Capillary Gel Electrophoresis of Proteins: Historical overview and recent advances

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ABSTRACT

This review summarizes the fundamental principles, basic methodologies, strength and weaknesses of capillary gel electrophoresis of proteins by providing both a short historical overview and highlighting new developments and applications in biopharmaceutical, biomedical as well as food and agriculture fields. The subsets of the method including native capillary gel electrophoresis, SDS capillary gel electrophoresis, capillary gel isoelectric focusing, capillary gel isotachophoresis and capillary affinity gel electrophoresis of proteins are all critically reviewed. Relevant protein labeling techniques are also addressed.

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1. Introduction

Proteins are important biomacromolecules playing crucial roles as enzymes, enzyme substrates and inhibitors/activators, hormones, hormone and drug receptors, antigens, antibodies, transporters, and other functional and structural elements in all living organisms. Some protein subtypes such as monoclonal and multi-specific antibodies, fusion proteins, antibody-drug conjugates, nanobodies, etc., recently became very important drug candidates in the biopharmaceutical industry requiring high sensitivity and high throughput bioanalytical methods for their analysis [1,2].

In capillary gel electrophoresis (CGE) the electomigration of proteins is based on their charge to hydrodynamic volume ratios, also influenced by the reticulations of the sieving matrix, i.e., biased towards size based migration, especially in cases when their surface charge densities are similar. The most frequently used sieving matrices are hydrophilic linear polymers such as non-cross-linked polyacrylamide, polyethylene oxide, polyethylene glycol, dextran and alkylated cellulose derivatives. The separation process in capillary gel electrophoresis can be fully automated because of the use of replaceable sieving matrices. Significant reduction or even elimination of the electroosmotic flow as well as prevention of solute adsorption onto the inner capillary surface is usually required and addressed by the application of covalent or dynamic coatings (interested readers can refer to some recent reviews on the subject [3,4]).

2. Native capillary gel electrophoresis (nCGE) of proteins

Analysis of proteins in their intact state is of high importance, especially in protein-protein interaction studies [5]. Gel electrophoresis separates native proteins based on their charge to hydrodynamic volume ratio, but the electromigration of the solute molecules is also influenced by their size if comparable to the pore size of the gel matrix. Please note that proteins will only electromigrate in gels towards the detector in normal polarity mode if the pH of the background electrolyte is lower than the pI of the molecule (i.e., positively charged) and vice versa, in reversed polarity mode if the pI of the protein is lower than the pH of the background electrolyte (i.e., negatively charged).

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Abbreviations	
nCGE	native capillary gel electrophoresis
CGE	capillary gel electrophoresis
SDS-CGE	sodium dodecyl sulfate capillary gel electrophoresis
cgIEF	capillary gel isoelectric focusing
cAGE	capillary affinity gel electrophoresis
IgG	Immunoglobulin G
LPA	linear polyacrylamide,
PEO	polyethylene oxide,
PVA	polyvinyl alcohol
LIF	laser induced fluorescence

Native proteins with 20−40 kDa were separated using capillary gel electrophoresis by Wu and Regnier with linear polyacrylamide gel matrices in the concentration range of 3.5−5%. Their results suggested that these gel composition filled columns did not fully discriminate just on the basis of size in the molecular weight range examined, but the separation was mostly based on the net charge to hydrodynamic ratios of the proteins [6]. Miksik et al. developed a Pluronic F127 sieving media based capillary gel electrophoresis method for the separation of collagen type I cyanogen bromide fragments in their native forms [7]. The electrophoretic separation was comparable or even better than that of those obtained by other separation methods such as HPLC, as shown in Fig. 1.

Karube and coworkers performed a simultaneous comparative capillary gel electrophoresis study of proteins for both in their native forms and sodium dodecyl sulfate (SDS) complexes. The analysis was accomplished in 20 min on a microchip with 36 parallel microchannels [8]. Ferguson plot analysis confirmed that the electrophoretic mobility of native globular proteins corresponded to their charge to size ratio and molecular weight in native and SDS-CGE separation modes, respectively.

3. Size separation of proteins by SDS capillary gel electrophoresis (SDS-CGE)

One of the frequently applied electric field mediated separation methods to analyze proteins by their size is sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE). The technique is based on the assumption that after denaturation, the SDS micelle covered polypeptide chains have comparable surface charge density, thus, the resulting electromigration differences are based on their sizes [9]. In reducing analysis mode, reducing agents such as mercaptoethanol, dithiothreitol, etc., brake the disulfide bridges of proteins enabling to analyze their subunits. As a matter of fact, SDS-CGE is considered as the automated instrumental version of the old fashion sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) [10]. In the early days, sieving medium filled capillary columns employed either covalently cross-linked gel compositions [11−13] or entangled polymer networks [14−16]. From the beginning of the new millennium, transiently cross-linked sugar based polymers are most frequently used [13]. It is important to note, however, that the term CE-SDS is still frequently, but mistakenly used for sodium dodecyl sulfate capillary gel electrophoresis. This misconception was originated from the initial attempts in the late 1990's to separate SDS-protein complexes by entangled polymer solution filled capillaries, which per definition were not considered as gels. Nevertheless, in the past 20 years, transiently cross-linked borate - dextran polymers have almost been exclusively used in the field in sodium dodecyl sulfate

capillary gel electrophoresis mode.

The first capillary gel electrophoresis based size separation of proteins was demonstrated by Karger and coworkers in the late 1980's [11]. They used acrylamide monomer cross-linked by N,N'-methylenebisacrylamide in a final gel composition of 7.5% T/3.3% C 8 (also containing 8 M urea and 0.1% SDS) and obtained baseline separation of the insulin A and B chains (Fig. 2A). The sieving characteristics of the separation matrix was proved by Ferguson plot analysis using the mixture of 4 standard proteins revealing a linear relationship between the logarithmic electrophoretic mobility and the gel concentration, as shown in Fig. 2B.

After these successful early demonstrations, full applicability of the method was proved for size based protein separation by analyzing a large number of otherwise well characterized proteins in a comparative study using SDS-polyacrylamide slab gel electrophoresis and SDS-CGE [17]. The standard calibration plots in Fig. 3 reveal adequate molecular weight *vs.* migration distance (slab)/ time (CGE) correlation with a similar number of outliers including proteins possessing extra high glycosylation and/or a large number of acidic or basic amino acid residues.

Unfortunately, the initial attempts of using covalently cross-linked polyacrylamide gels for SDS-CGE (similar to that of with slab gels), while featured good separation performance, frequent problems occurred including bubble formation in the gel filled column shutting down electric conductance and precipitation of the analytes at the injection end of the capillary, just to mention the most difficult ones to address. To alleviate these issues, several laboratories in the early 90's started to follow the successful utilization of replaceable entangled polymers made nucleic acid analysis successful (e.g., sequencing [18]) in narrow bore capillaries by using linear, non-cross-linked polymer matrices to separate proteins [16,19,20] (Fig. 4).

Among other hydrophilic polymers, polyethylene glycol and polyethylene oxide based matrices gained significant attention in CE based protein size separation [14,21−23]. Benedek and coworkers performed a detailed polymer chain length vs. separation performance study revealing that the 100,000 Da PEO matrix provided very good resolution with short separation time [15]. Linear poly[N-(acryloylamino)ethoxyethanol], featuring significantly higher hydrophilicity, was also attempted for the use in protein analysis by capillary electrophoresis showing greater hydrolysis resistance than that of its linear acrylamide polymer based counterpart [24]. Another advantage of these sieving matrices was their applicability for the analysis of both native and denatured proteins [25]. Polyvinylalcohol in the concentration range of 4−6% was another good sieving polymer for size based separation of SDS-proteins. With the use of such linear entangled polymer networks, as low as 4% molecular mass differences were resolved [16,26]. Regarding entangled polymer solutions, the entanglement threshold was key for all linear polymer based sieving matrices to provide adequate sieving capabilities. Interestingly, however, it was found that decrease in the capillary ID affected the apparent entanglement threshold, shifting to a regime where, e.g., <1% PVA already provided adequate molecular sieving. The phenomenon was explained by the greater surface to volume ratio based higher free surface silanol concentration, acting as nucleation sites promoting hydrogen bonds and polymer aggregation [27].

