reagents and abbreviations	Company
Asparagine	Sigma®
Aspartic Acid	Sigma®
Asparaginase	Changzhou Qianhong Bio-Pharma Co., LTA
Bovine Serum Albumin (BSA)	Sigma-Aldrich®
Trichloroacetic Acid (TCA)	PanReacQuímica
Ammonium Sulfate	Sigma®
Nessler's Reagent	Sigma-Aldrich®
Trizma base	Sigma-Aldrich®
Ammonium Persulfate	Sigma®
Bromophenol Blue	Sigma®
Glycerol 99 %	Sigma®
Glycine	Fisher Scientific
Tetramethylethylenediamine (TEMED)	Sigma®
Acrylamide/Bisacrylamide (30 %/0.8 % (w/v))	Bio-Rad
Coomassie Blue	Merk
Dithiothreitol (DTT)	Sigma®
Poloxamer 407	Sigma®
Formic acid	Aldrich®
Acetonitrile	Sigma®
Sodium Dodecyl Sulfate	Sigma-Aldrich®
Ammonium Bicarbonate	Sigma®
Ethanol Absolute	PanReac AppliChem
Zein	Sigma®
Methanol	Fisher Scientific
Acetic Acid glacial	Chem-Lab
Lowry Reagent	Sigma®
Folin Reagent	Sigma®
Deoxycholate (DOC)	Sigma®

Phenylmethylsulforyl Fluoride (PMSF)	Amresco®
Plus Protein All Blue Standard	BIO-RAD
Vegetable oil	Pingo Doce
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)	Sigma-Aldrich®
Dimethyl Dulphoxide (DMSO)	Fisher Scientific
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich®
Glutamine	Sigma-Aldrich®
Hepes	Sigma-Aldrich®
Fetal Bovine Serum (FBS)	Biochrom
Sodium Pyruvate	Sigma-Aldrich®

Table III – Equipment used in the work.

Equipment	Company
Homogenizer	SPX® APV2000
Ultra Sounds	Sonics® Vibra-Cell™
Lyophilizer	Labconco®, FreeZone 2.5
Incubation Bath	Grant OLS 200
ZetaSizer	Malver Instruments, Nano-ZS
Microplate Reader	BioTek®, Synergi MX
Mass spectrometer	Finnigan LXQ
HPLC system	Finnigan Surveyor – Thermo Electron Corporation
HPLC Columns	• Synergi Hydro-RP 80Å, size - 150 x 4.60mm 4μ, Phenomenex® • Kinetex™ 2.6μm C18 100Å, LC Column 100 x 4.6mm, Phenomenex®
Dip cooler	Ru-200 Techne
Centrifuge	• Beckman Coulter, Alegra® X-15R • Centurion Scientific, K3 system
Electrophorese System	BioRad

Methods

3.1. Determination of the purity degree of asparaginase

The purity degree of asparaginase was determined in a 15 % SDS-PAGE. Three concentrations of asparaginase (5, 10 and 15 μg) and the molecular marker Precision Plus Protein All Blue Standard were loaded to the SDS-PAGE gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue and then destained until the background disappear. The destained gel was then analyzed with an image processing program, ImajeJ, in order to compare the intensities of bands of the molecular marker with a known amount of protein with the band corresponding to the monomer of the enzyme. Comparing the intensities of both bands, it was possible to determine a relative intensity per μg of protein and calculate the purity degree of enzyme.

The purity degree of asparagine was also assessed by MALDI–TOF. The samples were dissolved on 30:70 acetonitrile/TFA mixture with 0.1 % TFA final concentration in water. The matrix used was sinapic acid in a satured solution prepared with 30:70 acetonitrile/TFA mixture. MALDI-TOF mass spectra were acquired on a Bruker Autoflex instrument (Bruker Daltonics GmbH) equipped with a 337 nm nitrogen laser. Samples were spotted onto a ground steel target plate (Bruker part n° 209519) and analyzed in the linear mode using factory-configured instrument parameters suitable for a 0-2kDa m/z range: source 19,5kV, extraction 18,3kV and reflector 1,891kV. Time delay between laser pulse and ion extraction was set to 80 ns, and the laser frequency was 25Hz. Laser power was adjusted to result in a strong analyte signal minimal matrix interference and usually did not exceed 30 μJ. The experiments were performed in linear negative mode.

3.2. Enzymatic Assay of Asparaginase

The reaction mixture was performed using several concentrations of asparagine and asparaginase (Table IV), to a final volume of 200 μL in 50 mM Tris buffer, pH 8.6. Typically, the reaction was carried out at 37 and 40 °C for 30 min and stopped by addition of 50 μL of 20 % TCA. In another set of reactions the temperature was kept at 37 °C and the incubation time increased to 240 min (Cappelletti *et al.*, 2008; Stecher *et al.*, 1999). When samples were prepared for HPLC-MS analysis, the Tris buffer was substitute by ultra-pure water and the reaction was stopped by cold for 5 min.

Table IV – Asparaginase and Asparagine concentrations for the enzymatic assay.

Tested [Asparaginase] (mg/mL)	Tested [Asparagine] (mM)
0.0005	5
0.0010	10
0.0025	20
0.005	30
0.010	40
0.025	50
0.050	75
	100
	150
	200

3.2.1. Ammonia quantification by Nesslerization

Ammonia quantification was determined by a stopped assay using Nessler's reagent. Before the couple with Nessler's reagent, the reaction mixtures were centrifuged during 2 min for 1000 rpm. The ammonia produced in 10 µL of the supernatant was determined by the addition of 990 µL Nessler's reagent. The optical density of the solution was read at 436 nm and the ammonia concentration was determined on the basis of a standard curve previously obtained with ammonium sulfate as a standard. One unit (U) is the amount of enzyme catalyzing the production of 1 µmol of ammonia under the conditions of the assay (Cappelletti *et al.*, 2008; Stecher *et al.*, 1999).

3.2.2. Asparagine and Aspartic Acid quantification by HPLC-MS without derivatization

Mass spectrometer method

Mass spectrometer analysis was performed on Finnigan LXQ in a positive ionization mode. The asparagine and aspartic acid solutions were prepared in ultra-pure water with 20 % of acetonitrile and then filtered with 0.2 µm filters. High flow source conditions were optimized for each amino acid with a flow rate of 5 uL/min. As both amino acids have similar structure, the source conditions are the same. 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 25.01 V.

HPLC-MS method

A reverse-phase HPLC system linked with mass spectrometer was used to isolate and quantify asparagine and aspartic acid. During methods' optimization were tested two columns: the Kinetex™ C18 (Phenomenex®) and the Synergi Hydro-RP (Phenomenex®). After optimization it was selected the column Synergi Hydro-RP with ammonium bicarbonate 0.01M, pH adjusted to 6 with formic acid, and acetonitrile as elution solvents. A sample volume of 25 µL was injected with a flow rate of 0.3 mL/min for 20 min using the mobile phase gradient described on Table V. Calibration standard solutions containing the two amino acids at varied concentrations (0.1 mM – 15 mM for asparagine and 0.1 mM – 10 mM for aspartic acid) were prepared in ultra-pure water.

Table V - Mobile phase gradient for the HPLC-MS determination of asparagine and aspartic acid

Time (min)	Eluents	
	Ammonium Bicarbonate 0.01M, pH 6	Acetonitrile
0	95 %	5 %
8	95 %	5 %
15	40 %	60 %
20	95 %	5 %

3.3. Bovine Serum Albumin based Nanoparticles

3.3.1. Nanoparticles prepared by sonication

The BSA, BSA:Asparaginase and BSA:Asparaginase:Pol407 nanoparticles were prepared by sonication using Sonics® Vibra-Cell™. For BSA nanoparticles, BSA was dissolved in PBS, pH 7.4, at a final concentration of 10 mg/mL. For BSA:Asparaginase nanoparticles, both components were dissolved in PBS, pH 7.4, at a final concentration of 10 mg/mL with a percentage of enzyme of 2 % (w/w) and for BSA:Asparaginase:Pol407 nanoparticles, the Poloxamer 407 was added to the previous BSA:Asparaginase solution with a final concentration of 5 mg/mL. The aqueous phase was sonicated

with 5 % (v/v) of vegetable oil during 6 min with cycles of 10 s (8 s of ultrasounds pulses on plus 2 s of ultrasounds pulses off).

To prepare the BSA:Asparaginase and BSA:Asparaginase:Pol407 nanoparticles with zein, the maize protein was dissolved in a final volume of 100 μL of ethanol 70 % and added to the vegetable oil.

The enzyme was added to the formulation at different stages, before the sonication cycles or after 69 s of sonication. After synthesis, the phase with the nanoparticles was collected by centrifugation (1000 g during 45 min, at 4 °C) using centricon tubes (molecular weight cut-off of 100 kDa) (Amicon Ultra-15, Millipore). After separation, the free protein in the aqueous phase was quantified by Lowry method (Loureiro et al., 2014).

3.3.2. Nanoparticles prepared by homogenization

The preparation of BSA, BSA:Asparaginase and BSA:Asparaginase:Pol407 nanoparticles was achieved by emulsion using a high-pressure homogenizer. BSA and asparaginase were dissolved in PBS, pH 7.4, at a final concentration of 10 mg/mL with a percentage of enzyme of 2 % (w/w) and emulsified with 5 % (v/v) vegetable oil by subjecting the mixture to 26 cycles of high pressure homogenization (240 and 580 bar).

To prepare the PEGylated nanoparticles, was added Poloxamer 407 on a concentration of 5 mg/mL dissolved on PBS to the initial BSA solution. The enzyme was added to the formulation both, before the homogenization cycles or after 5 homogenization cycles. The nanoparticles were collected by centrifugation and the free protein in the aqueous phase quantified (Loureiro *et al.*, 2014).

3.3.3. Nanoparticle characterization

The nanoemulsions were dispersed in ultra-pure water and analyzed at 25 °C for their size distribution, charge and Z-potential. The parameters were determined by photon correlation spectroscopy, using dynamic light scattering. The values for viscosity and refractive index were taken as 0.8872 cP and 1.330, respectively. Each sample was measured in triplicate and results are presented as mean value ± standard deviation.

3.3.4. Particle formation efficiency

The particle formation efficiency was determined using the Lowry Method (Table VI). After the steps described on Table VI, the sample was quantitated spectrophotometrically at 750 nm, comparing the absorbance values with a BSA standard curve (Peterson, 1977).

Table VI – Steps for the execution of Lowry Method.

Step 1	Add 50 μL of 1.5 mg/mL DOC to 400 μL of sample/standard solutions
Step 2	Vortex during 10 s
Step 3	Add 50 μL of 72 % TCA to the sample
Step 4	Centrifuge at 2000 rpm for 10 min
Step 5	Discard the supernatant
Step 6	Add 400 μL of H_20 and 400 μL of Lowry reagent
Step 7	Vortex the sample for 20 s
Step 8	Add 200 μL of Folin reagent to the sample
Step 9	Vortex the sample immediately for 30 s
Step 10	Read the absorbance at 750 nm.

3.4. SDS-Denaturing Electrophorese

An SDS-PAGE was used to evaluate the purity of asparaginase and the integrity of the enzyme free on solution or immobilized on nanoparticles, during the storage time at 4 °C. The resolving and stacking gel were prepared as described on Table VII, according to the desired acrylamide concentration (10 and 15 %). Samples were prepared by mixing equals volumes of sample and loading buffer with β-mercaptoethanol. Samples were boiled for 10 min before loading. Electrophoresis was performed at a constant voltage of 80 V.

To determine the relative molecular weights of proteins bands, it was used the Precision Plus Protein All Blue Standard marker from BIO-RAD that contain ten sharp bands of the following molecular weights: 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kD.

Table VII - Composition of resolving and stacking gel in SDS-PAGE

	Stacking gel	Resolving Gel		
Acrylamide percentage	4 %	10 %	12.5 %	15 %
Acrylamide/Bis-acrylamide (30 %/0.8 %(w/v))	0.7 mL	3.3 mL	4.125 mL	4.95 mL
0.5 M Tris-HCl (pH 6.8)	1.3 mL	-	-	-
1.5 M Tris-HCl (pH 8.8)	-	2.5 mL	2.5 mL	2.5 mL
10 % SDS	50 µL	0.1 mL	0.1 mL	0.1 mL
Water	2.92 mL	4.045 mL	3.22 mL	2.395 mL
*10 % (w/v) AP	25 µL	50 µL	50 µL	50 µL
*TEMED	5 µL	5 µL	5 µL	5 µL

*added right before each use

3.4.1. Staining of the electrophoretic gel

After running, the gel was stained with Coomassie-Blue. The staining was made by incubating the gel with a solution of 0.125 % (w/v) Coomassie Brilliant Blue R-250, 50 % (v/v) methanol, 40 % of water and 10 % (v/v) acetic acid, during 1 hour. To destain the gel was soaked in 10 % acetic acid, 50 % methanol and 40 % water until the Coomassie background disappear.

3.5. Effect of BSA on asparaginase

To study the effect of BSA on the stability of the enzyme, it were prepared several solutions with different percentages of asparaginase and BSA dissolved in PBS, with a final concentration of 10 mg/mL. The solutions were stored at 4 °C, and the activity determined over time (Table VIII). To these solutions it was added antibiotic to inhibit the growth of microorganisms that had the capacity to alter the activity of enzyme. The activity of asparaginase was determined every week for 107 days. Samples were ran in 15% SDS-PAGE to verify the enzyme structure over time.

The activity of the enzyme was determined at 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.

Table VIII – Percentage of asparaginase and BSA solutions on PBS

Sample	BSA/Asparaginase ratio (%)
A	0/100
B	10/90
C	20/80
D	30/70
E	40/60
F	50/50
G	60/40
H	70/30
I	80/20
J	90/10
K	98/2
L	100/0

3.6. Cellular viability assay

3.6.1. Cell culture maintenance

RAW 264.7 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 two times per week. Cellular subcultures were performed when confluence reached values close to 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 5 min at 160 g. The supernatant was discard and 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.

3.6.2. Cell viability assessed by MTT assay

The MTT assay is a colo rimetric method used to measure the toxicity of different substances in cells. The principle of this assay is that in viable cells, mitochondrial activity is constant and therefore an

increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt (MTT) into formazan crystals. The amount of formazan reflects the reductive potential of the cytoplasm and therefore cell viability (Figure 12).

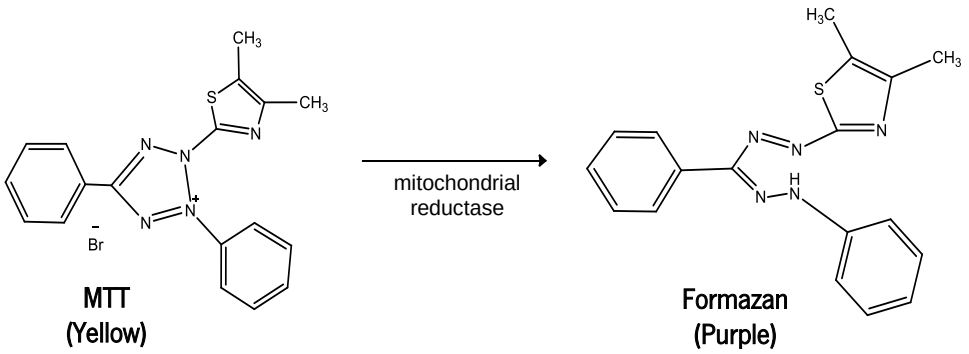

Figure 12 - Representation of the enzymatic conversion of MTT to formazan.

Cells were seeded at a density of 25000 cells/well on a 96-well tissue culture plate the day before the experiments. RAW cells were exposed to 4 concentrations (25, 50, 100 and 200 µg/mL) of nanoparticles with asparaginase and to 2 concentrations of free asparaginase (2 and 4 µg/mL). The two concentrations of free enzyme correspond to the amount of enzyme present on the highest concentrations of nanoparticles tested. Cells incubated with DMSO (30 % of the total volume) and cells without the addition of the compounds were used as controls; control of death and control of life 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 MTT viability assay. After incubation the medium with the particles and enzyme was removed and 110 µL of medium with MTT (5 mg/mL) was added to each well, and cells were 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).

Chapter 4: Results and Discussion

4.1. Kinetic study of free *E. coli* Asparaginase II

4.1.1. Study of Asparaginase II purity by SDS-PAGE and MALDI-TOFF

Purity and integrity of enzymes is very important to get accurate and reproducible sensitive results because the impurities may interfere with conditions of the assay and significantly affect the results. The first step of the work was to evaluate the Asparaginase II purity from Changzhou Qianhong Bio-Pharma Co., which could influence the enzyme activity.

A number of different techniques can be used to assess sample purity, depending on sample availability, required accuracy and sensitivity. The techniques used to determine the purity degree of asparaginase were Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectroscopy.

4.1.1.1. Study of purity by MALDI-TOF

Mass spectrometry is a good technique for determining the identity of protein because it provides an accurate direct measurement of protein mass over a wide mass range. MALDI-TOF technique allows to verify the presence of impurities as well as their mass, giving the possibility of identify the contaminants. It also allows the comparison of the measured mass of the enzyme with the predicted mass, confirming the identity of the enzyme.

Analyzing the spectrum obtained by MALDI-TOF (Figure 13) was possible to identify 5 major peaks, corresponding to compounds with mass-charge ratio (m/z) of 665.290, 7838.265, 11506.196, 17244.988 and 34513.132 Da. Considering the protein sequence of asparaginase monomer obtained from 'Protein Data Bank', the molecular weight calculated was 34593.9 Da. Thus the peak with the higher molecular weight (34513.132 Da) corresponds to the asparaginase monomer, showing a difference of 0.23 % from the theoretical value.

The peaks with lower molecular weight could be impurities of the sample or degradation products of the enzyme. For the impurities, the determination of its sequence could give additional information about the contaminants nature and how they may affect the enzyme activity, however this was not take in account during this study.

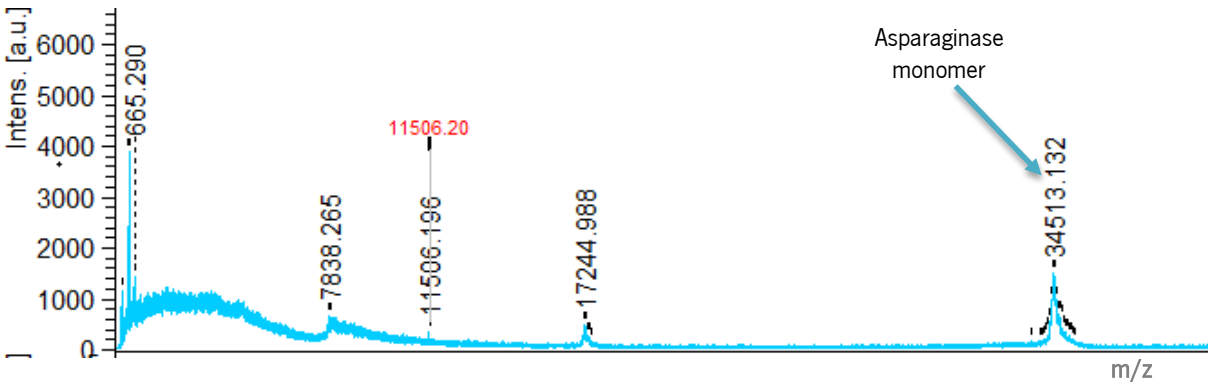

Figure 13 – MALDI-TOF spectrum of lyophilized asparaginase stored at 4 °C.

4.1.1.2. Study of purity by SDS-PAGE

SDS-PAGE staining with Coomassie Blue is a straightforward assessment of sample purity, as this technique is easy, rapid and inexpensive. To determine the purity of asparaginase, 3 samples corresponding to different enzyme concentrations were ran in a 15 % SDS-PAGE. Samples were boiled for 10 min and the disulfide linkages were reduced with β-mercaptoethanol to unfold the proteins' quaternary and tertiary structures.

To determine the purity of the enzyme with more accuracy, were applied three different concentration of asparaginase to the gel: 5, 10 and 15 μg (Figure 14). Analyzing the gel, it was possible to observe an intense band with a molecular weight approximately of 34 kDa corresponding to the enzyme monomer and other less intense bands with molecular weights bellow 34 kDa.

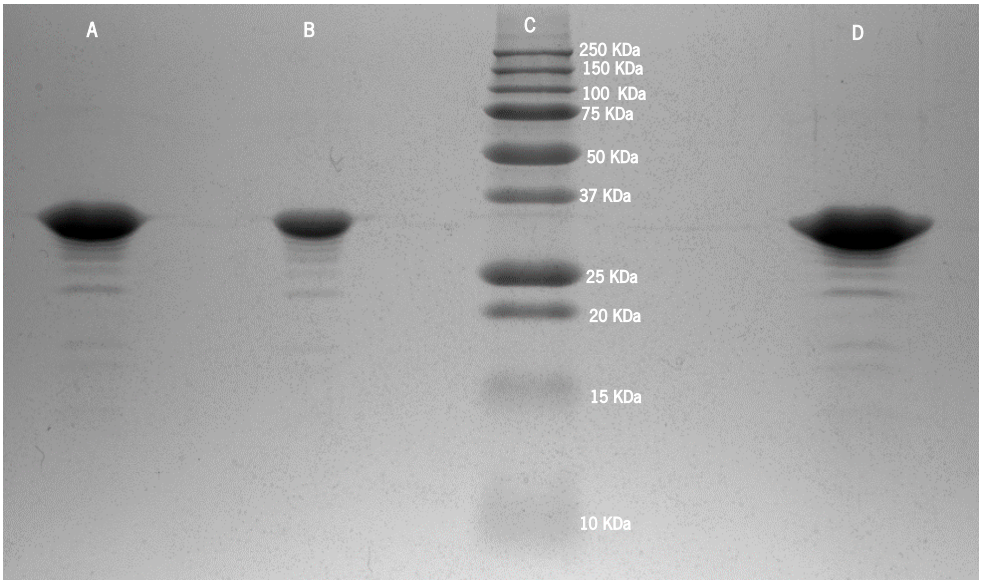

Figure 14 – Analysis of asparaginase purity. 15 % SDS-PAGE stained with Coomassie Blue. Lane A: 10 μg of asparaginase; Lane B: 5 μg of asparaginase; Lane C: Protein molecular weight marker; Lane D: 15 μg of asparaginase.

Different results regarding the purity of asparaginase were obtained with the two techniques. In the MALDI-TOF spectra any impurities were observed between the peak of enzymes' monomer (34513.132 Da) and the 17244.98 Da peak. Nevertheless, several weak bands with molecular weights between 25 and 34 kDa were detected in SDS-PAGE. Although the electrophoresis was performed in denaturing conditions (SDS and β-mercaptoethanol) the bands bellow 34 kDa may correspond to different conformations of asparaginase monomer, which alters the position of the protein during running and thus may not correspond to impurities. The gel was repeated several times to confirm the presence of the lower bands and as they continued to appear, they were considered as impurities.

The purity degree of asparaginase was determined using the image processing program, ImageJ. The intensity of the band corresponding to the enzyme was compared with the intensity of several bands of the protein molecular weight marker of known protein concentrations. Thus, comparing the intensity of the bands, the degree of asparaginase purity determined was around 96%.

4.1.2. Determination of asparaginase kinetic parameters

Asparaginase activity was determined by direct Nesslerization of ammonia produced by the enzymatic hydrolysis of asparagine by asparaginase. 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 (Figure 15).

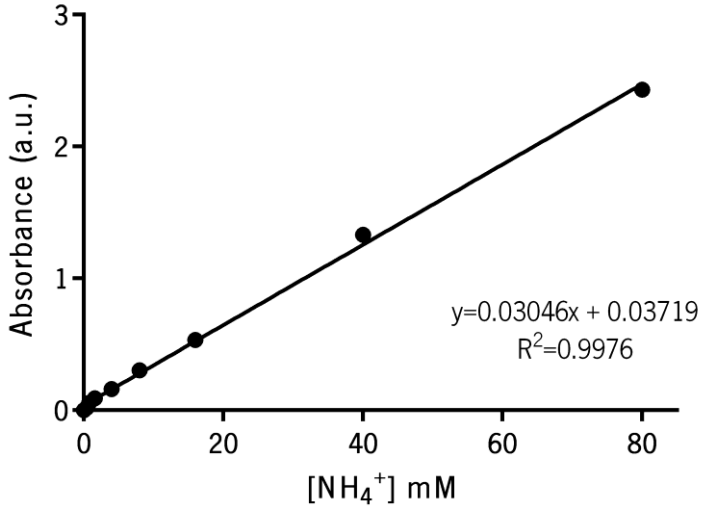

Figure 15 – Calibration curve prepared with ammonium sulfate for the determination of asparaginase activity. The absorbance of the solutions after Nesslerization were measured at 436 nm.

In order to explain how asparaginase can enhance the kinetic rate of the hydrolysis of asparagine and how reaction rates depends on enzyme and substrate concentration, the different activities were analyzed according to the Michaelis-Menten model (equation 1) and the kinetic parameters determined.

$$v = \frac{V_{max}\ [\mathrm{S}]}{K_M + [\mathrm{S}]}, \qquad \textbf{Equation 1}$$

where v is the steady-state rate of the enzymatic reaction, [S] is the initial concentration of the substrate, V_{max} is the limiting value of v at saturating concentrations of the substrate and K_M is the Michaelis-Menten constant.

For the determination of the kinetics parameters V_{max} and K_M, it was tested a concentration range of asparagine between 0-200 mM and five different concentrations of asparaginase: 0.001; 0.005; 0.010; 0.025 and 0.050 g/L. Two different temperatures were chosen, 37 and 40 °C that correspond to body normal temperature and temperature associated with inflammatory states, respectively. The kinetic parameters of asparaginase were determined using the Lineweaver-Burk equation (equation 2), a linear regression of Michaelis-Menten model.

$$\frac{1}{v} = \frac{K_M}{V_{max}[\mathrm{S}]} + \frac{1}{V_{max}} \qquad \textbf{Equation 2}$$

V_{max} and K_M are important values that characterize a particular enzyme-substrate system under specific conditions. V_{max} represents the maximum rate achieved by the system at saturating substrate concentrations. K_M represents the substrate concentration at half the maximum rate, namely the substrate concentration at which half the enzyme active sites are filled by substrate molecules. The Michaelis-Menten constant depends on temperature, the nature of substrate, ionic strength and other reactions conditions.

For the kinetics parameters determination, the absolute activity of asparaginase was plotted as μmol of ammonia produced per min in function of the substrate concentration. Figure 16 represents the Michaelis-Menten plot when asparaginase was incubated at 37 °C and Figure 17 when incubated at 40 °C.

