# Ionic Liquids as versatile supports in Bioseparations

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## 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, obtaining in some cases efficiencies over 90%.

## 1. Introduction

Anti-interleukin-8 is an antibody mainly used to treat diseases such as inflammatory bowel disorders (US patent 5702946), and asthma (US patent 5874080).

Generally, the processes underlying the purification of valuable compounds, such as monoclonal antibodies, carry high financial demands, given that since their main purpose is to be used as therapeutic agents[1–8], they must possess a high level of purity. Therefore, it is absolutely crucial to study and develop new purification techniques that will guarantee a higher financial viability of the process.

Ionic liquids are organic salts, generally composed by a large organic cation and an organic or inorganic anion, which melt bellow a conventional temperature of 100ºC. The combination of different cations and anions confer several properties to ILs, such as conductivity, viscosity and the ability to form biphasic systems which is a feature that can be

used in separations[9], hence being considered designer solvents[10]. Another important aspect of these organic salts' chemistry is the fact that they are also non-volatile[11], bypassing any problems that could arise related to the loss of solvent through evaporation, ultimately being classified the green solvents[12,13]. Given that IL-based materials have been studied as alternatives to separation processes, poly(ionic liquids) emerged as one of the possible solutions, due not only to the fact that they are in solid state but also their high chemical and thermal stability.

Poly(ionic liquids) (PILs) are a subclass of polyelectrolytes consisting of repeated ionic liquid (IL) monomers connected through a polymeric backbone, forming a macromolecular structure[14]. Poly(ionic liquids) have proven to be extremely interesting solutions with various applications in many scientific fields, due to their 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[15], that can be incorporate in PILs. Since PILs can combine different properties, mainly due to the variation of the ILs that are polymerized in their structure, they have emerged as new polyelectrolytes allowing their implementation in many fields, more specifically gas separation, catalysis and sorption[15,16].

However, up until now only one study has reported the use of PILs as separation matrices for biological products, namely bacteriophage M13 KE[17]. In this experimental work, PILs were synthesized and successfully used as a resin for ion-exchange chromatography. This novel adsorption matrix allowed a recovery yield of 70.2%, which is comparable to the recovery yield correspondent to other conventional chromatography operations. Also, these PILs were regenerated and successfully reused, which is an extremely important characteristic, since it validates the economic viability of the production process while assuring its environmental-friendliness.

This experimental work was aimed at the synthesis of PILs as well as their utilization as separation matrices for mAbs purification, in a negative-mode ion exchange chromatography.

## 2. Materials and Methods

### 2.1. Materials

Bromoethane (≥99%), 1-vinylimidazole (≥99%), (2-dimethylamino)ethyl methacrylate (≥99%), divinylbenzene (80%), ethyleneglycol dimethacrylate (98%), acetonitrile (99.8%), acetone (99.8%), ethyl acetate (≥99.5%) and 2-hydroxy-2-methylpropiophenone (97%) were purchased from Sigma-Aldrich. Lithium bis(trifluoromethylsulfonyl)imide salt (LiTFSI 99%) was supplied by IoLiTec GmbH. Methanol (99.99%) was purchased from Fischer Chemical. These chemicals were not further purified. The water was doubled distilled, passed through a reverse osmosis system and further treated with a Milli-Q plus 185 water purification equipment.

### 2.2. Imidazolium-based ionic liquids

In order to synthesize the 1-vinyl-3-ethylimidazolium bromide, equimolar quantities of 1-vinylimidazole and bromoethane were mixed, at 40ºC for 24h at 500 rpm. Then, the product obtained was submitted to three washing steps with ethyl acetate and, since the monomer is in solid state at room temperature,

it was filtrated. The retentate was later dried under vaccum (1Pa) and subjected to vigorous stirring at room temperature for at least two days.

### 2.3. Ammonium-based ionic liquids

In order to synthesize the (dimethylethylamino) ethyl methacrylate bromide, equimolar quantities of 2-(dimethylamino)ethyl methacrylate and bromoethane were mixed, at 25ºC for 15 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.

