Ionic Liquids as versatile supports in Bioseparations

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Resumo

Os anticorpos monoclonais são ferramentas extremamente importantes na prevenção e no tratamento de diversas doenças, tais como diferentes tipos de cancro, doenças autoimunes, entre outras. No entanto, a produção e purificação de anticorpos é bastante complexa e dispendiosa, pelo que, nos últimos anos, a necessidade de encontrar soluções mais económicas e ambientalmente viáveis tem vindo a aumentar. O desenvolvimento de novos materiais baseados em líquidos iónicos é uma área ainda pouco explorada, mas bastante promissora. Devido à facilidade de "desenhar" as suas propriedades conjugando diferentes aniões e catiões, os líquidos iónicos têm mostrado ser especialmente úteis em processos de separação. Estudos recentes revelaram que polímeros de líquidos iónicos são uma solução vantajosa para a purificação de bacteriófagos, obtendo rendimentos comparáveis aos obtidos por métodos de purificação convencionais, como cromatografia de troca aniónica.

Neste trabalho, foi avaliada a possibilidade de usar polímeros de líquidos iónicos como matrizes em cromatografia de troca iónica para a purificação de anticorpos em modo negativo, isto é, para a captura específica das impurezas. Células CHO foram cultivadas num meio livre de soro, de modo a produzir anticorpos monoclonais (mais especificamente, anti-interleucina-8), juntamente com proteínas do hospedeiro. O anticorpo foi purificado recorrendo a cromatografia de afinidade e as impurezas separadas foram usadas nos estudos de adsorção usando matrizes de polímero de líquido iónico. Paralelamente, foram sintetizados e caracterizados vários polímeros de líquido iónico usando diferentes aniões (brometo e TFSI), catiões (imidazólio e metacrilato) e agentes reticulantes (DVB e EGDMA), obtendo-se em alguns casos eficiências acima dos 90%.

Palavras-Chave

Anticorpos monoclonais; anti-interleucina-8; CHO DP-12; polímeros de líquido iónico; imidazólios; amónios; cromatografia de troca iónica.

Abstract

Antibodies are highly valuable tools to prevent and treat many kinds of illnesses, such as different types of cancers, autoimmune diseases among others. However, their production and purification processes are quite complex and thus costly. Therefore, the urge to find economically and environmentally viable solutions to purify these proteins has increased exponentially in the recent years. In particular, new materials that allow superior performance of traditional separation and purification techniques have been developed. Due to easy tunability of their properties, ionic liquid-based materials have shown to be promising materials in a large range of fields. For instance, recent studies showed that poly(ionic liquids) are a possible solution for purification of bacteriophages, resulting in yields which are comparable to those obtained using conventional chromatography techniques, such as ion-exchange chromatography .

In this work, the feasibility of PILs as ion-exchange matrices for antibodies purification in a negative mode, meaning impurities are captured, was assessed. CHO cells were cultured in a serum-free medium, to produce monoclonal antibodies (specifically, anti-interleukin-8), as well as host cell proteins. Hence, the antibody was purified resorting to affinity chromatography, with the impurities being retrieved, in order to assess the capture step of the purification process using matrices of PILs. Additionally, PILs were synthesized and further characterized, as different anions (bromide and TFSI), cations (imidazolium and methacrylate) and crosslinkers (DVB and EGDMA) were evaluated, with efficiencies over 90%.

Keywords

Monoclonal antibodies; anti-interleukin-8; CHO DP-12; poly(ionic liquids); imidazolium-based; ammonium-based ionic liquids materials; ion-exchange chromatography.

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List of Abbreviations

[C_4mim][Cl] – 1-butyl-3-methylimidazolium chloride

[C_2mim][TFSI] – 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide

[C_8mim]Cl – 1-methyl-3-octylimidazolium chloride

AEX – Anion-exchange chromatography

ATPSs – Aqueous Two Phase Systems

BSA – Bovine serum albumin

CALB – Lipase B from *Candida Antarctica*

CDRs – Complementary determining regions

CEX – Cation-exchange chromatography

CHO cells – Chinese Hamster Ovary cells

Cpg – Controlled porous glass

CV – Column volume

DMEAEM – (dimethylethylamino)ethyl methacrylate

DTT – Dithiothreitol

DVB - Divinylbenzene

EDTA – Ethylenediaminetetraacetic acid

EGDMA – Ethylene glycol dimethacrylate

Fabs – antigen binding site fragments

F**c** - crystallisation

FDA – Food and Drug Administration

FT – Flow-through

GVHD – Graft-verus-host disease

HCP – Host cell proteins

HIC – Hydrophobic interaction chromatography

IEC – Ion-exchange chromatography

IFX – Infliximab

IgG – immunoglobulin G

IL-8 – Interleukin-8

ILs – Ionic liquids

LiTFSI - Bis(trifluoromethane)sulfonimide lithium salt

mAbs – Monoclonal Antibodies

MTX - Methrotrexate

NMR – Nuclear Magnetic Resonance

PEG – Polyethylene glycol

pI – Isoelectric point

PIL – Poly(ionic liquid)

PPG – Polypropylene glycol

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Scanning Electronic Microscopy

Si – Silica

SPA – Staphylococcal protein A

TFSI - Bis(trifluoromethane)sulfonimide

TNF – α – Tumour necrosis factor α

VEIM – 1-vinyl-3-ethylimidazolium

1. Introduction

1.1. Antibodies

1.1.1. Historical Perspective and Evolution

Monoclonal antibodies (mAbs) have emerged as extremely important therapeutic molecules since 1980s. However, their production has limitations, considering that it was of paramount importance to achieve a certain specificity to avoid rejection of the treatment[1]. Furthermore, in 1975, as will be explored, Köhler and Milstein fused murine myeloma cells with splenic B cells from an immunized mouse to create cells with the ability to produce highly specific mAbs, developing the hybridoma technology[2]. Thus, in 1986, the first ever therapeutic mAb approved for clinical uses, the orthoclone OKT3, was derived by this technique, being used for the prevention of kidney transplant rejection[3]. This antibody was the first chimeric mAb, marking the turning point for mAbs as therapeutic agents. Later on, humanized and fully human antibodies started being produced and approved by the FDA as therapies. Every year, new mAbs are approved as drugs to fight life threatening diseases, such as autoimmune diseases, cancer, and many others[1].

1.1.2. Market

Initially, as the first mAbs could cause immunogenic reactions since these were not humanized, the sales growth of these products was slow until the 90s. However, once the first chimeric antibody was approved, in the late 90s[3], the market changed, with the global sales revenue for these therapeutic products climbing up to $98 billion[4] until 2017.

Whereas by 2013 the approval of mAbs each year had become steady (1.6 new mAbs per year), 31 new mAbs were introduced in the market for the past 5 years, thus increasing the average of mAbs approved by year to more than 6 mAbs per year, from 2013 to 2018.

Regarding antibody manufacturing, Chinese Hamster Ovary cells (CHO) are the main production organism of mAbs, with around 60% of these antibodies being produced in CHO cell cultures [4], as they heavily facilitate the manufacturing of human mAbs, which, in turn, comprise 54% of the therapeutic mAbs (**Figure 1**)[4,5].

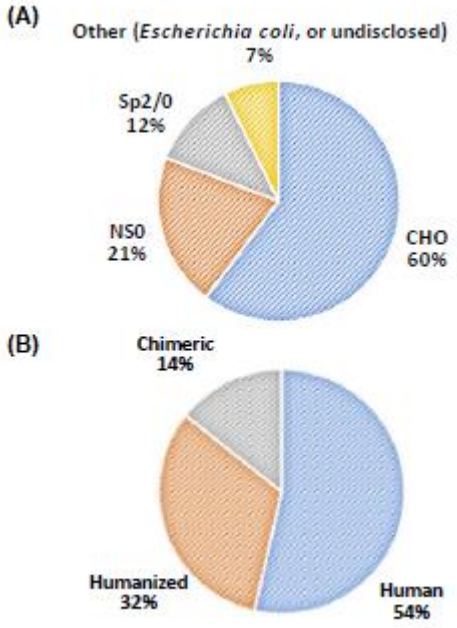


Figure 1 - Representation of (A) the mAbs production organism, as well as (B) the antibodies origin sequence (adapted from Grilo *et al.* 2018).

1.1.3. General Considerations

Antibodies, also designated as immunoglobulins, are soluble proteins produced by plasma cells (such as B lymphocytes) as an immunological response[6]. These glycoproteins are comprised of four polypeptides: two identical large heavy chains (55 kDa, approximately) and two identical light chains (25 kDa approximately). These peptides are bound through disulphide and non-covalent bonds, giving rise to a Y-shaped molecule of, approximately, 150 kDa[7] (**Figure 2**). Both heavy and light chains have variable regions and constant regions. The variable regions are located on the top of the arms of the molecule and the constant regions remain similar between the same class of antibodies[8]. Furthermore, three regions in each chain are hypervariable and are termed complementary determining regions (CDRs), where the antigens bind specifically[8]. The variability among the antigen-binding regions allows different antibodies to bind to different antigens, meaning that they respond to different stimuli[9].

1.1.4. Immunoglobulin G

Immunoglobulin G (IgG) is one of the most common proteins in the blood serum and its structure (**Figure 2**) resembles that described above. The region connecting the stem and the arms, denominated "hinge", is highly flexible but prone to proteolysis, exerted by the proteolytic enzymes such as papain and pepsin. When this phenomenon occurs, the protein is digested into smaller fragments. Papain cleaves the antibody above the hinge region giving rise to two Fabs (antigen binding site fragments), which correspond to the arms, and one Fc (where c stands for crystallization, as this fragment crystallizes rapidly), which corresponds to the stem[9,10].

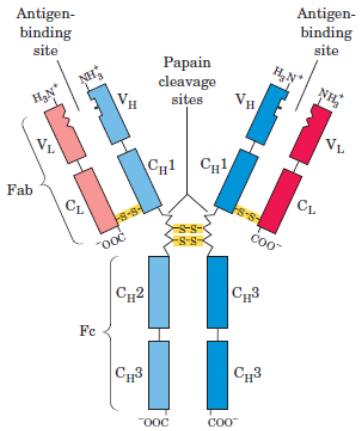


Figure 2 - *Immunoglobulin G structure. V stands for variable region, whilst C stands for conserved region; H represents Heavy Chain and L represents Light Chain[10].*

Mechanism of immune response

Immunoglobulin G plays a key-role in the immune system, being the principal antibody involved in secondary immune responses. When this protein interacts with invading organisms, it triggers a response in specific cells, such as leukocytes, activating and directing them to act against the foreign structures. Moreover, several IgGs bind to the invader and their Fc regions bind to a macrophage (Fc receptors), initiating the phagocytosis process[10] (**Figure 3**).

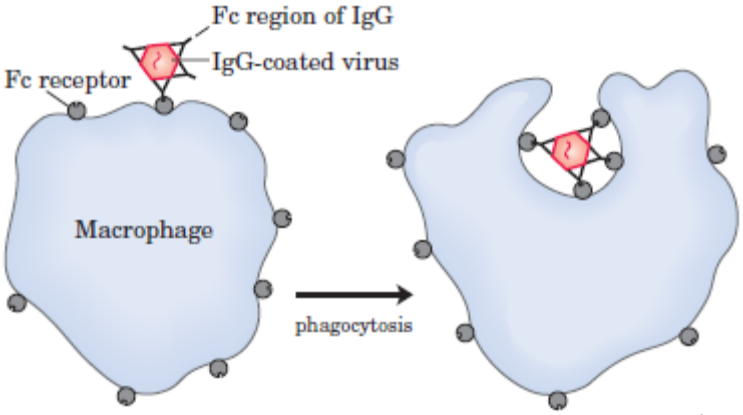


Figure 3 - *Phagocytosis of an IgG coated virus by a macrophage[10].*

To disclose the process of recognition of the invader by the antibody, the interaction between and the antigen and an antibody must be understood. The antigens which promote an immune response are designated immunogens. However, the antibody does not react with the whole antigen; instead it recognizes small sequences denominated the epitopes[6]. Thus, the antibodies specifically bind to the antigenic epitopes through the antigen binding sites located in the Fab fragments[6] (**Figure 4**).

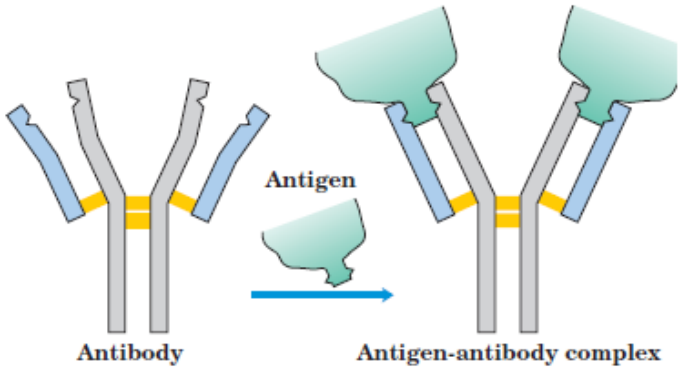


Figure 4 - *Binding of an antigen to its specific binding site in an antibody[10].*

1.1.5. Polyclonal and monoclonal antibodies

Depending on the production method, antibodies may be polyclonal or monoclonal. Antigens possess various epitopes, allowing their recognition by different lymphocytes. Each lymphocyte cell is stimulated to differentiate into plasma cells, which will then produce distinct antibodies that recognise the same antigen but binding at different epitopes. Therefore, the antibodies present in this mixture are named polyclonal antibodies, being produced by multiple B cells clones, resulting in different specificities and sites of action[7].

Monoclonal antibodies differ from polyclonal antibodies, given that they are produced by a single clone of B cells, thus resulting in a high antigen specificity. These antibodies are exquisite “devices” that may be applied in therapies or diagnostic techniques, since they recognize one specific epitope[11]. In 1975, Kohler and Milstein considered that the existing cultures of myeloma cells were not enough to produce specific antibodies in a large scale. Therefore, hybridoma technology was developed, in which a mouse was immunized with a pathogen and its splenic B cells were fused with myeloma cells. As a result, specialized cell lines were obtained that were both immortal and able to produce the desired antibody. It was concluded that these cells were able to generate great amounts of the specific monoclonal antibody desired[2].

Monoclonal antibodies (mAbs) are of paramount importance in several scientific fields, such as medicine, analytical biotechnology, among others. In fact, they can be used as inhibitors of disease progression either by necrosis or inactivation of the target cells[12], given that they have highly specific action

sites. Additionally, as they can guide certain molecules to the disease site, they can act as a diagnostic tool (immunoassays) or as a therapeutic agent[13]. Hence, theoretically, a monoclonal antibody can be designed to intervene in any kind of disease. According to this principle, many studies were performed in order to assess the effect of the antibodies in several diseases[11], which will be further on explored in this work.

