SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Effect of measurement parameters and separation medium composition during capillary gel electrophoresis of therapeutic proteins with sodium dodecyl sulfate

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1. Introduction and objective

Of the 10 pharmaceutical products with the largest market share today, eight are biotherapeutic protein products. Therapeutic proteins, such as monoclonal antibodies and fusion proteins, can be added to or substituted for traditional small molecule drug therapies for the treatment of hematological and solid tumors, autoimmune and inflammatory diseases, for optimized treatment. The characterization of these therapeutic proteins is a major challenge for the pharmaceutical industry, and monitoring of the purity of therapeutic proteins and detection of possible structural variations is important for quality control. In the characterization of biologic pharmaceuticals, various separation methods of capillary electrophoresis (CE) are routinely used for quality control and to assess protein size and charge heterogeneity in the pharmaceutical industry.

Capillary gel electrophoresis (CGE) allows the separation of polyionic macromolecules by their hydrodynamic volume. Initially, cross-linked polyacrylamide gels were used to separate proteins by size, which were later replaced by hydrophilic linear polymer matrices. Nowadays, the pharmaceutical industry mostly uses dextran gels reversibly cross-linked with borate ions for high-resolution size separation of proteins, which is also considered a standard method.

Due to the increasing number of proteins in biotherapeutics, there is a growing need for improved separation techniques, which are also suitable, for example, for the characterization of highly glycosylated proteins. Sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE), which nowadays uses a dextran matrix reversibly cross-linked with tetrahydroxyborate ion as separation medium, is a highly efficient bioanalytical method with fast analysis time and low sample requirements, making it an excellent tool for the efficient characterization of therapeutic proteins.

My aim was to optimize the method for more efficient separation, including the investigation of how the measurement parameters (e.g. temperature) and the composition of the separation medium (e.g. boric acid, dextran concentration) affected the qualitative analysis of therapeutic proteins. In my work, particular emphasis has been placed on the study of the mechanism of dextran-borate cross-linking. I studied the effect of monomer (dextran) and cross-linker (borate) concentration variation on SDS-CGE separation of protein standards and monoclonal antibody samples. For the background electrolytes, I determined physical and electrokinetic parameters important for the separation, such as viscosity and electroosmotic flow. For a

more detailed study of migration properties, I applied the Ferguson method, which can help to select the right gel composition for a given protein mixture sample. The obtained electropherograms were evaluated to achieve detailed information on the performance of the separations, such as theoretical plate number, resolution and selectivity factor determination. I investigated the temperature-dependent migration of proteins to determine the activation energy requirements of the separations and to optimize resolution. The correlation between the activation energy and the molecular weight of the sample molecule was explored in the gel system of interest, as well as the correlation between SDS protein complex mobility and molecular weight.

The aim of my thesis is to demonstrate 1) that optimization of the measurement parameters in the analysis of therapeutic proteins by capillary gel electrophoresis can lead to more efficient separation and is essential for many new types of glycoproteins, and 2) that exploring the properties of the dextran-borate gel-buffer system by studying the variation in composition allows a deeper understanding of the mechanism of separation, thus helping to address the challenges encountered in the separation of glycoproteins.

2. Results

2.1 Physical and electrokinetic parameters

As a first step in my work, I determined the boundary values of dextran and boric acid concentrations for a 50 µm diameter capillary with a total length of 30 cm (20 cm effective length), taking into account the viscosity and conductivity of the gel for easy handling.
Above 10% (w/V) dextran concentration, the increasing viscosity does not allow adequate gel exchange in the narrow diameter capillary, taking into account the pressure limits of the measuring instrument. Below 2% concentration, the molecular sieving capacity is significantly reduced in the range of 20 - 225 kDa tested.
At higher >4% (w/V) boric acid concentrations, the applied field strength of 500 V/cm and column length of 30 cm significantly increased the electric current, resulting in undesirable Joule heat generation. Below 2% (w/V) boric acid concentration, the analysis time increased significantly due to the increase in the reverse EOF. As a consequence, gels in the concentration ranges of 2-10% (w/V) dextran (D) and 2-4% (w/V) boric acid (B) were used for my experiments.