In addition to the above mentioned frequent bubble formation issues, covalently cross-linked polyacrylamide also had high UV light absorption, not supporting good detection sensitivity. Polyethylene oxide (PEO) and dextran (a glucose polymer) based sieving matrices, on the other hand, featured low UV absorption, therefore, proved beneficial for protein analysis by capillary gel electrophoresis with high sensitivity UV detection even at the low 200 nm range [28]. These polymer matrices also had low
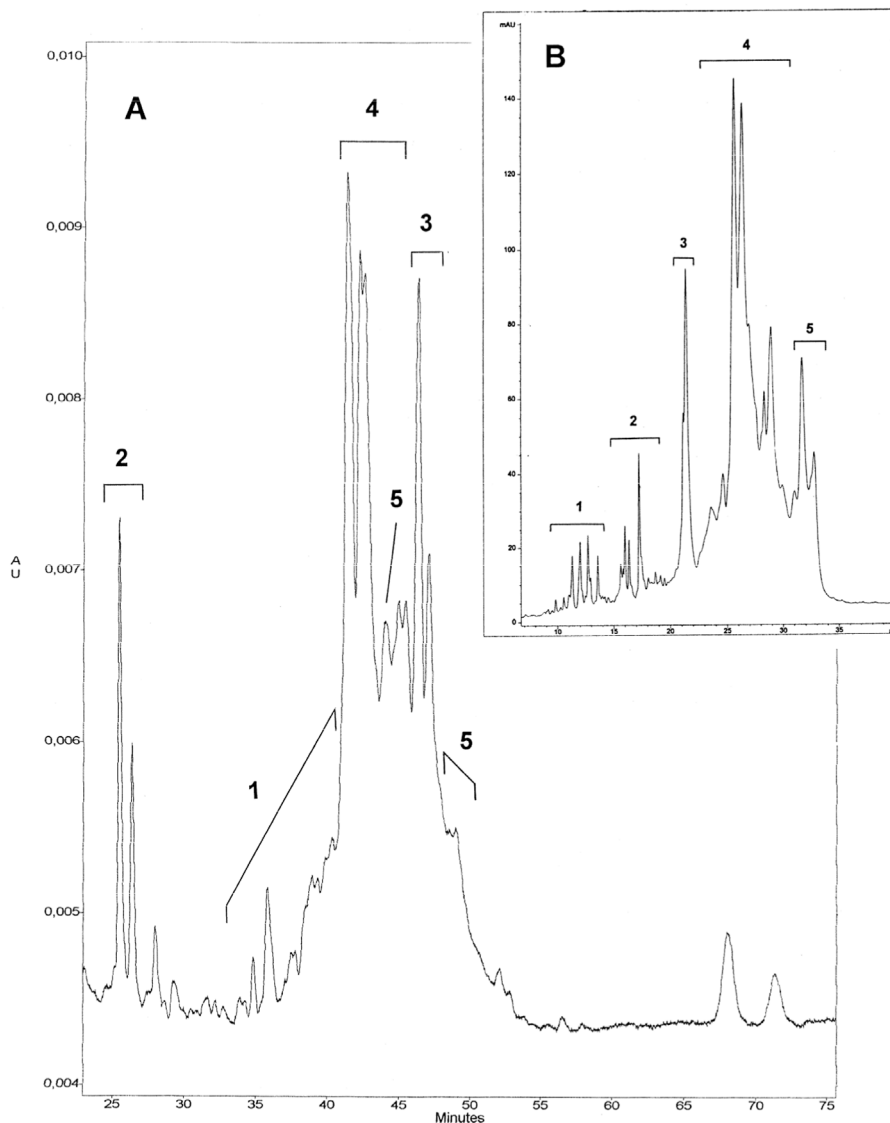
Fig. 1. Separation of collagen cyanogen bromide peptides by (A) capillary gel electrophoresis (Pluronic F127) and (B) high-performance liquid chromatography, showing comparable resolving power. Peak groups: 1: $\alpha_2(I)CB_2$, $\alpha_1(I)CB_2$, $\alpha_1(I)CB_5$; 2: $\alpha_1(I)CB_4$, $\alpha_1(III)CB_3$, $\alpha_1(III)CB_6$, $\alpha_1(I)CB_3$, $\alpha_1(III)CB_4$; 3: $\alpha_1(I)CB_6$; 4: $\alpha_1(III)CB_5$, $\alpha_1(I)CB_7$, $\alpha_1(I)CB_8$, $\alpha_2(I)CB_4$ and incomplete cleavage products; and 5: $\alpha_2(I)CB_{3,5}$, $[\alpha_1(III)CB_9]_3$. With permission from Ref. [7].
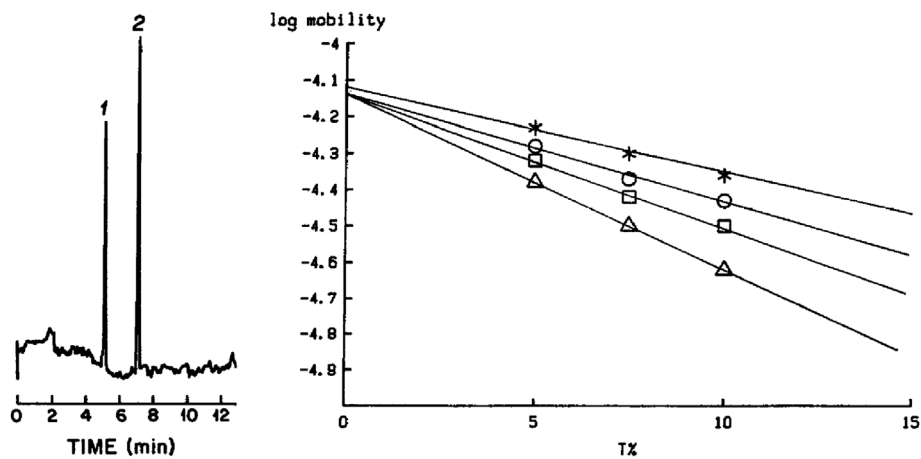