B-cell lymphoma consists in a type of cancer which proliferates in the lymph nodes. Rituximab was developed as a response to this kind of illnesses, becoming widely used into many chemotherapy treatments[14]. Ocrelizumab and ofatumumab are also used to treat this disorder[15]. However, rituximab has been increasingly applied as a treatment of autoimmune disorders, such as rheumatoid arthritis, given that its action has proven to be effective[16].

Crohn's disease is a chronic inflammatory illness, which affects the gastrointestinal tract[17]. Due to the emergence of the monoclonal antibodies and their high specificity, the infliximab (IFX) was formulated. This antibody is a chimeric human/murine monoclonal immunoglobulin G that binds particularly to tumour necrosis factor α (TNF- α)[18]. IFX has shown to be effective in the treatment of this disease[19].

Graft-versus-host disease (GVHD) occurs as an immune response towards a transplanted tissue or organ, causing its rejection[20]. To avoid this complication, monoclonal antibodies may be used, preventing the development of disease or as a treatment option for patients[21]. Basiliximab is a monoclonal antibody and, with a combination of cyclosportine, methotrexate and mycophenolate mofetil, was used to assess its effects regarding the GVHD. Hence, as demonstrated in Chen *et al.* (2003), the application of this antibody is favourable, since the displayed severity of this disorder was decreased[22].

Monoclonal antibodies can also target tumours and their neighbouring cells and, by blocking the binding of ligands or by signalling certain molecules, may decrease the high growth rates of carcinogenic cells, lead to their apoptosis or even expose the tumours to other therapeutic agents[23].

Hence, it can be stated that the use of monoclonal antibodies has widely increased over the years, given that their characteristics are interesting, from a therapeutic point of view, as referred to above.

Furthermore, to use antibodies as therapeutic agents, their production should take into account high levels of purity inherent to the product, allowing the removal of all impurities such as other proteins, DNA and other contaminants[24].

1.1.6. Purification of antibodies

Affinity chromatography

Various techniques may be used in the primary purification step of antibodies. Protein A chromatography, for example, is widely used as a capture step, in order to rapidly reduce the volume of the sample as well as to separate the antibody[25]. Affinity chromatography relies on the highly specific interactions between certain molecules. For instance, staphylococcal protein A (SPA) has high affinity towards IgG and this interaction played a crucial role in the development of affinity systems as platforms for protein purification[26]. SPA has five homologous IgG binding domains, which strongly interact with Fc-part of IgG1, IgG2, IgG4, but not with IgG3 (appears to have a weak interaction). This protein facilitates the purification of mAbs, given that it binds specifically to IgG. Therefore, this technique presents many advantages, such as a high selectivity for antibodies and a high yield of purification[25]. On the other hand, it is also time-consuming, highly expensive and within the column the intraparticle diffusion is slow and the pressure drop is high[27]. Hence, other techniques are being studied, in order to overcome these drawbacks inherent to the process. These may include alternative modes of chromatography, such as ion-exchange chromatography, multimodal chromatography, or non-chromatographic alternatives, such as aqueous two-phase extraction.

Ion-exchange chromatography

Regarding the chromatographic alternatives, ion-exchange has been widely used as capture and polishing steps in protein purification downstream processing. Ion-exchange chromatography (IEC) is widely used for analytical and preparative purposes, given that proteins and other macromolecules are charged , thus allowing their separation and purification by ionic interactions[28].

The IEC depends on the attraction of oppositely charged molecules. To purify a protein, for instance, its charge may be manipulated, by changes in the pH and according to the protein's isoelectric point (pI). The matrix of this type of chromatography is frequently in the porous form, in order to allow a high surface area and therefore a high adsorption. Depending on the charges of the molecule of interest at a given pH, the exchangers will have different charges and designations. Furthermore, a cation-exchanger adsorbs positively charged molecules, thus being negatively charged. Oppositely, the anion-exchanger is positively charged, hence adsorbing negatively charged molecules (**Figure 5**)[10,29,30]. The binding of any charged species to the adsorption matrix can be defined by the mass action law, as demonstrated in Boardman and Partridge (1955)[31].

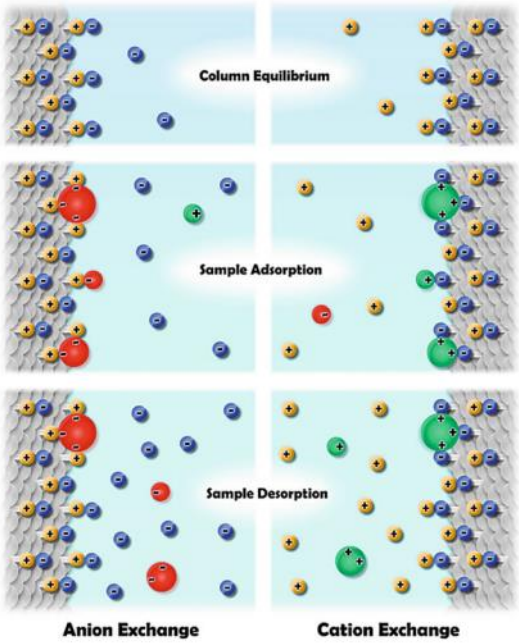


Figure 5 – *Scheme of a chromatographic workflow[28].*

Cation-exchange chromatography (CEX) is the non-affinity unit operations preferred to carry out the capture step in the downstream processing of mAbs. Indeed, CEX is already used in several commercial processes, including for instance the mAb-product Humira® (adalimumab)[32]. Anion-exchange chromatography (AEX) is also widely used for polishing of mAbs[33]. This step is designed to capture remaining impurities such as host cell proteins while the antibodies do not interact and are recovered in the flow-through fraction. Other modes of interaction beside ion-exchange have also been studied to achieve the purification of monoclonal antibodies, such as chromatography on phenylboronate resin[34]. This multimodal technique allowed the direct capture of the IgG, leading to a high recovery yield as well as a high clearance of the impurities. Thus, it can be used as a capture step or as a pre-purification step, to undergo further purification.

Tao *et al.* (2014) assessed whether the cation-exchange (CEX) chromatography would be a good alternative to Protein A chromatography as a capture step of monoclonal antibodies. These authors concluded that the CEX chromatography (newer generation with high capacity) overcame the drawback inherent to Protein A chromatography, thus constituting a viable option[35].

Urmann *et al.* (2010) evaluated the effects of different resins used in IEC with removing contaminant the host cell proteins (HCP) more efficiently than others, thus keeping the antibody in solution. According to the obtained results, this technique is considered a valuable tool as a capture step in the purification of antibodies process[36].

Xu *et al.* (2012) used IEC as a polishing step (cation-exchange chromatography followed by anion-exchange – AEX – chromatography), after protein A affinity chromatography. As a result, a robust process of monoclonal antibodies was obtained, in which the hcp level decreased, and the DNA, endotoxins and viruses were completely eliminated[33].

Considering the studies about IEC, it can be stated that this technique is a valuable asset to purify the highly-demanded antibodies.

Aqueous Two-Phase Systems

Regarding the non-chromatographic alternatives, extraction in aqueous two-phase systems (ATPSs) is widely used to separate molecules of interest, given that they are biocompatible and have a high water content, facilitating the preservation of the molecule's activity[13]. Traditionally, these systems may be composed by two polymers (for example, polyethylene glycol – PEG – and dextran) or by one polymer and one salt (citrate, phosphate, among others). The formation of phases occurs at certain conditions and when the amount of phase forming compounds present exceeds a critical concentration[37].

In Azevedo *et al.* (2007), an optimization of ATPSs has been developed as a primary step to purify IgG from both CHO and hybridoma cell cultures supernatant by varying the concentrations of PEG (with molecular weights of 6.000 and 20.000) and sodium chloride (NaCl). As a result, the system with 12% of PEG and 15% NaCl achieved an IgG recovery yields from the CHO and hybridoma cell cultures of 88% and 90%[13], respectively. Other types of ATPS systems have also been used by this group of researchers and others, in which promising results were also obtaining thus showing that ATPSs can be effectively used to separate antibodies.

Furthermore, chromatography was also successfully coupled to the ATPSs, in which the upper phase of the ATPS was loaded on a cation exchange column, in order to achieve higher purification[38]. The results showed that the recovery yield could be increased from 82% to 96% by combining these two extraction techniques. In another study, an ATPS with 10% (w/w) PEG 3350 and 12% (w/w) citrate achieved a recovery of IgG with a yield of 97% and protein purity of 72%. Then, the lower salt-rich phase was directly processed by hydrophobic interaction chromatography (HIC) in a phenyl-Sepharose column[39]. The resulting fractions from HIC were processed by size-exclusion chromatography in a Superose 6 column as a final polishing step. As a result, a yield of 90% was achieved, as well as a 100% pure IgG solution.

1.2. Ionic Liquids

1.2.1. General Considerations

Ionic liquids (ILs) are organic salts, generally composed by a large organic cation and an organic or inorganic anion, that melt below a conventional temperature of 100ºC. The combination between different cations and anions allows ILs to display different properties, such as conductivity, viscosity, ability to form biphasic systems (used in separations)[40], hence being considered as "designer solvents"[41]. It is possible to conceptually foresee the preparation of 10^6 ILs. Another important aspect in ILs chemistry is that they are non-volatile[42], avoiding problems related to the loss of solvent through evaporation, thus being included in the green solvents[43]. Some of these compounds are in the liquid form at room temperature, thus being called Room Temperature Ionic Liquids[44,45].

According to their properties, ILs have been used in many different processes of separation of biological products. ILs and their derived materials can be highly advantageous when comparing to volatile organic solvents. For example, unlike what was observed for polar organic solvents, some ILs do not lead to the inactivation of enzymes[46]. Moreover, many proteins keep their activity, even when dissolved in aqueous solutions of ILs, and some even increase their stability[47]. Indeed, the α-chemotrypsin when in the presence of ILs, has a 17-fold increase in its stability, comparing to conventional solvents. Although ILs are more expensive, they can be recovered and reused[48].

1.2.2. Studies using Ionic Liquids and Biological Compounds

Among the diversity of ILs structures, the most studied ones are the imidazolium-based ILs. These ionic liquids, and their aqueous solutions, were applied in many processes involving proteins[49–56]. According to Roosen *et al.* (2008), ILs increased selectivity, stability and activity of certain proteins, such as lipase B from *Candida Antarctica* (CALB)[52]. For instance, protein stabilization can be achieved due to the establishment of hydrophobic interactions and hydrogen bonds between the protein and the IL[57].

The stability of α-chemotrypsin was assessed in the presence of 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([C_2mim][TFSI]), in comparison to other solvents, such as water, sorbitol (3 M) and 1-propanol. The obtained results confirmed that the protein was more stable in the presence of ILs, corroborating what was previously stated about these fluids[49].

Myoglobin native structure is easily altered and forms fibrils, when the temperature assumes values between 55 and 75ºC[50]. Aqueous solutions of 1-butyl-3-methylimidazolium chloride ([C_4mim][Cl]) (0.2 M) allow myoglobin to maintain the native conformation and consequently the biological activity inherent to the structure, while also achieving stabilization[53].

Lipase B from *Candida Antarctica* (CALB) belongs to the α/β-hydrolases class[54]. The use of CALB in hydrolysis reactions in aqueous solutions of 1-decyl-3-methylimidazolium chloride ([C_{10}mim]Cl) allows a 10-fold increase in the enzymatic activity[56]. However, in the presence of 1-ethyl-3-methylimidazolium chloride

([C_2mim]Cl) the protein activity is higher than when in the presence of 1-methyl-3-octylimidazolium chloride ([C_8mim]Cl, illustrating the complexity and specificity of the behaviour displayed by these fluids [55].

1.2.3. Poly(ionic liquids)

Poly(ionic liquids) (PILs) are a subclass of polyelectrolytes which consist in repeated IL monomers connected through a polymeric backbone, forming a macromolecular structure[58]. Thus, PILs advantageously combine the unique properties of ILs with the solid state properties of polymers.

Poly(ionic liquids) to provided extremely interesting solutions in many fields, given that they have a polymeric nature, which allows the formation of several functional materials, namely films, membranes, among others. Moreover, there are many advantageous properties of ILs, such as their high ionic conductivity, electrochemical stability and also the easy tunability of their characteristics[59]. Since PILs can combine different properties, mainly due to the variation of ILs which are polymerized, they have emerged as new class of polyelectrolytes allowing their implementation in many scientific areas, more specifically gas separation, catalysis and sorption[59,60]. However, only one work reports the use of PILs as separation matrices for biological products, namely bacteriophage M13[61]. In this experiment, PILs were synthesized and successfully used as a resin for ion-exchange chromatography. This novel adsorption matrix allowed a recovery yield of 70.2%, comparable to other conventional chromatography operation. Also, PILs were regenerated and successfully reused. This is an extremely important characteristic, since it adds economic viability to the processes.

Therefore, it is highly relevant to evaluate PILs as a separation matrix of many other biological products.

1.2.4. Extraction/Purification Studies

Ionic Liquids have emerged as promising chemicals for the extraction and separation of biological compounds. Some studies regarding the uses of ILs as solvents, co-solvents, cosurfactants, electrolytes and adjuvants have been developed, as well as their use as basis for separation materials[62], such as membranes and adsorption matrices, as formerly stated. Therefore, the reported processes inherent to the use of ILs and its derivates consist of solid-liquid extractions, liquid-liquid extractions, IL-modified materials and IL-based crystallization. More than 60% of studies regarding the protein extraction and separation are conducted resorting to the use of IL-based ATPS[62].

Since many ILs do not alter protein conformation and allow the maintenance of protein stabilization and biological activity (as referred to previously in 1.2.2. Studies using Ionic Liquids and Biological Compounds), these green solvents were also tested using ATPSs, in order to evaluate their ability to separate proteins from different origins.

As demonstrated in Lin *et al.* (2013), eight different ionic liquids were tested, in order to acknowledge which of these ILs would be the best to extract the bovine serum albumin (BSA)[63]. They concluded that depending on the anion and cation, the extraction efficiencies were distinct, with 1-methyl-3-octylimidazolium bromide ([C_8mim]Br) achieving the highest extraction yield with a value of 96.6%.

Although imidazolium-based ILs have been the most studied family of ILs, several studies aimed at the assessment of other families of ILs, namely the cholinium, phosphonium and ammonium-based. In **Figure 6**, the general structures of these families of ILs are represented.