Subsequently, by setting up a concentration series, I selected 16 gels of different compositions to be used in my experiments, containing 10, 7.5, 5 and 2% (w/V) dextran and 4, 3.3, 2.7 and 2% (w/V) boric acid. For these gels, I determined some important physical and electrokinetic parameters such as viscosity, electroosmotic flow and current, which were also necessary to determine the concentration limits mentioned above.

The viscosity of the gels varied with the concentration of dextran and boric acid. The 10%D/4%B gel showed the highest viscosity (100 mPa*s) while the 2%D/2%B gel showed the lowest (10.7 mPa*s). As the amount of boric acid decreased, the viscosity also decreased, for example to 85 mPa*s for 10%D/2%B. Decreasing the concentration of dextran and increasing the amount of boric acid also increased the viscosity, but still remained significantly lower than for gels with high dextran content.

The lowest EOF ($-0.18\pm0.001 \times 10^{-9}$ m^2/Vs) was observed for the 10%D/4%B gel, which is a consequence of the high viscosity and ionic strength. In contrast, the 2%D/2%B formula had the highest EOF flux: $-4.92\pm0.03 \times 10^{-9}$ m^2/Vs.

Higher currents were observed for gels with 4% boric acid content than for gels with 2% boric acid composition. The 2%D/4%B gel had the highest current (37.53 μA), while the 10%D/2%B gel had the lowest current (22.58 μA).

2.2 Temperature-dependent electromigration of proteins

Measurements of the standard protein ladder (20 - 225 kDa) and test protein mixtures (10 kDa internal standard, PSA specific nanobody (14.26 kDa), omalizumab light (23.98 kDa) and heavy (49.37 kDa) chains) were performed at 5°C intervals using separation temperatures between 15 and 60°C.

For the standard protein ladder, the mobility of each peak increased steadily with increasing separation temperature: $\mu_{20\ kDa}$= 3.71E-09 - 4.66E-08 [m2/Vs], while the resolution decreased: $Rs_{20\ kDa\text{-}35\ kDa}$ = 7.14 - 4.95.

The resolution between the peaks of the test protein mixture changed differently with increasing temperature, in contrast to the regularity observed for the protein ladder peaks. The resolution between the 10 kDa and PSA specific nanobody peaks increased (Rs = 4.11 to 7.94) with increasing temperature, almost doubling its value at the highest temperature. The opposite occurred between the PSA specific nanobody and omalizumab light chain, where the resolution dropped by more than half at 60°C compared to 15°C: Rs=1.99 and Rs=5.78, respectively. For the omalizumab light chain and heavy chain, the resolution was highest at a separation temperature of 35°C (Rs=11.39), and decreased by about 30% with further temperature increase (Rs=8.46).

Using the Arrhenius method [1], I determined the activation energy (Ea) of the separated proteins required to pass through the gel. The Ea values of the protein ladder components exhibited a convex decreasing property with increasing molecular weight: $Ea_{20\ kDa}$ = 44.76 kJ/mol and $Ea_{225\ kDa}$ = 41.91 kJ/mol.

No regularity was observed in the Ea demand of the components of the protein mixture, in contrast to the protein ladder. The heavy chain with a molecular weight of 49.37 kDa had the lowest Ea, but the nanobody with only 14.26 kDa had almost the same Ea. The SDS-CGE analysis of the biotherapeutic protein test mixture showed no correlation between the activation energy requirements and molecular weight of the different sample components, emphasizing the importance of optimizing the separation temperature for the sample components of interest in order to achieve the highest possible resolution.

To better understand the migration behavior of SDS-protein complexes during their separation in dextran-borate gels, I also investigated the relationship between mobility and molecular weight using the possible molecular weight equivalents of the molecular radius.

I plotted the electrophoretic mobility values of SDS-protein complexes as a function of the square root of $Mw^{-1/3}$ (i.e., $Mw^{-1/6}$), assuming that their shape is cylindrical. This approach resulted in an average r^2 value of 0.999. On the basis of this almost perfect linear relationship, I concluded that under isothermal conditions there is a linear relationship between the electrophoretic mobility of SDS - protein complexes and the -1/6 power of the molecular weight.

