Better Separation Resolution of New Biopharmaceutical Modalities through Fine Tuning of the Temperature with CE-SDS

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Introduction

New protein based biopharmaceutical modalities such as nanobodies, bispecific antibodies and fusion proteins represent a promising class of therapeutic agents with high precision and efficacy. In addition, these new modalities may overcome some of the limitations of monospecific mAb therapeutics and consequently change the treatment landscape for various diseases. Nanobodies are important in many fields of medicine, including oncology, inflammatory, infectious and neurological diseases, as well as in imaging [1]. Pre-clinical studies and early phase clinical trials have demonstrated that multispecific antibodies may offer effective strategies for solid tumors [2], while fusion proteins are very powerful in the treatment of autoimmune diseases [3]. Purity assessment of these therapeutic entities and revelation of any structural ambiguities possibly introduced during the manufacturing process are of high importance for the biopharmaceutical industry and also represents regulatory agency requirements.

The PA 800 Plus Pharmaceutical Analysis System

One of the well-established techniques for rapid characterization, release and stability tests for protein therapeutics is sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE, also referred to as CE-SDS). Automated capillary electrophoresis instruments, such as the PA 800 Plus Pharmaceutical Analysis system, offer rapid analysis time and high reproducibility [4], thus represent an excellent tool of choice for SDS-CGE. The sample preparation protocol for CGE-SDS is practically the same as for conventional polyacrylamide slab gel electrophoresis (SDS-PAGE), i.e., based on the fact that 1 g of protein binds 1.4 g SDS in an adequately uniform fashion. This leads to very similar charge to hydrodynamic volume ratios of the protein – SDS complexes, thus, necessitates the use of a sieving matrix for their size based separation. It is also well

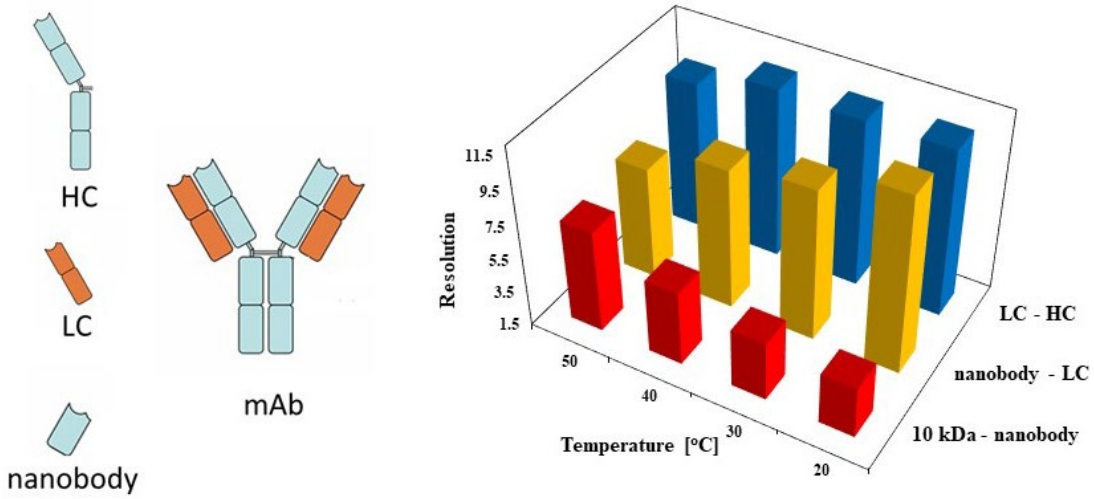

Figure 1. Effect of the separation temperature in CE-SDS on the resolution of some biotherapeutics. With increasing temperature, the resolution between the 10 kDa protein and the PSA specific nanobody is increasing (Red), while it is decreasing between the nanobody and the light chain (LC) of Xolair (Yellow). There is a moderate decrease in resolution between the light (LC) and heavy (HC) chains of Xolair with elevating temperature (Blue).

known that hydrophilic post translational modifications, such as glycosylation, may alter this binding ratio by decreasing the overall charge of the complex and increasing its hydrodynamic volume. Both effects cause drop in electrophoretic mobility leading to molecular mass overestimation.