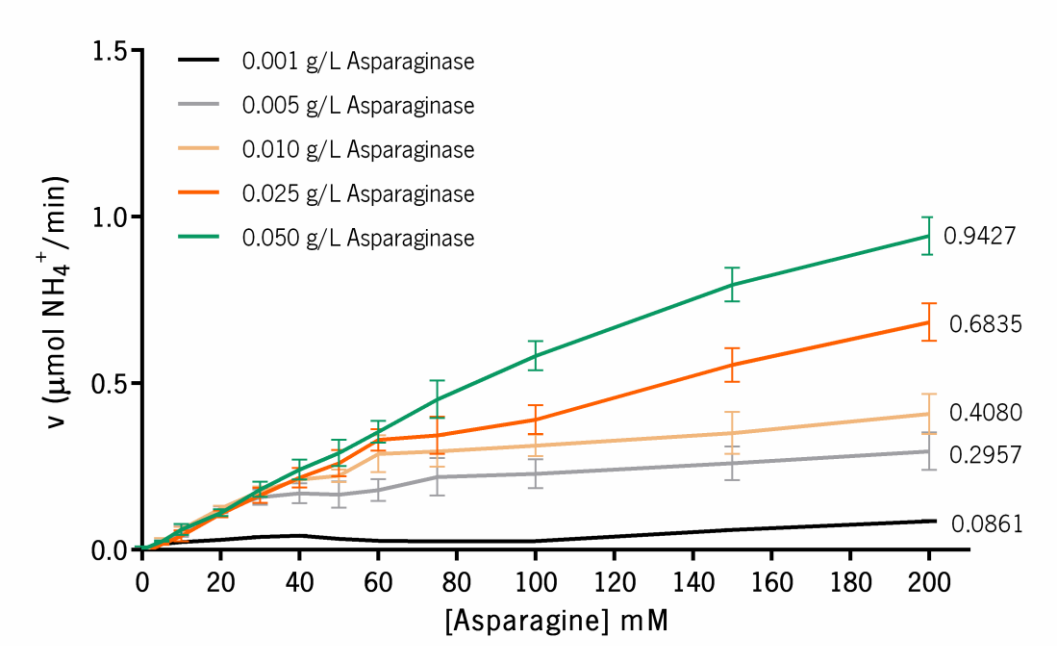

Figure 16 – Michaelis-Menten plot for different concentrations of asparagine (0.001; 0.005; 0.010; 0.025 and 0.050 g/L). The reaction was performed for 30 min at 37 °C with asparagine concentrations between 0 and 200 mM, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

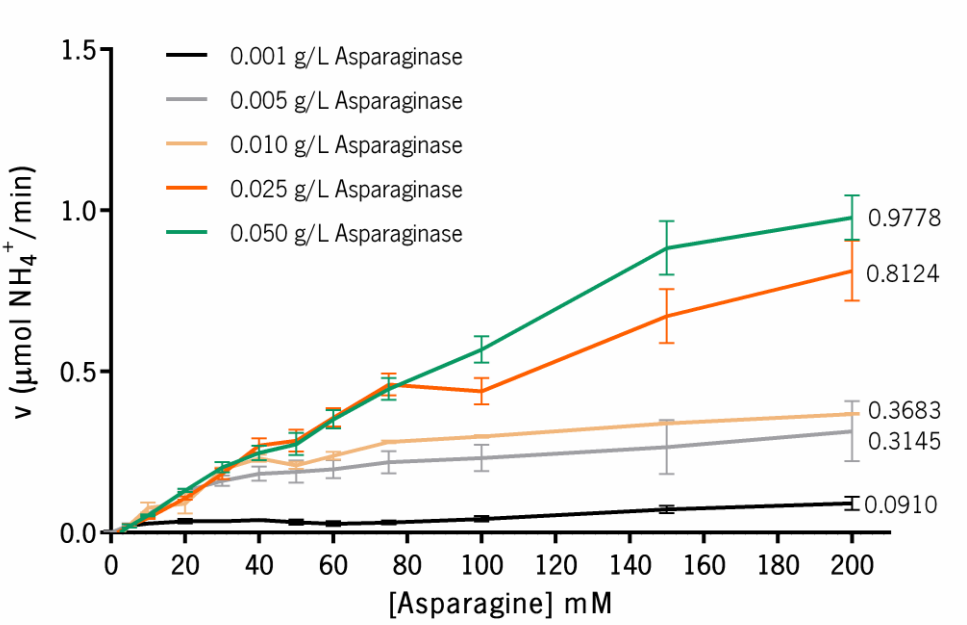

Figure 17 – Michaelis-Menten plot for different concentrations of asparagine (0.001; 0.005; 0.010; 0.025 and 0.050 g/L). The reaction was performed for 30 min at 40 °C with asparagine concentrations between 0 and 200 mM, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

For both temperatures and except for the lowest enzyme concentration (0.001 g/L), asparaginase exhibited a hyperbolic response to asparagine. For the two highest concentrations of asparaginase, 0.050 and 0.025 g/L, the reaction demonstrated a first order kinetic in almost all tested concentrations of asparagine. For these two enzyme concentrations the velocity of the reaction was dependent on asparagine concentration, with an increase on the reaction velocity as the substrate concentration increased

For the concentrations of 0.010 and 0.005 g/L of asparaginase, a two phase profile was observed. A first order kinetics for asparagine concentrations lower than 40 mM for 0.005 g/L and lower than 60 mM for 0.010 g/L of asparaginase was observed when the reaction was incubated at 37 °C. At 40 °C, there was a shift of the first order kinetic to concentrations lower than 30 and 40 mM, for 0.005 and 0.010 g/L of asparaginase, respectively. For the highest concentrations of substrate, the reaction showed a zero order kinetics because the velocity of the reaction was almost invariably even altering the substrate concentration. Meaning that for high concentrations of asparagine, the maximum velocity of reaction was achieved because the reaction system is saturated and the enzyme molecules were all in the enzyme-substrate complex form.

Generally it was observed a directly proportional increase between the velocity of the reaction and the enzyme concentration tested on the assay. On this way, the highest enzyme concentration (0.050 g/L) hydrolyzed asparagine into ammonia and aspartic acid more rapidly, with a velocity of reaction of 0.9427±0.056 and 0.9778±0.069 μmol NH_4^+/min for 37 and 40 °C, respectively.

The kinetic parameters were determined using Lineweaver-Burk linear regression as exemplified on Figure 18, that represent the Michaelis-Menten and the Lineweaver-Burk plot for the enzyme concentration of 0.050 g/L for the reaction performed at 37 °C. The kinetic parameters for all the enzyme concentrations were determined using the Lineweaver-Burk linear regression and are represented on Table IX.

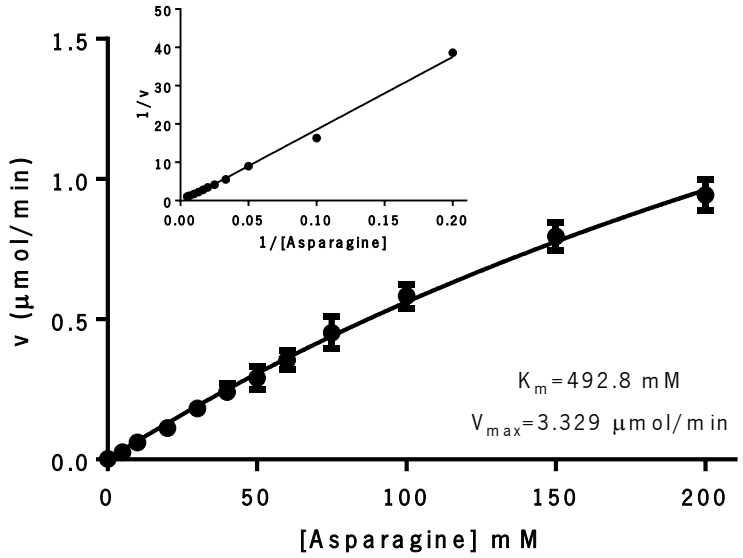

Figure 18 – Michaelis-Menten and Lineweaver-Burk plot for an asparaginase concentration of 0.050 g/L. Reaction was performed for 30 min at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

Table IX - Kinetic parameters for the hydrolysis of asparagine by asparaginase. Reaction performed at 37 °C, in 50 mM Tris buffer, pH 8.6.

Kinetic parameters	[Asparaginase] g/L			
	0.005	0.010	0.025	0.050
K_m (mM)	44.73±6.27	59.63±6.97	248.31±40.40	492.82±63.95
V_{max} (µmol/min)	0.34±0.018	0.52±0.026	1.50±0.16	3.33±0.34

Analyzing the results on Table IX, it was observed that the asparaginase activity was proportional to enzyme concentration on the reactional mixture, since the V_{max} value increased with increasing asparaginase concentration. For the asparaginase concentration of 0.005 g/L was achieved a V_{max} of 0.3438±0.01832 µmol NH_4^+/min while for the concentration of 0.050 g/L the V_{max} increased about ten times to 3.329±0.3398 µmol NH_4^+/min.

For the Michaelis-Menten constant, it was verified a direct relation between the K_M value and the enzyme concentration as higher K_M values were determined for higher asparaginase concentrations. Comparing the lowest and the highest enzyme concentrations (Table IX), there was a 10 times increase

on the K_M value when enzyme concentration increased 10 times. The Michaelis-Menten constant is an important parameter of enzyme-substrate interactions and is independent of enzyme concentration, so this value should be constant even if the enzyme concentration changes. Despite the differences, when comparing the kinetics for each enzyme concentration the only K_M values that were significantly different were the determined for asparaginase 0.025 and 0.050 g/L. The concentration of 0.050 g/L of asparaginase was the one that had less affinity for asparagine, since exhibited the higher K_M value. The difference on K_M could be related with the enzymes' tetrameric structure. Asparaginase is a tetrameric enzyme, composed by four identical subunits who communicate with each other. It is classified as a dimer of intimate dimers since the active sites are located at the interfaces of the subunits and just only when the four subunits are connected with each other is that the enzyme is active. Others reasons that could explain that differences on K_M are the impurities presents on the asparaginase formulation and the effect of aspartic acid on asparaginase, previously described by Homer (1972) and by Jayaram *et al.* (1986). Those authors reported that aspartic acid, a product of the hydrolysis of asparagine, had the capacity of inhibit asparaginase by binding, not only to the active site but also to a second site with lower intrinsic affinity.

The values of K_M determined using the Lineweaver Burk adjust were different from the reported by Ho *et al.* in 1970. Ho and coworkers, although using the same reaction conditions and an enzyme concentration of 0.005 g/L, determined a K_M of 1.15×10^{-2} mM for the reaction of hydrolysis of asparagine by asparaginase from *E.coli*. This value was smaller when comparing with the K_M value here determined for the same enzyme concentration (Table IX).

The kinetic parameters for the enzyme concentration of 0.001 g/L were not determined because it was not observed the hyperbolic profile characteristic of the Michaelis-Menten model. To better analyze the kinetic profile of this asparaginase concentration, the specific activities for all enzyme concentrations were plotted against the substrate concentrations (Figure 19).

For high asparagine concentrations (150 and 200 mM) there was a decrease on the specific activity of asparaginase as the enzyme concentration increase. The specific activity for the lowest asparaginase concentration (0.001 g/L) was 448.21±57.90 μmol NH_4^+/(mg.min) when incubated with 200 mM of asparagine, while it was 98.20±5.86 μmol NH_4^+/(mg.min), 4.5 times lower, for the 0.050 g/L concentration. For substrate concentrations lower than 40 mM, the tendency on the effect of substrate concentration on enzymes' specific activity was maintained.

For the 0.001 g/L asparaginase concentration, an oscillating profile for the enzyme specific activity was obtained. Between the 0 and 40 mM of asparagine the specific activity of asparaginase increase up to 224.56±5.57 μmol NH_4^+/(mg.min). Between 40 and 75 mM of asparagine decreased until 132.77±6.87 μmol NH_4^+/(mg.min). And increased again until reach a specific activity of 448.21±57.90 μmol NH_4^+/(mg.min) for the highest substrate concentration.

This oscillating profile for the 0.001 g/L asparaginase concentration could be related with an inhibitory effect of asparagine for the lowest enzyme concentration. Calculations about the reason between the moles of asparagine and moles of asparaginase on the reaction mixture, demonstrated that when the enzyme and the substrate are present in a ratio of n(asparagine)/n(asparaginase) between $1.0x10^6$ - $3.0x10^6$ it seems that the asparagine affects the asparaginase activity. Probably by inducing some alteration on the enzyme structure which affects the catalytic centers resulting in a decrease of asparaginase activity.

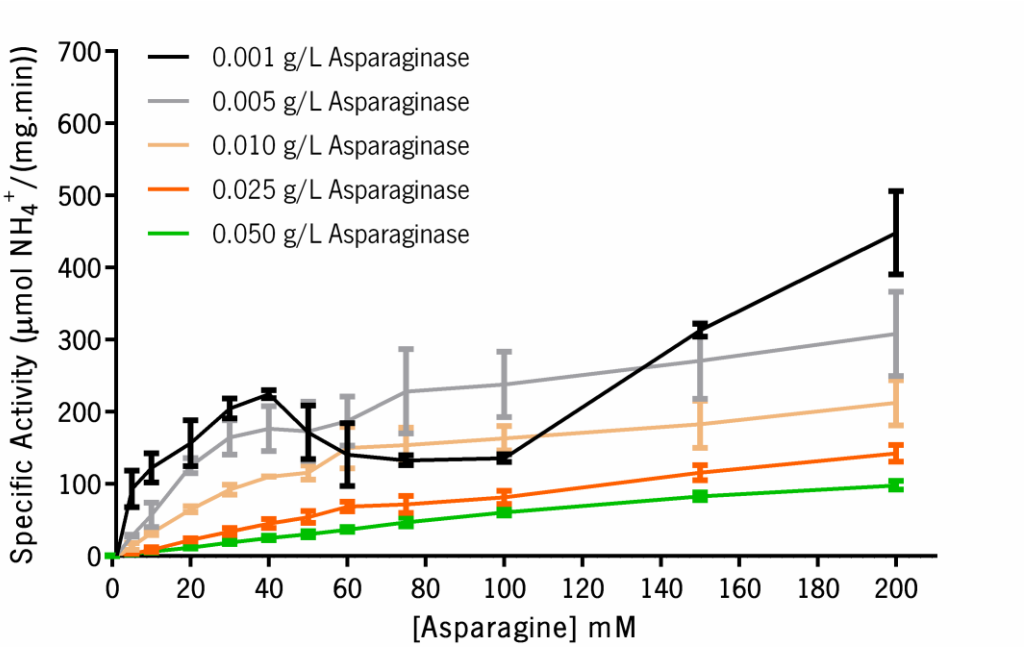

Figure 19 – Effect of asparaginase concentration and asparagine concentration on the specific activity of asparaginase. The reaction was performed for 30 min at 37 °C with asparagine concentrations between 0 and 200 mM, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

4.1.3. Effect of temperature on the activity of asparaginase

Temperature can affect an enzyme in two ways. It can promote the denaturation of the enzyme if the temperature is too high and it can directly affect the velocity of the reaction. Like most chemical reactions, the rate of a reaction catalyzed by an enzyme, increases as the temperature is raised due to the increase in molecules velocity and kinetic energy. With faster velocities, there will be less time between collisions, which result in more molecules reaching the activation energy and an increases in the reaction rate.

Two temperatures, 37 and 40 °C, were tested to study the effect of temperature on asparaginase activity in a situation of acute inflammation. The five asparaginase concentrations were tested but as the effect of temperature was similar, only the results for the 0.050 and 0.005 mg/mL concentrations are represented on Figure 20. The effect of the temperature on the enzyme kinetic it is demonstrated on Table X, where the kinetic parameters for each temperature are shown.

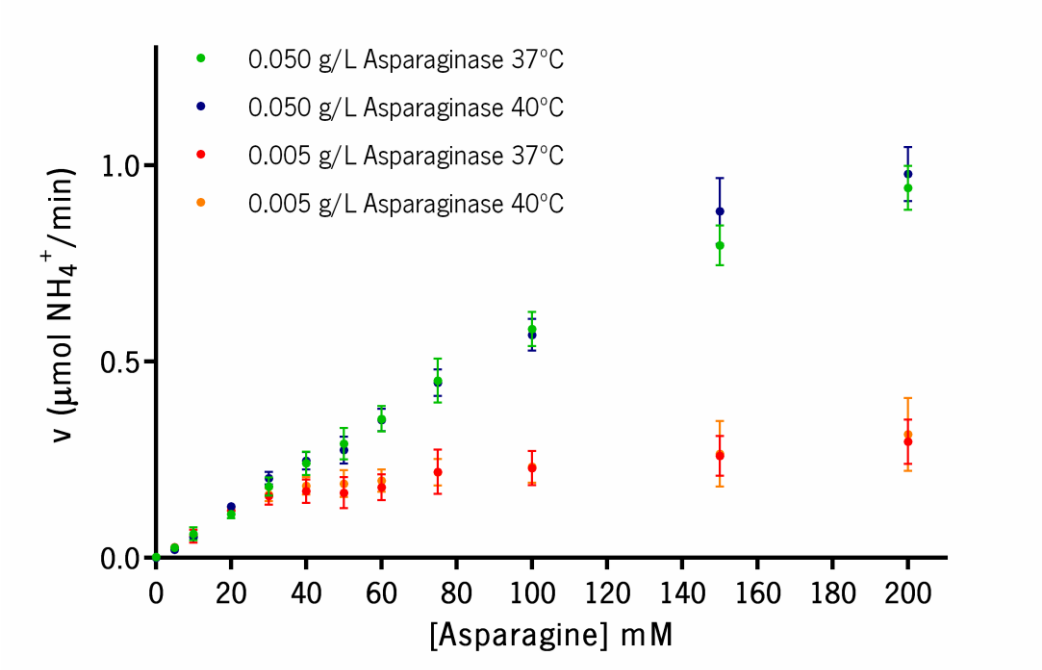

Figure 20 – Effect of temperature on the velocity of the hydrolysis of asparagine, at 37 or 40 °C. The reaction was performed for 30 min, at 37 and 40 °C, with asparagine concentrations between 0 and 200 mM, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

Table X - Kinetic parameters for the hydrolysis of asparagine by asparaginase. Reaction performed at 37 and 40 °C, in 50 mM Tris buffer, pH 8.6.

Kinetic parameters	Temperature (°C)	[Asparaginase] g/L 0.005	0.010	0.025	0.050
K_m (mM)	37	44.73±6.27	59.63±6.97	248.31±40.40	492.82±63.95
	40	42.37±6.16	52.79±10.93	278.22±62.87	654.86±204.11
V_{max} (µmol/min)	37	0.34±0.018	0.52±0.026	1.50±0.16	3.33±0.34
	40	0.35±0.019	0.46±0.039	1.92±0.30	4.35±1.12

Analyzing the results of the activity of asparaginase at 37 and 40 °C there was, in general, an increase between 0 and 10 % on the velocity of asparaginase at 40 °C, when comparing to the activity of asparaginase at 37 °C. The results on Table X indicated that for the lowest enzyme concentrations, an increase on temperature leaded to a slightly decrease on the K_M while, for the highest enzyme concentrations, this rise on temperature increased the K_M value. So, considering the highest enzyme concentration of 0.050 g/L, it was observed that the increase in the V_{max} of the reaction, due to the increase on temperature, did not give any advantages to the reaction kinetic since the affinity of the enzyme for the substrate decreased.

Generally, the results demonstrated that an increase on body temperature will not affect significantly the activity of asparaginase and the rate of hydrolysis of asparagine by asparaginase will be maintained.

4.1.4. Determination of asparaginase absolute activity over time

Two different assays were performed to analyze de asparaginase activity over time. The first assay intended to study the effect of asparaginase concentration on the velocity of the reaction over time, at 37 °C – Figure 21. For this assay, five different concentrations of asparaginase were tested while maintaining the concentration of asparagine at 100 mM. The second assay was to study the effect of asparagine concentration on asparaginase activity over time at 37 °C – Figure 22. For this test it was used a constant concentration of asparaginase of 0.010 g/L and concentrations of asparagine between 5 and 200 mM.

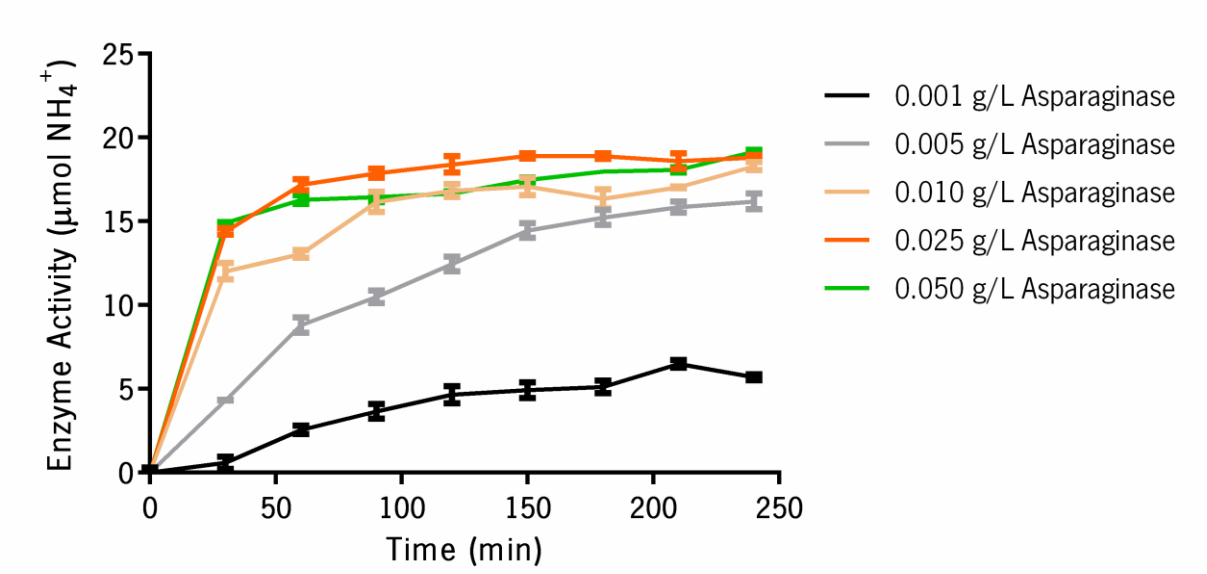

Figure 21 – Effect of the enzyme concentration on asparaginase activity over time. Five concentrations of asparaginase were tested: 0.001, 0.005, 0.010, 0.025 and 0.050 g/L. The reaction was performed for 240 min, at 37 °C, with 100 mM asparagine, in 50 Mm Tris buffer, pH 8.6. Samples were taken at 30 min intervals, for a total of 240 min. The absorbance of the solutions after Nesslerization were measured at 436 nm.

Figure 21 show four different profiles of enzyme activity over time. With this approach it was possible to determine the velocity of the reaction at a specific time point by the analysis of the slope of the curve at that specific point. For the two highest enzyme concentrations it was verified a similar profile with an initial velocity of 0.483 µmol NH_4^+/min. After the first 30 min, the reaction slow down and the activity reached a plateau phase due to the total depletion of asparagine on the reaction mixture. For the asparaginase concentration of 0.010 g/L, the profile was similar to 0.050 and 0.025 g/L asparaginase profiles but the plateau phase was just reached after 90 min of incubation. This was according expected since with less enzyme more time to consume all the substrate is needed. The two lowest asparaginase concentrations (0.001 and 0.005 g/L) showed a more linear profile. For these two concentrations, all the enzyme may be complexed with the substrate since the hydrolysis of asparagine occurred at a constant rate. The concentration of 0.005 g/L of asparaginase almost reach the plateau phase after incubation during 240 min at 37 °C.

Maintaining constant the enzyme concentration at 0.010 g/L there was an increase on ammonia concentration with the increase of asparagine, for the incubations times higher than 120 min at 37 °C (Figure 22). All the tested conditions reach the plateau phase, which indicates a total conversion of asparaginase into aspartic acid and ammonia. Concentration between 5 and 50 mM of asparagine

reached the plateau phase after 30 min of incubation while the highest concentrations of substrate needed about 90 min for the enzyme to hydrolyze all asparagine.

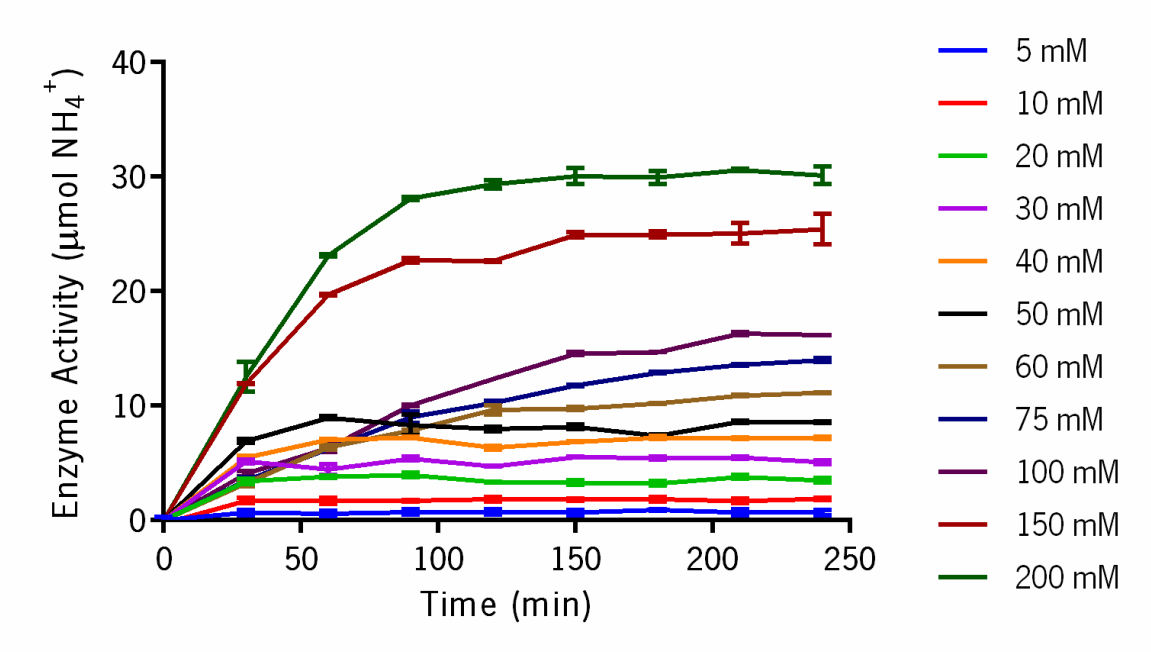

Figure 22 – Effect of the asparagine concentration on asparaginase activity over time. The reaction was performed for 240 min, at 37 °C, with 0.010 g/L of asparaginase in 50 mM Tris buffer, pH 8.6. The concentrations of asparagine varied between 5-200 mM. Samples were taken at 30 min intervals, for a total of 240 min. The absorbance of the solutions after Nesslerization were measured at 436 nm.

4.2. Effect of BSA on free Asparaginase

To study the effect of BSA on the stability of asparaginase, several solutions with different percentages of asparaginase and BSA ranging from 0 to 100 %, were prepared. The BSA/Asparaginase solutions were stored at 4 °C and enzyme activity was studied over time. This study intended to analyze which BSA/Asparaginase proportion conferred higher enzyme stability along time, and thus was the most suited for the preparation of BSA/Asparaginase nanoparticles.

The solutions were stored during 107 days and the activity was determined every weak for that period (Figure 23).