### 2.4. Anion exchange reaction

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 24h (for 1-vinyl-3-ethylimidazolium bromide) or 12h (for (dimethylethylamino)ethyl methacrylate bromide, in absence of light) at 500 rpm. The resulting monomers were washed with distilled water for at least 4 times.

### 2.5. NMR analysis

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.

### 2.6. Polymerization

After verification of the structure of the compounds obtained in step **2.5.** by NMR analysis, each one of the monomers was mixed with 30% of one crosslinker (either divinylbenzene -DVB- or ethylene glycol dimethacrylate – EGDMA) and with 5% of a photoinitiator (2-hydroxy-2-methylpropiopheno--ne). The resulting solutions were polymerized under UV light for 5 minutes and the obtained polymer ground with a coffee grinder. PILs were then washed with acetone to remove any unreacted precursors and subsequently stored at 4ºC.

### 2.7. PILs characterisation

#### 2.7.1. SEM

Scanning Electron Microscopy was performed in order to qualitatively assess the homogeneity of PILs grinding.

#### 2.7.2. 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.

### 2.8. Cell culture and supernatant characterisation

CHO DP-12 cells were cultured using ProCHO5™, supplemented with 4 mM L-glutamine (Gibco®), 2.1 g/L $NaHCO_3$, 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). Cell passage was performed on the 6$^{th}$ day of the culture and the supernatant was collected on the 8$^{th}$ day to be further studied.

The isoelectric points of the proteins in the supernatant were then assessed, in order to determine the pH at which the experiments would occur.

### 2.9. Chromatographic assays

To study whether PILs were able to be used as separation matrices in a negative-mode ion-exchange chromatography, cell culture impurities were retrieved. Chromatographic assays were performed using 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, which weighted 200 mg. The experiments were conducted at a rate of 0.2 mL/min. The PIL was equilibrated with 20 column volumes (CVs) of adsorption buffer (20 mM Tris-HCl pH 7.5 or 20 mM Tris-HCl pH 8.5) and the sample loop (1 mL) was washed and emptied three times its volume. The samples were then injected and washed, thus following the elution step, using 20 mM Tris-HCl, 1.0 M NaCl (pH 7.5 or 8.5) as an elution buffer. Flow-through and eluted fractions were collected on a Frac-95 fraction collector to qualitatively analyse their purity through SDS-PAGE. Cell culture samples were diafiltered against the adsorption buffer, resorting to Amicon® Ultra-0.5 Centrifugal Filter Devices prior to injection.

Blank runs were performed as well in order to take into account the effect of the buffers in each run. The peak areas of each chromatogram (both blank and sample) were determined and then adsorption yields of every experiment were calculated, considering that the total area of the reduced chromatogram (sample minus the blank) was proportional to the total amount of protein loaded.

## 3. Results and discussion

Monoclonal antibodies have been 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. Furthermore, ILs and PILs have become relevant in separation of many compounds and their ability to purify bioactive compounds should be assessed. In order to do so, PILs synthesis is essential. The alkylation of 1-vinylimidazole or 2-(dimethylamino)ethyl methacrylate with a halocarbon (bromoethane) is designated as quaternization reaction.

Subsequently, the addition of the salt lithium bis(trifluoromethylsulfonyl)imide to the obtained monomers will allow the exchange of the anions, thus resulting in monomers with a different anion (TFSI) and a salt (lithium bromide – LiBr), through a reaction termed anion metathesis. This anion was chosen, given that it is highly hydrophobic, conferring different properties to ILs and consequently PILs. ILs were then polymerized with two different cross-linkers (DVB and EGDMA) and grounded resorting to a coffee grinder (**Figure 1**). PILs

