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## Research Article

# Development of a capillary gel electrophoresis method for monitoring disulfide isomer heterogeneity in IgG2 antibodies

A CGE method for monitoring the disulfide isomer distribution characteristic of IgG2 MAbs is presented. Disulfide heterogeneity of MAbs has been studied using various chromatographic and electrophoretic methods. Although CGE operates using a different selectivity mechanism from that of sorption chromatographic techniques, similar trends are present in the data, which allow the CGE method to be used as a complementary method for studying disulfide isomer distribution. This article focuses on the optimization of a capillary-based gel electrophoresis method that can be used to support antibody development including bioprocess optimization, antibody characterization, release, and formulation stability assessment.

### Keywords:

Antibody IgG2 / CE / Disulfide isomer / Protein characterization

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## 1 Introduction

Therapeutic antibodies have been the fastest growing segment of the biopharmaceutical market with greater than 450 antibody-based candidates currently under development [1]. Twenty-one of these antibody-based therapeutics have been approved in the US with seven of the marketed MAbs thus far seeing greater than \$1B USD in annual sales. To successfully move an antibody through development, it must be “well characterized” to ensure product quality and consistency [2]. Biopharmaceuticals in general are very heterogeneous products that are likely to have numerous chemical and post-translational modifications, often requiring several orthogonal analytical methods to adequately characterize the complex product. Modifications may include deamidation, oxidation, aggregation, N-terminal cyclization, proteolytic cleavage, glycosylation, sialylation, C-terminal lysine heterogeneity, and disulfide bond scrambling [3–5].

The antibodies that have been marketed are mainly of the IgG class, which can be broken up into four subclasses including IgG1, IgG2, IgG3, and IgG4. The majority of approved IgG antibodies are of the IgG1 class, although several IgG2 and IgG4 candidates have also received regu-

latory approval [6]. As a result, much of the analytical data available in the literature only address the IgG1 subclass. IgG2 type antibodies typically lack effector function, which is a reason why development of these molecules is of interest to several pharmaceutical/biotech companies [7]. Apparent separation efficiencies of intact MAb are typically much better for the IgG1 class as compared with the IgG2 class, which are generally more hydrophobic [8]. IgG2 antibodies typically yield broad peaks with reduced resolution relative to IgG1 antibodies under identical separation conditions prompting investigation of structural motifs that could have such an impact on separation performance.

Each of the subclasses (IgG1, IgG2, IgG3, and IgG4) differs in their primary structure and connectivity in the hinge region (Table 1) [9]. Despite these differences, there is a large degree of homology between the IgG subclasses. Recent reports have identified alternative disulfide linked isoforms to that of the classical structure for human IgG2 [10]. As shown in the literature, several orthogonal analytical methods with optimized conditions are required to separate these isomers including RP chromatography, ion-exchange chromatography (IEX), CGE, and peptide mapping to identify the different isomers and ultimately yield a more complete understanding of the product [11–16].

Over the past decade, CGE has replaced SDS-PAGE as the primary method for size-based protein analysis [13, 17–26]. Advantages of the capillary technique include automation, enhanced precision, high-speed analysis, improved resolution for closely migrating species, and on-line quantitative detection. Protein samples are heated in the presence of SDS at concentrations greater than the CMC. At high SDS concentrations, SDS coats the protein in a ratio of approximately 1.4 g

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**Abbreviations:** IPA, isopropyl alcohol; MP, mobile phase; NR, non-reduced

**Table 1.** IgG subclass properties

IgG subclass	MW (kDa)	Amino acids in hinge	Disulfide bonds in hinge
IgG1	~146	15	2
IgG2	~146	12	4
IgG3	~170	62	11
IgG4	~146	12	2

of SDS *per gram* of protein yielding a uniform negative charge. Thus, the surface charge is based solely on the number of SDS micelles bound to the protein and not the native protein charge. By this measure, all proteins will have roughly the same mass-to-charge ratio thus they will have the same electrophoretic mobility. The separation of size-based variants occurs due to the presence of a sizing matrix within the capillary yielding a separation that is based on hydrodynamic radius. This matrix is composed of a linear or branched polymer such as polyacrylamide, PEG, polyethylene oxide, or dextran [27–29]. EOF must be suppressed in this application by using a high buffer concentration (Beckman CE-SDS buffer), a buffer with a dynamic coating (Bio-Rad CE-SDS buffer), or by using coated capillaries to achieve a separation that is solely based on hydrodynamic radius as the analytes migrate through the gel sieving matrix. CGE can be used for the analysis of reduced proteins by including an appropriate reducing agent with the sample preparation (2-mercaptoethanol) or for the analysis of non-reduced (NR) proteins by including an alkylating agent to the sample preparation (iodoacetamide) to prevent disulfide rearrangement.

