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Proteins/peptides purification by a three-well OFFGEL electrophoresis with immobilized ultra narrow pH gradient gels

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Abstract

Purification and desalting of protein and peptide samples by a three-well OFFGEL electrophoresis with immobilized ultra narrow pH gradient gels is proposed as a fast preparative strategy for proteomics. The gist of this strategy is to separate the proteins and peptides according to their isoelectric point and to isolate those of a given p*I* value equal to the mean pH value of the gel. The present approach has been demonstrated both on protein mixtures and a digested *Escherichia coli* protein extract. UV-Vis spectroscopy, MALDI-MS, SDS-PAGE and LC-MS/MS were employed for the quantitative and qualitative characterization of the separation results. The electrophoretic methodology has been simulated by finite element methods.

Keywords: electrophoresis, immobilized ultra narrow pH gradient gel, mass spectrometry, LC-MS/MS, proteomics, protein desalting

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1. Introduction

Proteomics presents very demanding challenges for bioanalytical chemistry, due to the highly complex samples and the wide dynamic range of analyte concentrations. To date, the most widely used protocol is the bottom-up proteomics approach [1, 2], where proteins are digested and the generated peptides are separated by liquid chromatography (LC) and identified by tandem mass spectrometry (MS/MS).[3, 4] Alternatively, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is used in top-down proteomic approaches [5], which includes a high resolution separation of proteins according to their p*I* in the first dimension by isoelectric focusing (IEF) and then according to their molecular weight (MW) in the second dimension after denaturation by sodium dodecylsulfate. The 2D-PAGE has a high capacity for the fractionation of complex mixtures of proteins, permitting to resolve hundreds or even thousands of sample spots [6-8].

IEF separates proteins or peptides according to their isoelectric points (p*I*) in a stable pH gradient under the application of an electric field [9]. The pH gradient was originally established with a mixture of carrier ampholytes. Nowadays, the most widely used pH gradient media are the immobilized pH gradient (IPG) gels where a series of ampholytes with specific p*I* values and high buffering powers are precisely organized along the gel to generate a linear pH gradient. IEF can also be performed in an OFFGEL manner [10] in a multiwell device [11], where the gel is placed under an array of wells that are filled with sample solutions. Proteins or peptides are fractionated according to their isoelectric points, while the separated components are directly recovered in liquid fractions on top of the gel and thus easily analyzed by other techniques such as LC-MS [12-14]. In addition of being a high-resolution separation technique, IEF electrophoresis also provides information on the sample isoelectric point that is valuable for identification. For instance, it has been suggested that a

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confident identification of peptides can be obtained by employing both the information of molecular weight from MS and p*I* from IEF even without tandem MS[15].

Considering the complexity of proteomic samples, new simple strategies and methods are still needed to pre-fractionate, separate, detect and identify the relevant species[16] even if many techniques for preparative purposes, *e.g.* fractionation, simplification and purification of tissue extracts, have already been developed [17-20]. The proteomic samples, depending on their nature, may contain a high concentration of salts or detergents, causing a major problem for mass spectrometry analysis. The use of microchip[21] and OFFGEL flow cells[22] were proposed in order to overcome the time-consuming desalting techniques, such as dialysis,[23] gel filtration[24] or precipitation techniques[25]. To decrease the experimental time and to improve the protein recovery, a multielectrode setup was introduced recently for OFFGEL electrophoresis [26].

Herein, we have developed an electrophoretic separation method with an immobilized ultra narrow pH gradient (UNPG) gel centred on a mean pH value, which in comparison to the previous OFFGEL reports is used in a miniaturized version as a preparative proteomic technique for fast sample purification and desalting. Proteins/peptides can be separated according to their electrophoretic mobility at the mean pH value and fractionated according to their charge at this pH. Immobilized UNPG gels can be obtained similarly to IPG gels by polymerization of acidic or basic monomers (Immobilines) in different ratios or simply obtained by cutting an area with a defined pH value from a commercially available IPG plate gel.

Using UNPG gels, ampholytes were rapidly fractionated by a three-well OFFGEL device into 3 fractions: the cathodic one containing proteins or peptides having a p*I* larger than the mean pH value of the gel in the reservoir where the cathode, the anodic

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one for species with a p*I* smaller than the mean pH of the gel and a third one in the middle reservoir for species that have a p*I* contained into the narrow pH range. This concept was first demonstrated by separating a mixture of four proteins followed with protein identification by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The protein recovery was measured by UV-Vis spectroscopy. Additionally, purification of an *Escherichia coli* (*E. coli*) extract was performed, demonstrating that the proposed methodology can be applied to complex samples. Furthermore, an *E. coli* protein extract was digested and the peptide mixture was firstly separated in the three-well device and then analysed by LC-MS/MS analysis. Moreover, it was shown that the three-well device could be used for partial desalting. All the results suggested that electrophoretic separation with UNPG gels provides an efficient strategy for fast purification of protein mixtures, and can thereby be used as a preparative technique for proteomics.

