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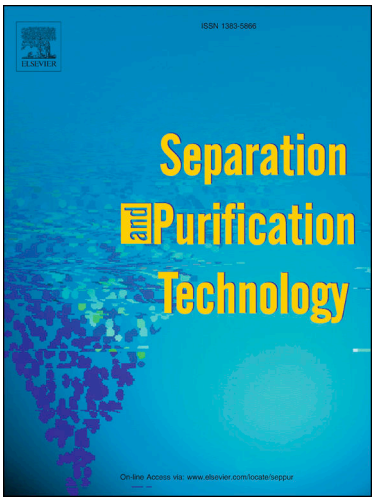


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# Screening polymeric ionic liquids for chromatography-based purification of bacteriophage M13

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*Abstract:*

M13 bacteriophage is a key instrument in phage display applications, as well as a possible antibacterial therapeutic agent due to its highly restrictive bacterial pathogenesis, and other applications. The traditional phage purification process is usually achieved by gradient ultracentrifugation or a combination of precipitation, centrifugation and microfiltration. These approaches easily lead to long process times, high operational costs, phage aggregation and consequent product loss (approximately 60%). This work is thus focused on an alternative potential large-scale process to achieve high yield and purity while minimizing the operational costs.

Electrostatic-based separation processes are also common biomolecules purification techniques. Although anion exchange chromatography has been used before to purify several viral particles, this technique has been poorly reported for the purification of M13 phage. In a recent work, our group has demonstrated the use of a predominant anion exchange process, where a polymeric ionic liquid (PIL) was used as an alternative separation matrix for M13 bacteriophage. In this work, a variety of system parameters was studied, including chemical structure of the cation and the anion, the crosslinker nature and its concentration, either in batch adsorption/elution or chromatographic operation mode. The PIL-based chromatographic operation mode revealed to be a suitable separation process for M13 from directly filtered *E. coli* supernatant, reaching over 70% M13 recovery and 4.6 purification factor in a single step. To our knowledge, this is the first time that PILs have been reported as separation agents for bioproducts from complex mixtures.

**Keywords**: Polymeric ionic liquids, Bacteriophage M13, Batch adsorption, Chromatography, Anion-exchange

## 1. Introduction

M13 bacteriophage is a single stranded DNA virus that infects *Escherichia coli* via the F pilus and, with 1 μm in length and 7 nm in diameter, is one of the smallest filamentous phages known [1–3]. Structurally, the M13 genome is 6407 nucleotides long and codes for 11 viral proteins, with 2700 copies of the major coat protein pVIII and five copies of each of the minor coat proteins on the ends [4]. This protein shell is composed of a negatively-charged region at the amino terminal end, which is rich in acidic amino acid residues that interact with the solvent and give the phage a low isoelectric point (pI) of 4.5 [5,6]. Due to the simplicity in manipulation and the exceptional stability to pH and temperature, M13 bacteriophage is frequently used in phage display technologies [7,8]. Peptides and proteins derived from phage display are being employed as sensors [9], drug delivery and discovery [10,11], assembly of materials [12] and to target and aid in imaging cancer cells [13]. Furthermore, reports also indicate the use of this bacteriophage in high-sensitivity lateral-flow immunoassays [14] and, at a larger scale, as biocontrol agents against foodborne pathogens to evaluate food safety [15].

In spite of the rapid growth of phage technology, only recently phage purification caught interest. Traditionally, density gradient ultracentrifugation, in cesium chloride or sucrose, or successive rounds of high-speed ultracentrifugation have been used in the purification of bacteriophages [16,17]. An alternative downstream operation is a multi-step platform combining precipitation, centrifugation, and microfiltration. This combination results in an extensive process, with high operation and maintenance costs and product loss due to degradation [17]. Therefore, there is an increase demand for a large scale and cost-effective phage purification platform with high yield and purity.

In the last few years, there has been an outbreak of new methods for phage purification using chromatography, namely the use of ion exchange chromatography. This technique is very popular for the purification of biological products, and can be applied to the purification of peptides, proteins, oligonucleotides, nucleic acids, and other charged molecules, and it has been described for the purification of viral particles [18,19]. However, for the purification of M13 bacteriophages, this method was only reported twice. In these reports, two types of anion-exchange matrices have been examined: quaternary amine (Q) and diethyl amine (DEAE) [17,20]. Both works explore the use of commercially available chromatographic media that are usually used for the purification of proteins. Since the diffusion of bacteriophages in solution is much slower than proteins and the binding occurs primarily on the bead surface, the chromatographic conditions must be optimized [18]. Therefore, there is a crucial need to further

develop media structure and chemistry as well as the development of novel chromatographic matrices.

Ionic liquids (ILs) have been used in a variety of different areas and, due to their unique interactions, might also provide interesting opportunities in the bioseparations field. ILs are generally described as salts composed of ions - organic cations and either organic or inorganic anions - which present a melting point below 100°C [21]. These compounds have attracted much attention due to their interesting characteristics such as low vapor pressure, a wide temperature range for liquid phase, high thermal and chemical stability, but perhaps their most interesting property is the easy tune of their properties by the fine tuning of the anion and cation chemical structure [22]. Therefore, the large variety of possible combinations of cations and anions [23,24] yields a versatile platform of diverse materials that can be advantageously used in the field of chromatography [25]. Rito-Palomares *et. al*. [26] recently reported a pioneering study on the use of an IL-based two aqueous phase system for the primary recovery of bacteriophage M13.