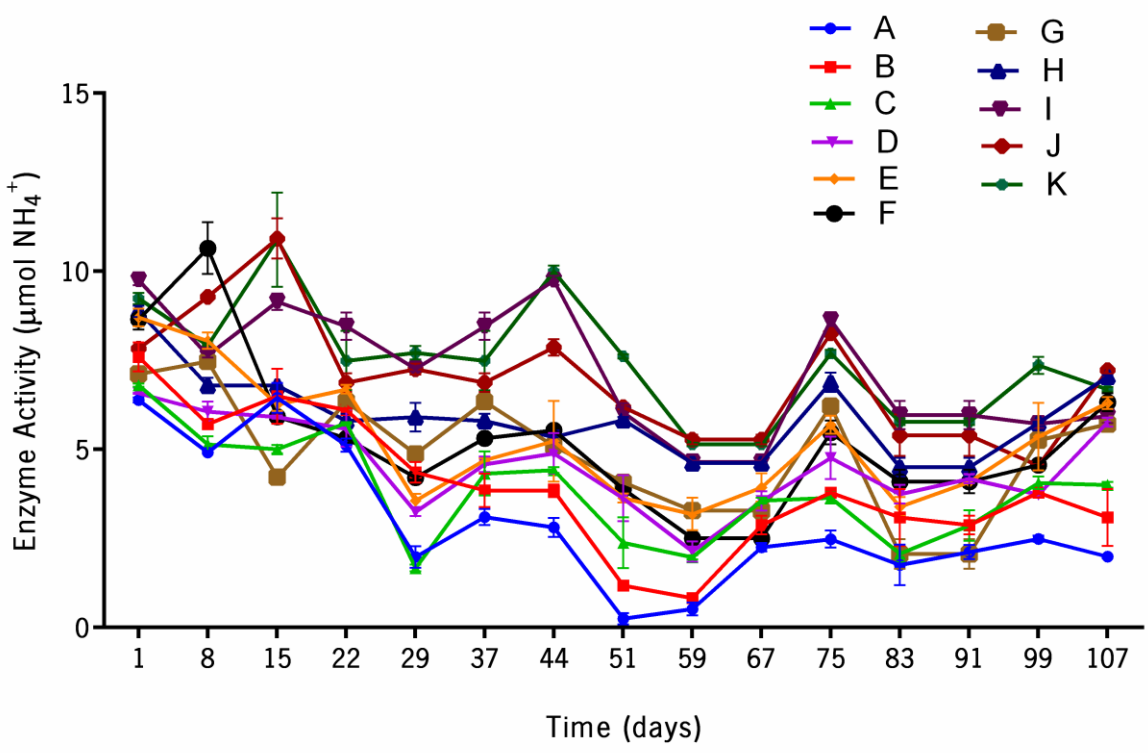

Figure 23 – Asparaginase activity over time of several solutions (A - 0 % BSA/ 100 % asparaginase solution; B - 10 % BSA/90 % asparaginase solution; C - 20 % BSA/80 % asparaginase solution; D – 30 % BSA/70 % asparaginase solution; E – 40 % BSA/60 % asparaginase solution; F – 50 % BSA /50 % asparaginase solution; G – 60 % BSA/40 % asparaginase solution; H – 70 % BSA /30 % asparaginase solution; I – 80 % BSA/20 % asparaginase solution; J – 90 % BSA/10 % asparaginase solution; K – 98 % BSA/2 % asparaginase solution) with different proportions of BSA and asparaginase. The reaction was performed for 240 min, at 37 °C, with 0.010 g/L of asparaginase in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

Analyzing the results of the activity of asparaginase over 107 days, it was possible to determine the enzyme half-life time in some BSA/Asparaginase samples. For others samples the half-life time was estimated because during the 107 days of storage the enzyme did not achieved half of the initial activity (Table XI).

Table XI - Half-life time of asparaginase, when prepared with different percentages of BSA on PBS*

Solution	BSA/Asparaginase ratio (%)	Initial activity of asparaginase (μmol ammonia)	Half-life time of asparaginase
A	0/100	6.39±0.071	46.70
B	10/90	7.59±0.40	57.01
C	20/80	6.80±-0.044	72.08
D	30/70	6.57±0.016	130.88**
E	40/60	8.70±0.24	89.18
F	50/50	8.65±0.29	81.58
G	60/40	7.12±0.14	105.13
H	70/30	8.87±0.15	144.64**
I	80/20	9.75±0.15	120.75**
J	90/10	7.83±0.045	147.52**
K	98/2	9.23±0.16	148.95**
L	100/0	0	-

*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 absorbance of the solutions after Nesslerization were measured at 436 nm.

**estimated half-time of asparaginase

Generally, it was observed an increase in the half-life time of asparaginase with the increase of percentage of BSA in solution. The only exception was verified for the sample 30 % BSA/70 % asparaginase and 70 % BSA/30 % asparaginase which showed a high asparaginase activity relatively to the closest BSA/Asparaginase ratios. Comparing all the tested BSA/Asparaginase ratios, the ratio which stabilized the enzyme for a longer period was the 98 % BSA/2 % asparaginase. This proportion was thus chosen for the preparation of the BSA:Asparaginase nanoparticles.

The protective effect of BSA has been previously described for acetylcholinesterase (AChE EC3.1.1.7) from *Drosophila.* BSA had the capacity to protect this enzyme against denaturation after incubation at 4 °C, during 5 h. At low concentrations of acetylcolinesterase, the enzyme lost half of the initial activity after incubation at 4 °C while, when BSA was added, the activity was not affected by the incubation time at low temperatures (Nasseau *et al.*, 2001).

The effect of storage time on enzyme structure was analyzed every week in a 15 % SDS-PAGE gel (Figure 24 and 25). The appearance of a band with a molecular weight slightly inferior to the molecular weight of the monomer of asparaginase was observed with increasing the storage time at 4 °C

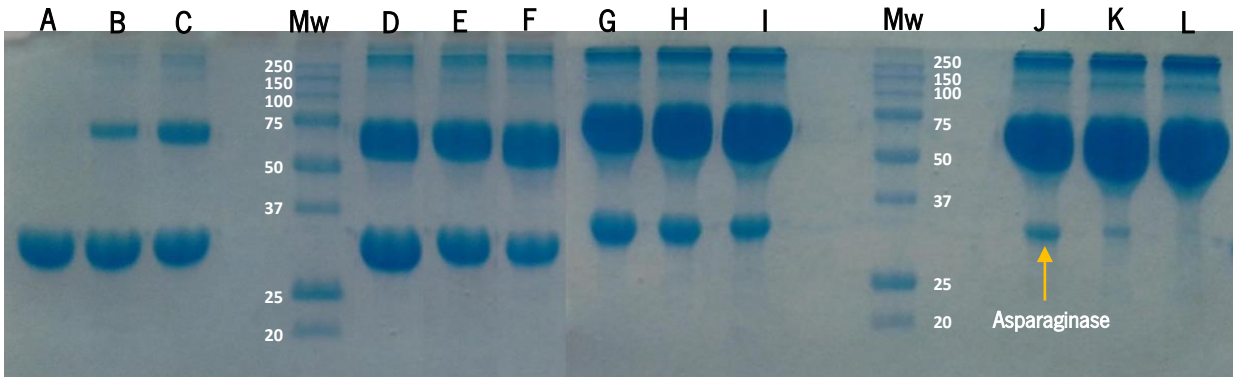

Figure 24 –Effect of storage time at 4 °C in asparaginase stability measured for several BSA/Asparaginase ratio solutions. Samples correspond to the first day of storage, at 4 °C. 15 % SDS-PAGE stained with Coomassie Blue.

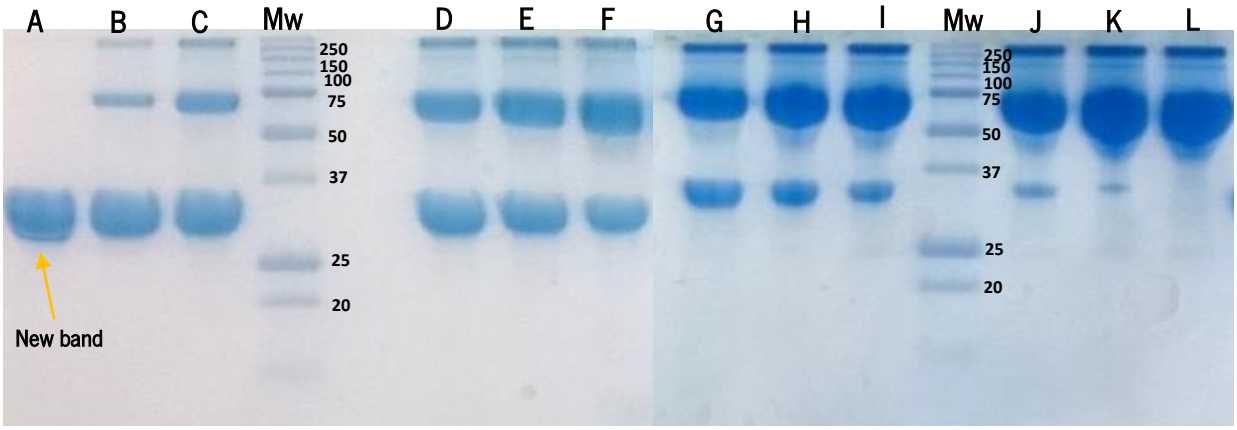

Figure 25 - Effect of storage time at 4 °C in asparaginase stability measured for several BSA/Asparaginase ratio solutions. Samples correspond to day 22 of storage, at 4 °C. 15 % SDS-PAGE stained with Coomassie Blue.

The new band appeared first for the 0 % BSA/100 % asparaginase solution after 22 days at 4 °C and then for the remaining samples with exception for 90 % BSA/10 % asparaginase and 98 % BSA/2 % asparaginase. However after storage for 107 days, all samples presented the new band. It seems that, during storage, the asparaginase suffered a denaturing/cleavage process that caused the loss of a small fragment of the monomer, which could explain the reduction on enzyme activity. This process seems to be retarded in the presence of increasing concentrations of BSA thus stabilizing the enzyme during storage at 4 °C.

This study confirmed the protective effect of BSA for asparaginase. BSA preserved the enzyme activity for a long period of time during storage at low temperatures.

4.3. Effect of storage time, reaction buffer, protease inhibitor, antibiotic and immobilization on asparaginase activity

The 98 % BSA/2 % asparaginase ratio was considered the BSA/enzyme proportion that most stabilize the enzyme and thus was used for the particles formulation. Nevertheless, it was necessary to study the effect of several parameters on enzyme activity, such as storage time, reaction buffer, presence of protease inhibitor, presence of antibiotic and effect of immobilization.

The behavior of free enzyme on an aqueous solution was characterized for a period of 124 days at 4 °C (Figure 26). The activity was measured once a week during four months and it was observed an overall decrease on asparaginase activity over time. Comparing the activity at the day 1 and day 124, there was a decrease of 32 % in enzyme activity.

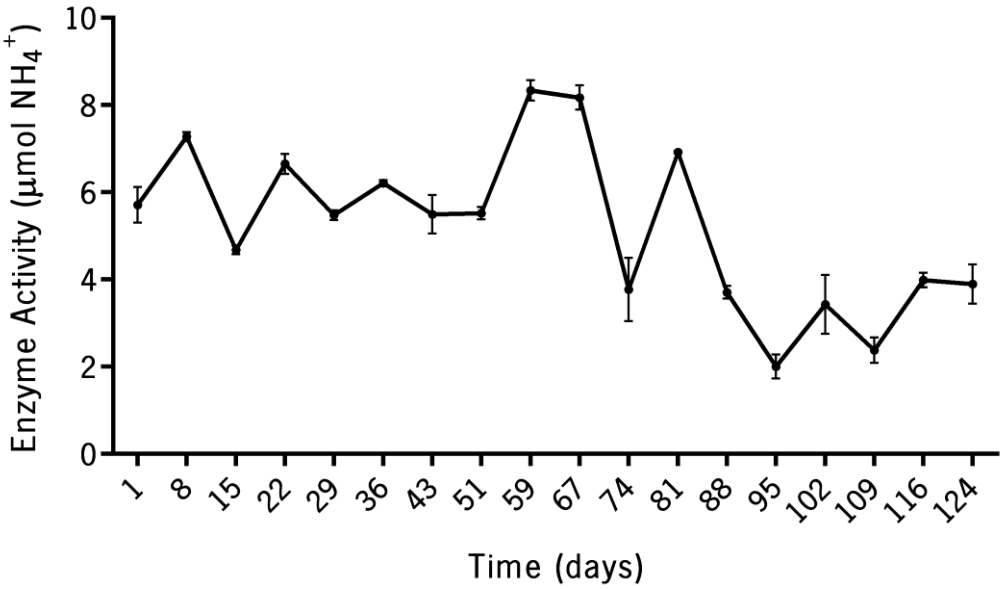

Figure 26 – Effect of the storage at 4 °C on asparaginase activity over time. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8. 6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

The effect of PBS on asparaginase activity was evaluated over time. The PBS buffer was used during the synthesis of nanoparticles. To study the effect of PBS it was prepared a solution of 10 mg/mL asparaginase on PBS, pH 7.4, and storage at 4 °C. The activity of asparaginase in PBS buffer was compared with the activity when asparaginase was in an aqueous solution (Figure 27). Despite the activity at day 1 and at day 74, the asparaginase activity when enzyme was in PBS was significantly lower than the asparaginase activity when enzyme was in ultra-pure water (p-value≤0.0001). The differences

between activities may be due to the different pH between ultra-pure water and PBS and to the alteration of the ionic strength of the system. Both pH and ionic strength may interfere with the enzyme protonation state which alters the enzyme-substrate interaction and, as a consequence, alters the catalytic activity of the enzyme (Acker and Auld, 2014). Although this differences, the asparaginase on a PBS solution maintain the activity for more than 88 days when stored at 4 °C.

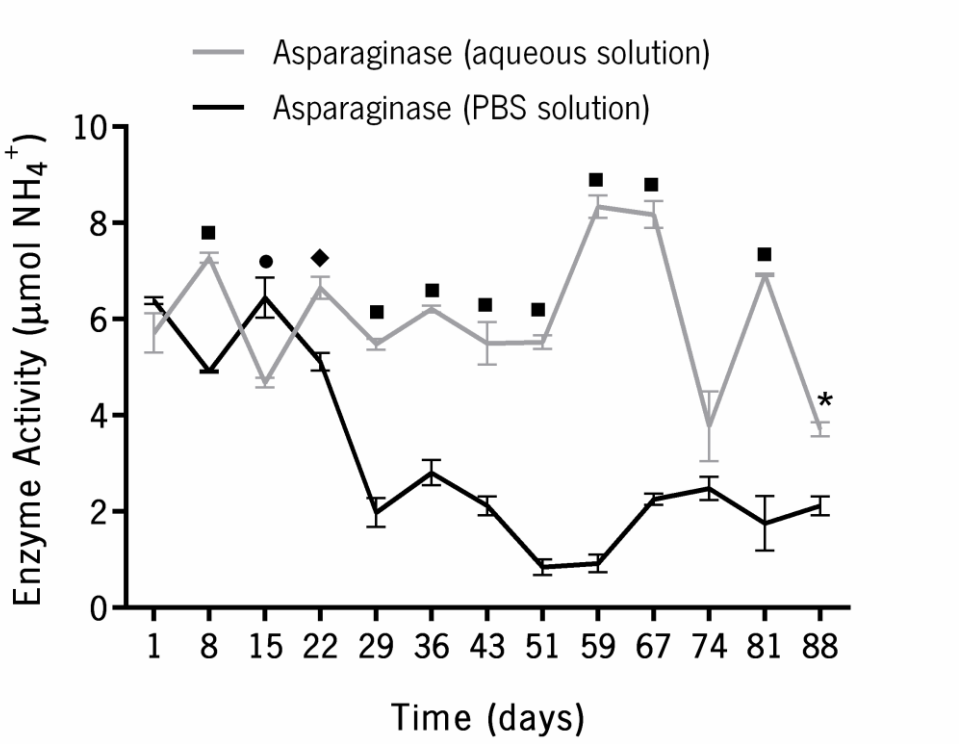

Figure 27 – Effect of PBS on the activity of asparaginase over time, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

Since the nanoparticles were principally composed by BSA, it was important to study the effect of BSA on asparaginase activity when prepared on a ratio of 98 % BSA/2 % asparaginase in PBS, pH 7.4, without antibiotic and protease inhibitor, when stored at 4°C (Figure 28). Asparaginase activity was measured once a week until the loss of enzyme activity.

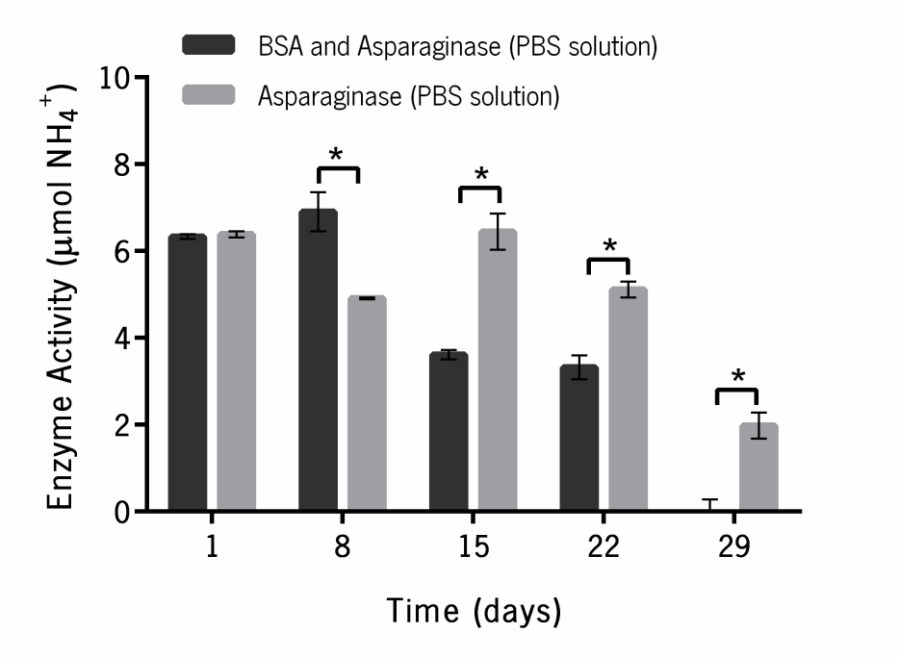

Figure 28 - Effect of BSA in the absence of antibiotic and protease inhibitor on the activity of asparaginase over time, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

Analyzing Figure 28, it was evident that when the enzyme was co-incubated with BSA to a proportion of 98 % BSA/2 % asparaginase, without antibiotic and protease inhibitor, it lost its activity in 29 days.

In order to understand what caused the loss of asparaginase activity, two different analysis were performed. First, samples were ran in a 15 % SDS-PAGE to see if the enzyme suffer any structural alteration or if it was cleaved, which will justify the loss of activity (Figure 29).

Comparing the asparaginase at day 1 (lane C) with the asparaginase at day 35 (lane D), it was observed that the storage during 35 days of an aqueous solution of asparaginase apparently did not affect the enzyme structure. Although, when comparing the asparaginase with BSA at day 1 (lane E) with the asparaginase with BSA at day 35 (lane F), it was verified that the enzyme, when incubated 35 days at 4 °C with BSA in PBS, it lost a fragment.

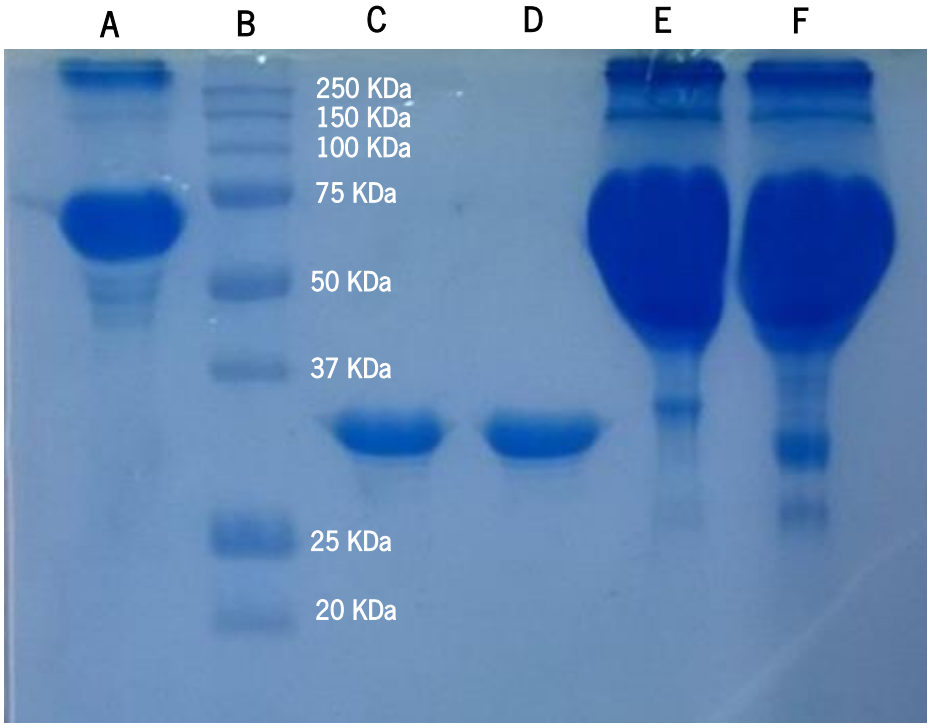

Figure 29 – Effect over asparaginase activity of BSA in the absence of antibiotic and protease inhibitor. 15 % SDS-PAGE stained with Coomassie Blue. Lane A) BSA Solution; Lane B) Molecular weight marker; Lane C) Aqueous solution of asparaginase – 0 days at 4 °C; Lane D) Aqueous solution of asparaginase – 35 days at 4 °C; Lane E) Solution of 0.2 mg/mL asparaginase and 9.8 mg/mL BSA – 0 days at 4°C; F) Solution of 0.2 mg/mL asparaginase and 9.8 mg/mL BSA – 35 days at 4 °C.

To determine the fragment mass/charge ratio, the 98 % BSA/2 % asparaginase % solution was analyzed by MALDI-TOF (Figure 30). With this technique was determined a mass/charge ratio of 31996.2 Da for the inactive enzyme, comparing to the value of 34513.1 Da for the active enzyme monomer. The loss of activity was related with the cleavage of a fragment with 2516.9 Da from the enzyme sequence that could correspond to a cleavage between the residues Val26 and Gly27 from the N-terminal or Leu21 and Leu22 from C-terminal.

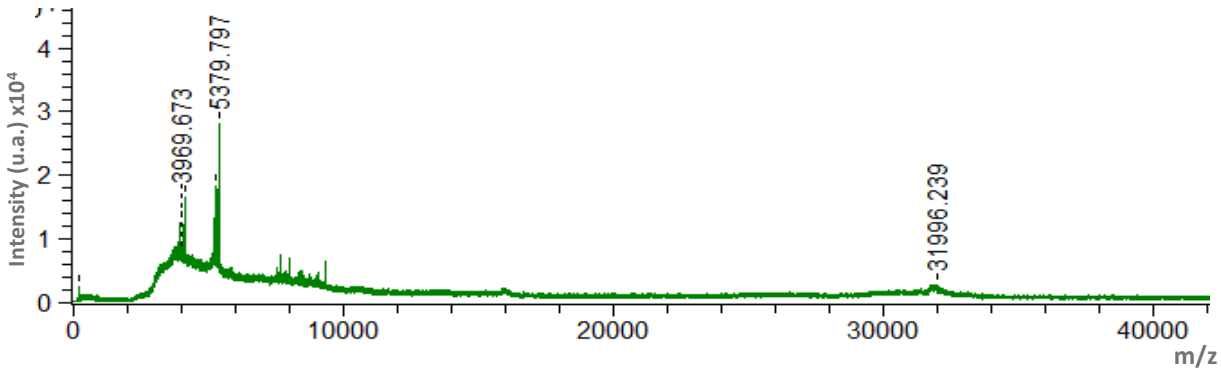

Figure 30 – MALDI-TOF spectrum of a solution of BSA and asparaginase, in PBS, after storage during 35 days at 4 °C.

When the solution 98 % BSA/2 % asparaginase was prepared in PBS but with antibiotic, the asparaginase activity was retained for a longer time (Figure 31), while the solution of asparaginase and BSA without antibiotic did not show any activity after 29 days at 4 °C. Even after storage during 91 days at 4 °C, the enzyme on the solution with antibiotic produced 4.997±1.145 μmol NH_4^+.

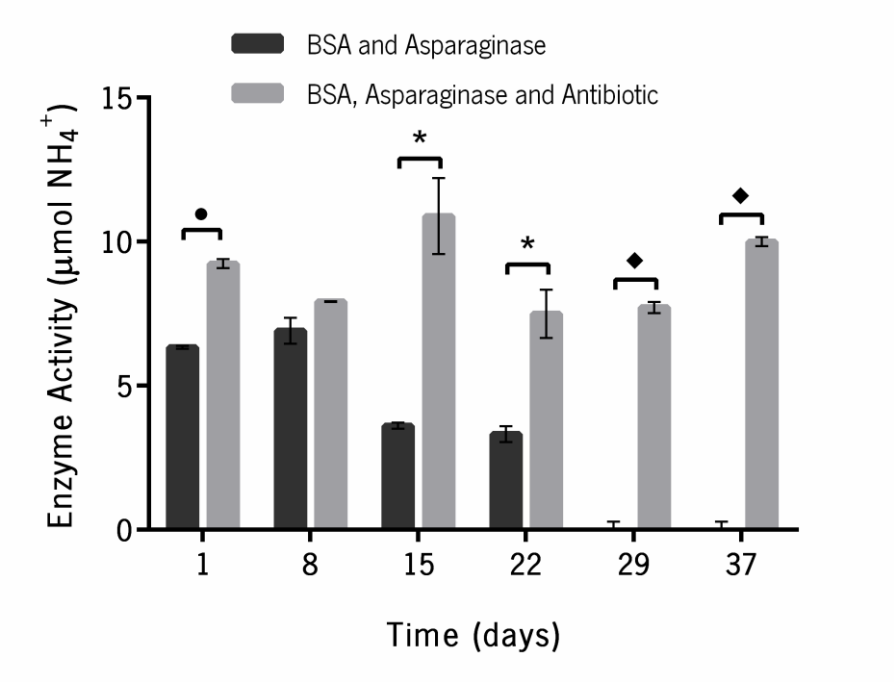

Figure 31 – Effect of the addition of antibiotic on asparaginase activity over time, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

It was also tested the presence of proteases to justify the loss of asparaginase activity. The BSA 98 % BSA/2 % asparaginase was prepared in the presence of a protease inhibitor (PMSF) but without antibiotic. The addition of PMSF, right after preparation did not had any significant effect on the maintenance of asparaginase activity over time.

BSA:Asparaginase particles were prepared with and without antibiotic. Like for the 98 % BSA/2 % asparaginase solution, it was observed a rapid loss of asparaginase activity when no antibiotic was added to the particles.

It was concluded that was necessary to add antibiotic to the formulation in order to inhibit the growth of microorganisms that had the capacity to cleave the enzyme which results in its inactivation.