Therefore, I propose the following equation to describe the migration of SDS-protein complexes in borate cross-linked dextran gels:

$$\mu = const_A \cdot Q \cdot Mw^{-1/6} \cdot e^{-E_a/RT} \qquad (1)$$

where μ is the mobility, $const_A$ is the sum of constants, Q is the net charge of the SDS-protein complex, MW is the molecular weight, Ea is the activation energy, R is the universal gas constant and T is the absolute temperature.

Considering the results obtained, I used 25°C separation temperature for all further experiments.

2.3 Investigation of protein migration by the Ferguson method

To investigate the sieving effect of dextran-boric acid gels, I measured the mobility of a standard protein ladder and a test protein mixture in gels with 2% and 4% boric acid concentrations. The Ferguson plots showed nonlinear concave curves instead of the expected linear relationship, especially for gels with higher dextran concentrations. I observed that as the molecular weight of the sample increased, the degree of curvature increased towards higher dextran concentrations. For gels containing 2% boric acid, the expected linearity was distorted, in contrast to gels containing 4% boric acid. The electroferograms reflected the effects of molecular sieving and electroosmotic flow for the different gel formulations.

When analyzing protein standards, I found that the 10%/2% D/B gel provided the highest resolution and apparent selectivity, even though it required longer separation times. To study the migration of SDS-protein complexes, I introduced a new equation to describe the linear relationship between mobility and molecular weight (Equation 1).

For the LC/ngHC peaks, the 10%/2% D/B gel gave the best resolution and apparent selectivity, although it was associated with longer separation times. When testing intact the mAb and its subunits, I observed that the 10% D/2% B gel provided the highest resolution.

Introducing three-dimensional (3D) Ferguson diagrams, varying the concentration of dextran (D) and borate (B) based on simultaneous changes in dextran, and setting up 16 different D/B ratio gels allowed a better understanding of the electromigration process of SDS-protein complexes. The protein standards and the new modality fusion protein etanercept also yielded concave 3D Ferguson diagrams. The interplay between the electroosmotic flow and matrix viscosity played a key role in the resulting migration time and resolution.
The 3D Ferguson diagrams allowed a rapid assessment of the migration behaviour of separated SDS-protein complexes as a function of dextran-borate concentration. Therefore, this approach can help to select the appropriate gel composition for a given protein mixture whose separation may not be adequate in a standard gel composition of 10% dextran, and 4% boric acid.

2.4 Effect of the ratio of monomer to cross-linking agent on the selectivity of the separation

At a constant dextran concentration, the rate of formation of 1:1 (BD-) and 1:2 (BD2-) bonds, which influence the degree of cross-linking, depends on the amount of borate. Increasing the boric acid concentration increases the number of bonds (cross-links) between the chains, resulting in a decrease in the average pore size of the gel and a more structured cross-link. However, at low dextran concentrations, excess boric acid in the buffer can lead to saturation of the 1:2 bonds and the resulting increased number of 1:1 bonds can cause repulsion of the dextran chains, which increases the pore size. The complex formation results in a negatively charged gel, the chains are aligned in the direction of the electric field, creating a more structured network (reduced configurational entropy), similar to agarose in slab gel electrophoresis.

Next, I investigated how the ratio of boric acid to dextran affects the selectivity of the separation. I developed a retention model to better understand the selectivity between glycosylated and non-glycosylated heavy chains, taking into account the interaction of dextran-borate adducts with glycans on the protein.

The apparent selectivity (α) in borate-dextran systems during SDS-CGE is the product of the molecular weight sieving selectivity (α_{MW}) and the selectivity based on the secondary equilibrium complex formation (α_C), i.e., the dextran-borate-glycan complex formation:

$$\alpha = \alpha_{MW} \times \alpha_C \qquad (2)$$

Glycans attached to the polypeptide chains of glycoproteins are not covered by SDS, so diol groups in the correct position on them can form complexes with borate. There are two ways in which complex formation can occur. With the free borate in the gel, whereby the surface charge density of the complex increases and the mobility difference decreases, selectivity

between non-glycosylated and glycosylated chains. On the other hand, the borate (1:1) adducts within the chain in the gel may also complex with the carbohydrate moiety of the glycoprotein. For the present experiments I used omalizumab, a glycosyltated therapeutic protein. In this case, the core fucose, terminal manose, sialic acid and terminal galactose structures with α oriented vicinal hydroxyl groups may all be involved in the complex formation reaction. This type of complex formation affects differential electromigration, as the heavy chain becomes temporarily immobilized during the secondary equilibrium bridge formation by attaching to a dextran structure cross-linked to the borate. The separation time window between the non-glycosylated heavy chain and the glycosylated heavy chain increases as a consequence, so they will migrate at different rates in the gel.