Poly(ionic liquids) (PILs) are polyelectrolytes that can be obtained from ionic liquids monomers polymerization or by post-polymerization processes [27]. This subclass of polyelectrolytes advantageously combine some of the properties of ILs with the improved mechanical stability and macromolecular architecture of polymers. In fact, PILs are solid in most cases, while ILs are in liquid state at near room temperature. Owing to their affinity properties, PILs are being used for new applications [27]. Notably, PILs are being studied as new separation and absorption phases in gas separation membranes, gas chromatography, capillary electrophoresis, and solid-phase microextraction to extract a specific component, such as a gas, metal ions and pharmaceuticals inorganic pollutants, from complex systems [27–29]. A novel boronate-functionalized graphene-coupled guanidinium type PILs monolith was developed for the separation of multiple types of glycoproteins [30]. This new type of monoliths improved the separation efficiency and allowed the separation of multiple types of glycoproteins.

The use of PILs as separation matrices for biological products was previously reported by our group. The separation of pure M13 bacteriophages based on an anion exchange process was successfully developed using a hydrophobic PIL, bearing a polycation based on imidazolium cation combined with bis(trifluoromethylsulfonyl)imide anion, poly (1–vinyl-3-ethyl imidazolium bis(trifluoromethylsulfonyl) imide) — poly(VEIM-TFSI) as a novel separation matrix for the purification of biological products [31]. In this work, the performance of different PILs for the selective separation of M13 bacteriophage was explored. Hydrophobic PILs, bearing a polycation based on imidazolium and acrylate cations combined with different anions and

different crosslinker agents, were prepared and their performance as anion exchange separation matrix for negatively charged M13 phages, in adsorption/elution optimization studies, evaluated. Two types of PILS were tested i) imidazolium based PILs and ii) ammonium based PILs. Since the monomers of the ammonium based PILs are less reactive, an acrylate group was used to activate the monomer. The effect of the anion was also tested using two anions.
The use of poly(VEIM-TFSI) as anion exchanger enabled the development of a rapid and simple method for the recovery of phage M13 from *E. coli* supernatant, with over 70% recovery and 4 purification factor after only one purification step, either in adsorption/elution assay or chromatographic operation mode.

## 2. Material and methods

### 2.1. Poly(ionic liquid) synthesis

The PILs used in the M13 purification were prepared by photopolymerization of the three different IL-monomers. The IL monomers chosen were 1-vinyl-3-ethylimidazolium bis(trifluoromethylsulfonyl)imide and 1-vinyl-3-tetradecylimidazolium bis(trifluoromethylsulfonyl)imide, both synthetized using the same methodology, and 2-(dimethylethylamino)ethyl methacrylate bis(trifluoromethylsulfonyl)imide, synthesized using a different methodology. The first two monomers, both containing the imidazolium cation but different alkyl chain lengths (ethyl and tetradecyl) were synthesized to evaluate the effect of the side chain, while the third monomer is a quaternary ammonium

The first step for the preparation of imidazolium-based PILs was the synthesis of the 1-vinyl-3-ethylimidazolium bromide ($C_2$imi) and 1-vinyl-3-tetradecylimidazolium bromide ($C_{14}$imi) IL monomers, as previously described [32]. Briefly, 1-vinylimidazole was mixed with an equimolar amount of bromoethane or bromotetradecane at 40°C for 24 h at constant and vigorous mixing (800 rpm). The product was then precipitated and thoroughly washed with ethyl acetate to remove any unreacted reagent. After filtration and drying under nitrogen flow, the monomer was dissolved in methanol and subjected to anion metathesis so that IL monomers bearing the TFSI anion were obtained. In order to perform the anion exchange, the monomer was mixed with an equimolar amount of LiTFSI, during 24 h at 500 rpm, to obtain the hydrophobic monomer 1-vinyl-3-ethyl imidazolium bis (trifluoromethanesulfonyl)imide (VEIM-TFSI) and 1-vinyl-3-

tetradecylimidazolium bis (trifluoromethanesulfonyl)imide, after drying under nitrogen atmosphere.

The cross-linked polymers were synthetized by mixing the monomer with different percentages of divinylbenzene (DVB) or ethyleneglycol dimethacrylate (EGDMA) as the cross-linker (x % (w/w) to the monomer), and 2-hydroxy-2-methylpropiophenone as the photo initiator (5% (w/w) to the monomer). The mixture was moved to a silicon mold (2 x 4 cm) and placed under UV light until complete polymerization. Afterwards, the polymer was washed with acetone, to remove any unreacted precursors, filtrated and dried in an oven at 60°C during 8 h. Finally, the dried material was ground, using a small blade coffee grinder, and stored at room temperature.

In order to synthesize the 2-(dimethylethylamino)ethyl methacrylate bromide, equimolar quantities of 2-(dimethylamino)ethyl methacrylate and bromoethane were mixed, at room temperature for 15h at 800 rpm, in the absence of light. The obtained product was submitted to three washing steps with ethyl acetate and, since the monomer is highly reactive making the filtration of the compound unviable, it was dried under vaccum (1Pa) under 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 formerly obtained monomers, equimolar portions of each monomer was mixed with LiTFSI and incubated at room temperature for 15 h at 500 rpm. The resulting monomers were washed with distilled water for at least 3 times and dried under vaccum (1 Pa) with vigorous stirring for, at least, two days.

The 2-(dimethylethylamino)ethyl methacrylate bis(trifluoromethylsulfonyl) monomer was mixed with 30% of DVB crosslinker and with 5% of a photoinitiator (2-hydroxy-2-methylpropiophenone). The resulting solution was polymerized under UV light until complete polymerization and the obtained polymer ground with a coffee grinder. PILs were then carefully washed with acetone to remove any unreacted precursors and subsequently stored at 4°C.

