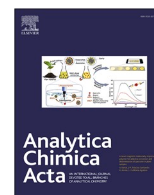
Analytica Chimica Acta 1360 (2025) 344147

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca



Rapid baseline hump-free analysis of therapeutic proteins in a wide molecular weight range by SDS - capillary agarose gel electrophoresis

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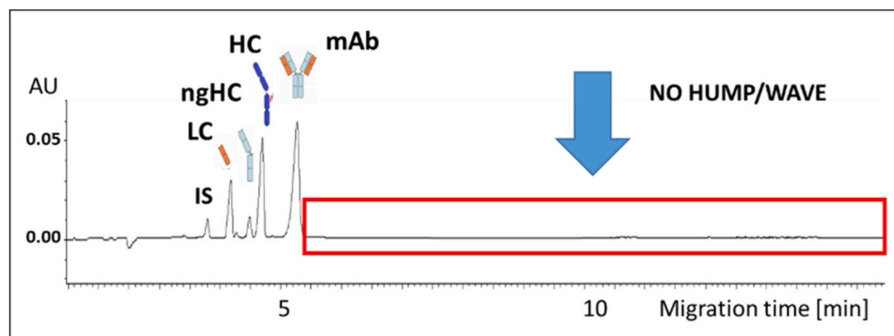
HIGHLIGHTS

- SDS-CAGE effectively eliminates the baseline disturbances that often occur in CE-SDS.
- Rapid purity testing and subunit integrity analysis of protein therapeutics are shown.
- Ultrafast analysis of a highly glycosylated fusion protein was achieved.
- Large MW proteins were effectively analyzed by SDS-CAGE without the baseline hump.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Capillary gel electrophoresis
Agarose
Sodium dodecyl sulfate
Therapeutic proteins
Baseline hump

ABSTRACT

Background: Sodium dodecyl sulfate capillary gel electrophoresis traditionally employs entangled polymer networks (CE-SDS) or borate cross-linked gels (SDS-CGE) for size-based protein analysis. However, the separation of SDS proteins in these transiently cross-linked sieving matrices requires long analysis times and in addition, baseline humps/waves frequently occur making peak identification and quantification challenging. The analytical biopharma community has been trying to solve this problem for a decade, making it clear that a novel gel composition was required to provide baseline hump-free separation of SDS-proteins by capillary electrophoresis of higher MW biopharmaceuticals.

Results: Using a transiently cross-linked agarose matrix shown in this paper enabled rapid separation of therapeutic proteins with excellent resolution, but more importantly, eliminated the commonly observed baseline disturbances of dextran-based gel formulations. Using capillaries as short as 10 cm effective length, the tetrahydroxyborate cross-linked agarose matrix supported fast analysis (~5 min) of an intact anti-SARS-CoV-2 therapeutic antibody and its subunits with excellent run-to-run migration time (RSD <0.3 %) and peak area (RSD <5 %) reproducibility. High resolution between the closely migrating non-glycosylated heavy chain and the heavy chain fragments was also obtained (RS = 1.65). The high molecular weight thyroglobulin (660 kDa), and the highly glycosylated fusion protein of etanercept were also successfully analyzed with borate-agarose-based gels, exploiting the stable baseline at the upper molecular weight range of the separation trace.

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https://doi.org/10.1016/j.aca.2025.344147
Received 5 March 2025; Received in revised form 25 April 2025; Accepted 1 May 2025
Available online 3 May 2025
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Significance: This paper reports for the first time on a baseline hump-free approach for rapid analysis of therapeutic proteins in a wide MW range by SDS capillary agarose gel electrophoresis. The unique potential of tetrahydroxyborate-stabilized agarose gels revolutionizes capillary SDS gel electrophoresis analysis of mAbs and more complex new modalities, offering a robust and efficient platform for high-resolution protein separation, paving the way for advancements in baseline hump-free therapeutic protein characterization and consequently good quantification.