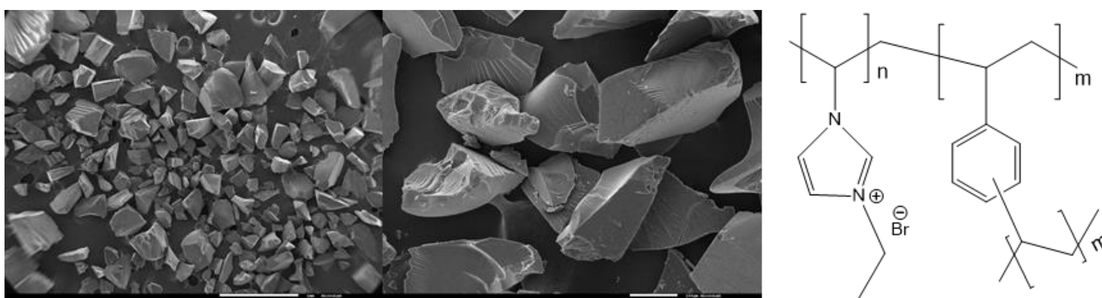


***Figure 1 –*** *(A) Representative SEM images of poly (VEIM-Br) with 30% DVB; (B) Chemical structure of poly(VEIM-Br) with 30% DVB.*

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.

PILs were packed into a column and used in an ÄKTA purifier system. As previously stated, the chromatograms were obtained and the peak areas calculated, in order to determine the adsorption yields and consequently the efficiency in the reduction of host cell proteins. Firstly, 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, thus following an icrease of salt concentration, to perform the ion-exchange chromatography[18]. The choice of the pH at which the experiments would occur was based on the results of the isoelectric focusing (data not shown). The efficiencies obtained for each PIL were the following: 81% 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 (**Figure 2**). 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 for 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 than bis(trifluoromethane)sulfonimide (TFSI), thus resulting in a stronger bond. For the purification of larger particles, such as bacteriophages[17], 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 in Sara Rosa *et al.*[19] 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.*[20], in which HCP removals of 83% and higher were achieved, resorting to affinity chromatography using boronic acid magnetic particles.

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 efficiencies obtained for each PIL were the following: 82.9% for poly(VEIM-Br) with 30% DVB; 92.7% for poly(VEIM-Br) with 30%

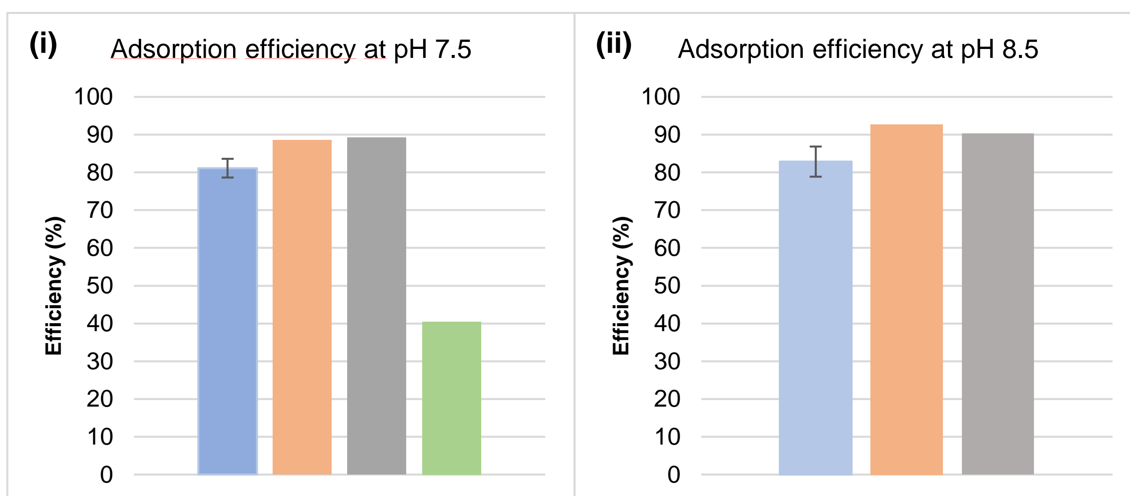


***Figure 2 -*** *Adsorption efficiencies obtained for each PIL at (i) pH 7.5 and (ii) pH 8.5. Poly(VEIM-Br) with 30% DVB is represented with light blue, poly(VEIM-Br) with 30% EGDMA with light orange, poly(DMEAEM-Br) with 30% EGDMA with light grey and poly(DMEAEM-TFSI) with 30% EGDMA with light green.*

EGDMA and 90.3% for poly(DMEAM-Br) with 30% EGDMA as shown in **Figure 2**.