Fig. 2. High-performance capillary SDS polyacrylamide gel electrophoresis of the two insulin chains (left panel, 1: A chain, MW 1640; 2: B chain, MW 3494) showing excellent resolution. The right panel depicts the Ferguson plot analysis of a protein mixture in SDS-CGE proving good sieving capability of the gel composition used. Symbols for the sample components: *: α-lactalbumin (MW 14,000); o: β-lactoglobulin (MW 18,400); □: trypsinogen (MW 23,700); Δ: pepsin (MW 34,500). With permission from Ref. [11].
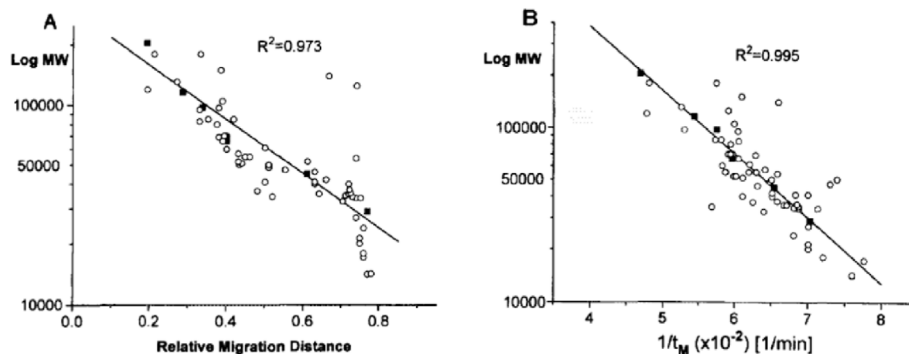
Fig. 3. Molecular weight estimation of >60 proteins using SDS-PAGE slab gel electrophoresis (A) and SDS-CGE (B). In both instances good correlation was found between the migration properties and the Mw of proteins with a similar number of outliers. With permission from Ref. [17].
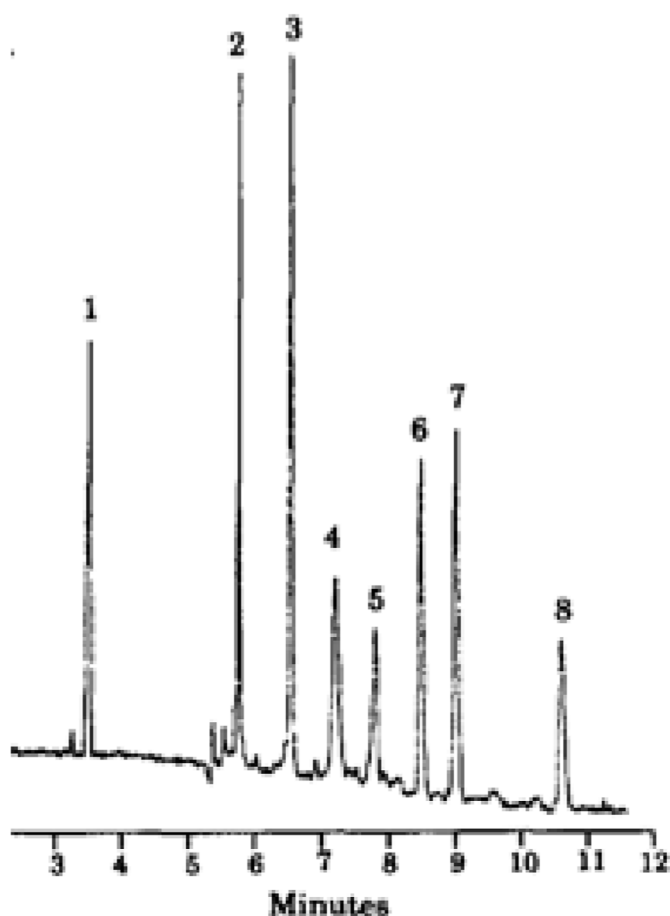

Fig. 4. High resolution protein separation by capillary electrophoresis using an entangled polymer solution. Peaks: 1) mellitic acid (reference marker), 2) α-lactalbumin (MW: 14,200), 3) carbonic anhydrase (MW: 29,000), 4) ovalbumin (MW: 45,000), 5) bovine serum albumin (MW: 66,000), 6) phosphorylase b (MW: 97,400), 7) β-galactosidase (MW: 116,000), 8) myosin (MW: 205,000). With permission from Ref. [16].

viscosities, allowing their easy replenishment in narrow bore separation capillaries to accommodate up to dozens of injections with excellent migration time reproducibility (<0.5% RSD).

The influence of separation parameters such as temperature, capillary length and ID have all been studied in SDS-CGE by several authors since the early 90's of the last century [22,29]. The effect of temperature was comparatively evaluated on the performance of

dextran and PEO based polymeric sieving matrices [30]. The electrophoretic mobility and peak efficiency were investigated for ovalbumin with increasing capillary temperatures in the range of 20 and 50°C. Under constant voltage separation conditions (isoelectric) the electrophoretic mobilities increased with elevated separation temperature in both sieving matrices, while the theoretical plate numbers only increased in the dextran gel but decreased with the PEO based sieving medium, as shown in Fig. 5.

Based on this interesting observations, the Arrhenius method was applied later to understand the activation energy requirement of the electromigration for different size SDS-protein complexes in transiently cross-linked dextran borate gels [31]. In the temperature range of 15−60 °C, no size dependent correlation was found whatsoever in the required activation energy values for the various molecular weight proteins. This reflection suggested the option for separation temperature optimization to achieve good resolution between any sample components of interest in hand. The same study revealed a reciprocal sixth root function relationship between the electrophoretic mobility (μ) and the molecular weight (M_W) of protein sample components, as delineated by equation (1), suggesting cylindrical molecular conformation during electromigration in borate cross-linked dextran gels.

$$\mu = \text{const}_A \bullet Q \bullet M_W^{-1/6} \bullet e^{-E_a/RT} \tag{1}$$

where Q is the charge of the SDS-protein complex, E_a is the activation energy, R is the universal gas constant and T is the absolute
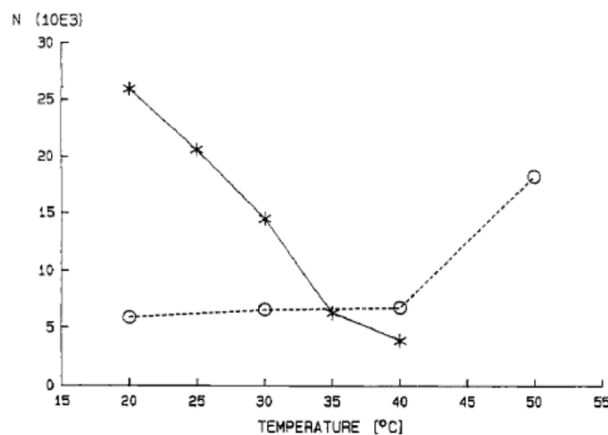

Fig. 5. Effect of the column temperature on the peak efficiency of ovalbumin with PEO (*) and dextran (o) based sieving matrices showing decreasing and increasing tendencies in the PEO and dextran gels, respectively. With permission from Ref. [30].

temperature. This $M_W^{-0.167}$ term was similar to the earlier results of Cooke and coworkers [22] reporting the value of $M_W^{-0.2}$ for the molecular weight term in the mobility equation for the electromigrating SDS-protein complexes in PEO based sieving matrices.

Recent progress in the field demonstrated the importance of SDS-CGE in the process analytical laboratories of the growing biopharmaceutical industry for fast molecular mass assessment and purity evaluation of recombinant protein therapeutics [32]. The technique was mainly utilized for the analysis of monoclonal antibodies [33−35] as it readily separates the main mAb fragments including the light chain (LC), the non-glycosylated heavy chain (ngHC) and the heavy chain (HC) subunits under reducing conditions, while also reveals most different size impurities in non-reducing separation mode [36]. Geurink et al. studied the effect of gel buffer dilution on the separation of viral vaccine proteins [37]. They proposed a multi-step process with multivariate design of experiments based on the identified critical method parameters and applied to SDS-CGE method development. In addition, hard to analyze membrane proteins could also be analyzed by capillary electrophoresis using dynamic sieving matrices [38] and also appropriate for the separation of genetic variants of bacterial protein patterns [39]. Furthermore, the biopharma industry successfully introduced SDS-CGE for purity determination of the capsid proteins of adeno associated viruses (AAV) [40,41]. The sensitivity of the technique can be significantly improved by labeling the samples with a fluorescent dye prior to analysis in conjunction with the use of laser induced fluorescence (LIF) detection [42].