The synthesis and purities of the IL monomers and PILs were followed by $^{1}$H NMR on a Bruker 400 MHz Ultra-Shield-Plus Magnet NMR instrument using d6 – DMSO as deuterated solvent. No impurities or unreacted monomers were found during the process and in the final material.

### 2.2. M13 bacteriophage production and titration

Bacteriophage M13 KE was propagated in *E. coli* strain XL1-Blue grown in SOB medium and purified as previously described [31]. Briefly, the supernatant was centrifuged to remove all bacterial cells, and precipitated to purify the viral particles from the supernatant impurities. After

resuspension of the pellet in 1% BSA and 15% glycerol in PBS 1x, the solution was centrifuged and the supernatant, containing the M13 bacteriophage particles, was filtered to be further used in optimization tests.

The concentration of M13 phage particles in produced supernatant, flow-through and elution fractions was determined by phage titration as plaque forming units per milliliter (#/mL), as described in the previous work [31].

### 2.3. Adsorption/Elution studies

Adsorption and elution studies were performed in 96-well MultiScreen-HV Filter Plate (0.45 µm; Millipore) as described in the previous work [31] using 50 mM Tris-HCl pH 7.5 as adsorption buffer and 1.5 M NaCl in 50 mM citrate buffer pH 4 as elution buffer. In the optimization studies, 50 µL of 3.7 x $10^{10}$ # of purified M13 bacteriophage/mL solution was diluted with 150 µL of adsorption buffer and incubated on the well with the PIL in study.

Adsorption and elution efficiencies and total recovery yield were calculated as determined in the previous work [31].

This adsorption/elution studies format was also used for M13 recovery directly from 0.45 µm filtered *E. coli* supernatant – the filtration step was added to assure that no bacteria would be present in the supernatant. 50 µL of 8.1 x $10^{11}$ # M13 bacteriophage/ mL of *E. coli* supernatant was diluted with 150 µL of adsorption buffer and incubated on the well with the PIL in study.

### 2.4. PIL chromatography column preparation

To prepare the PIL column, 100 mg of poly(VEIM-TFSI) crosslinked with 30% DVB as crosslinker agent was weighted and then added to a column with an inner diameter of 5 mm (Tricorn 5/20, GE Healthcare). The PIL chromatography column was washed with 1 mL of Milli-Q water for 3 times to reduce the amount of small PIL particles that could pass through the column filter and be detected by absorbance at 280 nm during the chromatographic runs. After adding the poly(VEIM-TFSI), the column was closed with the top adjustable cap and connected to an ÄKTA Purifier 10 system (GE Healthcare).

### 2.5. PIL-based chromatography

PIL-based chromatography was performed using the chromatographic column prepared as described in the previous section, and connected to an ÄKTA Purifier 10 system (GE Healthcare) under the control of UNICORN 5.11 software (GE Healthcare). The column was washed with MilliQ water until a conductivity inferior to 0.054 mS/cm was reached and no variation on controlled parameters was observed. The runs were performed using 50 mM Tris-HCl pH 7.5 and 1.5 M NaCl in 50 mM citrate buffer pH 4 as adsorption and elution buffer, respectively. The absorbance of the eluate was continuously measured at 280 nm by a UV detector positioned at the column outlet.

The column was equilibrated at 0.1 mL/min with 10 column volumes (CVs) of adsorption buffer. Then, 250 µL of 0.45 µm filtered *E. coli* supernatant ($9.9x10^{11}$ # M13 bacteriophage / mL) diluted with 750 µL of adsorption buffer was injected into the column by washing the loop with 3 times its volume. All unbound sample was washed out of the column with 10 CVs of adsorption buffer. The bound material was eluted with a step gradient of 40 CV. The system pressure oscillated between 0.4 and 0.6 MPa. The flow-through and eluate fractions were collected in 0.5 mL and 0.1 mL fractions, respectively, in 1.5 mL microtubes positioned on a Frac-950 collector (GE Healthcare). The collected fractions were analyzed by acrylamide gel electrophoresis, total protein amount and phage titration.

### 2.6. Analytical Methods

#### 2.6.1. Protein Gel Electrophoresis, SDS-PAGE

Protein purity of the different chromatographic fractions was analyzed through acrylamide gel electrophoresis, as described in [33]. Briefly, samples were first diluted in appropriate loading buffer and denatured in reducing conditions. Then, samples were applied in a 15% acrylamide gel and ran at 90 mV. Finally, the gels were stained using a conventional silver staining protocol, with constant agitation (60 rpm) at room temperature. First the bands were fixated using a solution of 30% (v/v) ethanol and 10% (v/v) acetic acid, for 2 h. Afterwards, the gels were washed with 30% (v/v) ethanol, for 10 minutes, and with 2 consecutive washes, with water, for 10 minutes. The gels were sensitized by soaking in 0.02% (w/v) sodium thiosulfate, for 10 minutes. After washing 3 times with Milli-Q water, the gels were incubated with a fresh 0.15% (w/v) silver nitrate solution, for 30 minutes. After washing with Milli-Q water, for 1 minute,

the bands were developed by incubating the gel in a developer solution (3% (w/v) sodium carbonate and 0.05% (v/v) formaldehyde). The reaction was stopped with a solution containing 5% (v/v) acetic acid in Milli-Q water, for 15 minutes. The SDS-PAGE gels were afterwards stored in water until scanned using a GS-800 calibrated densitometer, from Bio-Rad (California, USA).