4.4. Asparaginase Immobilization in Particles prepared by Ultrasounds and High Pressure Homogenization

For the nanoparticles preparation two different techniques were used. The ultrasounds consist of sound waves applied at a low frequency of approximately 16 kHz. When sound waves enter a liquid medium, sound is propagated as sinusoidal waves and energy is propagated through the system in the form of vibration. This vibration consists of cycles of compression and expansion which causes high pressures inside the liquid medium, resulting in thousands of bubbles and in a physical phenomenon called cavitation (Dolatowski *et al.*, 2007; Sango *et al.*, 2014). The high pressure homogenization is frequently used to provide particles with small size and very stable. During the high pressure homogenization, the liquid is forced to pass through a thin gap under high pressures. Thus, it creates a fast acceleration and, as the fluid exits the homogenization valve, the pressure undergoes an extreme drop. The effects caused by the high pressure homogenization cannot be produced just by one single physic phenomenon. At high pressure, it is a combination of high hydrostatic pressure, shear stress, cavitation collapse, strong impacts, high-speed friction and heating that is responsible for the emulsion formation (Pedras *et al.*, 2012).

4.4.1. Particles prepared by Ultrasounds

4.4.1.1. BSA:Asparaginase B and BSA:Asparaginase D particles

After the synthesis of the particles, and addiction of 1 % (v/v) of antibiotic, the activity of the immobilized asparaginase was evaluated for 2 months. Two types of particles were prepared depending on the moment of addition of asparaginase to the formulation. Both particles were prepared with 9.8 mg/mL BSA, 0.2 mg/mL asparaginase and 5 % (v/v) of vegetable oil. If the enzyme was added before sonication, particles were designated as BSA:Asparaginase B; if the enzyme was added during the sonication process the particles were designated as BSA:Asparaginase D. The activity of immobilized asparaginase was compared to the free asparaginase on a PBS solution (Figure 32), and the activity of both particles was compared between them (Figure 33).

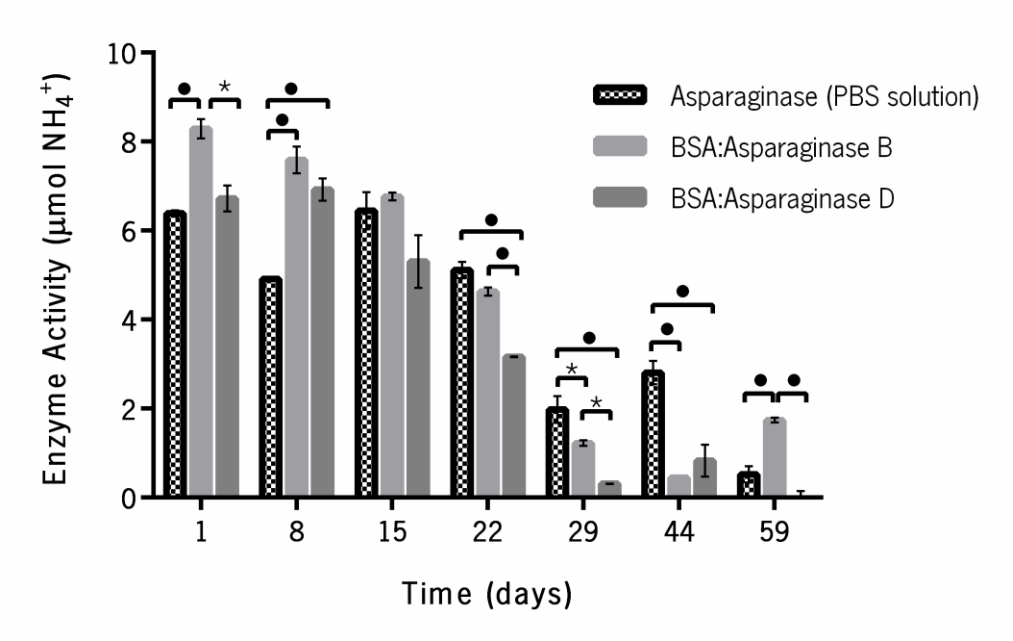

Figure 32 – Activity of asparaginase immobilized on BSA particles prepared by ultrasounds (BSA:Asparaginase B and BSA:Asparaginase D), and free asparaginase (10 mg/mL) on a PBS solution, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, at pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The efficiency of particle formation was determined by the quantification of the free protein after centrifugation, by Lowry Method. It was determined an efficiency of particle formation of 93.9 % for BSA:Asparaginase B and 94.69 % for BSA:Asparaginase D.

Over time, it was observed a decrease on free asparaginase activity when stored at 4°C. This decrease was principally triggered by the effect of PBS on the enzyme, as comproved by the results showed on section 4.3, where it was compared the asparaginase activity between the enzyme dissolved on an aqueous solution and on a PBS solution.

Relatively to the immobilized asparaginase, there was a general decrease on asparaginase activity over the storage time. At the end of first week, there was a significant increase on immobilized asparaginase activity when compared to the activity of free asparaginase. At day 8, immobilized asparaginase presented an activity increase in about 35 and 30% for the BSA:Asparaginase B and BSA:Asparaginase D particles, respectively.

Between day 22 and day 44 of storage at 4 °C, there was a shift on the tendency of compared asparaginases' activity. For this time period, the free asparaginase had more activity than the immobilized

enzyme. Nevertheless at day 59 the BSA:Asparaginase B particle presented three times more activity than the free asparaginase, while the BSA:Asparaginase D particles showed no activity. A direct comparison between the activities of the two particles is represented on Figure 33. For almost all storage time, the activity of BSA:Asparaginase B particles was higher than the activity of BSA:Asparaginase D particles. Though, the differences between the two particles were only statistically significant for four measurements, namely day 1, 22, 29 and 59. The results showed an advantage of adding the asparaginase before sonication because the activity along time was higher than when the enzymes was added during sonication cycles.

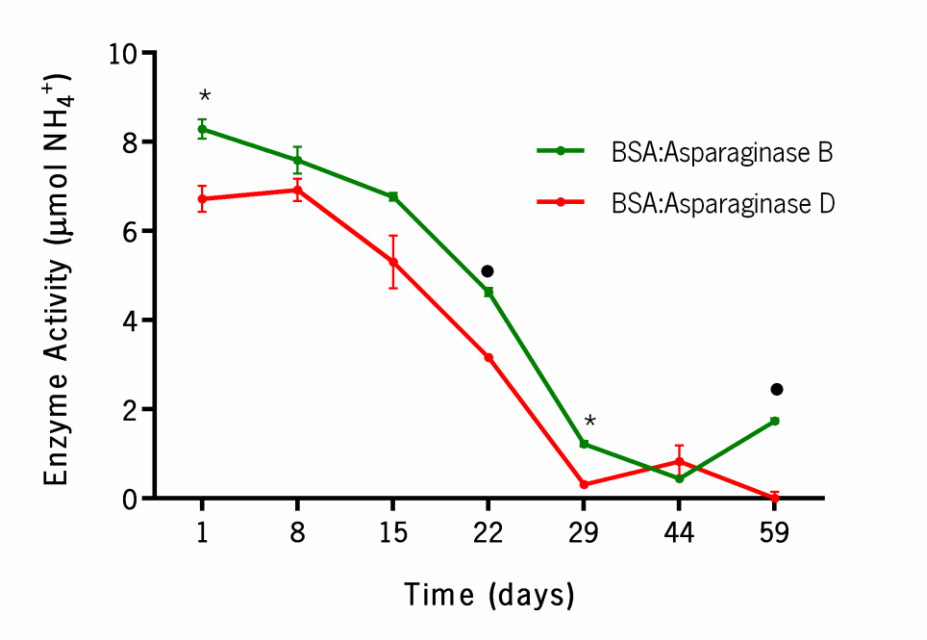

Figure 33 - Effect on asparaginase activity of adding the enzyme at different stages during the process of particles synthesis by ultrasounds. BSA:Asparaginase B – enzyme added before the sonication cycles. BSA:Asparaginase D – enzyme added during the sonication cycles, after 69 s. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The loss of activity during particles storage could be due to some damages provoked to the enzyme during the immobilization process, which could lead to enzyme instability. These damages may be caused by the high energy of the ultrasound waves that causes high pressures into the enzyme solution by the cavitation process. Although the enzyme solution were kept immersed in an ice-water bath at 0 °C, the cavitation process also produced extreme temperatures locally by the release of large magnitudes of energy, which can alter the performance of the activity right after synthesis or increase the enzyme instability over time.

The Z-average and polydispersity index (PDI) of particles were assessed by Photon correlation Spectroscopy whereas laser Doppler Anemometry was employed for Z-potential. The particles physical stability was evaluated over 2 months, by weekly measurements of Z-average, PDI and Z-potential of the particles stored at 4 °C. The physical stability of the BSA:Asparaginase B particles is represented on Figure 34 and the physical stability of BSA:Asparaginase D particles on Figure 35.

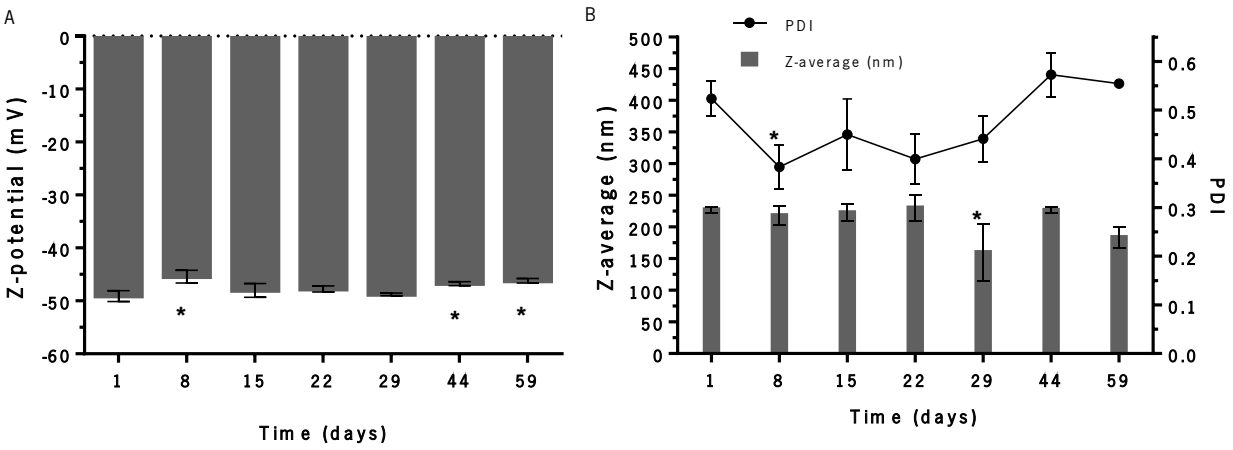

Figure 34 – Characterization of BSA:Asparaginase B particles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

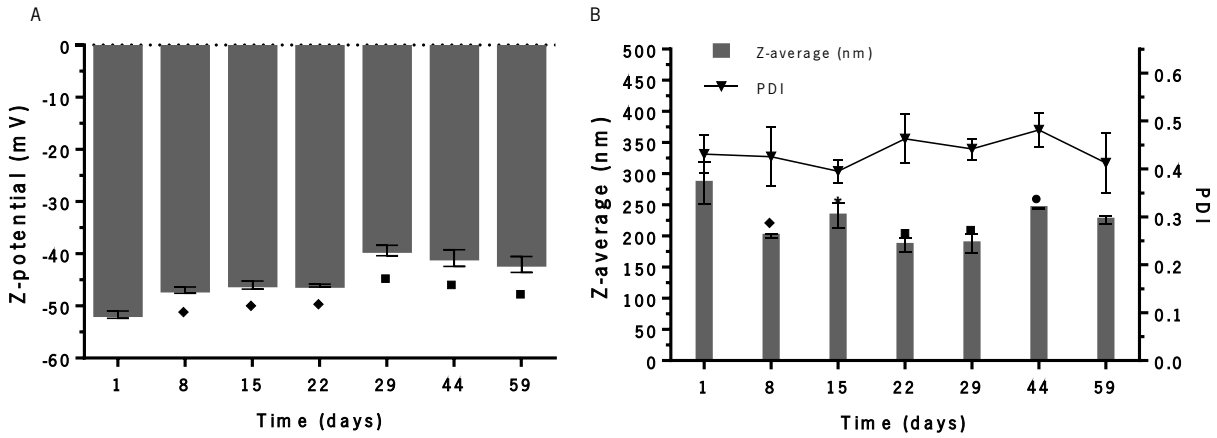

Figure 35 - Characterization of BSA:Asparaginase D particles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ●p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

On day 1, the BSA:Asparaginase B particle had a Z-average of 227.3±4.6 nm, a PDI of 0.5237±0.036 and a Z-potential of -49.10±1.0149 mV and the BSA:Asparaginase D a Z-average of 284.61±6.7 nm, a PDI of 0.4310±0.0388 and a Z-potential of -51.67±0.6807 mV.

It was also prepared BSA particles (10 mg/mL BSA and 5 % (v/v) of vegetable oil on the same conditions of ultrasounds cycles described previously) with a Z-average of 183.47±23.38 nm, a PDI of 0.4297±0.038 and a Z-potential of -28.8±0.9 mV. Comparing the BSA particles with and without the enzyme, it was observed that the addition of asparaginase leaded to a significant increase in the particles size (p-value≤0.05). For the PDI there was only a significant increase on the PDI value for the BSA:Asparaginase B (p-value ≤0.05). The addition of enzyme either before or during the ultrasounds cycles turned the particles significantly more negative (p-value≤0.0001). The negative Z-potential of the BSA particles could be due the carboxyl groups that acquired a negative charge when dissolved on PBS with a pH of 7.4. Since the asparaginase has a isoelectric point of 4.9 (Ehrman *et al.*, 1971) it was expected that the enzyme on PBS also acquired a negative charge. So, the more negative Z-potential could be justified by the addition of asparaginase to the formulation and it location at the surface of the particles.

Comparing both BSA:Asparaginase B and BSA:Asparaginase D particles at day 1, the differences between both Z-averages and PDI were not significant but the BSA:Asparaginase D particles were more negative (p-value ≤0.05). The more negative character of this particle could be due to a more superficial position of asparaginase when the enzyme was added during sonication.

BSA:Asparaginase B particles were stable over time with a slight variation on size at day 29 that can be explained by some errors during the size measurement since for the others days the size was maintained relatively constant (Figure 34). During the storage, the BSA:Asparaginase B particles slightly changed the superficial charge as well as the PDI when compared with day 1 (p-value ≤0.05).

For the BSA:Asparaginase D particles, although PDI did not significantly varied along time, they were more instable during storage at 4 °C. Comparing with day one, the Z-potential and size always showed significant differences during storage (p-value≤0.001).

Comparing activity, size, PDI and Z-potential the BSA:Asparaginase B particles seem to be more stable over time than the BSA:Asparaginase D particles.

4.4.1.2. BSA:Asparaginase:Pol407 B and BSA:Asparaginase:Pol407 D Particles

A nonionic copolymer Poloxamer 407 (Pol407) was added to the particles. Poloxamer 407 shows surfactant properties due to their amphiphilic structure and it is composed of a central hydrophobic block of polypropylene glycol flanked by two hydrophilic blocks of polyethylene glycol (Figure 36).

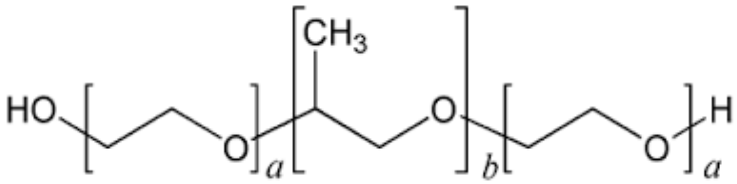

Figure 36 – Poloxamer 407 chemical structure (a=98, b=67).

To the particles formulations were added 5.0 mg/mL of Poloxamer 407 and, as previous, the enzyme was added at different stages during the particles formation by ultrasounds. The particles were designated as BSA:Asparaginase:Pol407 B when the enzyme was added before ultrasounds, and were designated as BSA:Asparaginase:Pol407 D when the enzyme was added during the ultrasounds cycles, after 69 s of sonication.

These particles had an efficiency of particle formation of 89.75 % and 89.42 % for the BSA:Asparaginase:Pol407 B and BSA:Asparaginase:Pol407 D, respectively.

The ability of particles to hydrolyze asparagine was followed along storage time at 4 °C (Figure 37). As for BSA:Asparaginase B and BSA:Asparaginase D particles, there was a decrease on asparaginase activity over time.

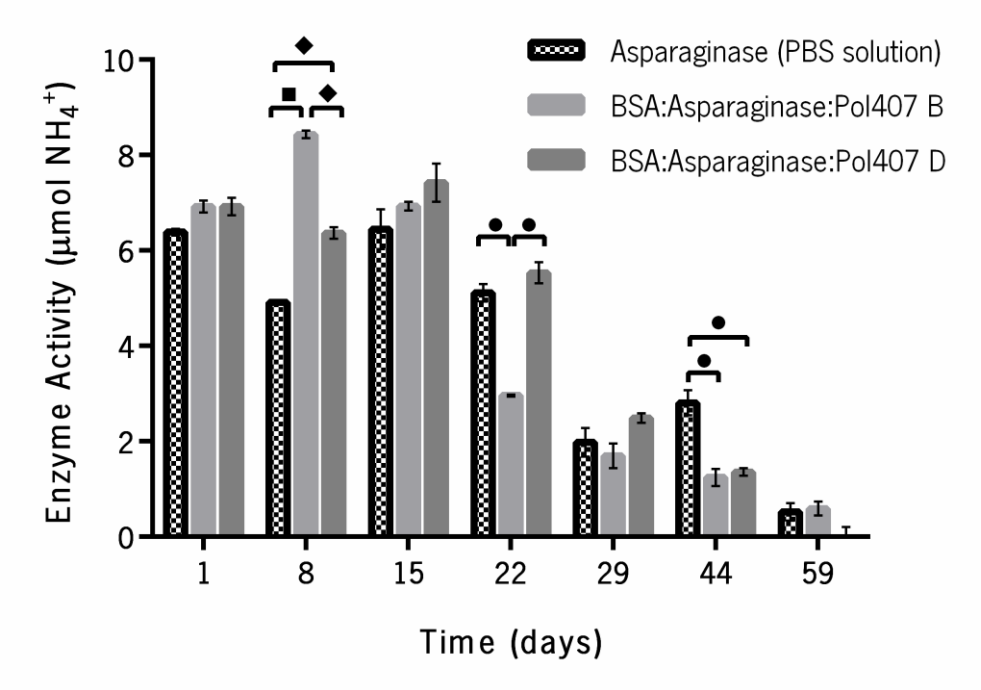

Figure 37 - Activity of asparaginase immobilized on BSA:Pol407 particles prepared by ultrasounds and free asparaginase (10 mg/mL) on a PBS solution, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

To evaluate the effect of adding the enzyme at different stages of the particles synthesis, the activities of BSA:Asparaginase:Pol407 particles were plotted against the storage time at 4 °C (Figure 38). Analyzing all time points, the BSA:Asparaginase:Pol407 D particles only showed a significant increase on asparaginase activity at day 22, while the BSA:Asparaginase:Pol407 B particles showed a significant increase on the asparaginase activity at day 8. Thus, considering the activity over the two months of storage, it was not observed any advantage in adding the enzyme during the particles synthesis process.

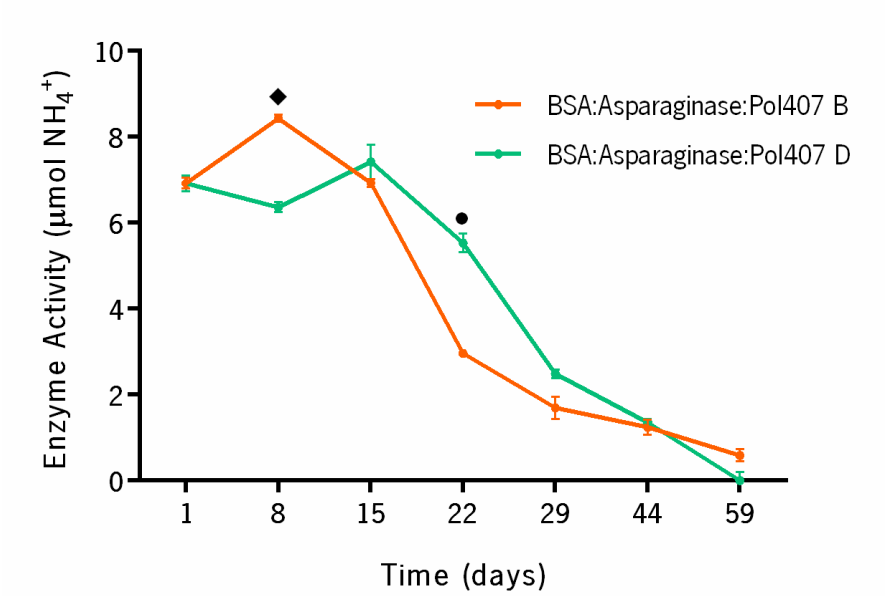

Figure 38 - Effect on asparaginase activity of adding the enzyme at different stages during the process of particles synthesis. BSA:Asparaginase:Pol407 B – enzyme added before the sonication cycles. BSA:Asparaginase:Pol407 D – enzyme added during the sonication cycles, after 69 s. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

Regarding the characteristics of the particles, the BSA:Asparaginase:Pol407 B had an initial Z-average size of 232.67±15.15 nm, a PDI of 0.313±0.0659 and a Z-potential of -3.013±0.261 mV (Figure 39). The BSA:Asparaginase:Pol407 D particles had an initial Z-average size of 284.667±74.122 nm, a PDI of 0.319±0.0593 and a Z-potential of -2.83±0.269 mV (Figure 40). The addition of asparaginase during the process of synthesis did not changed the characteristics of the particles, comparing to the BSA:Asparaginase:Pol407 B particles.

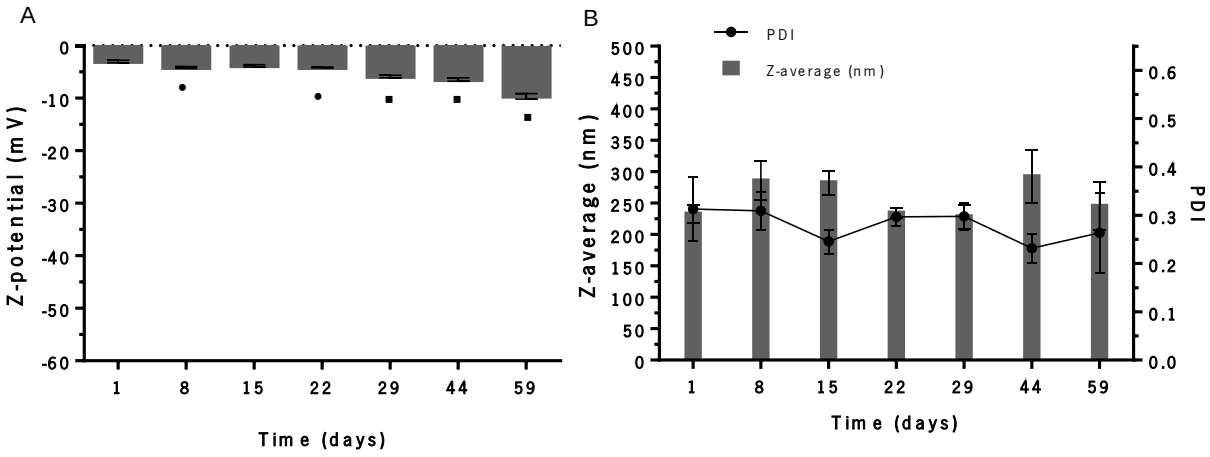

Figure 39 - Characterization of BSA:Asparaginase:Pol407 B particles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

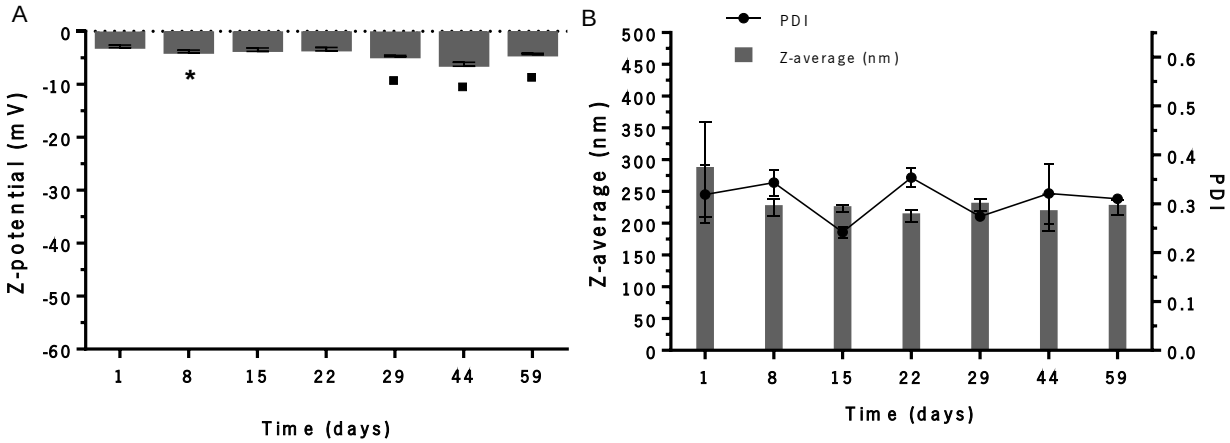

Figure 40 - Characterization of BSA:Asparaginase:Pol407 D particles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

Analyzing both Figure 39 and 40, it was evident the stabilizing effect of Poloxamer. With Poloxamer 407, the formulation become more stable over storage, in respect of size and PDI. Relatively to the Z-potential, both particles exhibited some instability during storage, with a significant increase on the net negative charge of the particles over time.

Poloxamer 407 is a surface active compound that, due to their amphiphilic structure, exhibits a tendency to accumulate at the interface between the aqueous and organic phase, lowering the surface tension and stabilizing the system. This compound is used not only to control the particle size, particle size distribution and shape but also to prevent particles to aggregate and coalescence.

Comparing to the BSA:Asparaginase particles, the addition of Poloxamer 407 did not affect the Z-average of the particles when prepared by ultrasounds, but lowered the PDI. With regard to the Z-potential of the BSA:Asparaginase particles, it was seen a reduction of the Z-potential after adding the Poloxamer 407 to the formulation. These reduction could be explained by the coating of the particle by the nonionic surfactant Poloxamer 407, which shield the surface charge making it less negative.

BSA:Pol407 particles were also prepared with 5 mg/mL Poloxamer. These particles had a Z-average of 237.3±9.3 nm, a PDI of 0.296 ± 0.022 and a Z-potential of -4.71±0.115 mV. The addition of asparaginase to the BSA:Pol407 particles did not change significantly the size and the PDI but altered the Z-potential (p-value ≤0.001).

4.4.1.3. BSA:Asparaginase:Zein B and BSA:Asparaginase:Zein D Particles

To stabilize the BSA:Asparaginase particles it was added zein to the formulation. This maize protein was chosen due to its hydrophobic properties in order to form a more stable core in the particles. To prepare the particles, the total amount of protein – 10 mg/mL – was maintained and thus the particles were prepared with 8.8 mg/mL BSA, 0.2 mg/mL asparaginase, 1.0 mg/mL zein and 5 % (v/v) of vegetable oil. The solution was sonicated for a total of 6 min (8 s on + 2 s off), with the enzyme added before the ultrasounds cycles, for BSA:Asparaginase:Zein B, or after 69 s of ultrasounds cycles, for BSA:Asparaginase:Zein D. The efficiency of particle formation was 93.96 % for BSA:Asparaginase:Zein B and 94.59 % for BSA:Asparaginase:Zein D.