SDS-PAGE and CGE have long been used for size-based purity analysis for all IgG subclasses. A recent report outlining the specific use of CGE for studying disulfide heterogeneity has been published [13]. The separation occurs in this case due to an alternative buffer matrix, which appears to be responsible for providing partial resolution of disulfide isomers by CGE although the mechanism leading to enhanced selectivity was not discussed [13]. Here, we describe the development of a generic platform CGE methodology for assessing disulfide heterogeneity across a portfolio of antibodies. We evaluate several parameters that were found critical to optimal resolution, long-term use, and transferability of the methodology across multiple laboratories. The optimized method conditions allow reproducible analysis of bioprocess development samples, formulation development samples, and QC samples to support a given project as it moves through development.

## 2 Materials and methods

### 2.1 Chemicals and materials

ProteomeLab SDS-MW sample buffer (100 mM Tris-HCl, 1%SDS pH 9, P/N 390960), SDS-MW gel buffer (P/N 391163), acidic wash solution (0.1N HCl, P/N 391646), basic

wash solution (0.1N NaOH, P/N 391988), and 10 kDa internal standard were obtained from Beckman Coulter (Fullerton, CA, USA) as part of the IgG Heterogeneity (P/N A10663) and SDS-MW (P/N 390953) kits. An additional sample buffer (eCAP SDS, 120 mM Tris/HCl/1%SDS pH 6, P/N 241525) was also obtained from Beckman Coulter. CE-SDS Gel buffer (P/N 148-5032) and 10% SDS solution (P/N 161-0416) from Bio-Rad (Hercules, CA, USA) were also used. Iodoacetamide (P/N A-3221), isopropyl alcohol (IPA), ACN, TFA, guanidine-HCl (Gd-HCl), and Tris buffer (pH 7.5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Endoproteinase Lys-C (P/N 125-02543) was purchased from Wako (Richmond, VA, USA). Protein Express Reagents and microchips were obtained from Caliper Life Sciences (Mountain View, CA, USA, P/N 760431). Bare fused silica capillaries of 50  $\mu$ m id were obtained from Polymicro Technologies (Phoenix, AZ, USA, P/N 2000017) and Beckman Coulter (P/N 338451). Agilent Poroshell C8 columns (5  $\mu$ m, 300  $\text{\AA}$ , 2.1  $\times$  75 mm, P/N 660750-906) were obtained from Agilent Technologies (Santa Clara, CA, USA). Vydac 214TP C4 columns (5  $\mu$ m, 300  $\text{\AA}$ , 2.1  $\times$  250 mm, P/N 214TP52) were obtained from Grace Davison Discovery Sciences (Deerfield, IL, USA). TSKgel DEAE-5PW columns (10  $\mu$ m, 1000  $\text{\AA}$ , 7.5  $\times$  75 mm, P/N 07164) were obtained from Tosoh Bioscience (Montgomeryville, PA, USA). Commercially available IgG MAbs were obtained from AmerisourceBergen (Chesterbrook, PA, USA), and were also produced in house (Pfizer).

### 2.2 Sample preparation

Unless noted otherwise, the CGE sample had a final volume of 100  $\mu$ L of protein at a 1 mg/mL concentration. The preparation consisted of 50  $\mu$ L sample buffer (ProteomeLab SDS-MW sample buffer), 5  $\mu$ L iodoacetamide (250 mM), and 2  $\mu$ L of 10 kDa internal standard with the remaining volume being made up with H<sub>2</sub>O to attain a concentration of 1 mg/mL. Sample preparations were heated in a water bath at 65°C for 10 min. The sample was placed in a PCR tube and analyzed. RP-HPLC samples were prepared at a concentration of 2 mg/mL using mobile phase (MP) A, and a 5  $\mu$ L injection of sample was made on to the column. Samples were prepared for peptide mapping by incubating approximately 80  $\mu$ g of protein (based on A<sub>280</sub>) in the presence of 8 M guanidine-HCl and 15 mM iodoacetamide for about 6 h at 37°C. The digestion reaction was prepared by combining 100  $\mu$ L of the denatured antibody mixture with 124  $\mu$ L H<sub>2</sub>O; 56  $\mu$ L of 1 M Tris buffer, pH 7.5; 280  $\mu$ L of 8 M urea; and 4  $\mu$ g of endoproteinase Lys-C [enzyme:substrate = 1:15 (w:w)], followed by incubation at 37°C for 5 h. Samples analyzed with microchip CE were prepared by diluting 5  $\mu$ L of sample (1 mg/mL) with 35  $\mu$ L of Protein Express sample buffer containing approximately 30 mM iodoacetamide and heated for 10 min at 65°C. After the heating, the sample was diluted with 70  $\mu$ L of H<sub>2</sub>O for analysis.

### 2.3 Instrumentation

A Beckman Coulter ProteomeLab PA800 instrument equipped with a PDA detector utilizing 32Karat software and Waters Empower Chromatography data collection system were used for all CE analyses unless otherwise noted. Agilent 1100 or 1200 LC systems utilizing Waters Empower Chromatography data collection system and control were used for all LC analyses unless otherwise noted. Microchip CE was performed using a Caliper GXII with LabChip GX software.