Abbreviations

SDS	Sodium dodecyl sulfate
CAGE	Capillary agarose gel electrophoresis
LC	Light chain
ngHC	Non-glycosylated heavy chain
HC	Heavy chain
mAb	Monoclonal antibody
EOF	Electroosmotic flow

1. Introduction

Checking the purity of biotherapeutics is a crucial part of the quality control process in the biopharmaceutical industry [1]. The traditional ''workhorse'' for size-based protein separation with electric field mediated techniques is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in slab gel format utilizing the phenomenon that the electrophoretic mobility values decrease linearly with the logarithmic protein molecular masses, resulting in separations based on the size (hydrodynamic volume) of the solute molecules [2]. The use of a sieving matrix is essential due to the 1.4 g SDS/gram protein binding ratio of the surfactant, rendering a very similar mass-to-charge ratio of the resulting complexes [3], except with some alterations for highly glycosylated proteins [4]. However, this technique is time-consuming, labor-intensive, the manual operations cause irreproducibility, and MW-based band identification is difficult [5]; therefore, not optimal for the QA/QC process in the biopharmaceutical industry. SDS-CGE (hackneyed as CE-SDS) has many advantages over SDS-PAGE, including on-column UV and/or fluorescent detection, automated operation, high resolving power, and excellent purity checking capability [6].

Variable sieving matrices, including entangled polymer solutions [7] and permanently [8] or transiently cross-linked [9] gels, as well as detection strategies such as UV [10], fluorescence [11,12], and MS [13,14] are available for SDS-CGE of proteins. However, it also has its difficulties such as bubble formation in permanently cross-linked gels, as well as baseline humps/waves, and even unknown peaks possibly showing up with some transiently cross-linked commercial matrices [15]. Multiple users reported issues related to baseline stability and drift with the use of the commercially available dextran borate gel buffer system, representing a major issue that complicates peak identification and integration [16,17]. Some of the suggested possible causes of these problems were Joule heating due to the high concentration of the background electrolyte components, electroosmotic flow (EOF), sample preparation, capillary conditioning, etc. [18], but no solution for the issue was reported for the time being.

Agarose represents an alternative sieving matrix to borate cross-linked dextran gels for size-based separation of SDS-proteins [19]. The linear double helical chains of agarose can be cross-linked by the tetrahydroxyborate anions present in high boric acid-containing background electrolytes. As our group reported earlier, the low melting temperature agarose-borate gel is an excellent sieving matrix for SDS-proteins in the molecular weight range from 6 kDa to 200 kDa [20]. In this paper, we explored the possibility of using borate-stabilized agarose matrix for SDS capillary gel electrophoresis analysis of highly glycosylated and high molecular weight proteins (up to 660 kDa) exploiting the excellent baseline stability feature of this sieving matrix at the higher MW separation range, i.e., no humps and waves, which would otherwise influence both the resolution and quantification capabilities.

2. Materials and methods

2.1. Chemicals

Agarose (ultra-low gelling temperature, low EOF), sodium dodecyl sulfate (SDS), boric acid, Tris, EDTA.Na_2, sodium hydroxide, hydrochloric acid, water (HPLC grade), glycerol, 2-mercaptoethanol, and thyroglobulin were from Sigma Aldrich (St. Louis, MO, USA). The 10 kDa size standard and the dextran-based SDS-MW gel buffer were from Bio-Science Kft (Budapest, Hungary). The PNGase F enzyme was from the Bio-Nanosystems Laboratory of Pannon University (Veszprem, Hungary). The anti-Covid19 mAb of bamlanivimab and the fusion protein of etanercept were kindly provided by the Semmelweis Hospital (Miskolc, Hungary).

2.2. Gel preparation

The background electrolyte contained 640 mM boric acid, adjusted to pH 8.0 with Tris-base, followed by the addition of 2 mM EDTA.Na_2, 10 % (v/v) glycerol, and 0.8 % (w/v) agarose. The gel buffer solution was first heated in a microwave oven (set to 1000 W) until boiling, repeated once, and then stirred overnight at 75 °C (250 RPM) to obtain a clear and homogenous medium. SDS was then added in the final concentration of 0.2 % (w/v) during slow mixing for an additional hour (100 RPM).

