Gradient-free determination of isoelectric points of proteins on chip

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The isoelectric point (pI) of a protein is a key characteristic that influences its overall electrostatic behaviour. The majority of conventional methods for the determination of the isoelectric point of a molecule rely on the use of spatial gradients in pH, although significant practical challenges are associated with such techniques, notably the difficulty in generating a stable and well controlled pH gradient. Here, we introduce a gradient-free approach, exploiting a microfluidic platform which allows us to perform rapid pH change on chip and probe the electrophoretic mobility of species in a controlled field. In particular, in this approach, the pH of the electrolyte solution is modulated in time rather than in space, as in the case for conventional determinations of the isoelectric point. To demonstrate the general approachability of this platform, we have measured the isoelectric points of representative set of seven proteins, bovine serum albumin, β-lactoglobulin, ribonuclease A, ovalbumin, human transferrin, ubiquitin and myoglobin in microlitre sample volumes. The ability to conduct measurements in free solution thus provides the basis for the rapid determination of isoelectric points of proteins under a wide variety of solution conditions and in small volumes.

1 Introduction

The isoelectric point (pI) is the value of the pH of a solution at which amphoteric molecules, such as proteins, have a vanishing net charge and hence no effective electrophoretic mobility. Knowledge of the value of pI of a protein is particularly useful for separation and purification, and for the characterisation of key physicochemical properties, such as surface charge and solubility, which are typically lower at values of the pH in the vicinity of the pI.

A range of conventional methods can be used to determine the isoelectric point of proteins, including isoelectric precipitation, techniques based on ion-exchange adsorption, zeta potential measurements, or a recently developed nanoparticle-based approach. However, the predominant way to separate proteins and investigate their pI is isoelectric focusing (IEF), in which a pH gradient is generated across a chamber when an electric field is applied. Proteins that are then introduced into the system migrate in the gradient until they reach their pI and start to precipitate. A significant challenge underlying this approach is the requirement to generate and maintain a spatially stable pH gradient of a known and well controlled magnitude. One possible way to characterise such pH gradients is the use of a set of molecular markers, such as fluorescently labelled peptides or proteins, of known pI to which the pI investigated sample can be added. This general principle of isoelectric focusing (IEF) can be applied using capillaries, gel slabs, IEF or macro scale Free Flow Isoelectric Focusing (FFIEF).

Microfluidic platforms are powerful technologies offering many advantages over bulk measurements, including high resolution, well controlled experimental conditions, low analyte volume requirement, short analysis time and low cost. One currently used micro-scale approach for determining pI is micro-fluidic Free-Flow Isoelectric Focusing (μFFIEF), which is based on the same principle as IEF with a pH gradient in the direction perpendicular to the advective flow in a microchannel. The advantages offered by μFFIEF are rapid focusing of protein mixtures and protein complexes, accurate control of laminar flow and negligible Joule heating, although the quantitative control of maintenance and characteristic of spatial pH gradients remains a limitation. IEF devices with spatial pH gradients have been created simply by mixing acidic and basic buffers, but this approach can be challenging to implement, because of difficulties in ensuring the stability of the gradient. A significant improvement...
in this context is the invention of a “natural” pH gradient,\(^2^9\) generated by the simultaneous use of several carrier ampholytes (CAs), amphoteric compounds with pH values close to each other. These compounds undergo a differential drift in an applied electric field and form a gradient,\(^3^0\) that is nonlinear.\(^3^1\) Moreover, CAs and often markers can also interact with the protein under investigation and thus affect its pl.\(^1^6,3^1\) Another potential drawback of CAs-gradients is the cathodic drift that can be due to the electromigration of CAs, electrolyte diffusion or electroosmosis,\(^3^0,3^2\) and, moreover, the CAs approach is most reliable typically for samples under low salt concentration conditions.\(^3^2\) Despite the limitations of CAs, including their relatively high cost,\(^3^3\) this µFFE gradient-model is constantly being improved\(^1^4,2^7\) and is now commonly used for protein separation as well as the determination of pl values.