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%, which, in comparison to the other two, was a considerable increase (**Figure 2**). 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[20]. 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. 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 3**). The gel showed that most of the proteins were captured and consequently eluted, since

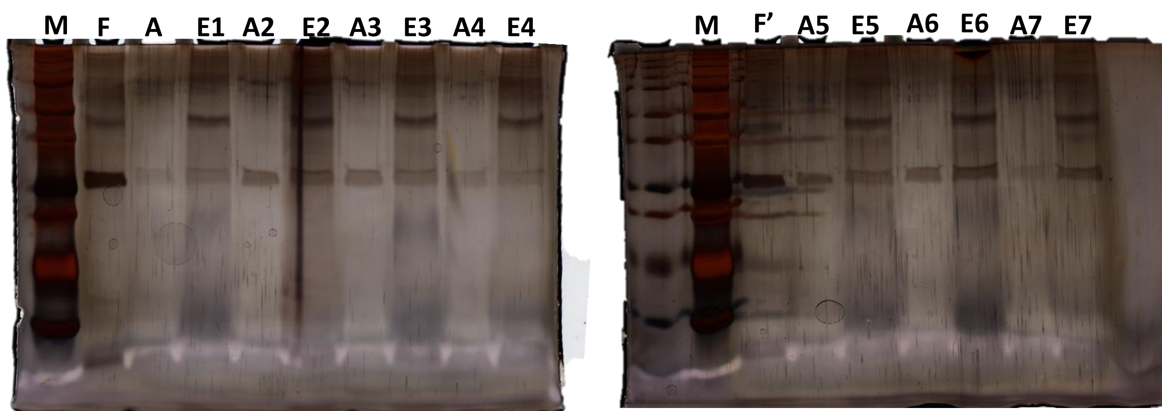


***Figure 3 -*** *Two experiments are presented in this figure: Runs at a pH of 7.5 – poly(VEIM-Br) with 30% EGDMA (lanes A2 and E2), poly(VEIM-Br) with 30%DVB (lanes A6 and E6) and poly(DMEAEM-Br) with 30% EGDMA (lanes A7 and E7) – and runs at a pH of 8.5 – poly(VEIM-Br) with 30% EGDMA (lanes A1 and 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.*

bands in the eluted fractions are considerably darker than the respective flow-through bands (**Figure 3**). 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 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). 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 4**). 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 4**). 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.

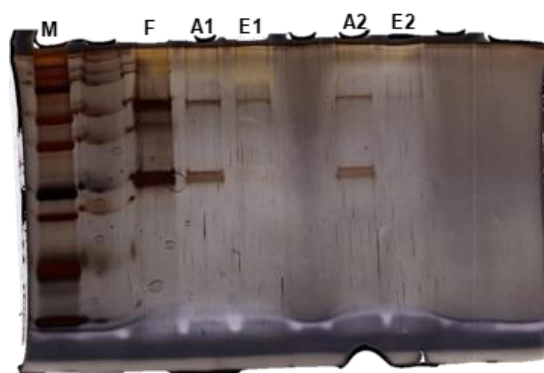

***Figure 4*** *- Gel with the samples of the preliminary assay, in which the Marker is represented by M, the feed is represented by 1, the adsorption steps are represented in lane A1 and A2, whilst the lanes E1 and E2 represent the elution step.*

## 4. Final remarks and future work

New materials have emerged as potential new separation matrices, such as poly(ionic liquids). 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, 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.

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