2.3. Sample preparation

Eighty microliters of 100 mM Tris-HCl, 2 % SDS (pH 9.0) was added to 10 μL of the diluted (10 mg/mL, working solution) bamlanivimab, followed by mixing with 2 μL of 10 kDa internal standard in a 200 μL PCR tube. For reduced conditions, 5 μL of 2-mercaptoethanol was added, while for non-reduced conditions, 5 μL of 40 mM iodoacetamide was used. The mixtures were denatured at 80 °C for 15 min. N-glycan removal from the etanercept and the heavy chain fragment of the bamlanivimab was accomplished by the addition of 2.0 μL of the PNGase F (200 mU) and keeping the reaction mixture at 50 °C for an hour. The resultant deglycosylated etanercept and bamlanivimab heavy chain-containing reaction mixture were mixed with the original (glycosylated) product and used in the study, respectively. The 1 mg/mL etanercept working solution was prepared using the same SDS-sample buffer and heating parameters described above and denatured in both its glycosylated and PNGase F-digested forms. The thyroglobulin sample (final concentration of 0.5 mg/mL) was prepared in its intact form and subjected to a longer but gentler denaturation at 70 °C for 20 min.

2.4. SDS-capillary gel electrophoresis

SDS-capillary gel electrophoresis separations were carried out by employing a PA 800+ Pharmaceutical Analysis System (Beckman Coulter, Brea, CA) with UV 214 absorbance detection using a 10 cm effective length bare fused silica capillary column (total length 30 cm,

50 μm i.d., 365 μm o.d.) in case of agarose-based gels, and 20 cm effective length with the use of the commercial dextran-based SDS-MW gel. Before each run, the capillary was conditioned by washing for 3 min with 0.1 M NaOH, 1 min with 0.1 M HCl, and 4 min with HPLC-grade water, then filled with the appropriate gel buffer system. Injection of the samples was accomplished by applying 5 kV electric potential for 20 s with the cathode at the injection side. The intact mAb and the mixture of its reduced subunits (this latter contained the PNGase F from the deglycosylation reaction), along with the 10 kDa internal standard, were injected consecutively by applying 5 kV for 20 s, to ensure no reducing agent would mix with the intact form. Separations were performed by applying 500 V/cm electric field, also with the cathode at the injection side, with the capillary temperature maintained at 25 °C. The acquired data was processed and analyzed by the PeakFit, version 4.12 program (SYSTAT Software Inc., San Jose, CA).

3. Results and discussion

In this paper, a borate cross-linked agarose gel formulation is introduced to eliminate any baseline disturbances, frequently observed with the use of conventional dextran-based sieving matrices during the separation of SDS-proteins. Rapid separation of the subunits and intact form of a monoclonal antibody (including the deglycosylated heavy chain fragment), as well as a highly glycosylated therapeutic fusion protein and a high molecular weight (~660 kDa) protein drug are also demonstrated.

Fig. 1 shows the sodium dodecyl sulfate capillary agarose gel electrophoresis (SDS-CAGE) separation of the subunits and the intact forms of the mAb therapeutics of bamlanivimab using 0.8 % agarose gel in Borate-Tris-EDTA-Glycerol background electrolyte. The inset highlights a zoomed-in section of the baseline-separated non-glycosylated heavy chain (ngHC, peak 4 at 4.48 min) and heavy chain (HC, Peak 5 at 4.68 min) peaks, with the intact mAb (peak 7) migrating at 5.25 min. Most importantly, this last peak was followed by a very steady baseline without any humps/waves up to 13 min, which is 2.5 times longer as the migration time of ~150 kDa mAb, i.e., it should readily cover the range of up to the few megaDalton MW range of other grater size new modality therapeutic proteins accommodating precise quantification otherwise problematic with the baseline hump. As an example, the separation of monomer and dimer forms of thyroglobulin (MW ~ 330 and 660 kDa, respectively) are demonstrated later in this paper. The bamlanivimab-related peaks of 2, 4–7, represent the light chain (LC), non-glycosylated heavy chain (ngHC), heavy chain (HC), light chain-heavy chain complex (LC/HC), and the intact mAb [21]. Peaks 1 and 3 correspond to the 10 kDa internal standard protein and the remaining PNGase F enzyme from the deglycosylation reaction, respectively. If this latter (PNGase F, MW ~ 36 kDa) is expected to interfere with any of the sample proteins, its immobilized form should be used during the deglycosylation process [22]. Important physicochemical properties of agarose- and dextran-based gels are depicted in Table 1. For comparative purposes, in addition to that of were reported in Ref. [18], separation of the same sample is shown with the use of a commercial dextran-based sieving matrix exhibiting various level baseline humps (Supplementary Fig. 1). It should also be noted that while the total analysis time using the commercial dextran gel was approximately 5x longer, the resolution between some key sample components, e.g., nonglycosylated heavy chain/heavy chain fragments, was 25 % better with the agarose-based gel composition (1.65 vs 1.32).

