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Review article

Recent advances in capillary gel electrophoresis for the analysis of proteins

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ABSTRACT

The purpose of this review is to highlight noteworthy advancements in the field of capillary gel electrophoresis for the separation and analysis of proteins from the period of 2015-2021. This review will provide an overview of the historical perspective and principles of the technique, introduce the challenges and limitations commonly faced, and highlight the advancements made to overcome these issues and broaden our knowledge of the method. Finally, applications of capillary gel electrophoresis and future directions for the technique will be presented.

Introduction

Proteins are an important class of macromolecules that play critical roles in all living organisms. They provide structural support, assist with growth, function in the immune system, and serve as catalysts, transport molecules, and storage compounds, amongst other uses [1]. It is this versatility that allows us to use these functional units of life to understand the balance between healthy and diseased states on a molecular level [2,3]. To study proteins, we must first be able to isolate and characterize them. Numerous analytical techniques to separate proteins exist with electrophoresis being one of the most well studied and widely used. While multiple different modes of electrophoresis have been developed, this review is focused on capillary gel electrophoresis (CGE), also referred to as capillary electrophoresis (CE) with sodium dodecyl sulfate (CE-SDS).

While early work on electrophoresis based on Faraday's laws of electrolysis date back to the nineteenth century [4], the rise of electrophoresis as a separation technique is generally attributed to Arne Tiselius, who successfully separated horse serum into albumin, α-, β-, and γ-globulin in the 1930's [2,5-7]. This discovery combined with Tiselius' work on adsorption and electrophoresis culminated in him receiving the Nobel Prize in Chemistry in 1948 [8]. In the decades following, different modes of electrophoresis were developed including zone electrophoresis, isoelectric focusing, and isotachophoresis [4]. Notable during this time was the upsurge of gel electrophoresis as a separation method that began with starch gels [9] and in 1970 yielded what is now modern SDS polyacrylamide gel electrophoresis (SDS-PAGE) [10]. The use of tubes with a narrow internal diameter goes back to the 1960s during which Stellan Hjertén demonstrated the use of a 3 mm internal diameter narrow bore tube that offset convective issues by rotating the narrow bore tube [11,12]. However, it wasn't until 1981 when Jorgenson and Lukacs created a method of zone electrophoresis in a capillary tube that the technique of CE started gaining traction. They used a 75 µm internal diameter tubular glass capillary with an on-column fluorescence detector under voltages of up to 30 kV to demonstrate the separation of amines, amino acids, and dipeptides [13]. Using smaller capillaries led to more efficient heat dissipation by increasing the surface area to volume ratio. This allowed for higher voltages to be applied to the system resulting in improved efficiency and shorter separation times [14]. Later it was shown that CE technology could employ gels as a separation media leading to capillary gel electrophoresis methods [15]. This work by Hjertén showed separation in capillary tubes of 0.05-0.30 mm internal diameter with both agarose and polyacrylamide gels. Proteins present in amounts as low as 0.01 µg/mL were separated and detected via an on capillary ultraviolet (UV) detection system. In the decades following these discoveries, CE and CE-SDS

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have undergone many advancements and have been applied in a wide range of fields.

The goal of this review is to focus on the recent advancements and applications of CE-SDS for the separation and analysis of proteins. This review is not meant to be comprehensive but rather highlight the most important advances in CE-SDS between 2015-2021. Included works are those that have improved our understanding of the sieving matrix, increased detection sensitivity, improved biotherapeutic characterization, and describe an efficient workflow for method development. Our intention is to consolidate recent findings to help other scientists determine if any of the advancements reviewed fit their needs and to provide a framework for future work to be built upon.

Principles of electrophoresis, CE, and CE-SDS

To appreciate the advances made to reach the current state of CE-SDS, it is imperative to understand the theory behind both electrophoresis and CE.

Capillary electrophoresis

When charged particles migrate under the influence of an electric field, the phenomenon is known as electrophoresis and the basic principles of electrophoresis and CE such as electrophoretic mobility (μ) and electroosmotic flow (EOF) have been covered in a previous review by our group [16]. Briefly, μ is dependent on the charge to size ratio of the analyte, with the mobility being directly proportional to the charge of the analyte (q) and inversely proportional to the hydrodynamic radius of the analyte (r) and the viscosity of the medium (η) [16]. The mathematical relation is described in Eq.1 below:

$$\mu = \frac{q}{6\pi\eta r} \tag{1}$$

In CE, separation is achieved within a narrow-bore capillary, typically 25 – 75 µm in internal diameter and with a total length of 30 – 100 cm [17]. Depending on the application, capillaries with lengths and diameters outside of these ranges can also be used. The capillary is filled with background electrolyte (BGE) by submerging it into a BGE reservoir. Electrodes that connect to a voltage source are also submerged into all reservoirs the capillary is placed in. It is important that both ends of the capillary are always in contact with the reservoirs during separation to maintain electrical neutrality. For sample injection, the capillary is inserted into a sample vial and the sample plug is injected into the inlet side either by voltage or pressure. The capillary is then returned to the BGE reservoir for the separation stage. Analytes are detected as they pass through a transparent detector window on the capillary and the results are displayed as an electropherogram with the signal on the y-axis and migration time on the x-axis [18]. Different modes of detection can be used such as laser induced fluorescence (LIF) and UV absorption in addition to mass spectrometry (MS) [19–21].

The exterior of the capillary is coated with polyimide to protect it and increase flexibility, except for the detection window where polyimide is removed to allow for optical detection of the analytes as they migrate. The interior of the capillary is bare fused silica that has an ionizable surface coated with silanol groups. The formation of the electrical double layer and rise of EOF has been described previously [16]. It is the combination of both the EOF and μ that influences the migration of analytes. While μ is unique to each analyte, the EOF is constant for all the analytes in a sample per separation.

The mobility of the analyte and EOF also plays an important role during sample injection, depending on the method chosen. The two methods of injection used are a pressure based hydrodynamic injection and an electrophoresis based electrokinetic injection. Hydrodynamic injection was initially achieved through siphoning [12]. With improved pneumatic systems of CE instruments, it is most commonly accomplished by applying pressure on either the capillary inlet (positive pressure) or the outlet (negative pressure) [22]. The volume of the sample injected into the capillary (V) is directly proportional to the inner diameter of the capillary (D), difference in pressure between the two ends of the capillary (ΔP), injection time (t) and inversely proportional to the viscosity (η) and capillary length (L) [12]. Eq. 2 shown below can be used to estimate the total amount of sample injected via hydrodynamic injection:

$$V = \frac{\Delta P D^4 \pi t}{128\eta L} \tag{2}$$

Electrokinetic injection is achieved by applying a voltage and allowing the analytes to migrate based on their mobility and/or the EOF. These sample loading methods have different advantages and disadvantages depending on the analysis being performed, the conductivity of the BGE, the nature of the sample, and the ionic strength of the sample matrix. Some important considerations are that with hydrodynamic injection a defined volume is injected and due to this, the volume of the capillary used is the limiting factor for the amount of sample to be loaded [22]. Furthermore, for samples with a high salt content this method is preferred [12]. On the other hand, electrokinetic injection might not be limited to capillary volume and is more selective than pressure loading, however, it can lead to a bias if there is a significant difference in the mobility of the analytes. Moreover, the number of cations, anions, and neutral ions injected will also differ and might lead to variation in injection of target analytes. Due to this, estimations for amount of analyte injected have to be done separately for each individual solute. The amount of each solute injected (Q) is given by Eq. 3 [12]:

$$Q = (\mu_{eo} + \mu_{ep})\, \pi r^2 E C t \tag{3}$$

Where μ_{eo} is the electroosmotic mobility, μ_{ep} is the electrophoretic mobility, r is the radius of the capillary, E is the strength of the electric field, C is the concentration of that particular solute, and t is the injection time. Irrespective of these issues, electrokinetic injection is still generally preferred in CE-SDS as it has a better ability to increase the sensitivity relative to hydrodynamic injection [22].

Closely related to injection is the concept of stacking, which is caused by electrophoretic phenomena [23,24]. Common techniques of sample stacking include Field-amplified sample stacking (FASS) and Field-amplified sample injection (FASI). In brief, the former uses hydrodynamic injection and the latter electrokinetic injection to insert a plug of lower conductivity relative to the BGE into the capillary that helps focus the analytes into narrow bands, subsequently yielding taller, sharper peaks which improves the detection sensitivity [23]. This is achieved due to electric field differences in the sample plug and BGE, causing the analytes to accelerate in the sample plug and slow down at the interface of the sample and BGE, leading to them being concentrated at this boundary. Other methods of stacking have also been developed, such as those based on pH junction [25,26], sweeping [27,28], and isotachophoresis [29]. Like the type of injection, the choice of stacking technique depends on many different factors.

Capillary gel electrophoresis

CE-SDS can be thought of as a combination of regular CE and SDS-PAGE. In essence, the BGE is replaced with a sieving gel matrix and the sample is treated with SDS and heated to denature the

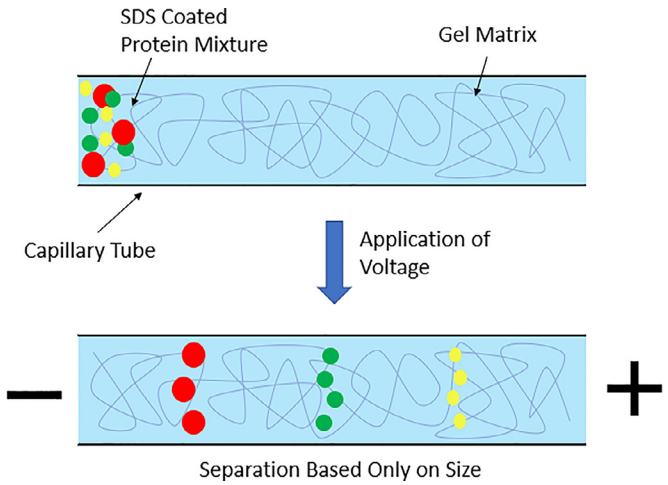

Fig. 1. Schematic representation of the size-based separation in CE-SDS.

proteins providing a consistent coating of about 1.4g SDS per gram of protein [30,31] and a uniform anionic charge to size ratio, or a uniform q/r ratio. Furthermore, when all the proteins in a sample are coated with SDS, the effects of the intrinsic charge on individual proteins are negligible. When substituted back in Eq. 1, it can be seen that following denaturation and SDS treatment, μ is now the same for all the analytes and consequently, separation is now based solely on the size of the analytes, with the gel acting as a molecular sieve. Either of the injection methods noted in Section 2.1 can be used, with electrokinetic injection still favored for increased sensitivity as well as the fact that it does not displace the gel inside the capillary. The polarity of the electrode is flipped so that the anode is on the outlet side, and upon the application of a voltage, separation is now driven only by the size of the proteins as they navigate the pores of the gel (Fig. 1). The nature of this matrix can allow for EOF suppression and prevents proteins from interacting with the walls of the capillary [17], however, EOFs may not be completely suppressed by lower viscosity gels. Coated capillaries can be used if this is a cause of concern, however they are liable to causing undesirable interaction with the migrating analytes.

The gels themselves are largely made up of a polymer, buffer, an organic compound, detergent, and a reducing agent [32]. The polymer is responsible for creating the network of pores that cause the sieving effect, the buffer helps in maintaining pH and conductivity, the organic component improves resolution, the detergent ensures that the protein to detergent ratio stays constant, and the reducing agent prevents the reformation of disulfide bonds in case of reduced CE-SDS [32]. Additives can also be added to the gels, a prominent example being chelating agents such as EDTA. The interaction between all the above components is complex and substituting compounds or varying their concentration creates numerous gel formulations that allows for freedom to tailor a formula that best suits the users interest. As CE-SDS is a size-based separation, it is imperative to select the optimal sieving matrix. A good matrix should be able to create a uniform network of pores, have minimal background noise, not bind migrating molecules, be robust, and be well-suited to the detection technique employed [33].

Broadly speaking, there are two classes of gels, crosslinked chemical gels and non-crosslinked physical gels [33]. While crosslinked gels like those made from polyacrylamide work well as slab gels in SDS-PAGE, issues such as bubble formation due to shrinkage of the gel within the capillary and clogging of the capillaries is common when used in CE-SDS applications [33,34]. In addition, the matrix is also highly susceptible to alkaline hydrolysis [35,36]. Furthermore, only a limited number of runs could be done with this system as once issues with injection or separation arise, the capillary must be replaced. To overcome this limitation, non-crosslinked gels were created, the first using linear polyacrylamide as the polymer [37]. Other examples of these water soluble polymers include dextran [32] and polyethylene glycol [38], amongst others. In these gels the network of pores in caused by physical entanglement of the polymer. At low concentrations, these polymers do not form a sieving matrix, but at and above a specific concentration termed as the overlap threshold concentration, the molecules of the polymer get entangled and form a porous network capable of sieving [12,33]. These interactions are transient with the linkages being constantly broken and renewed [33]. The most important virtue of using non-crosslinked gels is the ease of regeneration, thus extending the lifetime of the capillary and improving the reproducibility [34].

The advantages of using the capillary format for separation over traditional slab gel SDS-PAGE are numerous. These include easier set up with lower sample and reagent consumption, increased resolution, superior heat dissipation that permits the application of higher voltages and subsequently lower run times, and real time sample analysis with an on-capillary detection system [2,34,39,40]. When non-crosslinked gels are chosen as the separation matrix, the capillary can be regenerated between runs and be reused. CE-SDS is also highly automated [2,34,40,41], allowing for multiple injections and samples to be tested within a single experimental run. Finally, the reagents used are more environmentally friendly than those used in SDS-PAGE. The versatility of the technique through alteration of the separation matrix, injection, or detection method can help users find the optimal separation method for their intended application.

Limitations and challenges of CE-SDS

While CE-SDS outperforms SDS-PAGE under numerous parameters, there are still drawbacks and challenges to using this technique. The biggest challenge for CE-SDS, inherited from the capillary based parent electrophoresis technique is the relatively low concentration sensitivity of the most frequently used UV-absorption detection [23]. This is due to the narrow internal diameter of the capillary tubes, yielding a short path length and consequentially, a lower absorbance associated with online detection. To increase sensitivity, a commonly used detection method is LIF, which, generally, can detect peptides and proteins using their native fluorescence caused by aromatic amino acids that can be excited in the region of UV light [42,43], however, for wide applicability, it requires labeling or tagging of proteins to further increase sensitivity of all proteins, regardless of their specific aromatic amino acid composition. Challenges with this method include labels that only bind to specific sites or do not bind evenly, potentially resulting in a combination of labeled, partially labeled, and unlabeled target analytes [34]. Furthermore, the advantage of lower sample injection volumes can also be a disadvantage due to the lower amount of analyte in the capillary resulting in inferior sensitivity, especially when compared to traditional separation techniques such as HPLC [44]. Improving the detection limits of CE has been an area of intense focus, with a series of bi-annual reviews highlighting the most significant advancements made on this topic from the early 2000s onwards [23,45-50].