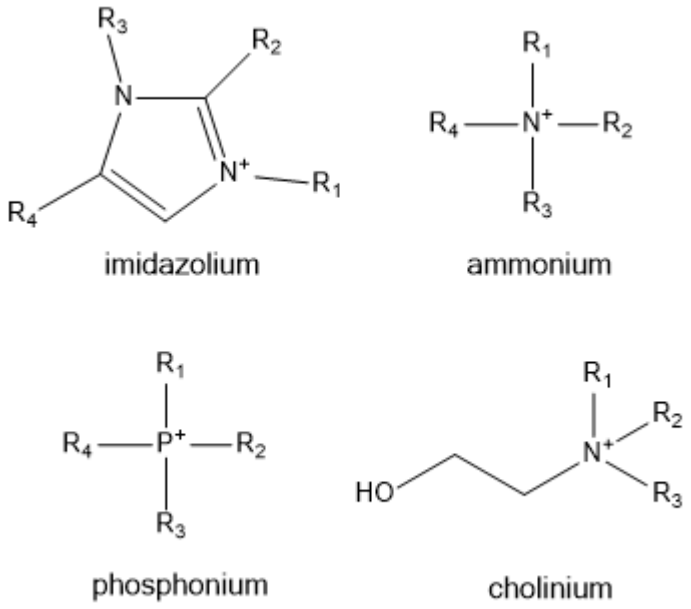


Figure 6 - *Structures of imidazolium, ammonium, phosphonium and cholinium-based ionic liquids.*

Considering cholinium-based ILs, some studies were conducted in order to extract BSA from purified samples as well as from a real bovine serum sample[64]. Extraction efficiencies from 92% to 100% were achieved and no protein denaturation occurred, indicating that the ILs can definitely be used for separation and purification purposes.

As formerly stated, phosphonium and ammonium-based ILs were also evaluated regarding the extraction of BSA. Pereira *et al.* (2015) used these two classes of ILs and performed extraction assays by using ATPS[65]. As a conclusion, extraction efficiencies of 100% were obtained, except for one IL. In the case of 100% extraction efficiencies, no protein denaturation was observed, thus showing that these ILs are viable options to extract BSA.

Ferreira *et al.* (2016) resorted to imidazolium and ammonium-based ILs as adjuvants in polymer-salt ATPS in order to extract IgG from pure solutions as well as from rabbit serum samples. With the addition of ILs, extraction efficiencies of 100% were obtained in only one-step and even higher recovery yields than the IL-free ATPS[66].

As aforementioned, ILs are truly task-specific solvents and thus through their synthesis, different ILs from those commercially available can be prepared. Mondal *et al.* (2016) prepared bio-based ILs, which means ILs were prepared with compounds derived from natural sources, being more biocompatible and biodegradable than most of the commonly used[67]. The ability to extract IgG from real rabbit serum samples was evaluated, through an ATPS with polypropylene glycol 400 (PPG 400). An extraction yield of 100% was achieved and 85% of the protein was recovered. Also, the majority of ILs did not change IgG native structure.

Taking into account that IEC is a valuable asset to purify mAbs and that ILs based-materials have shown to be more and more useful in capturing biological products by IEC, the purification of highly valuable and demanded products such as mAbs should be addressed.

2. Aim of the Study

Monoclonal antibodies have emerged as novel therapeutic agents to fight cancer, autoimmune diseases and infections, and consequently producing and purifying these proteins became essential. Thanks to the development of new technologies for the production of antibodies, such as the hydridoma technology[2], the production efficiency of these products has increased, providing more alternatives to cure a certain disease. Using monoclonal antibodies, a high specificity towards the action site of the protein is achieved, resulting in extremely viable tools to diagnose or to treat many life-threatening diseases. Despite their advantages and therapeutic potential, the manufacturing costs of mAbs are extremely high due to the absence of cost-effective extraction and purification methods, which is barring the access of these biomolecules to the general population. Thereupon, it is necessary to search and study alternative techniques which may be more efficient and economically viable to purify monoclonal antibodies. Poly(ionic liquids) emerged as possible solutions, since these polymeric compounds can be designed to obtain characteristics which are favourable in certain situations, allowing the separation and purification of many different molecules. Currently, antibody purification techniques are extremely costly and considering that these proteins have gained more interest as therapeutic agents, there is now a pressing need to explore alternatives to purify these molecules. Hence, since poly(ionic liquids) have shown to purify and separate different compounds or macromolecules, their ability to purify the valuable monoclonal antibodies must be assessed.

The main goal of this thesis was to develop a new purification process for monoclonal antibodies, in this case for antibodies against human Interleukin-8, produced using a serum-free medium and cultivating Chinese Hamster Ovary (CHO) cells in suspension. Interleukin-8 (IL-8) is an inflammatory cytokine, which promotes neutrophil chemotaxis and degranulation, affecting neutrophils function[68,69]. This peptide may be involved in the pathogenesis of inflammatory disorders, such as inflammatory bowel disease, gastric and colonic carcinomas and rheumatoid arthritis[70,71] (US patent 5702946).

As it has been stated, the downstream processing of the products is expensive, since it is based on the Affinity Chromatography. The aim of this work was to develop new chromatography matrices which captures the impurities, rather than the antibodies, thus purifying the antibody in negative mode. To achieve these aims, it was of paramount importance to accomplish the following steps:

- Synthesize the poly(ionic liquids) to be used and characterize them, in order to understand the mechanisms inherent to the process;
- Culture CHO DP-12 cells using a serum-free medium (ProCHO™5) and characterize the host cell proteins as well as the anti IL-8;
- Use the synthesized PILs as separation matrices to purify the antibody produced by the CHO cells.

3. Materials and Methods

3.1. Poly(ionic liquids) synthesis

3.1.1. Imidazolium-based ionic liquid monomers

In order to synthesize the 1-vinyl-3-ethylimidazolium bromide, equimolar quantities of 1-vinylimidazole and bromoethane were mixed, at 40ºC for 24 h at 500 rpm. Then, the obtained product was submitted to three washing steps with ethyl acetate and it was filtrated. The retentate was later dried under vaccum (1 Pa) and subjected to vigorous stirring at room temperature for at least two days. The same methodology was applied to synthesize 1-vinyl-3-hexylimidazolium bromide and 1-vinyl-3-tetradecylimidazolium bromide, using bromohexane and bromotetradecane, respectively, instead of bromoethane. Also, since the latter products were liquid at room temperature, the ethyl acetate evaporated using a Rotary Evaporator IKA RV10. The obtained products were dried under vacuum (1 Pa) and subjected to vigorous stirring at room temperature for at least two days.

With the purpose of exchanging the counter ion of the formerly obtained monomers, equimolar portions of the compounds and LiTFSI were mixed and incubated at room temperature for 24 h (for 1-vinyl-3-ethylimidazolium bromide and 1-vinyl-3-hexylimidazolium bromide) or 48 h (for 1-vinyl-3-tetradecylimidazolium bromide) at 500 rpm. The resulting monomers were washed with distilled water for at least 3 times. The monomers were further dried under vacuum (1 Pa) with vigorous stirring for, at least, two days.

After verification of the structure of the compounds obtained in the former steps by NMR analysis, each one of the monomers, 1-vinyl-3-ethylimidazolium bromide, 1-vinyl-3-ethylimidazolium bis(trifluoromethylsulfonyl) imide, 1-vinyl-3-hexylimidazolium bromide, 1-vinyl-3-hexylimidazolium bis(trifluoromethylsulfonyl) imide, 1-vinyl-3-tetradecylimidazolium bromide and 1-vinyl-3-tetradecylimidazolium bis(trifluoromethylsulfonyl) imide was mixed with 30% of one crosslinker (either divinylbenzene -DVB- or ethyleneglycol dimethacrylate – EGDMA) and with 5% of a photoinitiator (2-hydroxy-2-methylpropiophenone). The resulting solutions were polymerized under UV light for 5 min and the obtained polymer ground with a coffee grinder, followed by a bead mill. The PILs were then washed with acetone to remove any unreacted precursors and subsequently stored at 4ºC.

All the synthesized structures were analysed by ^{1}H NMR and ^{13}C NMR on a Bruker 400 MHz Ultra-Shield-Plus Magnet NMR instrument using DMSO as deuterated solvent.

3.1.2. Ammonium-based ionic liquid monomers

In order to synthesize the (dimethylethylamino)ethyl methacrylate bromide, equimolar quantities of (dimethylamino)ethyl methacrylate and bromoethane were mixed, at 25ºC for 12 h at 500 rpm, in the absence of light. Then, the obtained product was submitted to three washing steps with ethyl acetate and, due to the high reactivity of the monomer, it was not possible to filter the compound, but was later dried under vacuum (1 Pa) and subjected to vigorous stirring at room temperature for at least two days, in the absence of light.

With the purpose of exchanging the counter ion of the obtained monomers, equimolar portions of the compound and LiTFSI were mixed and incubated at room temperature for 12 h at 500 rpm. The resulting monomer was washed with distilled water for at least 3 times. The monomers were further dried under vacuum (1 Pa) with vigorous stirring for, at least, two days.

Each one of the monomers, (dimethylethylamino)ethyl methacrylate bromide and (dimethylethylamino)ethyl methacrylate bis(trifluoromethylsulfonyl), was mixed with 30% of one crosslinker (either divinylbenzene -DVB- or ethyleneglycol dimethacrylate – EGDMA) and with 5% of a photoinitiator (2-hydroxy-2-methylpropiophenone). The resulting solutions were polymerized under UV light for 5 min and the obtained polymer ground with a coffee grinder. The PILs were then washed with acetone to remove any unreacted precursors and subsequently stored at 4ºC.

All the synthesized structures were analysed by ^{1}H NMR and ^{13}C NMR on a Bruker 400 MHz Ultra-Shield-Plus Magnet NMR instrument using DMSO as deuterated solvent (**6.1 NMR spectra**).

3.2. PILs Characterisation

3.2.1. Zeta Potential Measurements

To determine the charge of PILs, each one of them was suspended in 1 mL of Milli-Q water and the suspension was placed inside a Folded Capillary Zeta Cell DTS1070. Resorting to a Zetasizer Nanoseries equipment from Malvern Instruments, Zeta Potential measurements were performed. These measurements were performed taking into account the type of material and the solvent.

3.3. Silica Beads Coating using PILs

As an alternative to the synthesized PILs, a commercially available PIL (poly(diallyldimethylammonium bis(trifluoromethane)sulfonimide)) was studied. The ability to coat particles of controlled porous silica was assessed. Firstly, the PIL was dissolved in acetone with a concentration of 5 mg/mL. Then, a heating mantle was heated until 60ºC, leading to the acetone's evaporation, thus supposedly leading to the coating of silica. This process was repeated several times, in order to fully coat the particles of silica and to evaluate whether the process was successful, SEM was performed in samples of silica with and without the coating (**Figure 7**).

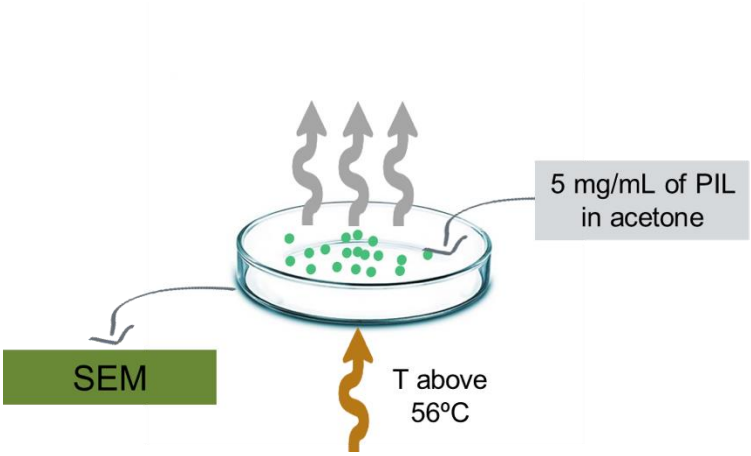


Figure 7 – *Scheme of the process of coating.*

3.4. Cell Structure

Anti IL-8-antibody-producing CHO DP-12 clone#1934 (ATCC CRL-12445) from the American Tissue Cell Collection (ATCC) (deposited by Genentech, Inc.) was used as model production cell line.

Cells were cultured using a protein-free medium designated as ProCHO™5 (Lonza). This medium is used to produce recombinant proteins expressed in CHO cells, given that it facilitates their downstream processing. With this culture medium, it is possible to obtain high density cultures without animal derived resources, resulting in less impurities within the culture itself. ProCHO™5 contains 0.1% Pluronic® whilst it does not contain L-glutamine, phenol red, hypoxanthine and thymidine. The medium was then supplemented with 4 mM L-glutamine (Gibco®), 2.1 g/L NaHCO3, 200 nM MTX, 10 mg/L recombinant human insulin, 0.07% (v/v) lipids (Lonza) and 1% (v/v) antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin).

TrypLE™ Select (1X) (Gibco®) was used to dissociate the adherent cells from the support. It is an animal origin free, recombinant enzyme for dissociating a wide range of adherent mammalian cells, including CHO, HEK 293, primary human keratinocytes, and embryonic stem cells. It contains EDTA and it is phenol-red free.

Cell passage was performed on the 6th day of the culture and the supernatant was collected on the 8th day.

3.5. Purification of mAb

Supernatant characterisation should be performed with and without the mAb in the culture, to be able to properly identify the existing impurities. Therefore, a step of mAb purification resorting to affinity chromatography was performed, resorting to ÄKTA Purifier system from Amersham Biosciences equipped with a Unicorn 5.1 data acquisition and processing software. The chosen column was Mab Select Xtra, the adsorption buffer was composed by 20 mM phosphate, 150 mM NaCl, pH 7.2 and the elution buffer was 0.1 M Citrate pH 3.66. The column was first equilibrated for 5 column volumes (CVs) of adsorption buffer. Then, the cell culture supernatant was injected at 1 mL/min, followed by the washing of the unbound or weakly retained molecules (8CVs of adsorption buffer). The mAb was eluted by changing the elution buffer for 5 CVs and consequently decreasing the pH.

Flow-through and the elution fractions were collected, resorting to a Frac-950 fraction collector. 1M Tris-HCl pH 8 was pipetted into Eppendorf tubes in which the elution fractions were collected, given that the pH of the elution buffer is too low. All fractions were properly aliquoted and kept at -20ºC for further analysis. However, the only fractions that will be discussed in this work are the flow-through fractions, as well as the crude supernatant.

For the assessment of the purification, SDS-PAGE was performed.

3.6. Supernatant characterization

3.6.1. Isoelectric focusing

Isoelectric focusing was performed in order to evaluate the isoelectric points (pI) of all the proteins in the cell culture, while comparing to the pI of the monoclonal antibodies. The marker (Broad pI Kit, pH 3-10, from GE Healthcare) and the samples of the cell culture with and without antibody were applied to a Phast Gel 3-9 and IEF was performed using a Pharmacia PhastSystem Separation, in which each run took 500Vh – 30 min. The prefocusing step took about 10 mins. Afterwards, in order to stain the gel, several solutions were used, such as 20% (w/v) trichloroacetic acid (Fischer Chemicals, 99.0%) to fix, 50% ethanol + 10% acetic acid (v/v),10% ethanol + 5% acetic acid (v/v) and Milli-Q water to wash, 8.3% glutaraldehyde as a sensitizer, 0.5% silver nitrate (v/v) and 0.015% formaldehyde in 2.5% sodium carbonate as a developer. As a final step, 5% acetic acid (v/v) is added as a stop reagent.

