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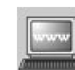
Research Article

Segmented field OFFGEL® electrophoresis

A multielectrode setup for protein OFFGEL electrophoresis that significantly improves protein separation efficiency has been developed. Here, the electric field is applied by segments between seven electrodes connected in series to six independent power supplies. The aim of this strategy is to distribute evenly the electric field along the multiwell system, and as a consequence to enhance electrophoresis in terms of separation time, resolution, and protein collection efficiency, while minimizing the overall potential difference and therefore the Joule heating. The performances were compared to a standard two-electrode setup for OFFGEL fractionation of a protein mixture, using UV-Vis spectroscopy for quantification and MALDI-MS for identification. The electrophoretic separation process was simulated, and optimized by solving the time-dependent Nernst–Planck differential equation.

Keywords:
Finite element simulations / Isoelectric focusing electrophoresis / Multielectrode setup / OFFGEL electrophoresis DOI 10.1002/elps.201200180



1 Introduction

Proteomics, the study of proteins involved in metabolic pathways and their interaction presents three main challenges to any analytical methodology [1, 2]: (i) high sensitivity, since protein concentrations vary over a wide dynamic range and since low abundant proteins play a relevant role in most biological processes [3, 4]; (ii) high-resolution power to separate, extract, and/or distinguish one or a group of proteins from a complex matrix; and (iii) a reasonably short experimental time. Improving any of these three points can certainly speed-up research and discovery in proteomics. 2D-PAGE has been the workhorse strategy for protein analysis during the last 30 years [5]. This approach is based on the orthogonal separation of proteins according to their p*I* in the first dimension and their molecular weight in the second dimension [6–8], allowing the separation up to thousands of proteins. However, 2D-PAGE suffers from a number of inherent limitations such as being time consuming, providing a low protein detection sensitivity after separation, or stemming from the comigration of proteins that introduces artifacts and makes the visualization of protein spots sometimes rather difficult. Moreover, proteins with extreme p*I* values (i.e. below 3 and above 10) or extreme molecular weight might be dismissed during the separation. Therefore, new strategies that alleviate the drawbacks presented by 2D-PAGE, or that become an alternative or complementary strategy for high-throughput protein analysis, are needed.

For instance, in 2002 Ros et al. introduced a new concept for protein separation so-called OFFGEL electrophoresis based on a preparative approach of IEF electrophoresis [9,10]. The idea behind is simply to place a frame containing several wells of identical dimensions over a hydrated IPG gel, in such a way that the pH gradient is divided between the wells, but without loosing connection through the whole gel. Then, an equivalent volume of buffered sample solution is loaded in each well (i.e. on top of the gel), and an electric field between two electrodes localized at the extreme sides of the frame is applied. As a consequence, the proteins present in the added solution are forced to migrate through the gel from one well to another according to their p*I*s. Once the proteins have been isoelectrofocused in one well (i.e. in the well where the pH is close to the protein p*I* value), the proteins are distributed between the gel below and the solution above, allowing the collection of protein fractions. Thanks to the different advantages that OFFGEL provides to proteomics (e.g. allowing an easier coupling of protein electrophoresis with sensitive protein detection techniques such as mass spectrometry) [11], it is now a widely used technique. A variety of samples have been analyzed by using OFFGEL electrophoresis, including *Escherichia coli* [12], human plasma [13], and eukaryotic samples [14]. Despite of this, protein electrophoresis

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Abbreviation: SA, sinapinic acid

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can be further improved by changing the paradigm of electric field application [15–17]. Usually, electrophoretic separations employ only two electrodes (i.e. located oppositely at each side of the gel) to apply an electric field to the separation media, which depending on its magnitude will affect drastically the protein separation efficiency (e.g. resolution power and experimental time), and also the protein collection efficiency in OFFGEL electrophoresis, for instance. Therefore, one strategy for improving protein electrophoretic separations and specially OFFGEL electrophoresis is to apply a higher and more homogeneous electric field across the whole system.

In the present study, a multielectrode setup is introduced for OFFGEL electrophoresis to provide a more efficient application of the electric field. The multielectrode setup consists of a lid with seven platinum electrodes placed over an OFFGEL frame with seven wells with, for demonstration, one electrode in each well. The separation of a mixture of five proteins by OFFGEL electrophoresis in both multielectrode and in two-electrode formats shows that the multielectrode setup yields a better protein separation resolution, as well as, a higher protein collection efficiency in a shorter time. Furthermore, analysis of an *E. coli* extract has demonstrated that the proposed methodology can successfully be applied to the fractionation of complex samples. Additionally, numerical simulations were performed to describe both electric field distribution and protein migration inside multi- and two-electrode OFFGEL separations corroborating the experimental results.