Considering the fact that the borate mediated pseudo cross-linking based transient type dextran gels are capable of high resolution size separation of SDS covered protein molecules [13], various monomer/cross-linker ratios were investigated to shed light on the basis of the electromigration process [43]. This study also included the introduction of three dimensional Ferguson plots. Interestingly, the resulting plots showed non-linear concave behavior suggesting non-classical sieving, as depicted in Fig. 6.

Recently, a general separation optimization scheme was established for SDS-CGE to address the analytical problem in hand, including specific gel compositions for initial screening (10%D/4%B), high resolution (10%D/2%B), separation speed (2%D/4%B) and analysis of highly glycosylated proteins (2%D/2%B), as depicted in Scheme 1.

An interesting correlation was found between the polymer chain distribution and separation efficiency with the use of dextran based sieving matrices [44] also suggesting free draining mechanism for higher molecular weight proteins [45]. Beckman et al. found that the use of sodium hexadecyl sulfate (SHS) to replace SDS in the gel-buffer system alleviated the fronting behavior of highly hydrophobic protein samples in SHS-CGE [46]. In a recent work, the
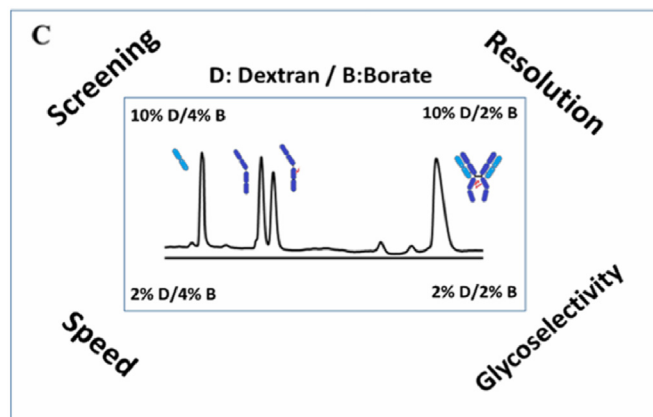
Scheme 1. SDS-CGE separation optimization scheme by varying the monomer/cross-linker ratios of the borate-dextran sieving matrix for initial screening, high resolution, separation speed and analysis of highly glycosylated proteins. With permission from Ref. [13].

separation efficiency in SDS-CGE was revisited in view of electromigration dispersion with the use of borate based co-ions that also served as a cross-linker. Both tailing and fronting peaks were observed for different molecular weight species at various boric acid concentrations suggesting an option for borate concentration based mobility matching to obtain the best possible peak shape and concomitantly high resolution. With the use of different concentration borate buffers in the inlet and outlet reservoirs, transient mobility matching was possible (Fig. 7), along with establishing the long sought pore-size gradient capillary gel electrophoresis option [47].

4. Charge variant analysis by capillary gel isoelectric focusing (cgIEF)

In isoelectric focusing, proteins or peptides are separated according to their isoelectric points (pI) in a pH gradient formed by carrier ampholytes (amphoteric electrolytes) upon the application of an electric potential [48,49]. The process includes the following steps: (1) the sample, mixed with carrier ampholytes, is filled into the capillary with the inlet and outlet ends immersed into a high-pH and low-pH solutions, respectively; (2) the electric field is applied to establish a pH gradient and the protein molecules with different pI values migrate until their net charge becomes zero [50]; (3) detection using a one or two step process. In the one-step cgIEF method the focusing and transport of the focused proteins occur simultaneously in a polymer buffer additive containing background
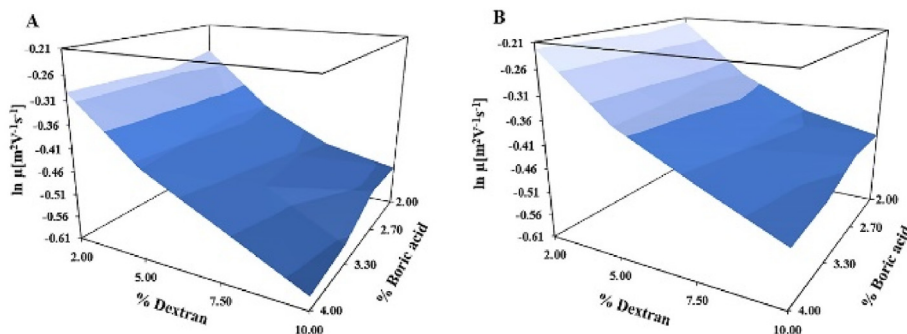

Fig. 6. Three dimensional Ferguson plots of regular (glycosylated, A) and de-*N*-glycosylated (B) etanercept subunits showing non-linear concave shapes, i.e., non-classical type sieving. With permission from Ref. [43].
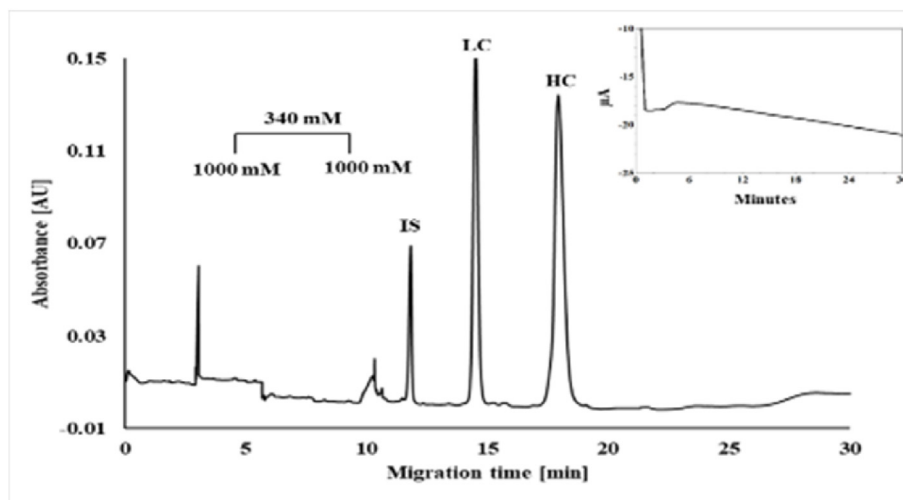
Fig. 7. Borate gradient mediated transient mobility matching for simultaneous peak symmetry optimization for the light and heavy chain fragments of omalizumab in SDS-CGE. With permission from Ref. [47].

electrolyte, like cellulose derivatives [51]. In the two-step process, on the other hand, the analyte molecules are first focused and then the distinct zones are transported by either chemical or hydrodynamic mobilization towards the detection zone [52,53]. In this latter case, EOF free capillary columns are required. CCD camera based detection after laser illumination [54,55] and spatial scanning LIF detection systems [56] can be used for real time imaging detection. In another recently introduced approach, the entire separation channel can be imaged [57], and even hyphenated to mass spectrometry [58].

On the application side, Zarabadi et al. analyzed salivary α-amylase isozymes in a rapid and automated manner within 6 min by UV whole column imaging detection (WCID) [59]. Full and empty adeno-associated virus capsid ratios of different serotypes were determined by using an automated capillary gel isoelectric focusing based rapid and high resolution method [60]. Based on the negatively charged DNA load, full capsids focused at the lower, while the empty ones at the higher pI regime. In another interesting application, capillary isoelectric focusing using linear polyacrylamide (LPA) and polyvinyl alcohol (PVA) coated capillaries was able to distinguish eight to twelve charge isoforms of high molecular-weight glutenin subunits (HMW-GS) with pI values between 4.72 and 6.98 from various wheat cultivars [61].