3.7. Protein characterization

3.7.1. Protein Gel Electrophoresis

In order to evaluate the purity of each sample as well as to assess qualitatively the presence of proteins, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed. 25 µL of each sample were mixed with 20 µL of Laemmli sample buffer and 5 µL of DTT 1 M. The marker Bio-Rad Precision Plus Protein™ Dual Color Molecular Ladder was used. The marker and the samples were loaded on to a polyacrylamide gel and ran for 2 h and at 90 mV using a running buffer. Gels were stained with and subsequently de-stained using a 30% ethanol and 10% acetic acid solution.

If protein concentration was low (<100 mg/L), a silver staining procedure was performed. Firstly, the gels were washed with 30% ethanol for 30 min, followed by two washing steps with Milli-Q water for 10 min each. The sensibilization of the gel occurred with a solution of 0.02% sodium thiosulfate for 15 min. After washing with Milli-Q water once again, gels were stained with a 0.15% silver nitrate solution for 30 min. The development was achieved with a 3% sodium carbonate and 0.05% formaldehyde and stopped with a 5% acetic acid solution for 15 min.

3.7.2. UV Absorbance

To determine proteins concentration, it is possible to analyse samples measuring the absorbance at 280 nm. In this work, these measurements were achieved resorting to a microplate reader from Molecular Devices. The calibration curve used in every experiment was prepared using BSA as a protein standard, with concentrations ranging between 10 mg/L and 1 g/L.

3.7.3. Bradford Method

The Bradford method was also used to determine proteins concentration, using the Coomassie assay reagent provided by Pierce. The protein standard used was BSA (Pierce). The calibration curve was obtained using a set of standards with the following BSA concentrations ranging between 25 and 400 mg/L. Then, 50 µL of the collected samples were added to each well, as well as 200 µL of Coomassie Blue solution. The plate was incubated for 10 min, after 30 s of mixing, at room temperature and the absorbance was measured at 595 nm in a microplate reader from Molecular Devices. For every sample, duplicates were made.

3.7.4. BCA Method

As an alternative to the Bradford method, the BCA Protein Assay Kit was also tested. The preparation of the calibration curve is identical, but the reagent that colours the samples differs. The working reagent (WR) is prepared by mixing fifty (50) parts of BCA Reagent A with one (1) part of BCA reagent B (50:1), both reagents provided by Pierce. Following this procedure, 25 µL of each standard or sample were pipetted into the wells and then 200 µL of WR were added (sample to WR ratio is 1:8). The microplate was then shaken for 30 s resorting to a Shaker-Incubator Stat Fax-2200 at velocity 1. Afterwards, the microplate was covered and incubated at 37ºC for 30 min. The absorbance was measured at 562 nm in a microplate reader from Molecular Devices.

3.8. Chromatographic assays

3.8.1. Microplate assays

Microplate assays were conducted using a 96-well MultiScreen-HV Filter Plate (0.45 µm; Millipore) linked to a MultiScreen® HTS Vacuum Manifold (Millipore) and filtered in a DirectStack™ mode. 20 mg of PIL were weighted and then poured into the wells of the filter plate. Afterwards, the PIL was equilibrated twice for 15 min, using 200 µL of the adsorption buffer, 20 mM Tris-HCl pH 7.5, resorting to a Shaker-Incubator Stat Fax-2200 at velocity 7 (around 1500 rpm). The buffer inside the wells was filtered and discarded. Prior to the adsorption phase, the samples of the impurities of the cell culture (200 µL each) were diafiltered against the adsorption buffer, resorting to Amicon® Ultra-0.5 Centrifugal Filter Devices, to equalize the buffer. Then, the samples were poured into the wells of the plate and incubated for 30 minutes at velocity 7. The samples were filtered and the content was kept for further analysis. Furthermore, 200 µL of the elution buffer were poured into the wells and incubated, once again, for 30 min at velocity 7. Then, the liquids were filtered and collected to assess the elution of the impurities. In order to facilitate the collection of the equilibrium, adsorption and elution phases, three distinct 96-well microplates (Greiner UV-Star® 96-well plates) were used[61].

3.8.2. ÄKTA Purifier system

Firstly, the impurities samples which resulted from the purification of the monoclonal antibody (**3.5 Purification of mAb**) were used in order to study whether the PILs were able to capture all the existing impurities. All these experiments were performed in an ÄKTA Purifier system from Amersham Biosciences equipped with a Unicorn 5.1 data acquisition and processing software.

A Tricorn 5/20 Column was used to support the PIL matrix. The column is packed by dry packing, which consists of weighting the PIL inside the column, placing the filters, inserting the top adapter and, with the help of a syringe, passing water through the column.

Before feed injection, PIL column was equilibrated with 20 column volumes (CVs) of adsorption buffer. Since the column was approximately 200 µL, the flow-through was injected at a rate of 0.2 mL/min. The sample loop (1 mL) was washed and emptied three times its volume. In a blank run, the sample was the adsorption buffer, whilst in a sample run, the cell culture sample was injected. After the injection, the column was washed in order to remove any unbound or weakly captured molecules, followed by the elution phase. Afterwards, the conductivity was increased by changing to the elution buffer 20 mM Tris-HCl, 1.0 M NaCl, for 20 CVs, in order to elute the impurities. Flow-through and eluted fractions were collected on a Frac-95 fraction collector to qualitatively analyse their purity through SDS-PAGE, UV absorbance, Bradford and BCA methods. Note that the injected samples were firstly diafiltered against the adsorption buffer, resorting to Amicon® Ultra-0.5 Centrifugal Filter Devices, to equalize the buffer. The obtained chromatograms were recovered and the peak areas calculated to compare and analyse the yields.

4. Results and Discussion

Monoclonal antibodies have emerged as new therapeutic agents and, since their purification is of paramount importance, new techniques have been developed to assure an efficient and economic process. On the other hand, ILs and PILs have become relevant in separation of a wide variety of compounds[60]. Therefore, in this work, PILs were synthesized and their ability to purify bioactive compounds assessed.

4.1. Synthesis and characterisation of poly(ionic liquids)

4.1.1. Imidazolium based poly(ionic liquids)

Firstly, the imidazolium-based ionic liquids were synthesized, given that these ILs are widely studied in purification processes. The quaternization reaction consists of an alkylation of 1-vinylimidazole with a halocarbon (bromoethane, bromohexane or bromotetradecane) (**Figure 8**).

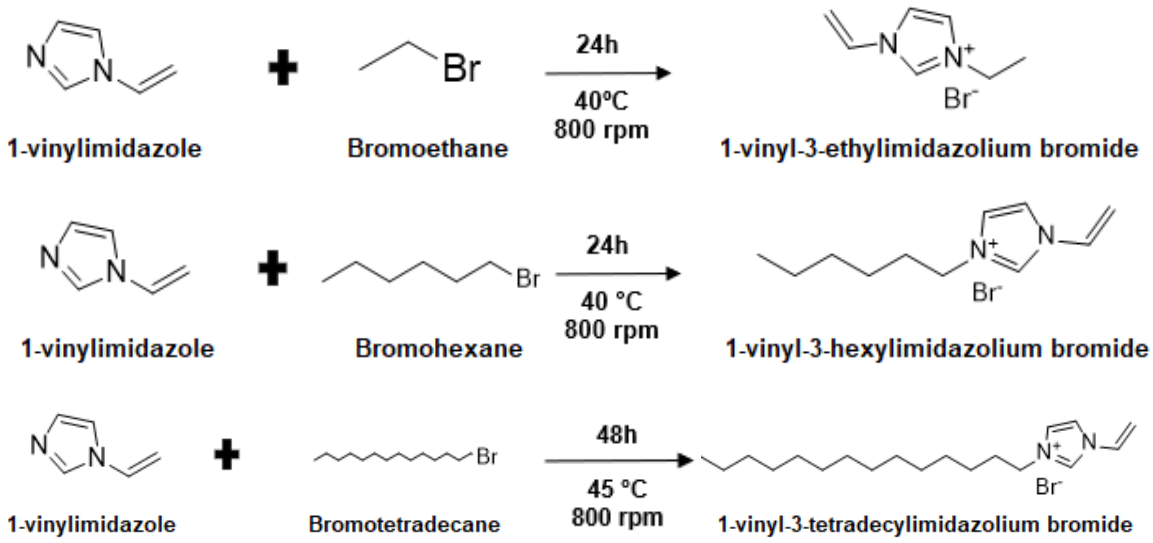


Figure 8 – *Imidazolium-based ionic liquids: Quaternization reaction that originates monomers with different alkyl chains.*

Subsequently, the addition of the lithium bis(trifluoromethylsulfonyl)imide salt to a solution of the obtained monomers allows the exchange of the anions, resulting in monomers with a different anion (TFSI) and a salt (lithium bromide – LiBr), through a reaction termed anion metathesis (**Figure 9**). This anion was chosen, given that it is highly hydrophobic, conferring different properties to ILs and consequently PILs. Thus, the effect of the anion in the efficiency of extraction processes was studied further on.

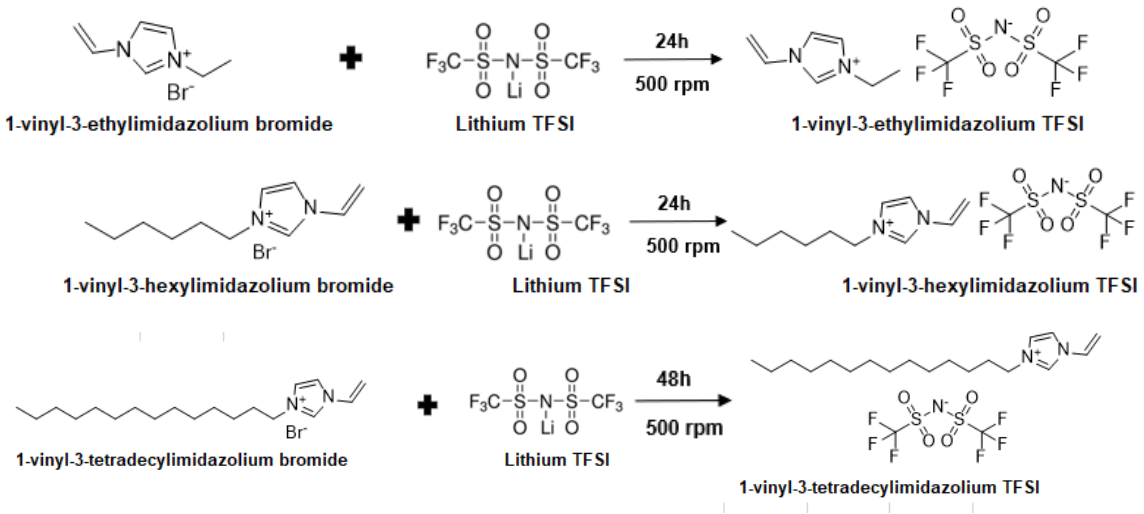


Figure 9 – *Imidazolium-based ionic liquids: Anion metathesis reaction, in which the counter ion is exchanged (from bromide to TFSI).*

To assess whether the monomers were successfully synthesized, 1-vinyl-3-ethylimidazolium bis(trifluoromethylsulfonyl)imide, 1-vinyl-3-hexylimidazolium bis(trifluoromethyl-sulfonyl)imide and 1-vinyl-3-tetradecylimidazolium bis(trifluoromethylsulfonyl)imide were subjected to an NMR analysis. Through the analysis of the NMR spectra, it was possible to conclude that the peaks observed in each spectrum of ^{1}H NMR correspond to the ones that were expected, considering the structure and the covalent bonds of every monomer. Similarly, regarding the spectra of ^{13}C NMR, the peaks are correlated to the links established between carbons and other atoms. Since all those expected bonds are represented in the spectra, it can be stated that these monomers were successfully produced (**6.1 NMR spectra**).

Once the monomer synthesis was validated, ILs were then polymerized with the photoinitiator and one cross-linker, resulting in repeating units of an IL monomer connected through a polymeric backbone (exemplified in **Figure 10**, using 1-vinyl-3-ethylimidazolium bromide and DVB as a crosslinker).

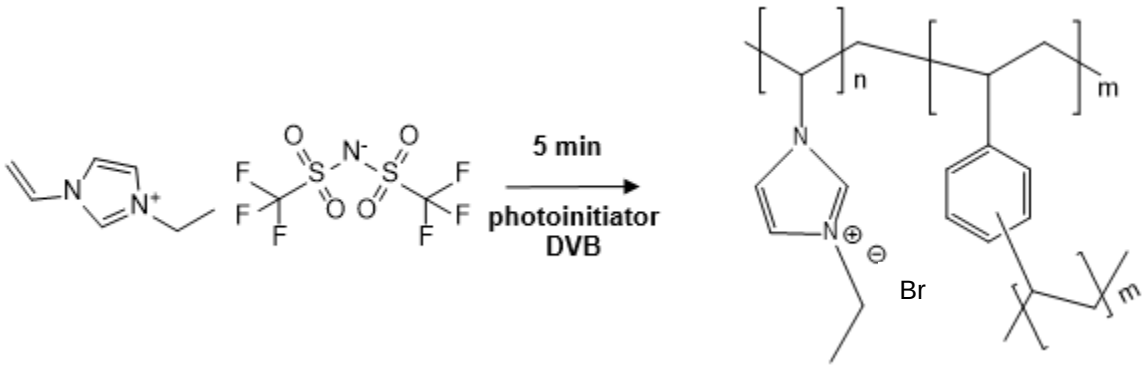


Figure 10 – *Imidazolium-based ionic liquids: Polymerization reaction of 1-vinyl-3-ethylimidazolium bromide with DVB as a cross-linker.*

After the polymerization step, the obtained film was grinded with a common coffee grinder and PILs particles were submitted to scanning electronic microscopy (SEM) to assess their homogeneity.

4.1.2. Ammonium-based poly(ionic liquids)

This class of ionic liquids was studied in order to consider the influence of the cation in the bioseparation. The synthesis was started with a quaternization reaction, similarly to the imidazolium-based ionic liquids. The alkylation of (dimethylamino)ethyl methacrylate with bromoethane is represented in **Figure 11**.