#### 2.6.2. Total protein concentration

Total protein concentration was determined by BCA protein assay (23225, ThermoFisher). The analysis was performed according to the manufacturer's instructions using the SpectraMax 384Plus microplate reader (Molecular devices, Sunnyvale, CA, USA). BSA was used as a standard curve with concentration between 2000-0 µg/mL.

## 3. Results and discussion

### 3.1. Influence of the PIL Chemical Structure

This study builds upon our first study where poly(VEIM-TFSI) was tested [31]. In that work it was shown that the anion exchange is the predominant effect in M13 bacteriophage purification and thus it is important to further study the interaction of M13 bacteriophage with the positively charged, polymeric backbone structure of the PIL. To better understand the influence of the PIL chemistry on the biological separation under study, several important features of the PIL, such as the chemical structure of the polycation backbone, the anion, the chemical structure of crosslinker and its content in the polymer network were investigated (Figure 1).

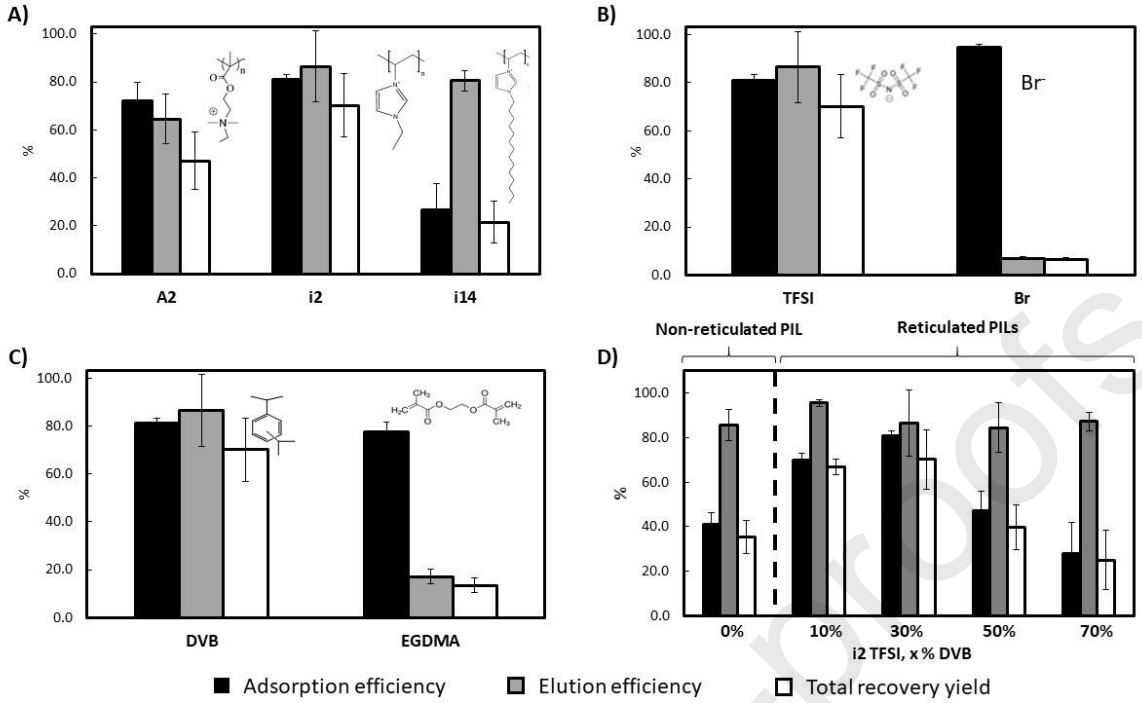


*Figure 1 – Study of the system parameters influence in a polymeric ionic liquid-based separation of M13 bacteriophage.* ***A) PIL cation****. Pure M13 bacteriophage recovery using different cations (2-(dimethylethylamino)ethyl – A2 -, 1-vinyl-3-ethyl imidazolium – i2 -, 1-vinyl-3-tetradecyl imidazolium - i14 - ) in poly(-TFSI), with 30% divinylbenzene (DVB) crosslinker agent, as the separation matrix;* ***B) PIL anion****. Pure M13 bacteriophage recovery using different anions (TFSI or bromide) in poly(VEIM), with 30% DVB crosslinker agent, as the separation matrix.* ***C) Crosslinker agent type****. Pure M13 bacteriophage recovery using 30% of different crosslinker agents (DVB or ethyleneglycol dimethylacrylate – EGDMA -) in poly(VEIM-TFSI) as the separation matrix;* ***D) Crosslinker agent percentage****. Pure M13 bacteriophage recovery using different DVB crosslinker agent percentages (0, 10, 30, 50 and 70%) in poly(VEIM-TFSI) as the separation matrix. The assays were performed using pre-purified M13 as feedstock with 50 mM Tris-HCl pH 7.5 as adsorption buffer and 1.5 M NaCl in 50 mM citrate buffer pH 4 as the elution buffer. Adsorption, elution, and total recovery yield percentages for the recovery of M13 phage particles in the elution fraction are shown. (n=3, mean ± standard deviation)*

First of all, the effect of PIL backbone chemical structure on the phage purification was also analyzed. Three different IL monomers, all bearing the TFSI as anion, were tested: 2-(dimethylethylamino)ethyl (A2), 1-vinyl-3-ethyl imidazolium (i2), and 1-vinyl-3-tetradecyl imidazolium (i14) (Figure 1A). These PILs were all crosslinked with 30% of DVB. When the 2 imidazolium-based PILs are compared it can be seen that the there is a significant effect of the alkyl side chain length: while for the 1-vinyl-3-ethyl imidazolium IL cation monomer an optimal adsorption efficiency of 81.0 ± 2.2 % was obtained, the use of the long alkyl chain length in 1-vinyl-3-tetradecyl imidazolium IL cation monomer dramatically decreases the adsorption efficiency to 26.6 ± 11%. This is probably related to the higher hydrophobicity of this last PIL. On the other hand, when the PIL based on 2-(dimethylethylamino)ethyl IL cation monomer was

used a slight decrease in the adsorption efficiency was observed to 72.0 ± 8.0%. Also, the more hydrophilic environment of this last PIL provides a lower elution efficiency, resulting in a lower total recovery yield, 47.0 ± 12.0% when compared to that of poly(VEIM-TFSI) where a value of 70.2 ± 13.2% was obtained.