Biopharma researchers/analysts currently face challenges in adopting gold-standard CE-SDS for purity/heterogeneity of new modalities where they may not obtain the desired separation resolution.

The industry gold standard CE-SDS method is utilizing a carefully designed borate cross-linked dextran gel as sieving matrix. This gel is optimized for the analysis of the size range of up to 225 kDa, matching the requirement of nanobody, mAb and fusion protein analysis [4-6]. The resolution of the method is dependent on the sieving matrix composition and the separation conditions applied, such as separation temperature [7]. In view of the effect of temperature on the electromigration of SDS-protein complexes in narrow bore capillary columns, particular attention is given to the activation energy required by the solute molecules to drift through the sieving gel. In capillary gel electrophoresis, the SDS-protein complexes reportedly migrate in cylindrical conformation [8], and their electrophoretic mobility (μ) is inversely proportional to sixth root of the molecular weight (Mw) as depicted in equation 1:

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

where Q is the net charge, E_a is the activation energy, R is the universal gas constant and T is the absolute temperature. Based on equation 1, the electrophoretic mobilities of the migrating SDS-protein complexes under isobaric/isothermal conditions are influenced by their activation energy requirement, i.e., the energy required by the solute molecules to overcome the obstacles of the separation medium [9]. In practice, the activation energy values can be derived from the slopes of the so called Arrhenius plots [10] of logarithmic electrophoretic mobility vs. reciprocal absolute temperature. For more details of the theory behind, please consult reference [8]. Nevertheless, one of the most important parameters in equation 1 is the separation temperature, which by individually affecting the migration velocity of the sample components can have a significant effect on peak resolution as shown in Figure 1.

In this Technical Note, a novel temperature optimization method is introduced to improve the separation performance of biotherapeutics in SDS-CGE. The separation between any proteins of interest can be easily fine-tuned by varying the separation temperature on a PA 800 Plus Pharmaceutical Analysis System. **Researchers can now rapidly accommodate these new modalities by simple temperature adjustments to obtain the desired resolution.**

Key Features

- The electric field mediated migration of any protein – SDS complexes is an activated process, therefore, different molecules require different temperature settings for their optimal resolution.
- Arrhenius plots can be used to study the temperature dependence of the separation performance and to understand the activation energy requirement for the electromigration of the SDS-protein complexes.
- Utilizing the activation energy concept, resolution of the components of interest for the sample mixture in hand can be optimized by simply fine tuning the separation temperature.
- Unlike other CE systems, the separation temperature in the PA 800 Plus Pharmaceutical Analysis System can be easily adjusted with high precision to provide the desired separation performance.

Experimental

Chemicals

The SDS-MW Analysis Assay kit (Part # 390953, Sciex, Brea, CA) with the SDS-MW Gel Buffer, Sample Buffer, 10 kDa protein standard and SDS-MW Size Standard mixture (10 to 225 kDa) was used in all experiments. Xolair (monoclonal antibody therapeutics) and the PSA specific nanobody were kindly provided by the University of Pannonia (Veszprem, Hungary). HPLC grade water, 2-mercaptoethanol and all other chemicals were from Sigma Aldrich (St. Louis, MO, USA).

Sample Preparation

The molecular weight sizing ladder sample contained 160 μg of SDS-MW Size Standard, 10 μg of internal standard (10 kDa) and 5 μl of 2-mercaptoethanol in a total volume of 85 μl. The

biopharma sample mixture contained 10 µg of 10 kDa protein standard, 16 µg of PSA specific nanobody, 30 µg of Xolair and 5 µL of 2-mercaptoethanol in 70 µl of sample buffer (100 mM Tris-HCl, 1% SDS, pH 9.0). All samples were denatured at 100°C for 3 minutes and cooled to room temperature before injection.