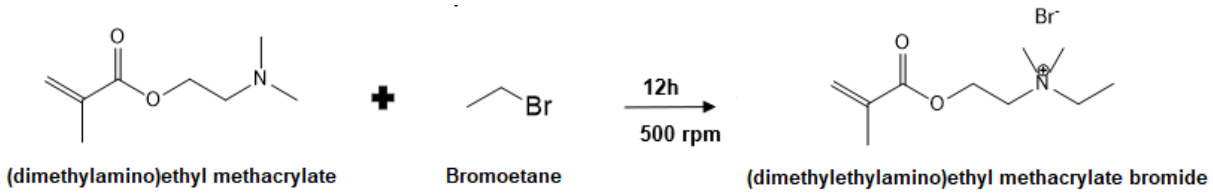


Figure 11 – *Ammonium-based ionic liquids: Quaternization reaction that originates monomers with different alkyl chains.*

Furthermore, with the addition of the salt lithium bis(trifluoromethylsulfonyl)imide the exchange of anions was facilitated, resulting in monomers with a different anion (TFSI) and a salt (lithium bromide – LiBr). This reaction is designated by anion metathesis (**Figure 12**). This anion was chosen given that it is highly hydrophobic, thus conferring different properties to ILs and consequently to PILs. The effect of this hydrophobicity can be taken into account in further studies.

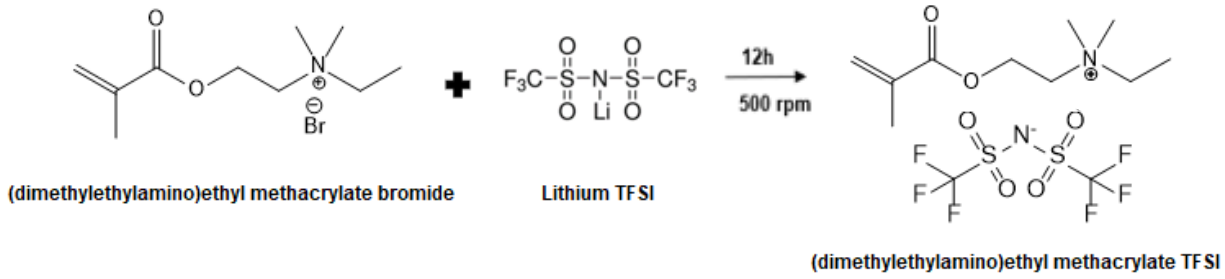
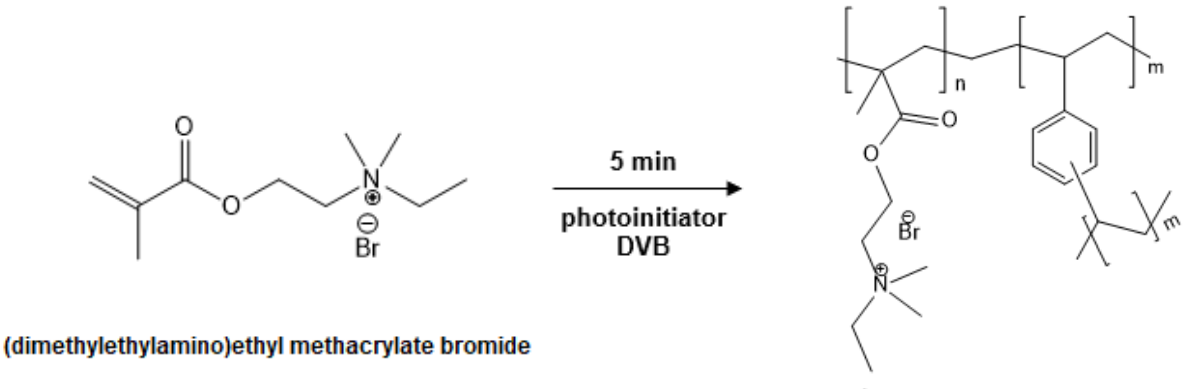


Figure 12 – *Ammonium-based ionic liquids: Anion metathesis reaction, in which the counter ion is exchanged (from bromide to TFSI).*

To assess whether the obtained monomers were successfully synthesized, they were subjected to an NMR analysis, just as described for the imidazolium-based ionic liquids. Given that the obtained spectra

for both ^{1}H NMR and ^{13}C NMR matched what was expected for each IL, it was possible to assume that the monomers were successfully synthesized.

After the validation of the synthesis, the ILs were polymerized with the photoinitiator and one crosslinker, resulting in repeating units of the same IL connected through a polymeric backbone, as it was previously described (**Figure 13**).



(dimethylethylamino)ethyl methacrylate bromide

Figure 13 - *Ammonium-based ionic liquids: Polymerization reaction of (dimethylethylamino)ethyl methacrylate bromide with DVB as a cross-linker.*

4.1.3. Scanning Electron Microscopy

To qualitatively assess the homogeneity of the PIL particles, as well as their surface, Scanning Electron Microscopy was performed (**Figure 14**).

The PIL particles sizes (**Figure 14A**), sizes show a disparity of values, as some particles are 1 mm or bigger and other particles are smaller than 100 µm. Subsequently, since it was concluded that the size distribution of the processed PILs was quite heterogenous, size homogenization as well as the increase of surface area was required to maximize their capacity to capture impurities. The samples were thus submitted to a grinding process using a bead mill, However, the qualitatively analysis (**Figure 14B**) revealed that the size distribution was still heterogenous, given that some particles were smaller than 10 µm, while others had, at least, 60 µm. Furthermore, some smaller particles aggregated, forming large clusters of PILs and decreasing their surface area, which became a major drawback of this process.

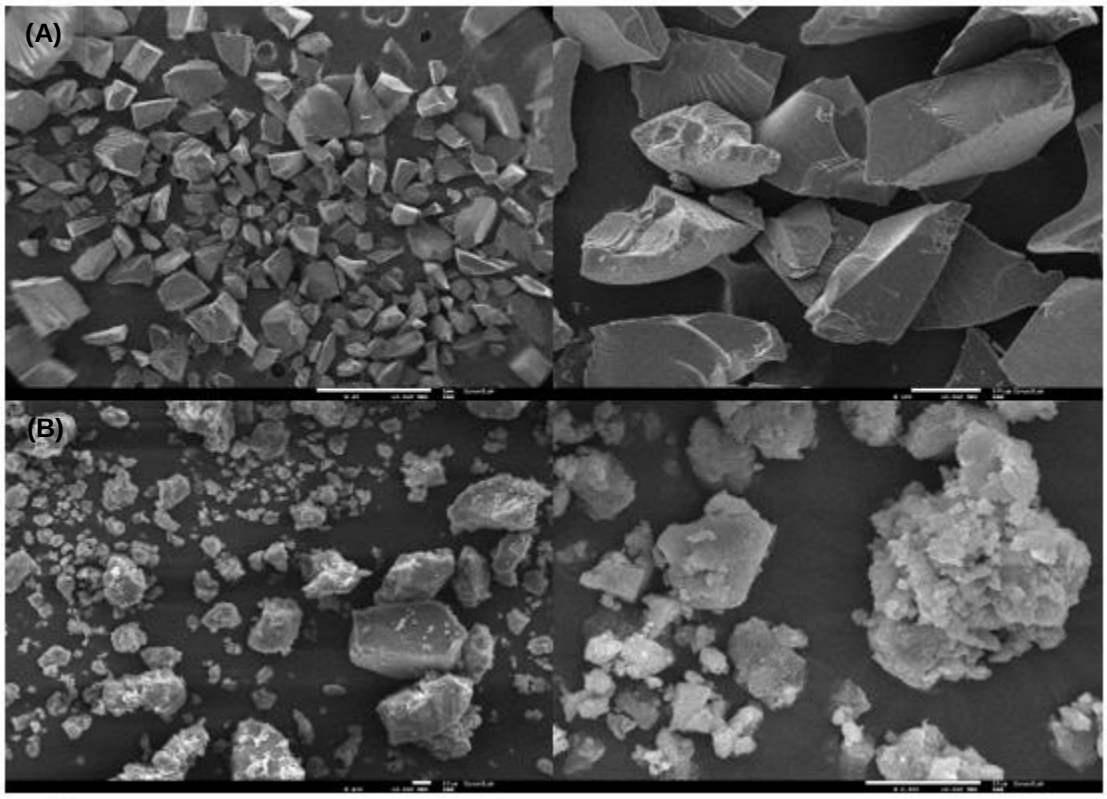


Figure 14 – *Images obtained by SEM for poly(VEIM-Br) with 30% DVB, where (A) represents PILs structure before the grinding resorting to the bead mill, whilst (B) represents the obtained PILs after using a bead mill.*

To assess if the grinding using a bead mill was a good alternative, PILs were tested and studied before and after the grinding (**4.4.1 Microplates**).

4.2. Depth coating using PILs

As an alternative to grounded PILs, it was attempted to coat controlled porous glass silica with poly(diallyldimethylammonium bis(trifluoromethane)sulfonimide). To qualitatively assess whether the coating of silica was successful, SEM was performed using silica particles before and after the coating (**Figure 15**).

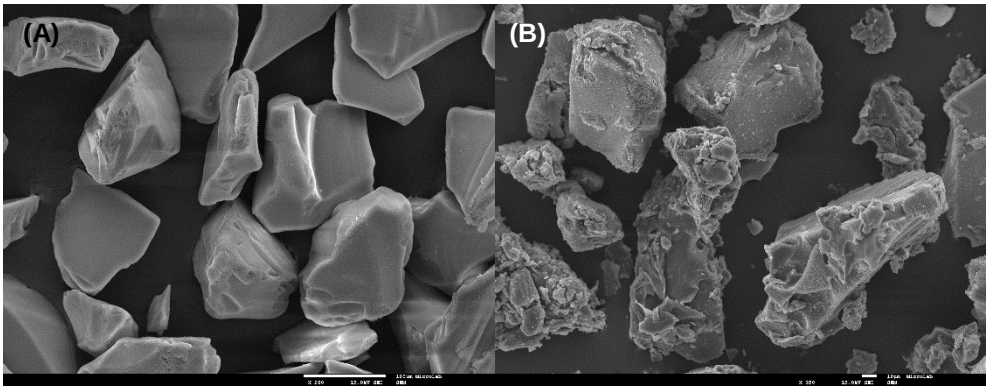


Figure 15 - *Images obtained by SEM to evaluate the process of coating. (A) represents silica before the coating and (B) represents silica after de coating.*

As can be seen in **Figure 15**, before the coating, the surface of silica was quite clean, while after the coating the surface became rough and edgy, indicating that PILs were deposited onto the surface of the silica. This coated silica will be further studied, in **4.4.1. Microplates**.

4.3. Supernatant characterisation

4.3.1. Fractionation of mAbs and HCPs

Despite that the CHO DP-12 cell culture aimed to produce anti IL-8, other proteins derived from the metabolism of cells (the host cell proteins – HCPs) were also produced. Given that the main goal of this work is to purify anti IL-8 by capturing the remaining proteins of the cell culture, referred to as impurities, there was the need to purify the antibodies, retrieving the impurities for further studies. Therefore, collected fractions were run in a gel, through SDS-PAGE, thus qualitatively showing whether the purification was successful or not.

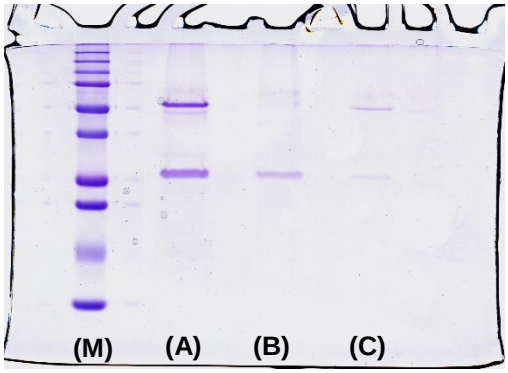


Figure 16 – *SDS-PAGE gel, in which fractions of (A) the feed, (B) adsorption phase and (C) elution phase were run. To adequately evaluate the results of the run, a marker (M) is needed.*

As expected, in the feed there was a high concentration of mAbs and some impurities. Furthermore, during the adsorption step, mAbs were captured, whilst the impurities were washed from the column and then collected. In the elution step, the mAb is eluted from the column. As can be seen in **Figure 16**, the bands which appeared (C) are consistent with what was expected, since they correspond to the molecular weights of reduced antibody chains (25 kDa and 50 kDa).

Moreover, the impurities that were not captured during the adsorption phase were collected in the flow-through fractions, aliquoted and kept at -20ºC before further use.

4.3.2. Isoelectric Focusing

In ion-exchange chromatography, the pH of the buffer is an important factor since it determines proteins charge. Furthermore, since it is desired to maximize the antibodies purification yield using PILs as separation matrices, the buffers should consider the antibodies pI as well as the pI of the impurities. Therefore, prior to the chromatographic assays, the isoelectric points of the impurities were determined (**Figure 17**). Samples of crude supernatant (lane B) as well as samples of impurities (lane A) were run on gel, to assess and characterize the proteins. The mAb isoelectric point was shown to be above pH 9 and the impurities had a vast range of pIs. Most of the impurities have pI lower than 6.55, given that they are below the band of Carbonic Anhydrase B Human, with a pI of 6.55. However, there are impurities, designated by ii, which have a similar pI to the mAbs pI (near pI 9.30). Hence, it is extremely difficult to completely purify the monoclonal antibody with just one step. Nevertheless, since many impurities have a relatively low pI, the considered pH for the buffer may be in the range of 7-8.5, to not interfere with the mAbs charge.

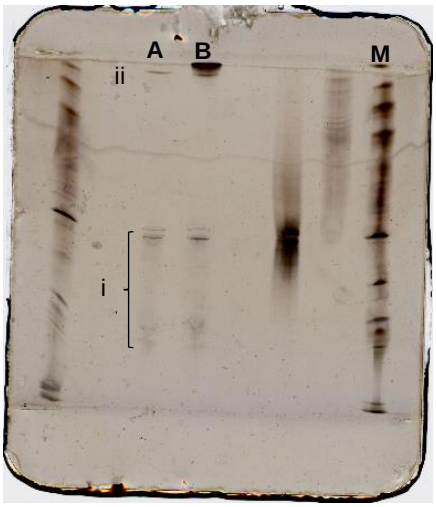


Figure 17 - *Isoelectric focusing gel, in which pI of (A) impurities of the cell culture and pI of (B) monoclonal antibodies together with the impurities were studied. The marker (M) from bottom to top is characterize by the following proteins: Amylglucosidase – pI 3.50, Soybean trypsin inhibitor – pI 4.55, β-lactoglobulin – pI 5.20, Carbonic anhydrase B bovine – pI 5.85, carbonic anhydrase B human – pI 6.55, myoglobin horse acidic band – pI 6.85, myoglobin horse basic band – pI 7.35, Lentil lectin (acidic) – pI 8.15, Lentil lectin (middle) – pI 8.45, Lentil lectin (basic) – pI 8.65 and Trypsinogen – pI 9.30.*

4.4. Chromatographic assays

PILs which were chosen to be tested as separation matrices were the following: poly(VEIM-Br) with 30% DVB, poly(VEIM-Br) with 30% EGDMA, poly(DMEAEM-Br) with 30% EGDMA and poly(DMEAEM-TFSI) with 30% EGDMA. Selection was made taking into consideration the study of three different conditions: the type of the cross-linker, the type of the cation (alkyl chain) and the type of the anion (bromide or TFSI). Zeta potential measurements confirmed that these PILs had a positively charged polymeric backbone. The following zeta potentials were measured: 23.00 ± 4.25 mV for poly(VEIM-Br) with 30% DVB, 38.80 ± 4.02 mV for poly(VEIM-Br) with 30% EGDMA; 18.20 ± 5.98 mV for poly(DMEAM-Br) with 30% EGDMA and, 26.50 ± 4.55 mV for poly(DMEAEM-TFSI) with 30% EGDMA. Therefore, given that it was clear that these PILs are positively charged, it was anticipated that these materials could emerge as new anion-exchangers for the purification of biological materials.