To evaluate the repeatability of the separation, and more importantly, the baseline stability using the agarose-borate sieving matrix, multiple injections were performed. Representative examples of the resulting traces are compared in Fig. 2, featuring the first, 4th, 8th, and 12th runs. Please note the humpless/waveless baselines after the mAb peak up to 15 min run time. The analyses also featured excellent migration time (RSD <0.3 %) and peak area percent (RSD <5 %) reproducibility.

Based on our encouraging results concerning the stable baseline, long beyond the 150 kDa size-range, a similar size but highly glycosylated (3 N-linked and 13 O-linked glycan moieties per subunit) therapeutic fusion protein (etanercept, MW ~ 150 kDa) and a high molecular weight protein drug (thyroglobulin, MW ~ 660 kDa) were analyzed. Fig. 3A depicts the analysis of the intact (upper trace) and PNGase F - digested (lower trace) form of etanercept. Please note that this particular endoglycosidase only removes the N-linked glycans (one site in the Fc region and two sites in the TNFα receptor region), but does not affect the O-linked ones. The O-linked carbohydrates are mostly located in the hinge region and the longer migration time of the PNGase F digested form of this fusion protein is caused by the non-SDS binding remaining O-linked oligosaccharides, which also increase the hydrodynamic

Table 1
Important physicochemical properties of agarose- and dextran-based gels.

Physicochemical properties	viscosity [mPa s]	current [μA]	EOF [m^2/V s]
0.8 % agarose/4 % boric acid	8.9	36	−5.71E-09
10 % Dextran 2 M/4 % boric acid	100	27	−1.80E-10

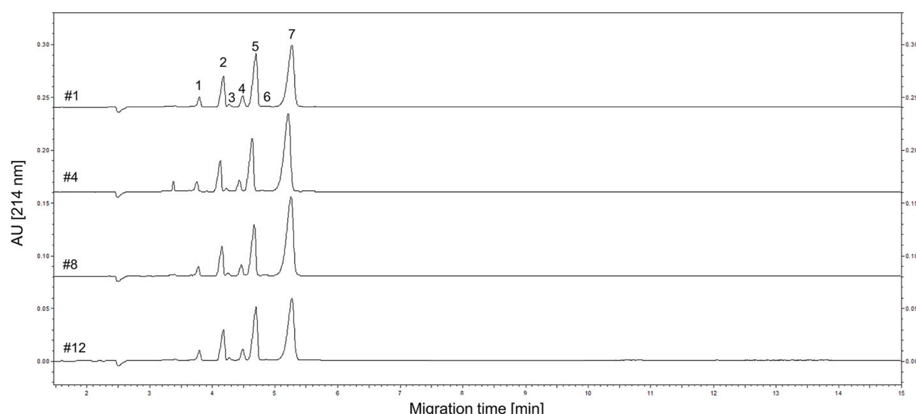

Fig. 1. Sodium dodecyl sulfate – capillary agarose gel electrophoresis (SDS-CAGE) analysis of bamlanivimab. Peaks: 1–10 kDa standard, 2 – LC (~25 kDa), 3 – PNGase F (~36 kDa), 5 – HC (~50 kDa), 6 – LC/HC (~75 kDa), 7 – Intact mAb (~150 kDa). Conditions: 0.8 % (w/v) agarose in Borate-Tris-EDTA-Glycerin background electrolyte; Capillary: 10 cm effective length bare fused silica column (total length: 30 cm, 50 μm i.d./365 μm o.d.); Detection: UV absorbance at 214 nm; Applied electric field strength: 500 V/cm (with the cathode at the injection side); Capillary temperature: 25 °C; Injection: electrokinetic at 5 kV for 20 s (with the cathode at the injection side).

