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Protein Separation by Capillary Gel Electrophoresis: A Review

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Abstract

Capillary gel electrophoresis (CGE) has been used for protein separation for more than two decades. Due to the technology advancement, current CGE methods are becoming more and more robust and reliable for protein analysis, and some of the methods have been routinely used for the analysis of protein-based pharmaceuticals and quality controls. In light of this progress, we survey 147 papers related to CGE separations of proteins and present an overview of this technology. We first introduce briefly the early development of CGE. We then review the methodology, in which we specifically describe the matrices, coatings, and detection strategies used in CGE. CGE using microfabricated channels and incorporation of CGE with two-dimensional protein separations are also discussed in this section. We finally present a few representative applications of CGE for separating proteins in real-world samples.

Keywords

Capillary gel electrophoresis; Proteins; Capillary electrophoresis; Capillary sieving electrophoresis

1. Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, see Table I for a list of acronyms used in this paper) has been used for size-based separations of proteins for over four decades [1, 2], and it is still the workhorse for protein separations and analyses in most biological research laboratories. The basic procedures of this method include: 1) preparing a gel and assembling the gel apparatus, 2) mixing a protein sample with a buffer containing SDS and cooking the mixture at an elevated temperature, 3) loading the protein-SDS mixture into the gel inside the gel apparatus and performing the electrophoresis, and 4) fixing, staining/de-staining, and quantitating the separated proteins. If SDS is allowed to react with sample proteins completely, the reactions should produce SDS-protein complexes having similar charge densities (or mass-to-charge ratios). When these complexes are electrophoretically separated, their mobilities will depend on their sizes, with smaller proteins having greater mobilities. The mobility values decrease linearly with the logarithm of protein molecular masses, which is the basic separation principle of SDS-PAGE. However, the technique is time-consuming and labor-intensive. The many manual operations (*e.g.*, gel preparation, sample loading, staining/de-staining, etc.) are believed to be sources of SDS-PAGE irreproducibilities.

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SDS-capillary gel electrophoresis (SDS-CGE), also called capillary sieving electrophoresis (CSE) or capillary gel electrophoresis (CGE), shows many advantages over classical SDS-PAGE. These advantages include on-column detection, automated operation, great resolving power, and capability of accurate protein quantification and molecular weight determination [3–8]. The first papers on CGE were published in the 1980s [9, 10]. As in slab-gels, agarose and cross-linked polyacrylamide (CPA) was used as sieving matrices, and these matrices were prepared directly inside the capillary columns. In the early 1990s [11], linear polyacrylamide (LPA) was introduced to replace CPA, but an in-capillary polymerization procedure was still used for the gel preparation. The lifetimes of these columns were limited (usually less than 10 runs) [12], and the run-to-run reproducibility was poor. Currently, replaceable and water-soluble linear or slightly branched polymers, such as linear polyacrylamide [11–13], poly(ethylene glycol) [11], poly(ethylene oxide) [14], dextran [15–17], pullulan [18, 19] and cross-linked polyacrylamide [20–22] are used as sieving matrices for CGE [5, 11, 23–25]. Availability of these polymer matrices has led to improved reproducibility and robustness of this methodology.

Recently, CGE has been recognized and established [26] as an important tool in biopharmaceutical industry to support analytical characterization, process development, and quality control of therapeutic recombinant monoclonal antibodies (rMAbs) [26–29]. In an effort to make CGE-based methods accepted by biotechnology companies, scientists in various pharmaceutical industries and regulatory authorities conducted cross-laboratory research to examine the reliability and robustness of the method [30, 31]. It is expected that some CGE methods will soon be used in pharmaceutical and biotechnological industries. In light of this advancement, we write this paper to review briefly the progress of CGE for protein analysis. We focus mainly on the methodology and application aspects of CGE. In the methodology aspect, we review the common sieving matrices, wall coatings, and detection strategies used in CGE. CGE performed in microfabricated channels and CGE as one dimension in two-dimensional (2D) separations are also discussed. In the application aspect, we present a few separations related to or closely related to practical uses. Table II provides a summary of literatures on CGE of proteins based on the sieving matrices used.

2. Methodology

The basic apparatus for CGE is identical to that of capillary zone electrophoresis (CZE) and consists of a capillary column, an on-column detector, and a high voltage power supply. The major difference between the two techniques is the separation media: a sieving matrix is employed in CGE while a background electrolyte solution is utilized in CZE.

2.1. Sieving Matrices for CGE

Polyacrylamide (PA) has been widely used in slab gel electrophoresis of proteins, and consequently it is frequently utilized in CGE. Initially, PA gels were synthesized in-situ inside capillaries [10, 32, 33]. Typically, a capillary column was prepared by mixing acrylamide (monomer), N,N'-methylenebis(acrylamide) (Bis, cross-linker), ammonium peroxy-disulfate or ammonium persulfate (radical initiator), N,N,N',N'-tetramethylethylenediamine (TEMED, catalyst) and other background electrolytes, introducing the mixture into the capillary, and allowing the solution to polymerize inside the capillary. While this worked in general, problems occasionally arose when PA shrank during polymerization, breaking PA gel into segments and/or forming bubbles inside the column. Additionally, a good column could work well for only the first a few runs, as large molecules and particulate materials accumulated at the injection end of the column, which deteriorated and eventually shut down the separation.

To address this issue, a replaceable sieving polymer – a low viscous LPA solution – was prepared. This sieving matrix was successfully used for DNA sequencing [34], as well as for protein sizing [12]. Because the sieving polymer inside a separation column could be replaced after each run, the run-to-run reproducibility was improved considerably.

