Catarina de Brito Paixão

Produção e purificação de biofármacos antileucémicos utilizando nanomateriais

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Química, realizada sob a orientação científica de Doutora Márcia Carvalho Neves, Investigadora Auxiliar do Departamento de Química, e do CICECO, da Universidade de Aveiro, e coorientação de Doutora Ana Mafalda Rodrigues Almeida Rocha, Investigadora no MOSTMICRO-ITQB, do Instituto de Tecnologia Química e Biológica António Xavier da Universidade Nova de Lisboa.

No amount of experimentation can ever prove me right.
A single experiment can prove me wrong.
-Albert Einstein

Dedico este trabalho ao meu Pai.

o júri

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resumo   Os biofármacos são uma alternativa natural aos fármacos quimicamente sintetizados. Estes podem ter como base enzimas, hormonas, anticorpos monoclonais, citocinas, entre outros; e já são aplicados no tratamento de diversas doenças que não respondem às terapias comuns.

A enzima L-asparaginase (L-ASNase) é um biofármaco atualmente usado no tratamento de diversas leucemias, nomeadamente a Leucemia Linfoblástica Aguda (LLA), ou doença de Hodgkin. No caso específico da LLA, tipo de leucemia que ataca maioritariamente crianças e jovens, a L-ASNase atua como um inibidor do crescimento das células cancerígenas. Porém, a L-ASNase atualmente comercializada, e proveniente de *Escherichia coli*, contém uma atividade elevada de L-glutaminase, provocando efeitos secundários, como edema, febre, diabetes e hemorragias, nos pacientes. Deste modo, a busca por novos microorganismos que produzam L-ASNase com efeitos colaterais diminuídos é de extrema importância. Neste trabalho, produziu-se uma L-ASNase recombinante por *Bacillus Subtillis*. Posteriormente, e uma vez que os métodos convencionais para a purificação desta enzima são muito dispendiosos, desenvolveu-se uma nova técnica de purificação para a L-ASNase mais económica através da utilização de líquidos iónicos suportados (LIS).

Foram sintetizados e caracterizados três LIS e testaram-se diferentes condições experimentais de forma a otimizar o processo de purificação da L-ASNase, nomeadamente a concentração do extrato celular e a razão material/extrato celular. Por fim, efetuou-se um ensaio em semi-contínuo, com as condições já otimizadas.

Os resultados obtidos indicam que as condições ótimas de purificação da L-ASNase ocorrem com uma concentração de proteína total do extrato celular de 16.7 mg/mL (concentração de proteína total obtida após a lise celular) e uma massa de 50 mg de material por cada mL de extrato celular, correspondentes a uma atividade específica de L-ASNase de 0.0382 U/mg e fator de purificação de 3.46.

keywords

abstract

Biopharmaceuticals are a natural alternative to chemically synthesized pharmaceuticals. They can be based on enzymes, hormones, monoclonal antibodies, cytokines, among others; and are already applied in the treatment of many diseases that do not reply to common therapies. The enzyme L-asparaginase (L-ASNase) is a biopharmaceutical already used in treatments of many leukemias, namely Acute Lymphoblastic Leukemia (ALL), or Hodgkin's disease. Particularly, in ALL, type of leukemia that attacks mostly children and young people, L-ASNase acts as an inhibitor of the carcinogen cells growth. However, the L-ASNase commercially available, and from *Escherichia coli,* contains a high activity of L-glutaminase, causing side effects, for example edema, fever, diabetes and haemorrhages, in the patients. Therefore, the search for new microorganisms that produce L-ASNase with decreased secondary effects is of extreme importance. In this work, a recombinant L-ASNase was produced from *Bacillus Subtilis*. After that, as the conventional methods for the purification of this enzyme are very expensive, it was developed a new purification technique for L-ASNase, more economical, through the usage of supported ionic liquids (SIL).

Three SILs were synthesized and characterized, and different experimental conditions were tested, in order to optimize the purification process of L-ASNase, namely the cell extract total protein concentration and the material/cell extract ratio. At last, a semi-continuous assay was performed with the optimized conditions.

The results obtained demonstrate that optimum experimental conditions for L-ASNase purification occur at a cell extract concentration of 16.7 mg/mL (original total protein concentration after cell lysis) and 50 mg of material per each mL of cell extract, corresponding to a specific activity of L-ASNase of 0.0382 U/mg and a purification fold of 3.46.

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Nomenclature

ABS – Aqueous Biphasic Systems

ALL – Acute Lymphoblastic Leukemia

ATR-FTIR – Attenuated Total Reflectance – Fourier Transform Infrared

BA – Bounding Amount

BSA – Bovine Serum Albumin

B. subtilis – Bacillus subtilis

CPMAS – Cross Polarization Magic Angle Spinning

E. coli – Escherichia coli

ELS – Electrophoretic Light Scattering

ILs – Ionic Liquids

L-ASNase – L-Asparaginase

Mr-SILs – Merrifield resin Supported Ionic Liquids

NMR – Nuclear Magnetic Resonance

N_{1114} – N,N-Dimethylbutylamine

N_{1222} – Triethylamine

N_{1444} – Tributylamine

OD – Optical Density

PBS – Phosphate Buffered Saline

pI – Isoelectric point

PILs – Polvionic Ionic Liquids

PZC – Point of Zero Charge

RTILs – Room-Temperature Ionic Liquids

S_{BET} – BET (Brunauer, Emmett and Teller) Surface area

SDS-PAGE – Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

SEM – Scanning Electron Microscopy

SILs – Supported Ionic Liquids

SILMs – Supported Ionic Liquid Membranes

SSF – Solid State Fermentation

SmF – Submerged Fermentation

SILs – Supported Ionic Liquids

SPR – Surface Plasmon Resonance

TCA – Trichloroacetic acid

TSILs – Task Specific Ionic Liquids

1. Introduction

1.1. Biopharmaceuticals

Biopharmaceuticals are products used for medicinal purposes and consist of sugars, proteins, nucleic acids, living cells, or tissues. They can be obtained from biological sources, like humans, animals, or microorganisms, being obtained by biological processes, like extraction from living systems or production by recombinant DNA [1]. This means that a specific organism (specially plants, animals, or microorganisms) can be genetically modified in order to develop specific desired characteristics [1].

Due to the fact that these products can be found in human body, biopharmaceuticals turn out to be more compatible with human body compounds, making them capable of curing diseases, instead of only treat symptoms, and without (or very low) side effects when compared to conventional drugs [2].

The first biopharmaceutical showed up in 1982, the recombinant human insulin [3]. It was produced from synthetic biology, by inducing pre-determinate genes in bacteria, animal, or plant cells. Nowadays there are several types of biopharmaceuticals being developed to treat several diseases like cancer, viral infections, diabetes, hepatitis and multiple sclerosis (Figure 1) [4,5].

The majority of biopharmaceutical products are in the category of vaccines and monoclonal antibodies, existing also a large number of hormones, cytokines, cell therapy and antisense drugs based products [5].
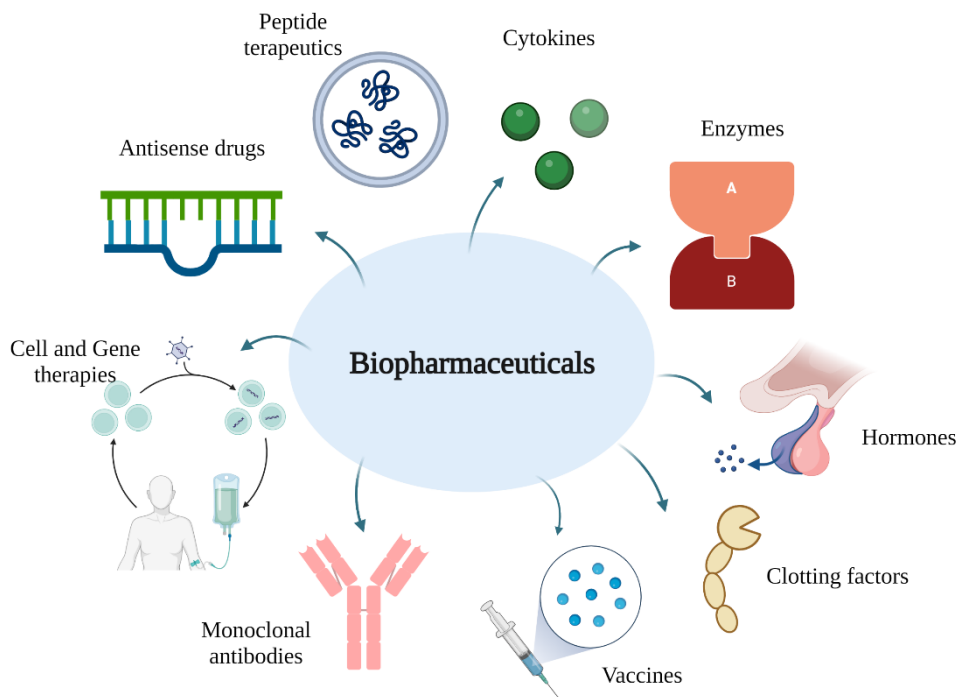
Figure 1 - Different biopharmaceuticals applied nowadays.

Biopharmaceuticals can be used in the prevention, diagnosis or even in the therapy of different diseases [1]. The prevention, for example through vaccines, resembles a pathogen and is usually made from inactivated microbes, live attenuated microbes, toxoids (toxins), and or part of surface antigens (subunits). It decreases significantly infectious diseases, such as measles, tetanus, and polio, however, can impact an increase of non-infectious diseases, such as cancers, cardiovascular diseases, metabolic diseases, and neurodegenerative diseases. The diagnosis with monoclonal antibodies is used to detect the presence of a substance for which the monoclonal antibodies are specific, in some cancers and infectious diseases. Is it very useful in immunohistochemistry (to detect antigens in fixed sections) and immunofluorescence tests (to detect the substance in frozen tissue sections or in live cells). In therapy, recombinant proteins or antibodies are used with the purpose of treating diseases in an effective way, acting in its pathophysiology.

Compared with chemical drugs, biopharmaceuticals have a more complex production, multiple ways of administration, and different pharmacokinetics, presenting a high selectivity and low nonspecific toxicity. However, these pharmaceuticals, present a high cost [4].

1.2. L-Asparaginase (L-ASNase)

One of the most promising biopharmaceuticals being used as a therapeutic in chemotherapy for the treatment of leukemia is L-Asparaginase (L-ASNase). L-ASNase is an enzyme that catalyses the hydrolysis of L-asparagine (Figure 2), which is an essential amino acid for leukemic cells [6].
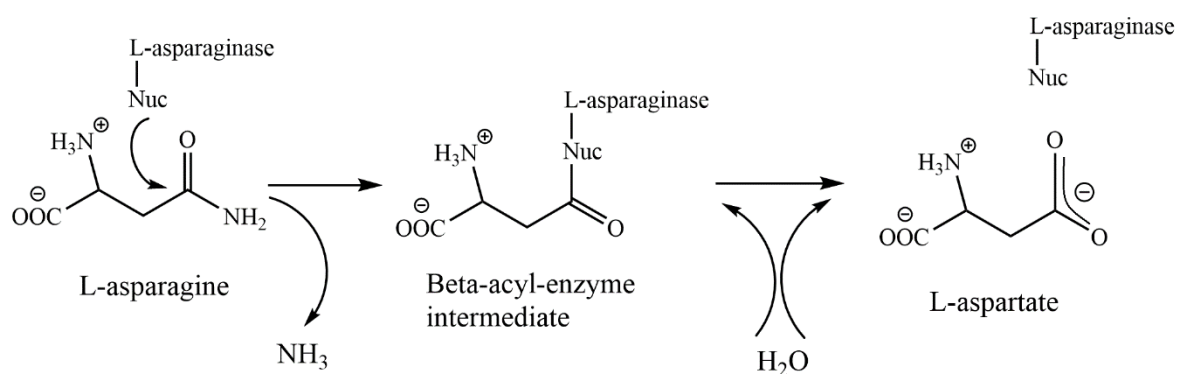
Figure 2 - Reaction mechanism of L-Asparaginase (adapted from [6]).

L-ASNase can be found in diverse organisms, such as animals (except humans), plants, and microorganisms (bacteria, fungi, algae, yeast, and actinomycetes) [6].

In 2017, it was studied the possibility of isolating L-ASNase endophytes from a fungus present in some common Malaysian medicinal plants, such as *Pereskia bleo* (also known as Seven star needle), *Murraya koenigii* (curry plant), *Oldenlandia diffusa* (chinese herb) and *Cymbopogon*

citratus (lemon grass) [7]. In every case, leaf tissues contained more endophytes than stem tissues, where some of them had an L-ASNase activity in between 0.010 and 0.025 μM^{-1} mL^{-1} min^{-1} [7].

Another study used the roots of *Withania somnifera*, a traditional medicinal plant used in Ayurveda as a rejuvenating tonic, as a source of L-ASNase [8].

However, the most researched source of L-ASNase are the microorganisms, since its production methods are easier than other sources [9]. The main bacterial sources of L-ASNase are *Escherichia coli*, *Erwinia chrysanthemi* and *Erwinia carotovora* [6], since they are easier to manipulate genetically. Bacterial L-ASNase can be categorized in type I (cytoplasmic) and type II (periplasmic) [10]. The main difference between both types, besides their cellular location [9], is that type I L-ASNase has enzymatic activity on both L-glutamine and L-asparagine, but lower affinity for L-asparagine [10], comparing to type II, that has high specific activity on L-asparagine [10]. This last one has been used, for more than three decades, as an antitumor agent in the treatment of acute lymphoblastic leukemia (ALL), as detailed forward.

1.3. Applications of L-ASNase

1.3.1. Pharmaceutical industry

L-ASNase is the first therapeutic enzyme with antineoplastic properties that has been broadly studied by researchers and scientists far and wide. It was firstly observed in 1904, and a few years later, in 1922, found in blood serum of guinea pig. gave another step in this research and proved guinea pig serum capacity as a tumour inhibitor [6]. In 1961, another discover demonstrated the ability of L-ASNase as a tumour agent in guinea pig serum with substrate specificity [6]. In 1967, it was identified two isozymes of L-ASNase: type I and type II. Both of them are characterized by enzymatic activity for both L-asparagine and L-glutamine, but type II displays higher specific action against L-asparagine and, more precisely, shows antitumor activity, being able to become a chemotherapeutic agent that is already used, in combination with other drugs, in the treatment of certain diseases, such as ALL, Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticulosarcoma, and melanosarcoma (malignant melanoma) [6].