2.Materials and methods

2.1 Materials

OFFGEL: Immobiline® DryPlate, linear pH range from 4.0 to 7.0 was purchased from Amersham Biosciences (Uppsala, Sweden). **Proteins and solutions:** α-casein (p*I* 4.6) from bovine milk was from Fluka (Buchs, Switzerland). α-lactalbumin from bovine milk type I (p*I* 5.02), β-lactoglobulin A from bovin milk (p*I* 5.1), myoglobin from horse skeletal muscle (p*I* 7.0, 7.4) and cytochrome C from horse heart (p*I* 9.6) were obtained from Sigma-Aldrich (Schnelldorf, Switzerland), as well as, sinapinic acid, acetonitrile, trifluoroacetic acid, methanol and acetic acid of the purest grade (>99.9 %). Deionized (DI) water was purified by an alpha Q Millipore system (Zug, Switzerland) and used in all aqueous solutions. **Digestion and LC-MS/MS:** 1,4-dithio-DL-threitol (DTT, > 99.5%), urea and iodoacetamide (IAA) were from Fluka

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(Buchs, Switzerland). Trypsin from bovine pancreas was from AppliChem (Darmstadt, Germany). **SDS:** sodium dodecyl sulfate (SDS), Trizma base, bromophenol blue, ammonium persulfate (APS), N,N,N',N'-tetramethylenediamine (TEMED), acrylamide and bis-acrylamide solution were all from Sigma (Buchs, Switzerland). Silver staining kit for protein visualization was purchased from Amersham Biosciences (Uppsala, Sweden).

2.2 UV-Vis spectroscopy

The UV-Visible absorption spectra were obtained with a standard spectrophotometer (Perkin Elmer, model Lambda XLS+) using quartz cells with a path length of 1 cm. A calibration bicinchoninic acid (BCA) protein assay kit for determining protein concentrations was obtained from Thermo Scientific (Rockford, USA).

2.3 Soluble *E. coli* protein extract preparation

An over-night 200 mL culture of *E. coli* (strain DH5α) was collected by 10 min centrifugation at 5000 *g* and 4 °C. The cell pellet (0.6 g wet weight) was re-suspended in 3 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA) and cells were disrupted by sonication (10 x 10 pulses of 1 s at 30 W). Cell debris were removed by 10 min centrifugation at 2000 *g*. Ultracentrifugation (1 h at 100000 *g* at 4 °C) was applied to the total cell extract to remove membranes and membrane-bound proteins. The supernatant was collected and was considered as the soluble fraction of *E. coli* proteins.

2.4 Tryptic digestion

30 µL of Urea (18 M) was added to 70 µL of *E. coli* protein extract with the initial concentration of 10 mg/mL. 5 µL of 200 mM DTT in 50 mM Tris-HCl was added to the extract mixture and incubated for 1 h at room temperature in the dark. Afterwards, 20 µL of 200 mM IAA in 50 mM Tris-HCl was added and the mixture was incubated

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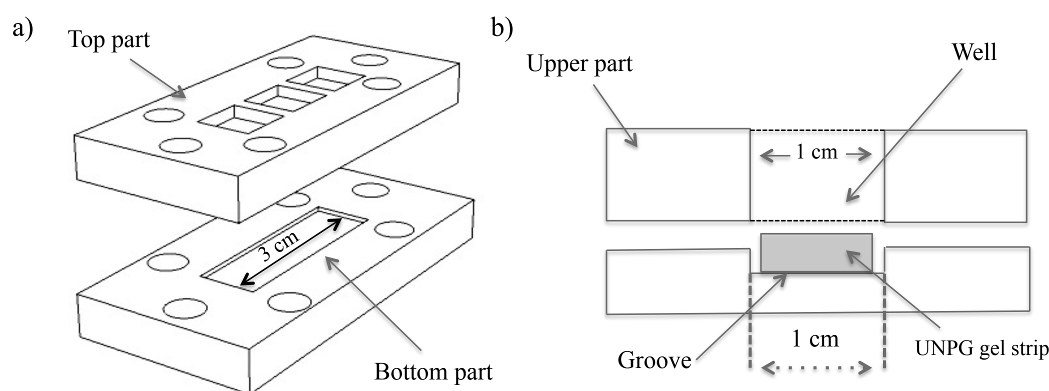
for 1 h at room temperature in the dark. To consume any unreacted iodoacetamide, 20 µL of 200 mM DTT in 50 mM Tris-HCl was added. To reduce the urea concentration to ≈ 0.6 M, 775 µL of 50 mM Tris-HCl solution was added. Finally, the trypsin was added to the solution with a final trypsin:protein ratio of 1:50 (w/w). The digestion was performed at 37 °C overnight.