As a result of not chemical but biological processing, protein therapeutics such as monoclonal antibodies, antibody–drug conjugates (ADCs), fusion proteins and such usually exist in different charge variant forms, thus, should be rigorously monitored at different stages of the manufacturing process. Isoelectric focusing is one of the best approaches to characterize protein charge heterogeneities. Heger and coworkers applied imaged capillary isoelectric focusing (icIEF) for the analysis of three classes of protein therapeutics, a biosimilar for the monoclonal antibody trastuzumab, a recombinant human erythropoietin (rhEPO) and a fusion protein [62]. The reported platform icIEF method was utilized without modification for the characterization of the different protein therapeutics, thus, saved valuable time and resources in method development and quality control. In another work, high resolution capillary isoelectric focusing with WCID was applied for the analysis of rhEPO glycoforms from different sources [63]. Fig. 8 shows the workflow of a novel microchip based system, where imaging based UV detection was combined with instant mass spectrometry detection [64]. The additional MS analysis helped to identify abundant glycoforms in the sample.

Zhang et al. developed an imaged capillary isoelectric focusing method with native conditions where they replaced the denaturant (e.g., urea) containing matrix with non-detergent sulfobetaine and taurine (NDSB-T) [65], a combination to support stabilization and to obtain high separation power, while still maintaining protein integrity. Their approach featured significantly improved repeatability and accuracy for denaturant-incompatible antibodies. A porous layer open-tubular capillary column was developed by Yan and coworkers with an immobilized pH gradient (PLOT-IPG) for cIEF of proteins [66]. First, the PLOT capillary was prepared by in situ polymerization of acrylamide, glycidyl methacrylate and *N,N′*-methylenebisacrylamide, followed by immobilization of Pharmalyte 4.5−6.0 (narrow pH range) or Pharmalyte 3.0−10.0 (wide pH range) in the PLOT capillary column. They applied this new type of capillary column for the analysis of human serum proteins. The obtained sensitivity and resolution in the narrow pH range approach were better than those with the wide pH range one (Fig. 9). The same group also prepared a monolithic capillary column with immobilized pH gradient (M-IPG) for cIEF where Pharmalyte 3−10 was covalently bound onto the monolith in the capillary column [67].

Kim and coworkers reported on a capacitively coupled contactless conductivity detection (C^4D) approach associated with a microfluidic capillary isoelectric focusing device for protein analysis (μCIEF) [68]. Fig. 10 shows the μCIEF-C^4D assay procedure. Protein separation and mobilization were also monitored by a fluorescence microscope to confirm the C^4D signal. A mixture of three different green fluorescence proteins including R-phycoerythrin (pI 5.01), GFP-F64L (pI 5.48), and RK-GFP (pI 6.02) were separated. With the use of this microfluidic device, the average resolution obtained was 2.06. More importantly, the developed label-free μCIEF-C^4D technique can be utilized as a portable, electronics-only protein-analysis tool.

5. Capillary affinity gel electrophoresis (cAGE) of proteins

Capillary affinity gel electrophoresis (cAGE) utilizes the inclusion of affinity-type ligand molecules in the sieving matrix either chemically attaching or non-covalently incorporating the binding agent into the separation gel matrix. The affinity ligands within the gel or polymer solution inside the capillary provide the means of
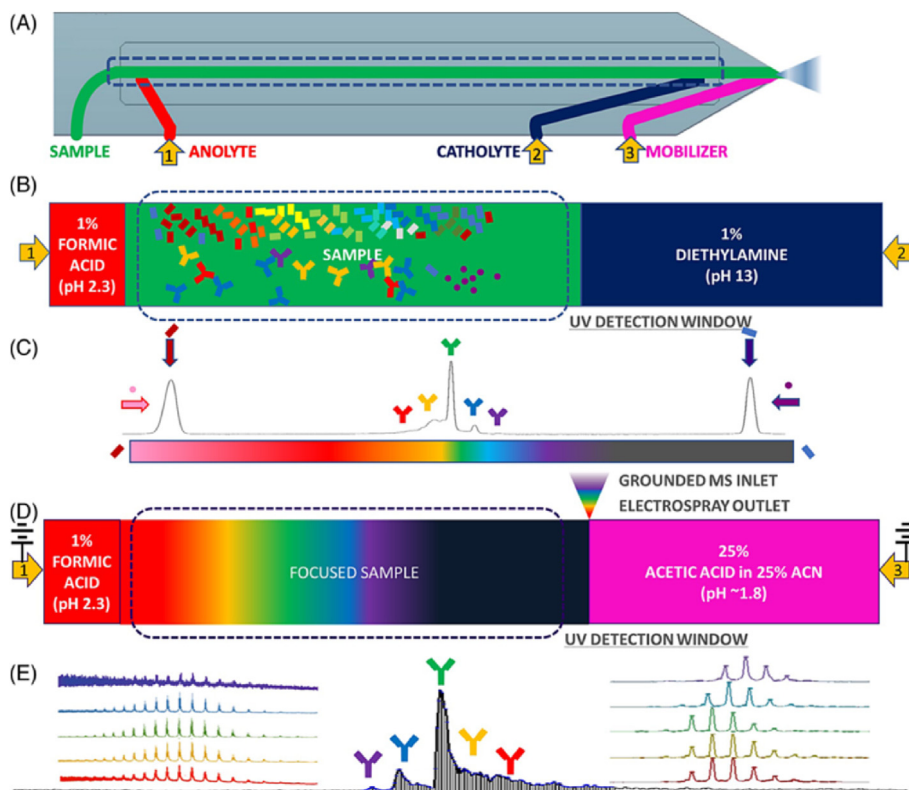
Fig. 8. icIEF-MS workflow for the analysis of a protein biotherapeutic. (A) The microfluidic separation and ionization chip with electrospray outlet; (B) Path for separation showing primed anolyte, sample, and catholyte solutions in the channels; (C) Focused sample UV absorbance electropherogram with highlighted pI markers, sample, ampholyte gradient, and stackers; (D) Path for mobilization initiation and ESI of the separated sample; (E) Time-resolved base peak intensity plot with inset normalized raw (right) and deconvoluted (left) mass spectra. With permission from Ref. [64].
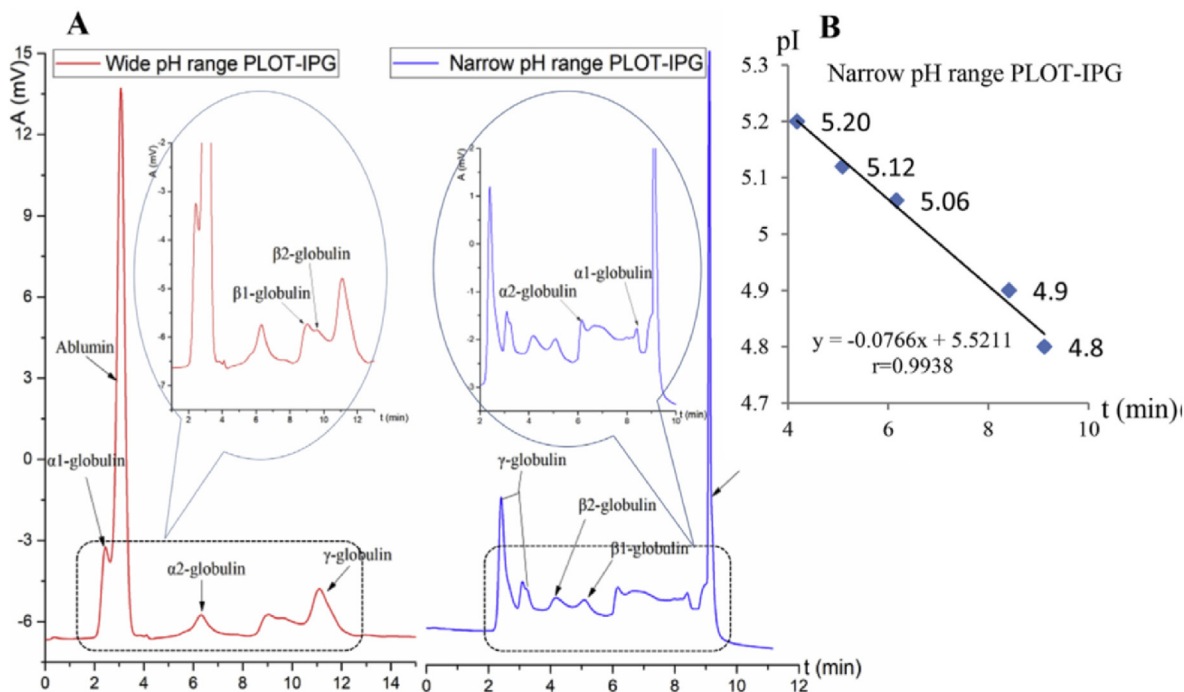