The activity of immobilized asparaginase on the BSA:Asparaginase:Zein particles was compared with the activity of free asparaginase (Figure 41).

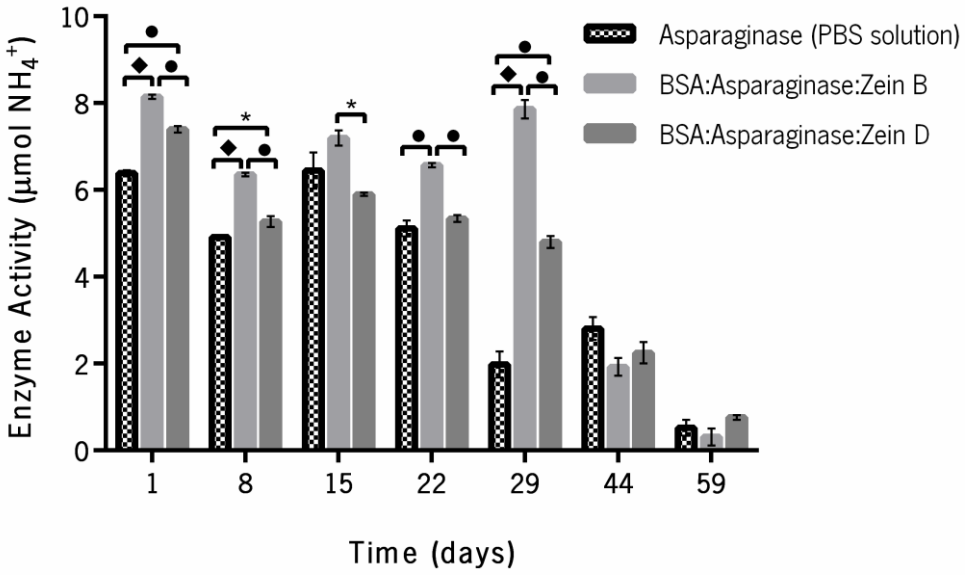

Figure 41 - Activity of asparaginase immobilized on BSA:Zein particles, prepared by ultrasounds, and free asparaginase (10 mg/mL) on a PBS solution, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ●p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

It was evident by the analysis of Figure 41 that the activity of immobilized asparaginase was significantly higher than the activity of free asparaginase until day 44. From this point and until the end of the storage time, the activity between the free asparaginases and the particles did not show any significant difference. At the end of storage time, both BSA:Asparaginase:Zein particles still demonstrated

some activity. The inclusion of zein in the particles formulation maintained the levels of asparaginase activity higher and for a longer period.

To compare the activities of both particles, the activities were plotted against the storage time at 4 °C, on Figure 42. Along time, the activity of BSA:Asparaginase:Zein B particles was generally always higher than the activity of BSA:Asparaginase:Zein D particles. This could be explained by a different location of asparaginase in the particle when added after 69 s of ultrasounds cycles. In BSA:Asparaginase:Zein D, the enzyme could have less freedom for the conformational change necessary for the enzyme activity, or the microenvironment in which the enzyme molecule is located could not be so favorable for the reaction.

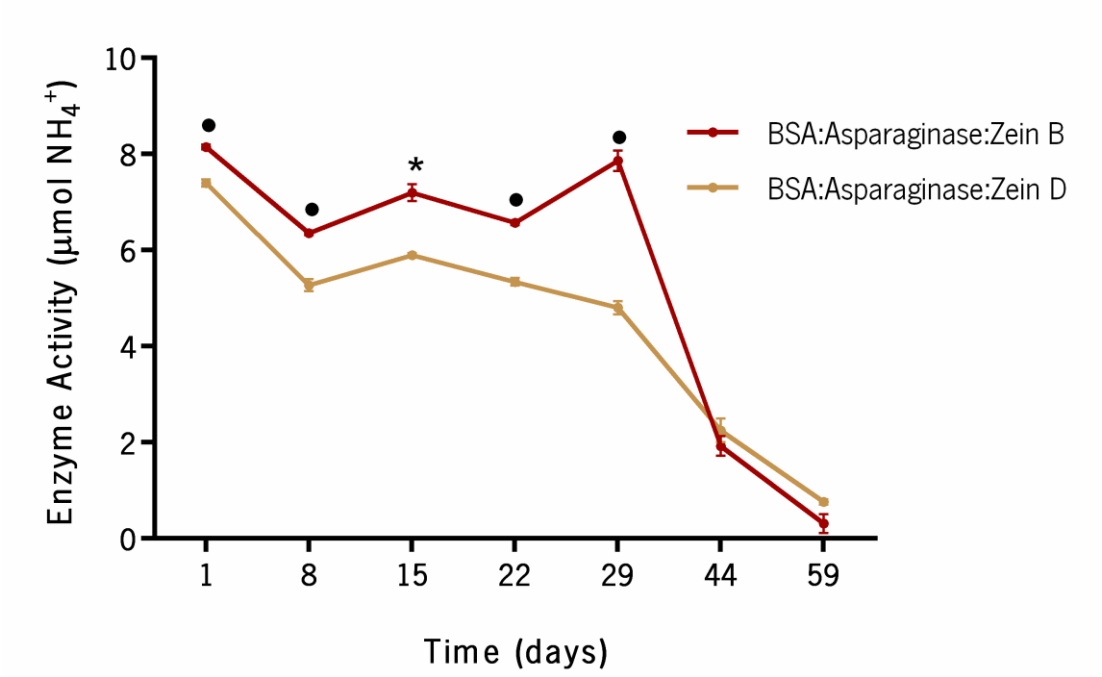

Figure 42 - Effect on asparaginase activity of adding the enzyme at different stages during the process of particles synthesis. BSA:Asparaginase:Zein B – enzyme added before the sonication cycles. BSA:Asparaginase:Zein D – enzyme added during the sonication cycles, after 69 s. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

To analyze the stability of both formulations, the characteristics of BSA:Asparaginase:Zein B (Figure 43) and BSA:Asparaginase:Zein D (Figure 44) particles were measured during the 2 months of storage at 4 °C.

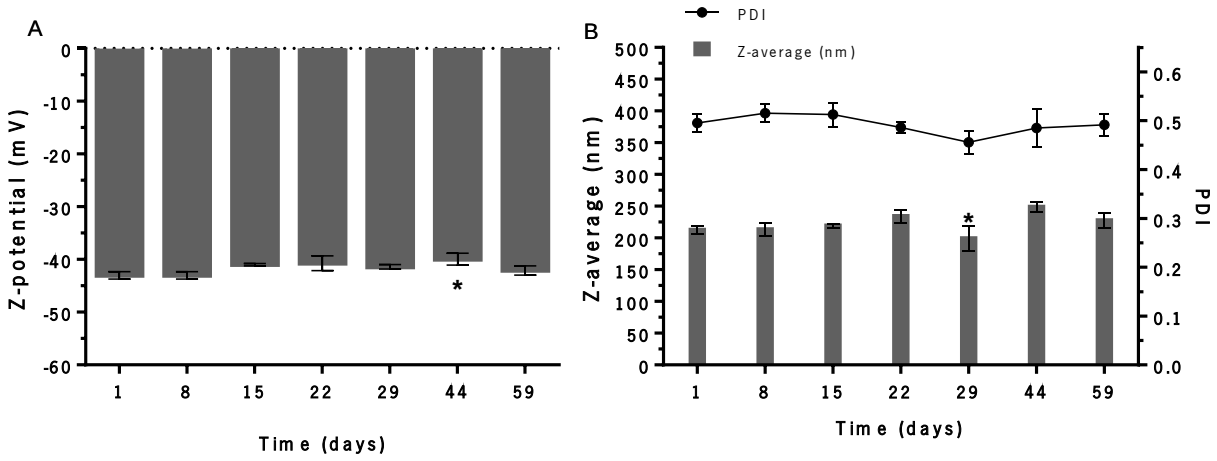

Figure 43 - Characterization of BSA:Asparaginase:Zein B particles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

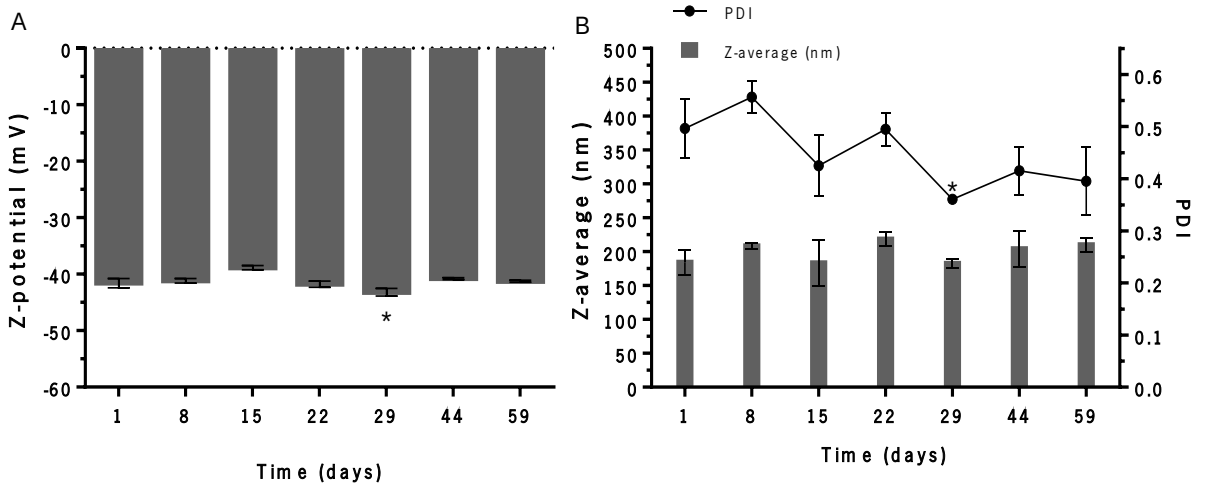

Figure 44 -Characterization of BSA:Asparaginase:Zein D particles, during storage at 4°C: A) Z-potential; B) Z-average and PDI Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

At day 1, BSA:Asparaginase:Zein B particles showed a Z-average size of 212.27±6.39 nm, a PDI of 0.495±0.0177 and a Z-potential of -43.03±0.702 mV. The BSA:Asparaginase:Zein D particles showed a Z-average size of 189.27±18.94 nm, a PDI of 0.4967±0.0571 and a Z-potential of -41.60 ±0.819 mV. The addition of the enzyme at different stages during the process of particle synthesis did not change significantly the characteristics of size, PDI and charge of the particles.

It was prepared BSA:Zein particles composed by 9 mg/mL BSA, 1mg/mL zein and 5 % (v/v) of vegetable oil, in the same conditions of ultrasounds cycles. These particles showed a Z-average of 149±18.358 nm, a PDI of 0.429±0.0125 and a Z-potential of -21.4±0.3606 mV. Comparing with BSA:Asparaginase:Zein particles, the addition of enzyme during the ultrasounds cycles did not change significantly the characteristics of size and PDI of the particles but when the enzyme was added before the ultrasounds cycles, it was observed a significant increase in the size and PDI of the particles (p-value≤0.01). The addition of enzyme to the BSA:Zein particles resulted in an increase of particles net negative charge. At pH of 7.6 the enzyme is deprotonated thus exhibits a negative charge.

Comparing the characteristics of BSA:Asparaginase with BSA:Asparaginase:Zein particles, was observed that the addition of zein to the formulation leaded to a significant decrease on the Z-average size of the particles. The PDI was no affected but the Z-potential also significantly changed, becoming less negative.

4.4.1.4. BSA:Asparaginase:Zein:Pol407 B and BSA:Asparaginase:Zein:Pol407 D Nanoparticles

The surfactant, Poloxamer 407 was added to the formulation BSA:Asparaginase:Zein. The new particles were prepared with 8.8 mg/mL BSA, 0.2 mg/mL asparaginase, 1.0 mg/mL zein, 5.0 mg/mL Poloxamer 407 and 5 % (v/v) of vegetable. The solution was then sonicated for a total of 6 min (8 s on + 2 s off), with the asparaginase added before the ultrasounds cycles, for BSA:Asparaginase:Zein:Pol407 B, or after 69 s of ultrasounds cycles, for BSA:Asparaginase:Zein:Pol407 D.

The activity of immobilized asparaginase over time was compared with the activity of the free enzyme (Figure 45). The activity of immobilized asparaginase was maintained during the first 15 days of storage however from this time point, there was a constant decrease on enzyme activity until day 59. After 59 days of storage at 4 °C, the only particle that had the capacity to hydrolyze asparagine was the BSA:Asparaginase:Zein:Pol407 B particles. Comparing the activities of immobilized asparaginases with the activity of free asparaginase, it was higher at day 8 for both particles and at day 29 for the BSA:Asparaginase:Zein:Pol407 B.

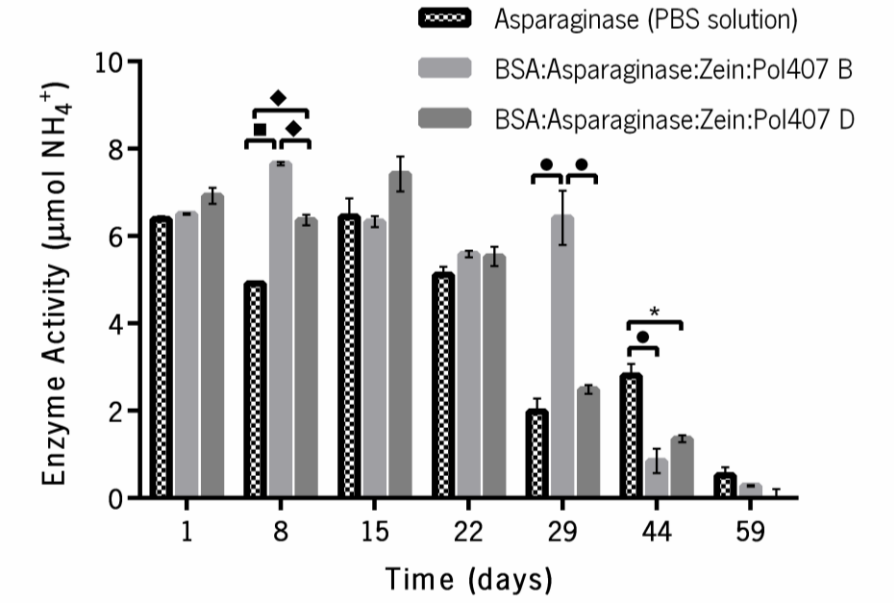

Figure 45 - Activity of asparaginase immobilized on BSA:Zein:Pol407 particles, prepared by ultrasounds, and asparaginase 10 mg/mL on a PBS solution, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The activity of BSA:Asparaginase:Zein:Pol407 B and BSA:Asparaginase:Zein:Pol407 D particles were directly compared on Figure 46. The differences between the two particles were only significant at day 8 and 29. However at the end of storage only the BSA:Asparaginase:Zein:Pol407 B had activity.

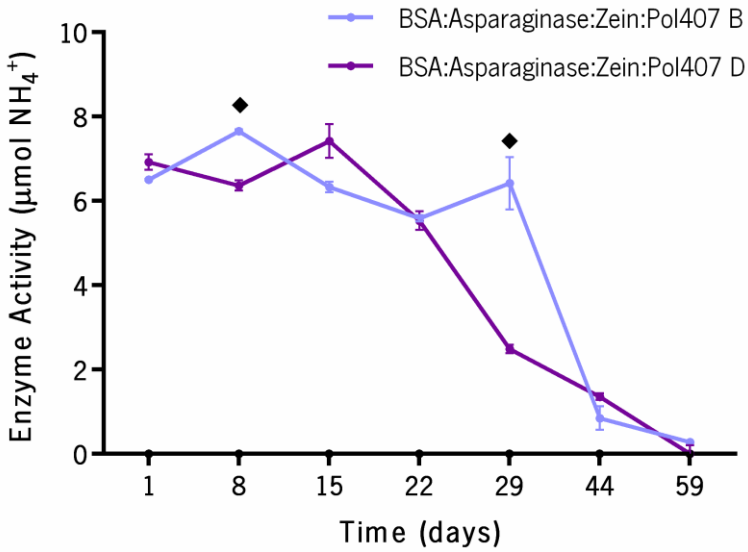

Figure 46 - Effect on asparaginase activity of adding the enzyme at different stages during the process of particles synthesis. BSA:Asparaginase:Zein:Pol407 B – enzyme added before the sonication cycles. BSA:Asparaginase:Zein:Pol407 D – enzyme added during the sonication cycles, after 69 s. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The BSA:Asparaginase:Zein:Pol407 B particles had a Z-average of 182.73±5.65 nm, a PDI of 0.444±0.0243 and a Z-potential of -8.83±0.5977 mV (Figure 47). While, the BSA:Asparaginase:Zein:Pol407 D had a Z-average size of 184.43±1.387 nm, a PDI of 0.4167±0.0064 and a Z-potential of -10.207±0.208 mV (Figure 48).

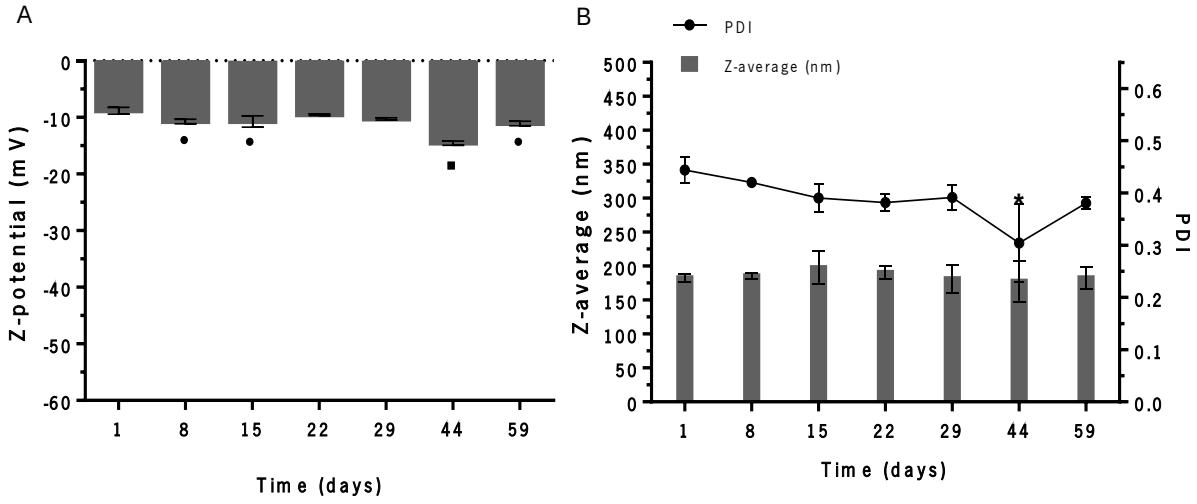

Figure 47 – Characterization of BSA:Asparaginase:Zein:Pol407 B particles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

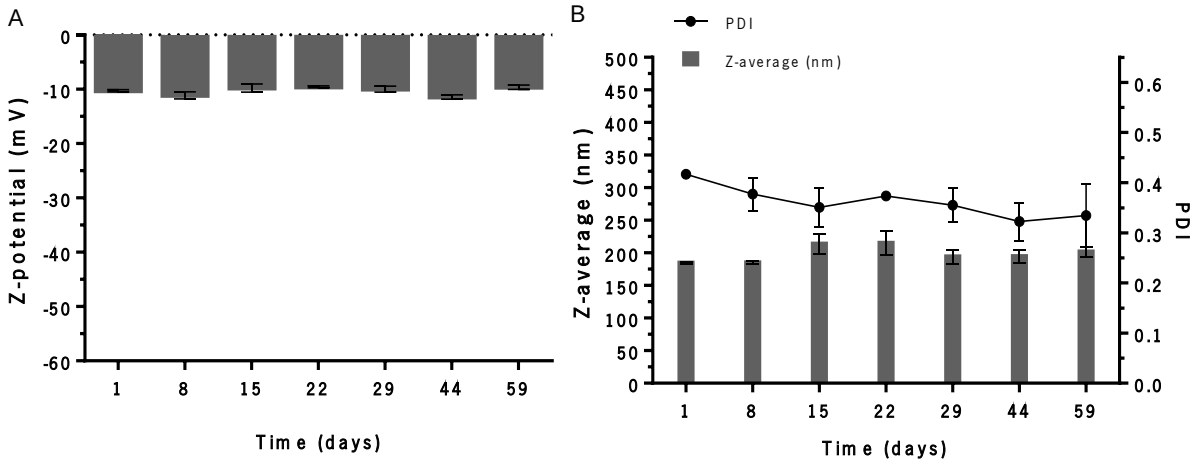

Figure 48 – Characterization of BSA:Asparaginase:Zein:Pol407 B particles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001, compared to the results obtained at day 1.

The addition of the enzyme during the ultrasounds cycles did not change significantly the size and PDI of the particles comparing with BSA:Asparaginase:Zein:Pol407 B, although the Z-potential become significantly more negative. The alteration on Z-potential can be due to the presence of more asparaginases located at the surface of the particle or less Poloxamer 407 molecules covering the particle surface.

It was prepared BSA:Zein:Pol407 B particles, with 9.0 mg/mL BSA, 1.0 mg/mL zein, 5.0 mg/mL Poloxamer 407 and 5 % (v/v) of vegetable oil, sonicated on the same conditions that the particles prepared before. The particles had a Z-average size of 177.75±6.576 nm, a PDI of 0.4153±0.0604 and a Z-potential of -10.367±0.6506 mV.

Comparing the characteristics of the BSA:Zein:Pol407 particles with the BSA:Asparaginase:Zein:Pol407 D particles, no significant differences were observed by the addition of asparaginase during sonication. However when the enzyme was added before sonication it decreased the Z-potential of the BSA:Asparaginase:Zein:Pol407 B particles (p-value≤0.05).

The effect of adding Poloxamer on the particles physical characteristics was only evident on the Z-potential, particles with less net negative charge, and on the PDI, particles population more homogenous.

By the analysis of Figure 47 and Figure 48, it was evident that BSA:Asparaginase:Zein:Pol407 D particles were more stable than the BSA:Asparaginase:Zein:Pol407 B, since no significant changes were verified during the 2 months of storage time at 4 °C.

4.4.2. Nanoparticles prepared by Homogenization

4.4.2.1. BSA:Asparaginase B and BSA:Asparaginase D Nanoparticles

Some of the particles described previously were also prepared by a high pressure homogenization process. The formulation used for the synthesis of BSA:Asparaginase nanoparticles was 9.8 mg/mL BSA, 0.2 mg/mL asparaginase and 0.5 % (v/v) of vegetable oil, in PBS. Two different nanoparticles were prepared, BSA:Asparaginase B nanoparticles, when the enzyme was added before homogenization, and BSA:Asparaginase D when the enzyme was added after 5 homogenization cycles.

The efficiency of particle formation for BSA:Asparaginase B was 95.2 % and 91.6 % for the BSA:Asparaginase D.

The activity of these nanoparticles along two months of storage is represented on Figure 49. The activity of immobilized asparaginase was maintained until day 15 and then started to gradually decrease until day 59. Relatively to the free asparaginase, the immobilization did not showed any significant improvement on the enzyme activity. The decrease on enzyme activity after immobilization could be related with harsh conditions employed during the immobilization process (high pressure and a temperature close to 50 °C), with diffusional problems between the substrate and the enzyme, with the steric hindrance that block the substrate access to the enzyme and loss of enzyme conformational freedom (Garcia-Galan *et al.*, 2011; Zhang *et al.*, 2013).

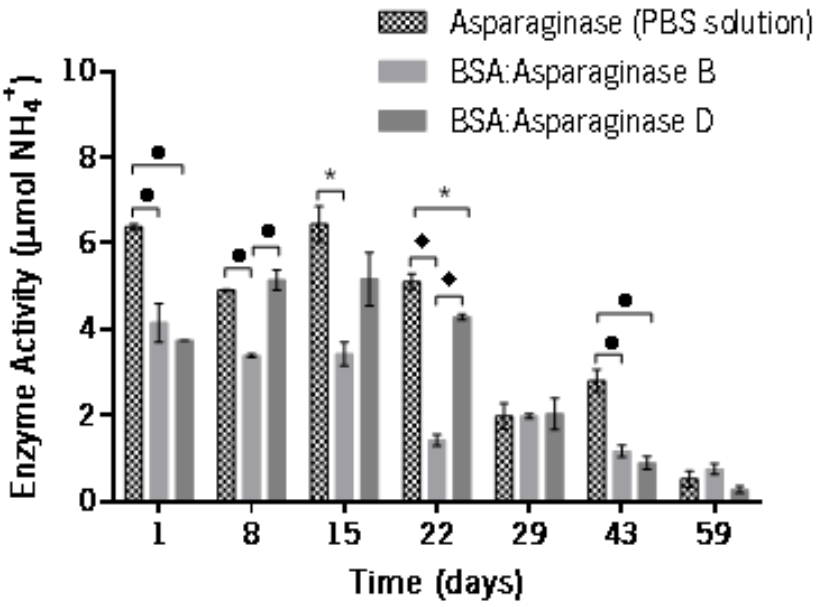

Figure 49 - Activity of asparaginase immobilized on BSA nanoparticles prepared by homogenization (BSA:Asparaginase B and BSA:Asparaginase D), and free asparaginase (10 mg/mL) on a PBS solution, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

Comparing the two formulations (Figure 50), it was observed an improvement on the activity when the asparaginase was added during the homogenization cycles. This improvement was more pronounced during the 8-22 days of storage and it was significantly higher at day 8 and 22. For the last month of the storage, the enzyme immobilized on both nanoparticles did not showed any significant difference on activity.

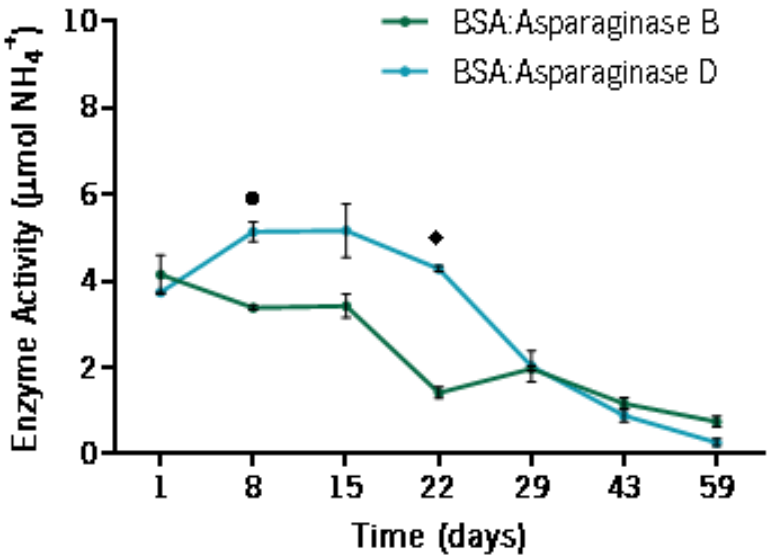

Figure 50 - Effect on asparaginase activity of adding the enzyme at different stages during the process of nanoparticles synthesis by homogenization. BSA:Asparaginase B – enzyme added before the homogenization cycles. BSA:Asparaginase D – enzyme added after 5 homogenization cycles. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

With this method to prepare nanoparticles, it was possible to obtain a nanoemulsion composed by only one population with a Z-average size lower than 200 nm. Relatively to the characteristics of the nanoparticles, the Z-average, PDI and Z-potential were accessed every weak for two months.