### 2.4 Methods

The analysis methods were based on that described in the Beckman Coulter IgG Purity/Heterogeneity kit with some modifications to enhance resolution and reproducibility. The analysis methods included a short capillary method (50  $\mu\text{m}$  id  $\times$  30.2 cm total length, 20.2 cm effective length) for higher throughput and a long capillary method (50  $\mu\text{m}$  id  $\times$  58.2 cm total length, 48.2 cm effective length) that provided enhanced resolution. Final method conditions are given in Tables 2 and 3. The cartridge and samples were kept at a temperature of 15°C. The PDA detector was set to a wavelength of 220 nm with a bandwidth of 10 nm. The reference channel was set to 350 nm with a 10 nm bandwidth. The RP method used an Agilent Poroshell C8 column with a gradient from 24% MP B to 40% MP B in 30 min at a flow rate of 0.5 mL/min. MP A consisted of 2% IPA and 0.1% TFA in H<sub>2</sub>O while MP B consisted of 70% IPA, 20% ACN, and 0.1% TFA in H<sub>2</sub>O. A column temperature of 80°C was used with detection at 215 nm. The digested samples were applied to a Vydac C4 column set at 60°C and analyzed using an Agilent 1100 HPLC. A linear gradient from 0 to 45% B in 120 min at a flow rate of 0.2 mL/min was used to elute the peptides. MP A was 0.1% TFA in H<sub>2</sub>O while MP B consisted of 0.1% TFA in ACN. The eluent from the HPLC was directed into a Q-TOF Micro

(Waters, Milford, MA, USA) electrospray TOF mass spectrometer for peak identification. The IEX method employed a Tosoh Bioscience TSKgel DEAE-5PW column with a gradient of 0–20% MP B in 40 min. For IEX, MP A consisted of 50 mM Tris-HCl (pH 9.0) while MP B consisted of 50 mM Tris-HCl (pH 9.0) with 0.5 M NaCl. The column temperature for the IEX separation was ambient with detection at 280 nm. The microchip CE (Caliper GXII) method consisted of the standard (unmodified) HT Antibody 200 script.

## 3 Results and discussion

### 3.1 Method optimization

CGE has been deployed in development and QC laboratories as a replacement for SDS-PAGE. As a part of method development, several buffers were used for the analysis of reduced and NR antibodies. A typical separation of IgG2 MAbs using NR CGE analysis is shown in Fig. 1. Figure 1A shows a separation of a NR IgG2 MAb using the Beckman ProteomeLab CE-SDS buffer that is commonly used for CGE separations in pharmaceutical labs. An alternative buffer (Bio-Rad CE-SDS buffer) is shown in Fig. 1B. It is evident in this case that the electropherograms are quite different as a result of using different dextran-based CE-SDS buffers. An initial observation with the Bio-Rad CE-SDS buffer was that it provided improved resolution in the high molecular weight region, but sensitivity and resolution were lost in the lower molecular weight region making it difficult to detect a ~23 kDa fragment (light chain) that is usually present in antibody samples at a low abundance.

To understand the difference in the separation, buffer composition was examined. Both buffers contain Tris-Borate buffer with approximately 10% dextran (MW = 2 million). The Beckman buffer is composed of a higher concentration of Tris-Borate buffer to suppress the EOF allowing a separation based solely on hydrodynamic radius [30]. This buffer also contains a small percentage of glycerol (solubilizing agent). The Bio-Rad buffer uses a lower concentration of Tris-Borate buffer and suppresses the EOF by another mechanism. According to a patent, a small percentage of the dextran is converted to triethanolamine dextran, which serves as a dynamic capillary coating [31]. The positively charged polymer coats the capillary surface to effectively eliminate EOF. With a lower buffer concentration the current is also reduced with the Bio-Rad CE-SDS buffer allowing quicker analysis times (20 min *versus* 35 min). Interaction with the charged polymer on the capillary surface or a slightly different sieving matrix could be responsible for the improved resolving power around the intact IgG2 peak with the Bio-Rad CE-SDS buffer.

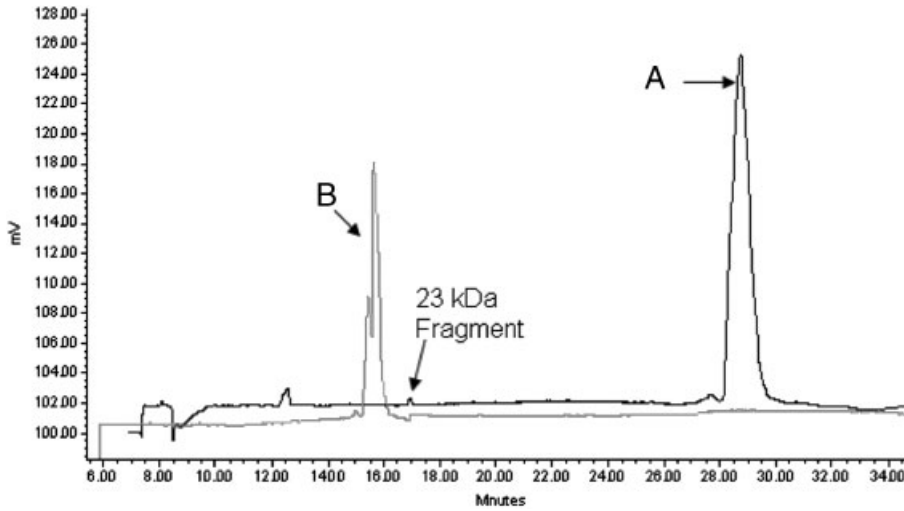
As previously mentioned, recent reports have identified the presence of disulfide heterogeneity in the IgG2 class of MAbs. A recent study employed CGE as a tool to provide electrophoretic evidence of the presence of disulfide-medi-