It should be noted that, when LPA was developed, its low viscosity (or replaceability) was emphasized. This might be why CPA was rarely investigated as a replaceable sieving matrix initially, because common sense tells that a cross-linked polymer would have a high viscosity. In 2005, Lu et al. [35] noticed that, if the degree of cross-linking was carefully controlled, CPA was an excellent replaceable sieving matrix – superior over LPA for protein separations in many aspects. Using this sieving matrix, CGE was capable of resolving proteins ranging from ~4–250 kD in less than 20 min.

When PA sieving matrices are used to run CGE, capillary walls often need to be coated for achieving high quality separations. Poly(N,N-dimethylacrylamide)-grafted PA, a derivative of PA, was prepared by Zhang et al. [36] in 2006, and when this polymer was used to sieve proteins, capillary wall coating could be avoided. This is because poly(N,N-dimethylacrylamide)-grafted PA is capable of coating capillary walls dynamically.

Various polysaccharides form another important type of sieving matrices for protein separations. One advantage of polysaccharides is that these polymers do not absorb as much UV light as PA does. Ganzler et al. [11] separated SDS-protein complexes using dextran and poly(ethylene glycol) (PEG). The separated proteins were detected at 214 nm in which dextran and PEG are transparent. These matrices had moderate viscosities and could be conveniently replenished. Luo et al. [17] performed high-throughput protein analysis by multiplexed SDS-CGE, and Xu et al. [37] realized separation and characterization of SDS-protein complexes on a microchip with UV adsorption detection using similar matrices. Hydroxypropyl cellulose (HPC) is another polysaccharide sieving matrix used in CGE. For example, Hu et al. [38] developed a CGE-laser-induced fluorescence (LIF) method for separating proteins from HT29 cancer cells. Pullulan [24, 39, 40] and hydroxyethyl cellulose [41] were used for CGE, as well.

Other polymers have also been utilized for protein sieving. Yu et al. [42] used poly(vinyl alcohol) (PVA) to perform on-line protein concentration and separation. Bernard et al. [43] used poly(2-ethyl-2-oxazoline) for CGE and achieved separation efficiencies of ~10 million plates per meter. Hu et al. [8] used polyethylene oxide (PEO) to analyze the protein contents in a single HT29 cancer cell and obtained protein profiles similar to those determined by other methods.

Using dynamic light-scattering, Sumitomo et al. [44] evaluated the mesh size and homogeneity of three sieving polymer solutions, PA, PEO and HPC. Based on their experimental results, these authors concluded that an optimal sieving polymer for separating proteins ranging from 14.3 to 97.2 kD is a homogeneous polymer network with a mesh size of less than 10 nm. Sumitomo et al. also stated that PEO in solution can aggregate, degrade into smaller pieces, and form polymer-micelle complexes with SDS. This disturbs protein-SDS complexation and impairs the protein separation efficiency. Recently, the same group surveyed the composition of the separation buffers, and results showed that Tris-CHES buffer was able to suppress SDS adsorption to PEO and achieve separation of six proteins [45].

Commercial sieving kits are now available to run CGE. These kits are largely from Beckman-Coulter (www.beckmancoulter.com), Agilent Technologies (www.agilent.com) and Bio-Rad Laboratories (www.bio-rad.com) and they are optimized for their CGE instruments. Beckman SDS Gel was demonstrated to be capable of sizing membrane

proteins [46], protein biotoxins [47], and antibodies [48], but coated capillaries or channels were usually needed to achieve good separations [49]. Using Bio-Rad CE-SDS run buffer, Na et al. [50, 51] used uncoated fused-silica capillaries for CGE and separated poly(ethylene glycol)-modified proteins. This kit was also employed for quantitative analysis of antibodies [52], RuBisCo in spinach [53], and water-/salt-proteins from bovine and ostrich meat [54]. Agilent commercialized the first microchip capillary electrophoresis system (Agilent 2100 Bioanalyzer), along with its microchips. Agilent 2100 Kit was provided with this instrument and utilized for analysis of half-antibody [55] and glycoproteins by microchip CGE [56, 57]. In these applications, the gel was pipetted into the designated reservoirs on a chip and propelled, by use of a syringe, into the chip channels.

2.2. Capillary Coatings

The interior walls of capillaries used in CGE are often coated for two purposes: reducing protein-wall interactions and suppressing electroosmotic flow (EOF). An uncoated wall can interact with proteins electrostatically (if part of the protein molecule is positively charged) and/or hydrophobically (if a portion of the protein molecule is hydrophobic), and these interactions deteriorate separation efficiencies. In CGE, the strengths of these interactions are greatly reduced because proteins have reacted with SDS forming hydrophilic and negatively charged SDS-protein complexes. Therefore, wall coating in CGE is used primarily for EOF suppressions.

Running CGE at low or zero EOF is important for achieving reproducible results. If one uses an uncoated capillary to run CGE, the EOF will carry the sieving matrix from anode to cathode while SDS-protein complexes migrate in the opposite direction. Some of the proteins will never pass the detector, unless the EOF is so large that it brings all SDS-protein complexes to the detector. Usually, this condition cannot be guaranteed. Another problem associated with EOF is its instability as the wall conditions change. The fluctuation of EOF causes the migration time change, and subsequently the separations become irreproducible. Including an internal standard in samples can mitigate this problem, as long as the standard does not interfere with protein peak detections. Pugsley et al. [58] developed a dye (fluorescently-labeled aspartic acid) that worked well as an internal standard, because it migrates faster than all fluorescently-labeled SDS-protein complexes.

Numerous approaches have been explored to control/suppress EOF, and the most commonly used approach is to derivatize capillary walls via either dynamic coatings [59–62] or covalent coatings [63–65]. Progress in the field of polymeric coatings can be found in a number of reviews [66–68].