An alternative to the CAs approach is the use of an immobilized spatial pH gradient (IPG), in which monomeric buffering species are covalently linked to a polyacrylamide gel, this overcomes many of the drawbacks of the CAs approach, and many successful examples have been demonstrated.\(^3^2,3^4\) This approach however, requires the casting of polyacrylamide gel with a spatial pH gradient, a process which can be challenging to automate and standardise to achieve a highly controlled linear gradient.\(^3^5\) In addition to the gradient generated by carrier ampholytes or IPGs, there are less common CAs-free methods, such as thermally generated gradients,\(^3^6,3^7\) electrolysis-induced pH gradients\(^3^6,3^8\) and the promising technique of gradients created using diffusion potentials.\(^3^9\)

Although micron scale approaches for pl determination have great potential, and present many advantages over bulk techniques, the control of spatial pH gradients remains a challenge. To overcome this limitation, we introduce here a gradient-free method based on microfluidic Free-Flow Electrophoresis (µFFE) to determine the pl of a protein. We developed an approach that exploits temporal rather than spatial pH gradients in combination with µFFE as a tool to determine the isoelectric point of proteins. In this approach, the separation of a mixture of molecules is achieved by exploiting the difference in their charge-to-size ratios, which allows us to control the movement of molecules along the main advective direction of a separation chamber while applying an electric field perpendicular to this direction. The positively charged molecules deflect towards the negative cathode, whereas negatively charged species migrate towards the positive anode.\(^4^0\)

In order to measure the deflection in the electric fields, the proteins studied here were initially labelled with ortho-pthalaldehyde (OPA) and imaged using an inverted fluorescence microscope. The analyte solution was introduced into the chip under native conditions (2 mM phosphate buffer pH 7.7) and the pH was only changed once the sample was within the microfluidic device (Fig. 1a); this process avoided the need to modulate the pH of the labelled protein off the chip prior its introduction to the device. To quantify accurately the electrophoretic mobility of the proteins under an applied electric field, we also monitored the DC current through the device, that together with the knowledge of the conductance of the buffer solution, allowed the magnitude of the electric field to be quantified.\(^4^1\) We then tested a series of buffers with different pH values to determine the pl from the dependence of the protein mobility on the pH, this approach has been extensively used in the macro scale.\(^1^2,4^2,4^3\) We also investigated the influence of Tween-20, a biocompatible surfactant that aids in maintaining solubility of the protein samples close to their pl values, and also showed the effect of OPA labelling, on the isoelectric point value of proteins.

2 Materials and methods

2.1 Preparation of protein samples and buffer systems

Bovine serum albumin (BSA), β-lactoglobulin (BLG), ribonuclease A (RNase A), ovalbumin (OVA), human serum transferrin (TF), ubiquitin (UB), myoglobin (MYO), ortho-pthalaldehyde, β-mercaptoethanol (BME), mono- and di-basic sodium phosphate, phosphoric acid, sodium hydroxide and the surfactant Tween-20 were obtained from Sigma-Aldrich (Gillingham, UK) and used without further purification.

A dye stock solution was prepared by dissolving OPA and BME in a ratio of 1:1.5 in 50 mM phosphate buffer at pH 8, to obtain final concentrations of 60 mM OPA and 90 mM BME. To prepare the analyte samples 2 mg of protein were dissolved in 990 μL of 2 mM phosphate buffer pH 7.7 (containing 0 v/v%, 0.01 v/v%, 0.1 v/v%, 1 v/v%, 2 v/v% of Tween-20) and then 10 μL of the dye stock solution were added, to give a final protein
2.2 Microfluidic device fabrication

A device master was first fabricated using standard UV lithography techniques. Briefly, a silicon wafer was spin coated with a 25 µm layer of negative photoresist SU8-3025 (Microchem, Massachusetts, USA) and baked on a hotplate at 98 °C for 5 min. The wafer was then exposed to UV light through the photomask (15 s, 16 mW) and post-baked at 98 °C for 5 min and unexposed photoresist was removed by developing with propylene glycol mono methyl ether acetate (PGMEA) (Sigma-Aldrich) (Gillingham, UK).