**Table 2.** Short-capillary method (50  $\mu\text{m}$  id, 20.2 cm effective length), cartridge temperature is 15°C

Step #	Event	Value	Duration	Comment
1	Rinse – pressure	70 psi	3 min	0.1 N NaOH rinse
2	Rinse – pressure	70 psi	1 min	Water rinse
3	Rinse – pressure	70 psi	10 min	SDS gel rinse
4	Wait		2	H <sub>2</sub> O dip
5	Wait		2	H <sub>2</sub> O dip
6	Inject – voltage	–5 kV	20 s <sup>a)</sup>	Sample injection
7	Wait		2	H <sub>2</sub> O dip
8	Separate	–15 kV	30 min	separation <sup>b)</sup>

a) Sample injection time was adjusted based on initial sample concentration and subsequent salt concentration.

b) 20 psi pressure was applied at the capillary inlet and outlet during the separation.

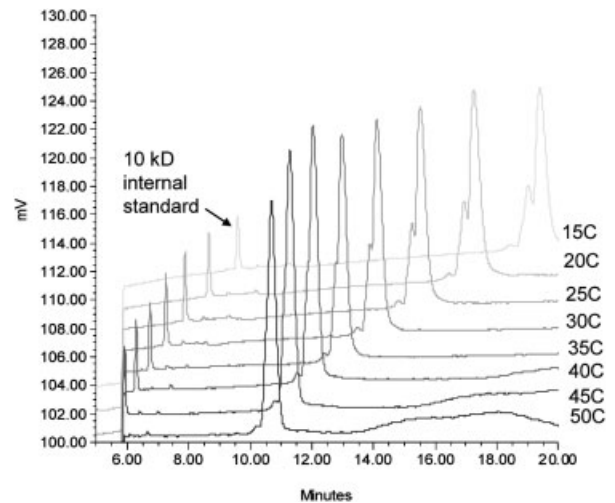


**Figure 1.** Evaluation of CE-SDS buffer for NR IgG2 separation (20.2 cm effective separation length). (A) Generated using Beckman ProteomeLab CE-SDS buffer, (B) generated using Bio-Rad CE-SDS buffer.

ated isoforms within IgG2s [13]. The buffer used in these studies to show partial resolution of disulfide isoforms was the Bio-Rad CE-SDS buffer, which generated similar separations to those reported here. In the present study, the lack of corresponding multiple peaks for IgG1 samples suggested that the peak multiplicity observed for IgG2 samples resulted from disulfide heterogeneity.

The CGE methodology has been optimized here to provide reproducible results comparable to that of orthogonal techniques that have been shown to also provide a separation of disulfide isomers including RP and IEX chromatography. Several parameters were studied to provide enhanced resolution of the disulfide isomers as well as provide a robust method that could be easily transferred to other development and commercial sites. The parameters that have been evaluated include preconditioning procedures, cartridge temperature, sample preparation, injection, capillary length, and applied voltage. Initially the basic preconditioning setup prior to sample injection and separation described within the Beckman IgG Heterogeneity kit was used. This included a sequential flush of 0.1 N NaOH, 0.1 N HCl, H<sub>2</sub>O, and CE-SDS buffer. The expected profile was obtained using the Bio-Rad buffer, but the resolution diminished after a few days and/or after ~30 injections, eventually resulting in only a single peak. This result looked similar to that obtained with the Beckman ProteomeLab CE-SDS buffer in Fig. 1A. In order to circumvent this reproducibility issue over time, the 0.1 N HCl flush was eliminated. This allowed the positively charged triethanolamine dextran to dynamically coat the negatively charged silanol groups on the capillary surface. This change to the method has allowed the use of a single capillary providing reproducible disulfide isomer separations for hundreds of injections over the course of 6 months.

To improve resolution, capillary cartridge temperature was examined. Overlays of the analysis at several different temperatures ranging from 15 to 50°C are shown in Fig. 2. As the temperature was decreased from 50 to 15°C, the resolution improved with the drawback of peak broadening