Thus, in the systems outlined above, a part of the glycoprotein (P) will be in a non-complexed (f) state (R), while the other molecules will be in a complexed form (1-R). The apparent mobility (μ) of the glycoprotein can be expressed as the sum of the free and complexed forms:

$$\mu = R\mu^{f} + (1 - R)\mu^{c} \tag{3}$$

The fraction R (7,8) can be calculated from the complexation constant (K, equation 5) of the reaction (4). n is the number of glycoproteins complexed with the dextran-borate chain.

$$D_B + nP \rightleftarrows D_B\, P_n \tag{4}$$

$$K = \frac{[D_B\, P_n]}{[D_B][P]^n} \tag{5}$$

$$R = \frac{1}{1+K[D_B]} \qquad\qquad 1 - R = \frac{K[D_B]}{1+K[D_B]} \tag{6,7}$$

By combining equations 3, 6 and 7, the effective mobility of the glycosylated heavy chain can be expressed:

$$\mu_{HC} = \frac{\mu_{HC}^{f}}{1+K[D_B]} + \frac{\mu_{HC}^{c} K[D_B]}{1+K[D_B]} \tag{8}$$

The selectivity (α_C) is based on the secondary equilibrium complex formation between the glycosylated (HC) and non-glycosylated (ngHC) heavy chains can be expressed as follows, without taking into account the selectivity of molecular weight sieving (α_{MW}):

$$\alpha_C = \frac{\mu_{ngHC}}{\mu_{HC}} = \frac{\mu_{ngHC}(1+K[D_B])}{\mu_{HC}^{f} + \mu_{HC}^{c} K[D_B]} = \frac{\mu_{ngHC}(1+K[D_B])}{\mu_{HC}^{f}(1+K[D_B]\, \mu_{HC}^{c}/\mu_{HC}^{f})} \tag{9}$$

The mobility of the free glycosylated heavy chain is assumed to be much higher than that of the dextran-borate complex ($\mu_{HC}^{f} \gg \mu_{HC}^{c}$), so the selectivity can be expressed as follows:

$$\alpha_C = \frac{\mu_{ngHC}}{\mu_{HC}^{f}} (1 + K[D_B]) \tag{10}$$

if $K[D_B] \gg 1$ the selectivity equation can be simplified as follows:

$$\alpha_C = \frac{\mu_{ngHC}}{\mu_{HC}^{f}} K[D_B] \tag{11}$$

Furthermore, if $K[D_B] \ll 1$ the selectivity equation becomes:

$$\alpha_C = \frac{\mu_{ngHC}}{\mu_{HC}^{f}} \tag{12}$$

The resolution (Rs) can be expressed by the apparent selectivity (α), where E is the electric field strength, N is the theoretical number of plates and l is the effective length of the capillary [1].

$$Rs = \frac{1}{4}\left(\frac{\alpha-1}{\alpha}\right)\sqrt{N}\ \frac{\mu E}{l} \tag{13}$$

If α is close to one, there will be a significant effect on resolution, similar to that seen in chromatographic methods.

The facts established in the theoretical model have been supported by experimental results [2]. Accordingly, the separation selectivity between the non-glycosylated heavy chain and the normal (glycosylated) heavy chain was investigated using gels with different monomer/cross-linker ratios.

2.5 The study of the effect of electromigration dispersion

In electrophoretic separations, electromigration dispersion impairs separation efficiency, peak resolution and peak capacity by reducing peak symmetry. This problem can be eliminated by matching the background electrolyte co-ion and the sample component mobilities. In the case of the borate crosslinked electrophoresis polymer gels, such as agarose and dextran, which are most commonly used in capillary gel electrophoresis of therapeutic proteins, boric acid not only functions as a co-ion but also serves as a cross-linker to form a polymeric structure with the appropriate screening effect. I have investigated the effect of this very important buffer constituent with dual functionality on electromigration dispersion and, through it, on separation efficiency, the analysis of which has not been done so far in the literature. As a first step, I have put forward a theory, which I have supported with experimental results. For the sake of clarity, I have indicated the ions in the equilibrium equations by symbols referring to their names.