2 Materials and methods

2.1 Materials

IPG gels (Immobiline→ Drystrips, linear pH range from 3.0 to 10.0, 7 cm length) and silver and Coomassie blue staining kit protein visualization were purchased from Amersham Biosciences (Uppsala, Sweden). β-Lactoglobulin A and B (p*I* 5.1) from bovine milk, α-lactalbumin from bovine milk type I (p*I* 5.02), cytochrome C from horse heart (p*I* 9.6), myoglobin from horse skeletal muscle (p*I* 7.0, 7.4), and ribonuclease A (RNase A) from bovine pancreas (p*I* 9.45) were obtained from Sigma-Aldrich (Schnelldorf, Switzerland), as well as, sinapinic acid (SA), ACN TFA, methanol, and acetic acid of the purest grade (>99.9%). DI water was purified by an alpha Q Millipore system (Zug, Switzerland) and used in all aqueous solutions. The UV-Vis absorption spectra were obtained with a standard spectrophotometer (Perkin Elmer, model Lambda XLS+, Waltham, MA, USA) using quartz cells with a path length of 1 cm. A calibration BCA protein assay kit for determining protein concentrations was obtained from Thermo Scientific (Rockford, IL, USA). Common OFFGEL electrophoresis was performed with the Agilent 3100 OFFGEL fractionator (Waldbronn, Germany). For the multielectrode setup, six power supplies from Fug (Switzerland) were used.

2.2 OFFGEL electrophoresis using two- and multielectrode setups

Immobiline→ Drystrips with a 7 cm length and a pH gradient from 3 to 10 were used as gel media for OFFGEL electrophoresis, as it most suitably fit the length of the multielectrode device. In water after reswelling the IPG strip for 1 h, IPG strip was placed on a flat surface and then covered with the multiwell frame. All wells of the device were filled with DI water (ca. 150 μL), except the middle well (No. 4 in Fig. 1) where 150 μL of a solution containing β-lactoglobulin, α-lactalbumin, cytochrome C, myoglobin, and RNase A (the concentration of each protein was 26 μg/mL) was added. For OFFGEL electrophoresis with two electrodes, a commercial Agilent 3100 Fractionator was employed and the anode and cathode platinum electrodes were placed outside each opposite border of the plastic frame. Two electrodes were placed at the extremes of the multiwell frame in a two-electrode setup, using one of the power supplies from the multielectrode system. In the case of the multielectrode setup, one platinum electrode was placed inside each well in the OFFGEL frame allowing the use of seven electrodes powered individually, but connected in series with six power suppliers (see Fig. 1). The electrode placed in the first well of the multielectrode setup was connected to the ground (0 V) output of each power supply, acting as the more negative electrode in the whole system. The second electrode was connected to the high tension output of the first power supply and placed inside the second well, while the third and subsequent electrodes are connected to the high tension terminals of their respective power supply and placed in the

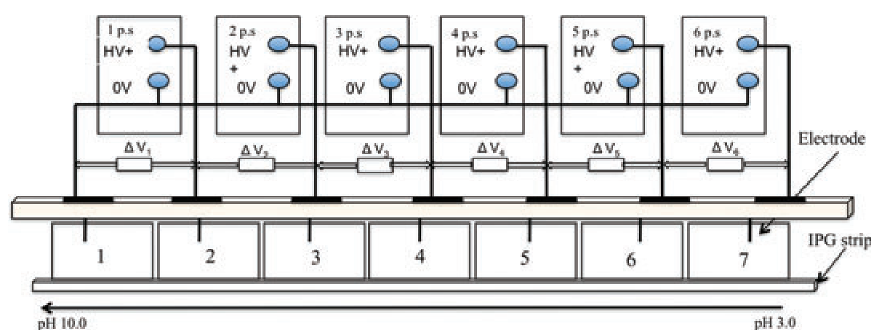

Figure 1. Schematic illustration of multielectrode setup, consisting of seven electrodes, which are connected in series with several power suppliers. The sample is placed in a chamber and covered with a lid with seven Pt electrodes.

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succeeding wells (see Fig. 1). Since all the Pt electrodes are connected in series, the potential difference along the system increases additively, providing a more positive electrode each time at the right side. In the present study, a progressive increment of the potential difference applied between neighboring electrodes was employed. Both the voltage and the current were monitored during all the experiments.

2.3 Soluble *E. coli* protein extract preparation

An overnight 200 mL culture of *E. coli* (strain DH5a) was collected by 10-min centrifugation at 5000 × *g* and 4°C. The cell pellet (0.6 g wet weight) was resuspended in 3 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA) and cells were disrupted by sonication (10 × 10 pulses of 1 s at 30 W). Cell debris were removed by 10 min centrifugation at 2000 × *g*. Ultracentrifugation (1 h at 100 000 × *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 MS analyses

The MS analyses were performed on a Microflex MALDI-TOF instrument (Bruker Daltonics) equipped with a 337 nm nitrogen laser. One microliter of the extracted protein solution was deposited on a steel target plate with 1 μL of SA matrix (15 mg/mL SA in 50% ACN, 0.1% TFA, and 49.9% water) and left to dry at room temperature.