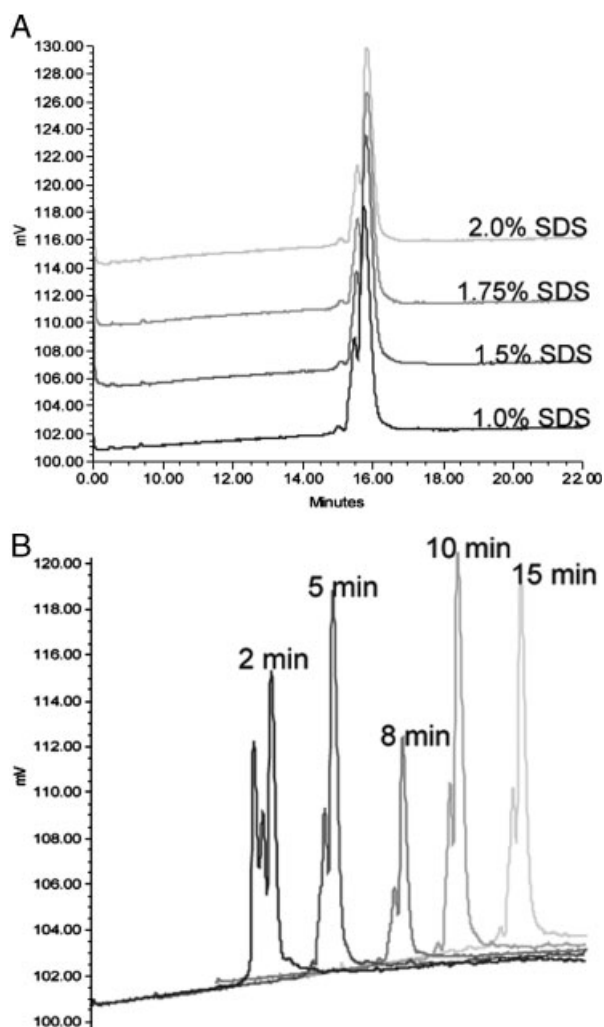
**Figure 2.** Evaluation of capillary cartridge temperature for IgG2 disulfide isomer analysis, resolution of the isomers improves by lowering the temperature.

and longer run times. As a primary goal of the optimization was to improve resolution, the 15°C cartridge temperature was selected for use in the final method.

As noted, sample buffer composition can impact the sample integrity resulting in increased fragmentation during the denaturation step at 65°C, which could potentially impact the separation of disulfide isomers [19, 23, 32, 33]. To assess if this had any impact on the profile obtained, two different sample preparation buffers were utilized. The eCAP SDS sample buffer (pH 6) and ProteomeLab SDS-MW sample buffer (pH 9) from Beckman were used in this instance. An identical separation (not shown) was obtained using both sample buffers indicating that sample buffer pH did not impact the disulfide isomer distribution or the separation. An additional concern with the multiple peaks of the disulfide isomers is that the sample has not been adequately denatured. To ensure that was not an issue, the

concentration of SDS was increased in the sample preparation. The Beckman ProteomeLab SDS-MW buffer has 1% SDS. The SDS concentration was increased by spiking the sample with 10% Bio-Rad SDS solution to have effective SDS concentrations of 1% (*i.e.* no additional SDS), 1.5, 1.75, and 2% SDS. With a denaturation time of 10 min at 65°C, all of the electropherograms were identical as shown in Fig. 3A. To also probe if the sample was completely denatured, the heating time at 65°C was also varied. As can be seen in Fig. 3B, a heating time of at least 8 min at 65°C should be used to ensure that the sample volumes of 200  $\mu$ L or less are adequately denatured. Larger sample volumes may require additional heating time to completely denature the protein.

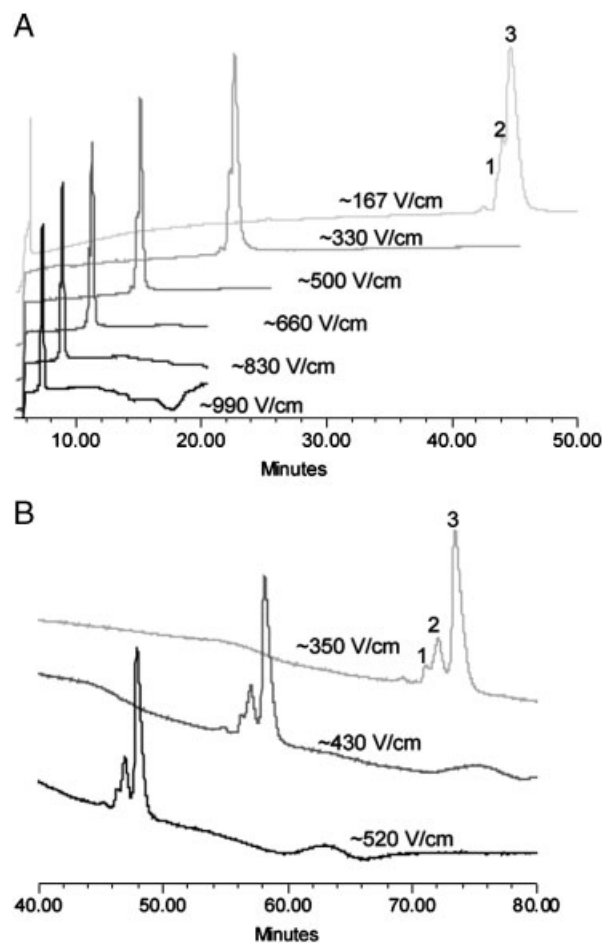
An increased electrokinetic injection time was required with the Bio-Rad CE-SDS buffer compared to similar