L-asparagine is an essential amino acid for protein synthesis and cell growth for many tumour cells and L-ASNase has the ability to convert L-asparagine to aspartate, which means that it can affect the main growth factors of malignant cells by depleting L-asparagine, and as result, the tumour cells die off, as shown in Figure 3.
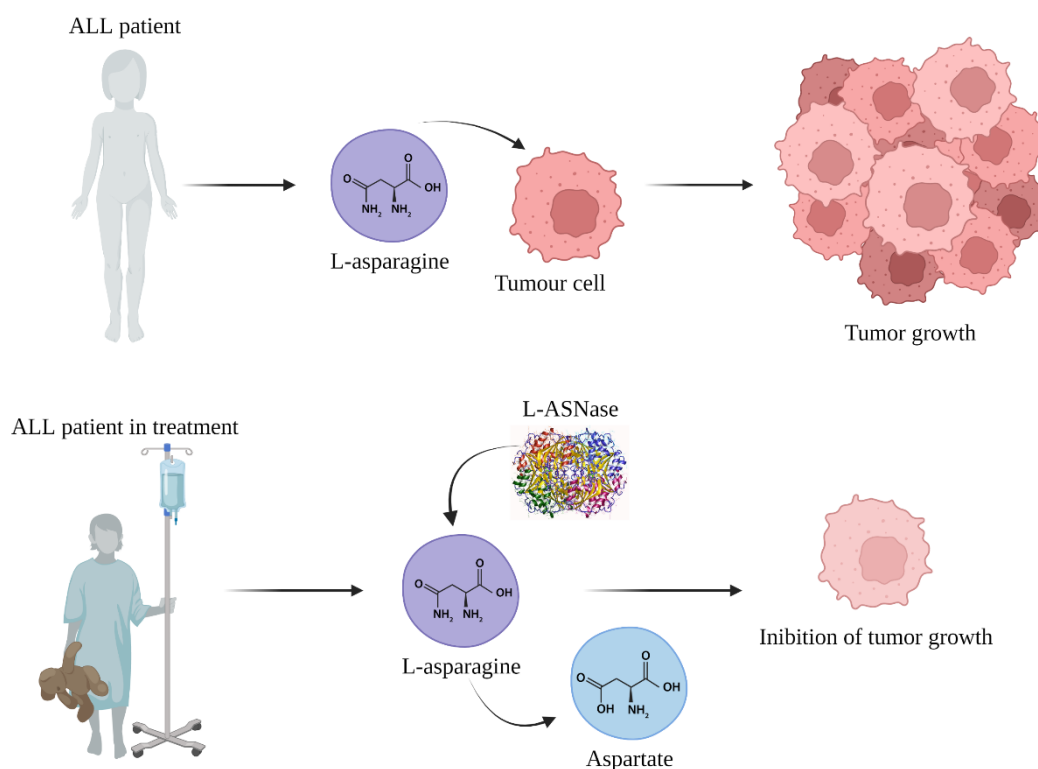
Figure 3 - Schematic illustration of the action of L-ASNase in an ALL patient.

Despite its potential antileukemic activity, the use of L-ASNase in treatment for leukemic patients causes certain side effects, with the possibility of lethality to normal cells, caused by L-glutaminase action [6]. The most common symptoms are edema, skin rases, fever, hepatic dysfunction, diabetes, leucopenia, pancreatitis, neurological seizures, and hemorrhage [6].

1.3.2. Biosensor

According to Kissinger [11], a biosensor is "*a device where a biological recognition element is built in (physically attached or confined) and is the primary selectivity element*", and, for that reason, a sensor that is used for biological purposes is not necessarily a biosensor, such as those for temperature, pressure, electrocardiograms, pH, Ca^{2+}, catecholamines, and so on [11]. Also defined as "*highly sophisticated chemical sensors incorporating some kind of biological material in a sensing layer intimately associated with a transducer*" [12], biosensors (Figure 4) are of high interest in every chemical analysis, since they have the capacity to recognize the presence of a specific analyte (electrochemical, optical, thermal or mass variation) and translate it, with an electrochemical, piezoelectric, optical, thermometric or magnetic transducer [13], to an electrical signal (most commonly) with an elevated level of sensitivity and precision [12].
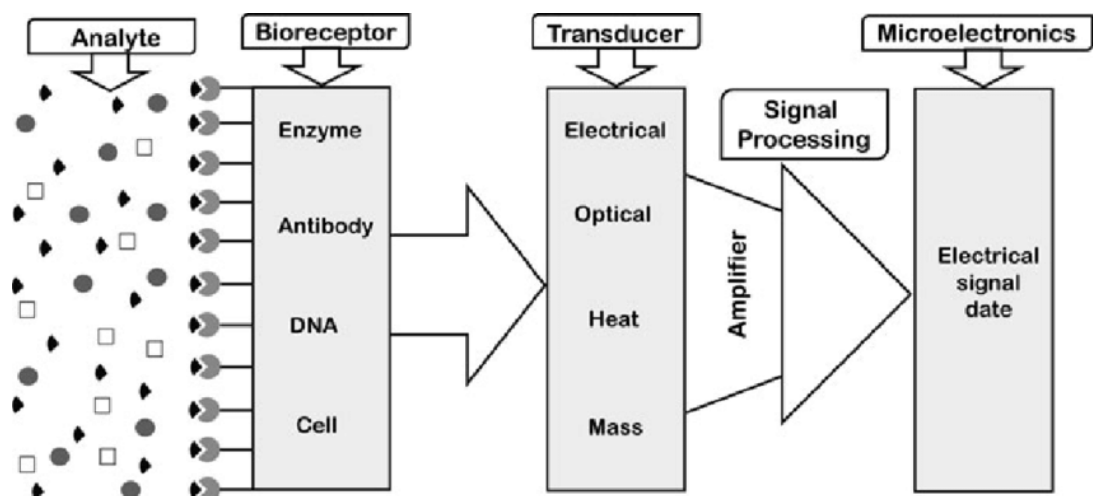
Figure 4 - Schematic diagram of a Biosensor (from [14]).

The first L-ASNase-based biosensor was conceived in 1983 with the purpose to detect ammonia, through the combination of an online gas dialyzer and a potentiometric ammonia gas detector, and was able to remove high levels of ammonia nitrogen background interference from physiological samples [13]. For the last decades, there has been an intense research through biosensors based in L-ASNase, especially in pharmaceutics, to identify L-asparagine in ALL treated patients' blood samples, and also in food industry, to identify the presence of L-asparagine in food.

Nowadays, ASNase-based biosensors (Figure 5) are mostly based on colorimetric detection, using the capacity of L-ASNase to catalyse L-asparagine hydrolysis, releasing L-aspartic acid and ammonium ions, that will generate a variation of the medium pH value, altering its colour [13].
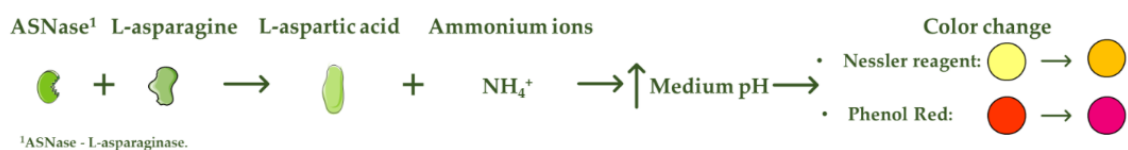
Figure 5 – Colorimetric L-asparaginase-based Biosensor (from [13]).

1.3.3. Food industry

The main reason of using L-ASNase in the food industry derives from the big concern about the carcinogenicity and neurotoxicity present in a large group of fried and oven-cooked foods, like cookies, bread, coffee, but especially French fries and potato chips, due to the presence of acrylamide combined with high temperature cooking [15]. The L-ASNase acts by mitigating this organic compound, reducing up to 97% of acrylamide formation, depending on the food and temperature,

6

without changing the appearance or taste of the final product [15]. This happens because L-asparagine is hydrolysed by L-ASNase, forming aspartate and ammonia, and, in that way, it will not react with the reducing sugar present in the food (browning process with Maillard reaction), not allowing the formation of acrylamide [15].

1.4. Production of L-ASNase

As mentioned before, L-ASNase can be produced from diverse sources, being the most known, *Escherichia coli* (*E. coli*). It is of high interest to produce L-ASNase using a recombinant strain by genetic engineering, which means to induce in the microorganism a 'command' that allows it to perform a specific action, such as the production of L-ASNase. *E. coli* and *Pichia pastoris* have been successfully used to originate recombined L-ASNase, being *E. coli* the one that shows the highest extracellular L-ASNase activity. However, due to its secondary effects when used in the treatment of ALL, different bacteria have been studied as a source of recombined L-ASNase, such as *Ganoderma austral* and *Bacillus Subtilis* (*B. Subtilis*) [16], which appears to be, in general, safe and with potential to produce L-ASNase [17]. Besides the source of production, there are also some strategies that improve the process of recombinant production of L-ASNase, such as optimization of nutrient, inducing condition, process parameters and signal peptide screening [17].

In 2017 there was a research about the production of L-ASNase from *B. Subtilis* 168, a combined strategy using signal peptide screening, promoter mutation, N-terminal deletion and fed-batch strategy in a fermenter with 3 L of capacity [17]. In the end, the enzymatic properties were analysed, resulting in 407.6 U/mL of extracellular L-ASNase (2.5 g/L of L-ASNase protein) with a productivity of 9.26 U/(mL.h) [17].

The main methods used for L-ASNase production are solid state fermentation (SSF) and submerged fermentation (SmF) [6]. The main difference between these two fermentations, is that SSF allows the growth of microorganisms in a solid material, like bran [18], while SmF takes place in flowing liquid substrates, for example broths [19]. Besides, SmF has also a high cost and low net yield, and SSF is cost-effective and has higher product yield [6]. In Figure 6 are described some advantages and disadvantages, when comparing one method to another (SSF vs SmF).

It is important to have into account that the conditions of enzymatic production vary between different organisms, and enzymes can be produced constitutively or after induction.

A study in 2006, where L-ASNase was produced from *Aspergillus niger* with SSF, reached a specific activity of 0.82 U/mg and a purification fold of 1.44 using SSF, being compared with SmF as more eco-friendly and less expensive [20].

Another study in 2012, showed a higher L-ASNase specific activity of 13.97 IU/mg for SmF production from *Penicillium sp.* [21].

Concerning its value as therapeutical agent, L-ASNase must be able to maintain stability in wide ranges of pH and temperature. Besides that, it should have a low value of Michaelis-Menten constant (K_m), which means a high affinity in physiological conditions, and low side effects [9].
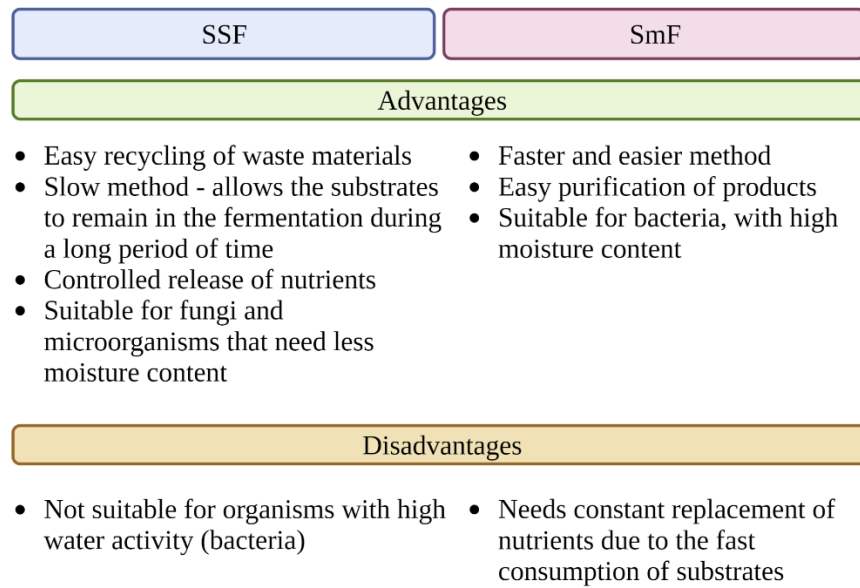
Figure 6 - Advantages and disadvantages of solid state fermentation and submerged fermentation [19].

1.5. Purification of L-ASNase

The purification is a really important step in L-ASNase management, especially for antileukemic drug purpose since it requires a high level of purity for this enzyme. In the case of intracellular L-ASNase production, extra steps are required, in comparison with extracellular production [6], since the extraction has to be done from the inside of the microorganism.

1.5.1. Conventional methods

The most common methods used to purify L-ASNase are non-chromatographic, like precipitation and centrifugation, or chromatographic techniques, such as ion exchange and gel filtration, as described in Figure 7 [15].
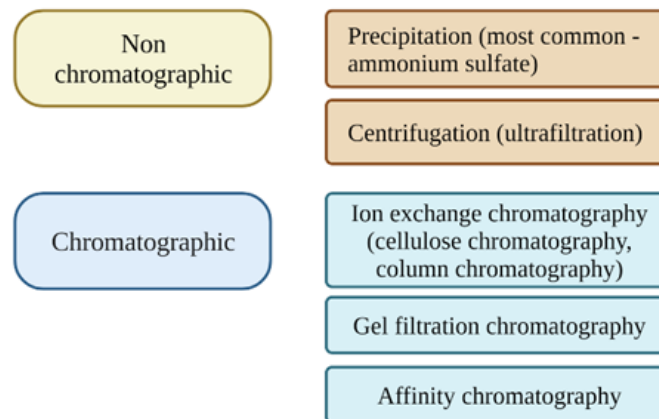
Figure 7 - Non chromatographic and chromatographic methods for L-ASNase purification [15].

Despite its high resolution and large usage, these conventional methods tend to be expensive, except the precipitation, and to have diffusional limitations. For that reason, it has surged the need of research for another alternative methods, more sustainable and with lower costs, without compromising the final purification fold [22].

1.5.2. Alternative methods

Aqueous Biphasic Systems (ABS) are a biocompatible liquid-liquid extraction technique, due to its high water composition [23]. In 2017, ABS composed by a polymer and a salt were used for the purification of L-ASNase from *E.coli*. The study resulted in a specific activity of 3.61 U/mg. Moreover combining a pre-purification step, using ammonium sulphate precipitation, with ABS a higher specific activity of 22.01 U/mg was achieved [24].

One way to make this enzyme more stable is to support it in a solid matrix. One of the materials that may be promising to overcome this drawback are supported ionic liquids (SILs).

1.5.2.1. Supported Ionic Liquids (SILs)

Ionic Liquids (ILs) are defined as salts composed by organic cations and organic/inorganic anions with a melting point below 100 °C [24,25], due to the charge distribution and the size of the ions, which is better than the common inorganic salts [26].

There are a lot of different types of ILs, such as room-temperature ILs (RTILs), task specific ILs (TSILs), polyionic ILs (PILs), and supported IL membranes (SILMs). They are used for different purposes, such as solvents, catalysts and reagents or both of them [25].

Supported Ionic Liquids (SILs) are ILs immobilized on some support, for example silica-based materials, magnetic nanoparticles, carbon, nanotube, among others. In this way, the material will be defined with the liquid properties instead of the material properties [27].

SILs are used to design new catalysts, and some other functional materials, with the capacity to achieve a homogeneous surface and high specificity [26].