A second issue that is also seen with SDS-PAGE is the uneven binding of SDS to certain molecules. The most prominent example is the lowered binding of SDS to carbohydrate or glycan groups attached to proteins. It is thought that only 0.2 g of SDS binds per gram of glycan [51], which is much lower than the 1.4 g of SDS that binds each gram of protein that we noted earlier [30,31]. This results in migration differences that might not be indicative

of the actual relative molecular mass (M_r) of this glycosylated protein. This is an important limitation to tackle as glycosylation plays an important role in developing new therapeutics, specifically with monoclonal antibodies (mAbs). It is also worth noting that other types of modifications such as phosphorylation have also been reported to demonstrate reduced SDS binding due to charge repulsion [52]. Research that characterizes the effects of glycosylation on migration as well as improving surfactant coating of proteins have been covered within this review.

Peak profiles and migration times must remain consistent across replicates for CE-SDS to be a useful analysis. Inconsistent injection amounts and variations in the sample or matrix preparation can affect the electric field within the capillary and subsequently give irreproducible peak areas, peak heights, and migration times for the same analyte [44]. Additionally, changes in the capillary surface and EOF can also affect reproducibility. The EOF is influenced by many parameters such as the ionic strength of the buffer, the pH, viscosity, and temperature, to name a few. A high EOF or inadequate suppression of even a relatively low EOF can have adverse effects. These include pumping out the analytes as they are being injected (for electrokinetic injection), a longer analysis time as μ is in the opposite direction, or even completely overcoming μ and failing to yield a separation profile. These effects are not limited to the analyte, as the separation matrix can also be carried toward the inlet side and pumped out of the capillary [34]. Coated capillaries can mitigate this problem. In most cases a coating isn't needed as commercially available gels are viscous enough that they offer adequate EOF suppression. These ready-to-use gel formulations have made it easy to separate proteins but optimization of the matrix might be required depending on the target analyte of interest, specifically in the case of non-mAb therapeutics [53]. However, the proprietary nature of these commercial gels makes them difficult to tweak, which might leave the user with the option of adjustment and optimization of the off-shelf CE-SDS products, thereby increasing the complexity of the process in terms of identifying the optimized formula in addition to the assessment of the assay requirements as listed in the analytical target profile (ATP) [53].

In addition to these challenges, there are other limitations with using CE-SDS. While it can be used to isolate specific proteins via microscale fraction collection, another technique is required to identify the proteins in these fractions, such as MALDI-MS [54,55]. On the other hand, one can both separate and employ multilane comparison with standards/ladder using SDS-Page which is not easy to reproduce in conventional CE-SDS [56]. However, the scale and analysis time in CE-SDS does offset this limitation. Moreover, commercial instruments such as the BioPhase 8800 by Sciex allow for multi-capillary analysis in parallel [57]. There is also a difference in the effective sieving range, with commercially available CE-SDS gels able to resolve protein sizes of 10 kDa to 225 [58] or 270 kDa [59]. In contrast to this, commercial gels to separate proteins as large as 500 kDa through SDS-PAGE are available [60].

While it may seem that the challenges and limitations presented above are a barrier for the routine use of CE-SDS, significant research has been done to overcome these issues. The most substantial advances made from the years of 2015-2021 will be presented in the following section.

Recent advances

The chosen works have been sub-sectioned based on the major topics the respective publications address. An overview of the papers critically examined, the primary topic addressed, the target analytes, and key takeaway is presented in Table 1.

Sieving matrix

Kits to run CE-SDS are commercially available with the sieving matrix (separation matrix) sold by Sciex (previously sold by Beckman Coulter) thought to be the most often used gel [17]. While the exact formulation of the gel is proprietary, it is known to contain dextran as the polymer and boric acid as part of the buffer system [32]. The chemistry between dextran and boric acid makes these gels ideal for separations of therapeutic candidates and this interaction has been reviewed in later sections. Furthermore, a recent review of this gel details the best applications for using this product for the development of CE-SDS methods [17]. While this work was based on the Sciex gel, the author does note that the working practices noted in the paper can be extrapolated and used for other gels as well. Another study compared commercially availability instruments sold by Sciex, Protein Simple, Agilent, PerkinElmer, and Prince Technologies for CE-SDS separations and a review of this work might help new users decide on the instrument that would best suit their needs [61]. As the entire principle of CE-SDS is based on the separation of proteins by M_r, it is crucial to choose an optimal sieving matrix for the intended application. Of practical interest is the separation of non-glycosylated and glycosylated heavy chains of antibodies. The glycan groups can be bulky and add to the M_r of the heavy chain. Moreover, we have noted how these groups do not bind SDS effectively, leading to inaccurate M_r estimations. However, there is also potential to use these glycans to enhance the separation between the two types of heavy chains.

A recent study by Filep and Guttman investigated the selectivity of these heavy chains on dextran-borate crosslinked gels [62]. It should be noted that while the authors use the term crosslinked, the linkage between the two constituents is continuously being formed and broken apart as it is an entangled gel. If present in the right spatial arrangement, borate ions form complexes with polyhydroxy compounds that contain 1,2 or 1,3 cis-diol groups [63]. This principle also applies to glycoproteins [64] and any free borate ions can complex with the glycan groups on the heavy chain. In the reviewed study [62] the authors created two gel formulations, the first with a higher dextran and borate concentration (10% dextran/4% boric acid) and the second with a lower concentration (2% of each component). For a mAb, they found that on the latter the resolution between the non-glycosylated and glycosylated heavy chains was 75% higher than the former. This is a significant finding as the initial thought might be that the gel with a higher dextran-borate concentration would have a higher resolution due to the greater sieving effect of the gel. However, the 2% dextran/2% boric acid gel likely has more intrachain 1:1 dextran-borate bonds that have a free $B(OH_2)$- group on each molecule to bind the glycoprotein moieties. This interaction briefly links the glycosylated heavy chain to the matrix, impeding migration and increasing the resolution between the peaks. On the other hand, the 10% dextran/4% boric acid gel likely has more 2:1 interchain bonds, with significantly less free borate groups to bind the glycan moiety. While the resolution between the heavy chains was improved, about 50% lower resolution was seen between the light chain and non-glycosylated heavy chain on the 2% dextran/2% boric acid gel. This was not surprising as the sieving effect and viscosity of this gel would be lower, as demonstrated through the shorter migration time, even with an EOF that is very likely higher than that of the 10% dextran/4% boric acid gel. It would thus appear that for comparison of non-glycosylated species, a higher dextran-borate concentration and ratio is better. To probe this relationship the authors evaluated a range of dextran and boric acid concentrations between the previously tested concentrations and created three dimensional plots displaying the selectivity between the light chain and non-glycosylated heavy chain as well as the non-glycosylated heavy chain and heavy chain combinations. These

Table 1
Overview of the papers that were critically reviewed.

Author	Primary Area of Focus	Target Analytes	Key Takeaway
Filep and Guttman [62]	Sieving Matrix Composition	mAb	By changing the ratio of dextran and boric acid in the sieving matrix the interaction between these components can be manipulated to optimize either the separation of glycosylated species from non-glycosylated species or non-glycosylated species from one another.
Guttman et al. [66]	Sieving Matrix Composition	mAb	Altering the dextran and boric acid concentration in the sieving matrix allows the gel to be optimized for various purposes such as screening, shorter analysis time, resolution, and selectivity of glycans.
Crihfield and Holland [67]	Sieving Matrix Composition	Human Serum Proteins	A nanogel composed of phospholipids was demonstrated to be thermally responsive by fluctuating between low and high viscosity in a temperature dependent manner. Between certain gel concentration and temperature ranges, a separation matrix that was able to separate proteins in the 20 – 80 kDa range was formed.
van Tricht et al. [72]	Sieving Matrix Composition	Viral Proteins	A simple yet effective method of diluting the commercial Sciex gel in Milli-Q water in conjunction with sample preparation enhancements was found to improve the resolution of the viral proteins tested.
Zhang and Meagher [74]	Sample Stacking	Viral Proteins	Sample stacking achieved by desalting the sample and pre-injecting a plug of water into the capillary was found to increase the sensitivity by three orders of magnitude relative to CE-SDS in the more conventional format.
Danish et al. [76]	Sample Tagging	Membrane Proteins	Molecular cloning was used to tag proteins with GFP and CE-SDS was run with LIF detection, revealing an LOD that is 5-fold lower than that obtained on a microplate reader.
Beckman et al. [80]	Detergent in Sample Buffer and Sieving Matrix	Recombinant Therapeutic Proteins (RTP), including mAbs	Using sodium hexadecyl sulfate (SHS) as the detergent during sample preparation and in the sieving matrix was found to increase the number of theoretical plates and resolution relative to using SDS, leading to the hypothesis that longer chain detergents improve separation of proteins that have a thermal stability that is very high, such as the RTP tested.
Guan et al. [82]	Detergent in Sample Buffer and Sieving Matrix	Recombinant mAbs	For reducing CE-SDS, preparing the sample in a buffer containing sodium tetradecyl sulfate (STS) and running it on a gel containing both SHS and SDS was found to improve protein denaturation by reducing aggregates of higher M_r.
Guan et al. [83]	Detergent in Sample Buffer and Sieving Matrix	Recombinant mAbs	For non-reducing CE-SDS, utilizing a sieving matrix containing SHS and SDS was found to reduce the formation of protein aggregates.
Filep and Guttman [85]	Effect of Temperature on Separation	mAb and Nanobody	A relationship between the size of the proteins in a M_r standard and the activation energy to move through the matrix was found. However, no such relationship between size and activation energy was seen for the biotherapeutic proteins, which differ more from each other than the proteins in the standard.
Wang et al. [91]	M_r Determination	Glycoproteins	This study provides a good framework for understanding the basis behind the inaccurate determination of M_r for glycoproteins under reducing and non-reducing CE-SDS and the contrast with SDS-PAGE.
Scheller et al. [92]	M_r Determination	Glycoproteins	Various factors such as interaction between carbohydrates, presence of N-glycans sites, sialyation, proline content, and hydrophobicity were found to potentially influence migration of either the glycosylated or deglycosylated proteins
Geurink et al. [53]	CE-SDS Method Development	Viral Proteins	A four-step framework for the analysis of viral proteins in vaccines using CE-SDS was described – 1) Test conditions described in commercially available kits 2) Optimize sample preparation 3) Optimize separation conditions and 4) Validate conditions from previous steps.
Filep et al. [99]	Applications of CE-SDS: New Antibody Modalities	Glycoengineered and Bispecific mAbs	The separation of newer and complex antibody modalities was evaluated with successful separation of a glycoengineered mAb and a bispecific mAb demonstrated.
Ouimet et al. [101]	Applications of CE-SDS: Protein-protein Interactions (PPIs)	Heat Shock Proteins	PPIs can be investigated using CE-SDS by crosslinking the analytes with formaldehyde before injection, with a crosslinking time of only ten minutes.
Ouimet et al. [103]	Applications of CE-SDS: PPIs	Heat Shock Proteins	Building on the above study, using glutaraldehyde to crosslink the sample reduced the treatment time to 10 seconds. Further adjustments to the capillary, separation matrix, and sample injection reduced the per sample analysis time to one minute.
Ouimet et al. [110]	Microchip-CGE (M-CGE)	Heat Shock Proteins and Enzymes	A device was developed for M-CGE that results in a high throughput method where each sample is analyzed in 10 seconds.
Arvin et al. [111]	Microfluidic System for Western Blotting	Actin and A431 Cell Lysate	Separated analytes are deposited directly onto a membrane following M-CGE, with the reagents needed for detection also added directly, resulting in assay completion within 90 minutes.
Smith et al. [114]	M-CGE Method Development	mAbs	Critical method parameters for sample preparation were identified and working ranges were developed that can be used for testing mAbs in cGMP and R&D environments.

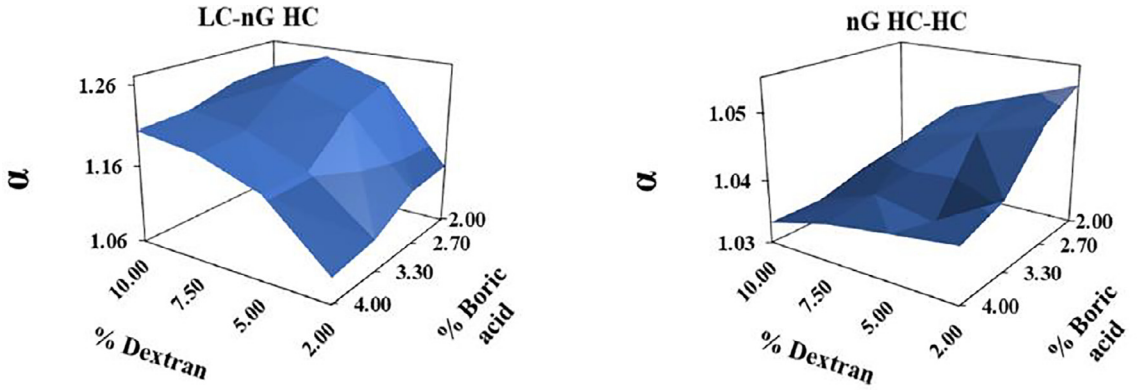

Fig. 2. Three-dimensional selectivity plots demonstrating the effect on selectivity between the light chain and non-glycosylated heavy chain (left) and the non-glycosylated heavy chain and glycosylated heavy chain (right) pairs of the tested mAb at varying dextran and boric acid concentrations. Reproduced from ref. [62] with permission.

plots are shown in Fig. 2. In brief, the plots expand on the findings noted above, with an excess amount of dextran at constant boric acid concentrations increasing the selectivity of the non-glycosylated subunits (Fig. 2, left), while the lowest amount of dextran and boric acid offers the best selectivity between the two heavy chain subunits (Fig. 2, right). These plots provide a good reference point for users when determining the optimal concentrations of dextran and boric acid. Finally, when comparing intact glycosylated and non-glycosylated antibodies, no resolution difference was seen using the above gels, likely due to the arrangement of the glycans between the two heavy chains, restricting access to the $B(OH_2)$- groups [65]. Overall, this study demonstrates the use of boric acid and dextran-based gels for the separation of glycosylated antibody subunits that can be applied to characterize therapeutics. This technique could also be used to separate other glycosylated proteins, dependent on if the glycan groups are spatially accessible when migrating through the matrix.