2.5 Electrophoretic purification with immobilized UNPG gels

A commercial gel plate with a linear pH range 4 – 7 (Immobiline DryPlate pH = 4 – 7, T = 4%, C = 3%) was cut at the desired mean pH value to obtain gel strips with an ultra narrow pH gradient ≈ 0.27 pH units per cm. After re-swelling in water for 1 hour, the strip was inserted in a simple three-well OFFGEL device (see Scheme 1) for separating the ampholytes according to their charge at the chosen pH and also for sample desalting.



Scheme 1. Schematic representation of a) the top and bottom view of the three-well device, and b) cross-section view of the three-well device with an UNPG strip placed in the groove of the bottom part.

During electrophoresis, Pt electrodes were placed at the outer wells. Each of the wells was filled with 150 µL of DI water. The sample was loaded in the middle well and electrophoresis was performed with a high voltage power supply of Pharmacia Biotech EPS 3500 XL (Sweden). It is very important to keep the sample in a concentration range equal to 1 – 10 mg/component/cm of gel in order to avoid

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overloading of the sample and possible electroosmotic flow [9]. The purification was performed either with 10 µL of a protein mixture, containing α-casein, *α*-lactalbumin, cytochrome C and myoglobin (26 µg/mL each) or with 30 µL of *E. coli* protein or its digest. Three-well OFFGEL electrophoresis in all the cases was performed under the following conditions: 30 min at 150 V and then 4-5 h at 400 V, the current limit was set at 1 mA. After fractionation with the immobilized UNPG gels, soluble fractions were collected from all the wells and further separated by MS analysis or by SDS-PAGE to obtain a two-dimensional map of the proteins present in the *E. coli* extract. SDS-PAGE was always run by 2 hours with a starting applied potential of 60 V and 100 V after 1 hour.

2.6 MALDI-MS analysis

The protein analyses were performed on a Microflex LRF MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser. 1 µL of the extracted protein fraction from the three-well device was deposited on a steel target plate to dry under ambient conditions. Afterwards, 1 µL of sinapinic acid (SA) matrix (15 mg/mL sinapinic acid in 50 % acetonitrile, 0.1 % trifluoroacetic acid and 49.9 % water) was added and left to dry at room temperature and pressure.

2.7 LC-MS/MS analysis

Liquid chromatography was performed on an ACCELA LC system (ThermoFisher Scientific, Reinach, Switzerland). 20 µL of *E. coli* digest was loaded on a C-18 reversed-phase packed column (2.0 mm ID × 150 mm, COSMOSIL, $5C_{18}$-MS-II) with a loop loading speed of 8.0 µL/s. The mobile phase consisted of A (ACN/TFA 99.95/0.05 (v/v)) and B (water/TFA 99.95/0.05 (v/v)). Gradient was from 5 to 50 % of solvent A in 66 min, followed by the increase from 50 to 95 % of A in 29 min. The mobile phase flow rate was always 200 µL/min. Electrospray ionization tandem MS

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(ESI-MS/MS) analysis were performed on a linear ion trap mass spectrometer of Thermo LTQ Velos (ThermoFisher Scientific, Reinach, Switzerland). MS/MS detection was operated in a positive ion mode with *m/z* 400-2000 scanning range and collision induced dissociation. LC-MS/MS data processing was performed with the Trans-Proteomic Pipeline (TPP), which is a set of integrated tools for MS/MS proteomics, developed at the Seattle Proteome Center of the Institute for Systems Biology in USA (http://tools.proteomecenter.org/wiki/index.php?title=Software:TPP). The peptide assignment was performed with X! Tandem as search engine and UniProtKB as database. Results validation was performed with the PeptideProphet of TPP.

2.8 Finite element simulations

The simulation of electrical properties of the three-well device was performed using commercial finite element package COMSOL Multiphysics (version 3.5a) operating under Ubuntu 8.04 environment installed on a four core MacPro with 2.66 GHz processing unit and 9.8 Gb of RAM. Details of geometrical arrangement of the model, mesh, boundary conditions and domain equations are summarized in the generated COMSOL report file (see Supporting Information, SI-1).

3.Results and discussion

3.1 Principle: electrophoretic separation with immobilized UNPG gels

The electrophoretic separation/desalting method using a polyacrylamide UNPG gel is based on the electrophoretic mobility difference of charged proteins or peptides loaded in the central well of the device as illustrated in scheme 2.