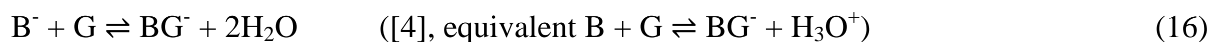
In aqueous solutions, boric acid ($B(OH)_3$: B) behaves as a monovalent acid, its dissociation and the distribution between boric acid (B) and the tetrahydroxyborate ion ($B(HO)_{4-}$: B^-) was described by Zittle as follows
[3]:

$$B + 2H_2O \rightleftharpoons B^- + H_3O^+ \quad (14)$$

The equilibrium constant of boric acid K_B (15a) can be used to express the concentration of tetrahydroxyborate (15b):

$$K_B = [B^-][H_3O^+]/[B] \qquad [B^-] = K_B[B]/[H_3O^+] \qquad (15a, b)$$

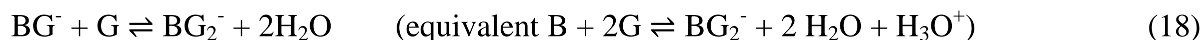
Tetrahydroxyborate ions react in equilibrium with polyols such as glycerol and glucose to form monomer (16) and dimer (18) adducts, with the extent of which is influenced by the relative concentration of polyol and tetrahydroxyborate ions. The reaction with glycerol to form monomer adduct is described by equation 16 and the formation of dimer adduct is described by equation 18. The equilibrium constants of the two reactions are illustrated in Equations 17a and 19a. Equations 17b and 19b are obtained by combining equations 15b, so I have inserted into the equations the concentration of the tetrahydroxyborate ion in terms of the equilibrium constant of boric acid.



$$B^- + G \rightleftharpoons BG^- + 2H_2O \qquad \text{([4], equivalent } B + G \rightleftharpoons BG^- + H_3O^+\text{)} \qquad (16)$$

$$K_{BG} = [BG^-]/[B^-][G] \qquad [BG^-] = K_{BG}[B^-][G] = K_{BG}[G]K_B[B]/[H_3O^+] \qquad (17a, b)$$

and



$$BG^- + G \rightleftharpoons BG_2^- + 2H_2O \qquad \text{(equivalent } B + 2G \rightleftharpoons BG_2^- + 2\,H_2O + H_3O^+\text{)} \qquad (18)$$

$$K_{BG2} = [BG_2^-]/[BG^-][G] \qquad [BG_2^-] = K_{BG2}[G][BG^-] = K_{BG2}K_{BG}[G]^2K_B[B]/[H_3O^+] \qquad (19a, b)$$

In the next step, the boric acid-dextran reaction is derived in a similar way to the boric acid-glycerol reaction. Boric acid can also form complexes with glucose units of dextran (D) polymer chains, thus forming monomeric (intra-chain, equation 20) and dimeric (interchain cross-linking, equation 22) adducts. These reactions reduce the mobility of the co-ion

complexes to virtually zero due to the practically negligible mobility of the dextran polymer [5].

The equilibrium constants K_{BD} and K_{BD2} are expressed in equations 21a and 23a. Equations 21b and 23b were obtained by combination with equation 15b.

$B^- + D \rightleftharpoons BD^- + 2H_2O$ (equivalent $B + D \rightleftharpoons BD^- + H_3O^+$) (20a,b)

$K_{BD} = [BD^-]/[B^-][D]$ $[BD^-] = K_{BD}[D][B^-] = K_{BD}[D]K_B[B]/[H_3O^+]$ (21a, b)

and

$BD^- + D \rightleftharpoons BD_2^- + 2H_2O$ (equivalent $B + 2D \rightleftharpoons BD_2^- + 2\ H_2O + H_3O^+$) (22a,b)

$K_{BD2} = [BD_2^-]/[BD^-][D]$ $[BD_2^-] = K_{BD2}[D][BD^-] = K_{BD2}K_{BD}[D]^2K_B[B]/[H_3O^+]$ (23a, b)

Furthermore, the monomer borate-glycerol adduct can form a complex with the dextran polymer (equation 24) using the K_{BGD} equilibrium constant described in equation 25a, which is combined with equation 15b to give equation 25b:

$BG^- + D \rightleftharpoons BGD^- + 2H_2O$ (equivalent $B + G + D \rightleftharpoons BGD^- + 2\ H_2O + H_3O^+$) (24a,b)

$K_{BGD} = [BGD^-]/[BG^-][D]$ $[BGD^-] = K_{BDG}[BG^-][D] = K_{BGD}K_{BG}[G][D]K_B[B]/[H_3O^+]$(25a, b)

The total concentration of borate-based negatively charged co-ions (CB-) in the background electrolyte can be expressed as the sum of the concentrations of each tetrahydroxyborate ion – polyol complexes in equations, 15b, 17b, 19b, 21b ,23b and 25b:

$C_B^- = [B^-] + [BG^-] + [BG_2^-] + [BD^-] + [BD_2^-] + [BGD^-]$ (26)

The mole fractions of the co-ions (R) are:

$$R_{B^-} = [B^-]/C_{B^-} \quad (27)$$

$$R_{BG^-} = [BG^-]/C_{B^-} \quad (28)$$

$$R_{BG2^-} = [BG_2^-]/C_{B^-} \quad (29)$$

$$R_{BD^-} = [BD^-]/C_{B^-} \quad (30)$$

$$R_{BD2^-} = [BD_2^-]/C_{B^-} \quad (31)$$

$$R_{BGD^-} = [BGD^-]/C_{B^-} \quad (32)$$

The effective mobility of a borate-based co-ion (μ_{eff} co-ion) can be determined by a linear combination of the corresponding ionic mobilities (μ^0) and its mole fraction (R)

$$\mu^{eff}_{co\text{-}ion} = \mu^0_{B^-} \cdot R_{B^-} + \mu^0_{BG^-} \cdot R_{BG^-} + \mu^0_{BG2^-} \cdot R_{BG2^-} + \mu^0_{BD^-} \cdot R_{BD^-} + \mu^0_{BD2^-} \cdot R_{BD2^-} + \mu^0_{BGD^-} \cdot R_{BGD^-} \quad (33)$$

Combining equations 15b, 17b, 19b, 21b, 23b, 25b and 27-32, we obtain the following result:

$$\mu^{eff}_{co\text{-}ion} = \frac{\mu^0_{B^-} + \mu^0_{BG^-} \cdot K_{BG}[G] + \mu^0_{BG2^-} \cdot K_{BG2}K_{BG}[G]^2 + \mu^0_{BD^-} \cdot K_{BD}[D] + \mu^0_{BD2^-} \cdot K_{BD2}K_D[D]^2 + \mu^0_{BGD^-} \cdot K_{BGD}[D]K_{BG}[G]}{1 + K_{BG}[G] + K_{BG2}K_{BG}[G]^2 + K_{BD}[D] + K_{BD2}K_D[D]^2 + K_{BGD}[D]K_{BG}[G]} \quad (34)$$

Again, it is important to emphasize that dextran-bound tetrahydroxyborates are virtually immobilized due to the negligible mobility of the large cross-linked 2 MDa polymer, i.e. $\mu_{BD}^{0-} \sim \mu_{BD2}^{0-} \sim \mu_{BGD}^{0-} \sim 0$. Consequently, the last three terms in the numerator of equation 34 approach zero at any tetrahydroxyborate concentration, which is an important conclusion for the evaluation of our results.

The two main factors of peak broadening and distortion during protein SDS-CGE are diffusion (σ_D^2) and electromigration dispersion (σ_{EMD}^2). All other variances are negligible, including adsorption, due to the repulsive effects between the negatively charged capillary wall and the SDS proteins. Joule heat generation is also negligible, due to the precise temperature control of modern capillary electrophoresis machines. This is further enhanced by constant detection zone and injection parameters. The total spatial variance of the peaks (σ_t^2) can be described by the following equation:

$$\sigma_t^2 = \sigma_D^2 + \sigma_{EMD}^2 \tag{35}$$

Based on the derivation presented above and the results presented in the previous chapters, it is evident that the electromigration dispersion of dextran gels stabilized with boric acid changes significantly with increasing boric acid concentration. To understand this phenomenon, I investigated the effect of borate concentration on the peak symmetry of SDS-protein complexes using 2% and 10% dextran-based gels (containing approximately 0.12 M and 0.60 M glucose building blocks).