**Figure 3.** Evaluation of sample preparation for IgG2 disulfide isomer analysis – (A) increasing the percentage of SDS in the sample preparation step does not result in additional denaturation of the IgG2 sample. (B) Increasing the denaturation time to at least 8 min at 65°C is required to adequately denature the IgG2 for a 200  $\mu$ L sample volume.

method conditions with the Beckman ProteomeLab CE-SDS buffer. Generally speaking, a 50% increase in injection time at  $-5$  kV should be used when running the same sample with the Bio-Rad CE-SDS buffer. Alternatively, pressure assisted (hydrodynamic injection) was found to have a deleterious effect on resolution at comparable sensitivities and was not explored further.

Having optimized the separation chemistry, capillary length and electric field strength were the final parameters to be optimized. It is not surprising that resolution can be enhanced by increasing the capillary length as resolution is roughly proportional to the square root of capillary length. This was indeed the case as shown in Fig. 4A and B. By doubling the column length, it was possible to partially resolve three isomer peaks of the same IgG2 molecule. The drawbacks of moving to a longer capillary method included longer injection times required to inject a sufficient amount of sample and the requirement of application of a higher electric field during the injection for acceptable sensitivity. Electric field strength of  $\sim 500$  V/cm ( $-15$  kV applied) is recommended while utilizing the short capillary to provide a short run time with acceptable resolution when compared



**Figure 4.** Evaluation of electric field strength (A) short capillary with 30.2 cm total length, (B) long capillary with 58.2 cm total length.

with other field strengths. An electric field strength of  $\sim 430$  V/cm ( $\sim 25$  kV applied) is recommended for the long capillary separation. Although a shorter run time with almost equivalent resolution was achieved with the application of  $\sim 30$  kV (field strength =  $\sim 520$  V/cm), this was at the high end of the instrument specification and may not be desirable for a routine analytical method. An additional drawback included a sloping baseline in the area of interest making reproducible peak integration more difficult.

The final method parameters for the short capillary CGE method that allowed a quick determination of disulfide isomer profiles can be found in Table 2. The long capillary method for improved resolution can be found in Table 3. It should be noted that the methodology does not provide equivalent resolution for all antibodies ( $\sim 25$ ) that have thus far been evaluated. The methodology exhibited enhanced resolution for some antibodies on both the short and long method allowing for a partial separation of three or four peaks. There were cases however where the optimized CGE methodology was only capable of resolving two disulfide isomer components.

**Table 3.** Long capillary method (50  $\mu$ m id, 48.2 cm effective length), cartridge temperature is 15°C

Step #	Event	Value	Duration	Comment
1	Rinse – pressure	70 psi	5 min	0.1 N NaOH rinse
2	Rinse – pressure	70 psi	2 min	Water rinse
3	Rinse – pressure	70 psi	15 min	SDS gel rinse
4	Wait		2	H <sub>2</sub> O dip
5	Wait		2	H <sub>2</sub> O dip
6	Inject – voltage	$-10$ kV	20 se <sup>a)</sup>	Sample injection
7	Wait		2	H <sub>2</sub> O dip
8	Separate	$-25$ kV	90 min	separation <sup>b)</sup>

a) Sample injection time was adjusted based on initial sample concentration and subsequent salt concentration.

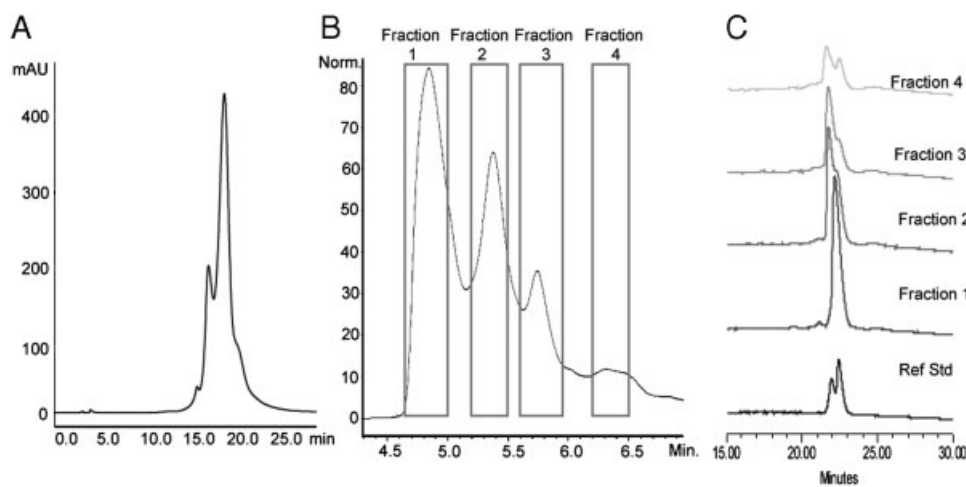
b) 20 psi pressure was applied at the capillary inlet and outlet during the separation.