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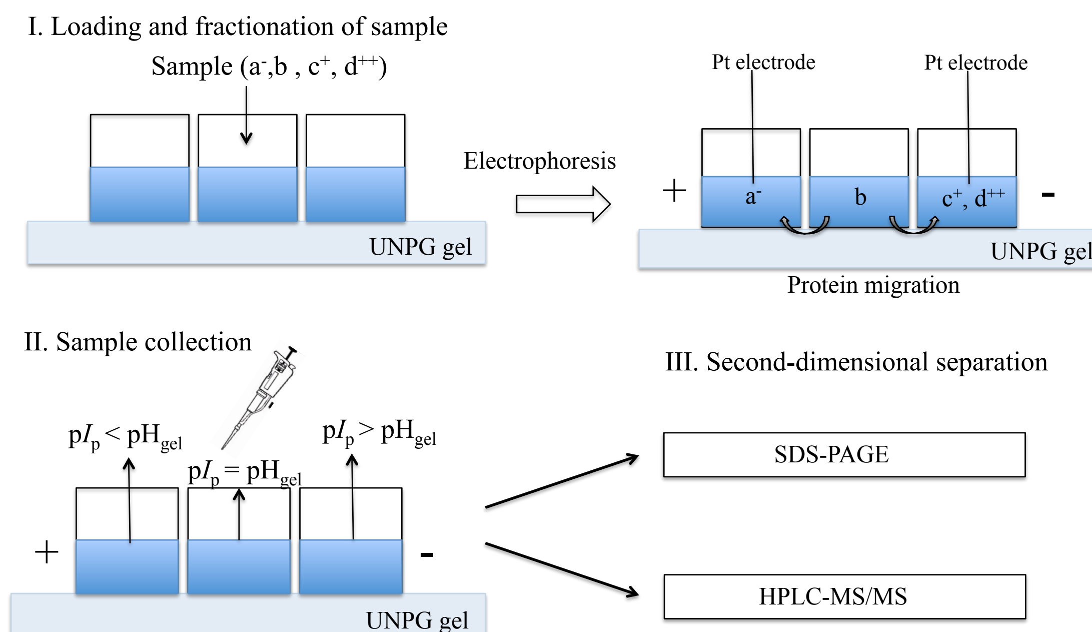

Scheme 2: Schematic representation of an UNPG gel electrophoretic separation method coupled with a second-dimension separation of HPLC-MS/MS or SDS-PAGE. pH_{gel}: the mean pH value of the UNPG gel; pI_p: the isoelectric point of proteins or peptides.

Upon application of an electric field, the proteins/peptides migrate in and over the polyacrylamide gel having a defined mean pH value. The proteins/peptides with p*I* higher than the mean pH of the gel will be positively charged (c^+, d^{++} in scheme 2) and therefore will migrate towards the cathode. Additionally, the proteins with larger net charge (d^{++}) or the smaller size will migrate faster than the others allowing a protein fractionation based on mobility. In contrast, the proteins/peptides (a^-) with a p*I* lower than the mean pH of the gel will be negatively charged and therefore migrate towards the anode. The proteins/peptides (b) with a p*I* equal or similar to the mean pH of the gel are globally neutral and then stay in the middle well without significant migration. Concentration gradient and diffusion can happen in the first and third well but not in the middle well because of isoelectric focusing. In addition to sample fractionation, the UNPG gel electrophoresis can also be used for sample desalting

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when the target proteins/peptides have a p*I* close to the mean pH of the gel, where samples will stay in the middle well and salts will migrate to the cathode or anode.

An important parameter for this methodology is the migration velocity (V_m) of the ampholytes determined both by their charge and their electrochemical mobility. V_m is classically defined as (eq.1):

$$V_m = -\frac{zF}{RT} D\nabla\phi = -zF\tilde{u}\nabla\phi = uE \qquad (1)$$

where z is the charge of the ampholyte at the mean pH value, F is the Faraday constant, R is the gas constant, T is the temperature, D is the diffusion coefficient of the ampholyte, $\tilde{u}$ is electrochemical mobility, u the electrophoretic mobility and E is the applied electric field [27].

Indeed, the migration of charged species during an electrophoretic separation follows the electric field according to the Nernst-Planck equation. In the present case, the separation is conducted in and above the gel in a three-well configuration. Figure 1 illustrates the distribution of electric field (E_n) demonstrating a significant inhomogeneity, especially near the well edges (see also a cross-section view on E_n distribution on Figure 1b). Even in the absence of an electrode in the central well where the sample is loaded, the electric field penetrates the liquid medium and further drives charged species into the gel, where the separation occurs according to the pH of the gel.