Fig. 9. cIEF analysis of human serum with the wide and narrow pH range porous layer open-tubular immobilized pH gradient (PLOT-IPG) column (A), as well as the linearity correlation of pI versus migration time (B). With permission from Ref. [66].
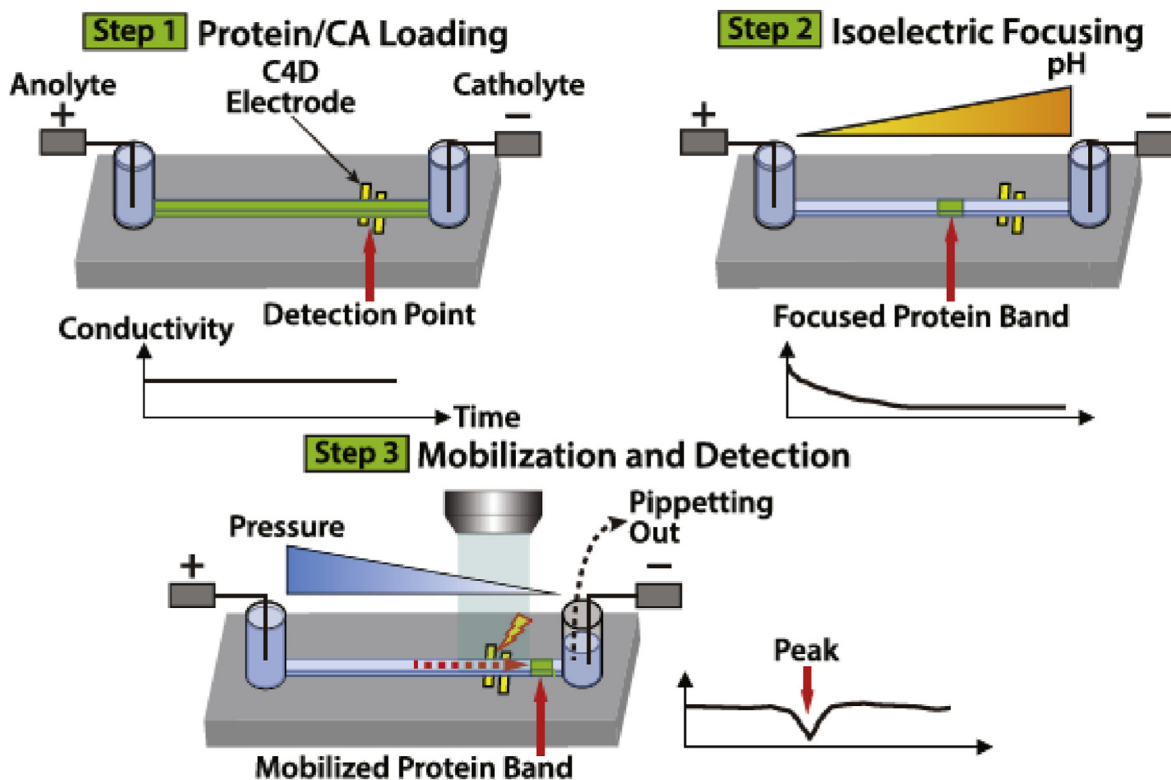
Fig. 10. The μCIEF-C^{4}D assay workflow. Step 1: loading the catholyte and anolyte solutions and the standard separation medium containing proteins after surface treatment of the microchannels; Step 2: isoelectric focusing; and Step 3: mobilization using hydrostatic pressure differences for C^{4}D detection. With permission from Ref. [68].

unique selectivity [69]. Affinity capillary electrophoresis is a useful tool for measuring binding constants, estimation of kinetic rate constants and determination of binding stoichiometries of receptor-ligand interactions in biochemical systems [70].

Haupt et al. developed an affinity capillary gel electrophoresis method utilizing the small ligand of iminodiacetate–Cu(II) that was covalently bound to a polyethylene glycol based replaceable polymer matrix. This metal chelate modified gel was used as a model system to study the interactions of different model proteins, namely ribonucleases, cytochromes *c*, chymotrypsin, and kallikrein [71]. Determination of the dissociation constants were performed using a modified Langmuir adsorption isotherm equation, which was applicable to fast on/off kinetic interactions. Shimura and Karger presented a highly sensitive affinity probe capillary electrophoresis (APCE) approach [72] using a tetramethylrhodamine-iodoacetamide labeled Fab' fragment of a mouse monoclonal antibody as affinity probe. The sample methionyl recombinant human growth hormone (met-rhGH) was mixed with the affinity probe and the associated complex was separated by capillary isoelectric focusing with laser induced fluorescence detection in the concentration of as low as 5×10^{-12} M. The same affinity probe capillary electrophoresis method was used later for the analysis of insulin [73]. Soper and coworkers used fluorescently labeled aptamer affinity probes (HD1 and HD22) for the analysis of thrombin by affinity microchip CGE [74]. Their poly(methyl methacrylate) (PMMA) microfluidic device was filled with a LPA sieving matrix to analyze the affinity complex. Separation of the free aptamer from the thrombin-aptamer complex was attained in less than 1 min. Baseline-resolved peaks with excellent resolution are shown in Fig. 11 with the use of HD22, due to its high binding affinity. Thrombin was successfully determined from the human plasma sample at the concentration level of 543.5 nM with the

developed method.