The characteristics of the BSA:Asparaginase B nanoparticles are represented on Figure 51. At day 1, the BSA:Asparaginase B nanoparticles had an Z-average size of 193.4±1.87 nm, a PDI of 0.130±0.00757 and a Z-potential of -36.57±1.498 mV. Regarding size, the nanoparticles were stable during the storage time with a significant increase in size only at day 44. The PDI showed a significant variation during storage, verifying an increase of the PDI over time to values bellow 0.2. The Z-potential exhibited some alterations at day 11 and 44, most likely due to errors during measurement, but for the remaining days this value was stable around -36 mV.

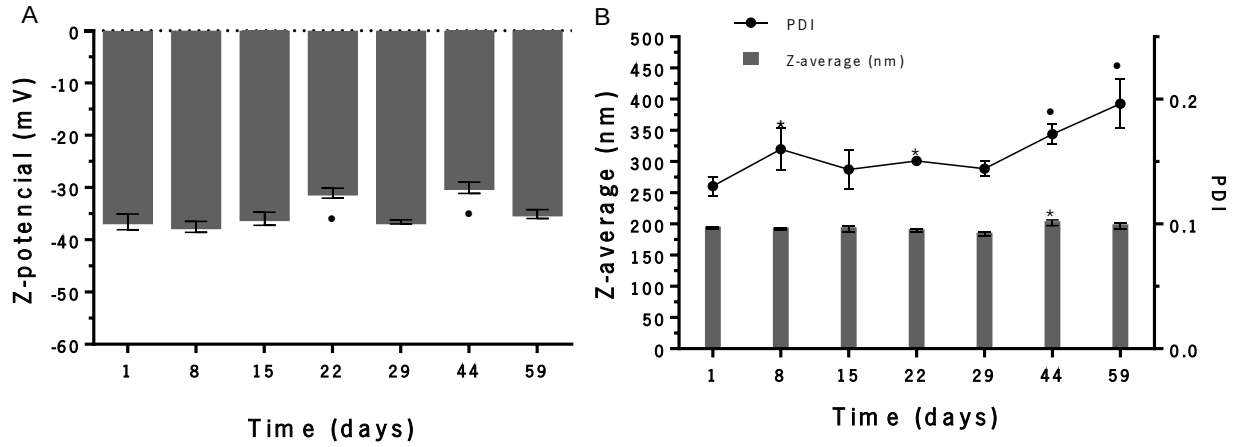

Figure 51 - Characterization of BSA:Asparaginase B nanoparticles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The BSA:Asparaginase D nanoparticles had a Z-average size of 196.2±1.267 nm, a PDI of 0.144±0.0215 and a Z-potential of -26.67±0.153 mV (Figure 52). In terms of stability, however there was no significant alteration of PDI during the two months storage, both size and Z-potential varied between day 22 and day 59.

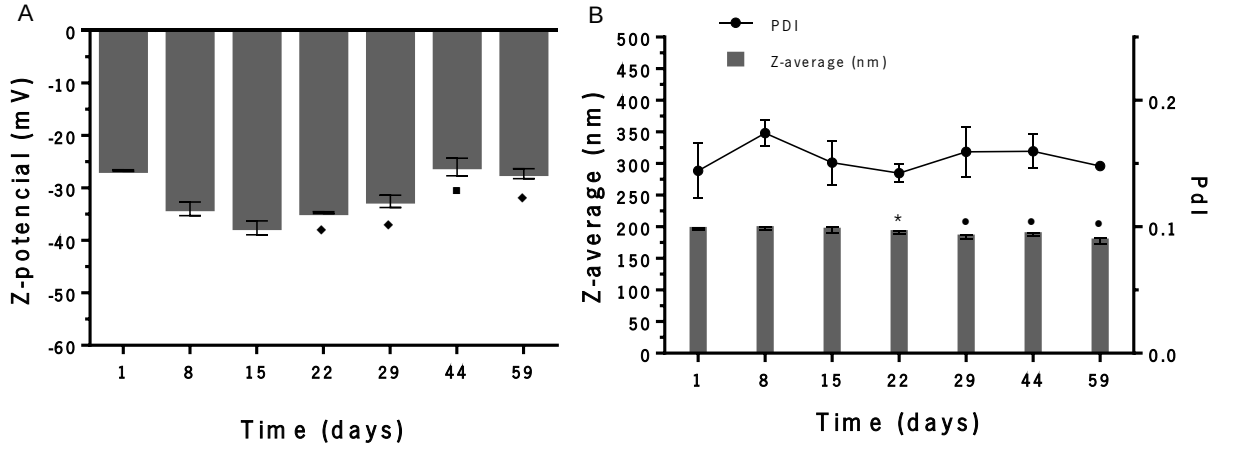

Figure 52 - Characterization of BSA:Asparaginase D nanoparticles, during storage at 4 °C: A) Zeta-potential; B) Z-average and PDI of nanoparticles. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

Comparing the characteristics of both nanoparticles, the addition of enzyme during the homogenization cycles only altered significantly the Z-potential. The BSA:Asparaginase D nanoparticles

had a lower net negative charge, which can be due to less enzyme immobilized on the nanoparticle or immobilized on a less superficial position on the nanoparticles.

4.4.2.2. BSA:Asparaginase:Pol407 B and BSA:Asparaginase:Pol407 D Nanoparticles

To study the effect of the addition of a surfactant to the nanoparticles prepared by homogenization, it was added to the previously formulation 5 mg/mL of Poloxamer 407. The enzyme was again added to the nanoparticles before the homogenization cycles, BSA:Asparaginase:Pol407 B, or after 5 homogenization cycles, BSA:Asparaginase:Pol407 D. The efficiency of particle formation with Poloxamer 407 were around 90% for both nanoparticles. It was obtained a nanoemulsion composed by only one population with a Z-average size of 84 nm, approximately.

The activity of both nanoparticles was determined weakly for two months and the activities are represented on Figure 53.

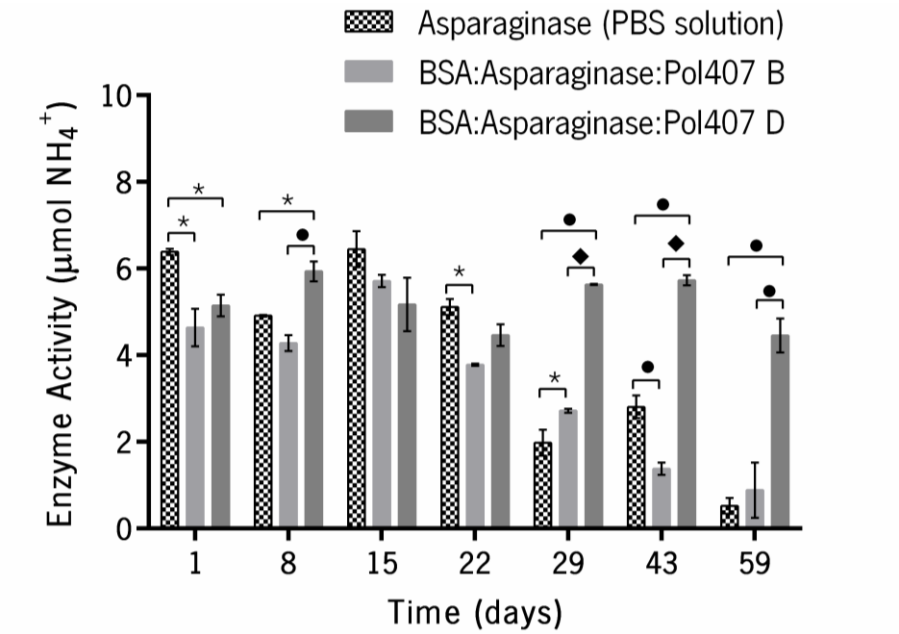

Figure 53 - Activity of asparaginase immobilized on BSA:Pol407 nanoparticles prepared by homogenization (BSA:Asparaginase:Pol407 B and BSA:Asparaginase:Pol407 D), and free asparaginase (10 mg/mL) on a PBS solution, after storage at 4 °C. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

For BSA:Asparaginase:Pol407 B it was observed a decrease on the asparaginase activity over time. Even though most of the formulations developed previously did not retained any activity or retained low levels of activity after two months at 4 °C, the BSA:Asparaginase:Pol407 D nanoparticles still had a high asparaginase activity at the end of storage time (4.46±0.391 μmol NH_4^+).

Comparing the activity of both nanoparticles (Figure 54), it was observed a significant improvement of the asparaginase activity when the enzyme was added to the nanoparticles during the homogenization process. During the two months of storage time, the enzyme only lost 13 % of its initial activity. Between all the developed nanoparticles, prepared by ultrasounds and prepared by homogenization, the BSA:Asparaginase:Pol407 D nanoparticles (homogenization) provided a major enzyme stability and showed the capacity to retain a high enzyme activity two months after synthesis.

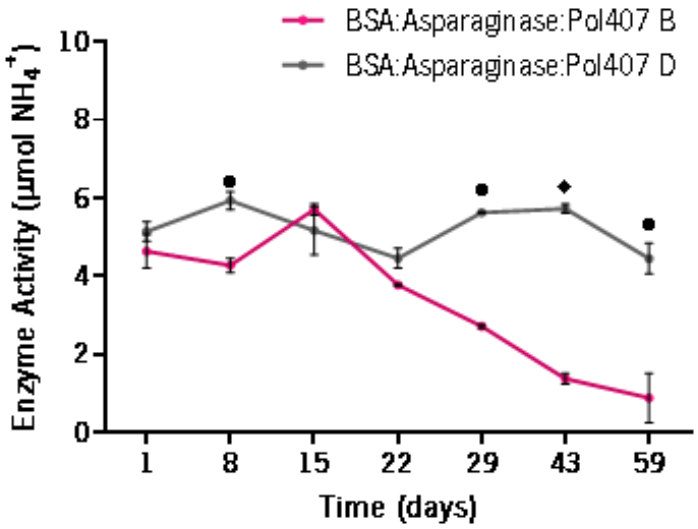

Figure 54 - Effect on asparaginase activity of adding the enzyme different stages during the process of nanoparticles synthesis by homogenization. BSA:Asparaginase:Pol407 B – enzyme added before the homogenization cycles. BSA:Asparaginase:Pol407 D – enzyme added after 5 homogenization cycles. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The addition of Poloxamer 407 changed the physical characteristics of the nanoparticles when compared with the nanoparticles prepared by homogenization without Poloxamer 407. The BSA:Asparaginase:Pol407 B nanoparticles presented an initial Z-average size of 82.22±0.967 nm, a PDI of 0.147±0.0138 and a Z-potential of -6.8±0.154 mV (Figure 55).

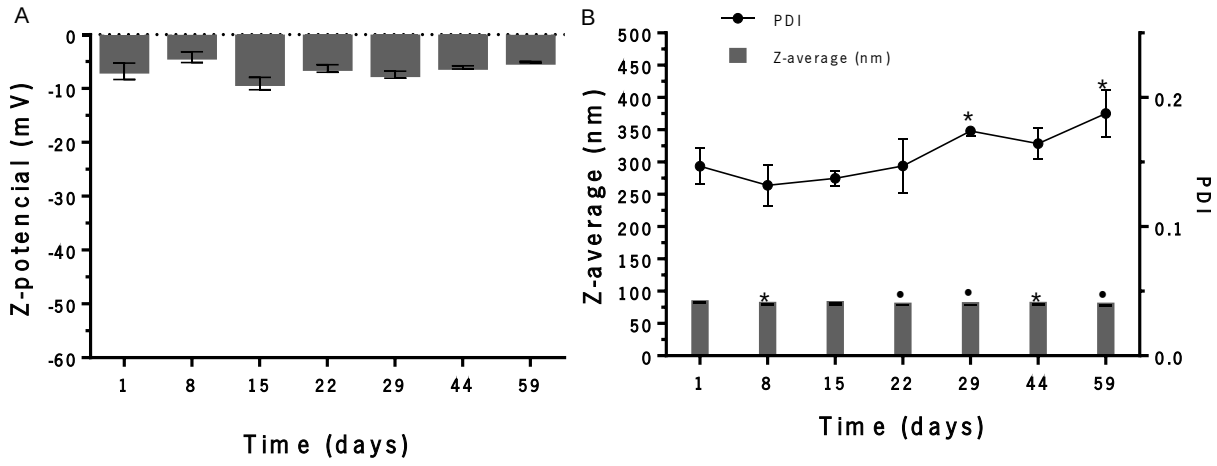

Figure 55 - Characterization of BSA:Asparaginase:Pol407 B nanoparticles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

Relatively to the Z-potential, the BSA:Asparaginase: Pol407 B nanoparticles were stable during all the storage time while the Z-average slightly changed weekly. The initial PDI was low but increased significantly over time until achieve a value of 0.1877±0.01803 mV.

The characteristics of BSA:Asparaginase:Pol407 D nanoparticles are represented on Figure 56. These nanoparticles had an initial Z-average size of 84.25±1.001 nm, a PDI of 0.125±0.0156 and a Z-potential of -2.31±1.05 mV.

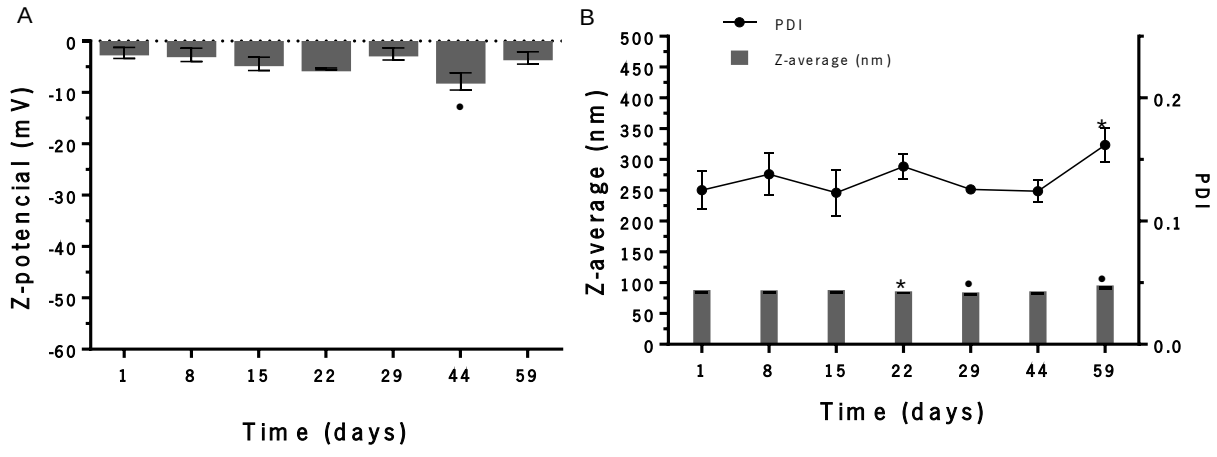

Figure 56 - Characterization of BSA:Asparaginase:Pol407 D nanoparticles, during storage at 4 °C: A) Z-potential; B) Z-average and PDI. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

It was also verified an increase of the PDI over time and the Z-potential was almost stable during the two months, with exception to day 44 where there was a higher variation on the net surface charge of the particle. The Z-average slightly change every week after 22 days at 4°C.

Comparing the characteristics of both nanoparticles, the addition of the enzyme during the homogenization cycles leaded to bigger nanoparticles, with lower PDI and less negative Z-potential. Comparing all the nanoparticles prepared by homogenization, the addition of Poloxamer 407 changed radically the characteristics of the nanoparticles. This surfactant demonstrated the capacity to decrease the size of the nanoparticles to less than half; to produce a more homogeneous nanoemulsion and to turn the Z-potential of the nanoparticles less negative.

4.4.3. Comparison of Asparaginase activity on different nanoparticles

4.4.3.1. Effect of the addition of Poloxamer 407

To study the impact of Poloxamer 407 on the catalytic properties of asparaginase, the activities of the enzyme immobilized on the different nanoparticles with and without Poloxamer 407 were plotted on Figure 57 and Figure 58. Figure 57 correspond to the effect of Poloxamer 407 on nanoparticles prepared by ultrasounds, while Figure 58 correspond to nanoparticles prepared by the homogenization method.

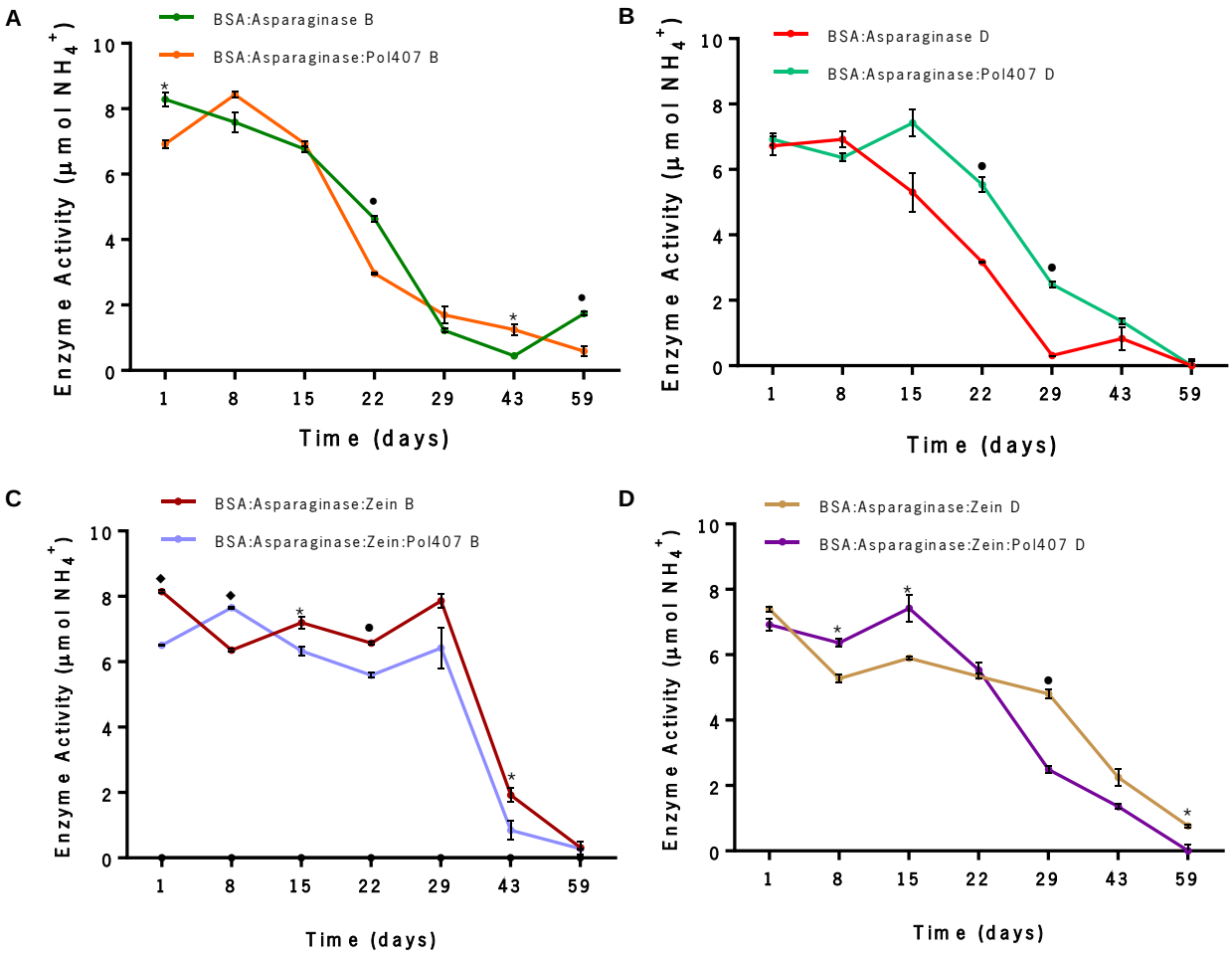

Figure 57 – Effect of the addition of Poloxamer 407 to the different nanoparticles prepared by ultrasounds: A) BSA:Asparaginase B; B) BSA:Asparaginase D; C) BSA:Asparaginase:Zein B; D) BSA:Asparaginase:Zein D. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The effect of Poloxamer 407 on the activity of immobilized asparaginase was different depending on the method used for the preparation of the nanoparticles. Considering the nanoparticles prepared by ultrasounds (Figure 57), for the BSA:Asparaginase nanoparticles it was observed an improvement on the activity of asparaginase by the addition of Poloxamer 407 only when the enzyme was added during the ultra-sounds cycles. When Poloxamer 407 was included to the BSA:Asparaginase:Zein nanoparticles, it was only observed an improvement on the asparaginase activity during the first 15 days of storage time for the BSA:Asparaginase:Zein:Pol407 D.

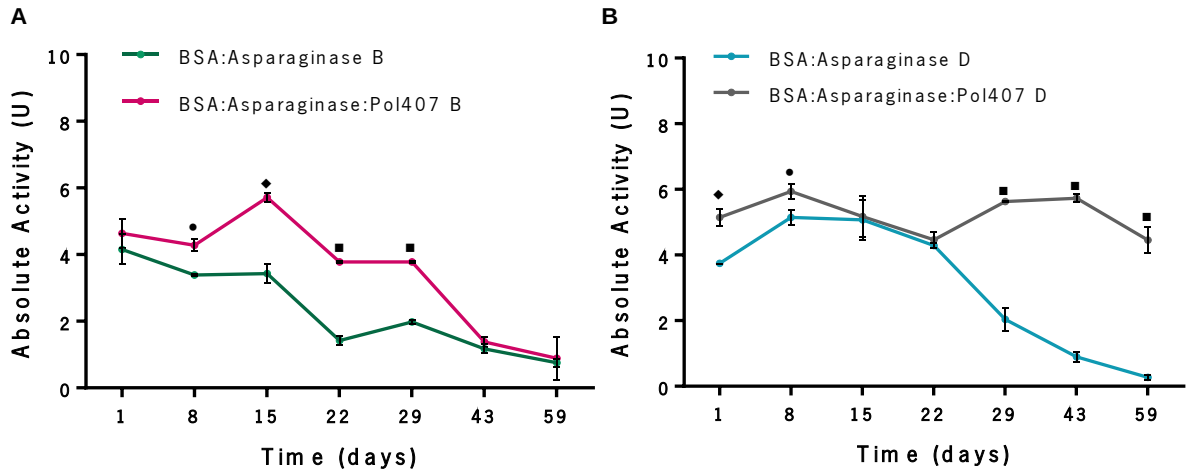

Figure 58 - Effect on asparaginase activity of the addition of Poloxamer 407 to the nanoparticles prepared by homogenization: A) BSA:Asparaginase B; B) BSA:Asparaginase D. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: *p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

Relatively to the nanoparticles prepared by homogenization (Figure 58), the addition of Poloxamer 407 improved the activity and the stability of the enzyme. For the BSA:Asparaginase B, it was observed an initial significant increase on the activity of immobilized asparaginase after the addition of this surfactant. For the BSA:Asparaginase D, it was observed a significant improvement on the activity of the immobilized asparaginase during the two months of storage.

Regarding the physical characteristics of the particles and the technique used during the particles synthesis, the addition of Poloxamer 407 decreased the PDI and turned the particles less negative relatively to the Z-potential (Figure 59). It also decreased the Z-average of the particles prepared by homogenization.

It was expected that the addition of Poloxamer 407 exert a protective effect and decrease protein inactivation during the storage time. Kim and coworkers previously reported the role of Poloxamer 407 on the stabilization of organophosphorus hydrolase. Poloxamer had the capacity to physically associate with the enzyme, due to interactions between the hydrophobic block of the Poloxamer and the hydrophobic amino acids of the enzyme. Theoretically, the Poloxamer will decrease the potential energy of the enzyme through the hydrophobic interactions, which will increase the native conformation stability and the activity of the enzyme (England, 1999; Kim *et al.*, 2014). The effect of increase the enzyme stability over time was only observed for asparaginase when immobilized on BSA:Asparaginase D

nanoparticles, prepared by homogenization. For the remaining nanoparticles, the addition of Poloxamer 407 did not inhibit the inactivation of the enzyme after two months of storage time.

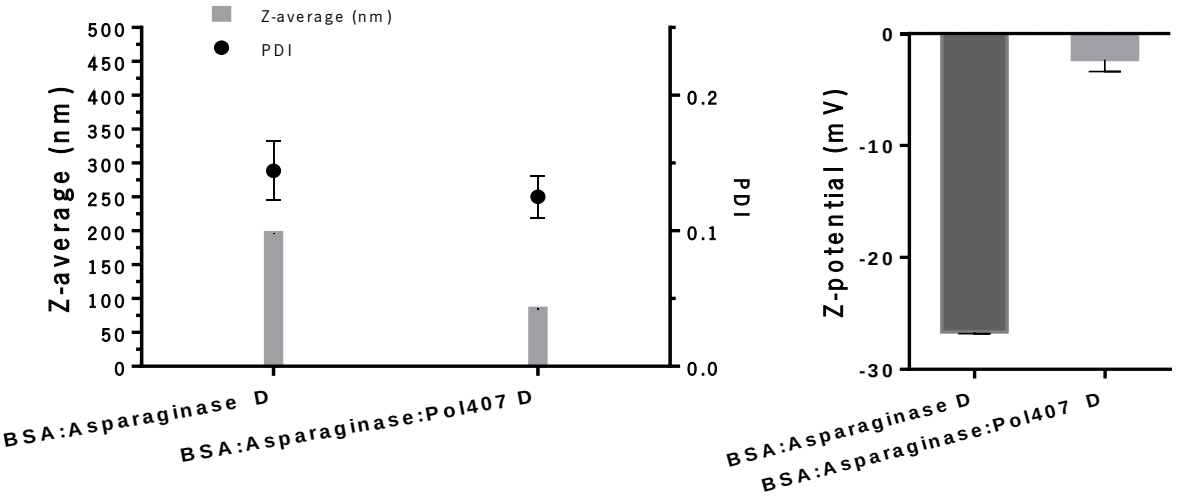

Figure 59 - Effect of Poloxamer 407 addition to the physical characteristics of the BSA:Asparaginase D nanoparticles, prepared by homogenization, one day after synthesis.

4.4.3.2. Effect of the addition of zein

To analyze the effect of adding zein to the particles, the activities of asparaginase immobilized on the particles with or without zein are represented on Figure 60. On the tested particles, zein was added to the formulations to act as a surfactant, in order to stabilize the particles and decrease the loss of enzyme activity.

Through the addition of zein, it was achieved an increase of the asparaginase activity for all the formulations presented on Figure 60 - BSA:Asparaginase B/D and BSA:Asparaginase:Pol407 B/D. This increase of activity was more pronounced during the first 29 days after particles synthesis by ultrasounds.