It was therefore expected that by using a buffer with pH above 6.55 (impurities pI) and below 9.30 (antibodies pI), the impurities would be negatively charged thus allowing their capture during the adsorption phase. Antibodies should have remained positively charged, thus not interacting with the matrix and being collected in the adsorption phase.

4.4.1. Microplates

4.4.1.1. Poly(ionic liquids)

As preliminary assays, PILs were tested in a microplate assay, where the main goal was to study different PILs and different conditions to optimize the purification of monoclonal antibodies. Hereupon, PILs grounded in a bead mill were tested. This approach was used in order to obtain smaller particles to increase the superficial area, thus increasing the adsorption efficiency. However, the evaluation of these materials became difficult, given that they would clog the filter, not allowing the passage of liquid.

PILs grounded with only a coffee grinder were then used to carry on the experiments. As explained previously, these experiments were carried on a batch mode, meaning that there was no continuous flow through the wells. Hence, firstly the PILs were equilibrated with adsorption buffer, the impurities sample was then added and finally the elution buffer was pipetted into the well, to elute what was captured by PILs. After the adsorption phase, the samples were collected and analysed by their absorbance measured at 280 nm, and by the Bradford and BCA methods. The same procedures were performed after the elution phase. To validate these methods, mass balances were made. The mass of protein in the unprocessed sample was determined, as well as the mass after the adsorption and after elution steps. The results of these mass balances indicated that throughout the experiment there was an increase in protein concentration, relatively to the initial sample. Since this happened in every measurement, it was of paramount importance to understand why the protein concentration supposedly increased. To do so, the same assay was performed with water in each of the wells, thus having a negative control to understand if it pointed out positive results

for the presence of proteins. Given that the results showed a false positive result, it was further interpreted as a possible slow solubilization of either the cross linker, or the monomers. Since the protocol for PILs synthesis had been previously tested in the lab to prepare membranes for gas separation, where full polymerization was observed, it was considered that probably the protocol was not optimized for these specific classes of PIL. In Patinha *et al.* (2017), Pyrrolidinium-based PILs polymerization was achieved with 1 min under the UV light[72], which supported the hypothesis that, in the present work, the synthesized PILs were under UV light for too long (5 mins), possibly causing their degradation. Here, to assess whether there were any visual changes in the final polymer comparing to the ones obtained and used throughout this work, polymerization was performed under the UV light for 10 mins. In fact, PILs became opaquer and the colour started to change, indicating that they were starting to degrade. Moreover, and given that the assays used to quantify proteins are sensitive to certain chemicals, such as imidazole, this degradation could explain the obtained contaminations while analysing the samples. On the other hand, considering that the impurities concentration was in the range of 30-70 mg/L, the amount of undesired proteins might have been too low to be determined, thus hinting possible errors related to the measurement.

4.4.1.2. Silica Beads coated with poly(diallyldimethylammonium bis(trifluoromethane)sulfonimide)

The coating of Silica Beads of controlled porous glass (cpg) silica was attempted resorting to a solution of a commercial PIL. Particles were submerged in a 5 mg/mL solution of poly(diallyldimethylammonium bis(trifluoromethane)sulfonimide), and then heated to 70ºC to evaporate the acetone and submerged again in the PIL solution. This process was repeated several times, to assure that the particles would be coated. In order to confirm if the coating was successful, particles were observed in SEM. Furthermore, a negative control test was conducted. A control experiment was performed in which Milli-Q water was passed through the coated silica. Afterwards, the obtained samples were tested for the presence of proteins. As expected, the presence of proteins was not detected. Therefore, to ensure that the contact with the aqueous solution did not wash away the PIL, CPG silica was kept and the particles were, once again, analysed in SEM. The comparison before and after the experiment is showed in **Figure 18**.

As can be seen from **Figure 18**, before the control, the particles of silica appeared to be covered in PIL. However, after the experiment, some PIL remained attached to the surface of silica, whilst the majority of the PIL aggregated, forming a big particle of PIL (C). This may be due to the fact that since the PIL was not covalently linked to the silica particles, the presence of water in the washing step caused the PIL to slowly solubilize and precipitate in aggregates. Consequently, these results invalided the use of these PIL particles, where no chemical bond is established between the PIL and the silica particles.

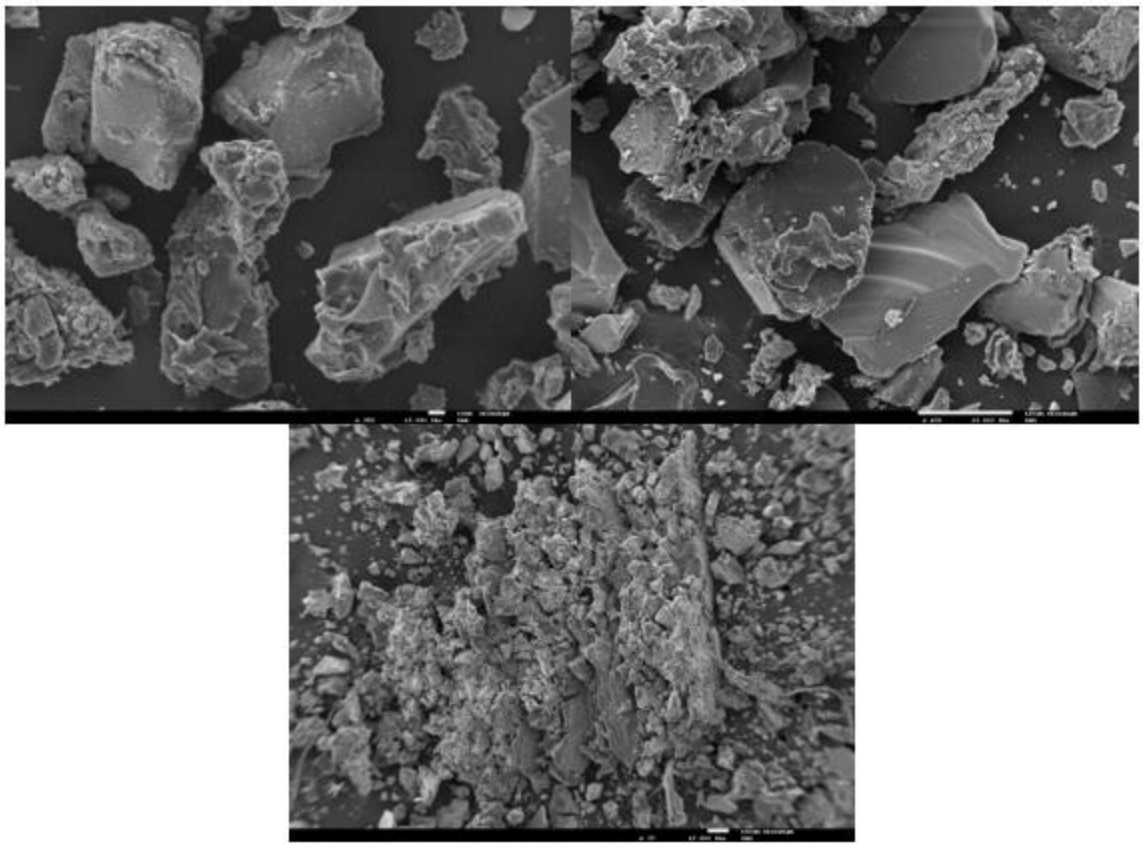


Figure 18 *– Images obtained by SEM which compare the coated silica (A) before and (B) after the microplate. (C) appears to be a representation of a PIL cluster.*

4.4.2. ÄKTA purifier system

To overcome the limitations brought by the experimental procedure aforementioned, PILs were packed into a column and used in an ÄKTA purifier system. The peak areas of the obtained chromatograms (both blank and sample) were calculated and the effect of the buffer along with the effect of the PIL itself were taken into account. Then, considering that the total area of the reduced chromatogram (sample minus the blank) was proportional to the total amount of protein loaded (it was formerly studied that all captured proteins were eluted), it was possible to calculate the adsorption yields of every experiment, and consequently the efficiency in the reduction of host cell proteins. The studied PILs were tested using two different pH conditions: pH 7.5 and pH 8.5.

Firstly, and considering what was found on the literature, experiments were conducted with an adsorption buffer composed of 20 mM Tris-HCl, pH 7.5, and an elution buffer composed of 20 mM Tris-HCl, 1.0 M NaCl, pH 7.5. These buffers were chosen following an increase of the salt concentration, to perform the ionic exchange[73] (**Figure 19**).

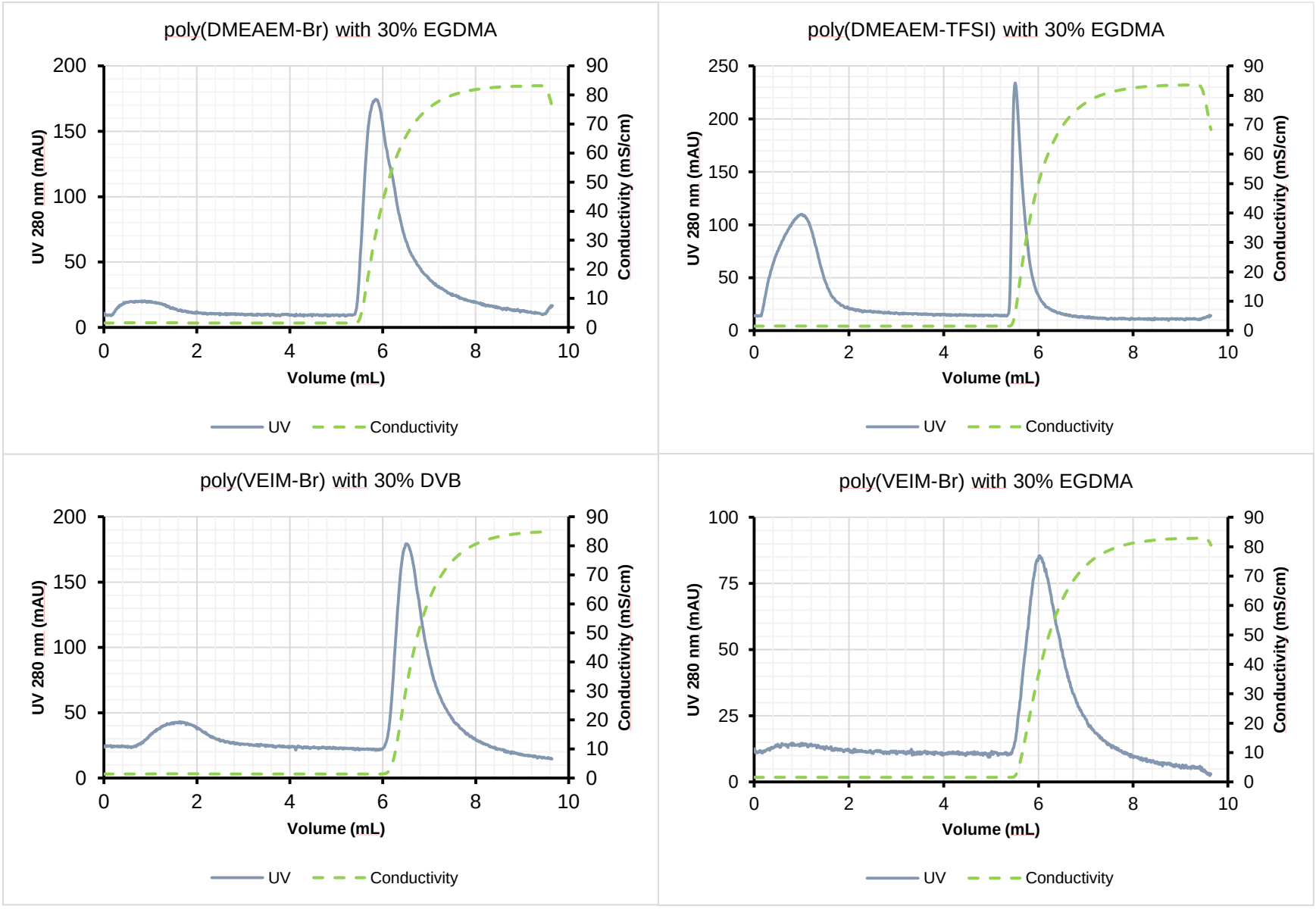


Figure 19 - *Chromatographic assays performed resorting to AKTA purifier system. These experiments were conducted at pH 7.5, with 20 mM Tris-HCl as adsorption buffer and 20 mM Tris-HCl + 1.0 M NaCl as elution buffer. In the xx axis is represented the volume, in mL, of the solutions through the column and in the yy axis there is the absorbance measured at 280 nm as well as the conductivity of the liquid, in mS/cm.*

In order to evaluate the efficiency of the process, given that proteins are detected at 280 nm, as stated before, the peak areas can be determined and therefore used to calculate the removal efficiency of the capturing step of the impurities from a cell culture supernatant. The efficiencies obtained for each PIL were the following: 81±2 % for poly(VEIM-Br) with 30% DVB; 86% for poly(VEIM-Br) with 30% EGDMA, 89% for poly(DMEAM-Br) with 30% EGDMA and 41% for poly(DMEAEM-TFSI) with 30% EGDMA. The values are represented in **Figure 20**, allowing a better comparison between the different PILs.