A dynamic coating, due to its simplicity, is a convenient way to modify capillary wall properties. It is normally produced by putting appropriate additives (often polymers) into SDS-SGE run buffers (or sieving matrices) and flushing the capillary columns with these run buffers before separation. Several polymers, including polydimethylacrylamide [61], epoxy poly(dimethylacrylamide) [69–71], and poly(-hydroxyethylacrylamide) [62] were used to create a dynamic coating. The exposure of silica surfaces to very dilute solutions of these polymers causes development of dense polymer layers via hydrogen bonding, electrostatic attractions and/or hydrophobic forces [72]. The molecular weight of the polymer has a strong impact on the stability of the coating since the adhesive forces/energies per chain increase in proportion to the number of monomer units [73]. Some CGE sieving matrices are excellent dynamic coating additives [8, 51, 58, 74]. With these matrices, bare capillaries can be used directly for protein separations.

Covalent coatings are generally more stable than dynamic coatings. These coatings are obtained by chemically bonding desired substances to capillary walls. One of the most

common coating protocols was introduced by Hjerten in 1985 [65]. Typically, 3-(trimethoxysilyl)propyl methacrylate is first attached to a capillary wall, leaving acrylic groups exposed on the wall surface. The capillary is then filled with a polymerizing solution containing acrylamide and a polymerization initiator. The free acrylic groups attached to a capillary wall serve as anchors for growing linear polymer chains. A problem of this coating is that linear molecules cannot cover the capillary wall completely. The poorly covered area will adsorb proteins and create EOF. To improve this situation, a CPA coating was developed by Gao and Liu in 2004 [75] and successfully used for SDS-CGE [35].

2.3. Microfabricated channels for CGE

Microfabricated (or microchip) devices are developed with a goal to perform and integrate multiple analytical processes (e.g. sample pretreatment, solution distribution/mixing, separation, detection, etc.) on a chip platform [76, 77]. Due to the short column length and high separation efficiency, microchip CGE is generally fast, typically from a few seconds to a few minutes. Yao et al. [78] is recognized as the first who performed SDS-PAGE in a microfabricated channel, and the separations were completed in less than 1 min. By combining an on-chip dye staining with an electrophoretic dilution step (similar to a destaining step), Bousse et al. [79] obtained excellent resolutions for microchip CGE of proteins. On the basis of this work, the first commercial microchip instrument was constructed by Agilent Technologies. In 2004, Han et al. [22] and Herr et al. [80] applied an in-channel photopolymerization approach to prepare CPA gels inside a microchip channel for SDS-PAGE, and a separation speed of <30 s per run was demonstrated. These authors also prepared a gradient CPA gel for on-chip protein sizing [20] and successfully implemented sample pre-concentration using these photo-patterned gels [21]. Huang et al. [81] combined isotachopheresis (ITP) to concentrate proteins for subsequent CGE. Xu et al. [82] performed on-line electrokinetic supercharging preconcentration on a microchip to improve method sensitivity. Tsai et al. [83] tested simultaneous separations of both native and SDS-denatured proteins on a single microchip with 36 microchannels. Herr et al. [84] recently integrated saliva pretreatment (mixing, incubation, and enrichment) with subsequent quantitative immunoassays and measured the concentration of endogenous MMP-8 in saliva. More recently, He and Herr [85] photopatterned different gels inside a microfluidic chamber for protein immunoblotting. Fig. 1 presents the immunoblotting chip. Gel-separation was first performed in the vertical dimension, and the separated proteins were then transferred to the immunoblotting gel in the horizontal dimension. Electric fields were applied to the chamber via the parallel microchannels, and the microchannel arrays were designed such that uniform electric fields were produced over the chamber area during separation and transfer steps in both the vertical and horizontal dimensions.

In 2005, Fruetel et al. [47] reported a hand-held microchip instrument called μ ChemLab™ that is capable of performing CZE and CGE in parallel. The instrument consisted of a microfluidics module, a dual channel LIF detection module, an integrated multichannel high-voltage power supply, and a main control board containing the laser diode drivers, user interface, and an embedded microprocessor (see Fig. 2A). It has an approximate volume of 7×8×4.5 cubic inches (see Fig. 2B).

Microchip devices were originally fabricated on glass substrates [86, 87]. Over the past decade, polymeric chips have attracted growing attention, due to the low material and fabrication costs. Polystyrene [88], polyesters [88], polycarbonate [89], poly(dimethylsiloxane) (PDMS) [90] and poly(methyl methacrylate) (PMMA) [91] were used to fabricate microchips. Hybrid materials are also used [49]. All these chips have been tested for CGE separation of proteins. Performance of microchip-based gel electrophoresis has been compared with that of capillary-based gel electrophoresis [41, 56, 57, 92–94]. In general, the performances are comparable, while microchip CGE provides faster separations.

2.4. CGE as One Separation Dimension in Two-Dimensional Protein Analyses

2D separation techniques are powerful tools for protein analysis, because the peak capacity of a 2D analysis is the multiplication product of the peak capacities of the two individual dimensions. To realize this resolving power, Chen et al. [90] constructed a 2D separation device using reconfigurable PDMS slabs in 2002. Four slabs were used to make channels and reservoirs to perform the first dimensional (1st-D) separation – isoelectric focusing (IEF). Then, the middle two slabs containing the IEF-resolved proteins were inserted into another two pieces of slabs which contained multiplexed channels for the second dimensional (2nd-D) separation – CGE. Because of their elastomeric nature, PDMS slabs could be attached and detached reversibly without fluid leaking. Although 2D separations were performed, high resolving power was not demonstrated using this device.

Griebel et al. [19] fabricated 300 parallel channels (64 mm long $\times$ 50 μ m wide $\times$ 50 μ m deep) on a PMMA chip. A 50- μ m opening was produced at one end of chip across all these parallel channels. To prepare for the separation, these parallel channels were filled with 15 % (w/v) pullulan. IEF (the 1st-D separation) was performed first on a separate device – a conventional immobilized pH gradients (IPG) strip. After IEF, the IPG strip was brought to the opening on the chip for parallel CGE (the 2nd-D) separations. However, 2D separation results were not disclosed in this paper.