Capillary SDS-Gel Electrophoresis

A PA800 Plus Pharmaceutical Analysis System (Part # A51963AE, Sciex) was used in all experiments in UV absorbance detection mode with a 214 nm filter. The EZ-CE cartridge (Part # A55625) with a 20 cm effective length (30 cm total length), 50 µm ID bare fused silica capillary was filled with the SDS-MW gel-buffer system. Capillary conditioning: 0.1 M NaOH rinse for 3 minutes, 0.1 M HCl rinse for 1 minute, HPLC grade water rinse for 5 minutes and SDS-MW gel buffer rinse for 5 minutes before each run. The applied electric field strength was 670 V/cm in all capillary electrophoresis analyses in reversed polarity mode (anode at the detection side). The samples were electrokinetically injected in triplicates (10 kV for 20 sec) and separated at 20, 30, 40 and 50 ± 0.1°C capillary cartridge temperatures. The 32Karat (version 10.1) software package was used for data acquisition and processing (Part # A79486AC, SCIEX)

Results and Discussion

Existing Methods to Separate AAV Full and Empty Capsids

The molecular weight standard mixture of a set of unmodified linear polypeptides in the size range of 10 kDa to 225 kDa was separated by SDS-CGE at capillary cartridge temperatures of 20, 30, 40 and 50°C. Panel A in Figure 2 compares the resulting electropherograms. As one would expect, the migration times decreased with increasing separation temperatures, due to the associated viscosity changes of the sieving matrix. It is important to note that in this sample, the individual polypeptides of the molecular weight ladder were assembled from the same oligopeptide building blocks with no post-translational modifications. Thus, they differed only by the number of the connected sections with very close physical – chemical properties. For activation energy determination, the Arrhenius diagrams were delineated by plotting the logarithmic electrophoretic mobilities as a function of the reciprocal absolute temperature as described in [8]. The individual mobility values were adjusted with the viscosity change of the sieving matrix with the increasing temperature (4%/5°C). The activation energy values, shown in Table 1, were derived by dividing the slope values by the universal gas constant. Closer inspection of the obtained numbers suggested size specific migration characteristics of the solute molecules.

The monotonic decrease of the activation energy values in Table 1 suggested predictable activation energy changes of the linear polypeptide Mw standards in the temperature range examined. Consequently, the resolution values among the migrating polypeptides changed very similarly in the entire temperature range due to their comparable molecular characteristics (linear polypeptides with no post translational modifications) as shown in Figure 2B.

Table 1. The activation energy values derived from the Arrhenius plots based on the separation traces in Figure 2A.