2.5 Finite element simulations

Numerical simulations were performed using finite element package COMSOL Multiphysics (version 3.5a) installed on a Mac Pro with four 2.66 GHz central processing units and 9.8 GB of RAM operating under Linux Ubuntu 8.04 platform. The mass-transport of charged species (i.e. proteins) was simulated in a 2D computational domain of an OFFGEL setup (see Fig. 1) utilizing Nernst–Planck without Electroneutrality and Conductive media DC application modes from Chemical Engineering and AC/DC modules, respectively. Numerical resolution of corresponding partial differential equations was obtained using direct solver UMFPACK in transient mode with relative error tolerance 10^{-6} and taking time steps from solver. In order to reduce computational efforts, the solution was computed sequentially, that is, solving mass-transport equations on top of the stored solution containing electric field distribution. The mesh size was adjusted down to the value of 10 μm at the corners of the wells and electrode edges. For more detailed information about the numerical model, please see Supporting Information.

3 Results and discussion

3.1 Numerical simulation of protein migration and electric field distribution in the OFFGEL device

The validity of a multielectrode approach for OFFGEL fractionation was examined simulating electrical properties of the gel and modeling protein separation for the OFFGEL setup geometry shown in Fig. 1. We have used finite element simulations for numerical analysis of Nernst–Planck equation (Eq. (1) describing mass-transport of species i [18] as:

$$\frac{\partial c_i}{\partial t} + div\left[-D_i \nabla c_i - \frac{Z_i F}{RT} D_i c_i \nabla \phi\right] = 0 \qquad (1)$$

and Laplace equation Eq. (2) determining electric field distribution within the computational domain:

$$\nabla(-\sigma \nabla \varphi) = 0 \qquad (2)$$

Herein, c_i, t and ϕ are the dependent variables, specifying concentration, time, and electric potential, while D_i, Z_i, F, R, T, and σ are constant values denoting diffusion coefficient, charge number, Faraday constant, gas constant, temperature, and electrical conductivity of the medium, respectively (see details in Supporting Information).

The Immobiline gel in our model is considered as a conductive media with an electrical conductivity determined by free, noncovalently bond ions in the matrix [19], while the sample is assumed to have no influence on electrical properties of the domain. Therefore, the conductivity is given as the function of local pH value, determined by the contribution from H^+ and OH^- ions:

$$\sigma = \frac{F^2}{RT}\left(D_{H^+} 10^{(3-\mathrm{pH})} + D_{\mathrm{OH}^-} 10^{(-14+\mathrm{pH})}\right). \qquad (3)$$

Although the conductivity variations within the gel arising during protein focusing have been reported [20], the quantitative description of these experimental observations are still unavailable and therefore this simple approximation of σ has been used in the present work. This function (see Fig. 2A) has a pronounced minimum value close to neutral pH resulting in a very resistive region at pH 5–9 giving rise to a sharp local increase of the electric field. As a consequence, the overall driving force for a charged species remains at very low values along the gel except in the low conductivity zone formed in the middle as shown on Fig. 2B. However, the significant improvement of an electric field distribution is observed when the potential difference is applied in a segmented manner using multielectrode arrangement (see Fig. 2B). As compared to a standard two-electrode configuration, the driving electrophoretic force is spread along the gel in a uniform manner that comprises lows and peaks of comparable intensity. Most likely, such changes in electric field distributions arise from variations of current density under well compartments and in between them.