IEF and CGE were incorporated in the above devices, but they were coupled in an off-line fashion. To implement on-line integration, Li et al. [89] integrated IEF with CGE on a polycarbonate microchip using PEO as their sieving matrix. Fig. 3 presents the channel layout of this chip: one horizontal channel intersected by eight parallel vertical channels. The IEF was performed in the horizontal channel, and SDS-PEO gel electrophoresis was performed in the vertical channels. Preferably, an SDS-PEO sieving matrix should be filled in the vertical channels before IEF was performed. However, the device as designed had a limitation in this regard. Because the 1st-D and the 2nd-D channels were directly connected, the SDS in the SDS-PEO matrix in the 2nd-D channels would bleed into the 1st-D channel due to molecular diffusion and electric field distortion at the channel intersections during IEF. The presence of SDS in the 1st-D channel would bind to proteins (which would add negative charges on the proteins), and therefore ruin the IEF. To circumvent this problem, the authors filled the 2nd-D channels with a matrix containing PEO but not SDS. The SDS required for the 2nd-D separation was electrokinetically introduced to the matrix after IEF was complete. During the SDS introduction, the protein bands focused based on their *pI* values were diffused/broadened before they were conjugated with SDS and electrokinetically injected to the 2nd-D channels. Thus, some IEF resolution was lost.

In 2008, Liu et al. [95] carried out IEF and parallel SDS gel electrophoresis on a similar device. PA gel plugs were patterned via photopolymerization at various locations to stop hydrodynamic flows between reservoirs/channels and thus prevent unwanted bleeding/mixing. These gel plugs may cause problems when channels require frequent washing.

It should be noted that the concept of this 2D separation chip had been discussed earlier [96], but 2D separation results were never published.

In a separate effort, Yang et al. [97] combined capillary isoelectric focusing (CIEF) with CGE in a linear format (see Fig. 4A) via a polyethersulfone dialysis hollow fiber interface. Fig. 4B illustrated the detailed structure of this interface. After hemoglobin variants were focused in the CIEF capillary, the catholyte in the reservoir on the methacrylate plate was replaced by a CGE buffer. The CGE buffer also served as a chemical mobilization solution for the CIEF. As voltages were applied to both capillaries, CIEF-resolved protein bands were chemically mobilized to the hollow fiber. At the same time, negatively charged SDS

continuously migrated into the hollow fiber and reacted with the proteins (forming SDS-protein complexes), and the SDS-protein complexes were subsequently injected into the CGE capillary for the 2nd-D separation. Because the CIEF-resolved proteins were continuously injected into the CGE capillary, some of the resolving power was sacrificed.

Additionally, Michels et al. [18] coupled CGE (the 1st-D) with MEKC (the 2nd-D) by connecting the exit-end of a CGE capillary to the sampling-end of an MEKC capillary. A small gap was left between the two capillaries and filled with an MEKC running buffer to facilitate electric field application and sample injection for MEKC. Two high voltage (HV) power supplies were used in this work. HV1 was used to execute sample injection and separation, and HV2 was utilized for MEKC. After a period of initial CGE separation, a fixed length (e.g., 10 s migration) of CGE-resolved protein band(s) was allowed to enter the gap. HV2 was turned on to apply a potential to the gap solution so that there was no electric field across the CGE capillary (to stop the CGE), while an electric field was created across the MEKC capillary to inject the proteins in the gap into the MEKC capillary and execute the MEKC separation. [Note: HV1 was on all the time.] When the MEKC separation was complete, HV2 was turned off for a given period of time (e.g., 10 s) so that more CGE-resolved proteins entered the gap. Then, HV2 was turned on again to execute the sample injection and MEKC separation. These operations were repeated until the proteins inside the CGE capillary were exhausted. This separation technique was successfully applied for separations of proteins from bacterium *Deinococcus radiodurans* [18] and proteins from single mammalian cells [40]. The method was later modified, and the separation speed was improved from 3–5 h per run to ~1 h per run [98].

In 2006, Shadpour et al. [91] incorporated CGE with MEKC on a PMMA device. Fig. 5A shows the channel layout of the microchip. By applying a vacuum to reservoir *D* while reservoirs *E* and *F* were sealed, a sieving matrix was aspirated into *d*₁ channel from reservoir *C*. As soon as the sieving matrix reached point *d*₂ (as shown in Fig. 5B), the vacuum on reservoir *D* was removed. An MEKC buffer was pressurized into *d*₃ channel from reservoirs *F* while reservoirs *A*–*C* were sealed. A protein mixture was injected into *d*₁ for CGE. As the first protein peak approached point *d*₂, appropriate voltages were applied to various reservoirs to stop CGE and effect MEKC. After MEKC was complete, voltages on the reservoirs were changed to stop MEKC and resume CGE for a short period of time (e.g., 0.5 second) to allow a fraction of CGE-resolved protein band to migrate toward point *d*₂. These operations were repeated until all CGE-resolved proteins were separated by MEKC. Complex proteins samples were analyzed using a similar chip and approach [99].

2.5. Detection Strategies

UV absorption is the most commonly used detection mode in CE, including CGE [15, 100, 101]. Proteins can be detected easily by a UV absorbance detector, because the peptide bonds between amino acids and aromatic side groups in protein molecules absorb UV light at 200–220 nm and 280 nm, respectively. Owing to the limited optical path length, the concentration sensitivities of UV absorption detection are normally low, especially when narrow capillaries are used.

LIF detectors are commonly used in CGE to improve concentration sensitivities. When an LIF detector is used, proteins need to be fluorescently “labeled”. Proteins have been covalently labeled by fluorescent dyes, such as naphthalene–2,3-dicarboxaldehyde (NDA) [102], 3-(2-furoyl) quinoline–2- carboxaldehyde (FQ) [8, 103, 104] and fluoresceinisoithiocyanate (FITC) [105, 106]. In 2007, Michels et al. [107] reported an improved fluorescent derivatization method for proteins analysis by CGE. In this assay, rMAbs were derivatized with FQ in the presence of cyanide (CN[−]). This technique

minimized sample preparation artifacts and greatly improved detection sensitivity of FQ-labeled rMAbs.