Devices for the measurements of pI5 were replicated from the master using a mixture of 1:10 curing agent : polydimethylsiloxane elastomer (PDMS) (Sylgard 184, Dow Corning), degassed in a vacuum desiccator for 30 min and baked at 65 °C for 90 min. Each PDMS device was provided with fluidic access by punching inlets/outlet with a 0.75 mm biopsy puncher (Harris UniCore), washed with isopropanol (IPA) (Sigma Aldrich) and dried with nitrogen gas. The device was then chemically bonded to a microscope glass slide (Thermo Scientific) by activating both surfaces with an oxygen plasma (10 s, 40 mW) (Electronic Diener Scientific) plastic syringe to avoid a significant increase in pressure which can damage the electrodes.

To quantify the deflection of the beam of the protein solution, the optical images acquired were analysed with ImageJ and custom software written in Python. After a series of the deflection measurements for all buffers were recorded with the different pH values, the device cell constant was calibrated for a KCl conductivity standard (Sigma-Aldrich) of 500 µS cm⁻¹ and for all tested buffers. Using a conductivity standard and the calculated conductance G, the cell constant K for each device was obtained as K = σ/G. The average cell constant of all the data presented in this paper was (49.6 ± 3.2 cm⁻¹). Recording the current, i, during the deflection measurements and from the conductance value obtained for the specific buffers, the effective voltage was calculated from V_e = i/G. Knowing V_e and the distance between the electrodes (w = 600 µm) allowed the electric field, E = V_e/w, to be calculated. The electrophoretic velocity v was determined by dividing the deflection δ by the residence time t, v = δ/t. In low aspect ratio channels the residence time at the centre of the channel can be approximated as the ratio of the device volume to the flow rate. Finally, to obtain the electrophoretic mobility µ, the migration velocity was divided by the electric field µ = v/E.

2.4 Quantitative labelling analysis

The proteins were labelled with a fluorogenic dye which reacts with protein primary amine groups (lysine residues, and the N-terminus). To determine the extent of modification with OPA, and any effect that this may have on the protein pI, we compared the fluorescence intensity for proteins labelled under our conditions (pre-labelled) to model substrates (BSA, BLG, UB and the amino acid lysine (Sigma-Aldrich)), which were labelled under the conditions we had previously shown permitted quantitative modification of all reactive groups, and thus determination of protein concentration from the fluorescence intensity.
The labelling solution was prepared by mixing 16 mg (12 mM) of OPA with 4 mL of 500 mM carbonate buffer pH 10.5 and 4 mL of water. As a next step 12.3 µL (18 mM) BME and 2 mL of 20 w/v% of the anionic surfactant sodium dodecyl sulphate (SDS) (Sigma-Aldrich) were then added to the solution. The vial containing the solution of the labelling cocktail was then wrapped in aluminium foil, left in the oven for 15 min at 65 °C, and filtered using MilliQ 0.22 µm filter (Merck Millipore).

Serial dilutions of BSA, BLG, UB, and lysine varying in concentration between 30 µM and 1.8 nM were prepared in 2 mM phosphate buffer solution at pH 8. Then, 2 mg mL⁻¹ of OPA-pre-labelled BSA, BLG and UB samples were prepared and diluted to 0.3 µM, and all the samples, and a background solution consisting of buffer and OPA, were placed in the wells of a half-area non-protein binding microplate (#3881, Corning) in triplicate. Using a CLARIOstar microplate reader (BMG LabTech), 50 µL of labelling cocktail was injected (430 µL s⁻¹) into each well containing 50 µL of unlabelled BSA, BLG, UB and lysine solutions to quantitatively label the samples, and the fluorescence intensity of each sample measured 3 s after dye injection. The fluorescence intensities of the pre-labelled BSA, BLG and UB solutions, corresponding to the “partially labelled” conditions used in this paper, were also measured, and the measurement for BSA repeated after 2 h (the maximum amount of time which passed during sample preparation and measurement for the experiments reported in this paper). The results were analysed using a custom Matlab program.