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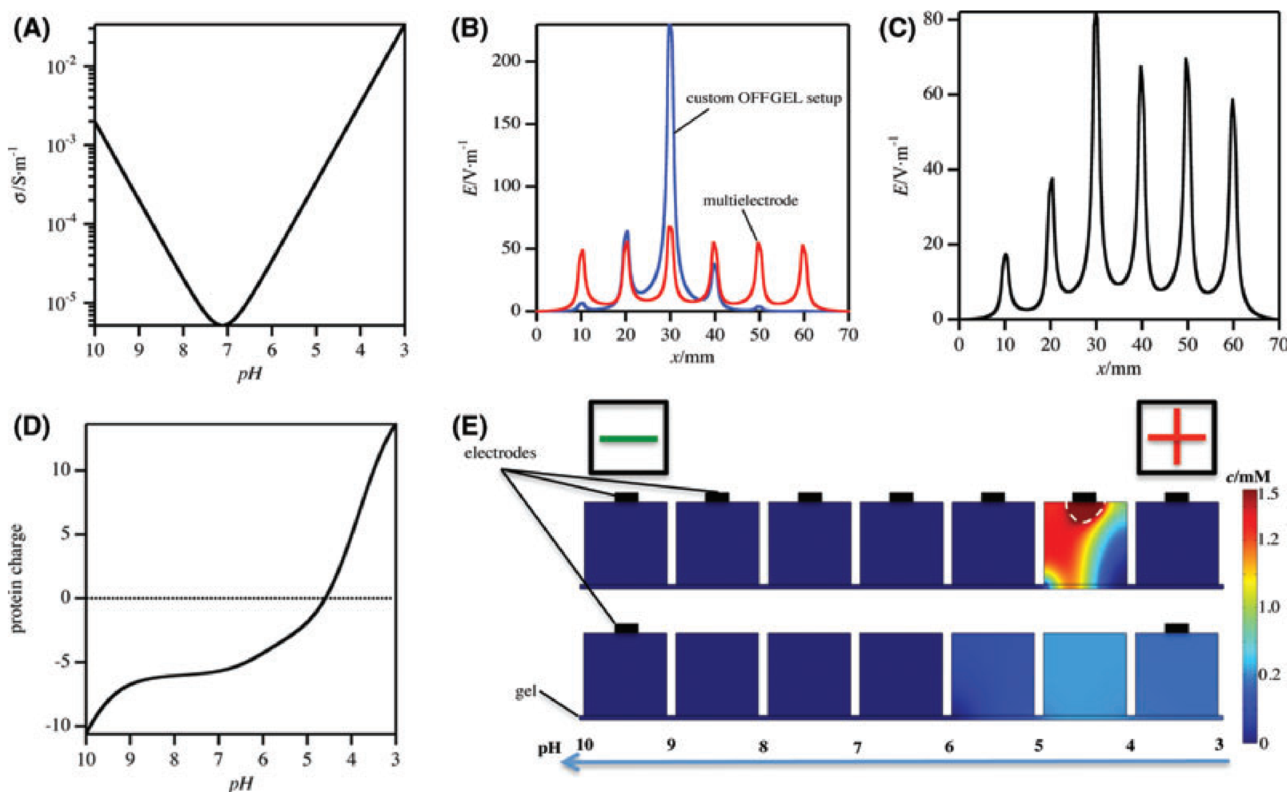

Figure 2. Finite element simulations of IEF using multielectrode approach. (A) Electrical conductivity (given in a logarithmic scale) in the immobilized pH gradient gel according to Eq. (3). Simulated electric field distributions along a gel medial cross-section for (B) a multielectrode setup with an optimized voltage program for general protein mixture separations, a two-electrode setup for common OFFGEL and (C) a multielectrode with an adjusted voltage program for the separation of proteins with acidic p*I*. (D) Calculated titration curve (overall charge versus pH) of α-lactalbumin. (E) Calculated protein concentration field for multi- (top) and two-electrode (bottom) arrangement (see scale bar on the right). The electrodes are schematically depicted on top of the well's compartments, "+" and "−" signs reveal the direction of the applied electric field; pH gradient scale is shown on the bottom.

Another advantage of a multielectrode system is the possibility to tune the potential program (i.e. the potential differences applied to each electrode) for electrophoretic separation of a particular sample mixture keeping overall potential difference constant. Figure 2C illustrates this feature exhibiting the simulated electric field distribution for the potential program adjusted for a separation of proteins with isoelectric points in acidic medium (see details in Supporting Information). As can be seen, the electric field lows and peaks reach higher values than in general case (Fig. 2B) in the acidic region of the immobilized pH gradient gel resulting in faster and more efficient separation of target proteins (vide infra).

In order to compare IEF efficiency for the common OFFGEL two-electrode arrangement with the multielectrode configuration, the isoelectric focusing of a test protein (α-lactalbumin) was simulated. Following previous numerical simulations for OFFGEL separations presented by Lam et al. [21], the net charge of a protein could be expressed, for instance, via Henderson–Hasselbach relation (Eq. (4)).

$$z(\mathrm{pH}) = -\sum_{i\varepsilon A^-} \frac{1}{1+\frac{10^{-\mathrm{pH}}}{K_i}} + \sum_{i\varepsilon A^+} \frac{1}{1+\frac{K_i}{10^{-\mathrm{pH}}}} \qquad (4)$$

where K_i is the ionization constant of the ionizable group of the corresponding amino acid, while A^- and A^+ denote the negative and positive charge of amino acids. The positive charge can be provided by histidine (H), arginine (R), lysine (K), and N-terminus. The negative charges are given by tyrosine (Y), aspartate (D), cysteine (C), glutamate (E), and by the charge of C-terminus [22]. It is important to notice that by employing this simple approach for the protein charge calculation some limitations can be introduced, since slightly variations from the use of macroscopic and microscopic dissociation constants can take place [23]. However, the aim of the present work is not to calculate precisely the p*I* of the proteins but to demonstrate the advantages that the multielectrode setup offers to OFFGEL electrophoresis. The simulated titration curve (net charge versus pH) of α-lactalbumin depicted in Fig. 2D has a well-defined isoelectric point (p*I* 5.02) and has a sufficient slope at p*I* value that was shown to be a key parameter in focusing dynamics; hence, a fast and efficient separation of this protein is expected.