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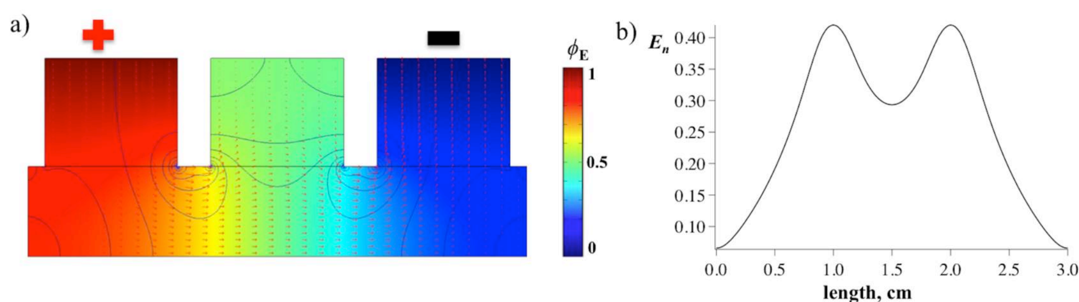

Figure 1: Simulated electrical properties in a three-well device. a) The overlay of color map illustrating electric potential distribution (**ϕ_E**) and contour plot depicting the isovalue lines for electric field (E_n) and b) the distribution of electric field along the central cross-section inside the UNPG gel. For simplicity, the electrodes are considered to be at the top surface of the outer reservoirs.

3.2 Purification on UNPG gels

Protein purification can be realized according to their charge with a very short UNPG gel strip. A mixture of four proteins with different p*I* values (*α*-casein (p*I* = 4.6), *α*-lactalbumin (p*I* = 5.02), cytochrome C (p*I* = 9.6) and myoglobin (p*I* = 7.0), 26 µg/mL each) was loaded in the middle well and separated on a UNPG gel (pH = 5-5.27). The applied potential difference between the electrodes was 150 V for 30 min and then increased up to 300 V for 3 h, the limiting current was 1 mA. Due to the fact that cytochrome C and myoglobin are coloured proteins, it was easy to visualize their migration inside the gel. At the end of the separation, the fractions were collected and analysed by MALDI-MS (see Figure 2). UV-Vis spectroscopy was employed to quantify the amount of proteins collected after fractionation. The results are summarized in Table1.

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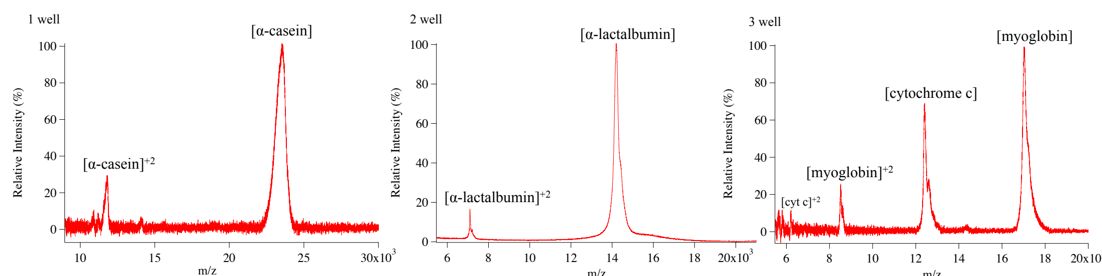

Figure 2. Mass spectra of the fractions from wells No. 1 (anodic side), 2 (middle well) and 3 (cathodic side) after electrophoresis performed with the three-well device over an UNPG gel (pH = 5-5.27). The samples were analyzed by MALDI-MS with sinapinic acid matrix.

According to the MS results shown in Figure 2 and the UV-Vis quantitative analysis summarized in table 1, all the proteins were completely fractionated according to their p*I* and reached the expected position (*i.e.* wells). For instance, *α*-lactalbumin was detected from the middle well, since the p*I* of this protein is within the pH range of the UNPG gel. Cytochrome C and myoglobin migrated to the third well (cathodic side), due to the fact that they were both positively charged. Similarly, the negatively charged *α*-casein migrated to the first well (anodic side). The protein recovery after separation was analysed by UV-Vis spectroscopy showing very high recovery in the central well used for purification. The recovery yields in the side wells are less due to the sample loss in the polyacrylamide gel and perhaps to the reaction occurring on the electrode. As depicted in Figure 1, the electric field near the well edges is low, which can lead to not efficient protein recovery. In order to increase the protein recovery in wells 1 and 3 longer experimental time is required.

This relatively high recovery resulted from the large solution to gel volume ratio; therefore increasing the amount of solution or decreasing the size of gel might lead to even higher protein recoveries. However, an increased solution-to-gel volume ratio can also lead to decreased buffer capacity of the system, and optimized ratio should be considered for both sample separation and recovery.

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Table 1. Protein recovery determined after electrophoretic separation in the three-well device over an UNPG gel (pH 5-5.27) using UV-spectroscopy.

Well No.	Identified proteins	Initial concentration of the proteins loaded	Amount of protein recovery	Protein recovery, %
1 (anodic side)	α-casein	26 μg/mL	13 μg/mL	62%
2 (middle)	α-lactalbumin	26 μg/mL	25 μg/mL	96%
3 (cathodic side)	myoglobin, cytochrome C	52 μg/mL*	35 μg/mL	67%

* This value corresponds to the summed concentration of the two present proteins.