The next step was to study the influence of the anion in the PIL. For that purpose, 1-vinyl-3-ethyl imidazolium as cation and crosslinked with 30% DVB was considered and 2 different anions, bis(trifluoromethylsulfonyl) imide – TFSI - and bromide, were tested. (Figure 1B). As expected, a smaller anion enhances the adsorption process (81.0 ± 2.2 % for TFSI and 94.6 ± 1.2% for bromide) since it facilitates the anion exchange process with the negatively charged M13 phage. However, this phenomena probably contributes to a higher number of cation-M13 electrostatic interactions that compromised the phage elution efficiency with 86.4 ± 14.9 % for the PIL bearing the TFSI anion and 7.0 ± 0.6 % for the PIL bearing the bromide anion. Higher amounts of salt, 2 M NaCl, were also tested to elute the M13 phage from poly(VEIM) bromide, but a significant recovery enhancement was not observed (data not shown).

The effect of two different crosslinker agents, DVB and EGDMA (Figure 1C), on the M13 bacteriophage purification, was studied using poly(VEIM-TFSI) with a fixed (30%) crosslinker agent content. DVB is a widely used crosslinker due to its versatility and ability to improve mechanical and thermal properties of the resulting material. In this specific application, DVB also provides solvent resistance, which is important during the whole separation process. On the other hand, EGDMA is also a widely used hydrophilic crosslinker and it also provides the possibility of hydrogen bonding. According to Figure 1A, the PIL containing EGMDA did not revealed to be an advantage for M13 bacteriophage adsorption efficiency, with 81.0 ± 2.2% for DVB and 77.4 ± 4.2% for EGDMA. Furthermore, when using the same elution buffer, the PIL synthetized with EGDMA led to a much lower elution efficiency when compared to the PIL synthetized with DVB as crosslinker, and the elution efficiency decreased from 86.4 ± 14.9 % to 17.2 ± 3.1 %, respectively. This is probably due to the more hydrophilic character of the PIL backbone provided by the EGDMA group, which hinders the elution of the large hydrophilic surface of the phage.

Finally, the percentage of the crosslinker agent present in the PIL was analyzed. For that purpose, different assays using poly(VEIM-TFSI) with different percentages (0, 10, 30, 50, 70%) of DVB as crosslinker agent were carried out (Figure 1D). It is important to refer that a high

percentage of the crosslinker agent means a low amount of IL monomer and thus low anion exchange capacity. Additionally, high crosslinker agent percentages yield a tighter polymer net, with smaller pore sizes, resulting in a more packed network. This can be confirmed through an increase in glass transition temperature [34]. It was also shown before that gas diffusion in crosslinked polymers decreases with increasing crosslinking content, due to the loss of free volume [35] and also a decrease in the pore size [36,37]. Therefore, small pore sizes and low charge density are expected with the increase of the crosslinker agent percentage.

Although the absence of crosslinker agent contributes to PIL with higher charge density and pore size when comparing to the same PIL with 10 and 30% DVB as crosslinker agent, the non-reticulated PIL was not the most effective for the M13 separation. In fact, with the increase of crosslinker percentage in the synthesized PIL there is a clear increase on the adsorption efficiency (40.9 ± 5.4% for non-reticulated PIL, 69.9 ± 3.2% for 10% DVB and 81.0 ± 2.2% for 30% DVB). Regarding the elution efficiency, it is possible to observe that it remains mostly constant for all the PILs in study, irrespective of the crosslinker content. Nevertheless, the increase of crosslinker agent percentage from 50 to 70% led to low adsorption efficiencies, 47.0 ± 8.8% and 28.1 ± 13.8%, respectively, and consequent lower M13 recovery yields. These results suggest that the best separation condition requires an equilibrium between the polymer charge density and pore size, for the M13 phage, where the use of an optimal content (30%) of DVB crosslinker agent enhances the purification efficiency.

In summary, the anion is the chemical feature of the PIL that most influences the overall recovery yield, although the chemical structure of the PIL backbone and the crosslinker nature also have significant effect in the PIL recovery. Among all the materials tested, the most effective PIL for the purification of M13 bacteriophage was the poly(VEIM-TFSI) crosslinked with 30% DVB, which presented 81.0 ± 2.2% adsorption yield, 86.4 ± 14.9% elution yield and 70.2 ± 13.2% total recovery yield.

### 3.2. M13 direct purification from *E. coli* supernatant

The performance of the poly(VEIM-TFSI) crosslinked with 30% DVB was then evaluated for the recovery of the M13 bacteriophage directly from the *E. coli* supernatant. The batch adsorption/elution approach was used, in order to study the influence of the supernatant impurities in the PIL-phage interaction. The same adsorption/elution studies conditions as described before were used with a 0.45 μm filtered *E. coli* supernatant.