Since it is a recent topic, there is no sure whether SILs will adsorb L-ASNase or the extract containing contaminants. In the last case, a simple discard of the solvent liquid would give the desired enzyme but, for the first one, it would be necessary to extract L-ASNase from the remaining solvent.

Nowadays, silica based SILs are known to be promising materials to be used in L-ASNase purification. However, silica gel SILs show an irregular morphology which causes the packaging of the particles, when used in continuous assays. To solve that, spherical silica or even polymeric spherical particles can be used as support for the functionalization with ionic liquids [28].

Based on the SILs potential, in this work, Merrifield resin supported ionic liquids (Mr-SILs) were applied to purify L-ASNase from a cell extract, produced by a recombinant bacteria *B. Subtilis Δ6* (Figure 8).
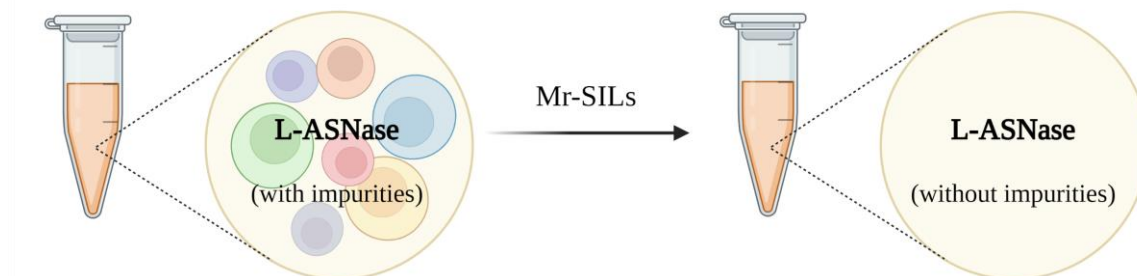
Figure 8 - Dissertation summary scheme: purification of L-ASNase present in a media with other proteins (here considered as impurities) using Mr-SILs.

2.   Experimental Section

2.1. Synthesis of Merrifield resin Supported Ionic Liquids (Mr-SILs)

For the present work, it was used the Merrifield resin as the solid support of the ionic liquids.

All the solvents and reactants used for the synthesis of the Merrifield resin supported ionic liquids are identified in Table 1.

Table 1 - Suppliers and purities of the reagents used in the Mr-SILs synthesis.

Reagent	Purity	Supplier	CAS
Merrifield Resin	-------------	Sigma-Aldrich	9003-70-7
(3-Chloropropyl)trimethoxysilane	98+ %	Acros Organics	2530-87-2
Toluene	>= 99.8 %	Fisher Chemical	108-88-3
Methanol	>= 99.9 %	Fisher Chemical	67-56-1
Ethanol	>= 99.8 %	Fisher Chemical	64-17-5
N,N-Dimethylbutylamine	99 %	Aldrich Chemistry	927-62-8
Triethylamine	-------------	CARLO ERBA Reagents	121-44-8
Tributylamine	>= 98.5 %	Sigma-Aldrich	102-82-9

These materials were synthesized according to two different methods.

The first method was based on literature, where the synthesis occurs in two steps. The first step is based on a reaction between the solid support, in this case Merrifield resin, and (3-chloropropyl)methoxysilane, which is used to add a chlorine to the support matrix, in order to 'make place' for other functional group to substitute. For that, 5 g of Merrifield resin was suspended in 50 mL of toluene and 5 mL of (3-chloropropyl)methoxysilane were added. The mixture was stirred under reflux (100 ºC) during 24 h. The solid material (Mr-intermediate) was isolated by filtration and washed with 100 mL of toluene, 200 mL of ethanol/water mixture (1:1), 500 mL of distilled water and 100 mL of methanol, by this order, and then dried at 50 ºC for 24 h. The second step has the objective to substitute the chlorine with the pretended cation of the ionic liquid. So, the 5 g of the material Mr-intermediate was dispersed in 50 mL of toluene and 5 mL of the pretended ionic liquid

(Table 2) was added. This mixture was stirred under reflux (110 ºC) during 24 h. The Mr-SILs materials were obtained and washed with 100 mL of toluene, 350 mL of methanol, 300 mL of distilled water and 150 mL of methanol, by this order, and dried at 50 ºC for 24 h.

However, in the production of these first method materials, there has surged an interesting question about the importance of the first step of the reaction in how it would be necessary to add a chlorine, since Merrifield resin already carries a chloromethyl group. This idea led to the second method, where only the synthesis second step was used to functionalize the Mr-SILs.

In the end, there were obtained two different types of materials, the two steps materials (Mr-SIL*) and the one step materials (Mr-SIL**), in order to evaluate whereas the first step has impact in the chemical, structural and morphological properties of the resultant supported ionic liquid.

Table 2 – Mr-SILs abbreviation and corresponding number of moles used in each synthesis.

Abbreviation	Cation source/ cation of the SIL	n of Mr-SIL (mol)
Mr-N$_{1114}$	N,N-Dymethylbutylamine /(N$_{1114}$)$^+$	0.0356
Mr-N$_{1222}$	Triethylamine /(N$_{1222}$)$^+$	0.0359
Mr-N$_{1444}$	Tributylamine /(N$_{1444}$)$^+$	0.0210

Besides the difference between the steps of the reaction, it was also studied how the chemical structure impacts the functionalization of the materials, due to the use of different ILs with different alkyl chains. This can be understood in Figure 9, analysing the structures of the different produced Mr-SILs.
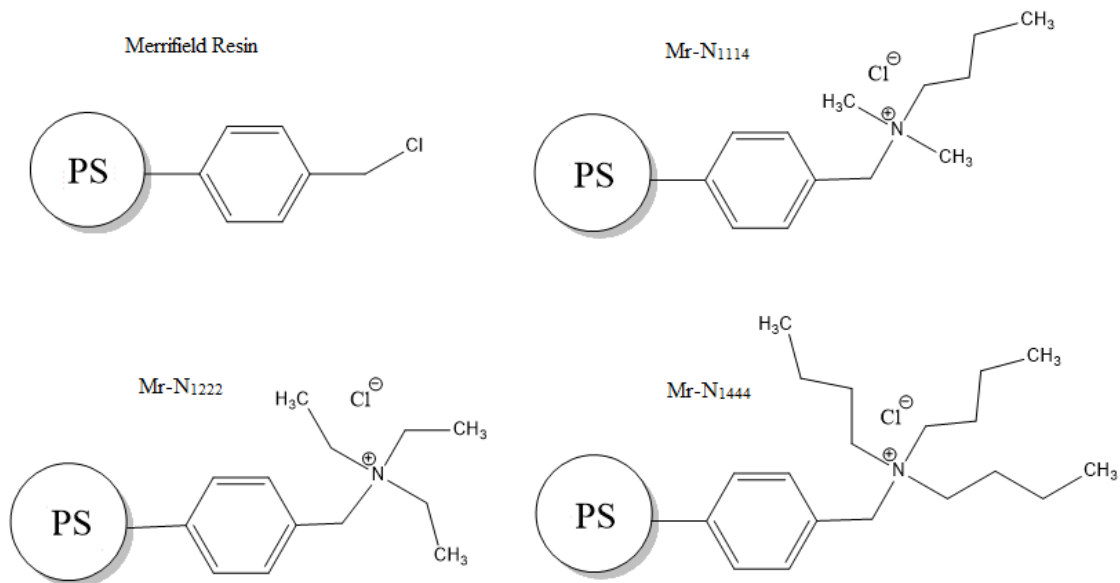
Figure 9 - Chemical structures of Merrifield Resin and the produced Mr-SILs.

2.2. SILs characterization

To evaluate and understand the main differences between the synthesized materials, the materials were characterized by several technics.

2.2.1. Elemental analysis

This method consists of determining the weight percentage of different elements, namely carbon, hydrogen, nitrogen, and sulphur. It was conducted by the equipment *Truspec 630-200-200*, with a sample size micro (up to 10 mg), a combustion furnace temperature of 1075 ºC and an afterburner temperature of 850 ºC. The detection method included a carbon, hydrogen and nitrogen-infrared adsorption.

2.2.2. Point of zero charge (PZC)

This method consists in analysing the zeta potential of particles at a nanoscale, through an Electrophoretic Light Scattering (ELS).

To perform this analysis, it was used the equipment *ZetaSizer Nano series*. Several suspensions of the Mr-SILs were prepared in distilled water and their pH were adjusted to several values with

HCl or NaOH solutions (0.01M). Then, the suspensions were sonicated and inserted into a folded capillary zeta cell (Figure 10). This procedure allows to obtain a zeta potential representation as function of the suspension pH, as shown in Figure 11.
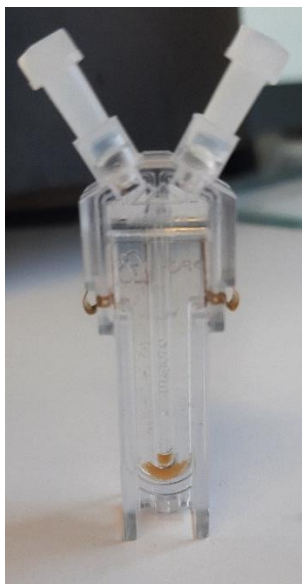
Figure 10 - Capillary zeta cell.
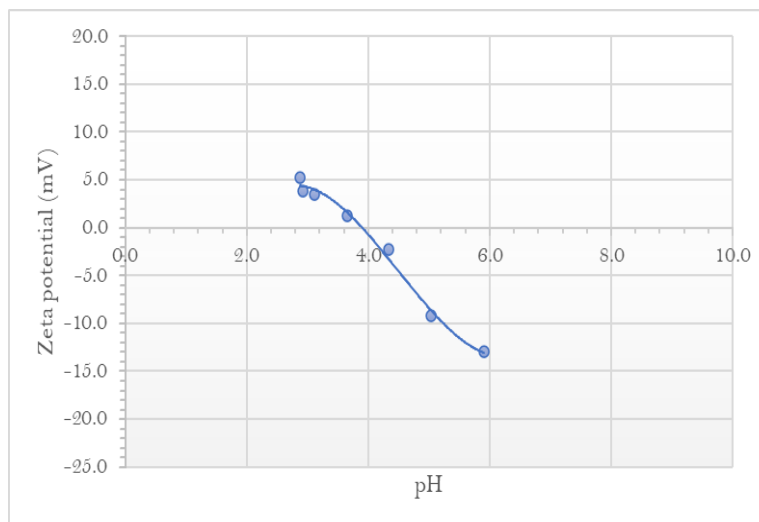
Figure 11 – Zeta potential values in function of pH for Merrifield Resin.

2.2.3. Attenuated total reflectance - Fourier-transform infrared (ATR-FTIR) spectroscopy

This is a very fast and precise method and has the perk of not destroying the samples.

For this work, it was performed an ATR-FTIR spectroscopy using a *Perkin Elmer* spectrometer, *FT-IR system Spectrum BX*. A sample of each Mr-SIL was analyzed with a single beam, in a range from 4000 to 400 cm^{-1}, with 256 scans and a resolution of 8.0 with an interval of 2.0.

2.2.4. ^{13}C CPMAS Solid State Nuclear Magnetic Resonance (^{13}C CPMAS NMR)

A solid state ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) was performed using a *Bruker Avance III – 400 MHz* spectrometer, *model DSX*. Each Mr-SIL spectra was obtained with 4 nm VTN probe at 9.7 T and cross polarization magic angle spinning (CPMAS) at 12 kHz and room temperature.

2.2.5. Scanning Electron Microscopy (SEM)

This method was performed using a *Hitashi SU-70* microscope with *EDX Bruker, model Quantax 400*. Each Mr-SIL sample was introduced in a small portion of carbon tape to be analyzed.

With the SEM results, it is possible to obtain information about the morphology of materials. Different resolutions can be used to have more than one perspective of the sample surface.

2.3. Production and purification of L-Asparaginase

All materials for the production and purification of L-ASNase are detailed in Table 3. The recombinant bacteria used in this study was *B. subtilis* KO7, kindly provided by Professor Valéria de Carvalho Santos Ebinuma from the Department of Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University – UNESP Brazil. Briefly, the ansB gene from *Aliivibrio fischeri* (also called *Vibrio fischer*) (NCBI:WP_011262105.1) was amplified using PCR of genomic DNA isolated from *A. fischeri* as a template. Specific primers were designed using Biobrick methodology. The PCR product was cloned in plasmid pBSoEXyIRxylA (V2) and used for thermal shock transformation in *B. subtilis* KO7.

Table 3 - Suppliers of reagents used in the production and purification of L-ASNase.

Reagent	Supplier	Purity
LB Broth	SIGMA-Aldrich	-----------------
D-(+)-Xylose	SIGMA - Aldrich	>= 99 %
Phosphate Buffered Saline tablet (PBS)	SIGMA - Aldrich	------------------
Tris(hydroxymethyl)-aminomethane, 99 %	Alfa Aesar	99 %
L-Asparagine, 99%	Acros Organics	99 %
Nessler's Reagent	PanReac AppliChem	------------------

2.3.1. Production of L-ASNase

All the materials and solutions used in this study were previously sterilized in autoclave (20 min at 121 ºC) and all the procedure was performed in a laminar flow chamber.

The pre-inoculum was prepared, adding 15 µL of erythromycin (1 mg/mL) and the recombinant bacteria (*B. subtilis* KO7) to 15 mL of LB broth, and then incubated overnight at 220 rpm and 37 ºC.

Subsequently, 100 mL of LB broth, 100 µL of erythromycin (1 mg/mL) and a pre-inoculum volume was added to an Erlenmeyer to start the inoculum with an optical density (OD) of 0.1. The inoculum was incubated in an orbital shaker at 37 ºC and 250 rpm.

When the inoculum reached an OD of 1, an aqueous solution of xylose (50%, m/V) was added to the inoculum (final xylose concentration of 0.75 % (m/V)), to induce the production of L-ASNase. The mixture was incubated in an orbital shaker during 24 h at 30 ºC and 210 rpm.

After the 24 h of incubation, the inoculum solution was distributed in falcons (50 mL of capacity) and centrifuged at room temperature (22 ºC) and 4700 rpm for 20 minutes. The supernatant was discharged and the remaining pellet containing the enzyme was dissolved in 10 mL of phosphate buffered saline (PBS) and homogenised.

Since L-ASNase production is intracellularly, the cellular lysis is necessary. The cells were disrupted by sonication with an amplitude of 60 % with 40 cycles of pulses of 5 s on, 10 s off, pausing at the 20th cycle to homogenise. After the lyses, the cell extract was centrifuged during 10 min at 4700 rpm. The supernatant was reserved containing L-ASNase and other contaminants.

2.3.2. Total protein concentration

The total protein of the cell extract was determined at 280 nm in a microplate reader. PBS was used as the blank sample (in this case PBS) and a bovine serum albumin (BSA) calibration curve previously determined was applied to determine the total protein concentration.