3.3 Sample desalting on UNPG gels

In addition to separation, protein/peptide samples can also be rapidly and easily desalted using the three-well device on an UNPG gel. A gel strip with a pH gradient in the range of 5-5.27 was introduced into the device and 100 μL of DI water was added into each well. 10 μL of the solution of *β*-lactoglobulin A (1 mg/mL, p*I* = 5.1) containing 1 M NaCl was loaded into the middle well and the following experimental conditions were employed for the sample desalting: voltage applied = 100 V, limiting current = 1 mA. The pH of the gel was chosen to fit the requirements of the electrophoretic separation principle (see section 3.1) that means the proteins with p*I* equal or similar to the mean pH of the gel would not migrate from the well where they were loaded. After the electric field was applied along the three-well system, the Na^+ and Cl^- ions penetrated inside the gel and started to migrate to the electrodes with an opposite charge, while redox reaction happened on the electrodes. In 1 hour the fractions from each well were collected and analysed by MALDI-MS (see Figure 3).

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Since no protein was detected in the well No. 1 and 3, only the mass spectrum of the middle well, containing the β-lactoglobulin A is shown.

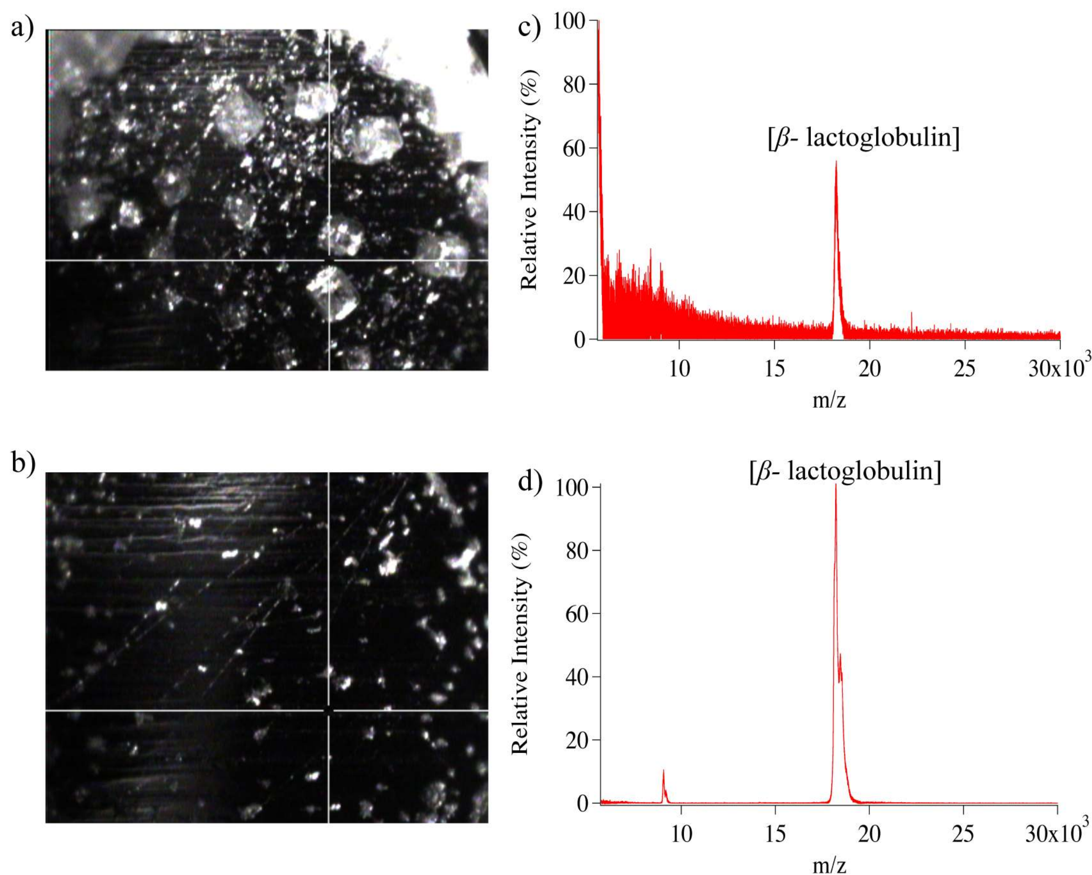

Figure 3: Optical image of the dried sample (1 mg/mL β-lactoglobulin A with 1 M NaCl) crystal on MALDI plate a) before and b) after the desalting by the three-well device with an UNPG gel (pH 5-5.27) and the obtained mass spectra of β-lactoglobulin A c) before and d) after the desalting. The samples were analyzed by MALDI-MS with sinapinic acid matrix.