Further work from Guttman et al. was carried out to investigate the relationship between the dextran and borate concentration and the subsequent effect on separation [66]. Using the same mAb and concentration range for dextran and boric acid as in the above reviewed publication, the present work evaluated not just separation of the antibody and its subunits, but also a M_r standard along with biophysical characteristics such as viscosity, electric current, and EOF. The paper presented the analysis of the boundary gels, defined as gels formulated using either the upper or lower limits of dextran (2% and 10% m/v) with either the upper or lower limit of boric acid (2% and 4% m/v). These limits were chosen based on efficient separation of the intended M_r range and ease of pumping the gel in and out of the capillary as well as mitigating undesired effects like joule heating and EOF. As expected, keeping the dextran concentration constant while increasing the boric acid concentration led to a lower EOF along with a higher viscosity and electric current measurement. At a fixed boric acid concentration, increasing dextran led to an increase in viscosity with a decrease in the electric current and EOF. To analyze the effect these factors had on mobility, EOF-corrected Ferguson plots were created with log μ on the y-axis and percent concentration of dextran on the x-axis. A nonlinear, concave shaped curve was seen for both the M_r standard and the mAb sample. In brief, for a larger M_r analyte, an increasing curvature was seen with higher dextran concentration, while with increasing boric acid concentration a decreasing curvature was observed relative to the lower boric acid concentration plot. These results demonstrate that the structure of the gel matrix is altered depending on the concentrations of the polymer and buffer (so called “crosslinker”), and a deeper analysis of the results offers useful insight into the type of dextran-boric acid adducts being formed. For example, with low borate, the curvature suggests that the borate is the limiting factor at a dextran concentration of 7.5%, while with higher borate complexation between borate and 10% dextran still takes place. Such plots, along with the three-dimensional plots presented earlier in this section [62], present a useful platform for method optimization. When analyzing the separation profiles, for both the M_r standard and the mAb, the 2% dextran/4% borate gel was found to produce the fastest analysis time, even faster than the 2% dextran/2% borate gel. This is an interesting finding as if only the sieving effect is considered the former gel is expected to form smaller pores and cause greater impediment to migration. However, the 2% dextran/2% borate gel has more than double the EOF directed towards the injection side, thereby increasing the time the analytes take to migrate against this EOF and reach the detection window. The same trend was seen at the 10% dextran concentration as well, although the actual migration times were higher relative to the 2% dextran gels due to the increase in viscosity. Consequently, the greatest separation time and resolution for non-glycosylated peaks was seen with the 10% dextran/2% borate gel for both the M_r standard and the mAb. Taking a deeper look at the mAb, the greatest resolution between the non-glycosylated and glycosylated heavy chain was seen on the 2% dextran/2% borate gel, in agreement with their earlier work [62], and was again attributed to the interaction of the carbohydrate moieties with the free borate ions in the intrachain adducts. Finally, the 10% dextran/4% borate gel was selected as the ideal formulation for screening purposes, based on the finding that the M_r of the intact mAb on this gel was closest to the actual M_r compared to the other gels, as well as the fact that the M_r of the mAb subunits were also close to the expected values. The electropherograms analyzing the mAb and its subunits are shown in Fig. 3. Each of the four boundary gels were demonstrated to be the best for a specific need, thus showcasing the importance of understanding the fundamental chemistry behind these gel compositions. Specifically, the interplay between factors such as viscosity, current, EOF, and pore size were explored in this study to build on previous knowledge of separation on boric acid and dextran-based gels.

The two studies presented above are of great importance to the CE community as dextran and borate-based gels are routinely used for separation but not much work has been published about the chemistry behind these gels. These studies provide researchers with valuable information that allow for selection and customization of a gel formulation based on their needs and provide practical value for the separation of proteins such as therapeutic mAbs. Future work can investigate the effect on the separation of non-

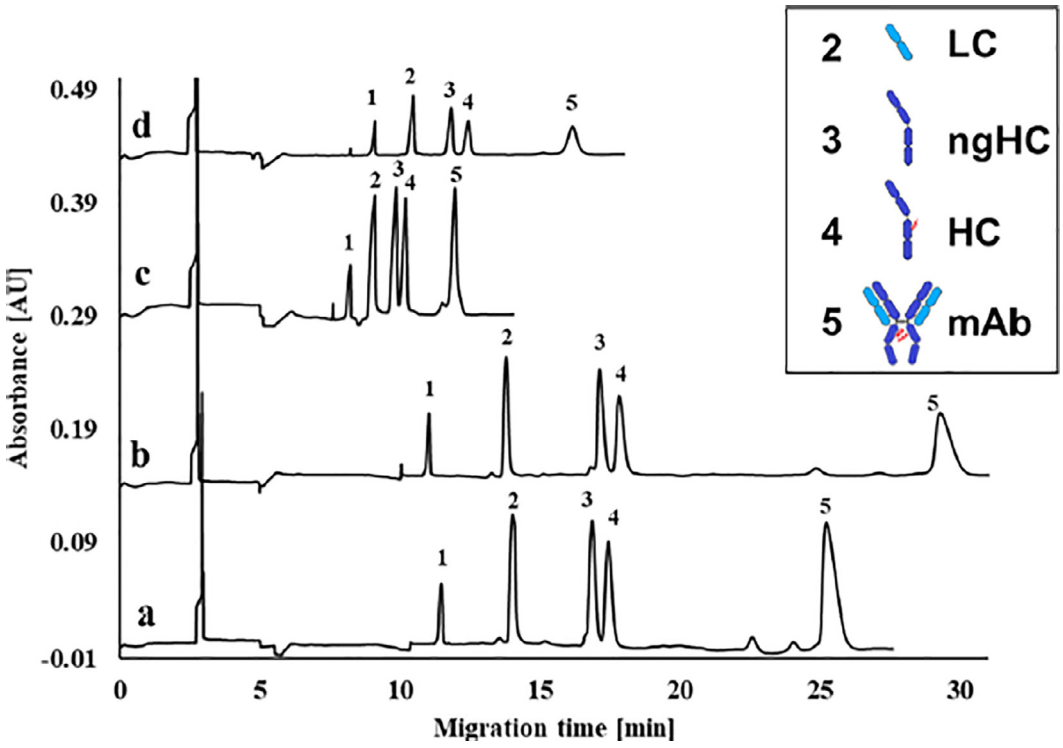

Fig. 3. Separation of intact mAb and reduced subunits on gels at the boundary of the dextran and boric acid concentrations. a) 10% dextran/4% borate b) 10% dextran/2% borate c) 2% dextran/4% borate and d) 2% dextran/2% borate. The first peak corresponds to the 10kDa internal standard. Key: LC – Light Chain, ngHC – Non-glycosylated Heavy Chain, HC – Heavy Chain, and mAb – Monoclonal Antibody. The red line indicates glycosylation. Reproduced from ref. [66] with permission.

mAb glycosylated therapeutics, as it would be interesting to see to what extent these gels can be used to increase the resolution between similarly sized proteins with different glycosylation levels.

Studies on other types of sieving matrices have also been carried out. A 2021 study by Crihfield and Holland investigated the use of a nanogel for capillary electrophoresis [67]. The novelty of this gel, which was used previously for the separation of DNA [68], is that changes in temperature cause great changes in the viscosity of the gel, such as a 480-fold increase when the temperature is raised by as little as 6°C, leading to the formation of a viscous, interconnected ribbon network at temperatures of 24-29°C [69,70] and a low viscosity solution comprised of nanodisks at lower temperatures. This makes it easier to pump the nanogel in and out of the capillary. The gel is a non-denaturing phospholipid nanogel made of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) in a DMPC:DHPC ratio of 2.5. The entire preparation was described in earlier work [71]. In this study, the authors found that at 27°C, nanogel concentrations of 20, 25, and 30% (m/v) along with a semipermanent capillary coating with 5% (m/v) DMPC:DHPC in a ratio of 0.5 were able to mitigate the EOF significantly and allow for the separation of proteins in the 20-80 kDa range. They demonstrated superior separation and quantification of three proteins in human serum with similar charge to size ratios over gel free CE, an unsurprising result as without separation based on other factors (such as sieving), proteins with similar charge to size ratios will be detected closer together in free solution. A comparison to traditional CE-SDS would have yielded value information about the nanogel.

While the use of such thermally responsive gels is unique, there is a lot to be desired in terms of practical applications. Firstly, the cartridge for the P/ACE MDQ Plus instrument used in this study does not provide temperature control over the entire capillary. While most of the capillary can be temperature regulated, the ends of the capillary are notably an exception. This is specifically an issue at the inlet side, as the nanogel will be most likely at a different conformation than the rest of the gel inside the capillary, depending on other factors like instrument and room temperature. The authors do not address this issue and further investigation is warranted for the injection of analytes into the unregulated temperature region of the capillary. Secondly, the separation range of 20-80 kDa is narrow, especially given that the analysis is non-denaturing. Furthermore, since the proteins are intact, the shape of the protein will also affect migration through the gel, possibly yielding skewed M_r estimations. In addition, without knowing the limit of detection (LOD) and quantification on these gels, it is difficult to assess the usefulness of the non-denaturing factor. If the limits are high and knowing that any individual protein fractions collected will have to be analyzed further with a second technique, it might be best to use other techniques or run CE-SDS. While the authors do not address the possibility of denaturing the proteins with SDS, it would be interesting to see whether this is possible and what the separation profile would look like. There may even be practical uses such as purity assessment of mAbs as the reduced and denatured light and heavy chains typically fall between the separation range of the nanogel. The authors also note that one advantage of their nanogel is that the capillary does not have to be covalently modified for reverse polarity separation. Overall, the novelty of this work is to be appreciated, but further research is needed to assess whether nanogels might be an alternative for the currently used separation matrices in CE-SDS.

Improvement of the sieving matrix does not have to be done by making the separation gel from scratch. It is possible to tweak the commercially available formulations in a simpler approach. A 2015 study from van Tricht et al. demonstrated that diluting the commercial separation matrix sold by Beckman Coulter (at the time, currently Sciex) can lead to an increased resolution and reduced analysis time for the study of viral influenza proteins [72]. One

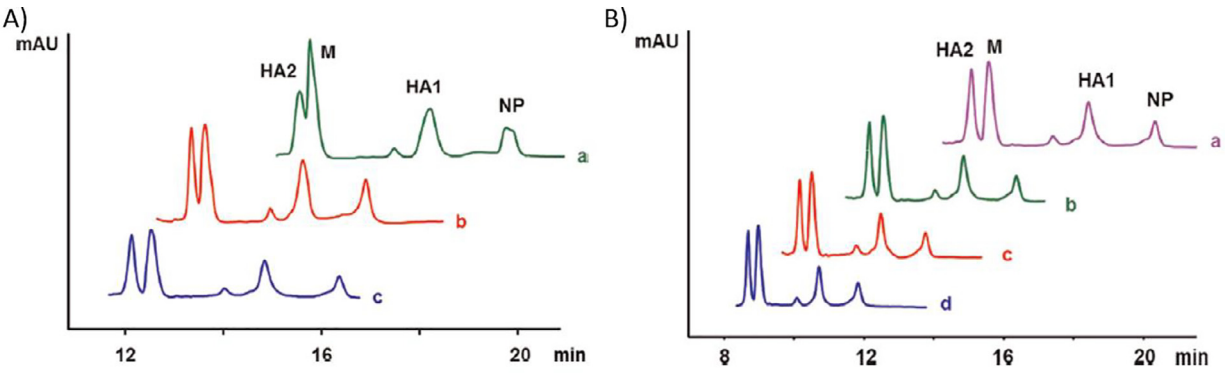

Fig. 4. Electropherograms demonstrating the effect of commercial gel dilution and separation voltage for separation of reduced and deglycosylated A/Victoria viral samples. All samples were electrokinetically injected for 100s at – 18 kV. A) Dilution of the separation gel to (a) 100% (b) 90% and (c) 70% of the original concentration. B) On a 70% separation gel, separation voltage of (a) -13.5 kV (b) -16.5 kV (c) -19.5 kV and d) -22.5 kV. Key: HA2 – Hemagglutinin Fragment 2, M – Matrix Protein, HA1 – Hemagglutinin Fragment 1, and NP – Nuclear Protein. Reproduced from ref. [72] with permission.

of the key findings of this work was that by diluting the separation gel in Milli-Q filtered water, higher voltages can be applied during the separation step and the resolution of proteins that migrate close together can be increased. Using the commercially recommended separation conditions and an optimal injection condition of 100s at -18 kV to inject their reduced and deglycosylated viral samples, the authors observed that the resolution between two similarly sized proteins, the matrix protein (M) and hemagglutinin fragment 1 (HA1), was only 0.6, which is not adequate for quantification. However, by diluting this gel, the resolution could be increased, with a maximum increase of more than two-fold to $\geq$ 1.3 between the M and HA1 peaks seen on a separation gel diluted to 70% of its original concentration (Fig. 4A). The fastest migration time for this gel was observed under a separation voltage of -22 kV, with all peaks being detected in about 12 minutes (Fig. 4B). To obtain these results, the sample preparation was optimized to increase the signal to noise ratio through an investigation of the concentration and choice of reducing agent as well as the incubation time and temperature for denaturation. The final selected parameters were a 10-minute incubation at 100°C using 0.9M 2-mercaptoethanol as the reducing agent. The overall time of the assay was also reduced by qualifying the implementation of a 1-hour deglycosylation step as opposed to the previously recommended overnight deglycosylation. The authors decided to use hydrodynamic injection in their final method as it is thought to be more precise [73], and an investigation into optimization revealed injection conditions of 100s injection at 100 mbar, separation conditions of -20 kV at 32.5°C, and the commercial gel diluted to 85% of its original concentration yields an electropherogram similar to that produced by using electrokinetic injection on a 70% gel at 25°C. This was the final method that was validated and successfully tested for quantification of influenza virus and virosome samples.

The brilliance of this work is in its simplicity. While the earlier reviewed articles in this section require the user to hand make their own gels, this paper provides a more straightforward improvement. Using their optimized methodology, the analysis time of 100 samples was reduced 60%, with CGE analysis taking 4 days as opposed to 10 days typically required by other techniques such as single-radial-immunodiffusion. Through method validation, the accuracy, linearity, and repeatability were shown to be comparable if not better than HPLC, providing further practical value over commonly used separation techniques. In terms of drawbacks, while not noted by the authors, it appears that the resolution between higher M_r peaks can be reduced under some of the tested conditions (Fig. 4B). Thus, the user must decide on the appropriate dilution, separation voltage, injection method, and other conditions depending on the sample of interest. For unknown samples, it would be easier to follow the guidelines set by the commercial kit as a starting point. The authors do point out that even diluting the gel by 10% did not allow the subunit peaks for a reduced mAb to be separated, further supporting the need to optimize the method for each different protein sample. This can be a tedious undertaking and one might resort to using the commercial formulation or another separation technique.