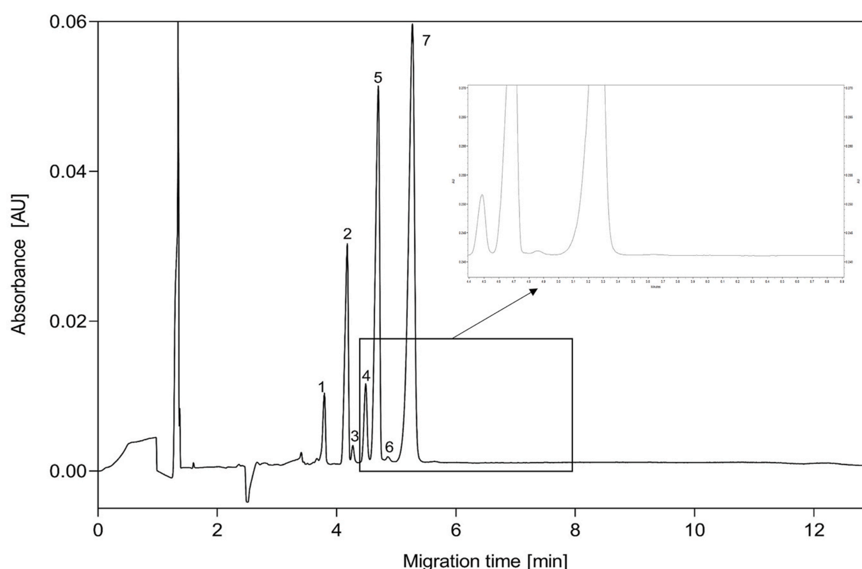

Fig. 2. Repeatability comparison of four representative SDS-CAGE traces of the intact and subunit forms of bamlanivimab up to 12 consecutive runs. Separation conditions and peak assignments are the same as specified in Fig. 1.