Figure 3 illustrates the desalting effect of the three-well device using the UNPG gel. Figure 3a and 3b show the MALDI plate image containing the protein sample with 1 M NaCl before and after the desalting. As it can be observed the crystals of sample after the desalting are smaller in comparison to those ones observed before desalting, suggesting the removal of NaCl. The desalting effect is also demonstrated by the mass spectra of β-lactoglobulin A. Figure 3d presents a much better MS signal for the protein in comparison to the one in Figure 3c. Indeed, the laser intensity was increased by 20% to get the mass spectrum in Figure 3c compared to that in Figure

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3d. These results show that the three-well device with an UNPG gel can provide effective and rapid desalting prior to further analysis.

3.4 Purification of *E. coli* extract on UNPG gels

To determine the capability of purifying complex samples and to show that the electrophoretic separation with UNPG gels could be used as a fractionation strategy prior to other separation techniques, such as SDS-PAGE, a three-well fractionation of an *E. coli* protein extract spiked with α-casein and cytochrome C was performed using a gel with a pH gradient of 5-5.27. The extract from *E. coli* was prepared with a protein concentration approximately of 30 μg/mL and added in each well of the device. The sample was spiked with cytochrome C and α-casein (3 μg/mL each) in order to observe the effect of such a complex matrix on the proteins fractionation. The fractions collected after fractionation in a three-well device were further separated by SDS-PAGE electrophoresis. The gels were silver stained to visualize the separated proteins (see Supporting Information, SI-2). SDS-PAGE of the pure *E. coli* extract presents a high degree of protein band overlapping, which is raised from the complexity of this protein mixture. After the fractionation in a three-well device using the ultra narrow pH gradient gel, the spiked α-casein (p*I* = 4.6) and cytochrome C (p*I* = 9.6) were separated according to the charges developed at chosen pH range. Indeed, clear and intense protein bands were visualized and detected in the expected positions (*i.e.* wells) that corroborates the capabilities of the three-well setup as a powerful tool for protein purification even in the presence of complex matrices. The fraction taken from the middle well with the pH close to 5.13 shows the highest protein population. Moreover, a well-resolved SDS-PAGE for each well has been obtained compared to the whole *E. coli* extract. Thus, it confirms that the electrophoretic separation in

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UNPG gels provides fast and efficient fractionation of proteins and can be applied to the analyses of complex samples.

3.5 Purification of digested *E. coli* extract followed by LC-MS/MS analysis

The purification of peptides derived from the tryptic digest of an *E. coli* protein extract was also carried out on an UNPG gel. An aliquot of the protein digest (30 µL) was loaded in the middle well of the three-well device on a gel strip with a pH gradient (pH= 6-6.27, mean pH=6.15). Then, 200 µL of water solution was added on top of each well. During the fractionation, the potential increased from 150 to 400 V in 6 hours with a limiting current of 1 mA. After electrophoresis, the peptide mixture was separated into three fractions, which were present in the liquid phase and can be easily collected for further analysis, for instance, by LC-MS/MS analysis. Each fraction was firstly lyophilized and re-dissolved in 30 µL water, then injected into the LC for LC-MS/MS analysis. The LC-MS/MS data of each fraction was searched against *Escherichia coli* database from UniProtKB by X! Tandem search engine and validated by Trans-Proteomic Pipeline (TPP) tools. The identified peptides from each well by LC-MS/MS are listed in supporting information SI-3. The threshold of probability values from TPP was varied for different LC-MS/MS data while the error rate was constant as 5%.

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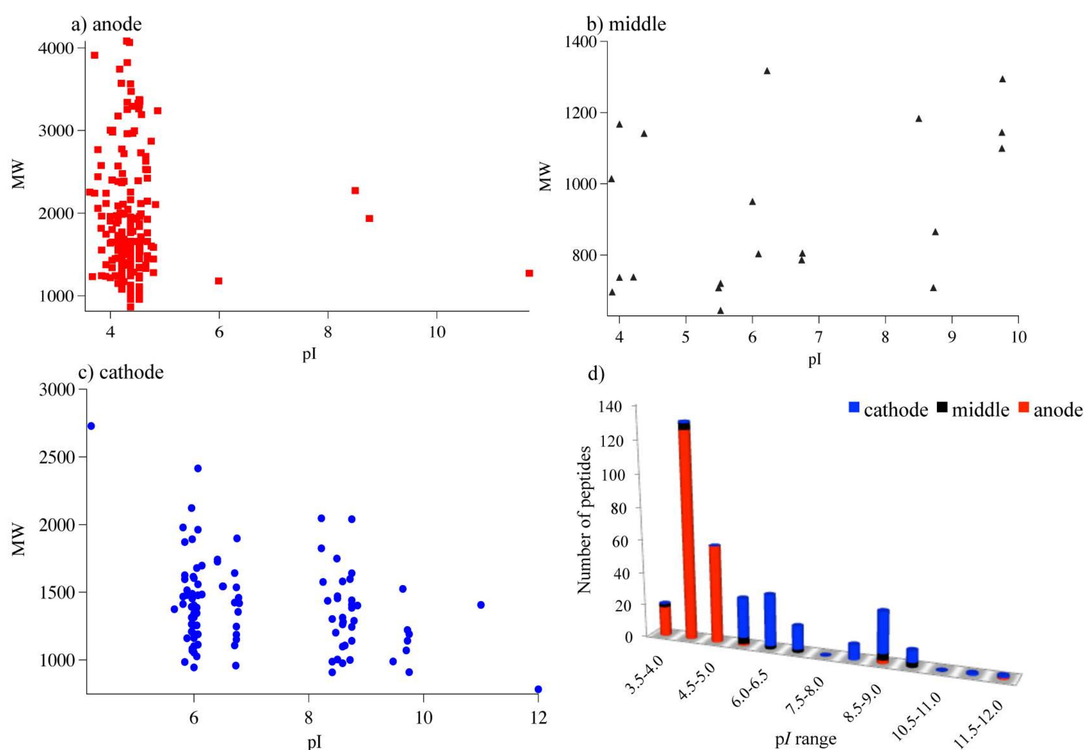