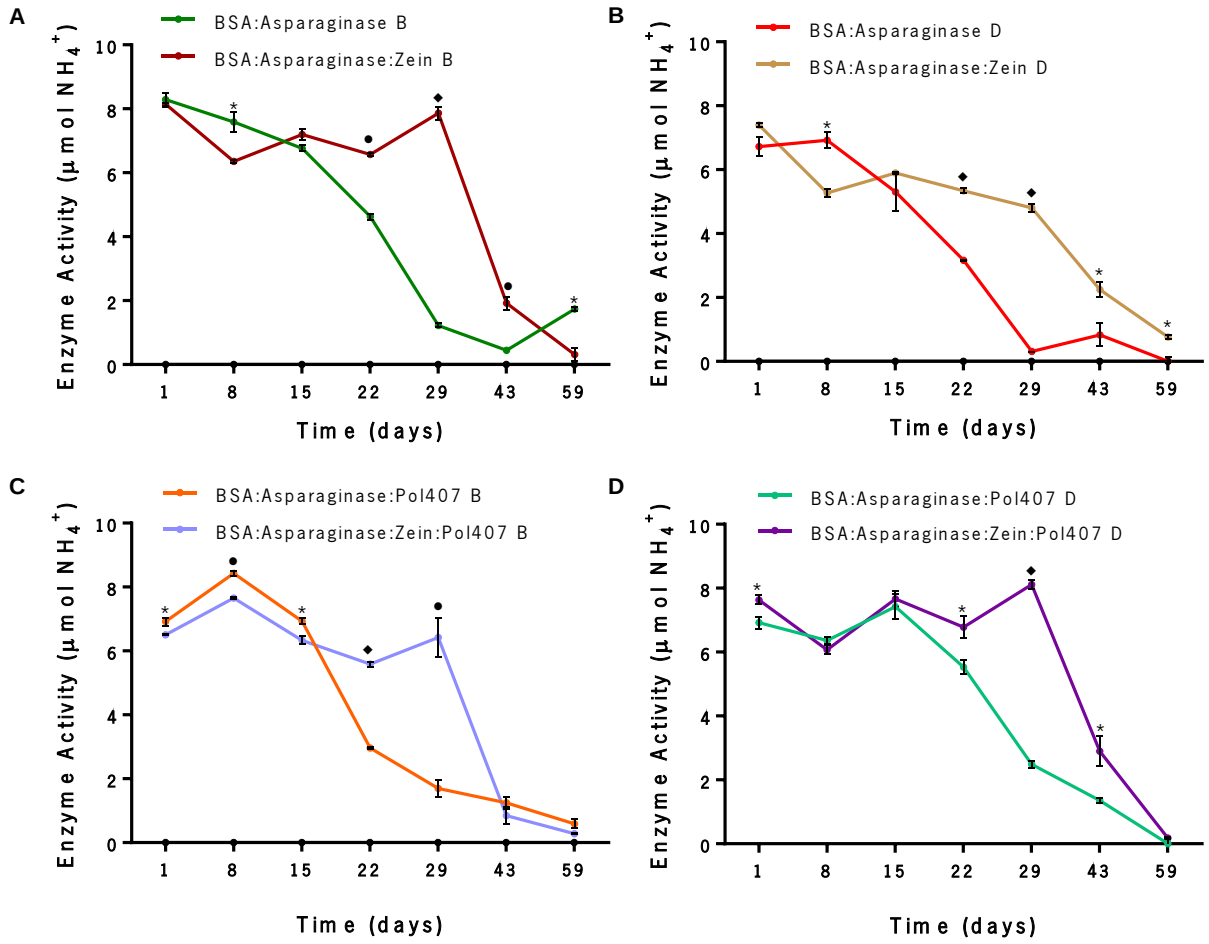

Figure 60 - Effect on asparaginase activity of the addition of zein to the different nanoparticles prepared by ultrasounds: A) BSA:Asparaginase B; B) BSA:Asparaginase D; C) BSA:Asparaginase:Pol407 B; D) BSA:Asparaginase:Pol407 D. The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min, at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm. Data were analyzed by one way-ANOVA: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The zein used for the synthesis of the particles was yellow zein, whose purity is around 88 - 90 %. This type of zein contains a high concentration of xanthophyll pigments, around 9 %, which are responsible for the yellow color of this maize protein (Podaralla and Perumal, 2012). This xanthophyll pigments include lutein, zeoxanthin and β-cryptoxanthin, and are known by their antioxidant capacities (Krinsky, 2002; Lim *et al.*, 1992). This antioxidant capacity is due to the unique structure of xanthophylls, that with the pattern of conjugated double bonds in the polyene backbone had the capacity to scavenge some reactive oxygen species like singlet molecular oxygen and peroxyl radicals (Stahl and Sies, 2003). The very high temperatures and pressures of collapsing gas bubbles during cavitation, lead to thermal

dissociation of water which generates hydrogen and hydroxyl radicals that may recombine to form hydrogen and hydrogen peroxide (Riesz *et al.,* 1985; Riesz *et al.*, 1992). The xanthopylls present on the zein may have the capacity to scavenge the free radicals formed during the preparation of the particles by ultrasounds, protecting the enzyme against this radicals.

The addition of zein did not changed significantly the properties of the particles with exception by a slight decrease on the net negative charge of the particles with zein, towards more positive values, and a slight decrease on the Z-average.

4.4.3.3. Effect of the Technique used for Particle Synthesis on Asparaginase Activity and Particle Properties

In order to evaluate which technique – ultrasounds and high pressure homogenization – originated particles which were more stable during storage, the activities BSA:Asparaginase:Pol407 D prepared by ultrasounds and homogenization were compared on Figure 61. These two particles were chosen for the study because the BSA.Asparaginase:Pol407 D (homogeneizer) was the only particle which maintained activity for longer times. The others particles prepared during asparaginase immobilization had similar activity profiles and thus are not presented here.

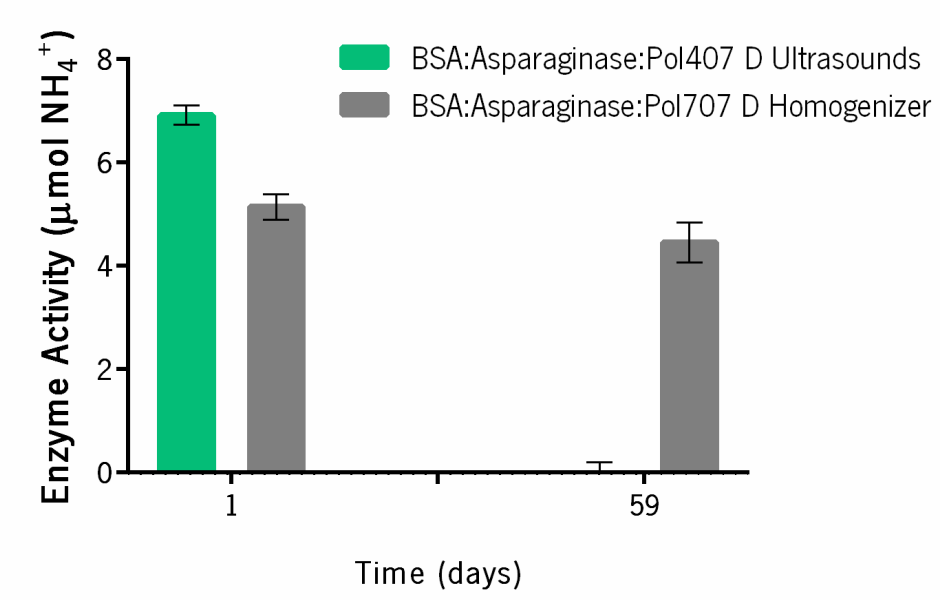

Figure 61 - Effect on asparaginase activity of the technique used for particles synthesis (ultrasounds or homogenizer). The reaction was performed with 0.001 g/L of asparaginase, 100 mM of asparagine for 240 min at 37 °C, in 50 mM Tris buffer, pH 8.6. The absorbance of the solutions after Nesslerization were measured at 436 nm.

When the particles were prepared by ultrasounds, it was observed a high asparaginase activity at day 1 relatively to the nanoparticles prepared by homogenization. Comparing both particles at day 1, the activity was 26 % lower when the nanoparticles were prepared by homogenization. Over time, it was observed a decrease on asparaginase activity except for the BSA:Asparaginase:Pol407 D (homogenizer) which had the capacity to hydrolyze 4.45±0.391 µmol of asparagine two months after nanoparticles' synthesis.

With respect to activities, the BSA:Asparaginase:Pol407 D (homogenizer) had the highest potential to be used in future applications, as they maintained a relatively constant asparaginase activity over time.

The physical characteristics of nanoparticles prepared by homogenization were significantly different from those with the same formulation but prepared by ultrasounds. The homogenization process had the capacity to produce smaller and more homogenous particles, principally the ones prepared with Poloxamer 407(Figure 62). The lower size and PDI are an advantages to a future application of this formulation, relatively to the one prepared by ultrasounds.

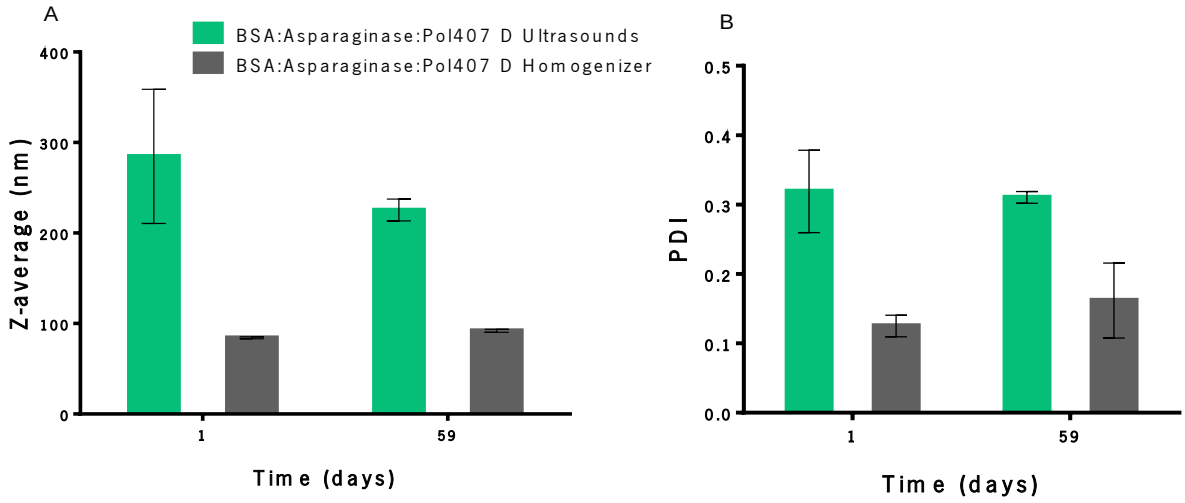

Figure 62 - Effect on Z-average (A) and PDI (B) of the technique used for the particles synthesis - ultrasounds or homogenizer.

The nanoparticles prepared by high pressure homogenization, particularly the BSA:Asparaginase:Pol407 D, had the best activity profile, best Z-average size and PDI to be used in future applications.

4.5. Determination of Asparaginase Activity by HPLC-MS

4.5.1. Protocol Optimization

There are several protocols described on literature to quantify the asparagine and aspartic acid amino acids. For the majority of the protocols it is only possible to quantify separately the two amino acids on a mixture after a derivatization process. This process has a lot of disadvantages like: interaction of the derivatization reagents with the sample medium that will affect the reaction efficiency; some derivatization protocols are very complex and time-consuming; the cost associated with the derivatization reaction; the formation of artifacts or several derivates from only one component and the reactions must be quantitative or at least reproducible (Frei and Santi, 1975). One of the goals of the work was the development of a protocol to quantify, in a mixture, both amino acids separately, without the derivatization reaction.

Initially, an aqueous solution with 20 % of acetonitrile of the individualized amino acids was injected on the mass spectrometer (MS) to determine the type of ionization that allowed the identification of the compounds. It was observed that the best ionization to identify the compounds was the positive mode ionization, and the asparagine presented m/z ratio of 132.89 (Figure 63 A) and the aspartic acid a m/z of 133.88 (Figure 63 B).

After confirming that both amino acids could be identified by mass spectroscopy, it was performed an analysis of the two amino acids by HPLC, to study the retention time of each amino acids individually and when mixed.

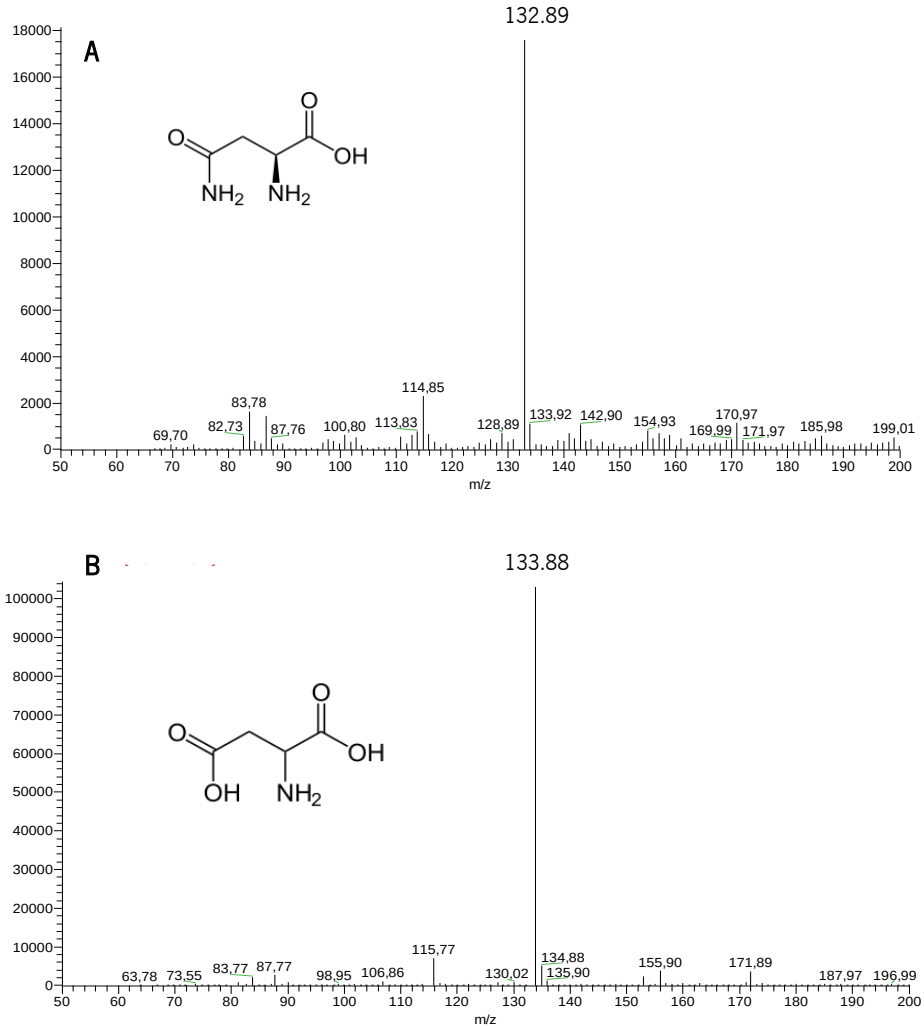

Figure 63 - Mass spectrum on positive ionization and chemical structure of A) asparagine; B) aspartic acid. Both amino acids were dissolved in acetonitrile 20 %.

A reverse-phase column, C18, with a flow rate of 0.2 mL/min and a sample volume of 25 µL was primarily tested. The mobile phase was 50 % of water with 0.1 % (v/v) formic acid and 50 % of acetonitrile during the 20 min of the analysis. A volume of 25 µL of each amino acid solution was injected and two chromatograms were obtained, both with the same retention time of 3.92 min (Figure 64). The protocol was not appropriated to quantify the two amino acids as the compounds were not eluted separately which lead to errors during the quantification.

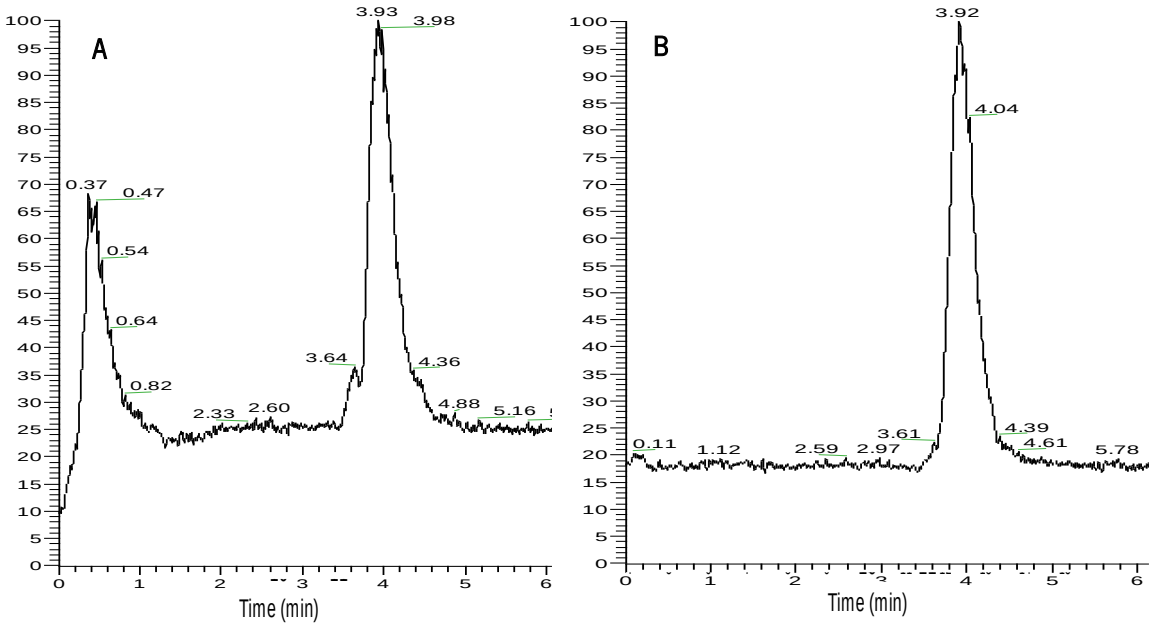

Figure 64 - Chromatograms of aqueous solutions of A) aspartic acid and B) asparagine, analyzed with a reverse phase C18 column and a mobile phase of 50 % water with formic acid and 50 % of acetonitrile.

After the first analysis, the mobile phase was altered two times in order to separate the two amino acids, keeping the other conditions constants. The eluents were also the same, water with 0.1 % (v/v) formic acid (A) and acetonitrile (B). The gradient of elution was: 0-14 min: 80 % A + 20 % B; 15-19 min: 40 % A + 60 % B; 20 min: 80 % A + 20 % B. With this alteration, there was a little difference between the retentions times of asparagine and aspartic acid (3.84 and 4.23 min, respectively) when analyzed isolated. However, when a mixture of both amino acids were analyzed, only a peak with a mean retention time of 3.89 min was observed, which means that asparagine and aspartic acid were eluted at the same time (Figure 65).

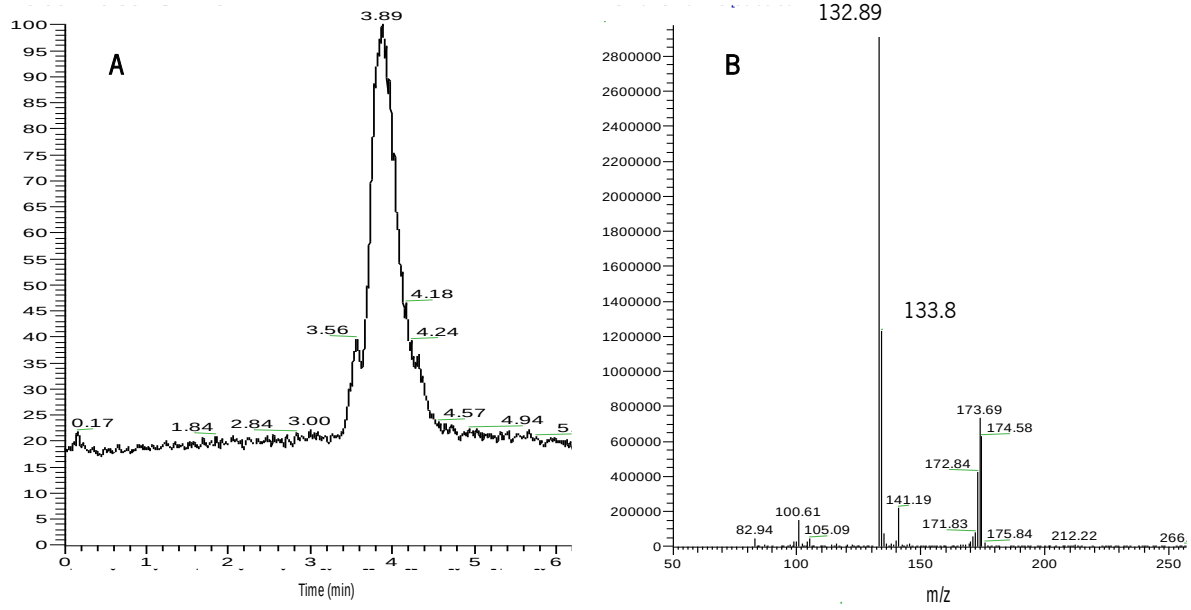

Figure 65 – A) Chromatogram of an aqueous mixture of asparagine and aspartic acid, analyzed with a reverse phase C18 column and a mobile phase of 50 % water with 0.1 % (v/v) formic acid and 50 % of acetonitrile; B) Mass spectrum of the compounds with a retention time of 3.89 min.

As both tested approaches did not allowed the separation of the two amino acids, it was tested a different column. It was chosen the Hydro C18 column from Phenomenex because it has a polar endcaped C18 phase that provides extreme retention of hydrophobic compounds and shows slight polar selectivity.

With the Hydro-C18 column, the aqueous solutions of asparagine and aspartic acid were analyzed with a flow rate of 0.2 mL/min and a gradient of elution of: 0-5 min: 80 % A + 20 % B; 5-15 min: 80 % A + 20 % B; 15-20 min: 40% A + 60 % B; 20 min: 80 % A + 20 % B (Figure 66). The volume injected of each solution was 25 µL. With this new column, the aspartic acid show a retention time of 7.17 min and asparagine of 5.64 min, although when a mixture of both amino acids was analyzed, the two compounds have the same retention time of 5.67 min. Maybe the two compounds were somehow interacting, altering in two minutes the retention time of aspartic acid.

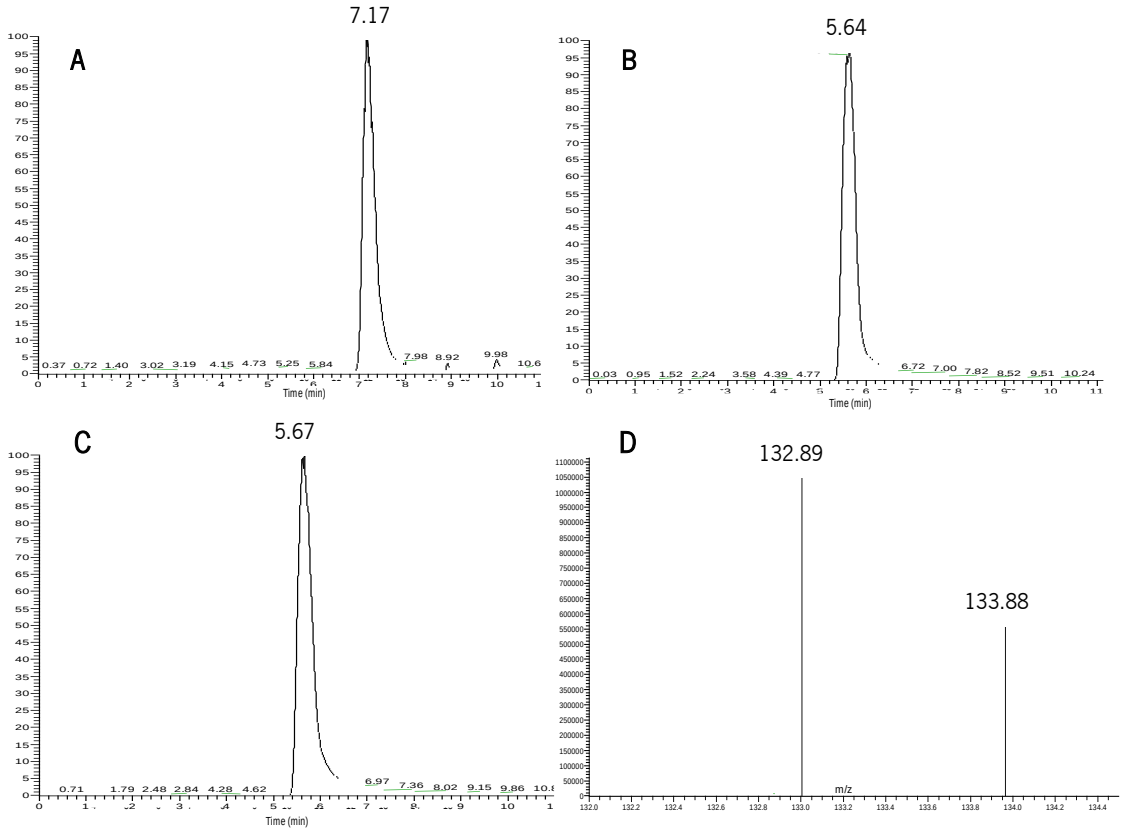

Figure 66 – Chromatogram of an aqueous solution of A) aspartic acid; B) asparagine; C) mixture of asparagine and aspartic acid; analyzed with a hydro-C18 column and a gradient of elution of: 0-5 min, 80 % A + 20 % B; 5-15 min, 80 % A + 20 % B; 15-20 min, 40 % A + 60 % B; 20 min, 80 % A + 20 % B.

It was necessary to change one more time the methodology. Comparing both tested columns, the Hydro-C18 allowed a better separation of the peaks with distinct retention times when the amino acids were analyze separately. Thus, it was selected for the new methodology. In the new approach, the eluents of the mobile phase were changed. The water with formic acid was replaced by a solution of ammonium bicarbonate, pH 6 adjusted with formic acid.

The eluent and the pH were chosen based on the pKa of the two amino acids (Table XII). At this pH asparagine and aspartic acid, charged differently which may help to separate the two components during the chromatography. Using an eluent with pH 6, the asparagine had the α-amino group positively charged and the α-carboxylic acid group negatively charged, while aspartic acid had both α-carboxylic acid group and side chain negatively charged and the α-amino group positively charged. So, the global charge of aspartic acid will be -1 while asparagine will have a neutral charge.

Table XII – pKa values of the ionizable groups of asparagine and aspartic acid.

Amino Acid	α-carboxylic acid	α-amino	Side chain
Asparagine	2.02	8.80	-
Aspartic Acid	2.10	9.82	3.86

With the Hydro-C18 column and acetonitrile (B) and ammonium bicarbonate (C) as eluents, it was tested an elution gradient of: 0-8 min: 95 % C + 5 % B; 8-15 min: 95 % C + 5 % B; 15-20 min: 40 % C + 60 % B; 20 min: 95 % C + 5 % B. The flow rate was maintained at 0.2 mL/min. With the changes in the eluent and in the elution gradient, it was possible to separate the two amino acids with retention times of 8.45 min for asparagine and 5.5 min for aspartic acid. Although, this difference decreased when was analyzed an aqueous mixture of both amino acids. Still, with the new protocol was possible to identify two peaks on the chromatogram of the mixture, one at 5.25 corresponding to aspartic acid and the other at 5.62 min corresponding to asparagine (Figure 67).