2.3.3. Quantification of L-ASNase activity

The activity of the enzyme in the cell extract was determined. Firstly, 500 µL of cell extract was incubated with 500 µL of Tris (50 mM, pH 8.6) and 50 µL of L-asparagine (189 mM) at 37 ºC and 400 rpm for 30 minutes. The reaction was stopped by adding 250 µL of trichloroacetic acid, TCA (1.5 M), and the sample was centrifugated during 10 min at 12000 rpm.

Nessler method was applied to determine the concentration of ammonia produced during the L-asparagine hydrolysis, which is directly proportional to the enzyme activity. 250 µL of the Nessler reagent was mixed with 500 µL of previous reaction and 500 µL of distilled water, and the mixture incubated during 30 min at room temperature. The mixture absorbance ate 436 nm was measured in a microplate reader. As a blank solution was used a mixture prepared under the same protocol but with PBS instead cell extract.

2.4. Purification of L-ASNase

This work aims the development of an alternative method to purify L-ASNase produced by *B. subtilis* KO7. Therefore, different experimental conditions, namely the total protein concentration and the ratio between the cell extract and the Mr-SILs, were studied. Cell extracts with total protein concentration of 3, 5, 7.5, 10 and 16.7 mg/mL, and 3, 20, 30 and 50 mg of Mr-SILs to 1 mL cell extract were applied.

The contact between the cell extract and the Mr-SILs happened in eppendorfs agitated in a trayster for 60 minutes. Then, the samples were centrifuged at 12000 rpm for 15 minutes to separate the Mr-SILs.

2.4.1. Total protein concentration

The procedure is identical to the one described in section 2.3.2., with the difference of the blank sample, which in this case was PBS in contact with the Mr-SILs in the same conditions of the other samples.

2.4.2. Quantification of L-ASNase activity

The procedure is identical to the one described in section 2.3.3.

2.4.3. L-ASNase specific activity

The specific activity is the relation between the L-ASNase activity and the total protein concentration of each sample according to Equation 1.

$$Specific\ activity = \frac{L-ASNase\ activity\ \left(^{U}/_{mL}\right)}{[Total\ protein]\ \left(^{mg}/_{mL}\right)} \qquad \text{Eq. 1}$$

2.4.4. Purification factor

The purification factor relates the initial specific L-ASNase activity (before the contact with the Mr-SILs) with the final L-ASNase specific activity (after contact with Mr-SILs), through the Equation 2, which tells the degree of purification achieved in the experiment.

$$Purification\ factor = \frac{Final\ specific\ activity}{Initial\ specific\ activity} \qquad \text{Eq. 2}$$

2.4.5. Semi-continuous assay

Instead of agitation in the trayster, the contact between the cell extract and the Mr-SILs occurred in a column, in a continuous way. In this method, the cell extract was passed through the Mr-SILs by gravity in a column, and different aliquots were taken and used as samples that go through the same procedure as the samples agitated in the trayster.

In this case, it was only tested one material with semi-continuous assay, to evaluate the efficacy of this method in comparison to the main one. Inside the column was inserted 500 mg of Mr-N$_{1114}$* and compressed in the bottom. Then, with the help of a peristaltic bomb, 10 mL of PBS were passed through the column, to humidify the Mr-SIL and 2 or 3 aliquots were taken to be used as blank samples. Without letting the Mr-SIL run out of PBS, 12 mL of cell extract were introduced into the column and 2 mL aliquots were collected, until all the cell extract passes through the Mr-SIL.

2.4.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is used to separate proteins according to their size and electrical charge, in a semi-qualitative way. The samples were diluted to a total protein of 6 mg/mL, then mixed with 20 μL of loading buffer, in a proportion of 1:1, and warmed up to 95 ºC for 5 minutes.

The SDS-PAGE occurred at 100 V during 1 h and 30 minutes. After that, the gel was carefully taken out of the setup, washed with distilled water, and stained with Coomassie Blue.

3.   Results and Discussion

3.1. Characterization of Mr-SILs

3.1.1. Elemental Analysis

The chemical composition in carbon, hydrogen and nitrogen obtain by elemental analysis for the materials are shown in Table 4. After analysing these results, it is possible to observe that the Mr-intermediate has practically the same percentage of these elements as Merrifield Resin, which confirms that the first step of the synthesis is useless, once it does not add or change the composition in these elements. Also, when comparing these elements composition for the materials prepared by the first method (Mr-SILs*) with the composition of the materials prepared by the second method (Mr-SILs**) no differences are observed. This reinforces the conclusion that the first step of the first method in unnecessary in the synthesis of Mr-SILs*.

Table 4 - Mr-SILs chemical composition obtained by elemental analysis.

Mr-SIL	% C	% H	% N
Merrifield Resin	83.594 ± 0.369	7.024 ± 0.248	0.110 ± 0.023
Mr-intermediate	83.538 ± 0.122	7.274 ± 0.290	0.078 ± 0.007
Mr-N_{1114}*	77.494 ± 0.588	8.688 ± 0.243	1.713 ± 0.033
Mr-N_{1222}*	80.525 ± 0.548	7.808 ± 0.106	1.183 ± 0.048
Mr-N_{1444}*	87.119 ± 0.346	7.612 ± 0.004	0.201 ± 0.005
Mr- N_{1114}**	79.464 ± 0.415	8.192 ± 0.062	1.681 ± 0.018
Mr-N_{1222}**	83.020 ± 0.144	8.092 ± 0.069	1.030 ± 0.015
Mr-N_{1444}**	86.266 ± 0.547	7.685 ± 0.098	0.269 ± 0.045

Moreover, it can also be observed that the SILs Mr-N_{1114} have a higher composition in nitrogen when compared with the other materials. This result is expected when compared with Mr-N_{1444} since a lower amount of cation precursor was used in the latter as shown in Table 2 (0.020 mol compared with 0.0356 mol). However, the almost same amount of cation precursor was used to synthesize Mr-N_{1222} (0.0359 mol) and in this case the composition in nitrogen is lower than in Mr-N_{1114}. A possible explanation can be related with steric effect, which means that each molecule will have a different volume, being possible a greater or lesser functionalization of the surface of the material. So, in this case the longer the alkyl chains the lower the functionalization of the material's surface will be possible.

To evaluate the degree of functionalization of each SIL by the correspondent IL two approaches were evaluated: the Bounding Amount (BA) and the molar amount of IL per weight of SIL material (Table 5).

The BA was calculated by Equation 3, where % (N) is the obtained nitrogen amount determined by elemental analysis taking into account the BET (Brunauer, Emmett and Teller) surface area of Merrifield Resin, S_{BET} (12.3 m^2/g)[29], where "1" is the number of nitrogen atoms per IL structure and M(N) is the nitrogen molecular weight.

$$BA = \frac{\frac{\%(N)}{1 \times M(N)}}{S_{BET}}$$ Eq. 3

The IL molar amount per SIL weight was calculated based on the corresponding ionic liquid structure, and on the nitrogen composition obtained by elemental analysis.

Table 5 - Bounding Amount (BA), IL molecular weight, mass of IL per mass of SIL, and the molar amount of IL per mass of SIL material for the synthetised Mr-SILs.

| Mr-SIL	BA (µmol/m^2)	IL molecular weight (g/mol)	IL mass / SIL mass (mg IL / g SIL)	Moles of IL / mass of SIL (mol IL / g SIL)
Mr-N$_{1114}$*	0.004972	115.22	140.84	1.222
Mr-N$_{1222}$*	0.003434	115.22	97.291	0.8444
Mr-N$_{1444}$*	0.000582	199.37	28.532	0.1431
Mr- N$_{1114}$**	0.004880	115.22	138.25	1.200
Mr-N$_{1222}$**	0.002989	115.22	84.667	0.7348
Mr-N$_{1444}$**	0.000781	199.37	38.280	0.1920

Analysing the obtained values, it is possible to notice that the Mr-N$_{1114}$ is the Mr-SIL with higher bounding amount and lower molar weight, which is actually the same as Mr-N$_{1222}$. With the relation between the IL mass and the SILs mass, it is possible to confirm that the Mr-N$_{1114}$ is the one with the highest level of functionalization.

3.1.2. Point of Zero Charge (PZC)

In Figure 12 and Figure 13 are represented the Zeta potential curves for each Mr-SIL material, where the isoelectric point can be determined by the interception of the fitting curve with the x-axis (Table 6). This point resembles the point of zero charge (PZC), where happens the change of the electrical charge of the Mr-SILs. Under the pH of each PZC, the Mr-SIL particles will have a surface positive electrical charge, and above that pH value, the particles will have a negative surface electrical charge.

Merrifield resin

Mr-N$_{1114}$ *

Mr-N$_{1222}$ *

Mr-N$_{1444}$ *
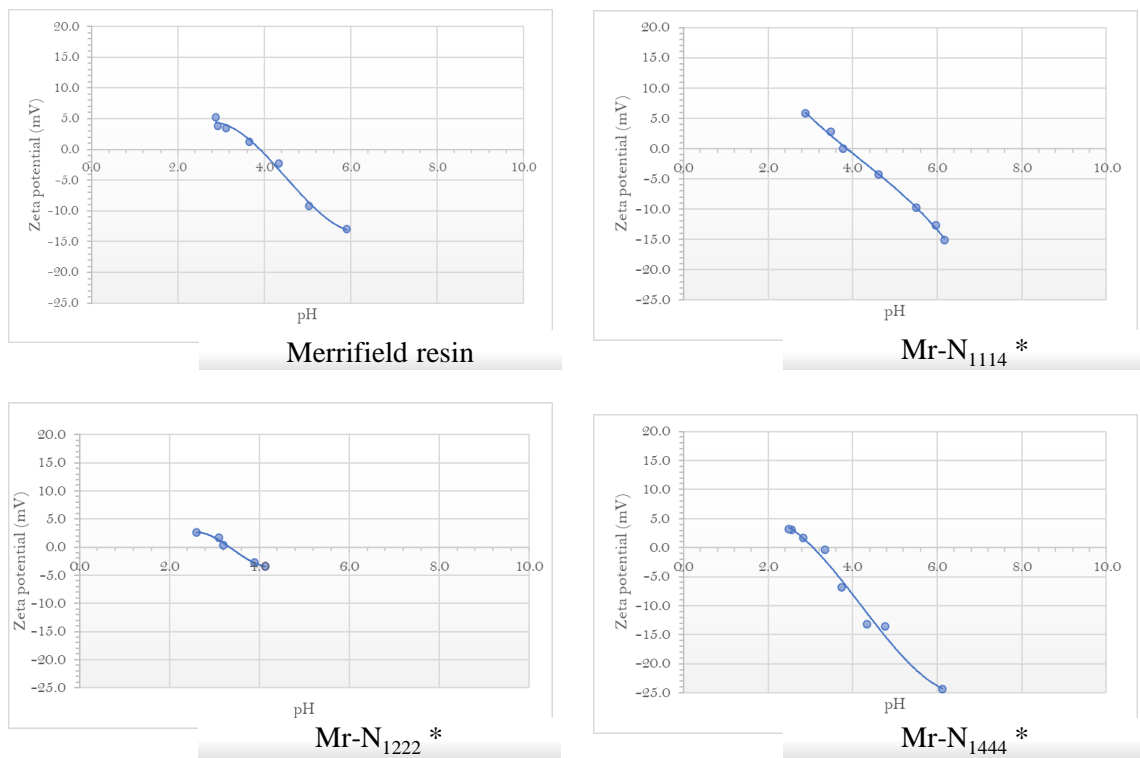
Figure 12 – Zeta potential values in function of pH for the materials prepared by the first method, Mr-SILs*.
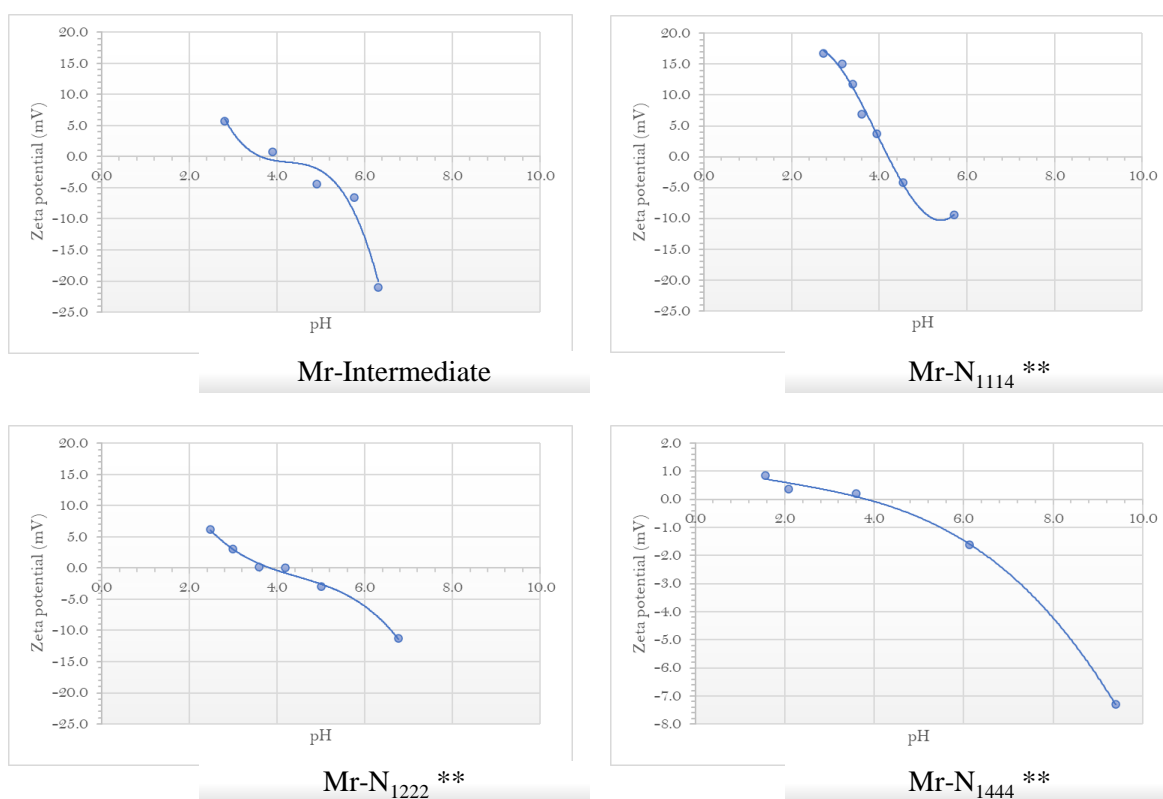
Figure 13 - Zeta potential values in function of pH for the materials prepared by the second method, Mr-SILs**.

Table 6 – Point of zero charge (PZC) for the synthesized Mr-SILs (obtained from Zetasizer Potential analysis).