6. Fluorescent labeling and sample preconcentration techniques for high sensitivity detection in capillary gel electrophoresis

Most proteins have native fluorescence characteristics due to such amino acid residues as tryptophan, however, the enhanced fluorescence properties of fluorophore labels are more effective for a number of applications [75]. Covalent bonding, noncovalent complexation and genetically engineered tags, such as expression with green fluorescent protein (GFP) are the most frequently used fluorophore labeling methods for proteins [75,76]. Covalent modification of peptides and proteins can be performed via the amine, carboxyl and thiol functional groups [77]. In case of labeling via the amine groups, multiple labeling of proteins can occur causing band broadening as the different derivatization rate molecules might migrate slightly differently. Noncovalent labeling is another way to improve the detection limit for protein analysis in CGE [78]. This labeling approach utilizes physical mechanisms, including hydrophobic interactions, electrostatic interactions, hydrogen bonding or groove binding. Noncovalent fluorophore labeling was reported earlier by Csapo et al. using Sypro dyes to obtain low femtomole level sensitivities in capillary dimensions (ultrathin layer gel electrophoresis). The instant, noncovalent fluorophore labeling reagent was added to the sample just prior to injection, skipping the time consuming pre-separation labeling process [79]. Mann et al. applied lab-on-a-chip technology for the analysis of tear proteins [80]. The detection was based on LIF with the use of an intercalating dye, which interacted with the protein/SDS complex. Finally, genetically engineered tagging is very specific since the labeled protein is generated by fusion between the protein of interest and
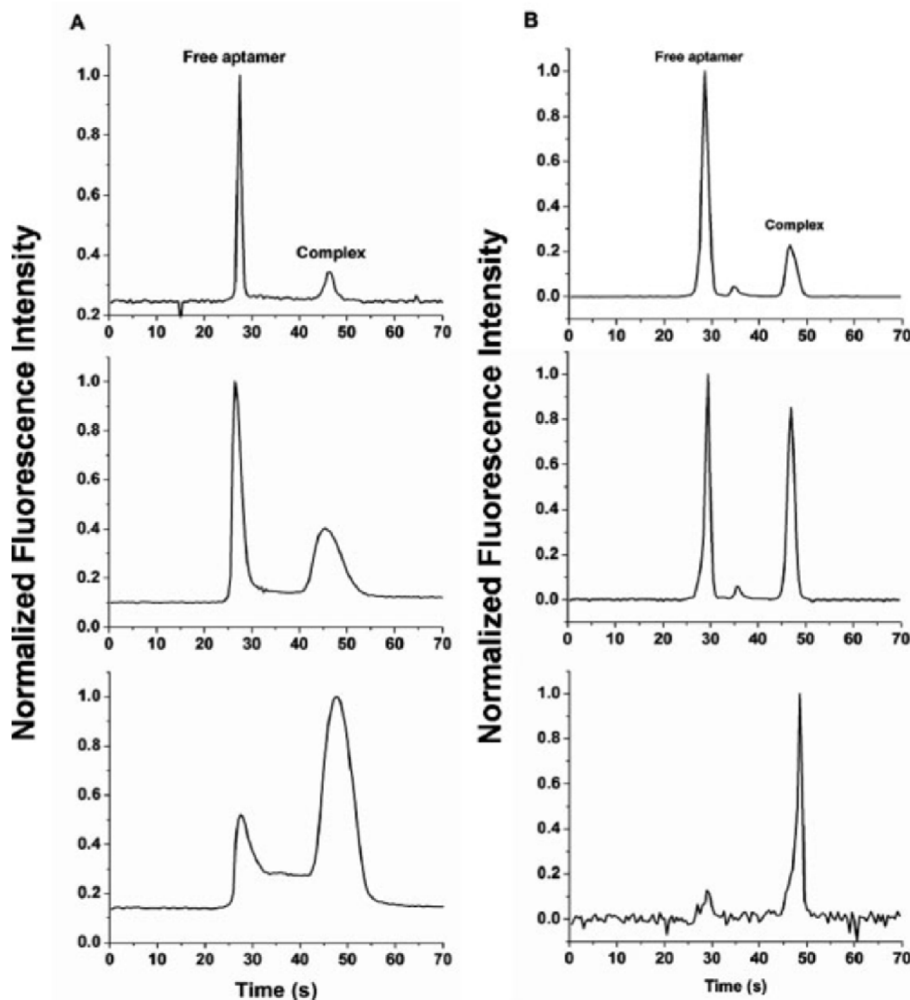
Fig. 11. Capillary affinity gel electrophoresis analysis of the mixtures of 75, 250, and 500 nM unlabeled thrombin incubated with Alexa Fluor 647 labeled HD1 (panel A) and HD22 (panel B) aptamer affinity probes prior to separation showing the corresponding increases in the peak areas of the complex with increasing thrombin concentrations (top-to-bottom). With permission from Ref. [74].

an autofluorescent protein tag [81,82]. The most frequently used fluorescence dyes applied in CGE with LIF detection of peptides and proteins are summarized in Table 1 with their excitation and emission wavelengths [77].

Labeling of proteins with 3-(2-furoyl)quinoline- 2-carboxaldehyde (FQ) and their separation in a replaceable polymer matrix was reported by several groups [84–86]. Important to note that in this instance, the possible multiple labeling phenomena of the sample

Table 1
Fluorescent dyes applied in capillary gel electrophoresis separation of peptides and proteins [83].

| Dye name	Structure/Type	Excitation max (nm)	Emission max (nm)
OPA	ortho-Phthalaldehyde	340	455
NDA	naphthalene-2,3-dicarboxaldehyde	419	493
FC	4-Phenylspiro[furan-2(3H), 1′-phthalan]-3,3′-dione	382	480
FQ	3-(2-Furoyl)quinoline2-carboxaldehyde	486	600
CBQCA	3-(4-Carboxybenzoyl)-2-quinolinecarboxaldehyde	465	550
6-AQC	6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate	248	398
FITC	Fluorescein isothiocyanate	491	516
NBD-F	4-Fluoro-7-nitrobenzofurazan	465	535
NBD-Cl	4-Chloro-7-nitrobenzofurazan	337	512
Chromeo 503	Pyrylium dye	503	600
Cy5	Sulfoindocyanine succinimidyl ester	649	666
ICG	Indocyanine green	789	814
NanoOrange	Merocyanine dye	470	570
Sypro Red	Merocyanine dye	300, 550	630
ANS	l-Anilinonaphthalene-8-sulfonate	350	505
Alexa Fluor 488	Sulfonated fluorescein derivative	490	525
Dylight 488	Sulfonated fluorescein derivative	493	518
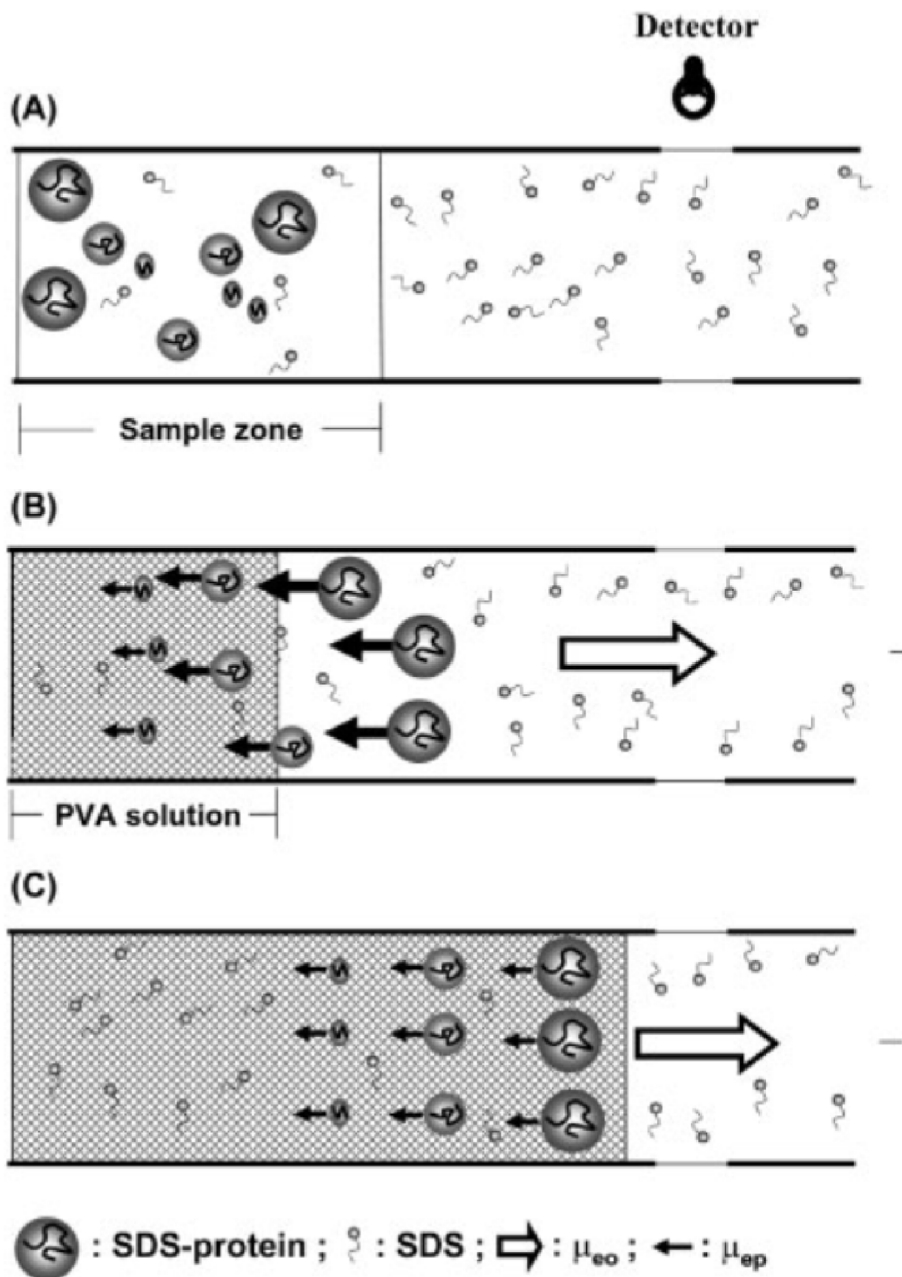
Fig. 12. Polymer solution mediated preconcentration in SDS-CGE. (A) Injection, (B) Stacking and (C) Separation of the stacked SDS protein complexes. With permission from Ref. [91].