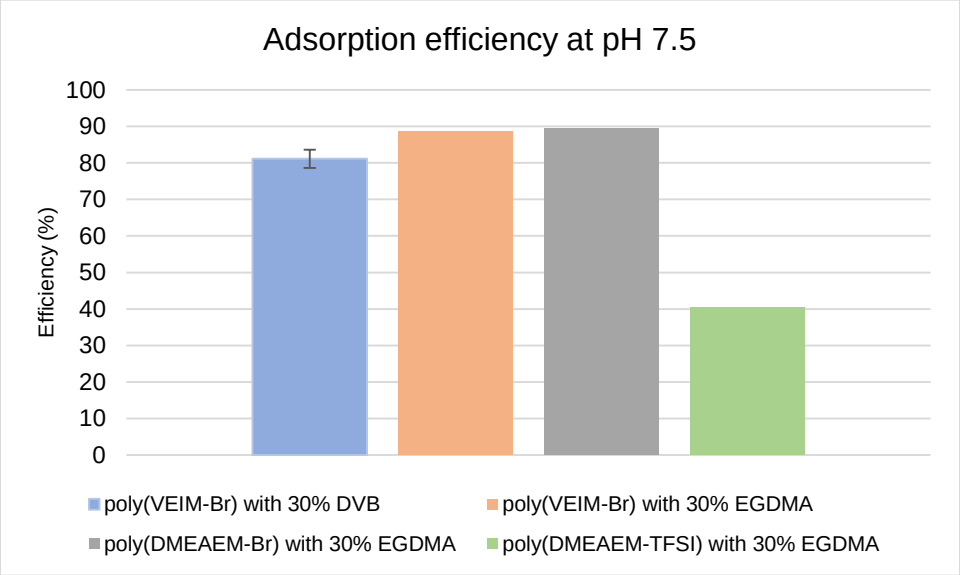


Figure 20 - *Adsorption efficiencies of the experiments performed at pH 7.5*

Therefore, at pH 7.5, the PILs with better performance were poly(VEIM-Br) with 30% EGDMA and poly(DMEAM-Br) with 30% EGDMA, the most hydrophilic PILs among the ones tested. Similar results were obtained with these two PILs, showing that maybe the impurities have more affinity for hydrophilic environments. Furthermore, the anion size might have also contributed to these results, as bromide presents a smaller size and a more localized charge than bis(trifluoromethane)sulfonimide (TFSI), thus resulting in a stronger interaction. For the purification of larger particles, such as bacteriophages[61], larger anions were found to be more efficient for the binding of phages, whereas bromide anions were too small for proper anion-exchange.

Additionally, the efficiencies regarding the capture of proteins appear to be higher than the ones obtained by Rosa *et al.*[74] with phenylboronic acid multimodal chromatography, in which 60% of soluble protein removal was achieved. However, PILs as separation matrices reach efficiencies similar to the ones obtained by Borlido *et al.*[75], in which HCP removals of 83% and higher were achieved, resorting to affinity chromatography using boronic acid magnetic particles.

The most hydrophobic PIL (poly(DMEAEM-TFSI) with 30% EGDMA), had the worst performance, confirming the hypothesis stated before. However, only speculations can be made since the results were not significant, due to the fact that duplicates were made only for poly(VEIM-Br) with 30% DVB.

Furthermore, in order to try to capture the impurities with similar pI to the anti-IL 8, the buffer pH was risen to 8.5. Given that the results of the poly(DMEAEM-TFSI) with 30% EGDMA were not good, this PIL was not tested.

The same analysis was performed for these charts, meaning that the peak areas were calculated in order to determine the adsorption efficiency in each case. The efficiencies obtained for each PIL were the following: 83±4% for poly(VEIM-Br) with 30% DVB; 93% for poly(VEIM-Br) with 30% EGDMA and 90% for poly(DMEAM-Br) with 30% EGDMA as shown in **Figure 22**.

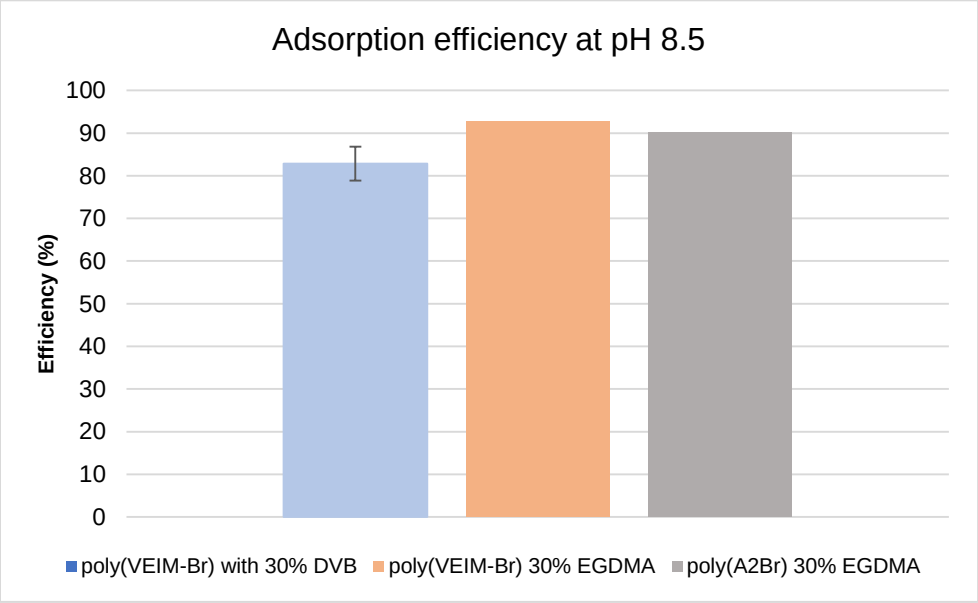


Figure 21 – *Adsorption efficiencies of the experiments performed at pH 8.5.*

The results concerning poly(VEIM-Br) with 30% DVB and poly(DMEAM-Br) with 30% EGDMA were not substantially different. The adsorption efficiency of poly(VEIM-Br) with 30% EGDMA rose 7.2%, which, in comparison to the other two, was a considerable increase. Once again, note that these results may not be significant, since no duplicates were made, except for the poly(VEIM-Br) with 30% DVB. Borlido *et al* also tested the boronic acid magnetic particles at pH 8.5 and achieved 99.5% of hcp removal yield[75]. This may be due to the fact that there are impurities which have a similar pH to mAbs, thus not being able to capture the culture inherent proteins. Nevertheless, many validations must be done regarding this method, in order to be comparable to the work of Borlido *et al*.

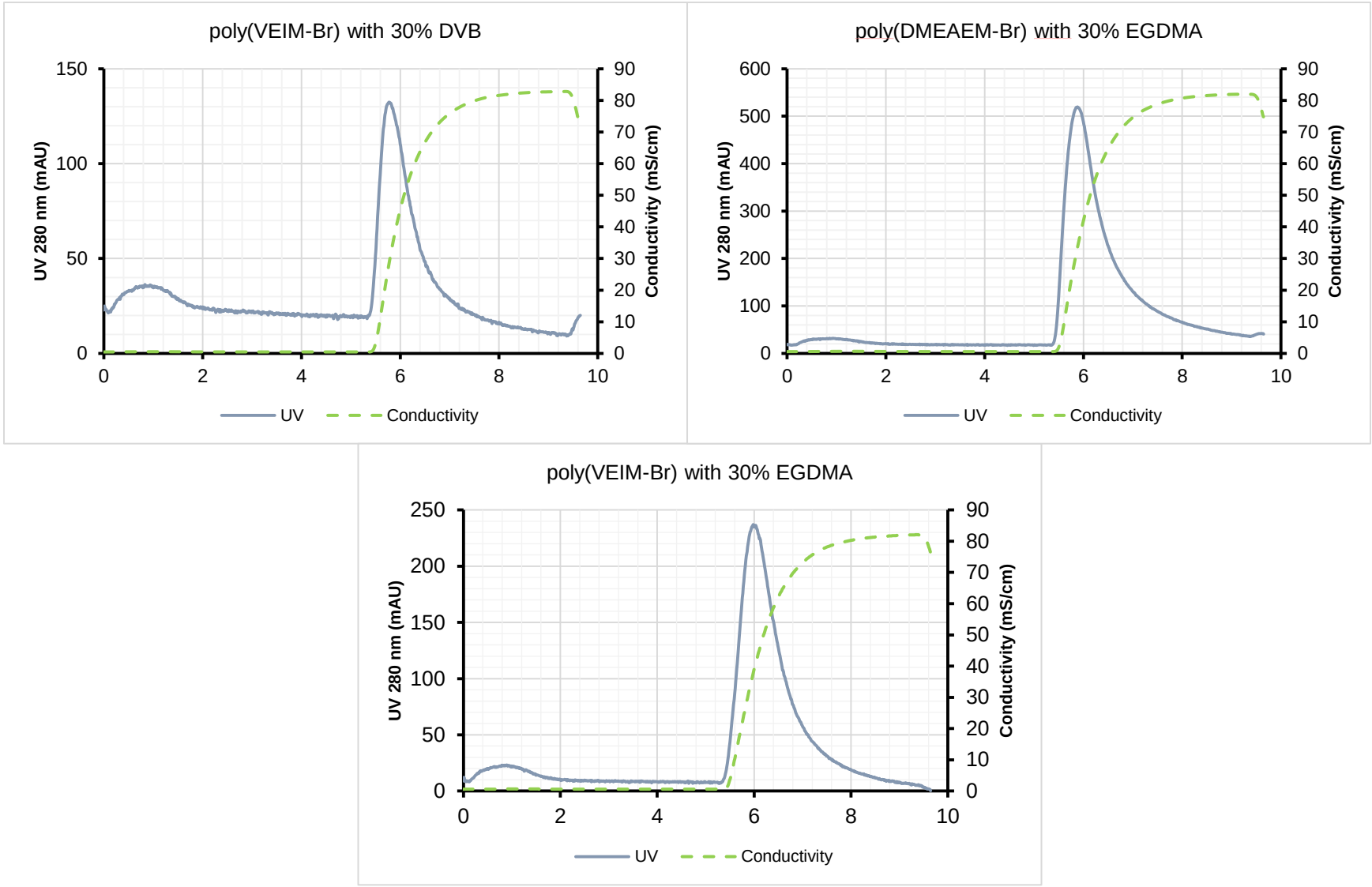


Figure 22 - *Chromatographic assays performed resorting to AKTA purifier system. These experiments were conducted at pH 8.5, with 20 mM Tris-HCl as adsorption buffer and 20 mM Tris-HCl, 1.0 M NaCl as elution buffer. In the xx axis is represented the volume, in mL, of the solutions through the column and in the yy axis there is the absorbance measured at 280 nm as well as the conductivity of the liquid, in mS/cm.*

As stated before, the adsorption and elution samples were collected and were later run in an SDS-PAGE gel, to verify that the proteins were successfully captured by the PIL matrix (**Figure 23**).

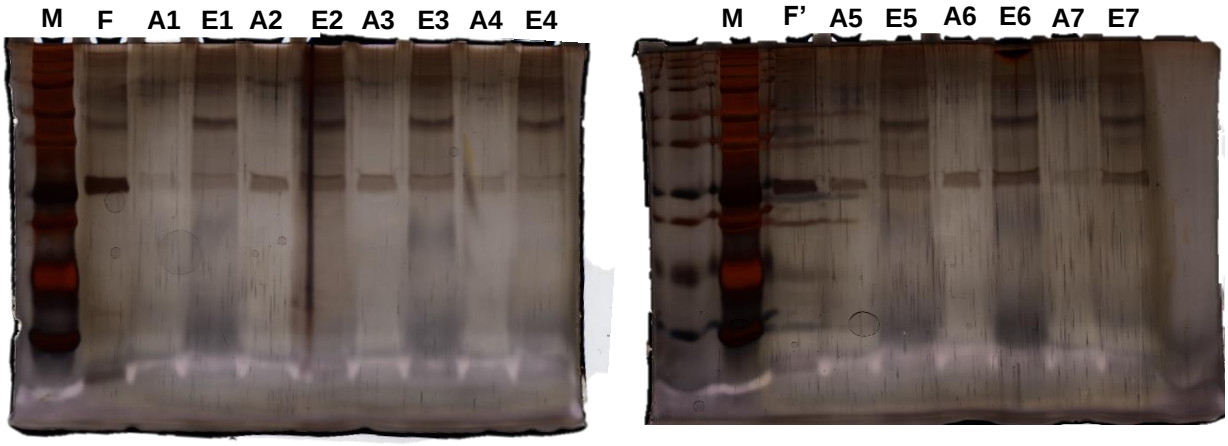


Figure 23 - *Two experiments are presented in this figure: Runs at a pH of 7.5 – poly(VEIM-Br) with 30% EGDMA (lanes A2 and E2), poly(VEIM-Br) with 30%DVB (lanes A6 and E6) and poly(DMEAEM-Br) with 30% EGDMA (lanes A7 and E7) – and runs at a pH of 8.5 – poly(VEIM-Br) with 30% EGDMA (lanes A1 and E1), poly(VEIM-Br) with 30% DVB (lanes A3 and E3, and A5 and E5) and poly(DMEAEM-Br) with 30% EGDMA (lanes A4 and E4). The feed for the run at pH 7.5 is represented in lane F', whilst the feed for the run at pH 8.5 is represented in lane F.*

The gel showed that most of the proteins were captured and consequently eluted, since bands in the eluted fractions are considerably darker than the respective flow-through bands (**Figure 23**). Comparing to the results obtained from the isoelectric focusing, there was a high probability that the proteins present in the FT pools (lanes A1, A2, A3, A4, A5, A6, A7) are the ones that presented a higher pI, close to 9.30, since they were not adsorbed. However, it is of paramount importance to better characterize these impurities and correlate their pI to a molecular weight, thus trying to identify them.

As a preliminary assay, a diafiltered supernatant, with anti IL-8, was passed through the poly(VEIM-Br) with 30% DVB, at pH 7.5, even though it was not possible to determine yields, given that the Bradford and BCA assays were not able to quantify the protein concentration (concentration below the detection limit). The chromatogram obtained is represented in **Figure 24**. Samples from both flow-through and elution fractions were once again collected, and analysed by SDS-PAGE to validate whether the antibody was present in the samples correspondent to the adsorption phase (i.e., in the columns flow-through) (**Figure 25**).