Mr-SIL	PZC
Merrifield Resin	3.9
Mr-intermediate	3.9
Mr-N$_{1114}$*	3.5
Mr-N$_{1222}$*	3.0
Mr-N$_{1444}$*	3.8
Mr- N$_{1114}$**	4.2
Mr-N$_{1222}$**	3.9
Mr-N$_{1444}$**	3.9

Analysing Table 6 it can be observed that the PZC values of Mr-SILs vary between 3.0 and 4.2. This analysis was quite difficult to perform since the Mr-SIL suspensions were not stable and ended up depositing, making analysis unfeasible. This could be the reason why the PZC values for the same material prepared by different methods are different. This is not in agreement with the other

characterization techniques used, which demonstrate that the materials prepared by the two methods are chemically and structurally identical.
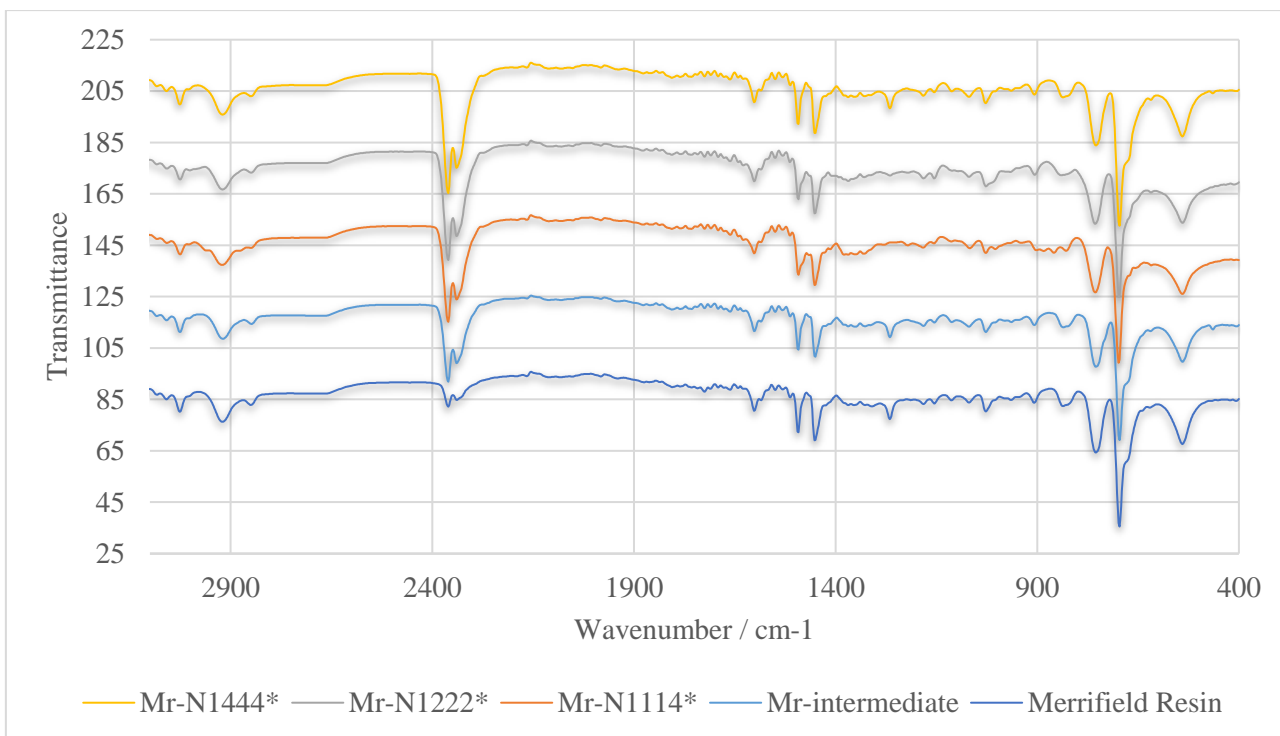
3.1.3. FTIR

Figure 14 – FTIR spectra of Mr-SILs*.
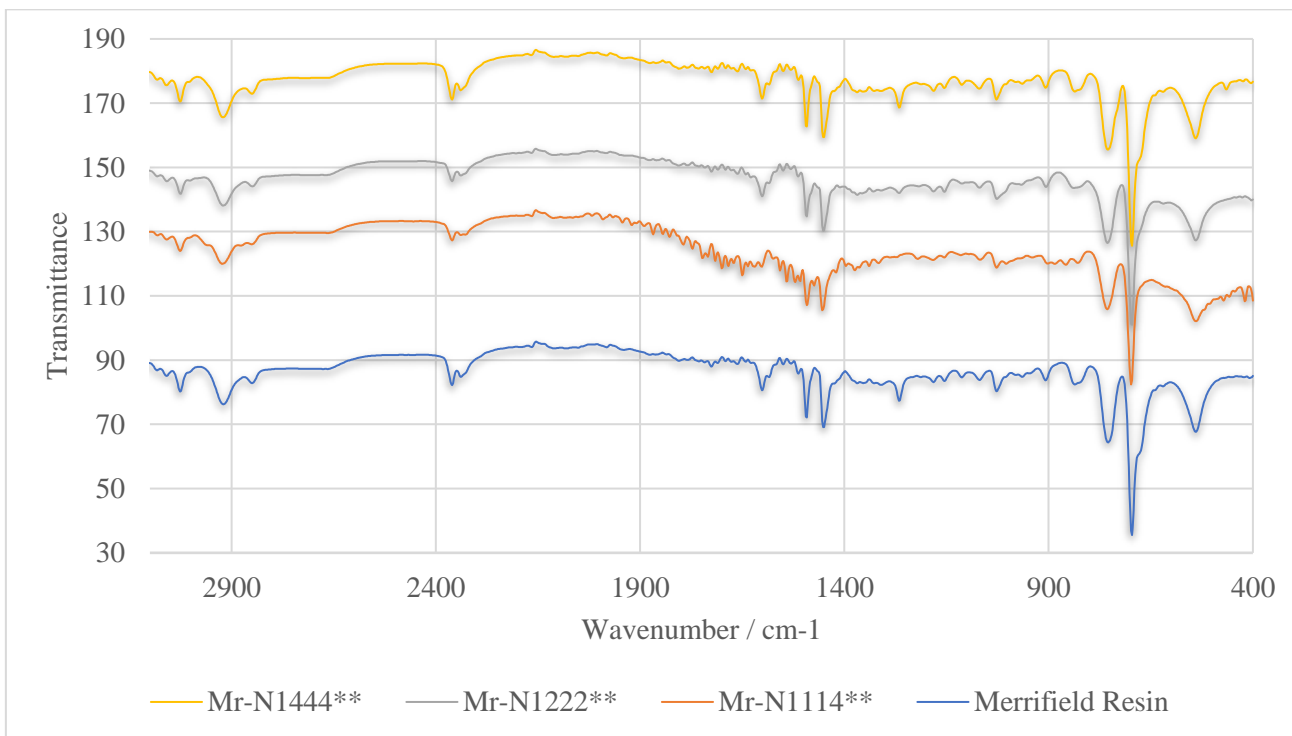
Figure 15 - FTIR spectra of Mr-SILs.**

By analysing Figure 14 and Figure 15, we could not verify differences between the spectra of the Merrifield resin and Mr-SIL materials. This may be justified by the low degree of surface functionalization by the ILs, as observed in silica materials [28].

3.1.4. ^{13}C CPMAS NMR

The obtained spectra of each NMR analysis are shown in Figure 16 and Figure 17.

Figure 16 - ^{13}C CPMAS solid state NMR spectra of Mr-SILs*.

Figure 17 – ^{13}C CPMAS solid state NMR spectra of Mr-SILs.**

According to Figure 16 and Figure 17, the Mr-SILs seem to be almost identical, which confirms the low functionalization of the materials, as seen in the other chara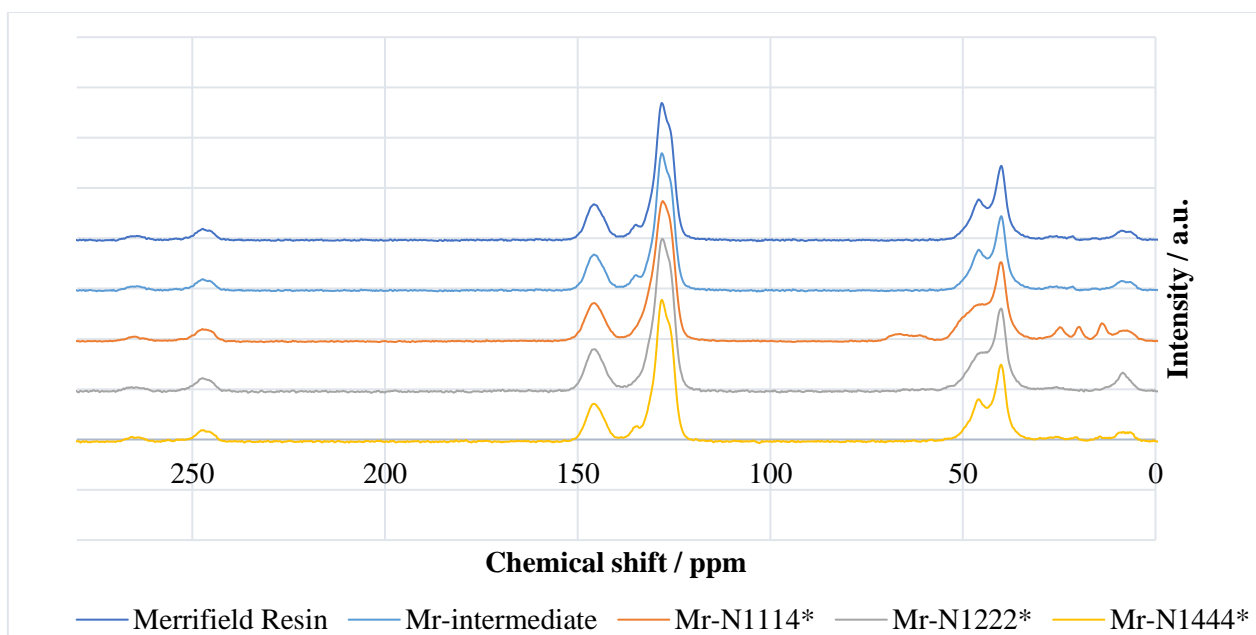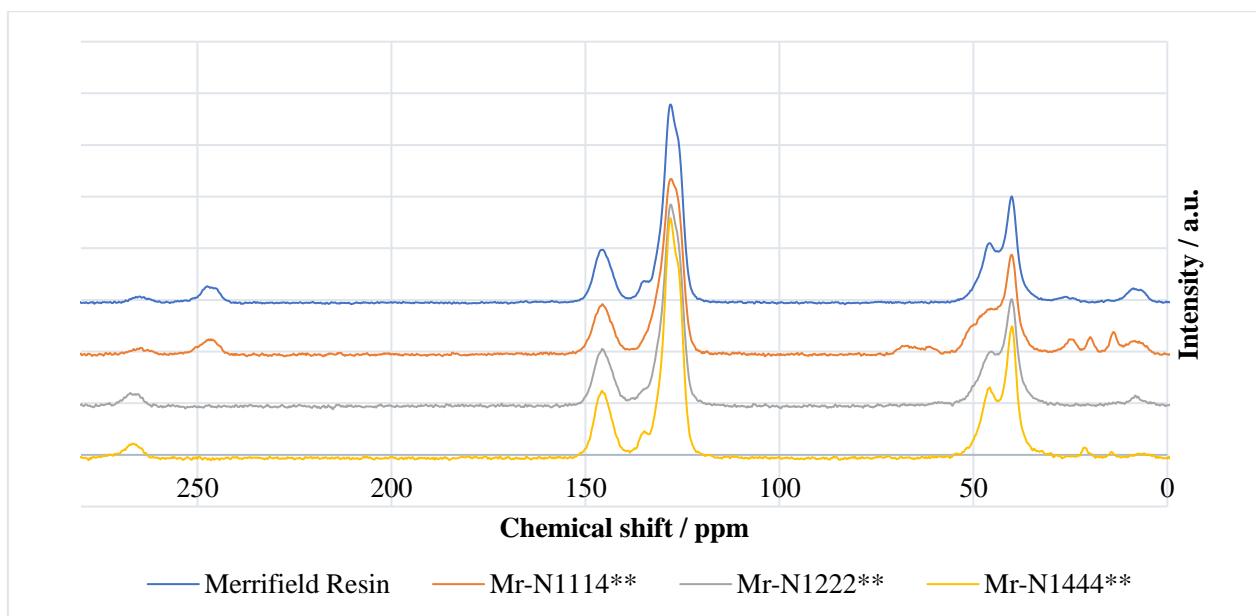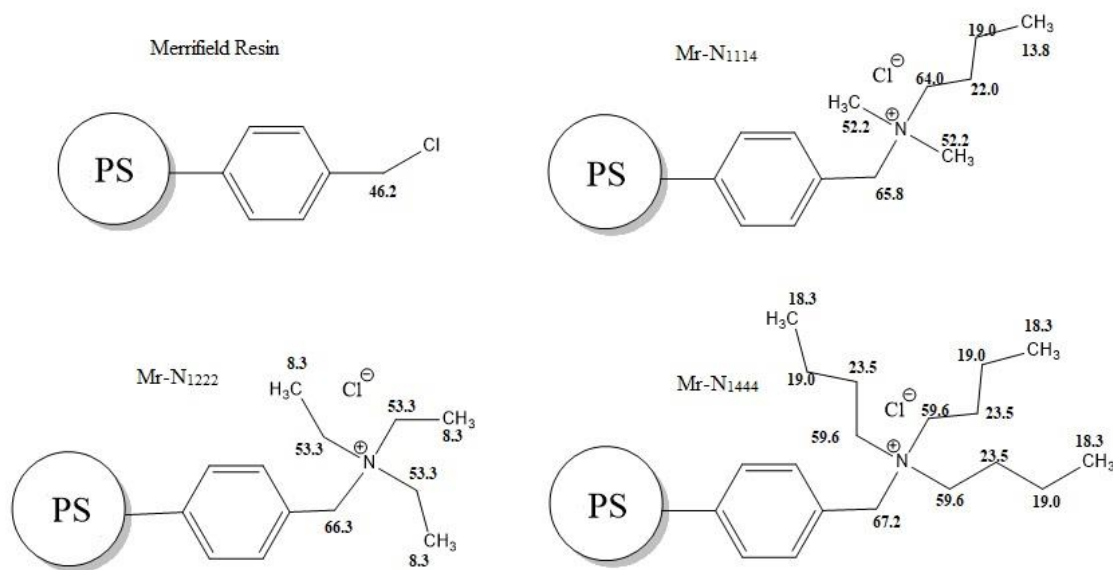cterization methods. The only different peaks observed in these results are in between 0 and 50 ppm and may correspond to the different alkyl groups of the Mr-SILs.

To help identify the chemical shifts associated with the carbons in Mr-SIL structures, the *ChemDraw* software (Figure 18) was used.

Figure 18 - Predicted NMR peaks of Mr-SILs in *ChemDraw* software.