Figure 2E displays the simulated concentration field of α-lactalbumin after IEF with both two- (bottom) and multielectrode (top) configurations. The differences in separation efficiency are clear: in a multiplex arrangement, the test

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protein is fairly focused mostly in one well with a collection efficiency of 97%, in contrast to the 45% achieve in two-electrode (and also standard OFFGEL) setup where α-lactalbumin was dispersed between three wells. Finally, the time required for completely focusing α-lactalbumin by using multielectrode setup was examined for three different voltage programs with an overall potential difference of 1 V (i.e. optimized potential program for general protein mixtures, adjusted voltage program for separation of proteins with acidic p*I*, and common two-electrode OFFGEL) (see details in Supporting Information). The fastest IEF process was achieved within 418 s for the adjusted voltage program for separation of proteins with acidic p*I*, while focusing with the optimized voltage program for general protein separations and with the common two-electrode setup took 1.26 and 62.17 times longer, respectively. As suggested by these numerical results, the multiplexed electrode approaches for IEF electrophoresis allows for faster, more efficient, and quantitative electrophoretic separations due to the enhanced electric field distribution. Additionally, when working with known protein mixtures that present mainly acidic p*I*s, for instance, or that due to their molecular weight will limit the required time for focusing all the proteins, a special voltage program can be designed.

3.2 OFFGEL electrophoresis with a multielectrode setup

A mixture of five different proteins (i.e. β-lactoglobulin, α-lactalbumin, cytochrome C, myoglobin, and RNase A, 26 μg/mL each) was separated by OFFGEL electrophoresis with the multielectrode setup shown in Fig. 1. For this protein separation, an IPG gel with a pH range from 3 to 10 was employed and covered with a multiwell system. The solution containing the protein mixture was added to the middle well (i.e. No. 4 in Fig. 1), since according to the numerical simulations a higher electric field is found in this region forcing a faster migration of the proteins at the first stages of the electrophoretic process. However, in principle the sample can be added to any well or to all of them and the same result should be obtained if the proper experimental time is used to achieve a complete separation. Then, the multielectrode setup was placed over the multiwell frame. The applied potential difference between the different neighboring electrodes (ΔV_i) can be seen in Fig. 1, where a clear progressive increment in ΔV_i was adjusted in such way that ΔV_i is always lower than ΔV_{i+1} by a value of 25 V. Since all the seven electrodes employed in the multielectrode setup were connected in series (see experimental part), the total potential difference applied through the whole system is equal to the sum of each ΔV_i and therefore equal to 675 V. The employed voltage program corresponds not to the optimized voltage program for a general protein mixture, but to the adjusted one for sample employed in the present work (vide supra). OFFGEL electrophoresis experiments with the multielectrode setup were performed under these conditions during a period of 3 h (see Table 1), after which the solution over the gel was collected and analyzed by MALDI-MS and UV-Vis spectroscopy.

Table 1. Experimental conditions employed during OFFGEL electrophoresis

	Voltage (V)	Current (μA)	Power (W)	Time (h)
Multielectrode setup	675	130[a)]	-	3
Two-electrode setup	675	110[a)]	-	3
Agilent fractionator 1	300	100[b)]	200	3
	1000			
	5000			
Agilent fractionator 2	5000	100[b)]	200	15

a) Initial measured values.
b) Defined limiting current values.

Figure 3 shows the mass spectra of fractions collected from wells No. 1, 3, 5, and 6 where all the added proteins where found after electrophoresis, since no protein presence was observed in the mass spectra of fractions from wells No. 2, 4, and 7 (see Supporting Information). According to the results shown in Fig. 3, all the proteins migrate completely from the middle well according to their p*I* and reached the expected position (i.e. well or wells) where the pH of the gel is close to their respective p*I* as seen in Table 2. For instance, α-lactalbumin was extracted from wells No. 5 and 6, as its p*I* is situated on the border between these two wells. The results shown in Fig. 3 demonstrate that the multielectrode setup offers to OFFGEL electrophoresis the possibility to achieve a complete protein separation in a short period of time (3 h), simply by applying a more homogeneous electric field as described with the numerical simulations. Moreover, the potential difference applied is lower in comparison to the one usually employed in “two-electrode” OFFGEL electrophoresis (vide infra).