Enhancement of detection sensitivity

As noted in Section 2.3, one of the biggest limitations of CE-SDS is the relatively low concentration sensitivity of the most frequently used UV-absorption detection. It is important to select the optimal mode of injection and injection conditions (voltage and time) to ensure the ideal amount of sample enters the capillary. Another method to increase the sensitivity is to tag the proteins with a fluorescent compound and use LIF detection instead of the more commonly used UV detection. Finally, preconcentration methods are routinely used to improve sensitivity, such as through stacking.

A 2017 study by Zhang and Meagher demonstrated that sensitivity of CE-SDS can be increased by three orders of magnitude for the separation of capsid proteins of Adeno-associated virus (AAV) via the use of head-column field-amplified sample stacking (HC FASS) [74]. In this work, the sample was desalted by exchanging the sample solution to reduce the conductivity of the buffer used for sample preparation. A water plug was hydrodynamically injected into the capillary tube for 24 seconds at 20 psi right before sample injection. The BGE used was the commercial gel from Beckman Coulter/Sciex. The conductivity of the water plug was 0.055 µS/cm and that of the sample following desalting was 0.81 µS/cm, while the conductivity of the BGE was much higher at 1.52 mS/cm. At the sample injection step, this led to a stronger electric field in the water plug and caused the analytes to move faster in the water plug and abruptly slow down at the interface between the water and the BGE, leading to the observed stacking effect. The approximated length of the water plug was calculated to be 10 mm, but the authors noted that residual EOF directed towards the inlet led to shortening and eventual complete pumping out of this plug under an applied voltage. The LOD of this technique was calculated to be 0.2 ng/mL with only 20 pg of the protein sample being loaded into the injection vial. This was calculated to be on par with CE-SDS used with LIF detection and SDS-PAGE with silver staining. However, unlike the former, the samples used did not

need to be tagged or modified in any way, while detection was in real time and on-line unlike the latter. Furthermore, this method uses a much lower concentration of protein than orthogonal approaches. When analyzed against regular CE-SDS methodology described by the Beckman Coulter application guide (PN A51970AD, Jan 2014), the authors found that even when the stacking method was used to load 6960-fold less protein, the separation profiles were similar although a lower sensitivity was seen with the stacking method. However, when corrected for sample concentration, the reported results were a 3500-fold sensitivity enhancement over conventional CE-SDS. Finally, the authors were able measure the purity of the capsid proteins with a low RSD of <1%, with only 25 ng of protein being added to the injection vial. This work demonstrates a simple stacking mechanism that can be used to improve the analysis of proteins, specifically those that might be present in low concentrations. Given that a lower amount of sample is required for detection compared to regular CE-SDS, this approach is also appealing when working with scarce or expensive samples. As demonstrated by the authors, this technique can also be used in industry for purity analysis, as it should be able to detect lower concentration impurities or degradation products that might not be seen with the non-stacking method. Nevertheless, even with the demonstrated advantages, questions remain on the exact mechanism behind the 3500-fold increase in detection sensitivity. As the conductivity of the sample is reduced due to desalting, the difference in conductivity between the sample and BGE is increased, which should lead to better stacking. Given this step and the pre-injection of the water plug, the authors do not clearly state how much each step contributes to the observed stacking effect.

Labeling the analytes with fluorescent tags combined with LIF detection provides a sensitivity that is higher than UV detection and comparable with SDS-PAGE using silver staining [75]. Danish et al. [76] were able to use LIF coupled with CE-SDS to separate and quantify membrane proteins that were tagged by green fluorescent protein (GFP). In brief, molecular cloning techniques were used to introduce GFP at the C-terminal of three membrane proteins and Chinese Hamster Ovary cells were transfected with the recombinant plasmids. In experiments to detect GFP alone, the authors found that the LOD using their CE-SDS method was 0.168 µg/mL, which is 5-fold lower than the LOD using fluorescence with a microplate reader. They also calculated a relative standard deviation (RSD) of <2% for the former method. Homogenizing the transfected cells and running the recombinant protein-GFP conjugates, the authors were able to determine the concentration of each of the membrane proteins. To confirm the results, binding assays using radioligands were used for the two membrane proteins. The results were mixed, with one of the proteins having a similar calculated concentration and the other having a 5-fold lower concentration on the binding assay. This was explained by the fact that only functional protein can bind in the radioligand assay and subsequently only protein that can bind is factored into the concentration calculation. In CE-SDS, all protein, even if unfolded or non-functional, are detected as part of the same peak as long as the M_r is the same. All things considered, the use of GFP was successful and provides a method to increase sensitivity. There are a few important findings to highlight in this paper. The first is that not only is the 5-fold sensitivity increase relative to the microplate a good step forward, but with the capillary format the proteins are separated before detection, thus making recognition of the target protein, impurities, degradation, and aggregates that much easier. Furthermore, using a gel matrix overcomes the drawback of gel free CE approaches where the analyte can interact with the inside of the capillary leading to poor reproducibility, as observed by the authors of the present study as well. The reported RSD of <2% is encouraging and increases confidence in the technique moving forward. While this paper provides practical support for protein tagging and LIF detection with CE-SDS, it does not overcome the biggest problem, which is conjugating the tag to the protein. The molecular cloning technique used here is tedious and must be simplified if it is to be adopted widely. Furthermore, GFP has a size of 26.9 kDa [76] which will cause an increase in migration time. The most convenient method would be to tag the proteins in a test tube using a fast and robust method. A technical note released by Sciex details a simple method to conjugate capsid proteins of AAV to a LIF detectable dye known as Chromeo P503 [77] which is almost 70-fold smaller at 393.29 Da [78]. The described two step "denature and label" methodology can be carried out in less than one hour and demonstrates an increased sensitivity relative to UV absorbance [77]. Such avenues are promising for the future of protein tagging but further experimentation is required to determine the suitability and robustness of these commercially available dyes with CE-SDS.

The sensitivity of the method is also influenced by the injection conditions, which in turn depend on factors such as the type of injection method used, the voltage, injection time, conductivity, and viscosity of the BGE. In Section 3.1 we saw that to get optimal results using electrokinetic and hydrodynamic injection on diluted commercial gels, the concentration of the gel and the temperature during separation had to be optimized for each injection type [72]. Five years after that study, follow up work from Geurink et al. takes a deeper dive into the chemistry behind those observations [53] . When the gel is diluted, the viscosity decreases, leading to an increase in the conductivity. However, the dilution also reduces the ionic strength of the gel, thereby decreasing the conductivity. These conflicting effects on the conductivity were studied and it was found that the net effect was a reduction in conductivity at all dilutions and temperatures evaluated, revealing a greater effect from the reduction in ionic strength. For electrokinetic and hydrodynamic injections, the effects of this are opposing. With a reduced conductivity, less sample is injected with electrokinetic injection. Hydrodynamic injection does not depend on conductivity but does correlate to the viscosity of the BGE. As the viscosity is reduced, more sample is injected relative to the undiluted commercial gel (at a given pressure and time). Regardless of the type of injection method, the effect on sample stacking is consistent. With a lowered conductivity and therefore a lowered conductivity difference between the sample and BGE, the stacking effect is lowered. This is a bigger problem with hydrodynamic injection as it can lead to a wider sample plug and subsequent peak broadening. Optimization is thus required for both injection conditions. This paper by Geurink et al. goes on to establish an approach to develop CE-SDS methods for different samples that has been examined further in Section 3.6. However, these findings are worth mentioning in this section as it provides a link amongst the concentration of the sieving matrix, injection method, and sensitivity.

Detergent

The use of SDS as the surfactant in electrophoresis is well established, starting with PAGE and then being translated over to CE. However, the biggest drawback to using SDS is that it does not bind evenly to all proteins, such as proteins modified with glycan residues, as mentioned in Section 2.3. Given that 1.4g of SDS is bound to a gram of protein [30,31] but only 0.2g of SDS is present on each gram of glycan [51], stark differences can be seen between the actual and apparent molecular mass on the gel. Attempting to replace SDS with other detergents has been routinely carried out for PAGE [79] and similar investigations have now been published for CGE as well.

A 2018 study from Beckman et al. investigated the use of detergents with varying alkyl chain lengths in the separation gel and sample buffer for the separation of recombinant therapeutic pro-

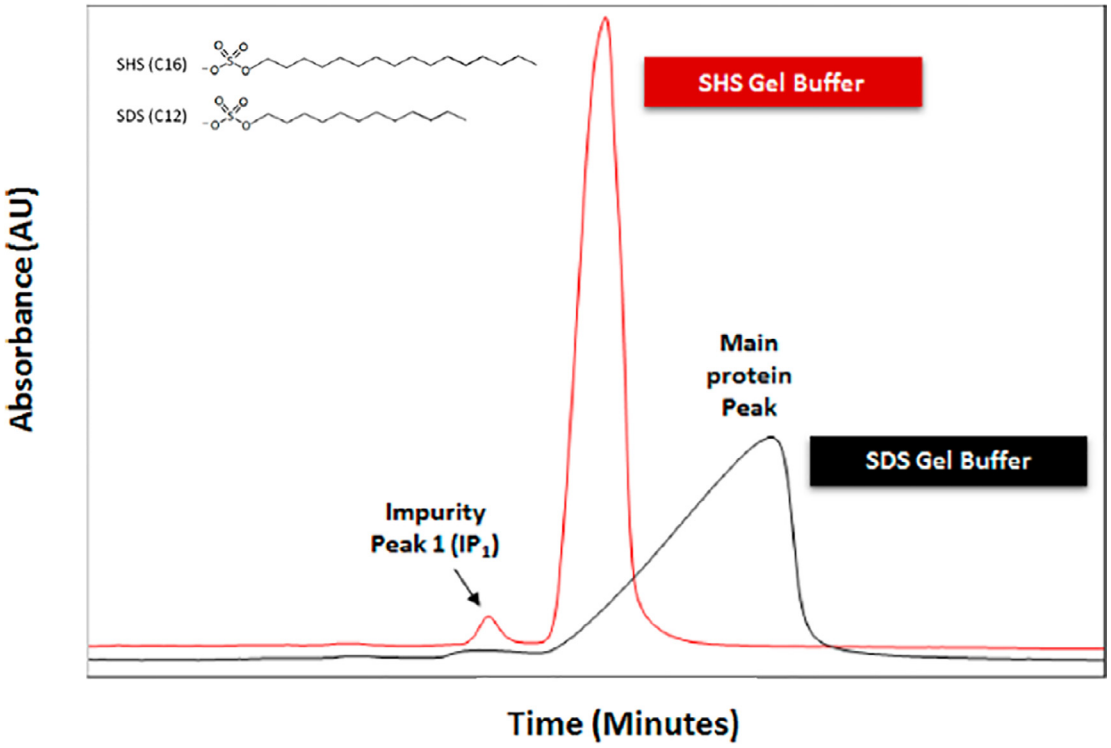

Fig. 5. Electropherogram of the Fc-Adnectin fusion protein on a separation gel composed of SDS alone (black) or SDS supplemented with 0.2% SHS (red). The molecular structures of the detergents are shown in the top left corner. Modified from ref. [80] with permission.

teins (RTP), with a focus on an Fc-Adnectin fusion protein that they refer to as RTP-1 [80] . Out of the different detergents tested, sodium hexadecyl sulfate (SHS) was found to improve the peak separation efficiency and impurity detection the most relative to SDS at a concentration of 0.2% (Fig. 5), as evidenced by a 2.3-fold and 8-fold increase in resolution and number of theoretical plates respectively. This was an interesting result as previous work had found that using SHS with PAGE reduced the peak separation efficiency of the model proteins tested [81], a finding that was seen by the authors of the current study as well [80]. They attribute the improved separation profile of RTP-1 with SHS to the Adnectin domain of the protein that is thermophilic and has a higher density of negative charge on one of its sides, thus requiring the more hydrophobic longer alkyl tail of SHS to create a protein-detergent interaction that is energetically more favorable for unfolding of the protein. It would seem that SHS can only be used to optimize CGE for specific types of protein and based on the Adnectic domain, the authors predict that proteins with very high thermal stability fall under this umbrella. However, this prediction is based on the three RTPs tested in this experiment and additional studies using different proteins are required. Interestingly, 0.2% SDS was needed in the gel matrix along with SHS for optimization as it was shown to improve the peak shape of the internal standard. This is based on the thought that longer chain detergents do not coat smaller analytes evenly as they form bigger micellar structures [81]. Even with an improvement to CGE performance, the biggest drawback to using longer chain hydrophobic detergents is that dissolution of the compound is more difficult than SDS in a hydrophilic environment, as evidenced by the need to sonicate the preparation in a water bath at 70°C [80]. This should be kept in mind for further optimization of this technique, as altering the components or their concentration can increase the difficulty of getting a homogeneous solution. Thus, if feasible, altering other aspects of the gel or parameters of the CE system might be a more straightforward approach.

Follow up work from the same group has demonstrated how using longer alkyl chain detergents can help reduce protein aggregation under both reducing [82] and non-reducing conditions [83]. In the former, a sieving matrix consisting of both 0.3% SHS and 0.2% SDS along with sample preparation using 0.5% sodium tetradecyl sulfate (STS) was found to be the best combination to reduce high M_r aggregates of recombinant mAbs caused due to incomplete denaturation [82]. An interesting finding the authors mention is the need for substituting SDS in both the sieving matrix and sample buffers, which they attribute to competitive binding of the surfactant molecules to the analytes leading to a dynamic equilibrium. In the second study, a combination of 0.3% SHS and 0.2% SDS in the gel matrix was shown to reduce aggregates under non-reducing conditions compared to regular CE-SDS and bring the calculated aggregate percentage in line with other reportable techniques, such as size exclusion chromatography [83]. One significant difference to highlight is that the addition of SHS or STS during sample preparation did not have a noteworthy effect on reducing aggregation, opposite to the dynamic equilibrium observed under reduced conditions [83]. While the nature of the non-reducing study is like the earlier one under reducing conditions, the former offers additional practical importance as non-reducing conditions are used to look for reduced fragments and impurities that can come about during the production or purification process [84] . In both above studies, varying the other parameters such as the pH, reducing agent, SDS concentration, etc. did not help reduce these aggregates, further indicating the usefulness of these longer alkyl chain detergents.

Based on this collective work, SHS along with SDS represents the best detergent choice in the separation matrix for analyzing specific proteins, with the application seemingly limited to proteins that do not unfold completely in the presence of SDS due to an increased energy requirement to reach this state. Currently, no other group has published research on using detergents other

than SDS in CGE, so it will be interesting to see if the technique can be standardized to model proteins, or if improvements can be made to aid in dissolution of SHS and other longer chain detergents. Nonetheless, this line of research offers useful insight into use of non-conventional detergents with higher hydrophobicity for the separation of specific types of proteins, specifically recombinant therapeutics, using CGE.

Temperature

Selecting the optimal temperature plays a role during both sample preparation and the separation process. An increase in temperature causes the viscosity of the matrix to decrease, leading to a decreased migration time, an increased mobility, and an increased EOF. The conductivity of the gel will also influence the temperature as a more conductive system will draw more current and can lead to joule heating and consequent band broadening due to ineffective heat dissipation.