3.1.5. SEM

According to different studies, Merrifield Resin shows a very smooth and homogeneous surface, but once functionalized with the ionic liquids, the surface tends to change their morphologically appearing to get a heterogeneous, kind of wrinkled, surface [30–32].

After the process of functionalization, the surface of the resin particles get some roughness, as shown in Figure 19b. This effect is not visible in all the Mr-SILs particles and is probably due to the process of synthesis namely the reflux in toluene.

Moreover, it can be observed in the Mr-SILs surface a small layer at the Merrifield resin surface, probably of IL. This is more visible in Figure 19h but this IL shell is visible in all the Mr-SILs materials.
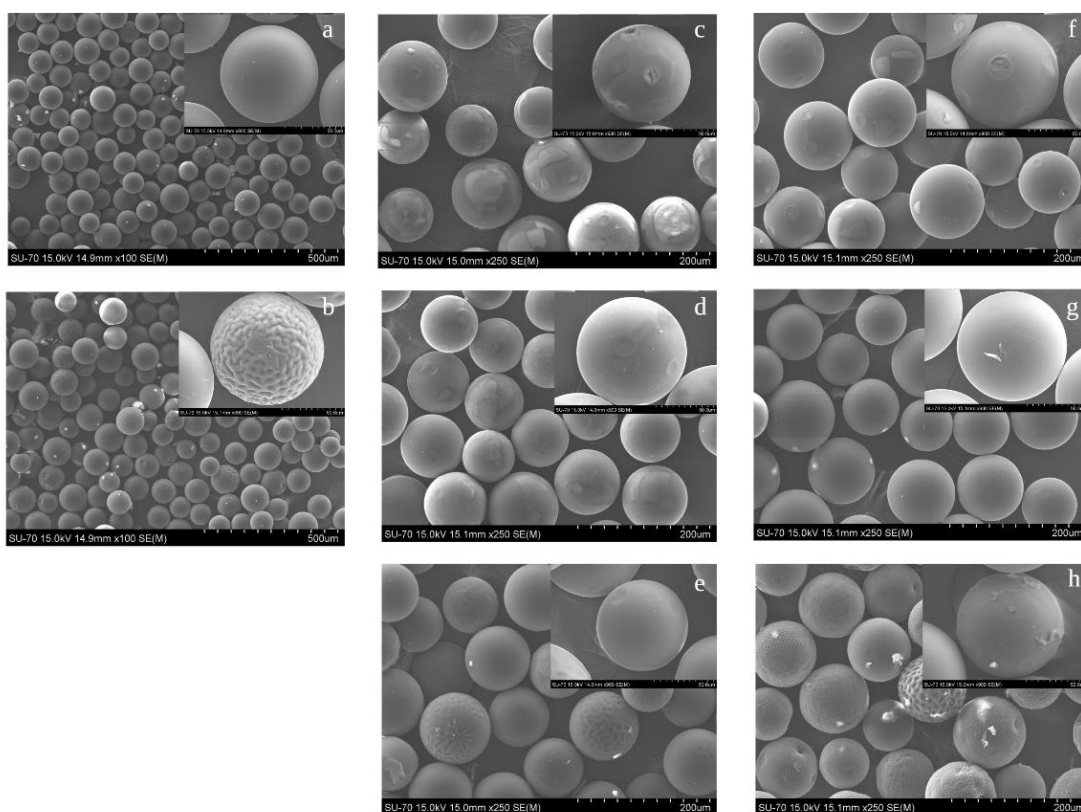
Figure 19 - SEM images of: a - Merrifield Resin; b - Mr-intermediate; c - Mr-N$_{1114}$*; d - Mr-N$_{1222}$*; e - Mr-N$_{1444}$*; f - Mr-N$_{1114}$**; g - Mr-N$_{1222}$**; h - Mr-N$_{1444}$**.

3.2.   Purification of L-ASNase

Figure 20 shows the comparation of different cell extract total protein concentrations for each Mr-SIL, whereas Figure 21 shows the different Mr-SILs for each cell extract concentration.

As seen in Figure 20, the more concentrated the cell extract, the higher is the L-ASNase specific activity. In this case, the highest total protein concentration is the original concentration itself (16.7 mg/mL). So, if possible, it would be interesting to try to produce a more concentrated cell extract and see if the L-ASNase activity continues to increase.

In other perspective, in Figure 21, for the same concentration, Merrifield Resin appears to be the material which confers the highest L-ASNase specific activity, but with a closer look, it is clear that all the materials have similar values for L-ASNase specific activities in each cell extract concentration, so Merrifield Resin is not necessarily the 'best' material, but practically the same as the others.
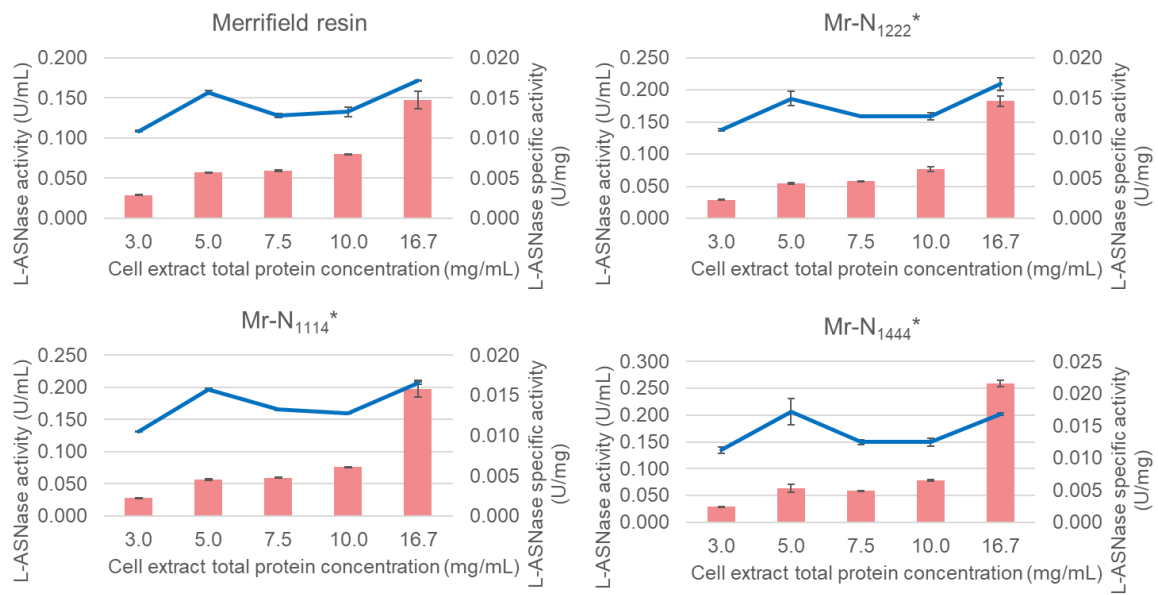
Figure 20 - Effect of cell extract total protein concentration on the purification of L-ASNase using for each Mr-SIL: L-ASNase activity (columns) and L-ASNase specific activity (line).
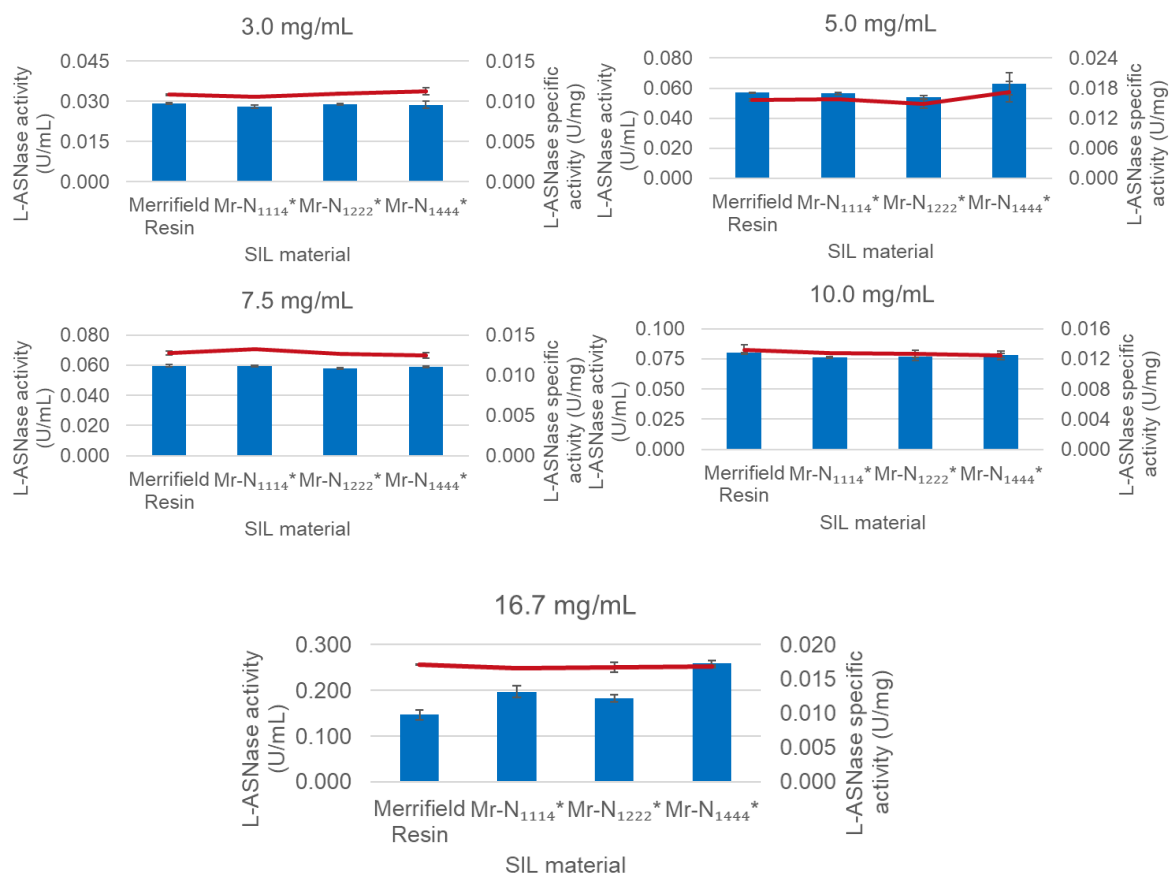
Figure 21 - Effect of cell extract total protein concentration on the purification of L-ASNase using for each concentration: L-ASNase activity (columns) and L-ASNase specific activity (line).

The purification factor (Equation 2) was calculated, for each cell extract and each material, and noted in Table 7.

Table 7 - Purification factor of L-ASNase obtained under the cell extract total concentration investigation.

	Purification factor			
Total protein concentration (mg/mL)	**Merrifield Resin**	**Mr-N$_{1114}$***	**Mr-N$_{1222}$***	**Mr-N$_{1444}$***
3	0.18	0.18	0.19	0.19
5	0.27	0.27	0.26	0.30
7.5	0.56	0.58	0.56	0.55
10	0.58	0.56	0.56	0.55
16.7	**1.33**	1.28	1.29	1.30

Figure 22 and Figure 23 show the effect of the amount of Mr-SIL in the L-ASNase purification. According to Figure 22, increasing the amount of Mr-SIL, the higher is the L-ASNase specific activity. On the other hand, in Figure 23, for each amount of Mr-SIL per each 1 mL of cell extract, the L-ASNase specific activity is, once again, similar for every material but, in the specific case of the higher Mr-SILs mass (50 mg), both Mr-N$_{1114}$* and Mr-N$_{1444}$* seem to be lightly higher.

The purification factor was also calculated for this condition and the results were noted in Table 8, where it can be noticed an increase, when comparing the purification factors of cell extract concentration optimization.
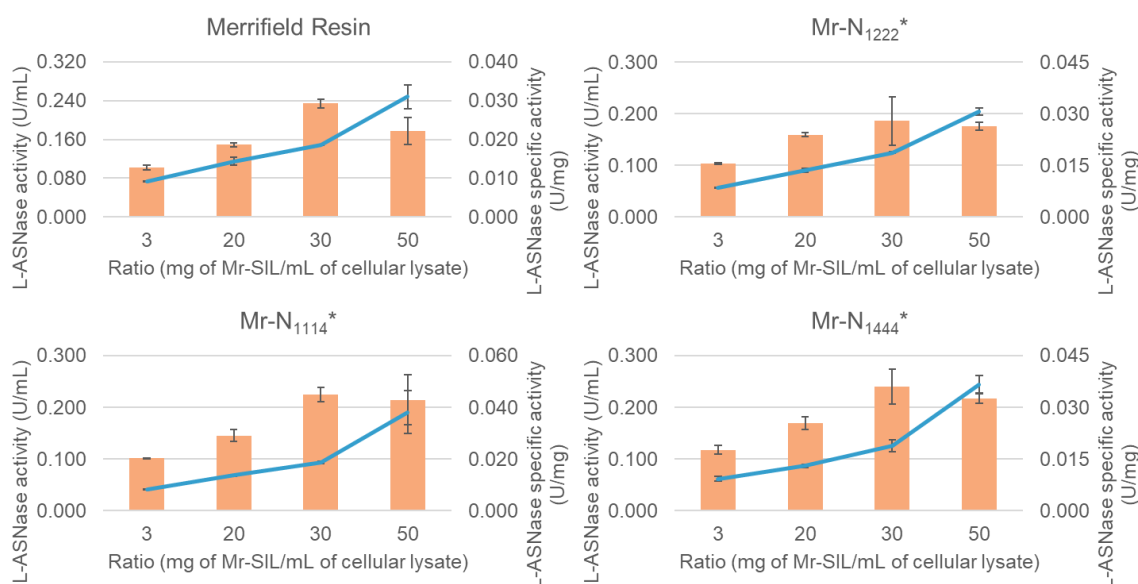
Figure 22 - Effect of Mr-SIL/cell extract ratio on the purification of L-ASNase using for each Mr-SIL: L-ASNase activity (columns) and L-ASNase specific activity (line).
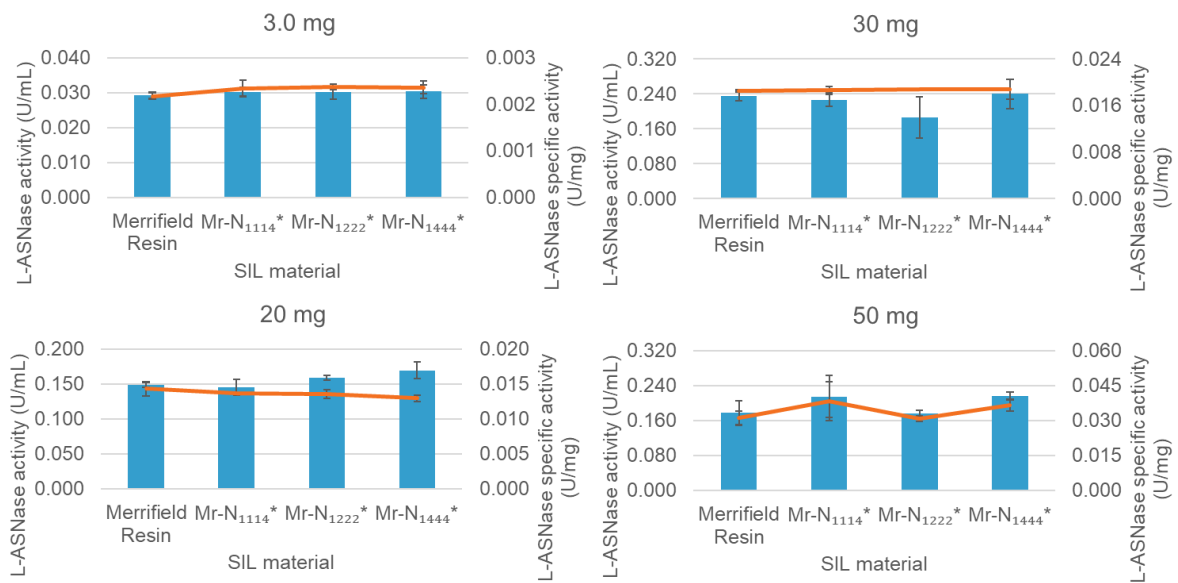
Figure 23 - Effect of Mr-SIL/cell extract ratio on the purification of L-ASNase using for each Mr-SIL mass: L-ASNase activity (columns) and L-ASNase specific activity (line).