In addition to the MALDI-MS analysis, UV-Vis spectroscopy was employed to quantify the amount of protein collected after electrophoresis and therefore establishes the effect of the multielectrode setup on the protein collection efficiency of OFFGEL. The results are summarized in Table 3. Basically, 100% recovery was obtained for RNase A and cytochrome C (well No. 1), Myoglobin (well No. 3), and β-lactoglobulin (well No. 5). α-Lactalbumin was 38% extracted from well No. 5 and 62% extracted from well No. 6. These results indicate that 100% of all the proteins after OFFGEL electrophoresis were found in the solution and no protein was present in the gel when a multielectrode setup was used. The latter was confirmed by the no visualization of proteins after Coomassie blue staining of the gels employed for OFFGEL electrophoresis when using the multielectrode setup (see Supporting Information).

In order to underline the advantages of the multielectrode setup, the separation of the same protein mixture (i.e. β-lactoglobulin, α-lactalbumin, cytochrome C, myoglobin, and RNase A, 26 μg/mL each) was performed using a two-electrode setup. All the experimental conditions employed

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Table 2. Table of common constituents in the protein sample and well number, where the separated proteins were detected by MALDI-MS

Protein	p*I*	MW (Da)	No. of well observed				No. of well expected
			Multielectrode	Two-electrode	Agilent fractionator 1	Agilent fractionator 2	
α-Lactalbumin	5.02	14 200	5, 6 (anodic side)	4, 5	5, 6	5, 6 (anodic side)	5, 6
β-Lactoglobulin	5.1	18 400	5	4, 5	5	5, 6	5
Myoglobin	7.0, 7.4	17 000	3	4	3, 2	3, 4	3
RNA A	9,45	13 600	1	1, 2, 4	1	1, 2	1
Cytochrome C	9.6	12 200	1 (cathode)	1, 4	Out	Out	1