Covalent labeling method has an intrinsic problem. A protein molecule usually has a number of sites that can react with a fluorescent labeling dye. Because these sites have different reactivities, it is challenging to make all sites to be labeled with the dye. This labeling reaction produces a mixture in which some proteins are un-labeled, some are fully-labeled, while the majority is partially-labeled. This mixture will cause peak-broadening or even multiple peaks in CE separations [108]. A postcolumn labeling method is often a good approach to address this problem. In 2009, Kaneta et al. [16] reported a postcolumn derivatization method for CGE separations of proteins. The method used a labeling dye of naphthalene-2, 3-dicarbaldehyde in the presence of 2-mercaptoethanol which played a role of a reducing agent in the derivatization reaction. Recently, these researchers replaced 2-mercaptoethanol with ethanethiol as the reducing agent and improved the method limits of detection by 1.4- to 4.5-fold [109].

Alternatively, proteins can be dynamically labeled with fluorescent dyes [110]. In 2001, Jin et al. [111] showed that SDS-protein complexes could be dynamically labeled with NanoOrange. NanoOrange does not fluoresce much in aqueous solutions, but as it binds to a protein-SDS complex, it fluoresces substantially. Sano et al. [112] took a similar approach for CGE analysis of collagenase. Chiu et al. [74] labeled proteins with SYPRO Red and accomplished LIF detection using a low-cost He-Ne laser. In 2007, Wu et al. [113] developed an elegant approach for protein labeling. First a pseudo SDS dye was synthesized by attaching an alkyl chain to an ionic fluorescent dye (e.g., FITC). Since the long carbon chain is equivalent to the dodecyl group while the negatively charged fluorescent group resembles the sulfate group of SDS, the pseudo-SDS dye has the same function as SDS when binding to proteins. As a mixture of SDS and pseudo-SDS dye reacts with proteins, protein molecules are dynamically labeled with some pseudo-SDS dye. Fig. 6 presents a schematic demonstration of pseudo-SDS dye-protein-SDS complex. Because each protein can be associated with many pseudo-SDS dye molecules, the detection sensitivity can be improved considerably. Using this approach, these authors obtained an LOD of 0.13 ng/mL and a dynamic range of ~5 orders of magnitude for CGE analysis of BSA.

Fluorescence imagers have also been used as detectors for SDS-CGE [19, 90, 114, 115]. A fluorescence imager is a great tool for early stage technology development since it allows researchers to see the migration of proteins inside a capillary or a microfabricated channel. The imaging area depends on the field of view of the imager but normally it will be about a few millimeters to a few centimeters in diameter.

Mass spectrometers (MS) are excellent detectors, because they are capable of identifying proteins. Coupling CGE with an MS, however, is challenging, because MS does not normally have access to CGE resolved proteins. In addition, the SDS in the sieving matrix interferes severely with MS detection. To address these issues, Lu et al. [116] developed an approach to couple SDS-CGE with matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS). Fig. 7 presents a schematic diagram of the experimental setup for this work. Basically, a PTFE membrane was used to collect CGE-resolved proteins (so that a MS detector will have access to these proteins). [Note: The collected proteins were actually SDS-protein complexes that could not be analyzed directly by MS.] After the collection, the SDS-protein complexes on the membrane were washed using an optimized solution to remove SDS while proteins were retained on the membrane. After SDS removal, a MALDI matrix was introduced onto the membrane for MALDI-TOF-MS analysis.

3. Applications

In the literatures we surveyed, a lot of the papers still dealt with standard (or commercially-purchased) proteins (see Table II). Here, we discuss only a few representative papers closely related to practical applications.

3.1. Proteins in Biological Fluids

Analysis of proteins in biological fluids is challenging due to the complexity of sample media. CGE offers a powerful tool to analyze these samples. In 2000, Lin *et al.* [46] used CGE to analyze erythrocyte membrane proteins in blood samples. The erythrocyte membrane samples were extracted from washed red cells, and spectrin in the samples was removed before CGE run. Erythrocyte membrane proteins in normal red cell indices or from healthy blood donors were utilized as controls. The same samples were analyzed by both CGE and SDS-PAGE, and similar profiles were obtained.

In 2008, Obubuafo *et al.* [117] analyzed thrombin, an important marker for various hemostasis-related diseases and conditions, by affinity microchip CGE for human plasma samples and also for rabbit plasma sample. The method employed a PMMA microchip and used LPA as sieving matrix. Two fluorescently labeled aptamer affinity probes, HD1 and HD22, which bind respectively to thrombin exosites I and II were investigated. HD22 affinity assays of thrombin produced baseline-resolved peaks with favorable efficiency due to its higher binding affinity, whereas HD1 assays showed poorer resolution of the free aptamer and complex peaks. Therefore, HD22 was selected in determining the level of thrombin in human plasma.

In 2011, Debaugnies *et al.* [118, 119] evaluated an automated CGE system, the Experion instrument from BioRad, for its ability to separate and quantify the erythrocyte membrane proteins. The major erythrocyte membrane proteins were extracted and purified from membrane ghosts by centrifugation, immunoprecipitation and electroelution. Analyses were performed using SDS-PAGE and SDS-CGE to establish a separation profile of the total ghosts. As the SDS-CGE method was able to achieve the same conclusion as with SDS-PAGE, except for the patient with elliptocytosis, Debaugnies *et al.* concluded that the new SDS-CGE method could be proposed as an automated alternative method to the labor-intensive SDS-PAGE analysis. Kaneta *et al.* [109] applied CGE with postcolumn derivatization/LIF detection to analyses of two biological samples, namely a cell lysate and a milk sample.