A 2020 study by Filep and Guttman investigated the relationship between the temperature and the migration of both biotherapeutic proteins and M_r standards on boric acid and dextran-based gels [85]. The selected range of temperatures was from 15 – 60°C at 5°C increments. The goal of this study was to gain insight into how the temperature can be optimized during separation based on the activation energy required by the SDS coated proteins to move through the gel. Arrhenius plots were created and used to calculate the activation energy that was then plotted against M_r. Surveying the M_r standards revealed that with an increase in M_r the activation energy decreases exponentially. This non-linearity can be explained by larger proteins needing to distort the matrix to migrate, which is not difficult for these bigger and highly charged molecules through the transiently linked separation matrix. A similar trend was seen in an older study on the migration of DNA through non-crosslinked CE gels when the activation energy was plotted against the fragment size [86]. This contrasts with a study of DNA migration through a crosslinked gel where there was a positive relationship between activation energy and fragment length as the gel is unable to distort meaningfully and subsequently more energy is required to push larger analytes through [87]. This trend would also be expected for proteins migrating through these chemically crosslinked gels. However, the same analysis using a mixture of therapeutic proteins revealed no discernible relationship between M_r and activation energy. This was attributed to the polypeptide constituents of the two samples. The standard is made of the same, unmodified polypeptides while therapeutic proteins are composed of various, differing polypeptides and modifications such as glycans that make it difficult to predict the activation energy based on the M_r of the molecule. This result makes this approach unviable in a practical setting such as biopharma where most of the proteins being studied are not made up of the same constituents. Outside of activation energy, the authors were able establish a linear relationship between μ and $\frac{1}{\sqrt[6]{MW}}$ with an r^2 of 0.999 calculated based on the assumption that molecules migrate with a cylindrical shape [88]. The r^2 value using this approach was higher than the corresponding r^2 values based on other models proposed by others [89,90]. This relationship can be used to determine μ once the M_r is known. It is important to keep in mind that this relationship was found on the commercially available SDS MW Gel Buffer from Sciex and may not hold true on other gels and systems, depending on other factors like EOF suppression, type of capillary used, etc. Furthermore, we have already seen that the link between activation energy and size does not hold true for therapeutic molecules, making it possible that this relationship also does not accurately describe similar compounds. The effects of glycosylation have been demonstrated previously, and such modifications will affect μ of the molecule in a non-M_r dependent manner.

Effects of glycosylation on M_r determination

Throughout this piece we have noted the drawback of glycoproteins not being bound by SDS evenly, which affects the charge to size ratio and subsequently, migration through the gel. In this section, work that was centered on comparing the accuracy of M_r determination of glycoproteins across CE-SDS, SDS-PAGE, and related techniques have been reviewed.

A 2019 study from Wang et al. sought to compare the migration of glycosylated proteins between CE-SDS and SDS-PAGE under both reducing and non-reducing conditions [91]. Eight experimental glycoproteins were chosen along with two non-glycosylated proteins as controls. Under reducing conditions, the authors found that the M_r of the proteins on SDS-PAGE were closer to the theoretical values compared to CE-SDS, where they calculated that each glycan site caused an average increase of more than 10 kDa from the expected M_r. To support the theory that this increase in apparent M_r in CE-SDS is because of glycosylation, the non-glycosylated controls were observed to run similar to the theoretical M_r. Furthermore, adding PNGase F to remove N-linked glycans under reducing conditions prior to CE-SDS revealed that the migration time was shifted with the proteins now migrating at a M_r closer to the theoretical expectation. Under non-reducing conditions, the M_r on SDS-PAGE was the same or lower than on reducing SDS-PAGE, an expected result as disulfide bonds might be holding the proteins in a tighter conformation, making traversing the pores easier. On the other hand, the M_r were increased on non-reducing CE-SDS compared to the reduced analysis. The non-glycosylated controls once again ran on par with the theoretical values, indicating that this migration shift is glycan dependent. The authors suggest that under these conditions, the glycans can interact with the separation gel, a theory put forward in Section 3.1 of this review [62]. As previously described, the interaction between matrix and carbohydrate would slow down the migration of the analytes. Under reducing conditions, the authors postulate that this interaction is limited, probably due to a shielding effect following denaturation. However, the apparent M_r on reducing CE-SDS is still greater than on reducing SDS-PAGE, suggesting this effect is not absolute. The effect of sialylation was also tested for a single protein and treatment with sialidase was found to not influence the migration time and calculated M_r. This was interesting as sialic acid is negatively charged under electrophoretic conditions and would in theory be expected to add to the negative charge on the molecule [92]. It is possible that removing sialic acid leads to better SDS binding, thus offsetting the loss of the negative charge by desialylation through detergent binding, although the authors conclude that the charge present on the glycans do not play a role in influencing mobility. This study provides a demonstration of the inaccuracy seen in the M_r determination of glycoproteins with CE-SDS, which is of critical importance to address as it is a barrier to true characterization of glycosylated analytes especially given the relevance of glycosylated therapeutic proteins such as mAbs. Further work is needed to understand the relationship between glycan modification and shift in migration time with CE-SDS.

An analogous study by Scheller et al. compared the effect of glycosylation not only across CE-SDS instrumentation and SDS-PAGE, but also across related methods like microchip CE-SDS and Simple Western [92]. Using these methods, seven proteins were run with and without their glycan groups. In brief, a range of differences in the reported M_r were seen both across methods and glycosylation condition. The results are presented in Table 2.

Most notable is the greater molecular mass for the majority of the glycosylated proteins on the CE-SDS and CE-SDS PLUS systems relative to the other techniques. Deglycosylation was found to bring the molecular mass closer to the theoretical amounts, confirming the effect of glycosylation for these results. Surveying the

Table 2
The molecular masses of the tested glycoproteins before and after treatment to remove the glycan groups across the respective techniques. Glyc – Glycosylated, deglyc – Deglycosylated, r-Mr – reference molecular mass. Reproduced from ref. [92] with permission.

		Mr (kDa)					
		CE-SDS	CE-SDS PLUS	SDS-PAGE	Wes	Labchip®	r-Mr (kDa)
Ovalbumin	glyc	43	44	45	45	46	44.3
	deglyc	33	35	42	39	41	42.7
α-2-macroglobulin	glyc	245	241	144	132	221	179
	deglyc	165	164	136	111	180	163.3
Matuzumab light chain	glyc	25	26	27	31	27	23.63
	deglyc	23	26	27	30	27	
Matuzumab heavy chain	glyc	63	67	57	56	68	49.66
	deglyc	53	57	53	51	61	
CD74	glyc	81	82	34	48	63	
	deglyc	34	35	23	31	32	19.3
EPO	glyc	97	101	40	56	74	
	deglyc	21	18	20	27	17	21
SynCAM1	glyc	343	345	74	123	194	
	deglyc	62	59	50	54	65	38.4
N-Cadherin	glyc	262	253	117 101	121	195	
	deglyc	134 108	127 103	108 94	99 82	164 143	89.2

Table 3
Properties of the glycoproteins that could potentially explain the differences in migration seen in the glycosylation/deglycosylation experiments. Reproduced from ref. [92] with permission.

	pI	GRAVY	Content Proline [%]	Neg. charges [%]	Pos. charges [%]	*N*-glycan sites	O-glycan sites
Ovalbumin	5.19	-0.001	3.6	12.2	9.1	1	0
α-2-macroglobulin	6.03	-0.195	5.3	10.7	9.2	8	0
Matuzumab light chain	6.34	-0.417	4.7	9.0	8.5	N/A	N/A
Matuzumab heavy chain	8.34	-0.552	7.6	9.4	10.3	N/A	N/A
CD74	6.65	-0.654	11.8	11.2	10.7	2	1
EPO	8.30	0.027	5.7	9.8	10.9	3	1
SynCAM1	5.14	-0.430	6.1	12.8	8.2	6	0
N-Cadherin	5.16	-0.405	9.0	11.8	8.4	8	0

migration of glycosylated analytes, SynCAM1 and *N*-Cadherin were found to have the biggest molecular mass difference between SDS-PAGE and the two CE-SDS techniques. One possible reason that has been put forward in Sections 3.1 and 3.2 is the interaction with the separation gel (47, 62). Given that SynCAM1 and *N*-Cadherin function in cell adhesion using carbohydrate-carbohydrate interactions [93], the significantly greater molecular mass on CE-SDS that normally uses dextran as the polymer is expected. On a similar note, N-glycan sites were also found to affect migration, with the three proteins containing the most N-glycan sites having the highest M_r on the CE-SDS techniques (Tables 2 and 3). Finally, the effect of sialyation was investigated. In the previous study Wang et al. [91] concluded that sialyation and charge on the proteins do not influence migration, but Scheller et al. are not as convinced. The latter observed that proteins that are expected to lower sialic acid content such as SynCAM1 and *N*-Cadherin have the highest reportable M_r (>200 kDa) on CE-SDS and CE-SDS PLUS. However, the M_r for these proteins are much higher than those reported by the three other techniques, so it is difficult to attribute this strictly to sialyation. Given that Wang et al. only investigated the effect of sialic acid residues for a single protein and this study makes observations without conducting experiments to remove sialic acid and observe migration, more work is needed to probe this relationship.

Interestingly, following deglycosylation there was a split between the reported molecular mass of the proteins and the theoretical mass, with some migrating at a time corresponding to a higher M_r than expected while others being around the same or lower. The authors examined properties that could explain these results, summarized in Table 3. The first is the hydrophobicity of the protein, indicated by the grand average of hydropathicity (GRAVY) score. In brief, a higher score, like those seen for α-2-macroglobulin, EPO, and ovalbumin correlate to a more hydrophobic protein, and potentially better SDS binding, leading to the similar or lower molecular mass seen with CE-SDS and CE-SDS PLUS relative to the reference molecular mass. The above three mentioned proteins are also made up of less proline, which can influence migration through the gel. The authors explain that proline is a heterocyclic amino acid which affects the protein being straightened out during denaturation due to kinks formed in the structure, leading to a greater Stokes radius and subsequently, a lower μ. The authors also use previous work to highlight the effect of pI and charge on migration [94,95], yet no such trend can be seen from the data presented in this study. Furthermore, as this comparison is of deglycosylated proteins, interaction between the polymer in the matrix and the carbohydrate groups should be non-existent, due to the removal of the aforementioned groups. However, the molecular masses still vary among the techniques used, suggesting that either the matrix or differences in sample preparation still influence the separation. The authors do not point this out, but they do note that making comparisons based on the separation matrix is not possible due to the proprietary nature of these gels.

The importance of this study is in providing researchers with an understanding of how the properties of glycoproteins dictate their separation on CE-SDS and related techniques. The effects of analyte hydrophobicity, N-glycans, separation matrix, and proline content were all found to influence the migration and subsequently the reportable molecular mass. Other traits like sialyation can also affect migration through the gel. It is not sufficient to look at these characteristics separately, as the interplay between all these properties influence separation and thus, researchers will have to choose the

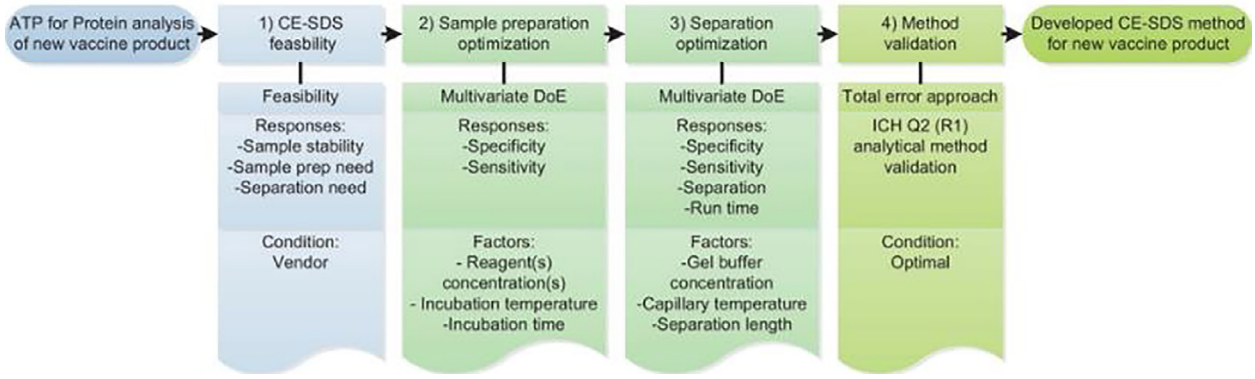

Fig. 6. Four steps to develop optimal CE-SDS methods for analysis of proteins in vaccine products and possibly other proteins. Reproduced from ref. [53] with permission.

best technique based on their analytes and needs. As noted by both Scheller et al. and Wang et al., more work is needed to understand the properties that influence migration of glycoproteins.

Method development

In this review, we have seen advancements in many different areas of CE-SDS. What we need is to tie all this information together in a way that those in the field have a framework to design their experiments. This was done by Geurink et al. [53], where a four-step approach to design CE-SDS methodology for the analysis of proteins in viral vaccines was proposed (Fig. 6). Briefly, the first step is to use the conditions suggested as part of the commercially sold kits. If there is need for optimization, steps two and three focus on the optimization of the sample preparation and separation conditions respectively. The authors identified incubation time, incubation temperature, and concentration of the reagents as the critical method parameters (CMPs) for step two and the dilution of the separation gel, the effective length of the capillary, and the temperature of the capillary system as the CMPs for step three. The final step is validating the conditions chosen in the prior steps. Using the proposed framework, the authors were able to design, validate, and successfully implement improved and differing methods for both qualitative and quantitative analysis of viral proteins used in vaccines. The authors report a LOD of 10 µg/mL of their inactivated polio vaccine in one of their studies and a limit of quantification (LOQ) of 0.50 mg/mL for mini-haemagglutinin in a separate study, though they note that the LOQ can be reduced further. All experiments were done using UV detection at 214 nm and hydrodynamic injection at 100mbar, based on previous work [72].

This study describes a straightforward workflow that users can apply to vaccine and possibly other protein samples as well. The vaccine focus stems from current and historical outbreaks of coronavirus (COVID-19), Ebola virus, and Zika virus. Establishing a method to optimize CE-SDS can streamline the development of vaccines. It will also increase the efficiency of other lines of work that use this technique. However, improvements can still be made, as noted by the authors themselves. In this study UV detection at 214 nm was used, but fluorescent detection of tagged proteins can also be investigated. Furthermore, this work is based on the commercially available separation gel sold by Sciex, but we have seen the potential of other gel formulations for CE-SDS separations. Admittedly, using a custom gel formulation would increase the overall time to define, transfer, and validate a method and might not be feasible for analysis of samples on shorter timelines, such as the yearly influenza vaccine. On the other hand, it might be useful for the analysis of certain proteins. The four steps proposed in this article are a good starting point and future work may add to this framework by adding steps for gel formulations, injection conditions, sample stacking, and the type of detector used.