Figure 4: (a, b, c) Isoelectric points plotted as a function of molecular weights for the peptides identified from different wells. (d). Number of identified peptides after OFFGEL fractionation and LC-MS/MS analysis and their isoelectric point distribution.

In Figure 4, the p*I* values of the identified peptides from different fractions are compared. From the 1st well, 206 peptides were identified, where only 3 of them had a p*I* greater than 6. Indeed, the probability scores for these 3 peptides calculated by TPP are quite low, (*i.e.* 0.4233, 0.5485, 0.5839), and among the lowest ones, indicating that these 3 peptides are highly possible to be false positive identifications. 21 peptides were identified from the fraction of the middle well, where the sample was initially loaded. As shown in Figure 4, the p*I* of these 21 peptides is randomly distributed, indicating that a small fraction of the loaded peptides could not migrate into other wells. 113 peptides were identified from the third well by LC-MS/MS. Only one peptide does not match the p*I* range and actually with a low probability value from TPP as 0.735. In total 322 peptides with the correct p*I* were identified by

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LC-MS/MS after pre-fractionation step.

All in all, the present results demonstrate that the purification in the three-well device is efficient, and can be easily applied in the analysis of complex samples. Indeed, by optimizing the length of the UNPG-pH strip and its pH, specific and general protein (or peptide) sample fractionations can be obtained.

4. Concluding remarks

Protein fractionation by OFFGEL electrophoresis with an immobilized ultra narrow pH gradient gel in a three-well format has been presented. In comparison to previous OFFGEL electrophoresis reports[11], the present work aims at developing a fast and efficient preparative technique for isolation of proteins with specific p*I* from complex biological mixtures in a miniaturized format. The latter opens the possibility to use this preparative technique in combination with a following separation method to provide a powerful tool for proteomics. By tuning the mean pH of the UNPG gel, a specific target protein can be purified from a mixture of proteins in a short period of time. Fractionation in the three-well device drastically decreases the experimental time: it takes only 4 h to fractionate a complex mixture such as *E.coli* extract, while the normal OFFGEL electrophoresis takes up to 8 h. Furthermore, the pH gradient in UNPG gels is very low (0.27 units) and close to a constant pH media. To date, only few studies made by Stoyanov *et al.* [28, 29] can be found in the literature related to the

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use of uniform pH gels for protein fractionation. The proposed approach is low cost, easy to use and can be prepared in any laboratory.

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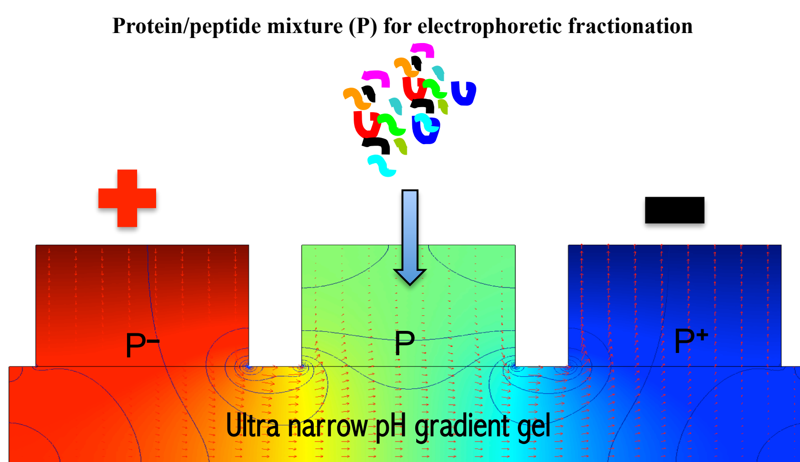

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