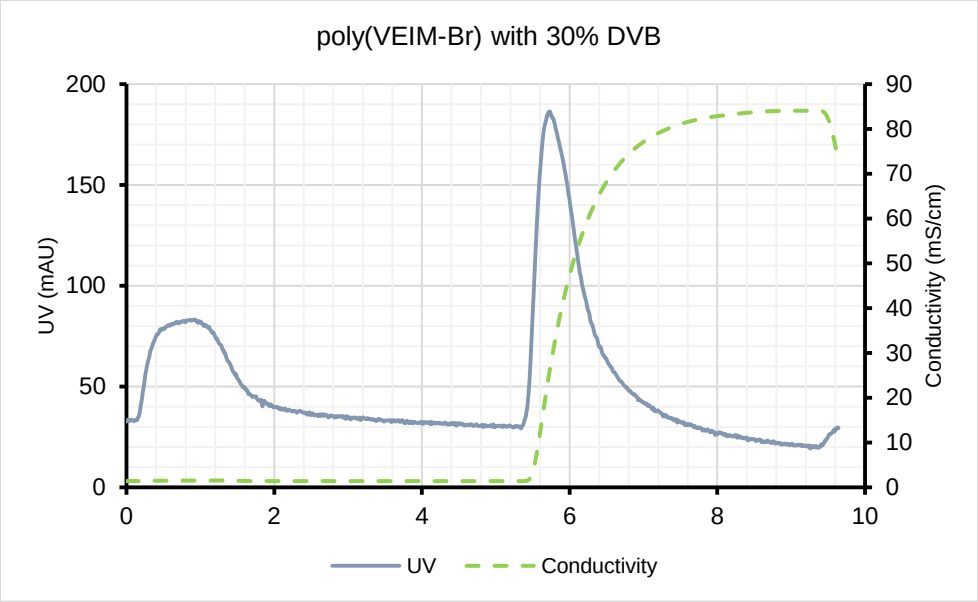


Figure 24 - *Chromatographic assay performed resorting to AKTA purifier system, in which a cell culture sample was injected. This experiments was conducted at pH 7.5, with 20 mM Tris-HCl as adsorption buffer and 20 mM Tris-HCl + 1.0 M NaCl as elution buffer. In the xx axis is represented the volume, in mL, of the solutions through the column and in the yy axis there is the absorbance measured at 280 nm as well as the conductivity of the liquid, in mS/cm.*

Considering the feed (F) sample, there were bands near the regions of 25 kDa and 50 kDa, which corresponded to the subunits of the mAb, when reduced (**Figure 25**). Contrarily to the tendency previously observed, the adsorption phase samples had darker bands with the molecular weights of approximately 25 kDa and 50 kDa, thus indicating that the monoclonal antibodies were not captured by the PIL matrix. However, the elution phase samples appeared to have fewer dark regions, maybe due to the fact that the antibody was in higher concentration than the impurities and due to the fact that there were different types of impurities, hence having different molecular weights.

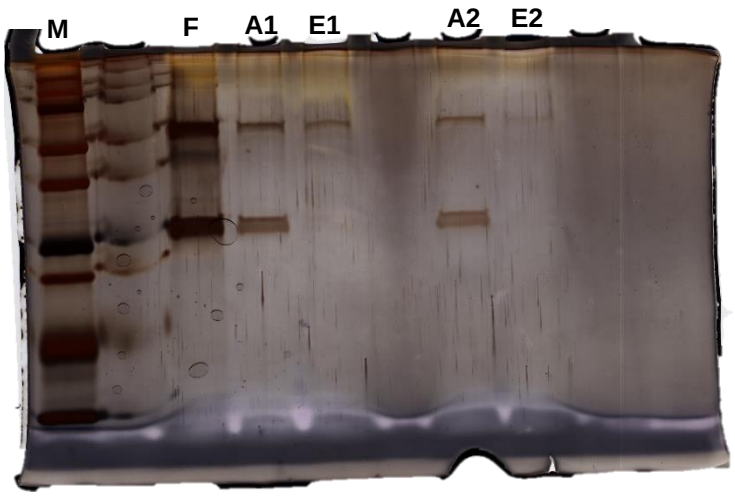


Figure 25 - *Gel with the samples of the preliminary assay, in which the Marker is represented by M, the feed is represented by 1, the adsorption steps are represented in lane A1 and A2, whilst the lanes E1 and E2 represent the elution step.*

Although these conditions were tested, many other conditions should be screened, using others PILs as well, in order to have a deeper understanding of the underlying mechanism.

5. Final remarks and future work

CHO DP-12 cells were cultivated in a serum-free medium (ProCHO™5) in order to produce monoclonal antibody. This antibody was further on purified and the impurities inherent to the cell culture were retrieved. In order to obtain a more sustainable process, the aim was to study alternatives to the affinity chromatography for the purification of mAbs.

Poly(ionic liquids) have emerged as new materials with potential as new separation matrices. These materials have been widely used for gaseous separation and there is one report in which PILs were used to purify M13 bacteriophages. Since the results were satisfying, in this work it was intended to study PILs further on, in order to understand whether they would be viable alternatives for mAbs purification.

To do so, firstly the ionic liquids were synthesized by quaternization reaction of 1-vinylimidazole and by quaternization of (dimethylamino)ethyl methacrylate followed by anion metathesis with lithium bis(trifluoromethylsulfonyl)imide. The obtained monomers were validated through NMR analysis. ILs were then polymerized, giving rise to poly(ionic liquids). These compounds were characterized by measuring their Zeta Potential, which varied between 18.2 and 38.8 mV.

Given that PILs were proven to be positively charged, an anion-exchange type of chromatography was performed, in order to assess their effectiveness as separation matrices. As the adsorption buffer pH was higher than the pI of most of the considered impurities and lower the pI of mAbs, the global charge of the impurities became negative, whilst mAbs remained positive. Hence, the capture of the impurities was facilitated, resulting in a higher purity of mAbs in the adsorption samples. Even though it was not possible to determine the mass of protein in each of the sample fractions, the determination of the adsorption yields was purely based on the obtained chromatograms, resorting to the peaks areas. Poly(VEIM-Br) with 30% EGDMA and poly(DMEAM-Br) with 30% EGDMA achieved the highest yields for pH 7.5 (86% and 89%, respectively) and 8.5 (93% and 90%, respectively). Considering that it is a single step purification and that it is not an affinity ligand, the results are promising.

Nevertheless, there is still a long way to go with this work, so it is imperative to overcome some of the drawbacks encountered so far. In order to avoid leaching, a new protocol of PILs synthesis should be performed. Achieving this, it will become possible to quantitatively estimate protein masses and therefore to determine protein yields, purification factors, purities, among others.

It is of paramount importance to further study the impurities, by performing 2D gel electrophoresis as well as considering that other impurities are present in the cell culture, such as genomic DNA. These impurities should be characterized as well, in order to achieve higher yields of purification.

Furthermore, it would be interesting to study the efficiencies of PILs, capturing impurities of a cell culture with higher protein concentration.

6. Annexes

6.1. NMR spectra

Prior to polymerization, ionic liquids synthesis was validated through ^{1}H NMR and ^{13}C NMR. The spectra regarding the imidazolium-based ionic liquids are represented in **Figure 26** and **Figure 27**.

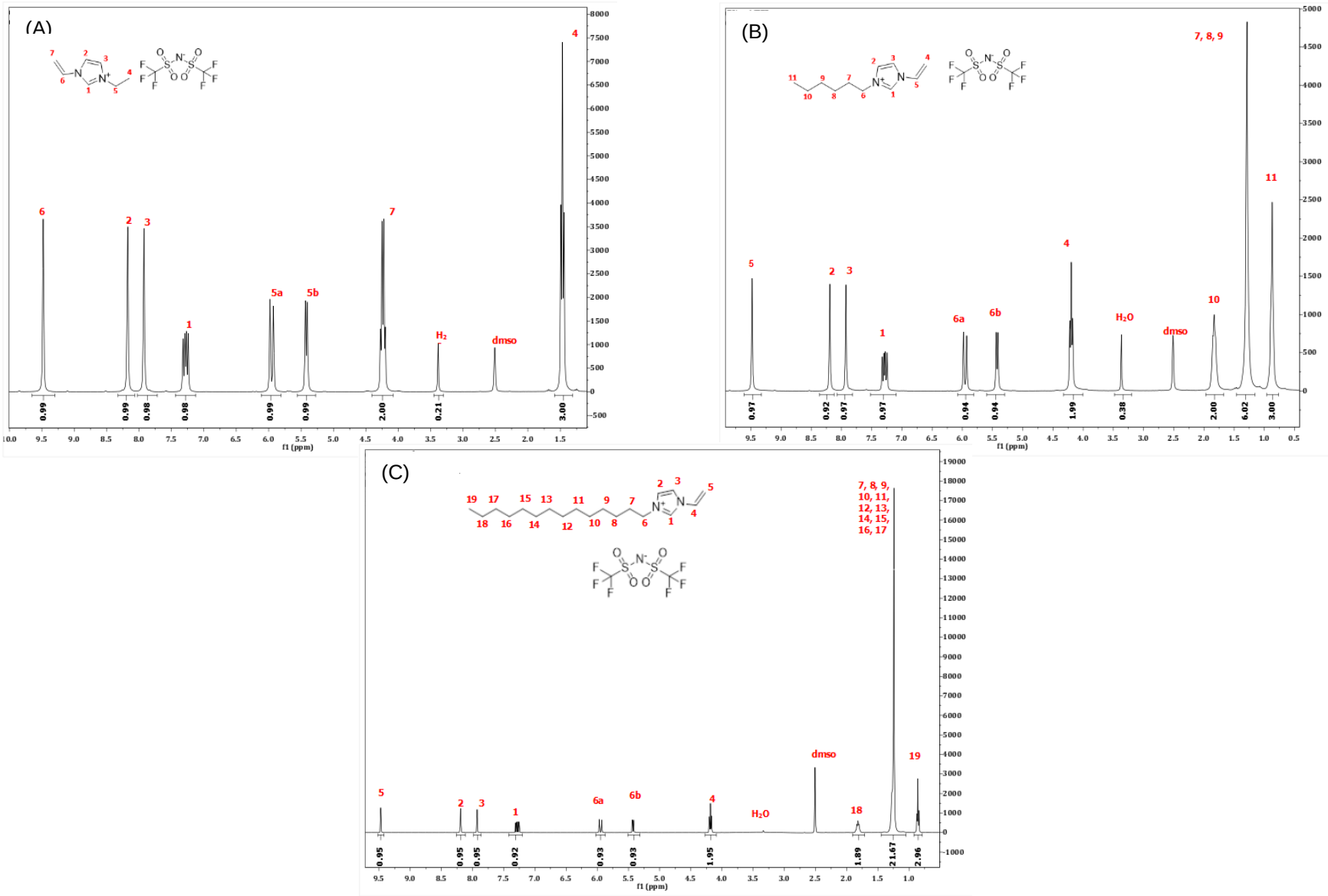


Figure 26 - *^{1}H NMR spectra of (A) 1-vinyl-3-ethylimidazolium bis(trifluoromethylsulfonyl)imide, (B) 1-vinyl-3-hexylimidazolium bis(trifluoromethylsulfonyl)imide and (C) 1-vinyl-3-tetradecylimidazolium bis(trifluoromethylsulfonyl)imide.*

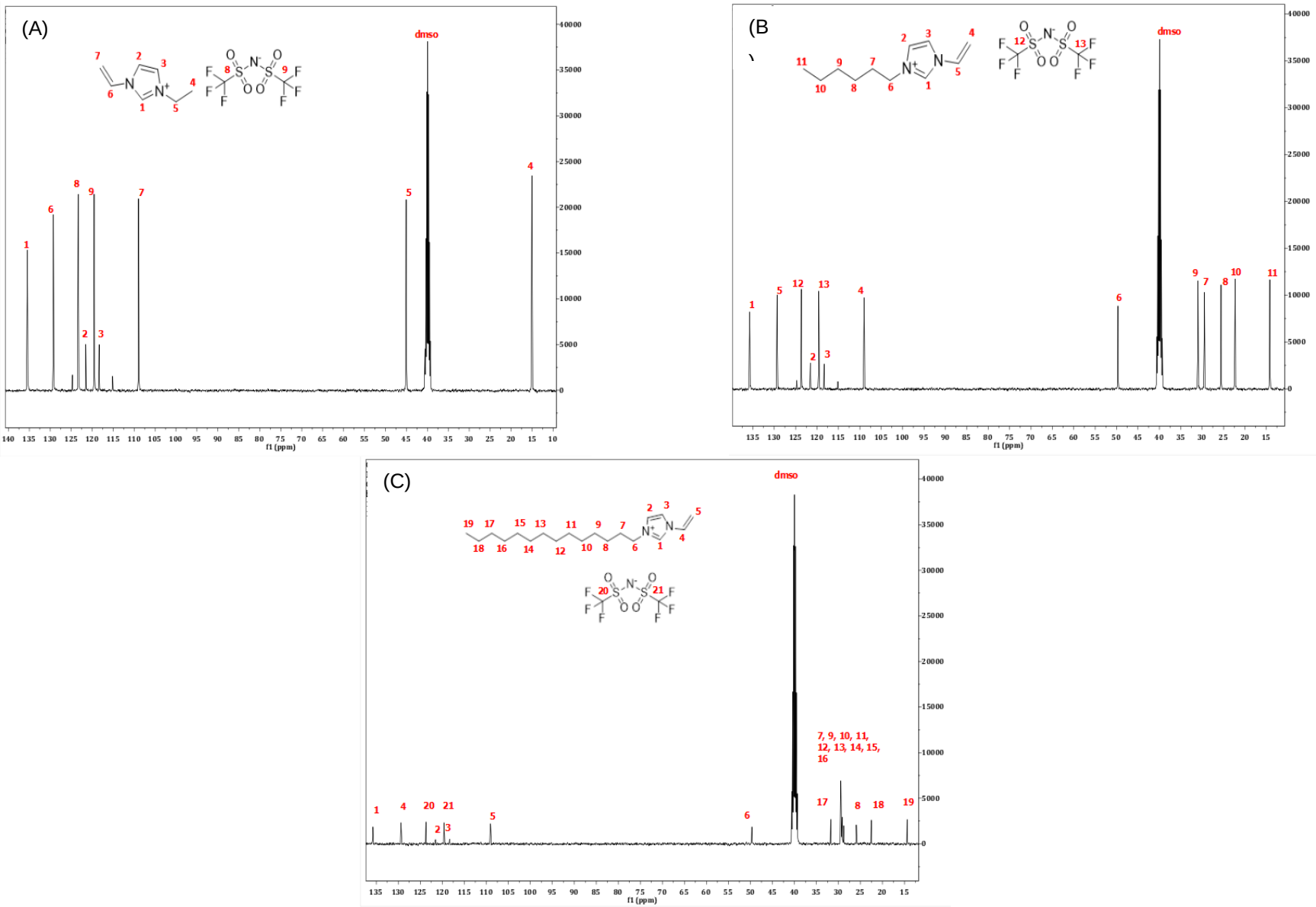


Figure 27 - *^{13}C NMR spectra of (A) 1-vinyl-3-ethylimidazolium bis(trifluoromethylsulfonyl)imide, (B) 1-vinyl-3-hexylimidazolium bis(trifluoromethylsulfonyl)imide and (C) 1-vinyl-3-tetradecylimidazolium bis(trifluoromethylsulfonyl)imide.*

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