### 3.2 Isomer identification

As previously mentioned, distinct disulfide-mediated isoforms have recently been identified for IgG2 MAbs [12, 16]. These isomers have alternative disulfide connectivity that is consistent with Cys residues in the hinge being linked to the C-terminal Cys of the LC and a Cys in the Fab of the HC [16]. Peak identification was confirmed *via* fraction collection using IEX or RP chromatography in conjunction with an endoproteinase Lys-C peptide mapping technique. IEX and RP chromatography were used to separate disulfide isomer fractions as shown in Fig. 5A and B. Even with optimized chromatographic conditions it has not yet been possible to get a baseline resolved separation of the disulfide isomers with chromatographic methodology. Thus, it was expected that there would be carryover in the individual fractions although the fractions should be significantly enriched for a given isomer.

RP fractions were digested using endoproteinase Lys-C in a non-reducing environment and subsequently analyzed *via* LC-MS to unambiguously assign disulfide isoforms in each fraction *via* observation of the unique peptide masses for each structure as summarized in Table 4. The first fraction collected in the RP run was characteristic of the B isomer. The second fraction collected was characteristic of the A/B isomer but also contained evidence of the B isomer (presumably due to carryover); however, the level was much reduced from fraction 1. The third fraction collected was characteristic of the A isomer with small amounts of the A/B and the B isomer also detected (seemingly due to carryover). The fourth fraction collected was characteristic of the A isomer but also had a small amount of modified B isomer present.

The RP fractions were subsequently analyzed by CGE as shown in Fig. 5C. For this particular antibody, only two peaks could be resolved by CGE. The second peak present in the reference standard CGE run can be confirmed as the B isomer based on the Lys-C digest identification of the first RP fraction as the B isomer. In this case the A and






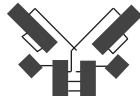


**Figure 5.** Use of CGE as an orthogonal method to analyze isomer fractions collected with by chromatography, (A) AEX profile, (B) RP profile showing the fraction cuts, (C) CGE overlay showing the reference standard and the different fractions collected by RP.

A/B isomer fractions are not pure as shown by CGE, which is also supported by the Lys-C peptide mapping technique (presumably due to carryover). The major peak in fraction 2 enriched for the A/B isomer has the same migration time as peak one in the reference standard run by CGE. The major peak present in fractions 3 and 4 that is characteristic of the A isomer has the same migration

time as peak one in the reference standard. For this antibody, peak 1 in the electropherogram is thus identified as the A/B and A isomer while peak 2 is identified as the B isomer.

**Table 4.** Peak identities *via* peptide mapping (endoproteinase Lys-C)

Isomer	Peptide identity	Schematic	Peptide MW (Da)
A	Hinge dimer		5354
	Classical interchain peptide	and 	10 096
A/B	Classical interchain peptide		10 096
	and		
	Non-classical A/B peptide(s) <sup>a)</sup>		15 451 16 064 16 677
B	Non-classical B peptide(s) <sup>b)</sup>		25 547 26 161 26 774

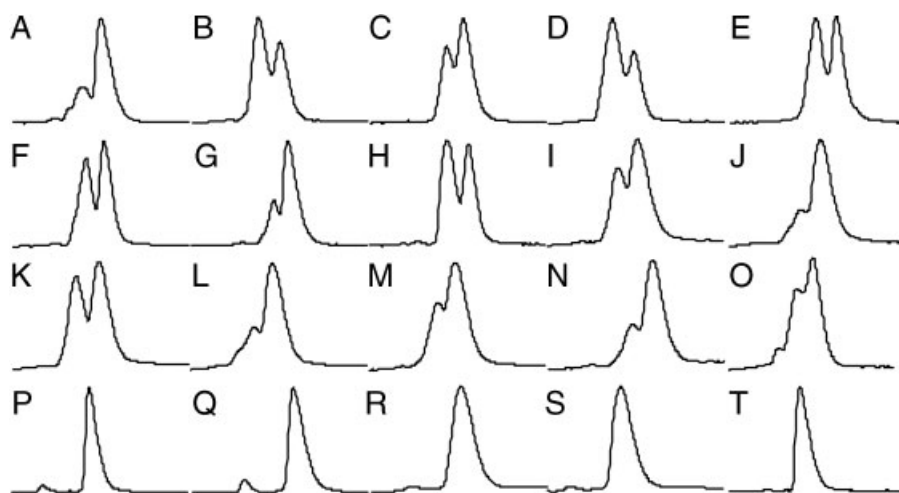
a) Multiple versions of non-classical A/B peptides are observed which arise from incomplete enzymatic digestion. Partial cleavages may contribute an additional 613 Da to one or both heavy chains of the A/B fragment.

b) Multiple versions of non-classical B peptides are observed which arise from incomplete enzymatic digestion. Partial cleavages may contribute an additional 613 Da to one or both heavy chains of the B fragment.