3 Results and discussion

3.1 Determination of the isoelectric point of BSA

BSA is a well characterized protein commonly used as a biophysical model system; we first set out to determine its isoelectric point using our gradient free µFFE approach. To do this, we recorded the deflection of streams of analyte co-flowing between streams of buffer of varying pH. The measurements were made using 2 mM phosphate buffers with different pH values (pH 3, 3.3, 3.8, 5.6, 6.6, 7.7). When the DC voltage (at 0.5 V intervals from 0 V to 4 V) was applied across the buffers with pH values higher than the pI of protein, the BSA molecules were negatively charged, and observed to move towards the anode (Fig. 1b, top, middle). In contrast, when buffers with pH values lower than the protein pI were used, the BSA molecules were positively charged and moved towards the cathode (Fig. 1b, bottom).

The deflection was measured four times in each device for each pH value, and was found to increase monotonously for enhancing voltages (Fig. 2a). When the maximum voltage (4 V) used in this study was reached, the highest average deflection (−17.8 ± 1.7 µm) was observed for solutions pH 7.7, while a lower average value (12.4 ± 2.3 µm) was observed for a solution with pH 3.0, which is closer to the pI for BSA. Moreover, deflection data for pH 7.7 were more reproducible than for pH 3. This fact can be explained by observing that BSA was always labelled with OPA off-chip in a buffer at pH 7.7. Hence during on-chip measurements at low pH (3, 3.3, 3.8) the buffer transitioned from pH 7.7 to lower pH at the nozzle, promoting BSA precipitation at pH = pI and the formation of a deposit, which has the propensity to affect the uniformity of the protein beam in the main channel. We explored two different paths (in this section, and in Section 3.2) to address this limiting factor both by modulating the flow rates and through the presence of a bio-compatible surfactant.

The flow rate during the deflection measurements was typically maintained at 250 µL h⁻¹; however, to avoid precipitation at the nozzle, for the low pH solutions, a higher flow rate (500 µL h⁻¹) was applied for the first 4 min of the equilibration step and afterwards decreased to 250 µL h⁻¹ and this procedure led to accurate measurements of the deflection. The beam deflection was found to correlate linearly with the simultaneously measured current (Fig. 2b); similarly, the electrophoretic velocity correlates linearly with the electric field (Fig. 2c) for pH 3.0 and 7.7 solutions and for all the other investigated pH values.

In order to estimate the protein isoelectric point several methods have been previously reported. Here we used linear interpolation. By using this approach, only the two points closest to the pI play a crucial role in analysing its value, although for a protein with unknown pI it is necessary to test a range of pH values. In our case values for the isoelectric point of BSA have been reported, so appropriate buffers were chosen accordingly. The pI obtained, in this way, which was based on
the average for three devices, was 4.6 ± 0.1, and was the
intersection of the curve and the $\mu = 0$ line (Fig. 2d). This result
is consistent with the values previously reported in the literature
(4.5–5.1).50–53 This confirms that our technique provides an
accurate measurement of the protein’s pI.

3.2 pI measurements in the presence of a bio-compatible
surfactant

To enhance the solubility of proteins close to their pI value and
eliminate adhesion problems at pH values close to the pI
(Fig. 3a–c), we explored the addition of a Tween-20 surfactant
to the BSA solutions during the labelling reaction at four different
centrations, 0.01, 0.1, 1 and 2 v/v% (Fig. 3e–h). This non-ionic
and non-toxic surfactant is highly bio-compatible, preserves the
native protein structure,54–56 increases protein solubility and
reduces protein adsorption on the device surfaces.7,57

It is commonly assumed that the presence of Tween-20 does
not affect the pIs of proteins.57 Here, with the microfluidic
measurement device, we set out to test this hypothesis. Addition
of 0.01 v/v% Tween-20 to the BSA solution (Fig. 3e) resulted in a
pI value (4.5 ± 0.1), that was essentially identical to that
determined for the measurement without Tween-20 (Fig. 2d).
Nevertheless, this amount of Tween-20 was not sufficient to
avoid the formation of any deposits in the solution. The
concentration of the surfactant was increased to 0.1 v/v%, a value commonly used in the literature,23,26,27 and with this
amount of Tween-20, we did not observe the formation of any
deposits of protein at any pH value (Fig. 3d). We measured an
isoelectric point of 4.7 ± 0.1 (Fig. 3f) in accordance with the
value obtained without the surfactant. This demonstrates that
0.1 v/v% Tween-20 concentration does not affect the protein pI
measurement while avoiding the formation of protein deposit.
At the concentration of Tween-20 of 1 v/v% (Fig. 3g) and 2 v/v%
(Fig. 3h, inset) the pI changed significantly (5.0 ± 0.1 (Fig. 3f)
and (5.1 ± 0.2) respectively in comparison to the one obtained without the
surfactant (Fig. 2d). The results, as shown in Fig. 3h, indicate
that by increasing the amount of the surfactant Tween-20 in the
analyte solution, a slow increase in pI was observed. These data
indicate, therefore, that the higher concentration of Tween-20
may affect the pI of protein. A possible reason behind this
observation could be related to the critical micelle concentration
of Tween-20 (CMC = 0.007–0.05 v/v%)55,56 above which surfactant
micelles formed,59 and thus non-ionic micelles interact hydro-
phobically with protein ions in the applied electric field can
induce changes in the electrophoretic mobilities57,60 or modulate
the deprotonation free energies of acidic residues.

3.3 Influence of labelling on pI determination

To permit visualisation with a standard epifluorescence optical
microscopy setup, we labelled BSA with a fluorogenic dye, ortho-
phthalaldehyde, which becomes fluorescent on reaction with
primary amine groups exposed on the protein surface, such as
lysine residues. However, this procedure removes positive
charges below the pK_a of lysine (10.5 ± 1.1).61 Therefore, at
pH values below the pK_a of lysine, the protein is more negatively
charged, and the value of the pI can be lowered.

Fig. 3 (a) The nozzle of the microfluidic device was blocked by the
precipitation of BSA at pH 3.0 and at a flow rate of 250 μL h^{-1}. (b) Formation
of a BSA deposit close to the nozzle at pH 3.0 and an initial flow rate 500 μL h^{-1} 2 min
after the start of the measurement. (c) Formation of a BSA deposit under the same conditions
as in (b), but after 4 min from the start of the measurement. The flow rate was then reduced to
250 μL h^{-1} and the system was equilibrated for 4 min to allow accurate measurement of the
beam deflection. In (a–c) Tween-20 was not added. (d) The stream of BSA flowing through the
microfluidic nozzle at a flow rate of 250 μL h^{-1}, but with the addition of 0.1 v/v% of Tween-20
to the solution. The mobility as a function of pH for three different devices for (e) 0.01 v/v%,
(f) 0.1 v/v%, (g) 1 v/v% of Tween-20 added to the protein solution during the preparation.
The data points and error bars show the mean and standard deviations obtained by averaging four
different measurements of the mobilities obtained for each pH and device. (h) Dependence of pI on
Tween-20 concentration (v/v%). The data points and error bars show the mean and standard
deviations obtained by averaging the pI measurements obtained for three
devices. The red square represents the mean of measurements performed without surfactant addition. The black line is a logarithmic fitting to the
data. The inset presents the mobility as a function of pH for three different
devices, measured at pH 3.8 and 5.6 and with 2 v/v% of Tween-20 addition to the protein solution.

We first determined the number of lysine residues that have
reacted with the OPA, under the conditions used in this study.
To obtain this information, we compared the fluorescence
intensity measured for BSA which had been pre-labelled with
OPA using our method, versus using another method46 where
lysine residues are quantitatively labelled with OPA as described
in the Methods section. From the point (Fig. 4, intersection of
the black/grey dashed lines) for quantitatively labelled BSA at
0.3 μM, we know the fluorescence intensity when all lysines
react with the dye. The fluorescence intensity of pre-labelled
BSA (Fig. 4, dark blue diamond) allowed us to calculate the percentage
of lysines which reacted with OPA during our experiment. We
showed that only 9 Lys (15%) reacted with OPA. We also showed
that the number of labelled Lys did not increase with the time of
experiment (Fig. 4, light blue diamond), but even slightly decreased, due to the instability of the isoindole fluorophore formed in this reaction. Additionally to confirm those data we repeated the experiment for BLG and UB, which had similar labelling efficiencies, at 10.5% and 10.6% primary amines respectively. It can thus be suggested that in our electrophoretic experiments, OPA labelling does not affect significantly the values of isoelectric points for proteins.

### 3.4 Rapid two-point pI measurement

We have next developed a procedure for the rapid estimation of the pI values of proteins without a full titration curve using the microfluidic platform, which could be a useful tool for separation or purification processes. By analysing literature data containing a variety of pIs of proteins, approximately 70% have a pI between 4 and 7, by measuring the deflections at two values of pH, one below and one above this range we can estimate the unknown pI of protein. The deflection measurements were made using 2 mM phosphate buffers with pH values (3.0 and 7.7) for BLG and (3.3 and 7.7) for all other proteins. By calculating the mean and standard error of three independent measurements we obtained a BSA pI of 5.1 ± 0.2 (Fig. 5a, dark blue). The value was slightly higher than the one determined by using six different buffer pH values (4.6 ± 0.1), however, it was in accordance with the values previously reported in the literature (4.5–5.1).

In the two-point procedure only the extreme two values (Fig. 2d) were considered in the calculation, so that the calculated pI was shifted towards higher values. Moreover, this method was also used to provide further confirmation of the minimal effect of protein labelling under our conditions on the measured protein pI. The same two-point μFFE experiment was performed in a label-free manner using a deep UV fluorescence setup. The pI value for label-free BSA* was 5.0 ± 0.2 (Fig. 5a, light blue), indistinguishable within error to the two-point measurement for labelled BSA. All the conditions and the statistical analysis for OPA-labeled and label-free approaches were described previously in Section 2.3.

We further explored the generality of this approach by measuring the pIs of β-lactoglobulin, ovalbumin, transferrin, ubiquitin and myoglobin (Fig. 5b). We also examined ribonuclease A, as an example of the protein which is outside the pH range (4–7) (Fig. 5c); by increasing the higher value of pH to 11.2. The obtained result (8.6 ± 0.2) was in accordance with previously reported values [8.6–9.6]. Similarly, the values measured for other proteins were in accordance with literature values: BLG 5.1 ± 0.2 (5.1–5.2), OVA 4.9 ± 0.3 (4.6–4.9), TF 5.4 ± 0.1 (5.2–5.5), UB 6.2 ± 0.1 (6.5–6.8) and MYO 6.5 ± 0.1 (6.8–7.0). By plotting the dependence of the literature and the experimental values of pIs (Fig. 5d), we observed a linear correlation with the high coefficient of $R^2 = 0.98$.

### 4 Conclusions

In this paper, we have reported a microfluidic device based on μFFE for the determination of protein isoelectric points, by varying the pH in time, rather than in space, allowing for reproducible measurement of pIs without the requirement for...
the generation of a pH gradient, which is often challenging in practice (μFFIEF). Using this approach we have obtained pIs for BSA, which are identical within experimental error with the values reported in the literature. The method also requires low voltages, and low sample consumption, and devices can be fabricated using inexpensive consumables. We also showed that it is possible to estimate pI values for a wide range of proteins (BLG, RNase A, OVA, TF, UB, MYO) from electrophoretic mobilities in free solution, measured at only two pH values. These results suggest that gradient-free determination of isoelectric points is rapid and accurate measurement of pI value on small volume of samples.

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