3. Summary

In my PhD thesis I investigated the electromigration of SDS-protein complexes in dextran gels cross-linked with borate of different composition and the effect of different measurement parameters using capillary electrophoresis with UV detection.

In a first step, I determined the applicable lower and upper boric acid and dextran boundary concentrations, taking into account the ease of gel exchange in the capillary, viscosity and conductivity, respectively. Subsequently, I determined the viscosity of the gels, the separation current and the resulting electroosmotic flow, which defines the separation profile, essential for understanding the migration behavior of SDS-protein complexes in dextran-boric acid gels. Previous studies have also shown that the choice of the appropriate temperature plays an important role in enhancing the separation efficiency. In view of this, Arrhenius diagrams were used to investigate the temperature dependence of protein separation and the activation energy (Ea) requirement for the separation. The gels and the measurement parameters were tested by separating and characterizing different therapeutic antibodies - monoclonal antibody, bispecific mAB, nanobody. The results obtained support the successful method development and optimization.

To test the basic filtering ability of the gel, 2.0, 5.0, 7.5 and 10.0 (w/V)% 2 million molecular weight dextran (D) monomer and 2.0, 2.7, 3.3 and 4, 0 (w/V)% borate (B) cross-linking agent were prepared by chemical gels to separate the ladder components of a protein in the range 20-225 kDa and the subunits and intact form of a therapeutic monoclonal antibody and a fusion protein by capillary electrophoresis.

To further study the migration properties, I used the Ferguson method, which can help to select the right gel composition for a given protein mixture. The obtained electrophorograms were evaluated to achieve detailed information on the performance of the separations, such as theoretical plate count, resolution and selectivity factor determination. By introducing the 3D Ferguson representation, it is easy to evaluate the migration properties of the separated protein molecule at different monomer and crosslinker concentrations, and thus to select the appropriate composition for a given protein mixture. My experiments investigated how the

ratio of boric acid to dextran affected the selectivity of the separation. I have developed a retention model to better understand the selectivity between the glycosylated and non-glycosylated heavy chains, taking into account the interaction of dextran-borate adducts with complex sugars on the protein. Dextran-borate-glycoprotein complex formation at lower monomer/crosslinker concentrations enhanced the resolution between the glycosylated and non-glycosylated heavy chains of the antibody. In light of these results, SDS gel formulations can be developed that can address the separation challenge of highly glycosylated new modality therapeutic proteins.

The decreasing resolution and peak capacity are increasingly causing problems in the electrophoretic separation of new types of therapeutic proteins, which is also contributed to by the changing peak shape (tailing and fronting effect) due to electromigration dispersion. My experiments to investigate electromigration dispersion have confirmed the need to improve peak shape by reducing this phenomenon in capillary gel electrophoresis. This can be achieved by choosing the right type of buffer co-ion component to match the mobility of the sample molecules under investigation. In order to avoid triangular distortions, it should be taken into account that in SDS-CGE systems using borate-crosslinked dextran gels, boric acid has a dual role: it is an ion with the same charge as the SDS-protein complex and it also participates in the formation of the molecular sieve crosslink.

4. List of publications

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Registry number: DEENK/97/2024.PL
Subject: PhD Publication List

Candidate: Csenge Boróka Filep
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10068626

List of publications related to the dissertation

1. **Filep, C. B.**, Guttman, A.: Electromigration Dispersion in Sodium Dodecyl Sulfate Capillary Gel Electrophoresis of Proteins.
   Anal. Chem. 94 (38), 13092-13099, 2022.
   DOI: http://dx.doi.org/10.1021/acs.analchem.2c02348
   IF: 7.4
2. **Filep, C. B.**, Guttman, A.: Capillary sodium dodecyl sulfate gel electrophoresis of proteins: introducing the three dimensional Ferguson method.
   Anal. Chim. Acta. 1183, 1-9, 2021.
   DOI: http://dx.doi.org/10.1016/j.aca.2021.338958
   IF: 6.911
3. **Filep, C. B.**, Guttman, A.: 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 (7), 3535-3541, 2021.
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Total IF of journals (all publications): 57,767
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