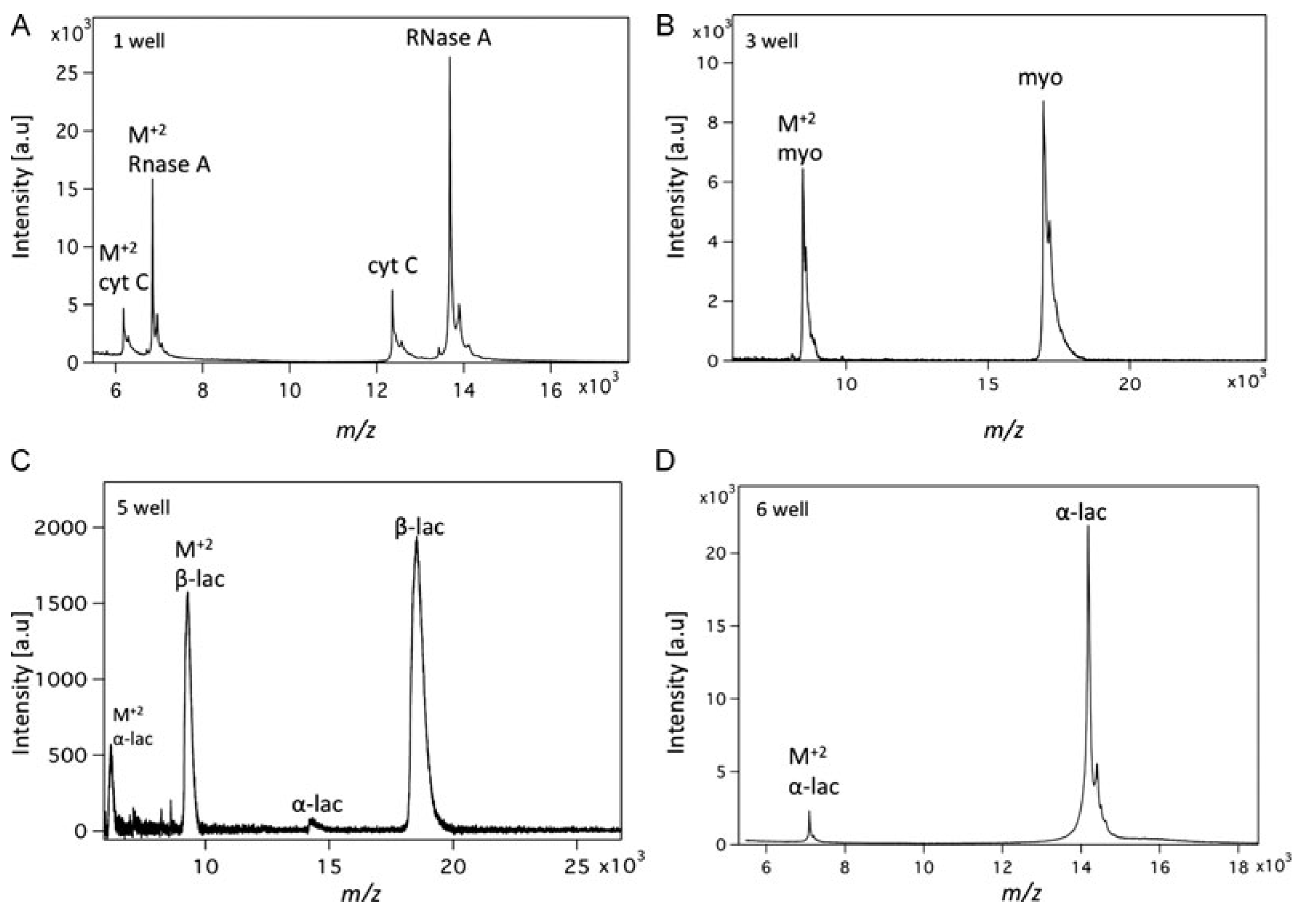

Figure 3. Mass spectra of fractions from wells No. 1 (A), 3 (B), 5 (C), and 6 (D) taken after OFFGEL electrophoresis performed with the multielectrode setup. The samples were deposited on a steel target plate with SA matrix.

with the multielectrode setup were also used for the OFFGEL electrophoresis with two electrodes (e.g. separation frame, length of the gel, amount and position of protein loaded, temperature) to have a reliable comparison between the multi- and two-electrode setups. Apart from the fact that the electric field was applied only between the two electrodes placed at both extremes of the multiwell frame, three different variations were tested regarding the potential program applied and the experimental conditions used (see Table 1).

As shown in Table 2, for the two-electrode system all the proteins were observed in the well where they were added (well No. 4), which suggests that in these conditions the separation was not efficient. This result is in good agreement with the UV experiments, where 48% of all the added proteins were recovered from well No. 4. After electrophoresis, the gel strip was stained using Coomassie Blue and the results showed the presence of a high amount of proteins inside the gel (see Supporting Information). These results show that under the employed conditions (e.g. potential applied 675 V), an experimental time of 3 h is not sufficient to properly separate all the proteins with a two-electrode setup. This is consistent with the numerical simulations, where a longer experimental

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Table 3. Protein recovery determined after OFFGEL separation using UV-spectroscopy

No. of well	Amount of protein recovery in μg/mL			
	Multi-electrode	Two-electrode	Agilent fractionator 1	Agilent fractionator 2
1	52 μg/mL[a)]	26 μg/mL[a)]	19 μg/mL	22 μg/mL
2		10 μg/mL	2 μg/mL	4 μg/mL
3	26 μg/mL		4 μg/mL	20 μg/mL
4		62 μg/mL[a)]		3 μg/mL
5	36 μg/mL[a)]	20 μg/mL[a)]	20 μg/mL[a)]	33 μg/mL[a)]
6	16 μg/mL		5 μg/mL	17 μg/mL[a)]

a) The value corresponds to the mixture of two or more proteins.

time is needed to complete the fractionation of the same protein sample under the application of the same overall voltage.

The same protein sample and gel were used in a subsequent experiment using an Agilent Fractionator 3100 (i.e. two-electrode setup) and an optimized potential step program (see Table 1, Agilent fractionator 1). Under these conditions, cytochrome C was not observed, as it migrated beyond the limits of the well setup to the cathode. This is due to the position of the electrodes beside the extreme wells rather than inside them and the extreme p*I* of cytochrome C (p*I* 9.6). However, a better protein separation was obtained thanks to the optimized potential program in comparison to the previous two-electrode experiment. Despite this, myoglobin was founded in two wells demonstrating that the separation efficiency achieved by using a two-electrode setup with a higher applied potential does not compete with that achieved by using the multielectrode setup at a lower applied potential. The concentration of proteins after the separation was measured using UV-Vis spectroscopy and summarized in Table 3. As it is clearly seen that the collection efficiencies obtained after separation are quite low compared with the collection efficiencies in the case of the multielectrode setup. A high amount of proteins were observed on the gel strip after the staining procedure.

Finally, an OFFGEL standard method (recommended by Agilent Technologies in the operator manual, Agilent fractionator 2 in Tables 1 and 2) was selected to perform the protein separation of the same sample. The time employed for such experiments was 15 h. However, β-lactoglobulin was still detected in several wells demonstrating not very effective resolution of the method. As in the previous experiment, cytochrome C migrates out of multiwell device due to the long experimental time employed. Analysis of the gel strip after the OFFGEL electrophoresis using a Coomassie Blue staining showed the presence of a high amount of proteins inside the gel. The concentrations of recovered proteins are higher in a comparison with that obtained under the method of Agilent fractionator 1, but still not as good as the one obtained with the multielectrode setup.