Peak	Mw (kDa)	Arrhenius Slope	Ea (kJ/mol)
1	10.00	-5420.23	44.86

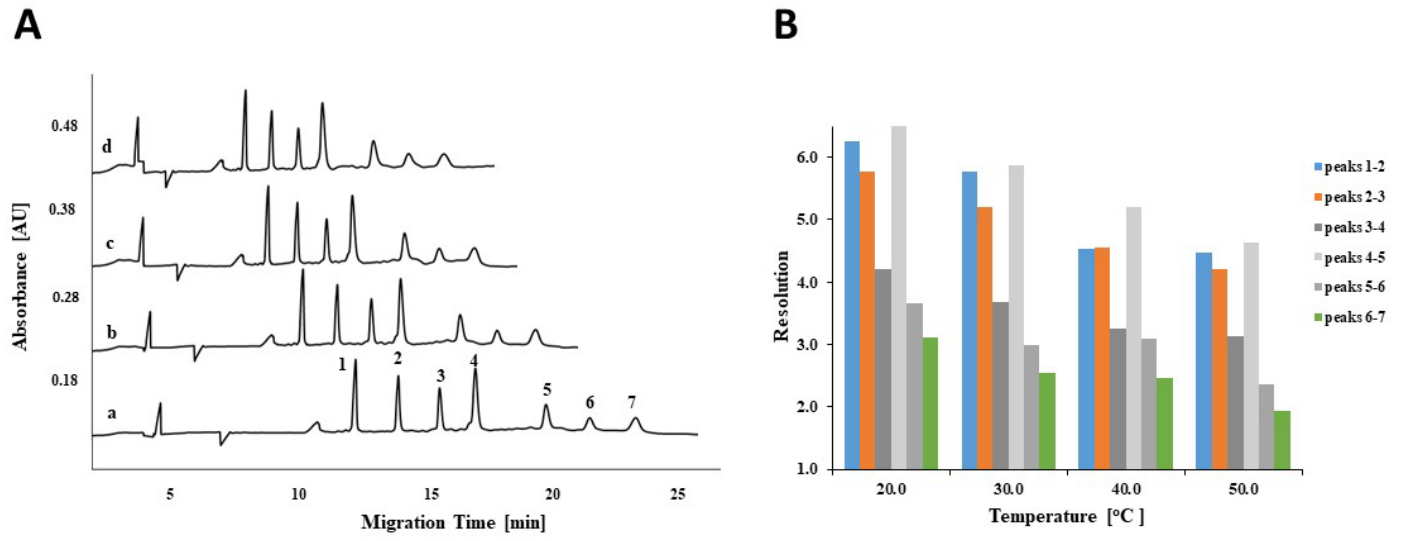

Figure 2. (A) Sodium dodecyl sulfate capillary gel electrophoresis analysis of the standard polypeptide molecular weight ladder at 20°C (trace a), 30°C (trace b), 40°C (trace c) and 50°C (trace d). Peaks: 1) 10 kDa, 2) 20 kDa, 3) 35 kDa, 4) 50 kDa, 5) 100 kDa, 6) 150 kDa and 7) 225 kDa polypeptides. Conditions: 20 cm effective separation length (30 cm total), SDS-MW Gel Buffer; 670 E: V/cm, Injection: 10 kV / 20 sec. (B) Temperature mediated resolution changes between the separated peaks as labeled in panel A .

2	20.00	-5393.12	44.66
3	35.00	-5351.91	44.32
4	50.00	-5298.89	43.89
5	100.00	-5190.00	42.98
6	150.00	-5100.31	42.27
7	225.00	-5030.52	41.70

Biopharma Sample Analysis

Next, the biopharma sample mixture containing the PSA specific nanobody (MW 14.26 kDa), the light and heavy chains of Xolair (a monoclonal antibody therapeutic, light chain: 23.89 kDa, heavy chain: 49.37 kDa) as well as the 10 kDa protein standard were subject to SDS-SGE analysis at the same temperature range of 20ºC – 50ºC, shown in Figure 3A. Arrhenius charts of the logarithmic mobility vs reciprocal absolute temperature values were plotted again and the activation energy values were calculated from the slopes as listed in Table 2. In contrast to Table 1, the activation energy values corresponding to the individual biopharma sample proteins did not result in a predictable function. Actually, all had quite different activation energy requirements, probably depending on their primary sequence and post translational modification mediated differences in surfactant binding.

In contrast to the results obtained in the Mw ladder separation, the resolution values between the biopharma sample mixture components revealed significant and non-predictable alterations with increasing temperature as one can visually observe in Figure 3B. The resolution between the 10 kDa protein and the PSA specific nanobody almost doubled with the increasing separation temperature from 20ºC to 50ºC, while the resolution between the PSA specific nanobody and the light chain of Xolair, as well as the light and heavy chains of the Xolair mAb, respectively dropped by 50% and 30% in the same temperature range.

Table 2. The activation energy values derived from the Arrhenius plots based on the separation traces in Figure 3A.

Peak	Mw (kDa)	Arrhenius Slope	Ea (kJ/mol)
1	10.00	-5359.34	44.56
2	14.26	-5163.24	42.93
3	23.89	-5236.61	43.54
4	49.37	-5129.02	42.64

Conclusions

Biopharma researchers/analysts, face a difficult time adopting gold-standard CE-SDS for purity/heterogeneity of new modalities can now rapidly accommodate these analytes by simple temperature adjustments that only the SCIEX PA 800 Plus provides.