Table 8 - Purification factor of L-ASNase obtained under the ratio optimization.

|	Purification factor			
SIL mass (mg)	**Merrifield Resin**	**Mr-N$_{1114}$***	**Mr-N$_{1222}$***	**Mr-N$_{1444}$***
3	0.27	0.29	0.30	0.29
20	1.30	1.24	1.23	1.18
30	1.68	1.69	1.70	1.71
50	2.81	**3.46**	2.78	3.32

After analysing all the purification factors, it can be concluded that the Mr-SILs are not adsorbing the L-ASNase, but the other impurities, since the purification factor is higher than 1, which means that the initial L-ASNase specific activity is lower than the final one. The zeta potential analysis (3.1.2.) also provides information about the electrical interactions between the Mr-SILs and the enzyme. As previously referred, the electrical charge under the PZC of each Mr-SIL is positive and above that pH value, it is negative. According to literature [33], the isoelectric point (pI) of L-ASNase, from *B. Subtilis*, is at pH 4.5 which means that under this value, the electrical charge of the enzyme is positive, and above this point it is negative (Figure 24). The considered pH used in the purification assays in this work is 8.6, point in which both Mr-SILs and the enzyme have negative electrical charges. With this, it is possible to conclude that the interactions between Mr-SILs and L-ASNase, if there are any, are not from electrical sources.
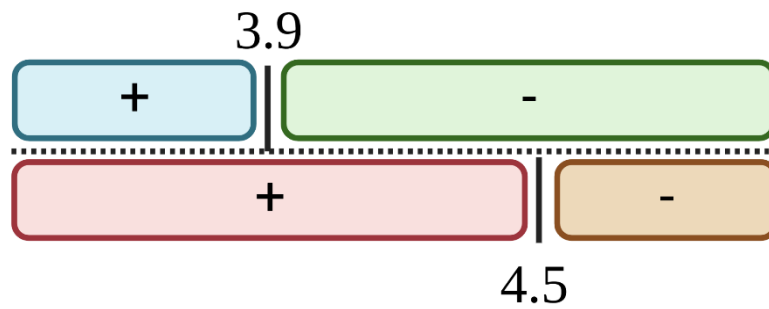
Figure 24 – Representation of the surface charge for Mr-N$_{1114}$ and L-ASNase (with PZC of 3.9 and pI of 4.5).

Once obtained the optimal experimental conditions (original cell extract concentration, 16.7 mg/mL, and 50 mg of Mr-SIL per each 1 mL of cell extract), Mr-SILs with only the second step of synthesis were tested, to see if there was any changes in L-ASNase purification (Figure 25).

When comparing all results (Table 8 and Table 9), it is possible to confirm that there are only slightly differences between the different Mr-SILs, but noticeable changes in purification factors, being the Mr-SILs* the higher ones.
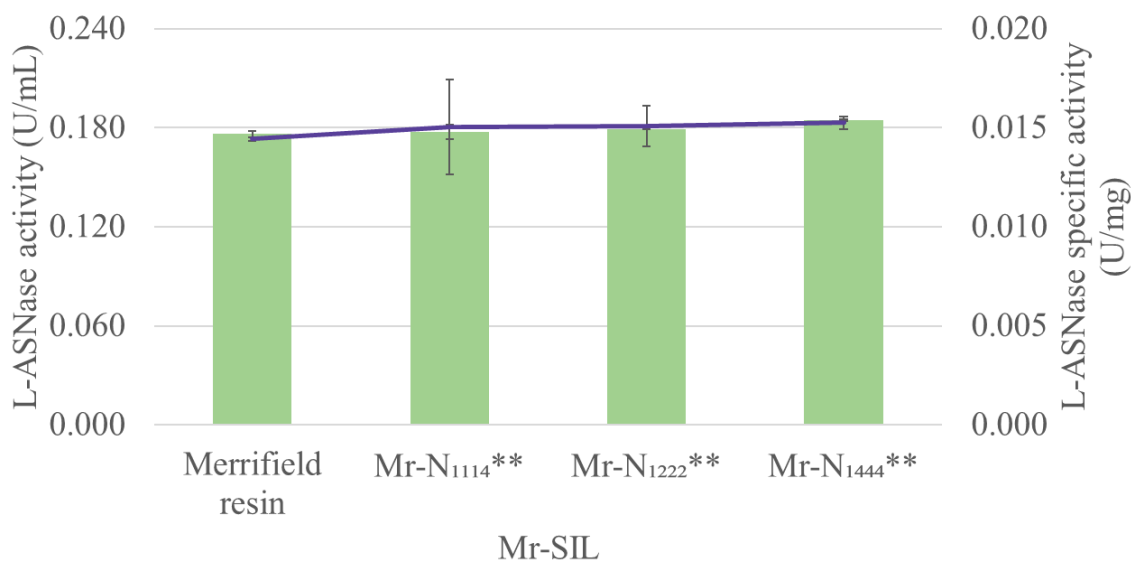
Figure 25 - Effect of Mr-SIL** on the purification of L-ASNase using for optimal conditions: L-ASNase activity (columns) and L-ASNase specific activity (line).

Table 9 - Purification factor of L-ASNase obtained under the optimal assay with Mr-SILs**.

Purification factor			
Merrifield Resin	**Mr-N$_{1114}$****	**Mr-N$_{1222}$****	**Mr-N$_{1444}$****
1.31	1.36	1.37	**1.38**

After the batch rehearses, it was tested the Mr-SIL with the best results, Mr-N$_{1114}$* in a column assay. In Figure 26, it is seen a variation of L-ASNase specific activity among the collected aliquots. So, to confirm that, the column assay was repeated, but without the peristaltic pump (only with gravity force) and with another cell extract (same fermentation conditions but different batch and different total protein concentration). The results can be observed in Figure 27.
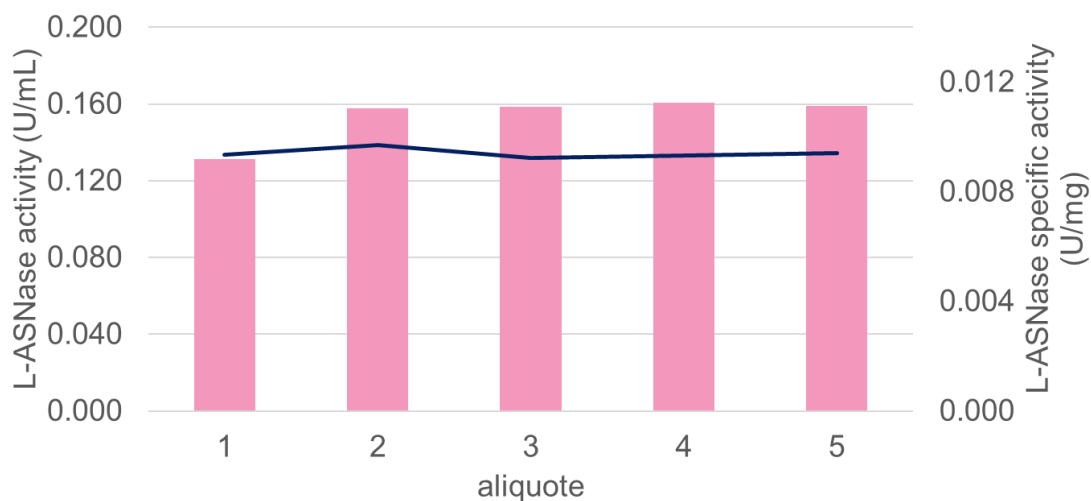
Figure 26 - Effect of semi-continuous method (with peristaltic pump) on the purification of L-ASNase using for Mr-N$_{1114}$*: L-ASNase activity (columns) and L-ASNase specific activity (line).

Table 10 - Purification factor of L-ASNase obtained under the column assay (with peristaltic pump) with Mr-N$_{1114}$*.

Purification factor				
1	**2**	**3**	**4**	**5**
0.16	0.16	0.16	0.16	0.16
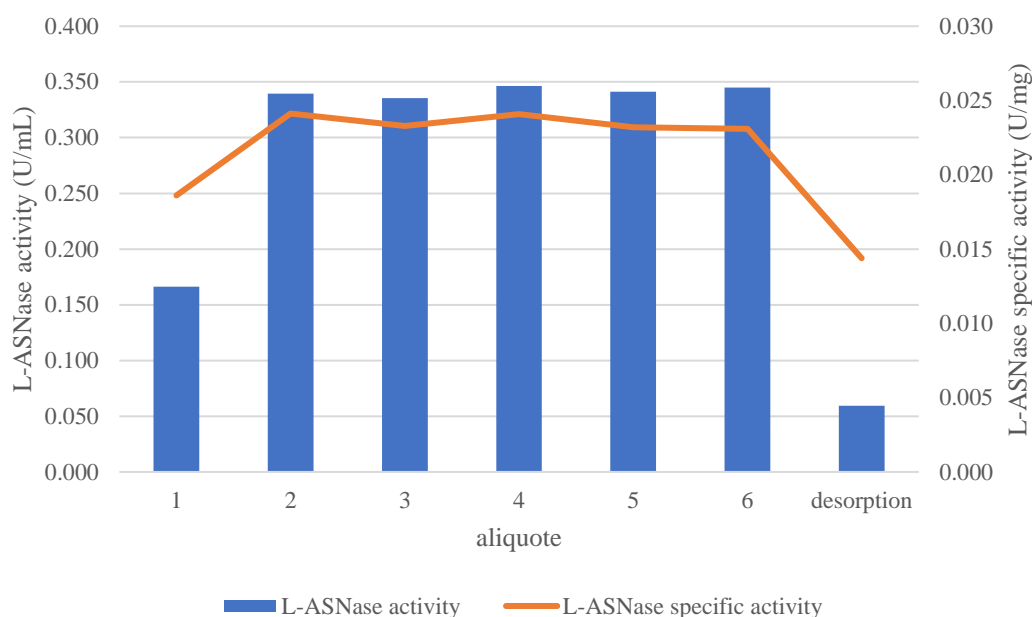
Figure 27 - Effect of semi-continuous method (with gravity force) on the purification of L-ASNase using for Mr-N$_{1114}$*: L-ASNase activity (columns) and L-ASNase specific activity (line).

Table 11 - Purification factor of L-ASNase obtained under the repeated column assay (with gravity force) with Mr-N$_{1114}$*.

Purification factor					
1	**2**	**3**	**4**	**5**	**6**
0.16	0.21	0.20	0.21	0.20	0.20

When compared the results from both column assays (Figure 26 and Figure 27), it is possible to observe that the second one (column assay with gravity force) proves that the enzyme stays adsorbed in the Mr-SIL, once the purification factor is less than one, which means that there is less L-ASNase in the aliquotes. However, when comparing the first column assay purification factors (Table 10) with the batch assay results previously obtained for Mr-N$_{1114}$*, it can be concluded that the column assay

has lower efficacy of purification of L-ASNase, probably because of the short contact time between the Mr-SIL and the cell extract.

3.2.1. SDS gel electrophoresis

This qualitative analysis permitted to analyse the presence of L-ASNase in each sample resultant from the rehearses.
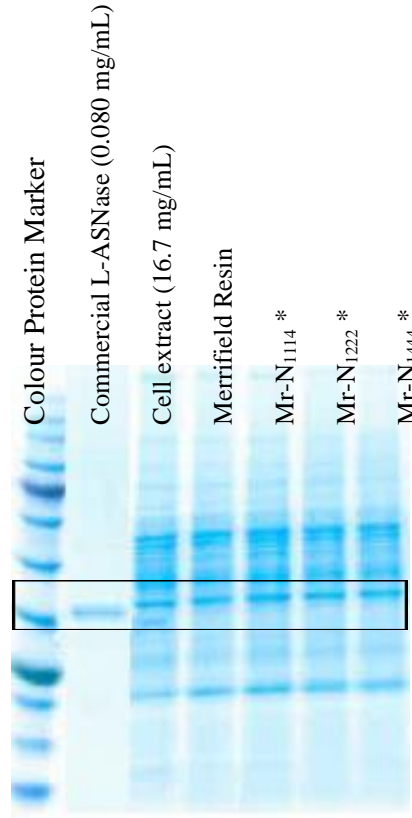
Figure 28 - SDS gel of cell extract total protein concentration batch assay (16.7 mg/mL).

Based on Figure 28, it can be concluded that the enzyme L-ASNase has a band size of 35 kDa, according to the NZY Colour Protein Marker I. It also shows the presence of other proteins, which represent the impurities of the cell extract. The other SDS gels present a similar image and for this reason are in Supporting Information.