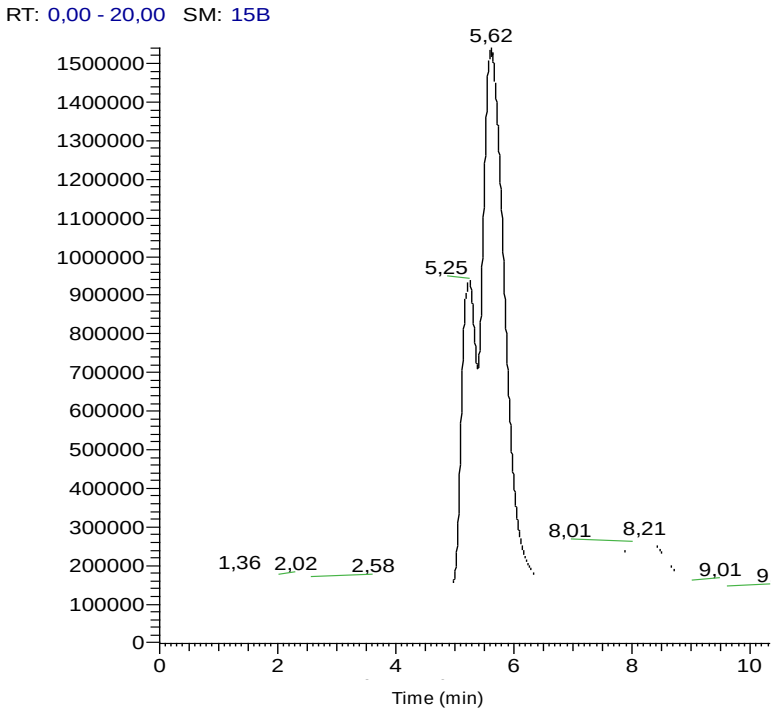

Figure 67 – Chromatogram of an aqueous mixture of asparagine and aspartic acid when analyzed with a Hydro-C18 column, with ammonium bicarbonate and acetonitrile as eluents and a flow rate of 0.2 mL/min.

To improve the separation between the two peaks, the analysis was performed on the same conditions but with a high flow rate of 0.3 mL/min. The parameters of the analysis of the HPLC and MS were automatically optimized for this flow rate: source voltage - 4.95 kV; source current - 0.60 μA; sheath gas - 40 arb; aux gas - 20 arb; sweep gas - 19.98 arb; capillary voltage - 17.77 V; capillary temperature - 274.88 °C and the tube lens voltage - 25.01 V. The elution gradient was maintained as in the last analysis: 0-8 min: 95 % C + 5 % B; 8-15 min: 95 % C + 5 % B; 15-20 min: 40 % C + 60 % B; 20 min: 95 % C + 5 % B.

When an aqueous solution of mixed asparagine and aspartic acid was analyzed under these conditions, it was possible to identify two separated peaks (Figure 68). The first with a retention time of 4.13 min corresponded to aspartic acid and the other with a retention time of 4.60 min to asparagine. Using this approach was possible to identify and separate the asparagine and aspartic acid molecules, when mixed on the same aqueous solution.

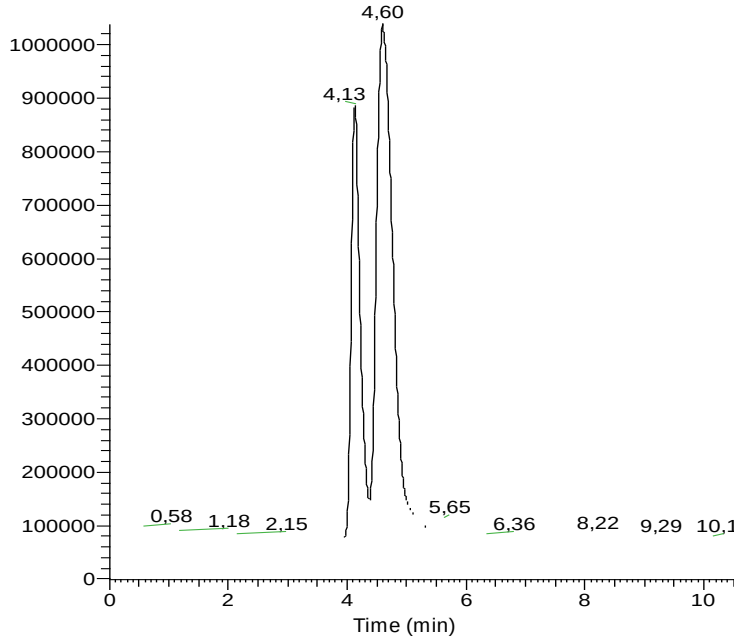

Figure 68 - Chromatogram of an aqueous mixture of asparagine and aspartic acid when analyzed with a Hydro-C18 column, with ammonium bicarbonate and acetonitrile as eluents and a flow rate of 0.3 mL/min.

4.5.2. Calibration Curves

After protocol optimization for the quantification of asparagine and aspartic acid, several solutions of both amino acids were prepared, in order to obtain calibration curves for each amino acid. The peak areas were plotted versus the amino acid concentration, and it was choosen a polinomial function for the

adjustment of the asparagine points and a linear funtion for the aspartic acid adjustment. The function adjusted to the asparagine (Figure 69 A) and aspartic acid (Figure 69 B) calibration curves was $y = -111677x^2 + 3.9647 \times 10^6 x$ and $y = 583946x$, respectively.

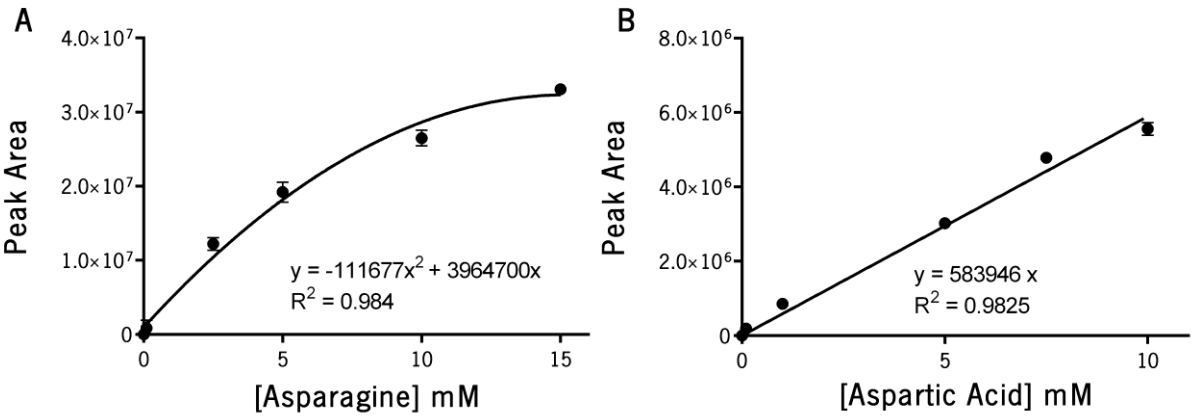

Figure 69 – Calibration curves of A) asparagine and B) aspartic acid, obtained by HPLC-MS with a Hydro-C18 column, with ammonium bicarbonate pH 6 (a) and acetonitrile (b) as eluents and a flow rate of 0.3 mL/min. The elution gradient was: 0-8 min: 95 % a + 5 % b; 8-15 min: 95 % a + 5 % b; 15-20 min: 40 % a + 60 % b; 20 min: 95 % a + 5 % b.

4.5.3. Determination of Asparaginase Activity by Aspartic Acid Quantification by HPLC-MS

After protocol otimization, the objective was to quantifie asparagine and aspartic acid resulting from the reaction of hydrolisis of asparagine by asparaginase. For the particles, the asparaginase concentration tested was 0.001 g/L and for the free enzyme was 0.001, 0.0015 and 0.002 g/L. The remain reaction conditions were 100 mM of asparagine, 37 °C, in ultra-pure water since Tris buffer affect the quantification of amino acids by HPLC. After 240 min of reaction, the enzyme was inactivated in a ice cold bath and removed from the reaction medium by ultra-centrifugation in eppendorfs with a membrane with a cut-off of 5000 Da. After ultra-centrifugation the membrane was washed with ultra-pure water to prevent amino acid entrapment in the membrane, and these water was also analysed.

The hydrolizes of asparagine into aspartic acid and ammonia of the samples free asparaginase, BSA:Asparaginase B, BSA:Asparaginase:Pol407 B, BSA:Asparaginase:Zein B and BSA:Asparaginase:Zein:Pol407 B particles, was determined by HPLC-MS and compared with the results of ammonia quantification by Nesselerization. All particles were synthesized on the day previously to the analysis.

The activities determined by aspartic acid quantification by HPLC-MS for the free enzyme and the enzyme immobilized on particles are shown on Table XIII. The asparagine quantification was not possible because the centrifugation necessary to remove the enzyme before the analysis altered the peak shape, which leaded to an incorrect quantification of this amino acid. To prevent this peak shape alteration, it was necessary to wash thoroughly the membrane previously centrifugation with ultra-pure water. When was discovered the reason that leaded to the peak shape alteration of asparagine, the sensibility of the HPLC-MS had been changed which make it necessary to repeat the calibration curve of asparagine. Due to the impossibility to repeat the calibration curve of asparagine, it was decided to determine the asparaginase activity only by the quantification of aspartic acid.

Table XIII – Asparaginase activity determined by the quantification of aspartic acid by HPLC-MS

Samples	[Aspartic Acid] (mM)	Asparaginase activity (μmol aspartic acid)
Asparaginase (0.001 g/L)	24.79	4.96
Asparaginase (0.0015 g/L)	44.91±3.91	8.98±0.78
Asparaginase (0.002 g/L)	61.22	12.24
BSA:Asparaginase B	52.52±1.95	10.5±0.39
BSA:Asparaginase:Pol407 B	37.93±0.96	7.60±0.19
BSA:Asparaginase:Zein B	40.44±1.95	8.07±0.39
BSA:Asparaginase:Zein:Pol407 B	39.91±2.89	7.98±0.58

Comparing the free asparaginase concentrations, it was observed an increase of the asparaginase activity with the increase of enzyme concentration on the reaction medium. For the same enzyme concentration of 0.001 g/L, it was observed that the activity of free asparagine was lower when compared to the activity of immobilized asparaginase. Analyzing the activities of the enzyme immobilized on the different particles, it was noticed that the activity of asparaginase immobilized on BSA:Asparaginase B had an activity significantly higher than the enzymes immobilized on the remaining particles. The particles with Poloxamer 407 showed a lower asparaginase activity may be due to the steric effect of the Poloxamer that may difficult the movement of the substrate to reach the enzymes' catalytical center.

The HPLC-MS protocol developed during this work, allowed the determination of asparaginase activity by aspartic acid quantification without a derivatization process.

4.6. Nanoparticles capacity to retain ammonia

In order to study the ability of particles with asparaginase to retain ammonia (main goal of this thesis), the hydrolysis products of asparagine (acid aspartic and ammonia) were simultaneously quantified after reaction with free asparaginase or immobilized asparaginase. The asparaginase concentration tested for the particles was 0.001 g/L and for the free asparaginase was 0.0015 g/L. It was chosen the 0.0015 g/L enzyme concentration for the free asparaginase since it was the concentration at which the conversion of asparagine into aspartic acid and ammonia was within the range of values determined for the particles. The ammonia obtained was quantified by Nesslerization while the aspartic acid was quantified using the developed HPLC-MS method (Table XIV).

Table XIV - Study of the capacity of the nanoparticles to retain ammonia at surface by electrostatic interactions.

Samples	Z-potential (mV)	[Aspartic Acid]* (mM)	[Ammonia]** (mM)	Ratio (aspartic acid/ammonia)
BSA:Asparaginase B	-43.90±0.79	52.52±1.95	27.35±0.43	1.92
BSA:Asparaginase:Pol407 B	-1.75±0.43	37.99±0.96	15.61±0.17	2.46
BSA:Asparaginase:Zein B	-41.90±2.20	40.36±1.95	19.52±0.23	2.07
BSA:Asparaginase:Zein:Pol407 B	-6.43±3.52	39.89±2.89	23.13±0.080	1.72
Asparaginase (0.0015 g/L)	-9.42±2.83	44.91±3.91	40.91±4.75	1.10

*Aspartic acid quantified by HPLC-MS
**Ammonia quantified by Nesslerization

One of the main objectives of the present work was the development and production of nanoparticles with the ability to retain the ammonia, resulting from the asparaginase activity, at the particles surface. The capacity to retain ammonia will result from the electrostatic interaction between the positively charged ammonia and the negatively charged groups located at the surface of the nanoparticles. It was expected that the particles with more negative Z-potential, as BSA:Asparaginase B and BSA:Asparaginase:Zein B, had a higher capacity to retain the ammonia than the particles with a less negative potential like the nanoparticles with Poloxamer 407.

Through the analysis of Table XIV, it was observed a higher aspartic acid concentration relatively to the ammonia concentration for all the tested samples, with the BSA:Asparaginase:POL407 B particles showing the higher aspartic acid/ammonia ratio. The ratio between the two products concentrations gave information about the particles ability to retain ammonia. If the value was higher than 1, the particle had the capacity to retain the ammonia resulting from the asparaginase activity. If this capacity to retain ammonia was due to electrostatic interactions, it was expected higher ratios for the BSA:Asparaginase B and BSA:Asparaginase:Zein B particles because of their more negative Z-potential. For the particles with zein, it was observed that relationship between the particles Z-potential and the aspartic acid/ammonia ratio. However, considering the particles without zein it was observed a higher ratio value for the particles with less negative Z-potential.

Neglecting the errors derived by the different sensitivities between the two methods used for the ammonia and aspartic acid quantification (absorbance spectroscopy and HPLC-MS, respectively), it was confirmed the ability of the particles with asparaginase to retain the ammonia, however it seems that this capacity it is not only related with the particles Z-potential. It was also observed some capacity to the free asparaginase to retain ammonia, although it was almost not significant.

4.7. Nanoparticles cytotoxicity by MTT assay

The first step towards understanding how the nanoparticles will react in the body involves cytotoxic studies on cell lines. The term cytotoxicity means the effect of chemical agents demonstrated by altered cellular morphology, failure of the cell to attach to surfaces, changes in the rate of cell growth, cell death, and cell disintegration. So, cytotoxicity testing is a rapid, standardized and sensitive mean to determine if the nanoparticles are biologically harmful to the cells. Cytotoxicity test methods are useful for screening because they serve to separate nontoxic from toxic materials, providing predictive evidence of the nanoparticles safety to the end-users (Miret *et al.*, 2006). Compared to animal studies, cellular testing is more ethically correct, is easier to reproduce and control, and is less expensive. In the case of cytotoxicity, it is important to recognize that cell cultures are sensitive to changes in their environment such as fluctuations in temperature, pH, and nutrient and waste concentrations, in addition to the concentration of the potentially toxic agent being tested. Therefore, controlling the experimental conditions is crucial to ensure that the measured cell death corresponds to the toxicity of the added nanoparticles (Lewinski *et al.,* 2008).

The majority of cytotoxicity assays used, measure cell death via colorimetric methods that can be categorized into tests that measure plasma membrane integrity and mitochondrial activity. The colorimetric method used in this cytotoxic assay was the MTT method that measure the cells metabolic activity and the cell line chosen was RAW 264.7

The RAW 264.7 is a monocyte macrophage cell line obtained from murine leukemia and it 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. In this way, any signs of toxicity will be potentially due to the proteic composition of the nanoparticles and not to the presence of asparaginase.

To assess the effect of the particles or the asparaginase on cells, four different concentrations of particles (25, 50, 100 and 200 μg/mL) and two of asparaginase (2 and 4 μg/mL) were incubated with RAW 264.7 cell line. The two concentrations of asparaginase correspond to the amount of enzyme present in the two highest concentrations of particles. After 24 h of incubation time, the metabolic activity of the cells was evaluated by the MTT assay (Figure 70).

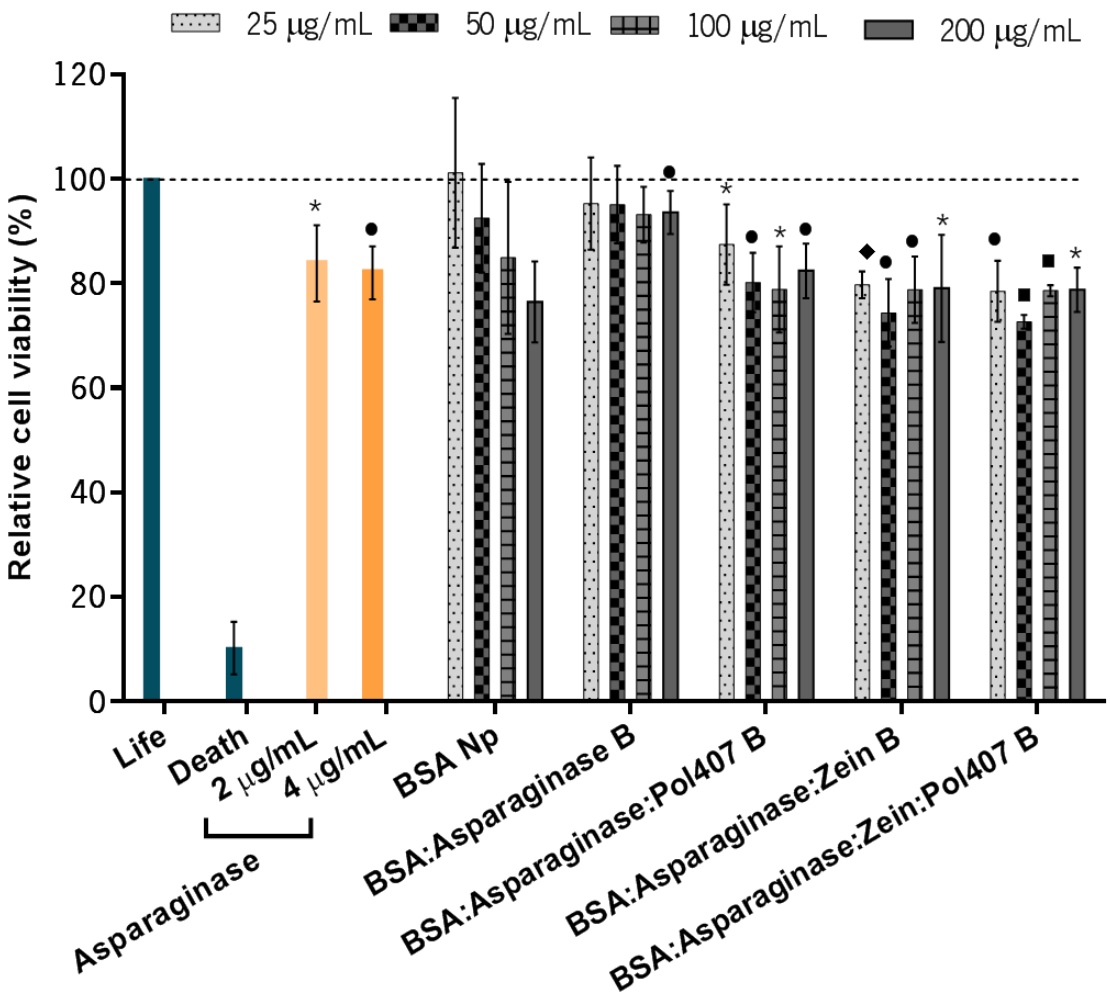

Figure 70 - Relative viability of RAW 264.7 macrophage cell line from leukemic mouse (evaluated by the MTT assay, after 24 h incubation in medium containing BSA, BSA:Asparaginase B, BSA:Asparaginase:Pol407 B, BSA:Asparaginase:Zein B, BSA:Asparaginase:Zein:Pol407 B particles and two concentrations of free asparaginase. The asparaginase concentrations correspond to the amount of enzyme present in the highest particles concentrations. Cells only incubated with medium were used as life control and cells incubated with 30 % DMSO as control of death. Data were determined in relation to the control cells. Results are the mean ± SD of triplicate of three independent experiments. Statistical significant differences from the control are indicated as: * p-value≤0.05, ● p-value≤0.01; ♦ p-value≤0.001; ■ p-value≤0.0001.

The particles selected were prepared by ultrasounds with enzyme added to the particle formulation before the ultrasound cycles. These particles demonstrated a better activity over time and were the same tested for the ability to retain ammonia at surface (see Results and Discussion: 4.6).

Analyzing Figure 70, it was possible to conclude about the cytotoxicity of the particles/enzyme on RAW 264.7 cells. Except for the BSA particles, the cytotoxic effect of the particles was not dose-

dependent as there were no significant differences in cytotoxicity between the tested concentrations. High concentrations of protein-based particles did not significantly affected cell viability.

Analyzing individually each particles and comparing cells viability when incubated with the particles with the life control, the cytotoxicity displayed by BSA:Asparaginase B nanoparticles was not significant, with percentages of cell viability higher than 93.2±5.31 %. For BSA particles without asparaginase, only the highest tested concentration (200 μg/mL) displayed significant levels of cytotoxicity with a viability lower than 80%. For this concentration, the addition of asparaginase to the formulation did not increased the cytotoxic effect of the nanoparticles, instead it did increased the viability of cells for the BSA:Asparaginase B and BSA:Asparaginase:Pol407 particles.

The remaining particles as well as the free asparaginase demonstrated significant differences on cell viability relatively to the life control for all tested concentrations. The particles where zein was included in the formulation, were the most cytotoxic for this cell line. Although the particles with zein were the most cytotoxic, the percentage of viable cells was always superior to 73 %, approximately. The increase in the cytotoxic effect of the particles with zein could be justified by the type of zein used during the synthesis and by the presence of little amounts of ethanol, necessary to dissolve zein before the particles preparation. The zein used was the yellow zein type, which is not the most pure form of zein. It is described that yellow zein has some non-protein contaminants, pigments like xanthophylls that are responsible for the yellow color and still difenuloylputnescine, a compound that gives the characteristic smell of zein (Sessa and Palmquist, 2009). The presence of ethanol on the formulation as well the presence of impurities and other compounds could explain the cytotoxic effect of the particles with zein.

However the RAW 264.7 cell line is able to produce its asparagine, the asparaginase had some cytotoxicity which was related not only with is ability to hydrolyze asparagine but also with its glutaminase activity. Generally, the developed particles to immobilize the asparaginase did not show significant cytotoxic effects.

Chapter 5: Conclusion and Future Perspectives

5. Conclusion and Future Perspectives

Initially the work was focused on the study of free asparaginase. For that, it was determined by SDS-PAGE an asparaginase purity degree of 96%. Considering the temperature of 37 °C and 0.005 g/L of asparaginase, it was determined a K_M value of 44.73 mM, very different from the value of 0.015 mM, described on literature. With the study about the effect of temperature (37 and 40 °C) on the kinetic properties of the free enzyme, it was observed two different profiles depending on the enzyme concentration tested. For low enzymes concentrations (0.005 g/L), it was verified a little decrease on the K_M value with the increase of the temperature, and almost no difference on the V_{max}. For the highest concentrations of asparaginase (0.050 g/L), it was verified an increase on V_{max} relatively to the temperature of 37 °C, although the enzyme lost affinity to the substrate since the K_M had increased. For high enzyme concentrations the increase of body temperature will not affect negatively the V_{max} of asparaginase hydrolysis but will affect the enzyme affinity for the substrate.

The second part of the work was dedicated to the development of BSA particles capable to immobilize the asparaginase and retain its activity over time, when stored at 4 °C. It was observed a protective effect of BSA for asparaginase during storage at low temperatures. Generally, there was an increase in asparaginase half-life increasing the percentage of BSA in solution. The 98 % BSA/2 % asparaginase ratio was chosen for the BSA/Asparaginase particles preparation as it gave to the enzyme a great stability during storage.

The particles for the enzyme immobilization were prepared by two different methods, ultrasounds and homogenization. All the particles prepared by ultrasounds lost all or almost all their activity after two months of storage at 4 °C. Neither the addition of surfactants, Poloxamer 407 nor zein, increased the nanoparticles capacity to retain the enzyme activity for longer times. Zein demonstrated some capacity to stabilize the asparaginase activity during the first month of storage, although during the second month the enzyme almost become inactive. For the particles prepared by homogenization, only the BSA:Asparaginase:Pol407 D nanoparticles showed an high capacity to hydrolyze asparagine after storage at 4 °C, losing only 13 % of its initial activity after two months. For these nanoparticles it was observed a stabilizing effect of Poloxamer 407, since the other nanoparticles lost almost their activity. The influence of the moment at which the enzyme was added was had into account during the study. The stabilizing effect of Poloxamer on the homogenization process was only evident when the enzyme was added to the formulation during the homogenization cycles.

Relatively to the physical properties of the particle, it was obtained particles with different sizes and PDIs, depending on the technique used. The best results were obtained for the particles prepared by homogenization. In terms of size these particles were within the nano-size range (size lower than 200 nm) and exhibited a PDI lower than 0.15, proving the nanoemulsion homogeneity. The low Z-average size and PDI are adequate for a future application of this nanoparticles.

The particles prepared by ultrasounds with the enzyme added before the sonication cycles were the chosen to the study the particles ability to retain ammonia and to be characterized regarding their potential cytotoxicity.

A new HPLC-MS method for the simultaneously quantification of asparagine and aspartic acid without a derivatization protocol was successfully developed. The asparaginase immobilization systems developed by ultrasounds showed a higher capacity to retain ammonia than the free asparaginase. This capacity seems not to be only related with the particles Z-potential. The BSA:Asparaginase:Pol407 B particle exhibited the higher ammonia retention capacity with an aspartic acid/ammonia ratio of 2.46.

The ability of particles to retain ammonia is of particular interest as this systems can be used to prevent the hyperammonemia caused during the ALL treatment with asparaginase. The hyperammonemia could be prevented since the ammonia will be retained by the particles and will not increase the plasma ammonia concentration.

The particles cytotoxicity to RAW 264.7 cell line was evaluated by MTT test. The particles which displayed higher cytotoxicity were the ones prepared with zein, although always with a viability higher than 73 % relatively to the live control. The less cytotoxic particles were BSA:Asparaginase B with a viability higher than 93 %. Generally it was not verified a substantial toxic effect of this particles to the cells.

Future perspectives involve further studies with the BSA:Asparaginase:Pol407 D nanoparticles prepared by homogenization. This nanoparticle was the best considering the activity over time, the size and PDI. As the immobilization method employed during this thesis lead to loss of enzyme activity two months after immobilization for most of the formulations, it will be interesting to test new immobilization methods in order to improve the stability of the immobilized asparaginase for longer times.

In future, new nanocarriers could be developed to improve the asparaginase pharmacokinetics and pharmacodynamics properties, resulting in the increase of plasma half-life, controlled/or site-specific release and reduced immunogenicity. The enzyme potential immunogenic epitopes also could be masked by liposomal, nanoemulsions and polymersomal based asparaginase nanoformulations. Other potential nanocarrier are the porous liposomes that could be developed by the incorporation of amphiphilic cyclodextrins into liposomes what will enhance the entrance of asparagine into the liposomes core where

the asparaginase will be immobilized. Regarding polymersomes, their ammonia retention capacity could be improved by the incorporation of poly-glutamic acid peptide that will allow the sequestering of the ammonia in the polymersome and reduce the hyperammonemia.

Chapter 6: Bibliography

6. Bibliography

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