This Technical Note shows the effect of temperature on the migration behavior of a set of linear polypeptides (Mw ladder) and several protein therapeutics using capillary SDS-gel electrophoresis in the temperature range of 20°C – 50°C. The activation energy concept was applied to shed light on the electromigration properties of the non-modified polypeptides and the therapeutic proteins with special respect of temperature-

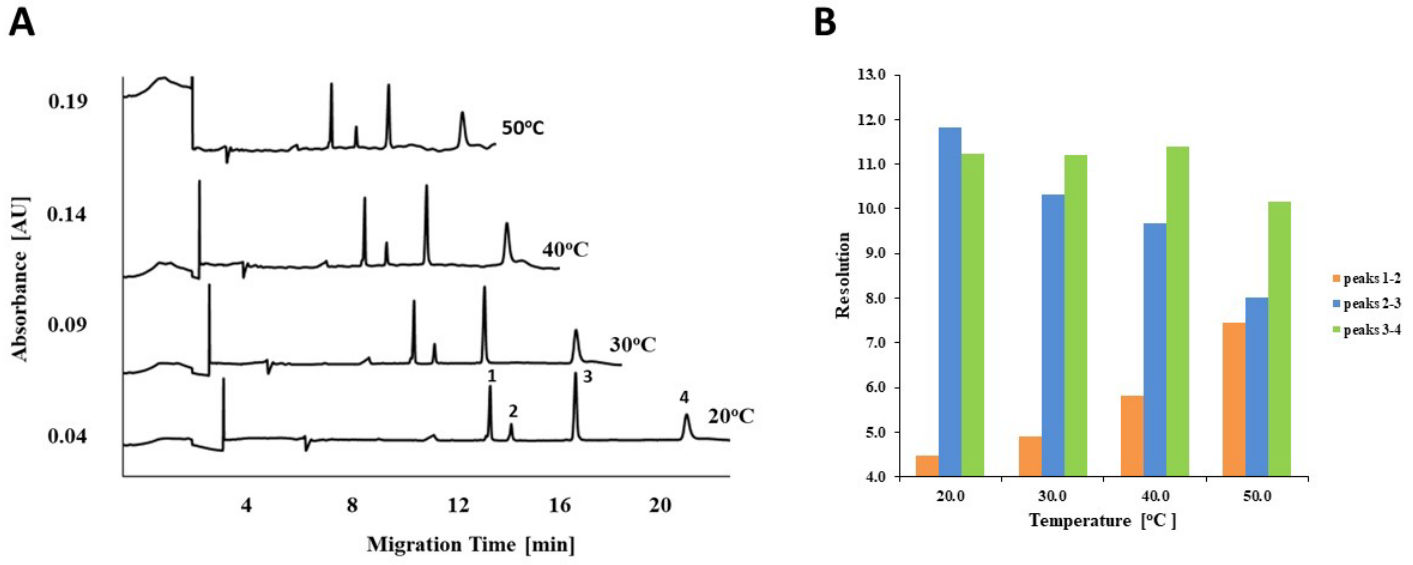

Figure 3. (A) Sodium dodecyl sulfate capillary gel electrophoresis analysis of the biopharma sample mixture at 20ºC (trace a), 30ºC (trace b), 40ºC (trace c) and 50ºC (trace d). Peaks: 1) 10 kDa protein standard (10.00 kDa), 2) PSA specific nanobody (14.26 kDa), 3) Xolair light chain (23.89 kDa), 4) Xolair heavy chain (49.37 kDa). Conditions were the same as in Figure 2.
(B) Effect of temperature on the resolution between the biopharma sample mixture components in SDS-CGE .

dependent resolution changes. While the resolution between the 10 kDa internal standard protein and the nanobody has significantly increased with elevating temperature, the resolution between the nanobody and the light and heavy chains of the monoclonal antibody therapeutic both decreased, but at different levels. Since the electromigration property of any protein – SDS complex is an activated process and each and every single molecule have their own activation energy requirement, one can adjust the separation temperature to fine tune the separation performance for the samples in hand. Unlike other CE systems, the separation temperature in the PA 800 Plus Pharmaceutical Analysis System can be easily adjusted with high precision to provide the desired separation performance.

Acknowledgment

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