Observing the SDS gel of the second column assay (with gravity force), in Figure 29, it can be noticed a slightly increase on the colour of some other protein band, and a slightly decrease on the colour of L-ASNase band, which can indicate that the Mr-SIL is starting to adsorb the enzyme and releasing the contaminants. This is interesting and may indicate that L-ASNase can also be absorbed in the Mr-SILs.
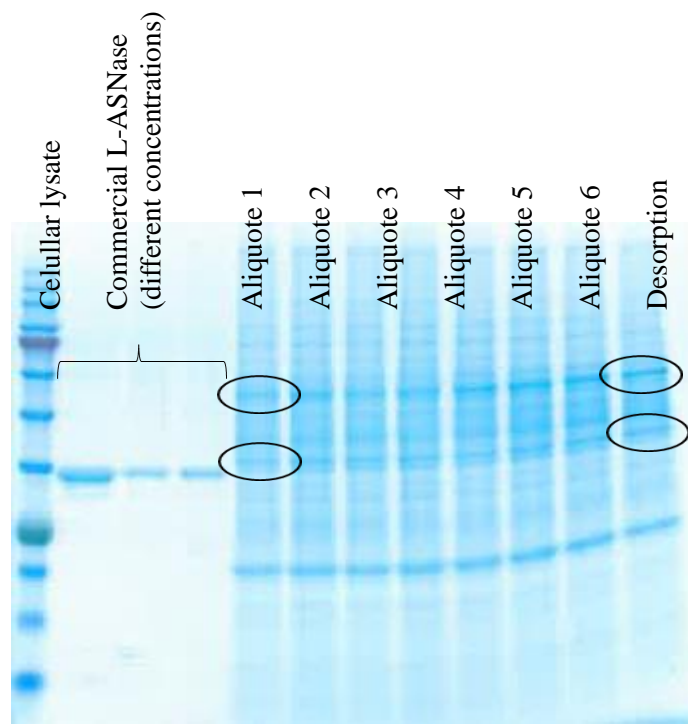
Figure 29 - SDS gel of column assay (gravity force).

4. Conclusions and future work

The aim of this dissertation was to produce and purify L-ASNase with less side effects. L-ASNase was produced in conditions already optimized by another research, and three different Mr-SILs were applied to purify L-ASNase. Cell extract total protein concentration and the solid/liquid ratio were optimized to increase the L-ASNase purity. Along that, the Mr-SILs were characterized by several techniques.

At the end of this research work, it was possible to conclude that the Mr-SILs can be synthetized by a single functionalization step. Regarding the use of Mr-SILs in the purification of L-ASNase it was concluded that the optimal conditions to purify L-ASNase occur with the higher total protein concentration of cell extract (in this case 16.7 mg/mL) and the higher ratio between the Mr-SILs mass and the cell extract volume (in this case, 50 mg of Mr-SIL per mL of cell extract). Mr-SILs demonstrated a higher affinity to the impurities, which turns out to be an advantage, since it is not necessary to desorb the pretended enzyme, L-ASNase, once it remains in solution. The higher purification fold obtained was 3.46, for 50 mg of Mr-N_{1114}* in contact with 1 mL of cell extract (16.7 mg/mL) for 60 minutes, in a batch mode. This value corresponds to a specific activity of 0.0382 U/mL.

Thereby, there is some interesting future work to develop around this work. In the batch mode, it would be interesting to study different pH values and contact times between the Mr-SILs and the cell extract, and also try to optimise temperature conditions.

Regarding the Merrifield resin supported ionic liquids, there is still a lot of research to do, namely trying to increase the functionalization degree, by adding a higher amount of precursor or test different cation sources with other functional groups, and study its influence in the purification of L-ASNase.

That way, these Mr-SILs could give an interesting step towards the purification of the L-ASNase, allowing it to be used safely, without causing side effects in the treatment of ALL, and other diseases.

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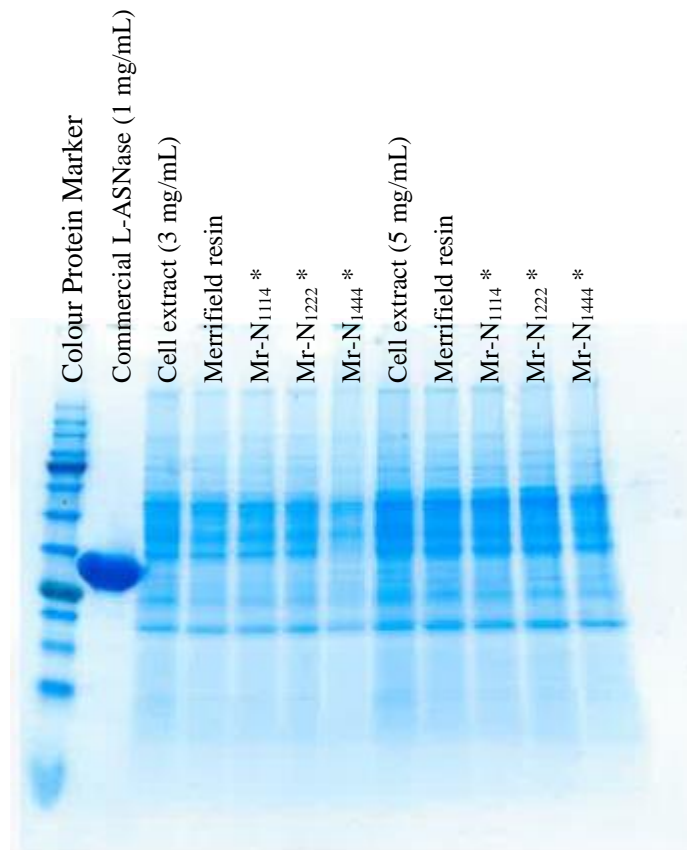
6. Supporting information

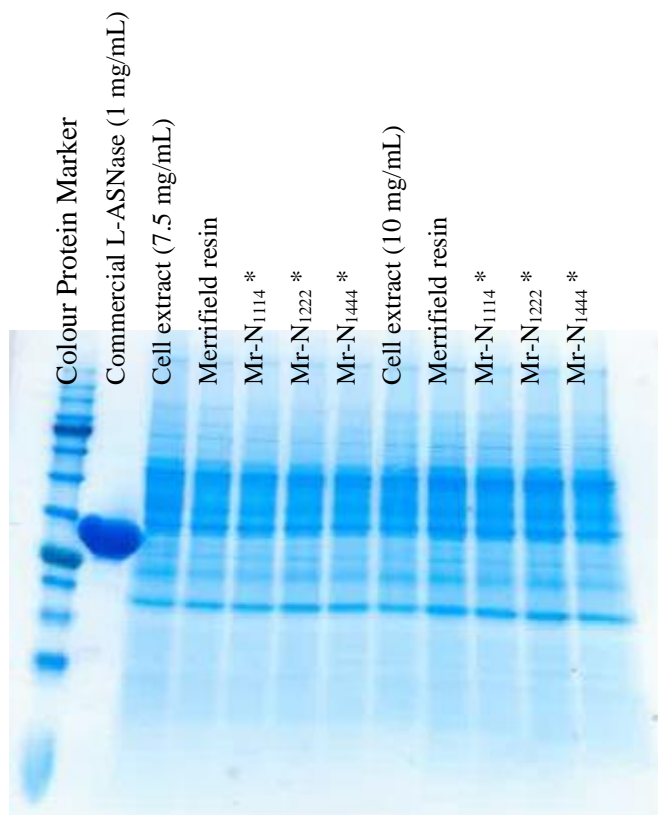**Figure S 1 - SDS gel of cell extract (3 mg/mL and 5 mg/mL) total protein concentration batch assays.**

Figure S 2 - SDS gel of cell extract (7.5 mg/mL and 10 mg/mL) total protein concentration batch assays.
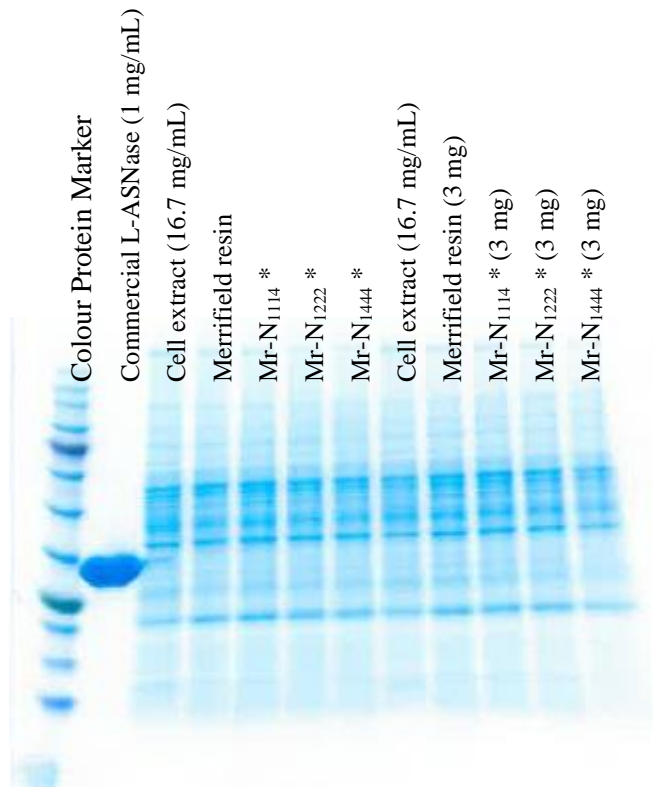
Figure S 3 - SDS gel of cell extract (16.7 mg/mL) total protein concentration and Mr-SIL/enzyme ratio (3 mg/mL) batch assays.
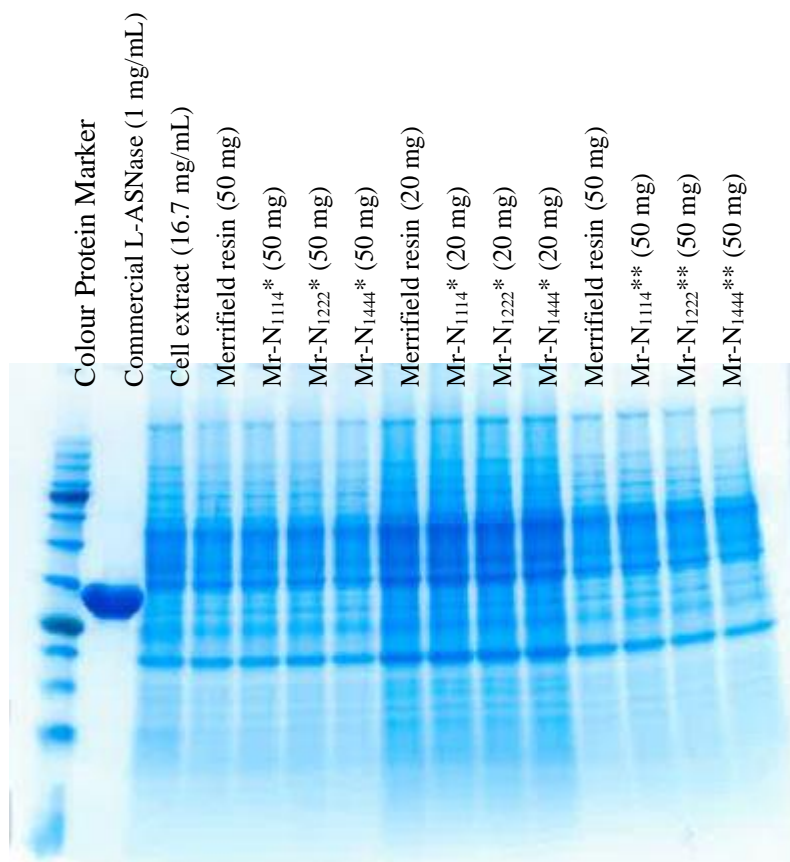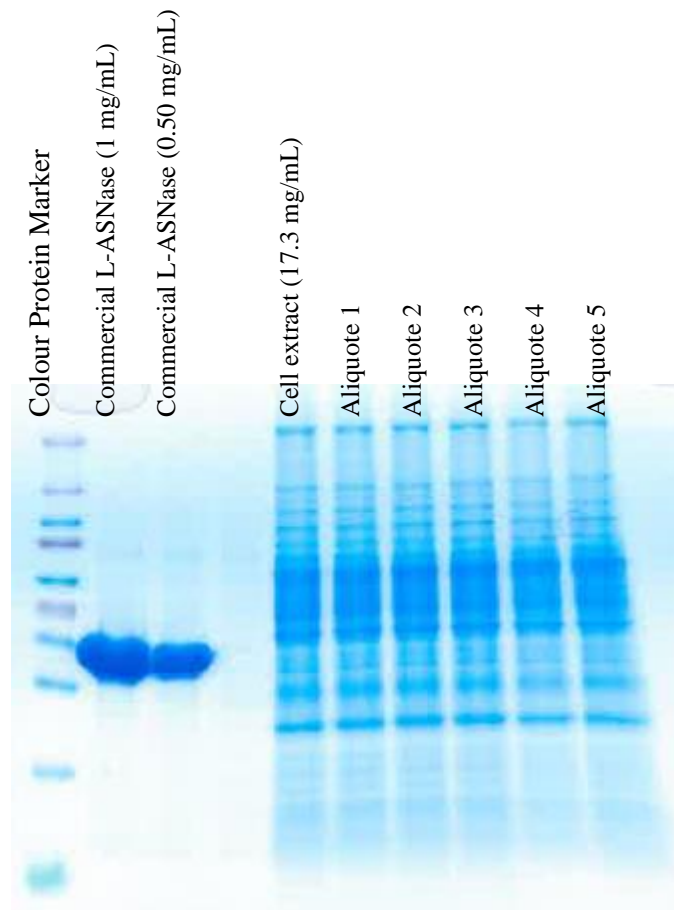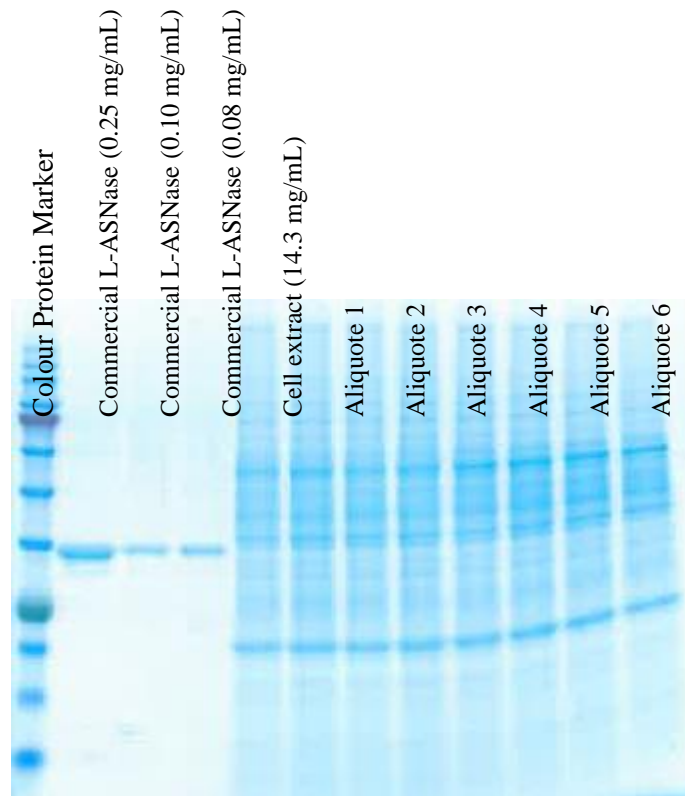

Figure S 4 - SDS gel of Mr-SIL/enzyme ratio (50 mg/mL and 20 mg/mL) and optimal Mr-SILs** batch assays.

Figure S 5 - SDS gel of column assay (peristaltic pump).

Figure S 6 - SDS gel of column assay (gravity force).