proteins did not influence the separation performance. However, unfortunately, the FQ labeling reaction utilizes highly poisonous potassium-cyanide, thus, due to safety concerns it is not preferred. Dovichi and coworkers performed SDS-CGE for various proteins in dextran sieving media and applied two different dyes, namely FQ and 6-(fluorescein-5-carboxamido) hexanoic acid succimidyl ester (FX) for covalent labeling of standard proteins and detected the fluorescence signals at 513 and 630 nm [87]. With the described method, they were able to detect the separated proteins at concentrations of less than 10^{-7} M. Pyrylium dyes such as Chromeo 465 and 503, which do not increase the charge state of the labeled species, were introduced as efficient tags for protein and peptide analysis in CGE [88,89]. This type of dyes produces highly fluorescent products for CGE-LIF detection, thus, picomolar detection limits can be routinely achieved. More

importantly, they have very low fluorescence in their unconjugated form, thus, no purification is required before analysis. SDS-CGE was used with LIF detection for the purity analysis and characterization of adeno-associated virus (AAV) gene therapy vectors, where the labeling of the viral capsid proteins was performed with Chromeo P503 dye [90].

Sample preconcentration techniques to improve detection sensitivity in capillary gel electrophoresis of proteins were mainly developed for SDS-CGE. In one instance, the polymer filled capillary was used for preconcentration (Fig. 12) by hydrodynamically injecting hundreds of nanoliters of SDS-protein complexes into the narrow bore column filled with the running buffer followed by immersing both ends of the separation capillary in polyvinyl alcohol (PVA) solution. When positive voltage was applied, the
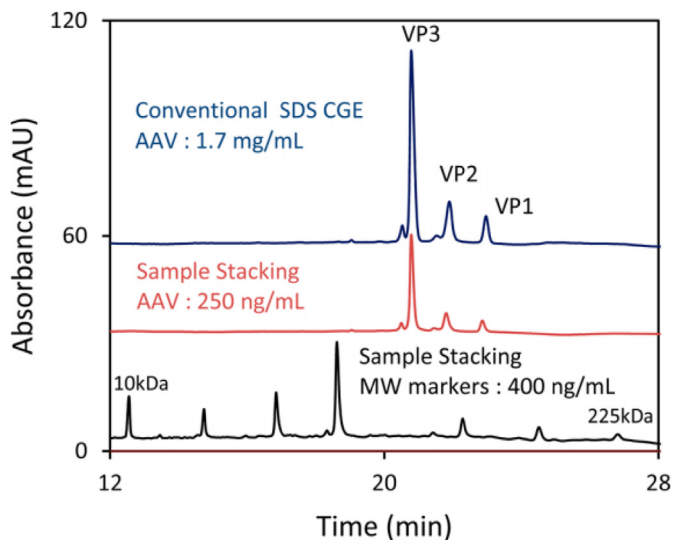
Fig. 13. Comparison of on-line sample stacking coupled SDS-CGE to conventional SDS-CGE. With permission from Ref. [92].

neutral PVA solution gradually filled the column from the inlet end driven by the EOF, and the proteins migrating towards the anode (inlet) were stacked in the PVA plug, which also acted as a sieving matrix. In particular, when a Tris-Tricine-HCl running buffer was applied, the PVA matrix was located between high mobility chloride and the low mobility Tricine ions resulting in stacking based preconcentration of the sample proteins by transient isotachophoresis (tITP). The limit of detection for BSA with PVA based tITP-SDS-CGE was 0.78 nM [91].

The Meagher group coupled on-line sample stacking with SDS-CGE, and achieved excellent detection sensitivity [92]. After pressure injecting of a short water plug into high conductivity running buffer filled uncoated fused silica capillary, protein-SDS complexes with an excess SDS were electrokinetically injected by applying a reverse electric field. Due to the higher potential drop in the low conductivity water plug, the protein-SDS complexes rapidly moved toward the anode (detection end) and slowed down at the boundary of the higher conductivity (i.e., lower potential drop) running buffer causing sample stacking. At the same time the EOF was directed toward the cathode (inlet) causing the excess high conductivity SDS zone moving out of the capillary, while the protein-SDS complexes moved into the capillary making the stacking effect more efficient. The limit of detection was 0.2 ng/mL (3.3 pM) for virus capsid proteins using a UV absorbance detector, a level comparable to silver staining SDS-PAGE and SDS-CGE with LIF (Fig. 13).

In-line coupled single drop microextraction (SDME) utilizing multi-layer of an acceptor drops at the inlet end of a separation capillary can also be efficiently utilized for preconcentration as recently reviewed in Ref. [93]. However, SDME of zwitterions such as proteins and peptides is challenging due to their low solubility in organic solvents, but by addition of a carrier like nonane-1-sulfonic acid (to the donor phase) [94] or Aliquat 336 (to the organic layer) [95] to form an ion pair complex significantly improved performance.

7. Conclusions and future prospective

The analysis of therapeutic proteins and protein biomarkers are of high importance in the biopharmaceutical industry and biomedical field. Different kinds of capillary gel electrophoresis

methods are important parts of the bioanalytical toolsets and some of them represent the gold standard in protein analysis such as SDS-CGE and cgIEF. Future trends of development include miniaturization and multiplexing of capillary gel electrophoresis based methods into high throughput lab-on-a-chip devices to fulfill the increasingly growing analytical needs, both for bioharma and clinical laboratory applications. Another important future strategy is the implementation of portable, handheld, point of care biomarker detection devices for the biomedical field. In addition, the newest trends in labeling and detection technology can further improve the sensitivity and accuracy of capillary gel electrophoresis based methods for protein analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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