3.2. Proteins in Food Products

Monitoring food safety and food quality has become increasingly important in recent years. Sample preparations are essential for these analyses. To examine the quality of seafood products, Sotelo *et al.* [120] applied CGE for analysis of myofibrillar proteins in fish and squid muscles. A Beckman-Coulter P/ACE 2000 capillary electrophoresis system was used in this work, and the manufacturer recommended procedure was followed. Myosin and actin contents in fish and squid muscles were measured, and these results were comparable to the results from a slab-gel SDS-PAGE system. While the resolving powers of the two methods were comparable, CGE had two significant advantages – automated operations and short separation times. However, P/ACE 2000 could only analyze one sample per run. When a batch of samples was to be analyzed, a technician could run all of them in a slab gel in one run, and the differences between samples were readily recognized by direct lane-to-lane comparisons. If these samples were analyzed serially by CGE, results comparisons were not as straightforward, especially when the reproducibility was poor.

Meat quality can be indicated by the profile and quantity of water-soluble and salt-soluble proteins. Vallejo-Cordoba et al. [54] employed CGE and analyzed these proteins in bovine and ostrich meats. Briefly, meats were mixed with water or saline buffer (typically, 0.6 M NaCl/0.01 M phosphate buffer, pH 6.0, 0.5% polyphosphates), blended and centrifuged. The filtered supernatant, sample buffer, benzoic acid (as internal standard) and mercaptoethanol were mixed, boiled and then cooled down. Proteins in this sample were injected for CGE analysis. CGE separations were carried out on a Bio-Rad CE system (BioFocus 3000), and the manufacturer recommended protocols were followed. Profiles and concentrations of water-soluble and salt-soluble proteins were measured successfully in this work.

Gomis et al. [121] analyzed cider proteins and determined their relative molecular masses. Various methods were hired to isolate cider proteins for CGE [122]. Chiu et al. [74] described a segmental filling method for analysis of SYPRO Red labeled SDS-proteins by CE-LIF with electroosmotic counterflow of PEO. This method was capable of determining casein in cow's milk below 0.5 mM.

3.3. Proteins in Agricultural Products

RuBisCo accounts for more than 50% of the soluble protein in chloroplasts and is a key enzyme in the photosynthetic fixation of carbon dioxide [123]. An accurate measurement of the quantity of RuBisCo subunits would provide an indication of a plant's physiological status. Nicolas et al. [53] established a CGE method for analysis of RuBisCo in Spinach leaves. To prepare samples for this method, spinach leaves were freshly harvested and ground in a chilled mortar with a portion of inert sand and some chilled buffer (100 mM Tris-hydrochloride, 0.1 mM EDTA and 1 mM ascorbic acid at pH 8.0). The homogenate was centrifuged, and the supernatant was desalted. This sample was diluted 1:1 with the CE-SDS protein sample buffer (CE-SDS Protein Kit: Bio-Rad, Hercules, CA, USA), and benzoic acid was added as an internal standard (CE-SDS Protein Kit) to a final concentration of 50 µg/mL. After SDS-protein complexes were formed, the sample was ready for analysis. An HP3D capillary electrophoretic system (Hewlett-Packard, Wilmington, DE, USA) was used in the work.

Chen et al. [124] also analyzed RuBisCo from leaves of *Vigna unguiculata*. Leaf tissues were ground to a fine powder in liquid nitrogen. Proteins were extracted from leaf tissue at 0–4 °C in 80 mM Tris buffer containing 0.1 M P-mercaptoethanol, 2% (w/v) SDS, and 15% (v/v) glycerol. The extract was centrifuged and the supernatant was used for protein analysis. CGE was performed with a Bio-Rad 3000 system. Proteins in soybean seeds were also analyzed using CGE by Gerber et al. [125]. Blazek and Caldwell [93] compared SDS-CGE with the lab-on-a-chip technology to quantify the relative amount of 7S and 11S fractions in twenty different soybean cultivars.

Marchetti-Deschmann et al. [126] recently evaluated a one-step single-grain wheat extraction process followed by a CGE-on-a-chip analysis for fast and reliable wheat variety control [119]. Based on the results of 15 different wheat varieties grown in Austria, Marchetti-Deschmann et al. concluded that the CGE-on-a-chip system was a promising alternative for the time-consuming and labor-intensive SDS-PAGE for high-throughput food analysis.

3.4. Proteins in Clinical and Pharmaceutical Studies

Recombinant immunoglobulin G4 (IgG4), as well as other IgG antibodies, is made up of two light chains and two heavy chains. In a normal human IgG4, disulfide bonds are formed between a light chain (L) and a heavy chain (H), and also between two HL dimers. In an abnormal IgG4, there are no disulfide bonds between HL dimers (the dimers are linked

together by only noncovalent interactions). Vasilyeva et al. [55] used an Agilent 2100 Bioanalyzer to quantitate these HL dimmers of abnormal IgG4 in rMAb samples. The microchip method described in this paper was suitable for analyzing samples containing HL from approximately 0.6% to at least 5.2% (may be extended up to 80%). The LOD and limit of quantitation (LOQ) were determined to be 0.05% and 0.59%, respectively. Good correlations were obtained between this method and conventional SDS-PAGE, and between this method and reversed-phase HPLC.

With the increasing therapeutic use of rMAbs, Analyzing the quality and purity of rMAbs becomes an important and routine task for rMAb manufacturers. Hunt and Nashabeh [26] developed a CGE method for analysis of rMAbs in biopharmaceutical industry. The method included precolumn protein labeling, CGE separation and LIF detection. 5-carboxytetramethylrhodamine succinimidyl ester was used as a labeling reagent. CGE separations were performed on a Bio-Rad BioFocus 3000 CE system equipped with a LIF detector. This method was validated according to the guidelines of the International Committee on Harmonization and had been used as part of a control system for the release of an rMAb pharmaceutical in Genentech, Inc. This method was optimized recently [30].