Applications of CE-SDS

In the biotech and pharmaceutical industry, CE-SDS is mainly used for biotherapeutic testing for product release, sample characterization, and stability testing [17,96,97]. Examples of these quality control tests included identity verification, assessing purity by screening for degradation products or aggregates, and determining post-translation modifications such as glycosylation, such as in the case of separating the non-glycosylated and glycosylated heavy chains for mAbs [96,97]. Furthermore, more than 96% of the biologic license applications received by the Food and Drug Agency in 2016-2017 used at least one CE method for product characterization [17], highlighting the usefulness of CE-SDS and related techniques in this field. Additionally, publications reviewed in this article have demonstrated the use of CE-SDS for the separation of viral and vaccine proteins such as for influenza [72] and AAV [74], as well as recombinant fusion proteins [80]. However, drug development is a continuous process and innovative products are constantly created. Some of these are termed as next-generation products and include a variety of types of antibodies, such as those that are glycoengineered, multi-specific, or have a lower M_r than your standard antibody [98]. The analytical methods used to support the characterization and testing of these compounds must also be able to keep up and be applicable to these complex therapeutics. Fortunately, recent work finds that the application of CE-SDS can go further than standard biotherapeutics. A study from Filep et al. demonstrates the use of CE-SDS to analyze complex modalities of antibodies such as glycoengineered and bispecific mAbs [99]. In this study, the authors were able to use CE-SDS to analyze both the individual subunits as well as the whole antibodies. While a standard CE-SDS method was able to separate the subunits of the glycoengineered mAb, the bispecific compound required a previously established temperature gradient during denaturation [100] as well as a longer capillary (increased effective length) to successfully separate the two different light chains from each other. This is an important finding as we have seen the need to be able to successfully separate different heavy chains in this review, but light chains pose a bigger challenge as they are smaller and migrate faster through the gel. Being able to baseline resolve two smaller and closely related peaks is of paramount importance. This work represents an encouraging step forward for separation of complex therapeutic compounds.

Another unique application of CE-SDS is its ability to study protein-protein interactions (PPIs). While these investigations have been carried out previously through affinity probe capillary electrophoresis (APCE), this technique suffers from limitations such as

interactions between the analytes and capillary wall and the need to maintain the linkage between proteins during migration, both of which are difficult to resolve under a single set of experimental conditions. A 2016 study by Ouimet et al. [101] demonstrated that CE-SDS can be used to investigate the PPIs by crosslinking the analytes of interest preceding injection into the capillary, referred to as protein crosslinking CE, or PXCE. The agent used to achieve the crosslinking was formaldehyde due to its versatility in binding multiple amino acids [102]. Ten minutes of formaldehyde treatment was shown to be successful to achieve crosslinking of multiple analytes including heat shock proteins and an antigen-antibody pair. Quantification is also possible with PXCE, with the authors successfully being able to calculate the dissociation and inhibitory constants. Follow up work from the same group was able to show an increase in the throughput of PXCE [103]. Switching the crosslinker from formaldehyde to glutaraldehyde reduced the crosslinker treatment time from 10 minutes to 10 seconds. Furthermore, the separation stage was also improved by using a capillary with a smaller internal diameter as well as a separation matrix with a lower viscosity, enabling the application of a stronger electric field during separation. By employing an overlapping multiple injection strategy, the analysis time for each sample was shown to be only a minute. Together these studies provide an improved approach to studying PPIs in a capillary relative to APCE, making the investigation of complexes of multiple analytes and examination of compounds with dissociation constants as low as a few nanomolar possible. Most importantly, this line of work has the potential to be used in drug discovery for target screening and candidate selection, which can lower the time for drugs to be taken from bench to bedside.

Microchip CGE

In Sections 3.1 through 3.7 we have critically analyzed CE-SDS in its most traditional and commonly used format. Nevertheless, the technique can still be improved. The use of microfluidic systems and microchips provide improvements over CE-SDS [104,105] akin those seen when comparing the traditional technique to SDS-PAGE, such as being higher throughput, reduction in sample and reagent requirements, and even lower analysis times. Earlier studies have also demonstrated that the sensitivity and purity analysis using microchip CGE (M-CGE) is comparable to CE-SDS [106]. However, while the use of M-CGE is not new, limited studies have been published on this line of work. In this section we will take a deeper look into promising work that demonstrates the potential of M-CGE as a protein separation technique.

Older studies had demonstrated the injection of droplets totaling only a few nanoliters in volume separated by an immiscible segment referred to as the carrier phase [107,108]. In these studies, the separation matrix was an aqueous solution as the EOF was required for sample injection, which is not feasible with gels due to EOF suppression. A first of its kind study demonstrating the use of microfluidics with CGE was published in 2013 that revealed a separation speed of just under a minute per injection [109]. A 2019 study by Ouimet et al. describes an even higher throughput M-CGE method that utilizes a novel approach for introducing sample droplets that are separated from each other by a less dense carrier or segmenting phase [110]. An illustration of this microchip device is provided in Fig. 7. As seen in the figure, droplets of about 5 nL were separated from each other in silicone oil (the segmenting phase) as it has a low density of just 0.93 g/mL. When injected into the capillary, the sample train is transported to a section under the oil draining reservoir which contains the BGE, which is a non-crosslinked gel utilizing dextran (3.5%, 1,500-2,800 kDa) as the polymer along with Tris (90 mM), borate (100 mm), SDS (13.8 mM), and EDTA (1mM). As the silicone oil has a lower density, it rises upwards and is removed from the sample train, leaving just the analyte in the sample droplet behind and preventing the oil from being injected into the separation channel. A high voltage is constantly applied in the reservoir to ensure that the sample droplets are directed towards the separation channel and do not enter the reservoir. The authors note that it is not possible to completely prevent the droplet from being diluted with the BGE as it crosses into the separation channel, as noted by the varying peak heights observed in the electropherograms. To verify the applicability of this method, the authors then tested their device for assaying PPIs, specifically building on previous work using heat shock proteins and their chaperones that was described in Section 3.7 [101,103]. A maximum of more than 600 injections and separations were possible from just 175 droplets before the microchip required reconditioning. The calculated time of analysis for each sample was just 10 seconds, a sixth of what was possible with PCXE [103]. Furthermore, the authors were able to successfully use this M-CGE method to test for inhibitory molecules as well as apply their device to non-gel CE, but a thorough discussion of this section of the study is out of scope of this review.

The device constructed in this study provides the highest throughput of any method seen in this review and was successfully used to study PPIs. Moreover, once set up the device is easy to use with hundreds of injections possible without needing to recondition the instrument. Yet, challenges and limitations still exist, as noted by the authors themselves. The first is the lower sensitivity of this method, an understandable result given the smaller scale of analysis. Thus, users might face difficulty working with dilute samples. On a similar note, samples that have a higher salt content would also have adverse effects such as an increase in joule heating. Finally, the issue seen due to dilution and subsequent variation in the peak heights provides a challenge to using this method quantitatively. Suggestions put forward to remedy these issues such as altering the volume of the sample droplets or the volume of the segmenting phase between the droplets can be counterintuitive as it would theoretically reduce the throughput of the technique. Given the recency and novelty of this method, such challenges are expected and will no doubt be improved upon in the future.

While the central theme was not M-CGE, an analogous study demonstrated the use of a microfluidic system for Western Blotting that allowed for blotting and immunodetection to be completed in less than 90 minutes [111]. In this study, the analytes were separated using M-CGE and directly deposited on the membrane as they exited the microchip, allowing for separation as well as analyte transfer to be completed in mere minutes [112,113]. The immunoassay detection was done using a flow method using a syringe to decrease the area the antibodies diffused into, making it easier to bind the proteins of interest and lowering detection time to just an hour. In addition to the lowered analysis time, this technique also reduced the amount of sample and antibody required significantly. While the focus of this study was not M-CGE, this work further demonstrates the potential of microfluidic and microchip systems for the separation and analysis of proteins.

Investigations using current instruments have also been conducted in a bid to establish M-CGE as a technique that can find routine use in a current good manufacturing practice (cGMP) environment, such as for stability and release tests as noted in Section 3.7. A 2017 study utilizing the HT Protein Express chip with the LabChip GXII system sold by PerkinElmer aimed to establish a M-CGE technique that could be used as a tool in both R&D and cGMP environments [114]. In this work, the sample preparation method was evaluated for two mAbs under both reducing and non-reducing conditions to identify CMPs during this step. The parameters evaluated were the denaturation time, denaturation temperature, volume of the appropriate sample buffer, the protein con-

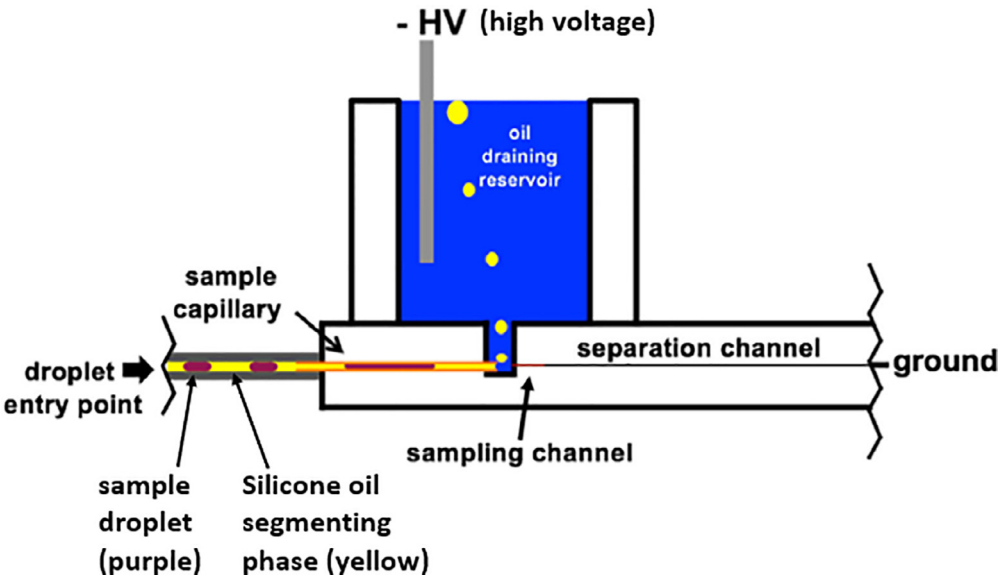

Fig. 7. Cross-sectional illustration of the oil drain microchip based on density differences between the higher density sample droplets (purple) and lower density segmenting phase composed of silicone oil (yellow). Modified from ref. [110] with permission.

centration, and the concentration of the reducing agent, which in this case was dithiothreitol (DTT). The authors identified that other than the concentration of DTT, changing the other parameters had significant impact on the purity of at least one of the mAbs under either, or both, of the reducing and non-reducing conditions. In addition to this, the amount of dye used was also found to be a CMP as it was seen to influence the baseline of the electropherograms. Using these findings, the authors were able to establish a working range for each of these CMPs during preparation of the mAb sample and validate their approach though experiments testing the accuracy, linearity, repeatability, reproducibility, and specificity. Additional considerations include testing samples no later than two hours after the sample preparation step as the purity results can be affected if the sample is allowed to sit for longer, presumably due to evaporative or degradative effects. Finally, the established method was successfully carried out for the testing of a further twelve mAbs, bringing the total number of therapeutics to be tested by this approach to fourteen. This work highlights that M-CGE can be used for the evaluation of commercial therapeutics, albeit only different mAbs, which are expected to yield fairly similar results, were tested in this work. It would be interesting to see if a similar method development approach can be taken for various non-mAb proteins, as this would greatly increase the likelihood of using M-CGE along with or instead of CE-SDS for testing purposes. Furthermore, qualitative data comparing this M-CGE method to currently used CE-SDS techniques is also required in order to truly assess the potential of this technique as a suitable, and improved, replacement.

Overall, the articles reviewed in this section demonstrate the potential of M-CGE as a powerful tool for both discovery research to find suitable drug molecules and targets as well as final testing of commercial products under cGMP guidance. Additional insight is needed before the routine implementation of these systems, with CE-SDS being a suitable technique for the time being.

Concluding remarks and future direction

CE-SDS is a robust technique for the separation of proteins and provides many advantages over traditional techniques such as SDS-PAGE as evidenced by the shorter analysis time, ease of automation, and higher throughput. These traits translate well into the biopharmaceutical field, and CE-SDS is routinely used for the characterization and release testing of drug products. The articles under the scope of this review cover advancements made in all facets of CE-SDS for protein analysis, including the sieving matrix, sample stacking, the novel use of longer chain detergents, fluorescent protein tagging, improved characterization of therapeutics relative to SDS-PAGE, method development, and the microchip CGE mode. It was most encouraging to see the progress made on increasing our understanding of the intricacies of the sieving matrix as the proprietary formulation of the commercial gels has proven to be a barrier for characterizing and improving these gels. However, there is still more work to be done by focusing on hyphenated techniques like CE-SDS with MS, which haven't received much attention, likely due to the complications caused by SDS through suppression of ionization and the signal [115,116]. Finally, given that CE-SDS is known to already have a superior resolution when compared to SDS-PAGE and that the biggest issue is the relatively low concentration sensitivity in the UV detection mode, we believe that improving the sensitivity of these gels should be the main area of focus moving forward. The use of fluorescent tags and sample stacking are promising avenues, but both face challenges that need additional work to overcome. For the former, the development of a quick and efficient universal protein tagging methodology is required while sample stacking is harder to achieve when using a viscous gel as the BGE, relative to gel free CE. It is our belief that the groundwork laid by the reviewed articles can be taken further to develop a method that yields improved separation relative to commercially available kits.

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.

CRediT authorship contribution statement

Rijul Bhimwal: Writing – original draft, Visualization. **Richard R. Rustandi:** Writing – review & editing. **Anne Payne:** Writing – review & editing. **Mohamed Dawod:** Conceptualization, Supervision, Writing – review & editing.