The results revealed to be in good agreement with the previous optimization studies, where 81.0 ± 2.2% and 77.3 ± 6.6% adsorption efficiency (1.6 ± 0.1 x109 #/mg of poly(VEIM-TFSI) crosslinked with 30% of DVB), 86.4 ± 14.9% and 97.9 ± 10.2% elution efficiency, and 70.2 ± 13.2% and 76.1 ± 13.2% total recovery yields were achieved from pre-purified M13 studies and filtered *E. coli* supernatant, respectively.

A purification factor of 4.2 ± 0.7 was determined as the ratio between the amount of M13 phage per total amount of protein in the elution fraction and the amount of M13 phage per total amount of protein in the initial *E. coli* filtered supernatant feedstock. The results obtained show a considerable increase on the purification performance, since 4.2 times more M13 particles per total amount of protein was observed when compared with the initial supernatant feedstock.

A SDS-PAGE analysis was performed in order to evaluate the protein purity in the initial *E. coli* filtered supernatant and in the flow-through and elution fractions (Figure 2). Since the M13 bacteriophage is composed by different proteins (pIX – 3.5 kDa -, pVII – 3.6 kDa -, pVIII – 5 kDa -, pVI – 13 kDa -, and pIII – 42.6 kDa that appear at the 60 kDa level [38]), only the fractions with high amount of M13, when all phage proteins bands are identified in that same lane, were considered.

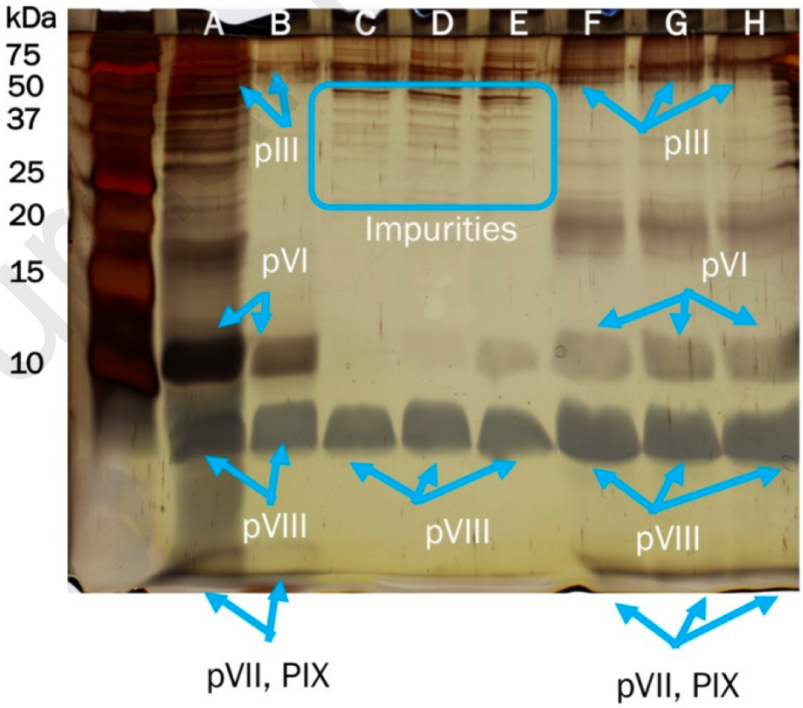

*Figure 2 -* ***Silver stained SDS-PAGE of M13 purification from E. coli supernatant using poly(VEIM-TFSI) as separation matrix in adsorption/elution studies****. Lanes ID: A: E. coli culture medium supernatant infected by M13; B: Pre-purified M13; C to E: flow-through triplicate fractions; F to H: elution triplicate fractions. M13 pVII, pIX, pVIII, pVI and pIII are indicated by solid blue arrows. Main supernatant impurities separated in flow-through fraction are also identified by a blue border. Precision Plus Protein™ Dual Color Standards is represented in the first left lane (kDa).*

As expected, the four main protein bands (illustrated in Figure 2) can be easily identified in the initial feedstock (lane A) and in the pre-purified M13 used in the previous batch optimization studies (lane B). Considering the differences between the flow-through and elution triplicate fractions, a series of impurities, between 25 and 50 kDa (identified in a blue box), mainly identified in the flow-through fractions, can be observed. On the other hand, the pVI, pVII and pIX protein bands are more distinguishable in the feedstock and elution fraction lanes, illustrating a higher concentration of the M13 bacteriophage in these samples. Therefore, M13 bacteriophage could be recovered directly from the *E. coli* supernatant in batch operation mode using poly(VEIM-TFSI) crosslinked with 30% DVB.

### 3.3. Poly(VEIM-TFSI) as an alternative chromatographic support

Taking into consideration that poly(VEIM-TFSI) crosslinked with 30% DVB has been proven to work as a novel separation matrix for the recovery of the M13 bacteriophage directly from the *E. coli* supernatant in adsorption/elution batch operation mode, as shown in the previous section, the possibility to use this polymer as a chromatographic support was explored.

In order to explore the use of poly(VEIM-TFSI) crosslinked with 30% DVB as a chromatographic matrix, 100 mg of this PIL were weighted and added to a column with inner diameter of 5 mm and connected to an ÄKTA Purifier 10 system. This approach would lead to the use of this separation support in a more conventional and well-established platform, with online monitoring of the system performance, for future biological separation applications. The chromatogram obtained is represented in Figure 3.