Guo *et al.* [52] developed a non-reduced SDS-CGE method and used it to study disulfide heterogeneity in IgG2 antibodies. This method was proved to be a powerful tool to get information on the backbone structure of IgG molecules. Zhang et al. [48] optimized a similar method to analyze mAb1 drug substance under both reduced and non-reduced conditions. Lancher et al. [127] established a generic method for monitoring disulfide isomer heterogeneity in IgG2 antibodies, and applied this method for purity analysis of reduced and non-reduced IgG2 mAbs [128]. Rustandi et al. [129] reported a wide range of applications of CGE for mAb product development, including purity analyses for product release, product-related impurities during process and cell-culture development, and product stability evaluation. Cherkaoui et al. [130] used CGE to evaluate the IgG structural integrity under various reduction conditions and track antibody reduction fragments.

Carbonyl-modified proteins are considered markers of oxidative damage in aged tissues and diseases such as Parkinson's, diabetes, emphysema, and atherosclerosis [131, 132]. Feng et al. [133] developed a carbonyl detection method based on the reaction of Alexa 488 hydrazide with carbonyls and on the separation of the Alexa 488-labeled compounds by CGE with a sheath flow cuvette. Because carbonyls on lipids, carbohydrates, and nucleic acids could also react with Alexa 488 [134], yielding products that would interfere with the detection of carbonyl-modified proteins, the Alexa 488-labeled proteins were further labeled with another fluorogenic reagent – FQ. FQ only reacted with proteins, and its fluorescence showed little spectral overlap with that of Alexa 488. Therefore, protein peaks with fluorescence characteristics of both Alexa 488 and FQ belonged to carbonylated proteins. The method was adequate for analyzing nanogram protein samples with femtomole levels of carbonyls.

Mellado et al. [135] described an application of CGE for the analysis of rotavirus virus-like particles. Particle's apparent molecular masses and quantities were determined, and these results were validated by comparing them with those obtained from traditional SDS-PAGE and MALDI-TOF-MS.

4. Conclusions

In conclusion, CGE is a powerful tool for protein analysis. Automated operation and short separation times are two most significant advantages of CGE over conventional slab gel electrophoresis. Reproducibility is still a shortcoming for CGE, although a lot of progress

has been made. Currently, CGE separations are performed usually in series, which makes lane-to-lane comparisons not as convenient as in multilane slab gel electrophoresis [120, 136]. Microchip CGE is a promising platform for high speed protein analysis. At the time being, however, most practical applications have been conducted using capillary-based systems. While UV absorption detection is still a popular detection scheme for CGE, LIF detection is gaining a lot of ground. The reason might be that reliable and affordable fluorescence labeling dyes are commercially available, and that multiple labeling is less an issue for CGE. CGE has been used as a separation dimension for 2D separations, but so far the resolving power of these schemes could not compete with that of conventional 2D gels. In terms of practical application, CGE has already been utilized for quality control and purity test of monoclonal antibody products. Other imminent applications include clinical diagnosis, food quality monitoring, etc. We expect CGE to be an important analytical technique in all these areas in the near future.[50, 117, 137–147]

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Biographies

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Fig. 1. Immunoblot chip

(a) Schematic design of the immunoblot chip for analysis of native proteins. The sample (2), sample waste (3), buffer (1, 4, 5, 6) and buffer waste (7, 8) reservoirs are indicated in sketch (not to scale). The middle region of the device (indicated as Chamber) has a length of 1.5 mm, a width of 1 mm and a depth of 20 μm . (b) Three separate zones inside the Chamber to facilitate protein immunoblotting: a large-pore-size protein loading gel on the top, a smaller-pore-size protein separation gel on the bottom-left and an antibody-functionalized blotting gel on the bottom-right. Colored dyes were used to visualize the different gel regions. Reprinted from ref. [85] with permission.

Fig. 2. μ ChemLab™ instrument

(a) The μ ChemLab instrument with the top off showing the separation platform, the control panel, the back of the LCD display, and the battery pack. The instrument is approximately 7"×8"×4.5" and weighs 6 lbs. (b) The separation platform houses the microfluidic chip in a compression manifold that connects the chip to eight fluid reservoirs, two sample injection ports, and a LIF detection module. The overall size of the platform is approximately 5"×3"×4". Reprinted from ref. [48] with permission.

Fig. 3. Schematic of a plastic microchip for 2-D protein separation
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(A)

(B)

Fig. 4. CIEF Integrated with CGE in a linear format

(A) Overall Arrangement of experimental setup. (B) Dialysis hollow fiber interface: (1) methacrylate plate, (2) capillaries, (3) Teflon tubes, (4) hollow fiber and (5) buffer reservoir. Reprinted from ref. [98] with permission.

Fig. 5. Microchip for 2D protein separation

(A) Geometrical layout of the microchip used for SDS- μ CGE-MEKC. (B) Fluorescence image of the sieving matrix/MEKC interface at the intersection of the SDS- μ CGE and MEKC dimensions. Reprinted from ref. [92] with permission.