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References

[1] J.M. Berg, J.L. Tymoczko, L. Stryer, Biochemistry, fifth ed., W. H. Freeman, New York, 2002.
[2] M. Dawod, N.E. Arvin, R.T. Kennedy, Recent advances in protein analysis by capillary and microchip electrophoresis, Analyst 142 (2017) 1847–1866, doi:10.1039/c7an00198c.
[3] P.R. Srinivas, Introduction to protein electrophoresis, in: B.T. Kurien, R.H. Scofield (Eds.), Protein Electrophoresis: Methods and Protocols, Humana Press, Totowa, New Jersey, 2012, pp. 23–28, doi:10.1007/978-1-61779-821-4_2.
[4] O. Vesterberg, History of electrophoretic methods, J. Chromatogr. A 480 (1989) 3–19, doi:10.1016/s0021-9673(01)84276-x.
[5] A. Tiselius, Electrophoresis of serum globulin. I, Biochem. J. 31 (1937) 313–317, doi:10.1042/bj0310313.
[6] A. Tiselius, Electrophoresis of serum globulin: electrophoretic analysis of normal and immune sera, Biochem. J. 31 (1937) 1464–1477, doi:10.1042/bj0311464.
[7] A. Tiselius, A new apparatus for electrophoretic analysis of colloidal mixtures, Trans. Faraday Soc. 33 (1937) 524–531, doi:10.1039/TF9373300524.
[8] A. Tunlid, Tiselius, Arne, in: eLS (Ed.) 2011. https://doi.org/10.1002/9780470015902.a0002931.
[9] O. Smithies, Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults, Biochem. J. 61 (1955) 629–641, doi:10.1042/bj0610629.
[10] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685, doi:10.1038/227680a0.
[11] S. Hjerten, Free zone electrophoresis, Chromatogr. Rev. 9 (1967) 122–219, doi:10.1016/0009-5907(67)80003-6.
[12] R. Weinberger, Practical Capillary Electrophoresis, second ed, Academic Press, Chappaqua, New York, 2000.
[13] J.W. Jorgenson, K.D. Lukacs, Free-zone electrophoresis in glass capillaries, Clin. Chem. 27 (1981) 1551–1553.
[14] J.W. Jorgenson, K.D. Lukacs, Capillary zone electrophoresis, Science 222 (1983) 266–272, doi:10.1126/science.6623076.
[15] S. Hjertén, High-performance electrophoresis: the electrophoretic counterpart of high-performance liquid chromatography, J. Chromatogr. A 270 (1983) 1–6, doi:10.1016/S0021-9673(01)96347-2.
[16] L. Farmerie, R.R. Rustandi, J.W. Loughney, M. Dawod, Recent advances in isoelectric focusing of proteins and peptides, J. Chromatogr. A 1651 (2021) 462274, doi:10.1016/j.chroma.2021.462274.
[17] C.E. Sänger–van de Griend, CE-SDS method development, validation, and best practice—an overview, Electrophoresis 40 (2019) 2361–2374, doi:10.1002/elps.201900094.
[18] Beckman Coulter, Introduction to capillary electrophoresis. https://ls.beckmancoulter.co.jp/files/appli_note/CEPrimer1.pdf. (Accessed 15 November 2021).
[19] S. Štěpánová, V. Kašička, Recent applications of capillary electromigration methods to separation and analysis of proteins, Anal. Chim. Acta 933 (2016) 23–42, doi:10.1016/j.aca.2016.06.006.
[20] S. Stepanova, V. Kasicka, Applications of capillary electromigration methods for separation and analysis of proteins (2017-mid 2021) - a review, Anal. Chim. Acta 1209 (2022) 339447, doi:10.1016/j.aca.2022.339447.
[21] I. Miksik, Coupling of CE-MS for protein and peptide analysis, J. Sep. Sci. 42 (2019) 385–397, doi:10.1002/jssc.201800817.
[22] M.C. Breadmore, Electrokinetic and hydrodynamic injection: making the right choice for capillary electrophoresis, Bioanalysis 1 (2009) 889–894, doi:10.4155/bio.09.73.
[23] M.C. Breadmore, W. Grochocki, U. Kalsoom, M.N. Alves, S.C. Phung, M.T. Rokh, J.M. Cabot, A. Ghiasvand, F. Li, A.I. Shallan, A.S.A. Keyon, A.A. Alhusban, H.H. See, A. Wuethrich, M. Dawod, J.P. Quirino, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2016-2018), Electrophoresis 40 (2019) 17–39, doi:10.1002/elps.201800384.
[24] A. Slampova, Z. Mala, P. Gebauer, Recent progress of sample stacking in capillary electrophoresis (2016-2018), Electrophoresis 40 (2019) 40–54, doi:10.1002/elps.201800261.
[25] G. Zhu, L. Sun, N.J. Dovichi, Dynamic pH junction preconcentration in capillary electrophoresis- electrospray ionization-mass spectrometry for proteomics analysis, Analyst 141 (2016) 5216–5220, doi:10.1039/c6an01140c.
[26] L. Wang, D. MacDonald, X. Huang, D.D. Chen, Capture efficiency of dynamic pH junction focusing in capillary electrophoresis, Electrophoresis 37 (2016) 1143–1150, doi:10.1002/elps.201600008.
[27] R.B. Yu, J.P. Quirino, Pseudophase microextraction for in-line sample concentration in micellar electrokinetic chromatography, Anal. Chim. Acta 1189 (2022) 339219, doi:10.1016/j.aca.2021.339219.
[28] L.Q. Peng, X. Dong, X.T. Zhen, J. Yang, Y. Chen, S.L. Wang, T. Xie, J. Cao, Simultaneous separation and concentration of neutral analytes by cyclodextrin assisted sweeping-micellar electrokinetic chromatography, Anal. Chim. Acta 1105 (2020) 224–230, doi:10.1016/j.aca.2020.01.037.
[29] Z. Mala, P. Gebauer, Recent progress in analytical capillary isotachophoresis, Electrophoresis 40 (2019) 55–64, doi:10.1002/elps.201800239.
[30] B.J. Smith, SDS polyacrylamide gel electrophoresis of proteins, in: J.M. Walker (Ed.), Proteins, Humana Press, Totowa, New Jersey, 1984, pp. 41–55, doi:10.1385/0-89603-062-8:41.
[31] G. Staikos, A. Dondos, Study of the sodium dodecyl sulphate–protein complexes: evidence of their wormlike conformation by treating them as random coil polymers, Colloid Polym. Sci. 287 (2009) 1001–1004, doi:10.1007/s00396-009-2059-3.
[32] Y. Liu, P.M. Reddy, C.K. Ratnayake, E.V. Koh, Methods and Compositions for Capillary Electrophoresis, Beckman Coulter, Inc., Fullerton, CA United States, 2008.
[33] M. Chung, D. Kim, A.E. Herr, Polymer sieving matrices in microanalytical electrophoresis, Analyst 139 (2014) 5635–5654, doi:10.1039/c4an01179a.
[34] Z. Zhu, J.J. Lu, S. Liu, Protein separation by capillary gel electrophoresis: a review, Anal. Chim. Acta 709 (2012) 21–31, doi:10.1016/j.aca.2011.10.022.
[35] A. Sartori, V. Barbier, J.L. Viovy, Sieving mechanisms in polymeric matrices, Electrophoresis 24 (2003) 421–440, doi:10.1002/elps.200390052.
[36] M. Chiari, P.G. Righetti, New types of separation matrices for electrophoresis, Electrophoresis 16 (1995) 1815–1829, doi:10.1002/elps.11501601300.
[37] A. Widhalm, C. Schwer, D. Blaas, E. Kenndler, Capillary zone electrophoresis with a linear, non-cross-linked polyacrylamide gel: separation of proteins according to molecular mass, J. Chromatogr. A 549 (1991) 446–451, doi:10.1016/S0021-9673(00)91458-4.
[38] K. Ganzler, K.S. Greve, A.S. Cohen, B.L. Karger, A. Guttman, N.C. Cooke, High-performance capillary electrophoresis of SDS-protein complexes using UV-transparent polymer networks, Anal. Chem. 64 (1992) 2665–2671, doi:10.1021/ac00046a003.
[39] A. Guttman, J. Nolan, Comparison of the separation of proteins by sodium dodecyl sulfate-slab gel electrophoresis and capillary sodium dodecyl sulfate-gel electrophoresis, Anal. Biochem. 221 (1994) 285–289, doi:10.1006/abio.1994.1413.
[40] D. Wu, F.E. Regnier, Sodium dodecyl sulfate-capillary gel electrophoresis of proteins using non-cross-linked polyacrylamide, J. Chromatogr. A 608 (1992) 349–356, doi:10.1016/0021-9673(92)87142-u.
[41] A. Guttman, Capillary sodium dodecyl sulfate-gel electrophoresis of proteins, Electrophoresis 17 (1996) 1333–1341, doi:10.1002/elps.1150170810.
[42] Z. Zhu, M. Lies, J. Silzel, Native fluorescence detection with a laser driven light source for protein analysis in capillary electrophoresis, Anal. Chim. Acta 1183 (2021) 338936, doi:10.1016/j.aca.2021.338936.
[43] X. Li, L. Yu, X. Shi, C. Rao, Y. Zhou, Capillary isoelectric focusing with UV fluorescence imaging detection enables direct charge heterogeneity characterization of erythropoietin drug products, J. Chromatogr. A 1643 (2021) 462043, doi:10.1016/j.chroma.2021.462043.
[44] M.C. Breadmore, Capillary and microchip electrophoresis: challenging the common conceptions, J. Chromatogr. A 1221 (2012) 42–55, doi:10.1016/j.chroma.2011.09.062.
[45] M.C. Breadmore, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips, Electrophoresis 28 (2007) 254–281, doi:10.1002/elps.200600463.
[46] M.C. Breadmore, J.R. Thabano, M. Dawod, A.A. Kazarian, J.P. Quirino, R.M. Guijt, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2006-2008), Electrophoresis 30 (2009) 230–248, doi:10.1002/elps.200800435.
[47] M.C. Breadmore, M. Dawod, J.P. Quirino, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2008-2010), Electrophoresis 32 (2011) 127–148, doi:10.1002/elps.201000412.
[48] M.C. Breadmore, A.I. Shallan, H.R. Rabanes, D. Gstoettenmayr, A.S. Abdul Keyon, A. Gaspar, M. Dawod, J.P. Quirino, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2010-2012), Electrophoresis 34 (2013) 29–54, doi:10.1002/elps.201200396.
[49] M.C. Breadmore, R.M. Tubaon, A.I. Shallan, S.C. Phung, A.S. Abdul Keyon, D. Gstoettenmayr, P. Prapatpong, A.A. Alhusban, L. Ranjbar, H.H. See, M. Dawod, J.P. Quirino, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2012-2014), Electrophoresis 36 (2015) 36–61, doi:10.1002/elps.201400420.
[50] M.C. Breadmore, A. Wuethrich, F. Li, S.C. Phung, U. Kalsoom, J.M. Cabot, M. Tehranirokh, A.I. Shallan, A.S. Abdul Keyon, H.H. See, M. Dawod, J.P. Quirino, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2014-2016), Electrophoresis 38 (2017) 33–59, doi:10.1002/elps.201600331.
[51] H.L. Bagger, S.V. Hoffmann, C.C. Fuglsang, P. Westh, Glycoprotein-surfactant interactions: a calorimetric and spectroscopic investigation of the phytase-SDS system, Biophys. Chem. 129 (2007) 251–258, doi:10.1016/j.bpc.2007.06.005.
[52] C. Lee, Y. Park, Y. Kim, A. Peterkofsky, Y. Seok, Phosphorylation-Dependent mobility shift of proteins on SDS-PAGE is due to decreased binding of SDS, Bull. Korean Chem. Soc. 34 (2013) 2063–2066, doi:10.5012/BKCS.2013.34.7.2063.