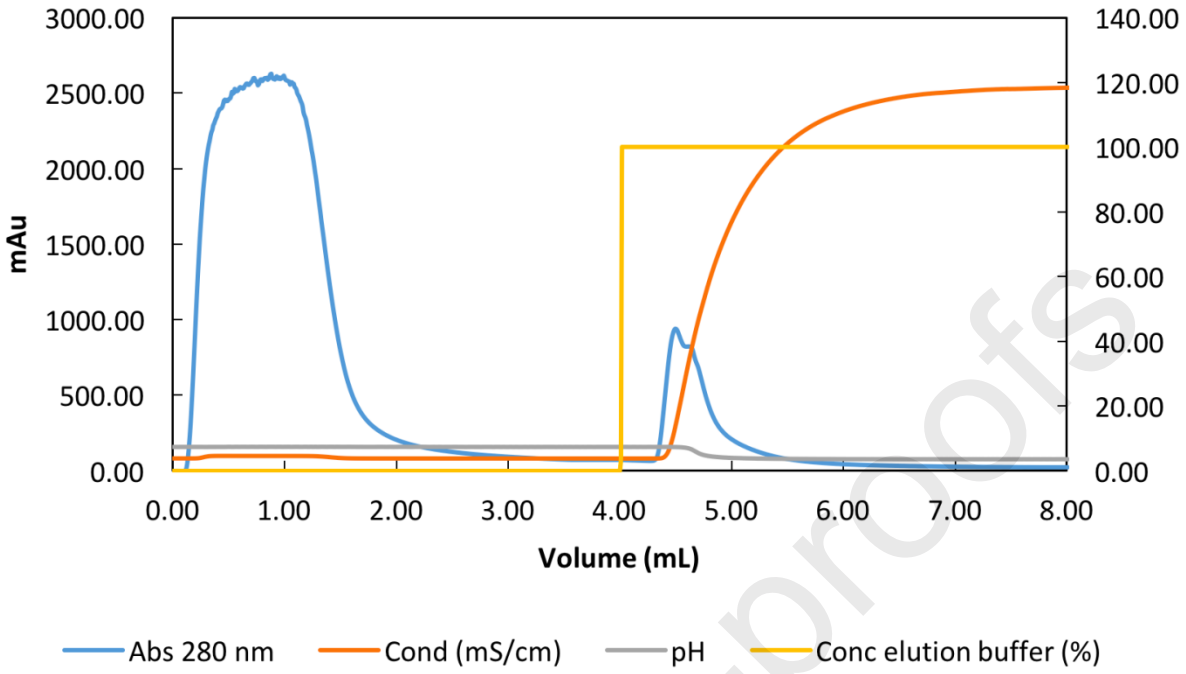


*Figure 3 –* ***Chromatogram illustrative of M13 bacteriophage purification from a filtered E. coli supernatant using poly(VEIM-TFSI) crosslinked with 30% DVB as chromatographic separation matrix****. 250 µL of filtered supernatant diluted in 750 µL of adsorption buffer (50 mM Tris-HCl pH 7.5) was injected into a pre-packed poly(VEIM-TFSI) column, pre-equilibrated with adsorption buffer. Unbound material was washed with 10 CV of adsorption buffer and step elution was performed with 40 CV of elution buffer (1.5 M NaCl in 50 mM citrate buffer pH 4). The absorbance at 280 nm, conductivity (mS/cm), pH and concentration of elution buffer B detected after the column outlet are represented by blue, orange, grey and yellow continuous lines, respectively.*

An increase in the absorbance, during the flow-through, was observed which reflects unbounded impurities that didn't adsorb to the polymer and thus flowed through the column unretained.

Also, a high amount of M13 bacteriophage bound to the separation matrix since 76.5 ± 1.3% M13 adsorption yield was achieved (2.2 ± 0.4 x109 #/mg of poly(VEIM-TFSI) crosslinked with 30% of DVB). When the elution buffer (1.5M NaCl in 50 mM citrate buffer pH 4) was applied, the decrease in the pH and the increase of conductivity caused the elution of 95.0 ± 2.8% of the bound M13 bacteriophages. Overall, a 72.7 ± 3.4 % total recovery yield and 4.6 ± 0.5 purification factor were achieved.

A new SDS-PAGE gel was performed in order to check the M13 purity in the eluted fractions (Figure 4). Again, the fractions with high amount of M13, when all phage proteins bands (pIX and pVII, pVIII, pVI, and pIII) are identified in that same lane, were considered.