Fig. 6. Schematic demonstration of pseudo-SDS dye-protein-SDS complex
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Fig. 7. Schematic diagram of experimental apparatus for SDS-CGE and protein collection
(a) SDS-CGE setup with membrane collector; (b) split view of membrane collector.
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Table I

List of Acronyms

Acronym	Representation	Acronym	Representation
2D	two-dimensional	LIF	laser induced fluorescence
Bis	<i>N, N'</i> -methylenebis(acrylamide)	LOD	limit of detection
CE	capillary electrophoresis	LPA	linear polyacrylamide
CGE	capillary gel electrophoresis	MALDI	matrix-assisted laser desorption ionization
CIEF	capillary isoelectric focusing	MEKC	micellarelectrokinetic chromatography
CPA	cross-linked polyacrylamide	MS	mass spectrometer
CSE	capillary sieving electrophoresis	NDA	naphthalene-2,3-dicarboxaldehyde
CZE	capillary zone electrophoresis	PA	polyacrylamide
EDTA	ethylenediaminetetraacetic acid	PAGE	polyacrylamide gel electrophoresis
EOF	electroosmotic flow	PDMA	polydimethylacrylamide
FITC	fluoresceinisoithiocyanate	PDMS	poly(dimethylsiloxane)
FQ	3-(2-furoyl) quinoline-2-carboxaldehyde	PEG	poly-(ethylene glycol)
HEC	hydroxyethylcellulose	PEO	poly-(ethylene oxide)
HPC	hydroxypropylcellulose	PEOX	poly(2-ethyl-2-oxazoline)
HPLC	High performance liquid chromatography	PMMA	poly(methyl methacrylate)
HRPN	hydrophilic replaceable polymer network	PVA	poly(vinyl alcohol)
HV	High voltage	rCPA	replaceable cross-linked polyacrylamide
IEF	isoelectric focusing	rMAbs	recombinant monoclonal antibodies
IgG	immunoglobulin G	SDS	sodium dodecyl sulfate
IPG	immobilized pH gradients	TOF	time-of-flight

Table II

Literature Summary Based on Sieving Matrices Used

Sieving Matrix	Analyte	Reference	Comment
In-capillary/channel prepared polyacrylamide (non-replaceable)	Standard proteins or mixtures	[10,20–22,80,83,90,97,115,139,146]	Gradient gel was prepared in ref. 20. Infrared laser desorption/ionization MS was interfaced with gel electrophoresis chip in ref. 139. 2D gel electrophoresis was performed on chip in ref. 90, 97 and 115.
	Human and bovine serum albumin	[9]	
	Interleukin-2 and growth factors	[80]	
Linear polyacrylamide	Standard proteins or mixtures	[11–13]	
	Thrombin	[117]	
	Cider proteins	[121,122]	
Beckman SDS gel	Apolipoproteins in human high-density lipoproteins	[100]	rMabs were labeled with FQ before separation and detection in ref. 107. 2D separation was performed in ref. 91 and 99. Performances of Beckman-Coulter ProteomeLab and Agilent 2100 Bioanalyzer were compared in ref. 93. Beckman SDS-gel goes with their instruments.
	Recombinant monoclonal antibodies (rMAbs)	[30,48,107,127–130]	
	Standard proteins	[49,91,145,147]	
	Protein from E. coli cell	[99]	
	Protein biotoxins	[47]	
	Proteins from soybean	[93,125]	
	Erythrocyte membrane proteins	[46]	
	Myofibrillar proteins (actin/myosin)	[120]	
Agilent 2100 kit	Standard proteins	[138]	Separations on chip were performed in ref. 55–57, 93, 94 and 138. Agilent SDS-gel goes with their instrument.
	Glycoproteins and de-N-glycosylated Human serum glycoproteins	[56,57,94]	
	Proteins from soybean cultivars	[93]	
	Monoclonal antibody	[55]	
Bio-Rad CE-SDS run buffer	Polyethylene glycolylated interferon (PEG-IFN)	[50,51]	Bio-Rad CE-SDS run buffer does not require coated capillaries for SDS-CGE.
	PEG-modified granulocyte-colony stimulating factor	[137]	
	RuBisCo	[53,124]	
	Monoclonal antibody	[52]	
	Water-/salt-soluble proteins from bovine and ostrich meat	[54]	
Slightly cross-linked polyacrylamide (replaceable)	Standard proteins or mixtures	[35,113,116]	MALDI-MS was interfaced with CGE in ref. 116.
	Proteins in crude cell extract	[35]	
	E. coli AcrA protein	[116]	
PEG/Dextran	Proteins in MCF-7 breast cancer cell	[58]	The work in ref. 58 was focused on selection of an internal standard for separation. 2D separation was performed in ref.
	Proteins in Barrett's Esophagus Tissue homogenate	[98]	

Sieving Matrix	Analyte	Reference	Comment
	Proteins in rat plasma	[11]	9898. Performances of PEG, dextran and LPA were compared in ref. 11. Detection limit of sub-pM were obtained in ref. 113.
	Proteins in E. coli cell extract	[11]	
	Standard proteins	[15,16,17,81,82,103,104]	
	Proteins from AtT-20 cellular homogenate	[141,142]	
	Proteins from Barretts esophagus homogenate	[143]	
	Tryptic digests	[144]	
	Carbonylated proteins from rat muscle	[133]	
Pullulan	Standard proteins	[18,19,24,39,40]	2D separation was performed in ref. 18, 19 and 40.
	Protein homogenate from D. radiodurans	[18]	
	Proteins from breast cancer cell	[40]	
Polyethylene oxide (PEO)	Proteins in HT29 human colon adenocarcinoma cell extract	[8]	Proteins were labeled with FQ before separation and LIF detection in ref. 8. Proteins were labeled with SYPRO Red before separation and LIF detection in ref. 74. 2D separation was performed in ref. 89 and 95.
	Casein in milk	[74]	
	Proteins from E. coli cell	[95]	
	Standard proteins	[89]	
Hydroxypropylcellulose (HPC)	Standard proteins or mixtures	[25,38]	
	Proteins in HT29 human colon adenocarcinoma cell extract	[38]	
Poly-N-hydroxyethylacrylamide	Standard proteins	[140]	An acid-labile surfactant was used to replace SDS in ref. 140.
Hydroxyethylcellulose (HEC)	Lanthanide chelate-labeled proteins	[41]	A time-resolved fluorescence detector was used in ref. 41.