[53] L. Geurink, E. van Tricht, J. Dudink, B. Pajic, C.E. Sanger-van de Griend, Four-step approach to efficiently develop capillary gel electrophoresis methods for viral vaccine protein analysis, Electrophoresis 42 (2021) 10–18, doi:10.1002/elps.202000107.
[54] M. Biacchi, N. Said, A. Beck, E. Leize-Wagner, Y.N. Francois, Top-down and middle-down approach by fraction collection enrichment using off-line capillary electrophoresis - mass spectrometry coupling: application to monoclonal antibody Fc/2 charge variants, J. Chromatogr. A 1498 (2017) 120–127, doi:10.1016/j.chroma.2017.02.064.
[55] Y.N. Francois, M. Biacchi, N. Said, C. Renard, A. Beck, R. Gahoual, E. Leize-Wagner, Characterization of cetuximab Fc/2 dimers by off-line CZE-MS, Anal. Chim. Acta 908 (2016) 168–176, doi:10.1016/j.aca.2015.12.033.
[56] T.M. Ngapo, M. Alexander, Capillary gel electrophoresis versus SDS PAGE of exudate from fresh pork, Meat Sci 53 (1999) 145–148, doi:10.1016/s0309-1740(99)00049-2.
[57] R. Estrada, Z. Demianova, T. Li, M. Santos, A. Guttman, S. Mollah, High-throughput multi-capillary SDS gel electrophoresis of proteins, 2021. https://sciex.com/tech-notes/ce/high-throughput-multi-capillary-sds-gel-electrophoresis-of-prote. (Accessed 17 November 2021).
[58] Sciex, SDS-MW analysis kit for the PA 800 plus pharmaceutical analysis system application guide., 2020. https://sciex.com/content/dam/SCIEX/pdf/customer-docs/application-guide/pa-800-plus-system-sds-mw-analysis-kit-application-guide.pdf. (Accessed 15 November 2021).
[59] ProteinSimple, Maurice CE-SDS application guide, 2019. https://www.proteinsimple.com/documents/Maurice_CE-SDS_Application_Guide.pdf. (Accessed 15 November 2021).
[60] Thermo Fisher Scientific, Unique protein gel chemistries for your every need. https://assets.thermofisher.com/TFS-Assets/BID/brochures/precast-protein-gels-brochure.pdf. (Accessed 15 November 2021).
[61] J. Kahle, K.J. Maul, H. Watzig, The next generation of capillary electrophoresis instruments: performance of CE-SDS protein analysis, Electrophoresis 39 (2018) 311–325, doi:10.1002/elps.201700278.
[62] C. Filep, A. Guttman, Effect of the monomer cross-linker ratio on the separation selectivity of monoclonal antibody subunits in sodium dodecyl sulfate capillary gel electrophoresis, Anal. Chem. 93 (2021) 3535–3541, doi:10.1021/acs.analchem.0c04927.
[63] K.R. Mitchelson, J. Cheng, Capillary electrophoresis with glycerol as an additive, in: K.R. Mitchelson, J. Cheng (Eds.), Capillary Electrophoresis of Nucleic Acids, Humana Press, 2001, pp. 259–277, doi:10.1385/1-59259-055-1:259.
[64] S.F. Contractor, M. Oakey, Use of borate buffers for purification of placental beta-glucuronidase on DEAE-cellulose, Anal. Biochem. 78 (1977) 279–282, doi:10.1016/0003-2697(77)90033-1.
[65] C.N. Scanlan, D.R. Burton, R.A. Dwek, Making autoantibodies safe, Proc. Natl. Acad. Sci. U.S.A 105 (2008) 4081–4082, doi:10.1073/pnas.0801192105.
[66] A. Guttman, C. Filep, B.L. Karger, Fundamentals of capillary electrophoretic migration and separation of SDS proteins in borate cross-linked dextran gels, Anal. Chem. 93 (2021) 9267–9276, doi:10.1021/acs.analchem.1c01636.
[67] C.L. Crihfield, L.A. Holland, Protein sieving with capillary nanogel electrophoresis, Anal. Chem. 93 (2021) 1537–1543, doi:10.1021/acs.analchem.0c03865.
[68] B.C. Durney, B.A. Bachert, H.S. Sloane, S. Lukomski, J.P. Landers, L.A. Holland, Reversible phospholipid nanogels for deoxyribonucleic acid fragment size determinations up to 1500 base pairs and integrated sample stacking, Anal. Chim. Acta 880 (2015) 136–144, doi:10.1016/j.aca.2015.03.009.
[69] M.P. Nieh, V.A. Raghunathan, C.J. Glinka, T.A. Harroun, G. Pabst, J. Katsaras, Magnetically alignable phase of phospholipid "bicelle" mixtures is a chiral nematic made up of wormlike micelles, Langmuir 20 (2004) 7893–7897, doi:10.1021/la048641l.
[70] T.A. Harroun, M. Koslowsky, M.P. Nieh, C.F. de Lannoy, V.A. Raghunathan, J. Katsaras, Comprehensive examination of mesophases formed by DMPC and DHPC mixtures, Langmuir 21 (2005) 5356–5361, doi:10.1021/la050018t.
[71] S.A. Archer-Hartmann, L.M. Sargent, D.T. Lowry, L.A. Holland, Microscale exoglycosidase processing and lectin capture of glycans with phospholipid assisted capillary electrophoresis separations, Anal. Chem. 83 (2011) 2740–2747, doi:10.1021/ac103362r.
[72] E. van Tricht, L. Geurink, B. Pajic, J. Nijenhuis, H. Backus, M. Germano, G.W. Somsen, C.E. Sanger-van de Griend, New capillary gel electrophoresis method for fast and accurate identification and quantification of multiple viral proteins in influenza vaccines, Talanta 144 (2015) 1030–1035, doi:10.1016/j.talanta.2015.07.047.
[73] C. Cianciulli, T. Hahne, H. Watzig, Capillary gel electrophoresis for precise protein quantitation, Electrophoresis 33 (2012) 3276–3280, doi:10.1002/elps.201200177.
[74] C.X. Zhang, M.M. Meagher, Sample stacking provides three orders of magnitude sensitivity enhancement in SDS capillary gel electrophoresis of adeno-associated virus capsid proteins, Anal. Chem. 89 (2017) 3285–3292, doi:10.1021/acs.analchem.6b02933.
[75] O. Salas-Solano, B. Tomlinson, S. Du, M. Parker, A. Strahan, S. Ma, Optimization and validation of a quantitative capillary electrophoresis sodium dodecyl sulfate method for quality control and stability monitoring of monoclonal antibodies, Anal. Chem. 78 (2006) 6583–6594, doi:10.1021/ac060828p.
[76] A. Danish, S.Y. Lee, C.E. Muller, Quantification of green fluorescent protein-(GFP-) tagged membrane proteins by capillary gel electrophoresis, Analyst 142 (2017) 3648–3655, doi:10.1039/c7an00981j.
[77] M. Santos, Sensitive AAV capsid protein impurity analysis by CE using easy to label fluorescent chromeo dye P503, 2019. https://sciex.com/content/dam/SCIEX/pdf/tech-notes/all/P503-to-label-AAV.pdf. (Accessed 15 November 2021).
[78] Active Motif, Chromeo P503. https://www.activemotif.com/documents/1641.pdf. (Accessed 15 November 2021).
[79] D.E. Otzen, Proteins in a brave new surfactant world, Curr. Opin. Colloid Interface Sci. 20 (2015) 161–169, doi:10.1016/j.cocis.2015.07.003.
[80] J. Beckman, Y. Song, Y. Gu, S. Voronov, N. Chennamsetty, S. Krystek, N. Mussa, Z.J. Li, Purity determination by capillary electrophoresis sodium hexadecyl sulfate (CE-SHS): a novel application for therapeutic protein characterization, Anal. Chem. 90 (2018) 2542–2547, doi:10.1021/acs.analchem.7b03831.
[81] M. Ospinal-Jimenez, D.C. Pozzo, Structural analysis of protein complexes with sodium alkyl sulfates by small-angle scattering and polyacrylamide gel electrophoresis, Langmuir 27 (2011) 928–935, doi:10.1021/la103495k.
[82] Q. Guan, J. Atsma, R. Tulsan, S. Voronov, J. Ding, J. Beckman, Z.J. Li, Minimization of artifact protein aggregation using tetradecyl sulfate and hexadecyl sulfate in capillary gel electrophoresis under reducing conditions, Electrophoresis 41 (2020) 1245–1252, doi:10.1002/elps.201900435.
[83] Q. Guan, R. Knihtila, J. Atsma, R. Tulsan, S. Singh, S. Kar, J. Beckman, J. Ding, Z.J. Li, Enhancement of covalent aggregate quantification of protein therapeutics by non-reducing capillary gel electrophoresis using sodium hexadecyl sulfate (CE-SHS), J. Chromatogr. B 1152 (2020) 122230, doi:10.1016/j.jchromb.2020.122230.
[84] K.M. Hutterer, R.W. Hong, J. Lull, X. Zhao, T. Wang, R. Pei, M.E. Le, O. Borisov, R. Piper, Y.D. Liu, K. Petty, I. Apostol, G.C. Flynn, Monoclonal antibody disulfide reduction during manufacturing: untangling process effects from product effects, MAbs 5 (2013) 608–613, doi:10.4161/mabs.24725.
[85] C. Filep, A. Guttman, The effect of temperature in sodium dodecyl sulfate capillary gel electrophoresis of protein therapeutics, Anal. Chem. 92 (2020) 4023–4028, doi:10.1021/acs.analchem.9b05566.
[86] Y. Fang, J.Z. Zhang, J.Y. Hou, H. Lu, N.J. Dovichi, Activation energy of the separation of DNA sequencing fragments in denaturing noncross-linked polyacrylamide by capillary electrophoresis, Electrophoresis 17 (1996) 1436–1442, doi:10.1002/elps.1150170907.
[87] H. Lu, E. Arriaga, D.Y. Chen, D. Figeys, N.J. Dovichi, Activation energy of single-stranded DNA moving through cross-linked polyacrylamide gels at 300 V/cm. Effect of temperature on sequencing rate in high-electric-field capillary gel electrophoresis, J. Chromatogr. A 680 (1994) 503–510, doi:10.1016/0021-9673(94)85149-2.
[88] L. He, B. Niemeyer, A novel correlation for protein diffusion coefficients based on molecular weight and radius of gyration, Biotechnol. Prog. 19 (2003) 544–548, doi:10.1021/bp0256059.
[89] R.E. Offord, Electrophoretic mobilities of peptides on paper and their use in the determination of amide groups, Nature 211 (1966) 591–593, doi:10.1038/211591a0.
[90] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Correlation of electrophoretic mobilities from capillary electrophoresis with physicochemical properties of proteins and peptides, Anal. Biochem. 197 (1991) 197–207, doi:10.1016/0003-2697(91)90379-8.
[91] A.L. Wang, M. Paciolla, M.J. Palmieri, G.G. Hao, Comparison of glycoprotein separation reveals greater impact of carbohydrates and disulfides on electrophoretic mobility for CE-SDS versus SDS-PAGE, J. Pharm. Biomed. Anal. 180 (2020) 113006, doi:10.1016/j.jpba.2019.113006.
[92] C. Scheller, F. Krebs, R. Wiesner, H. Watzig, I. Oltmann-Norden, A comparative study of CE-SDS, SDS-PAGE, and Simple Western-Precision, repeatability, and apparent molecular mass shifts by glycosylation, Electrophoresis 42 (2021) 1521–1531, doi:10.1002/elps.202100068.
[93] I. Bucior, S. Scheuring, A. Engel, M.M. Burger, Carbohydrate-carbohydrate interaction provides adhesion force and specificity for cellular recognition, J. Cell Biol. 165 (2004) 529–537, doi:10.1083/jcb.200309005.
[94] Y. Guan, Q. Zhu, D. Huang, S. Zhao, L.Jan Lo, J. Peng, An equation to estimate the difference between theoretically predicted and SDS PAGE-displayed molecular weights for an acidic peptide, Sci. Rep. 5 (2015) 13370, doi:10.1038/srep13370.
[95] A. Shirai, A. Matsuyama, Y. Yashiroda, A. Hashimoto, Y. Kawamura, R. Arai, Y. Komatsu, S. Horinouchi, M. Yoshida, Global analysis of gel mobility of proteins and its use in target identification, J. Biol. Chem. 283 (2008) 10745–10752, doi:10.1074/jbc.M709211200.
[96] R.R. Rustandi, M.W. Washabaugh, Y. Wang, Applications of CE SDS gel in development of biopharmaceutical antibody-based products, Electrophoresis 29 (2008) 3612–3620, doi:10.1002/elps.200700958.
[97] N.A. Lacher, R.K. Roberts, Y. He, H. Cargill, K.M. Kearns, H. Holovics, M.N. Ruesch, Development, validation, and implementation of capillary gel electrophoresis as a replacement for SDS-PAGE for purity analysis of IgG2 mAbs, J. Sep. Sci. 33 (2010) 218–227, doi:10.1002/jssc.200900597.
[98] K. Tsumoto, Y. Isozaki, H. Yagami, M. Tomita, Future perspectives of therapeutic monoclonal antibodies, Immunotherapy 11 (2019) 119–127, doi:10.2217/imt-2018-0130.
[99] C. Filep, M. Szigeti, R. Farsang, M. Haberger, D. Reusch, A. Guttman, Multilevel capillary gel electrophoresis characterization of new antibody modalities, Anal. Chim. Acta 1166 (2021) 338492, doi:10.1016/j.aca.2021.338492.
[100] M. Szigeti, A. Guttman, Sample preparation scale-up for deep N-glycomic analysis of human serum by capillary electrophoresis and CE-ESI-MS, Mol. Cell Proteomics 18 (2019) 2524–2531, doi:10.1074/mcp.TIR119.001669.
[101] C.M. Ouimet, H. Shao, J.N. Rauch, M. Dawod, B. Nordhues, C.A. Dickey, J.E. Gestwicki, R.T. Kennedy, Protein cross-linking capillary electrophoresis

for protein-protein interaction analysis, Anal. Chem. 88 (2016) 8272–8278, doi:10.1021/acs.analchem.6b02126.
[102] B.W. Sutherland, J. Toews, J. Kast, Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions, J. Mass Spectrom. 43 (2008) 699–715, doi:10.1002/jms.1415.
[103] C.M. Ouimet, M. Dawod, J. Grinias, V.A. Assimon, J. Lodge, A.K. Mapp, J.E. Gestwicki, R.T. Kennedy, Protein cross-linking capillary electrophoresis at increased throughput for a range of protein-protein interactions, Analyst 143 (2018) 1805–1812, doi:10.1039/C7AN02098H.
[104] R. Vitorino, S. Guedes, J.P.D. Costa, V. Kasicka, Microfluidics for peptidomics, proteomics, and cell analysis, Nanomaterials (Basel) 11 (2021), doi:10.3390/nano11051118.
[105] S. Stepanova, V. Kasicka, Analysis of proteins and peptides by electromigration methods in microchips, J. Sep. Sci. 40 (2017) 228–250, doi:10.1002/jssc.201600962.
[106] H. Cai, Y. Song, J. Zhang, T. Shi, Y. Fu, R. Li, N. Mussa, Z.J. Li, Optimization of microchip-based electrophoresis for monoclonal antibody product quality analysis revealed needs for extra surfactants during denaturation, J. Pharm. Biomed. Anal. 120 (2016) 46–56, doi:10.1016/j.jpba.2015.10.041.
[107] G.T. Roman, M. Wang, K.N. Shultz, C. Jennings, R.T. Kennedy, Sampling and electrophoretic analysis of segmented flow streams using virtual walls in a microfluidic device, Anal. Chem. 80 (2008) 8231–8238, doi:10.1021/ac801317t.
[108] M. Wang, G.T. Roman, M.L. Perry, R.T. Kennedy, Microfluidic chip for high efficiency electrophoretic analysis of segmented flow from a microdialysis probe and in vivo chemical monitoring, Anal. Chem. 81 (2009) 9072–9078, doi:10.1021/ac901731v.
[109] X. Niu, F. Pereira, J.B. Edel, A.J. de Mello, Droplet-interfaced microchip and capillary electrophoretic separations, Anal. Chem. 85 (2013) 8654–8660, doi:10.1021/ac401383y.
[110] C.M. Ouimet, C.I. D'Amico, R.T. Kennedy, Droplet sample introduction to microchip gel and zone electrophoresis for rapid analysis of protein-protein complexes and enzymatic reactions, Anal. Bioanal. Chem. 411 (2019) 6155–6163, doi:10.1007/s00216-019-02006-7.
[111] N.E. Arvin, M. Dawod, D.T. Lamb, J.P. Anderson, M.D. Furtaw, R.T. Kennedy, Fast immunoassay for microfluidic western blotting by direct deposition of reagents onto capture membrane, Anal. Methods 12 (2020) 1606–1616, doi:10.1039/d0ay00207k.
[112] S. Jin, G.J. Anderson, R.T. Kennedy, Western blotting using microchip electrophoresis interfaced to a protein capture membrane, Anal. Chem. 85 (2013) 6073–6079, doi:10.1021/ac400940x.
[113] S. Jin, M.D. Furtaw, H. Chen, D.T. Lamb, S.A. Ferguson, N.E. Arvin, M. Dawod, R.T. Kennedy, Multiplexed western blotting using microchip electrophoresis, Anal. Chem. 88 (2016) 6703–6710, doi:10.1021/acs.analchem.6b00705.
[114] M.T. Smith, S. Zhang, T. Adams, B. DiPaolo, J. Dally, Establishment and validation of a microfluidic capillary gel electrophoresis platform method for purity analysis of therapeutic monoclonal antibodies, Electrophoresis 38 (2017) 1353–1365, doi:10.1002/elps.201600519.
[115] K.L. Rundlett, D.W. Armstrong, Mechanism of signal suppression by anionic surfactants in capillary electrophoresis-electrospray ionization mass spectrometry, Anal. Chem. 68 (1996) 3493–3497, doi:10.1021/ac960472p.
[116] P. Hommerson, A.M. Khan, G.J. de Jong, G.W. Somsen, Comparison of electrospray ionization and atmospheric pressure photoionization for coupling of micellar electrokinetic chromatography with ion trap mass spectrometry, J. Chromatogr. A 1204 (2008) 197–203, doi:10.1016/j.chroma.2008.04.017.