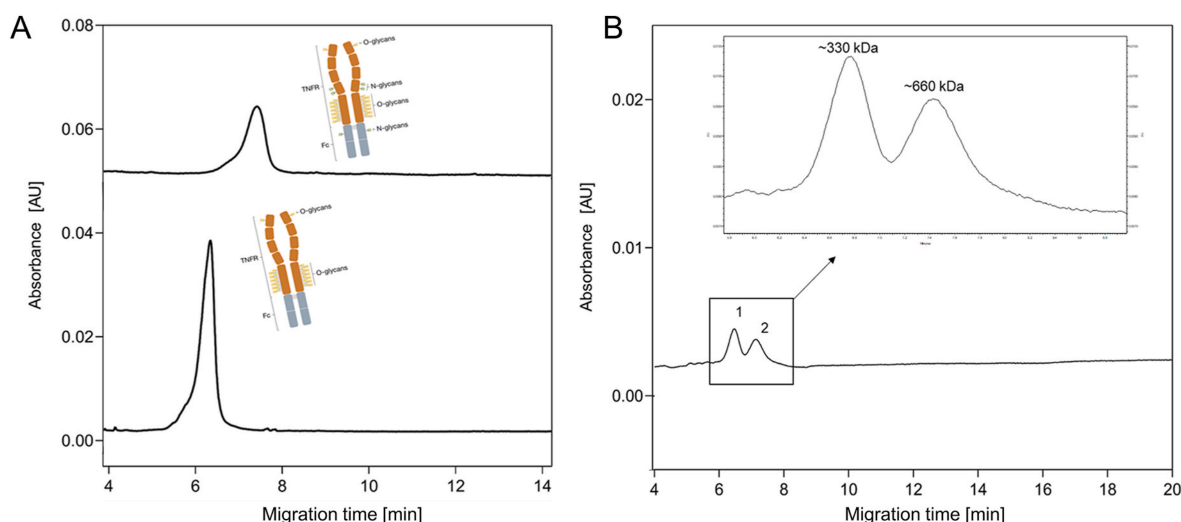

Fig. 3. (A) SDS-CAGE analysis of the intact (upper trace) and PNGase F-digested (lower trace) etanercept. The insets show the glycosylation sites and types. **(B)** Separation of the intact (peak 2, ~660 kDa) and subunit (peak 1, ~330 kDa) forms of thyroglobulin by SDS-CAGE. The small peaks under the bracket marked by the asterisk (*) are sample impurities. Separation conditions were the same as in Fig. 1.

volume of the complex. Both phenomena contribute to a higher mass-to-charge ratio and consequently longer migration time of etanercept (6.34 min) vs the similar MW intact form of bamlanivimab (5.31 min). Please note that the conserved (Asn-297) glycosylation of intact mAbs faces inward, so does not influence the resulting mass-to-charge ratio to a significant extent [9].

Fig. 3B shows the SDS-CAGE analysis of thyroglobulin, a high molecular weight two-subunit glycoprotein drug (~660 kDa, i.e., ~330 kDa per subunit), used to treat hypothyroidism. Approximately 10 % of the molecular weight of thyroglobulin is carbohydrate, mostly N-linked sugars [23]. In this instance, the migration times of the intact and subunit forms of the protein were 7.42 and 6.75 min, respectively. While these migration times are apparently not much longer than that of the 150 kDa mAb in Fig. 1, but please consider the above mentioned logarithmic relationship between the migration time and MW of the solute molecules in SDS gel electrophoresis. It is important to emphasize that the excellent baseline stability of this agarose-borate matrix enabled a comprehensive analysis of these high MW and highly glycosylated proteins by SDS-CAGE.

4. Conclusions

Baseline stability (humps/waves) and drift are reoccurring issues with dextran-based sieving matrices in SDS-CGE, making peak identification and integration challenging. In this paper, we demonstrated that the use of borate cross-linked agarose gel effectively alleviated the baseline disturbance problems, also enabling to obtain rapid and high-resolution separations. Proteins up to ~660 kDa MW were readily separated in less than 7.5 min, with a resolution of Rs = 1.65 between the non-glycosylated heavy chain and heavy chain fragments of the mAb sample of bamlanivimab. Etanercept, a highly glycosylated therapeutic fusion protein (13 O-glycosylation and 3 N-glycosylation sites), and thyroglobulin, a high molecular weight (~660 kDa) protein drug, were also successfully analyzed by baseline hump-free SDS-CAGE. Potential drawbacks of the borate cross-linked agarose matrices are that only low EOF and low melting point agaroses should be used to prevent

electroosmotic flow-mediated migration disturbances and solidification of the matrix in the capillary, respectively. On the other hand, since agarose is a sugar-based polymer similar to dextran, its stability over time and compatibility with various sample types were apparently alike.

Most importantly, the approach described in this paper offers a robust and efficient way for high-resolution protein analysis by SDS capillary agarose gel electrophoresis, paving the way for advancements in baseline hump-free therapeutic protein characterization and consequently good quantification for the biopharmaceutical industry.

CRediT authorship contribution statement

Dániel Sárközy: Validation, Methodology, Investigation, Formal analysis, Data curation. **Anna Farkas:** Methodology. **András Guttman:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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.

Acknowledgements

The support of the University of Debrecen Program for Scientific Publications and the ADVANCED 150780 grant of the National Research Development and Innovation Office of Hungary are greatly appreciated. The authors also thank for the stimulating discussions with Dr Cari Sänger - van de Griend. This is contribution #218 from the Horváth Csaba Memorial Laboratory of Bioseparation Sciences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2025.344147.

Data availability

Data will be made available on request.

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