3.3 OFFGEL fractionation of *E. coli* extract

To corroborate the capabilities of the multielectrode setup for fractionation of complex protein samples, OFFGEL electrophoresis of an *E. coli* extract were performed with a multi- and a two-electrode (Agilent OFFGEL fractionator) systems. The experimental conditions employed in both experiments were exactly the same (e.g. separation frame, length of the gel, temperature), except the experimental time and difference of potential applied in each case. The extract from *E. coli* was prepared in 0.5% IPG buffer with a protein concentration approximately of 40 μg/mL and added in each well of the multiwell device. The sample was spiked with 10 μg/mL solution of cytochrome C in order to observe the effect of a complex matrix such as *E. coli* on the separation of this model protein. The potential program applied with the multielectrode setup corresponds to the one shown previously in Table 2. In the case of the two-electrode setup, the standard method (Agilent fractionator 2) was used. After OFFGEL electrophoresis with both methodologies, soluble fractions from each well were collected and further separated by SDS-PAGE to obtain a complete 2D map (i.e. 2D-PAGE) of the present proteins in the *E. coli* extract. The SDS-PAGE experiments were run for 2 h with a starting applied potential of 60 V, after 1 h the applied potential was increased up to 100 V. After SDS-PAGE, the gels were silver stained under the same conditions (i.e. same staining kit and protocol) to first visualize the separated proteins and second to compare the protein separation efficiency between the two employed methodologies. Figure 4 shows the results obtained after silver staining of the two electrophoretic separations, where each track corresponds to the separation

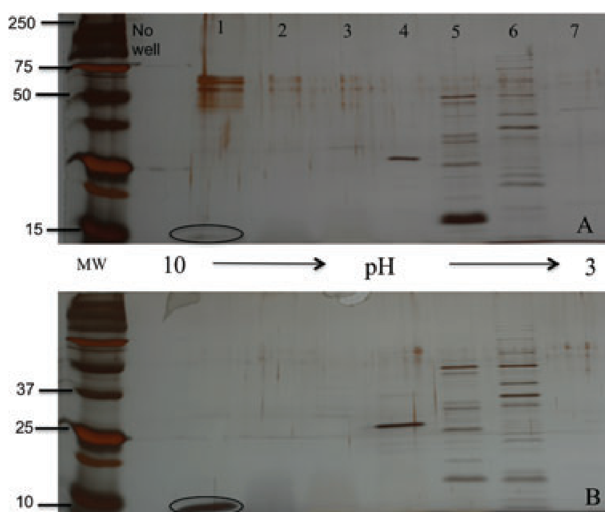

Figure 4. Silver stained 2D gels of an *E. coli* protein extract after fractionation on an IEF gel with a pH range between 3 and 10 (A) by Agilent OFFGEL Fractionator (15 h) and (B) by a multielectrode setup (3 h). Each well of SDS-PAGE matches the protein fractions collected from a respective well after OFFGEL separation. Prestained molecular weight protein markers appear on left side of the gel and are as follows from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa.

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of the proteins collected from each well. As it can be seen, the protein separation made with the multielectrode setup shows a lower number of protein bands, but with a higher intensity. The latter is most likely due to a better separation and focusing of the proteins present in the *E. coli* extract. For instance in Fig. 4A, a considerable protein population is observed in the region of proteins with high molecular weight and basic p*I*s, which is in disagreement with the literature [24], as there should not be a high amount of proteins with a basic p*I* values in the analyzed *E. coli* extract. The fact that the separation performed with the multielectrode setup does not show such protein population (see Fig. 4B) demonstrates that the present methodology can be applied to the analysis of complex samples avoiding the introduction of artifacts into the protein separations. Moreover, it can be seen that the spiked cytochrome C has been separated in both cases, but only with the multielectrode setup a clear and intense protein band is visualized that corroborates the capabilities of the multielectrode setup as a powerful tool for protein separation even in the presence of complex matrices. This result confirms that a higher protein separation and a higher protein collection efficiency is achieved by using the multielectrode setup concept, which additionally can drastically shortened the experimental time required for a complete protein separation.

4 Concluding remarks

A multielectrode concept for improving OFFGEL electrophoresis based on the application of a more homogeneous electric field has been developed. Numerical simulations showed that the applied electric field with two-electrode devices is not uniform along the separation media (e.g. IPG gel) and therefore long separation times are required for a complete protein separation. In contrast, the multielectrode setup provides a more uniform electric field offering faster and better protein IEF separations. To demonstrate experimentally this concept, OFFGEL electrophoresis were performed for the separation of ideal and complex samples with a multielectrode setup and compared with usual two-electrode OFFGEL electrophoresis. These results confirm that not only higher protein separation, but also better protein collection efficiency is achieved in a shorter time by using the multielectrode setup for OFFGEL experiments. We expect that the multielectrode concept will shorten and refine proteome research based on gel electrophoresis.

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