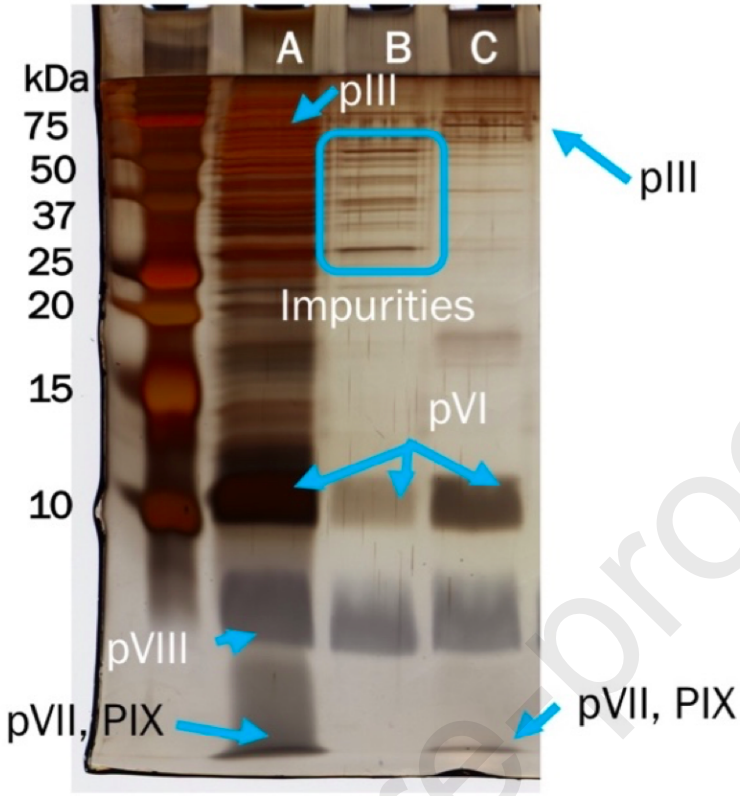


*Figure 4 -* ***Silver stained SDS-PAGE of M13 purification from E. coli supernatant using poly(VEIM-TFSI) as chromatographic support.*** *Lanes ID: A: E. coli culture medium supernatant infected by M13; B: chromatographic flow-through fraction; C: chromatographic elution fraction. M13 pVII, pIX, pVIII, pVI and pIII are indicated by solid blue arrows. Main supernatant impurities separated in flow-through fraction are also identified by a blue border. Precision Plus Protein™ Dual Color Standards is represented in the first left lane (kDa).*

The four main protein bands can be easily identified in the initial feedstock (lane A), as in the previously studied batch adsorption/elution experiments, and a clear series of impurities, between 25 and 50 kDa (identified in a blue box) that are mainly identified in the flow-through fraction, can be observed. On the other hand, the pVII, pIX protein bands are only visible in the feedstock and elution fractions lanes, indicating a much more significant presence of the M13 bacteriophage in these samples.

A comparative table with adsorption, elution, recovery yields and purification factors of the three study stages are represented in Table 1.

As observed in Table 1, the three operation modes presented similar adsorption, elution and recovery efficiencies. Also, chromatographic operation mode, an automatic, conventional and well-established platform, with online monitoring of the system performance, revealed to be a suitable separation process for M13 from directly filtered *E. coli* supernatant.

*Table 1–* ***Adsorption, elution and recovery yield (%) and purification factor in adsorption/elution and chromatographic separations of M13 bacteriophage in pure and filtered E. coli supernatant samples****.*

| Loading sample & operation mode | Adsorption yield (%) | Elution yield (%) | Recovery yield (%) | Purification factor* |
|---|---|---|---|---|
| Pre-purified M13– batch adsorption/elution | 81.0 ± 2.2 | 86.4 ± 14.9 | 70.2 ± 13.2 | - |
| *E. coli* supernatant – batch adsorption/elution | 77.3 ± 6.6 | 97.9 ± 10.2 | 76.1 ± 13.2 | 4.2 ± 0.7 |
| *E. coli* supernatant – chromatography | 76.5 ± 1.3 | 95.0 ± 2.8 | 72.7 ± 3.4 | 4.6 ± 0.5 |

This polymeric ionic liquid based separation revealed to be much more efficient than the conventional purification processes, which only recovers up to 36% of the viral particles by precipitation and centrifugation [17], and comparable to other published anion exchange chromatographies, such as 82.9% in an expanded bed or 74% using a pre-packed SepFast™ Super Q column [17,20].

## 4. Conclusions

Separation of M13 bacteriophages using a polymeric ionic liquid was successfully developed, by exploring this alternative separation matrix for both adsorption/elution and chromatographic operation modes. Using cross-linked poly(VEIM-TFSI) as the anion exchanger for M13 phage separation, it was achieved an excellent agreement between optimization assays and direct use of filtered *E. coli* supernatant, either in batch adsorption/elution mode or chromatographic operation mode. A total recovery yield of 72.7 ± 3.4% and a purification factor of 4.6 ± 0.5 were achieved in a single chromatographic process. Therefore, polymeric ionic liquid-based separation is an efficient method that can compete with conventional purification processes, which only recover up to 36% of the viral particles by precipitation and centrifugation [17]. Traditional chromatography processes, such as anion exchange chromatography, present

comparable results with the presented technology, with recoveries of 82.9% in an expanded bed or 74% using a pre-packed SepFast™ Super Q column [17,20]. Also, comparing to conventional bead-based columns, the PIL chromatographic approach presents a much more simple process with an easy and straightforward column packing that can be achieved in a few minutes due to the broad particle size distribution [31].

The use of polymeric ionic liquids as separation matrices also provides significant advantages when compared to ionic-liquid based aqueous biphasic systems since it allows the recovery of the M13 bacteriophage in a single fraction, in a defined elution buffer, without the need of any further treatment, as required in PEG or ionic liquid rich-phases [26,39].

This is the first time where PILs have been described as separation matrixes for complex biological samples, as *E. coli* supernatant. Therefore, the described results provide great encouragement to test these matrices for the purification of other biological products, such as smaller biomolecules like value added proteins as mAbs, either in bind-elute or flow-through operation modes. Also, the great number of possible cation-anion combinations, as well as the crosslinker agent type and percentage applied in the PIL synthesis, suggest PILs as customizable separation matrixes.

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## Conflict of interest

The authors declare no financial or commercial conflict of interest

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## Highlights

- Polymeric ionic liquid (PIL) as separation matrix for M13 phage was studied.
- PIL cation and anion, the crosslinker nature and its concentration were evaluated.
- M13 phage was successfully separated directly from filtered *E. coli*.
- Batch adsorption/elution and chromatographic operation mode were compared.
- The PIL-based chromatography reached over 70% M13 recovery in a single step.

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**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: