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Review

Current trends in capillary isoelectric focusing of proteins

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Abstract

Isoelectric focusing (IEF) in thin capillaries is reviewed here. After an introduction on the genesis and chemistry of the carrier ampholyte buffers, different approaches to IEF are discussed and evaluated. The classical approach consists on IEF under conditions of suppressed electroosmotic (EOF) flow, usually obtained by covalently bonding hydrophilic polymers to the inner capillary wall. The other approach consists of IEF in dynamically (and partially) coated capillaries, so as to allow a reduced EOF flow to coexist with the IEF process, so that focusing and transport of the train of stacked bands occurs simultaneously. The various experimental parameters: focusing, elution and detection steps, p*I* measurements, as well as typical drawbacks, such as isoelectric precipitation are evaluated. The review ends with some examples of analytical separations, at the moment mostly limited to focusing of native hemoglobins (normal and point mutants). These separations are compared with those obtained by slab-gel IEF and in immobilized pH gradients. © 1997 Elsevier Science B.V.

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Contents

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1. Introduction

Isoelectric focusing (IEF) represents perhaps the electrokinetic methodology with the highest resolving power (if one excludes two dimensional maps, as exemplified by IEF itself in the first dimension, followed by SDS-electrophoresis in a second, orthogonal direction). In IEF, amphoteric compounds are sorted in order of their isoelectric points (pI) in a steady-state pH gradient. Good resolution is favoured by both a low diffusion coefficient and a high mobility slope at the pI, conditions which are, with a few exceptions, well satisfied by all proteins. A high field strength and a shallow pH gradient further enhance resolution. There are two basic variants of IEF: (a) in soluble, amphoteric buffers [1] and (b) in insolubilized, non-amphoteric buffers (the latter technique known as immobilized pH gradients, IPG) [2]. The former methodology is the brain-child of Svensson-Rilbe who, in the early sixties, laid the theoretical foundations and the principle of the technique [3,4]. The additional, most important event, was the synthesis of the carrier ampholyte buffers (CA), carried-out by one of his pupils, O. Vesterberg [5]. In this review, we will deal with the former methodology, i.e., CA-driven IEF since IPGs have not been implemented as yet in capillaries. In fact, in IPGs, the buffers (acrylamido weak acids and bases) have to be grafted onto a support, at present only a polyacrylamide gel. In addition, the gradient is created artificially, outside the electric field (whereas in CA-IEF the pH gradient has to be generated and maintained by the electric field itself), and thus gel casting requires the use of a two-vessel gradient mixer, with the simultaneous pouring of a density and a pH gradient in a flat-gel slab format. As normally utilized, IPGs require additionally that the gel cassette is opened and that the polyacrylamide slab, with the grafted pH gradient, is exhaustively washed, so as to eliminate salts, ungrafted buffers and catalysts. Due to this sequence of manipulations, preparing an IPG in a capillary format does not seem to be an easy process.

CA-IEF allows a resolving power (or ΔpI, representing the difference in isoelectric points between two, just-resolved, protein bands) of the order of 0.01 pH unit [1]. Conversely IPGs, in shallow pH gradients, can be driven to an extraordinary resolving power, of the order of $\Delta pI = 0.001$ pH unit [2]. At present, five major reviews have appeared on IEF in capillaries (CIEF) and slab gels: by Mazzeo and Krull [6], by Righetti and Chiari [7], by Hjertèn [8], by Righetti and Gelfi [9] and by Righetti et al. [10]. Since these reviews are more of a general character than practically oriented, efforts will be concentrated, in this survey, on methodological aspects.

2. Carrier ampholyte buffers

Although Svensson-Rilbe [3,4] had derived the relevant theory pertaining to steady-state focusing, the methodology was not amenable to routine practice, due to lack of appropriate buffers covering the pH 3–10 interval. As the name implies, a unique set of buffers had to be utilized: they have to be amphoteric (so as to reach a focusing position in the column) and carrier as well. This last property is also fundamental: these buffers have to carry the current (thus be good conductors at $pH = pI$) and also carry a good buffering power. Both properties are satisfied when the protolytic groups, dissociating on either side of the pI value, are not too distant from the pI itself. Svensson has expressed this property as: $(pI - pK_{prox}) < 1$, where pK_{prox} indicates a protolytic group dissociating nearby the pI value. This last inequality can be visualized by plotting the pH/mobility curve of an ampholyte. Good carrier buffers have a steep slope of the pH/mobility curve in proximity of the pI value. Conversely, all poor

ampholytes (as an example, all mono-amino mono-carboxylic acids, such as Gly, Ala, etc.) exhibit a flat profile, extending over about four pH units, centred on the p*I* value. Svensson had been unable, in the early sixties, by examining thousands of organic chemicals, to obtain a reasonable number of such amphoteres covering adequately the pH 3–10 range. Moreover, there were essentially no chemicals with the desired properties in the pH 5–7 interval.

The remedy to this meagre situation came with the remarkable synthetic process of Vesterberg [5], who devised a chaotic organic synthesis for generating a multitude of ampholytes (he calculated that >600 species could be produced) covering the pH 3–10 region. He noted that oligoamines (e.g., tetraethylene tetramine, tetraethylene pentamine, pentaethylene hexamine) would provide a skeleton of amino groups having p*K* values fairly well distributed along the pH 3–10 region and with different values for the different amines. Additionally, oligoamines longer than four amino groups would exist as a mixture of linear and branched species, thus further increasing the heterogeneity of p*K* values. By properly mixing these three oligoamines (and possibly more) he could thus cover quite thoroughly the desired pH 3–10 region. Addition of an α-β unsaturated acid (mostly acrylic acid, but also small amounts of itaconic acid proved efficient for a good buffering action between pH 5 and 6) allowed the generation of this highly heterogeneous mixture (>600 species) of amphoteric buffers, ideal for good focusing. Note that by this synthetic process no salts were generated, since there was a direct addition of the double bond to primary and secondary amino groups; thus, in principle, these carrier ampholyte buffers could be used as such after the synthesis. Note additionally that the synthesis can only be performed at an appropriate ratio of amino/carboxyl groups (typically 2:1). If stoichiometric amounts of acrylic acid are added to fully saturate the amino groups in the oligoamines, a very narrow population of quite acidic compounds is produced. This synthesis has been the base of all subsequent attempts at generating carrier ampholytes, so that several brands are now available: Servalyt, Biolyte, Pharmalyte, etc.

A principal difference between IEF in a gel and in a capillary is that, in the latter, mobilization of the focused proteins past the detector has to be carried out if an on-line imaging detection system is not being used. Mainly three techniques are used: chemical and hydrodynamic flow mobilization (in coated capillaries) and mobilization utilizing the electroosmotic flow (in uncoated or partially coated capillaries). These techniques will be discussed below.

3. Focusing in internally coated capillaries

At any pH value above pH 2, the fused-silica surface will progressively acquire negative charges, due to ionization of weakly acidic silanol groups, which are fundamental constituents of any vitreous material. The EOF of silica surfaces seems to be a particular case in a very general phenomenon by which all surfaces (whether neutral or ionogenic) acquire a charge when in contact with an electrolyte solution. Close to the capillary wall (in the diffuse part of the double layer) there will be accordingly more positive than negative ions (electroneutrality will thus not prevail in the double layer). In an electric field, the hydrated, positively charged surface layer will move toward the negative pole, thus producing an electroosmotic flow (EOF), seen macroscopically as a bulk fluid movement. Per se, such EOF pump would not be deleterious to the analyte zone, since it has a flat profile (except in the few nm thickness of the double-layer); however, in the case of proteins, strong adsorption might ensue, due to multipoint attachment of positively charged species to the negative charges of the wall. In addition, particles migrating in directions opposite to the bulk liquid flow might never reach the detector. Hjertén [11], when analyzing the Helmholtz equation, correctly noted that the electroosmotic mobility (μ_{eo}) is inversely proportional to the viscosity (in the double layer). Thus, by coating the inner surface of the capillary with a hydrophilic, nonionic polymer, there will be two beneficial effects: the charges will be masked and in general suppressed (due to reaction of most silanols with Bind Silane) and, additionally, the viscosity in the double layer will be so high as to virtually eliminate EOF. Hjertén [12] achieved this coating by first reacting the wall with a bifunctional agent (γ-methacryloxypropyl trimethoxysilane) and then covalently affixing to the dangling double bonds a monolayer of linear polyacrylamide

(a procedure that had also been proposed by us in 1980 for binding soft gel matrices to glass slabs) [13]. Many other coating procedures have been described, and they are reviewed in [14].

3.1. Focusing step

Hjertén proposed the following methodology. The coated capillary is entirely filled with sample solution mixed into at least 1% CAs (the sample should better be desalted, so as to avoid pH gradient drift). One end of the tube is then pressed into a 1% agarose gel, prepared either in 20 m*M* NaOH or in 20 m*M* phosphoric acid (representing the cathodic and anodic solutions respectively). The gel plug thus inserted into the tube end prevents zone deformation by hydrodynamic flow in the tube during the subsequent focusing step. A constant voltage of 4000–6000 V is applied. When the steady-state has been attained, which occurs when the current has dropped to about 10–25% of the starting value, the voltage is switched off and elution started immediately, as illustrated below.

3.2. Elution and detection step

In such a system, due to suppression of EOF, the focused stack of carrier ampholytes and proteins is arrested; thus ways have to be found to mobilize the stack past the detector. Hjertén et al. [15,16] proposed a number of ways to achieve that: (a) to apply a mechanical pump to the capillary and generate a hydrodynamic flow (at the end of the IEF process); (b) to replace the base at the cathode with acid or the acid at the anode with base; (c) salt mobilization. This latter technique has attained wide popularity [17]: when a salt (e.g., NaCl) is added at the anolyte, mobilization will be towards the anode; conversely, if added to the catholyte, the train of bands will elute at the cathode. Fig. 1 gives a pictorial representation of this process: the upper part depicts the steady-state, characterized by a stationary pattern of focused proteins in an arrested pH gradient. In this stage, current is carried mostly by protons and hydroxyl ions moving from the anodic and cathodic compartments, respectively (and also by the to and fro movement of the CA buffers about the p*I* position).

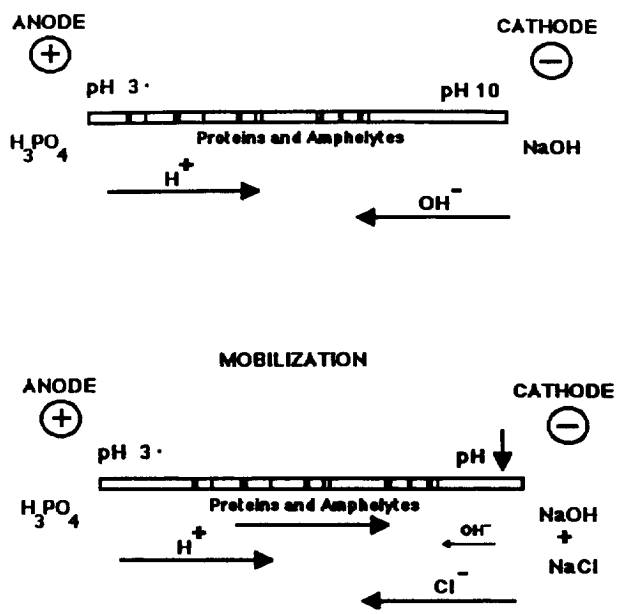

Fig. 1. Pictorial representation of the focusing (upper) and mobilization (lower) steps. In the upper drawing, the steady-state is shown as an arrested stack of proteins and CA buffers. Only protons and hydroxyl ions move from the respective electrodes, carrying most of the current. In the lower drawing, addition of NaCl to the cathode is shown to mobilize the stack of proteins and CA buffers towards and past the detector port (represented as a large vertical arrow close to the cathode; with permission from Bio Rad, Hercules, CA, USA).

Upon addition of NaCl at the cathodic reservoir, the stack of proteins and CA buffers is mobilized towards the cathodic side (past the detector). In general, for mobilization, 80 m*M* NaCl is added: it is suggested that the amount of salt should not be higher than 100 m*M* (to avoid Joule effects) and not lower than 20 m*M* (to avoid too long mobilization times). In general, the time required for mobilization is about 15 min at 360 V/cm. During mobilization, the current, which had reached a minimum at the end of the focusing stage (typically 1 μA), rises again to as high as 200 μA. It is during mobilization that the train of bands, titrated away from the p*I* by the cations or anions (other than protons or hydroxyl ions) entering the tube from one of the electrode reservoirs, transits in front of the detector and is registered as a spectrum of bands. Ideally, proteins and peptides should best be monitored at 210 (or even 190) nm, where the absorbance of the amido bond is 20–50 times higher than at 280 nm. However, at this low wavelength also the CA buffers produce a UV pattern (rather similar for Ampholine and Biolyte, quite different in the case of Pharmalytes) which could be mistaken as sample zones. Thus, in an IEF experiment, it is necessary to read the sample at 280 nm. As an alternate procedure, Zhu et al. [18] have proposed mobilization by replacing the catholyte with a p*I* 3.22 zwitterion, which they claimed would improve detection of acidic proteins. In addition, other amphoteric species, with selected p*I* values, could be used to mobilize only portions of the stack. Thus a p*I* 6.90 zwitterion was shown to improve, via selective mobilization detection of neutral and basic proteins. There is another important aspect regarding detection of analytes in IEF: it concerns the blind segment of the capillary, past the detector port till the electrode vessel. All sample ions focusing in this segment will be lost for the monitoring process, since mobilization has to go in the sense of pushing all analytes towards the detector from the opposite pole. A way to overcome this problem is; to have a pH plateau occupying the capillary segment going from the detector window to the neighbouring electrode (which could account for up to 20% of the separation space). It has been proposed to use an amount of 0.5% TEMED (the accelerator in acrylamide polymerization) admixed to the carrier ampholytes: upon focusing, TEMED will be arrested by a deprotonation mechanism at the cathodic end and occupy a capillary segment proportional to its starting concentration, thus forming an alkaline pH plateau [18].

3.3. Elution by vacuum or pressure under voltage

Chen and Wiktorowicz [19] have adopted commercially-available coated capillaries from J&W Scientific (Folsom, CA, USA) consisting on a 0.05 μm thick layer of dimethylpolysiloxane with a remarkable performance, in CIEF, of >400 runs. As a safety precaution, however, they adopted in each run a standard amount (0.4%) of methylcellulose (Sigma, St. Louis, MO, USA; having a viscosity of 1500 cps for a 2% solution). The idea here is that, were any slight loss of coating to occur, the added methylcellulose would mask any exposed site thus still permitting high reproducibility (which they claim to be better than ±0.1 pH unit, with relative standard deviations of the calculated p*I*s of less than 3% of the means) from run to run. These authors, for elution, have adopted a vacuum of 5 mmHg while still under high voltage. The applied vacuum causes the focused proteins to flow past the detector, while the voltage maintains the pH gradient and zone sharpness even in the presence of distorting effects due to laminar flow. First the entire tube is filled with NaOH (catholyte). Then, by hydrodynamic flow, ca. 2/3 of the capillary length are filled with carrier ampholytes. This is followed by a short sample plug, which is subsequently insulated from strong anolyte (which might, by contact denature some proteins). At this point liquid pumping is stopped and the focusing process takes place in between phosphoric acid as anolyte and NaOH as catholyte. Mobilization is again accomplished by a vacuum-driven hydrodynamic flow, under voltage. According to these authors, the sensitivity in protein detection at 280 nm is as low as 1.3 ng, while the signal linearity is in the range 1.3–10.7 ng (an eight-fold concentration span). Interestingly, this sensitivity is of the same order of magnitude as reported for silver staining of SDS-denatured protein zones and two to three orders of magnitude higher than conventional Coomassie Brilliant Blue staining. As an alternative, Hempe and Craver proposed mobilization under pressure [77].

3.4. pI Measurements

In its simplest approach, unknown p*I* values can be assessed by plotting the p*I* values of a set of markers, co-focused with the proteins under investigation, vs. their relative mobility upon elution. In the vacuum method proposed by Chen and Wiktorowicz [19] this plot is linear and thus a high precision (ca. ±0.1 pH unit) is obtained (see Fig. 2). According to these authors, p*I* values as low as 2.9 (for RNase T_1-wild type) and 2.75 (for unsulfated cholecystikinin flanking peptide) could be determined. In another approach, in the focusing of transferrin [20], Kilàr [21] has proposed a novel method for p*I* assessments: monitoring the current in the mobilization step. If one monitors simultaneously the peaks of the mobilized stack of proteins and the raising current due to passage of the salt wave in the capillary, one can correlate a given p*I* value (which should be known from the literature a priori, though) with a given current associated with the transit of a given peak at the detector port. The system can thus be standardized and used for constructing a calibration graph to be adopted in further work, without resorting to internal standards. One such a graph correlating current with p*I* values is shown in Fig. 3: this appears to be a precise method, since the error is given as only about 0.03 pH unit. Yao et al. [22] proposed a method for determining isoelectric points

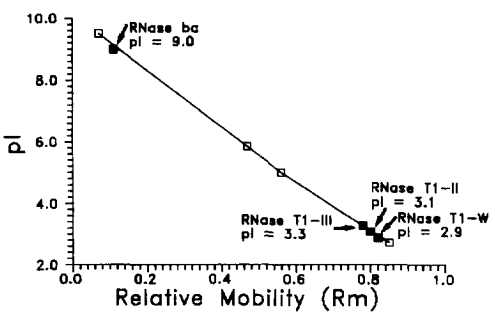

Fig. 2. Calibration graph for p*I* determination using a set of marker proteins. The markers (open squares) are: ribonuclease A (p*I* 9.45); carbonic anhydrase (p*I* 5.90); β-lactoglobulin (p*I* 5.1) and unsulfated cholecystikinin flanking peptide (p*I* 2.75). The four solid squares represent four unknown proteins, whose p*I*s have been determined by linear interpolation in the calibration graph (from Chen and Wiktorowicz, see Ref. [19], with permission).

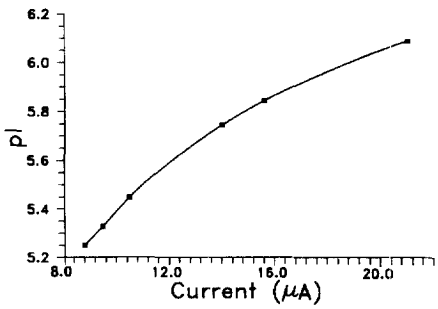

Fig. 3. Calibration graphs for p*I* determination using the current, during the mobilization step, as a parameter in capillary IEF. The six experimental points represent six forms of transferrin, containing different amounts of sialic acid and of iron (from Kilar, see Ref. [21], with permission).

of acidic and basic proteins in CZE by running the proteins of interest at a series of pH values, either above or below their p*I* values. From a plot of mobility vs. pH, the intercept at zero mobility yields the p*I* value of the protein. In order to prevent binding of cationic proteins to the wall, a dynamic polybrene (a quaternary amine polymer) coating is utilized. In another approach, Kleparnik et al. [23] propose an absolute determination of p*I* values based on the application of an additional electroosmotic and/or hydrodynamic flow of a background electrolyte. The mobilities of a given amphoteric species are measured at various pH values so as to find the pH at which the substance moves through the capillary at zero mobility under the influence of the additional flow only. Since the number of experimental points needed to find the precise point of zero mobility could be quite high, it is kept to a minimum value with the help of an iterative procedure based on the Regula Falsi algorithm. Finally, Slais and Friedl [24] suggested the use of amphoteric dyes (aminomethylated sulfonphthaleins) as p*I* markers in CIEF.

3.5. Isoelectric precipitation

Proteins have net negative and net positive charges at pH values above and below their p*I* values, respectively. This decreases the risk of aggregation,

which ultimately may lead to precipitation. However, at, or in the proximity of their p*I* value, proteins exhibit a minimum of total charge, thus a solvation minimum. This increases the risk of aggregation, further enhanced by the extremely low ionic strength conditions prevailing in IEF. When protein molecules precipitate, they probably aggregate by hydrophobic interactions. It seems therefore logical to try to suppress precipitation by supplementing the CA buffers with agents known to decrease hydrophobic interactions, such as ethylene glycol (10 to 40%, v/v) or detergents (1 to 4%, w/v). The detergents should be either non-ionic or zwitterionic, so as to be compatible with the focusing process; in addition, they should preferably by transparent at 280 nm, so as to minimize interference with protein detection. Examples of such detergents are G-3707 (from Atlas Chemie, Everberg, Belgium) and reduced Triton X-100 (Sigma). Both ethylene glycol and neutral detergents are mild agents and rarely change the p*I* of water soluble proteins significantly. According to Hjertèn [25], in fact, ethylene glycol often has a stabilizing effect on the structure of a protein. Urea, at high concentrations (6–8 *M*) is a well-known protein solubilizer, but it unfolds proteins, thus leading to loss of biological activity. Intrinsic membrane proteins, with their pronounced hydrophobic surface structure and great tendency to precipitate, can seldom be analyzed successfully by IEF, but a recent example has been given by Englund et al. [26] with the human red cell glucose transporter, solubilized with *n*-dodecyl octaoxyethylene. There are some simple ways to reduce protein interaction and precipitation: one is to use dilute protein solutions (aggregation is proportional to protein concentration); the other is to increase the CA buffer concentration (e.g., up to 4%), since this leads to an increase in total ionic strength. Küllertz and Fischer [27] have assessed the influence of a number of additives (glycerol, sorbose, saccharose, dextrans of mass from 1000 to 500 000 u, PVP from 10 000 to 360 000, HPMC and PEG, 1000 and 600 000) on the quality of the focusing pattern. Curiously, out of this vast number of compounds, only addition of 2% saccharose was found to improve resolution. Another, often overlooked factor, seems to play an important role on separation efficiency: the edge of the capillary. According to Cohen and Gruskha [28], straight-cut edges yield much more efficient electropherograms than do slanted-edge capillaries.

3.6. Detection system

As stated above, the standard detection system in IEF is at 280 nm, the typical absorption maximum of proteins. An important modification has been recently proposed in protein detection. Since in the mobilization process distortion of zones, loss of resolution and extra experimental time requirements occur, Wu and Pawliszyn [29–31] have proposed a universal concentration gradient imaging system not requiring a mobilization step. This system is based on the Schlieren shadowgraph methods and utilizes a He–Ne laser for probing the capillary content and a charge-couple device (CCD) as a detector. The capillary has to be square, not-covered by polymide and rather short (4 cm). Focusing is completed in 2 min, resolution is of the order of 0.02 pH units and the mass detection limit appears to be of the order of sub-picomole level. This system was used for scanning in situ the focusing of transferrin and the formation of its complexes with iron, after running a plug of free iron through a spectrum of focused, iron-free transferrin [32]. As an alternative, Wang and Hartwick [33] propose a whole column UV absorbance detection consisting of transporting the entire column past the detector (the capillary, of course, has to be UV transparent). Wu and Pawliszyn [34,78] have additionally described an absorption imaging detector, having a higher dynamic range and higher sensitivity. In this latest set-up, the optical configuration consists of a 5 mW argon ion laser (with either 496.5 nm or 514.5 nm lasing lines) probing the entire length of a 4 cm long, square glass capillary and using as a detector a 1204 pixel CCD camera. Focusing and imaging are usually ended in 2–4 min. The problem is that only coloured proteins (absorbing in this wavelength region) can be efficiently detected (e.g., hemoglobin, myoglobin and cytochrome C). The same authors [35] have extended their universal concentration gradient imaging system to the analysis of peptides obtained by tryptic digestion (e.g., of bovine and chicken cytochrome *c*). The advantage claimed is that even peptides not containing aromatic amino acids (such as Tyr and Trp) can be detected by this system. Detection of

peptides has also been claimed by Mazzeo et al. [36], who, however, had to resort to both anodic and cathodic mobilization for detecting neutral peptides. Finally, a mass spectrometer has been proposed as a detector after CIEF [37]: it should be noted that the technique becomes two-dimensional, since proteins are then mapped by both charge and mass.

4. Focusing in dynamically coated capillaries

Approaches from three groups have reported on the possibility of focusing in dynamically-coated capillaries. They will be reviewed below.

4.1. Dynamic coating with methyl cellulose

Mazzeo and Krull [38–40] noted that, more than eliminating completely EOF, one might try to reduce it to such an extent as to allow attainment of steady-state conditions; from there on, the bulk flow would keep mobilizing the arrested stack past the detection window. This approach would then obviate the need for performing salt, or vacuum, or hydrodynamic mobilization; focusing and elution being accomplished in one step. A simple way for modulating EOF is to add viscous polymer solutions. In their approach, the capillary is conditioned with 0.1% methyl cellulose, mixed with sample and CA buffers and used to fill the entire column. These authors have found, in addition, that under their experimental conditions anodic drift would be operative and destroy the separation of mildly acidic proteins (e.g., β-lactoglobulin A, with a p*I* of 5.1). It was found that increasing the anolyte concentration to 25 m*M* phosphoric acid allowed also the detection of acidic proteins. Finally, Mazzeo and Krull have investigated how deleterious different amounts of salt (NaCl) present in the sample would be to the focusing process. As little as 10 m*M* NaCl in the sample suffice to entirely destroy the separation; so, salt in the analyte must be kept below this level.

The conclusions of these authors about this technique are not terribly comforting. In their own words: 'using EOF driven capillary IEF, it may never be possible to achieve optimal resolution throughout the entire pH gradient'.

4.2. Dynamic coating with hydroxypropyl methylcellulose

In another system [41–43], the dynamic agent used for partial coating is hydroxypropyl methylcellulose (HPMC). In this approach, some interesting variants have been adopted. A new capillary is first rinsed for 20 min with 1 *M* NaOH and then for 10 min with 0.1 *M* NaOH containing 0.3% HPMC. It is during this last washing that conditioning of the capillary and partial coating with HPMC occurs. This etching procedure (in 1 *M* NaOH), followed by a short renewal of the dynamic coating (0.3% HPMC in 0.1 *M* NaOH) is shown to provide data of the highest reproducibility. The sample proteins are dissolved in 2.5% Ampholine solution, without any addition of HPMC. The anolyte is the standard 10 m*M* phosphoric acid solution, whereas the catholyte consists of 20 m*M* NaOH in presence of 0.1% HPMC. It appears that addition of HPMC to the catholyte, as well as a proper molarity level of the base in the cathodic reservoir, are of great importance on resolution and reproducibility in IEF, since the catholyte (NaOH) is the major contributor to the electroosmotic flow. Conversely, addition of HPMC to anolyte produces no improvement of the separation pattern. In the present method the sample is introduced as a plug, occupying only 10–50% of the capillary length at the anodic side, the remaining being filled with catholyte. Since the entire stack of proteins will eventually be displaced towards the cathode by the electroosmotic flow, this initial sample plug distribution allows more time for reaching a good focusing pattern prior to sample passage in front of the detector.

4.3. Dynamic coating with adsorbed surfactants or polymers

In yet another variant, Yao and Regnier [44] have proposed reduction of EOF via derivatization of capillaries with a hydrophobic coating (octadecyl silane) followed by adsorption of either a surfactant (Brij 35, PF-108) or a hydrophilic polymer [e.g., poly(vinyl alcohol), polyvinyl pyrrolidone, methyl cellulose, the latter being preferred]. The procedure is as follows: the capillary is first treated with 1 *M* NaOH for 30 min, followed by washings with

deionized water and methanol (30 min each). The residual methanol is evaporated in a GC oven at 90°C for 2 h, while flushing the capillary with a stream of nitrogen at 400 kPa. While still in the oven at 90°C, a solution of octadecyl trichlorosilane in 50% toluene is flushed through the capillary for 6 h. After silylation, the capillary is rinsed for 20 min with methanol and then with water for 30 min. Surfactant solutions (in general 0.4%) are pumped continuously through the capillary for an additional 6 h, in order to complete the coating process. Coating via adsorption of detergent (or methyl cellulose 4000) is shown to reduce the EOF of the native, untreated capillary, to approximately 1/20 to 1/30 of the original value. Yet, the residual EOF allows adequate flow to obviate the need of a separate mobilization step. Based on resolution of hemoglobin variants, proteins that varied 0.03 pH units in isoelectric point were resolvable.

Schwer [45] has recently evaluated these three approaches to CIEF. Her conclusions: (a) one-step (i.e., in case of dynamic coating) focusing affords fast analysis times but is best suitable for proteins with neutral p*I* values; (b) if linearity of the p*I* calibration curve is required or strongly basic proteins are to be analyzed, either hydrodynamic or chemical mobilization should be used; (c) for separation of proteins with only small p*I* differences, chemical mobilization offers the highest resolution.

5. Other means for generating pH gradients

There are a number of ways for changing the pH value along the length of a capillary: pH steps [46], pH pulses [47] and the creation of dynamic pH gradients [48,49]. In the latter technique, a pH gradient is generated by feeding the capillary with two different suitable ionic species from two separate electrode chambers via a three-way joint by simultaneous electromigration. The common feature of these studies was a direct control of the H^+ flow from the anodic chamber, which was relatively easy in acidic media, but more problematic in alkaline pH ranges. The problem of dynamic buffering of pH in neutral and alkaline media was then solved by employing a dynamic step of the buffering anionic system, consisting in a moving boundary of carbonate —> oxalate [50]. A mathematical description of separations under such dynamic pulses has also been provided by Gebauer et al. [51]. In a variant of this approach, Purghart and Games [52] have described the computer-controlled delivery of a main buffer (e.g., phosphate at pH 2.5) and a modifier from a reservoir (e.g., phosphate at pH 11) to a mixing chamber feeding a capillary for creating a pH gradient: a similar approach has also been adopted by Tsuda [53], who has proposed creation of a pH gradient via a solvent program delivery system, as routinely adopted in HPLC. It should be noted, however, that all these methods, per se, are not really used for separating amphoteric ions under steady state, i.e., under focusing, conditions: they are rather used in order to modulate the mobility of ions by titration during a conventional zone electrophoresis separation. Thus the usefulness of these methods for a true focusing system is rather limited. In addition, the experimental burden is increased, since one has to resort to multiple columns or to gradient programmers, still not available in any of the CZE instruments on the market. As an alternate procedure, pH gradients can be thermally engendered, by exploiting buffers, such as Tris, having a large temperature coefficient (dpH/dT). By varying the temperature of the capillary as a function of time or as a function of position during electrophoresis, a pH gradient can be generated in situ [54,55]. However, this approach does not seem to be easily applicable and in fact it was criticized long ago by Lundahl and Hjertèn [56]. Finally, Slais has published a theoretical treatment of focusing in a natural pH gradient in a tapered capillary [57]; additionally, Deml et al. [58] have proposed a continuous micropreparative trapping in carrier ampholyte-free IEF.

6. Sample preconcentration systems

Typical preconcentration steps commonly used in biochemical analysis (especially for macromolecules) such as lyophilization, ultrafiltration, partition between two polymer aqueous phases, osmotic removal of water and chromatographic adsorption–desorption, will entail large losses when the sample volume is 1–10 μl or less, as customary in CZE. Thus, Hjertèn's group [59–61] has proposed a number of

electrophoretic ways for concentrating biopolymers (especially peptides and proteins) while partially depleting them of strong electrolytes (often anathema in all IEF procedures). These methods are based on the fact that electrophoretic migration velocities decrease upon reducing the absolute value of the zeta potential of a solute and the pore size of the electrophoresis medium and upon increasing the cross-section of the electrophoretic chamber, the viscosity and the electrical conductivity of the electrophoresis medium. In addition, it is possible to utilize the zone-sharpening properties of displacement electrophoresis (isotachophoresis) in combination with a hydrodynamic counter flow to create a stationary zone where the sample solutes can be collected continuously. In practice, the whole electrophoresis tube is filled with the sample solution to be concentrated and then the sample is allowed to migrate against the end of the tube where a gradient of one of the above parameters (e.g., conductivity, viscosity) exists and is arranged in such a way as to continuously slow down sample electrophoretic migration. The sample will finally collect in a narrow tube region (typically 0.2–0.5 mm in width). A 400–1000 fold concentration is obtained when a 200 mm long tube is filled completely with the sample and still more if also an electrode vessel is loaded with sample. Liao et al. [60] have experimentally verified these assumptions. Also Mazereeuw et al. [62] have proposed an in-line isotachophoretic concentration process of very large injection volumes prior to CZE analysis. According to these authors, sample volumes up to 25 μl can be concentrated by this system. Since concentrating large volumes would take a relatively long time, depending also on migration path length in order to speed up the process, they have adopted a system of coupling a narrower bore to a larger bore capillary. Finally, after the ITP concentration step, the sample can be analysed by CZE via a junction connected to another electrolyte reservoir (i.e., one has to resort to a threepole column). An on-tube desalting technique has also been offered by Liao and Zhang [63], who propose an automatic substitution of the salts with ampholyte solution in a short prefocusing step prior to the final analytical IEF procedure.

While not directly applicable to IEF, the last two methods reported below offer remarkable sample concentration procedures, able to greatly increase its detection limits. According to Witte et al. [64], peptide-like solutes can be concentrated from very large injection volumes, up to 1.4 μl, without any loss of resolution as compared with regular sample loads (of barely a few nl). The isotachophoretic system here utilized is quite unusual: it is composed of the ammonium ion as leading ion, of acetic acid as terminator and of ε-amino caproic acid as background electrolyte. Van der Vlis et al. [65] propose instead a liquid–liquid electroextraction protocol, combined with isotachophoresis, able to greatly increase the sample detection limits down to 10^{-9}–10^{-10} mol/l even with simple UV detectors. The drawback here is that the analyte should be soluble in an organic phase which, due to high electric fields permitted, allows focusing of analytes into minute volumes.

7. Examples of some applications

7.1. Hemoglobin analysis

We will review here a number of hemoglobin (Hb) separations, since Hb is still one of the most popular proteins in clinical chemistry, due to the ease of separation and detection and to the fact that a vast number of spot mutations (ca. 600) have been described so far. In conventional focusing in gel slabs, a number of physico chemical applications were reported in the early seventies. Among them the first separation of valence hybrids, with the discovery of different p*I* values for the α-heme vs. the β-heme oxidized species [66]; the first report, by IEF, on subunit exchange and the separation of Hb asymmetrical hybrids [67]. More on the focusing of Hbs can be found in [1] and [2].

For a screening of homozygous β-thalassemia and of β-thalassemia trait globin chain IEF still represents one of the simplest and most reliable methods; however, the sample preparation for IEF analysis is still a lengthy and cumbersome process. Ideally, the method of choice would combine an IEF analysis performed on intact Hb species. By using umbilical cord blood where only three major Hb components are present (Hb F, Hb A and Hb F acetylated, F_{ac}) it should be possible to perform thalassemia screening

provided a good separation is obtained between Hb A and Hb F_{ac}, which have minute differences in p*I* values. Cossu et al. [68] reported a modified gel slab IEF technique by which a large screening of a population at risk for β-thalassemia was performed, by substituting the β/γ ratio obtained upon IEF of globin chains with the Hb A/Hb F_{ac} (or Hb A/Hb F) ratios obtained by IEF of intact Hbs from cord blood of newborns. In order to improve the separation, the pH 6–8 Ampholine was admixed with an equimolar mixture of separators, namely 0.2 *M* β-alanine and 0.2 *M* 6-amino caproic acid, which would flatten the pH gradient in the focusing region of the three major components, Hb A, F and F_{ac}. The method is simple, can unambiguously detect any thalassemic condition and can be easily performed on a routine basis for each delivery in a neonatal unit. In an extensive screening of newborns in the Sardinia island it was found that all the samples containing less that 10% Hb A at birth were associated with β-thalassemia heterozygosity [68]. Zhu et al. [69] and Molteni et al. [70] have also attempted similar separations by CIEF, but in their patterns F_{ac} is missing. We have recently reported, in CIEF, the same separations as in Ref. [68], by utilizing the same gradient-flattening principle, and the results have been excellent (see Fig. 4) [71]. By the same principle, we have also attempted CIEF separation of HbA from Hb A_{1c} (the glycated form of Hb A) [79], the latter component being of diagnostic value for the long term control of diabetic patients (glucose binds irreversibly to Hb molecules. The percent of Hb A_{1c} varies with the blood glucose concentration to which red blood cells have been exposed during their circulating lifetime). The separation of Hb A from Hb A_{1c} and the quantitation of the latter component was well achieved long ago by gel slab electrophoresis [72]. Fig. 5 gives the CIEF separation of HbA from Hb A_{1c} in the absence and in presence of pH-flattening. In another example, Harper et al. [73] have monitored the pattern of hemoglobin production in young calves subjected to multiple phlebotomies.

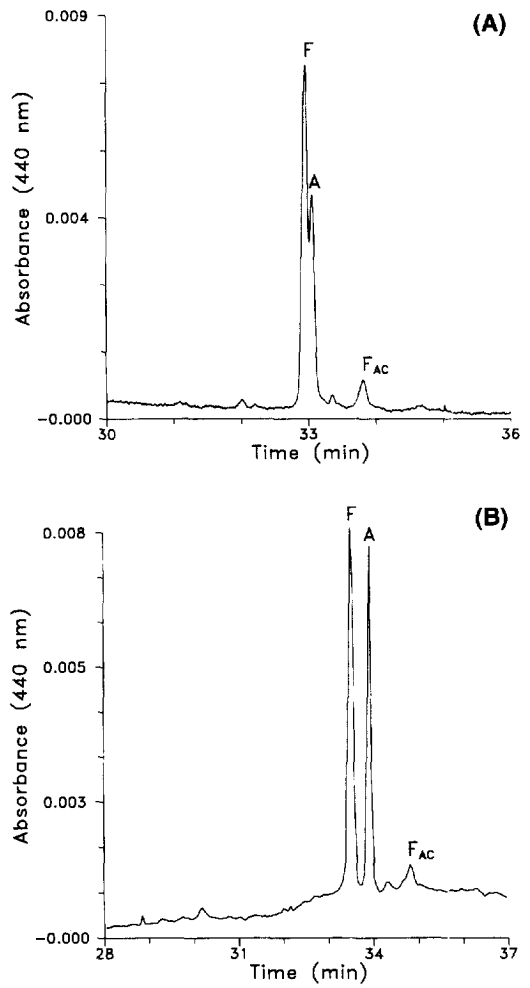

Fig. 4. Separation of Hb F, A and F_{ac} by capillary IEF. Background electrolyte: 5% Ampholine, pH 6–8, added with 0.5% TEMED (panel A) and additionally with 3% short-chain polyacrylamide and 50 m*M* β-Ala (panel B). Anolyte: 20 m*M* H_3PO_4; catholyte: 40 m*M* NaOH. Sample loading: by pressure, for 60 s. Focusing run: 20 kV constant at 7 μA (initial) to 1 μA (final current), 20 °C; capillary: coated with poly(AAEE), 25 μm I.D.; total length/effective length = 23.6/19.1; mobilization conditions: with 200 m*M* NaCl added to anolyte, 22 kV; detection at 415 nm (from Conti et al., see Ref. [71], with permission).

7.2. Other separations

There are not that many instances of other separations by capillary IEF. A few reports concern the analysis of transferrins in human serum [20,21]. Other reports deal with the dosage and analysis of a recombinant granulocyte macrophage colony stimulating factor [74] and of glycoforms of plasminogen activator [75]. Nelson [76] has described separations of bovine hemaglobins and serum albumin. A nice separation of monoclonal antibodies is shown in Fig. 6: as mobilization was obtained by

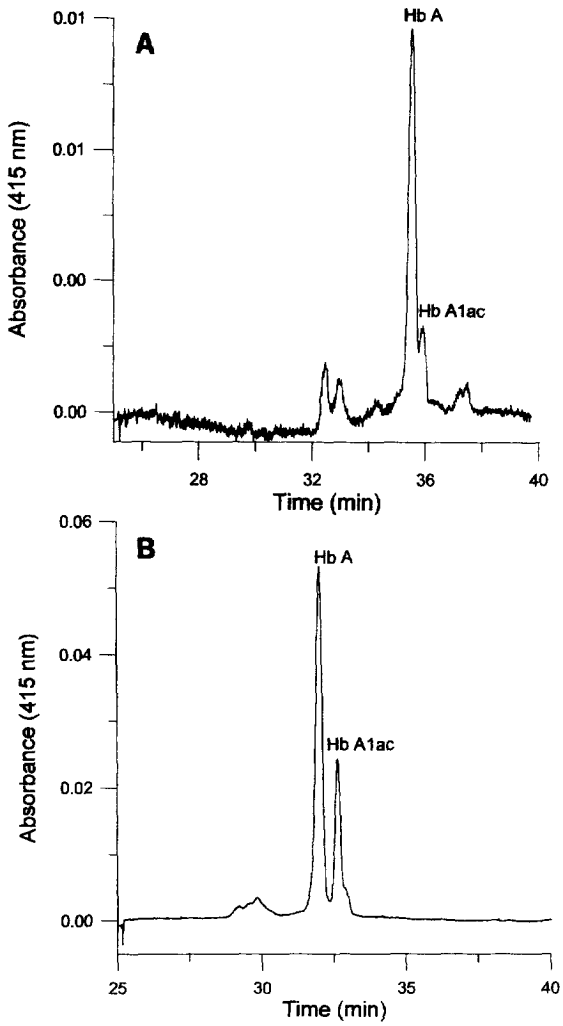

Fig. 5. Separation of Hb A from A_{1c} by capillary IEF in the absence (A) and in presence (B) of 3% short-chain polyacrylamide and an equimolar mixture of separators, 0.33 *M* β-Ala and 0.33 *M* 6-amino caproic acid. Background electrolyte: 5% Ampholine, pH 6–8, added with 0.5% TEMED. Anolyte: 20 m*M* H_3PO_4; catholyte: 40 m*M* NaOH. Sample loading: by pressure, for 60 s. Focusing run: 20 kV constant at 7 μA (initial) to 1 μA (final current), 20 °C; capillary: coated, 25 μm I.D.; total/effective length = 23.6/19.1; mobilization conditions: with 200 m*M* NaCl added to anolyte, 22 kV; detection at 415 nm (from Conti, Gelfi and Righetti, unpublished).

pressure, under voltage, it shows the importance of working under high voltage during this step. Finally, Moorhouse et al. [80] reported the capillary IEF separation of recombinant tissue-type plasminogen activator glycoforms.

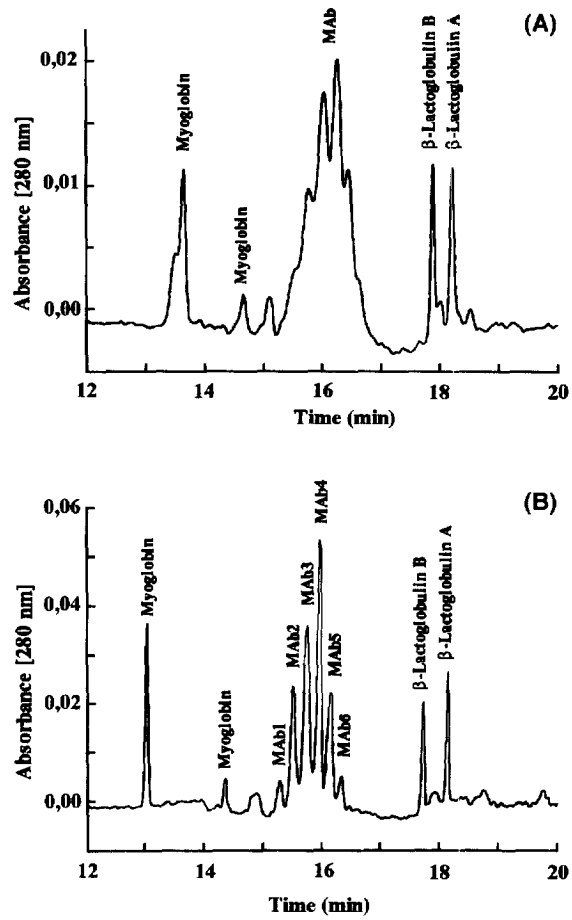

Fig. 6. CIEF of a mouse monoclonal antibody using pressure mobilization. Focusing for 2 min at 10 kV, followed by mobilization at low pressure (0.5 p.s.i.) at (A) 10 kV and (B) 20 kV. Concentration of the marker proteins: 50 ng/μl; concentration of desalted antibody: 0.5 μg/μl. Ampholyte solution: 4% Pharmalyte, pH 3–10, 1% TEMED in 0.8% methyl cellulose. Anolyte: 10 m*M* H_3PO_4; catholyte: 20 m*M* NaOH (from Schwer, see. Ref. [45], with permission).

8. Conclusions

Although IEF in the gel-slab format is a widely used technique in protein analysis (see Ref. [81] for an extensive, recent review), both in the conventional method and in the immobilized pH gradient version, CIEF is not yet a method used routinely, despite major advantages. This may be due to the fact that different methods for performing CIEF can be found in the literature, engendering uncertainties in the

users. We have compared and evaluated them and shown the advantages and limits of the different versions. Basically, however, if CIEF is performed in coated capillaries, the users need highly reliable, robustly coated capillaries giving top and reproducible performances. It is hoped that commercial products will be offered from different sources, tested for performance in CIEF.

9. List of abbreviations

CA	Carrier ampholytes
CCD	Charge-coupled device
CIEF	Capillary isoelectric focusing
EOF	Electroendoosmotic flow
F_{ac} (Hb)	Acetylated fetal hemoglobin
GC	Gas chromatography
Hb	Hemoglobin
Hb A_{1c}	Glycated hemoglobin
HPMC	Hydroxypropylmethyl cellulose
IEF	Isoelectric focusing
TEMED	N,N,N′,N′-Tetramethylethylenediamine

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