### 3.3 Separation mechanism

As described in previous manuscripts, the disulfide-mediated isoforms are structurally dissimilar around the hinge of the IgG2 [12, 16]. Structural differences in this case result in the generation of disulfide isomers that are more structurally compact than that of the classical A form as determined by Molecular Operating Environment modeling. The more structurally compact isomers should have a smaller apparent hydrodynamic radius based on *in silico* models, implying they should migrate faster through the gel based on solely a sieving mechanism. However, the observed movement through the gel is opposite of what is expected *via* size-based molecular sieving with the least compact isomer form (A) having the fastest migration through the gel followed by the A/B isomer and subsequently the B isomer. In addition, the resolution of CE-SDS gel is typically limited to 10% MW, thus the buffer should not have the capacity to resolve the isomers based on small differences in hydrodynamic radius alone due to insufficient sizing resolution.

The antibody is presumably coated with SDS during sample preparation procedure, which should mask structure-based non-specific interactions that could be responsible for resolving disulfide isomers by CGE. However, it was not possible to obtain an equivalent separation for all of the different antibodies analyzed by CGE under identical separation conditions. Despite having a primary sequence that is over 90% conserved, differences in the primary structure appear to be conveyed to the SDS-protein complex resulting in different disulfide isomer profiles (Fig. 6) for antibodies that appear identical under a separation based solely on hydrodynamic radius. It is evident from the figure



**Figure 6.** Evaluation of the short method for applicability as a generic isomer heterogeneity across a platform, IgG2 antibodies produced in house (A–N), IgG2 commercially available (O), IgG1 produced in house (P–S), IgG4 produced in house (T).

that all IgG2 antibodies do contain disulfide heterogeneity that is not present in IgG1 and IgG4 classes. It is also apparent that equivalent resolution was not achieved for all antibodies studied.

Both of these observations support an alternative mixed mode separation mechanism being responsible for resolving the disulfide isomers by CGE. In addition to the mechanism based on molecular sieving, the partial separation may be further mediated by ionic or adsorptive interactions of the different disulfide isomers with the CGE run buffer. Experiments will be performed in the future to obtain a better understanding of the separation mechanism to resolve disulfide isomers by CGE. Once the mechanism is completely understood, it should be possible to optimize buffer chemistries to further enhance and control the CGE separation.

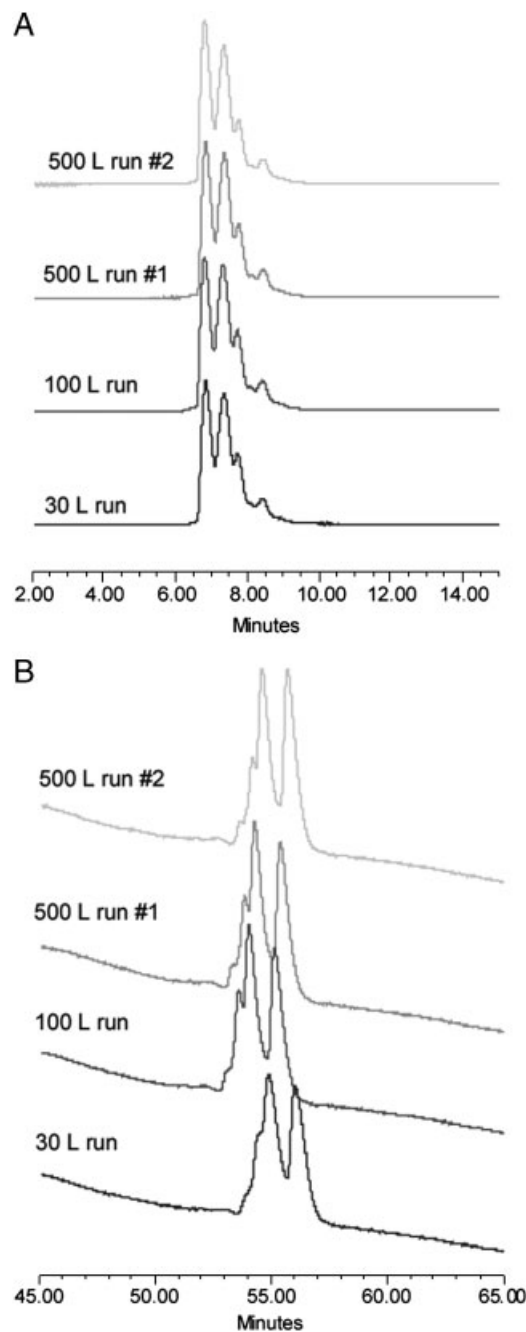
### 3.4 Applications

#### 3.4.1 Bioprocess development

Experiments are performed to try to maximize product titer while simultaneously minimizing undesired product quality attributes, including changes to the glycan profile. Another attribute that can potentially change if the process is modified is the disulfide isomer distribution. While it is not known at this time if there is any pharmacological consequence to the distribution of disulfide isomers *in vivo*, they have been shown in house to be sensitive to key process parameters including amino acid levels, types and amounts of metals, incubation time, and the processing time. Hence, monitoring disulfide isomer distribution constitutes a useful probe of process reproducibility and control. Orthogonal methods to separate the disulfide isomers are used in this case to provide information on the optimization of bioprocess conditions to ensure the manufacture of a MAB with the desired product quality attributes.

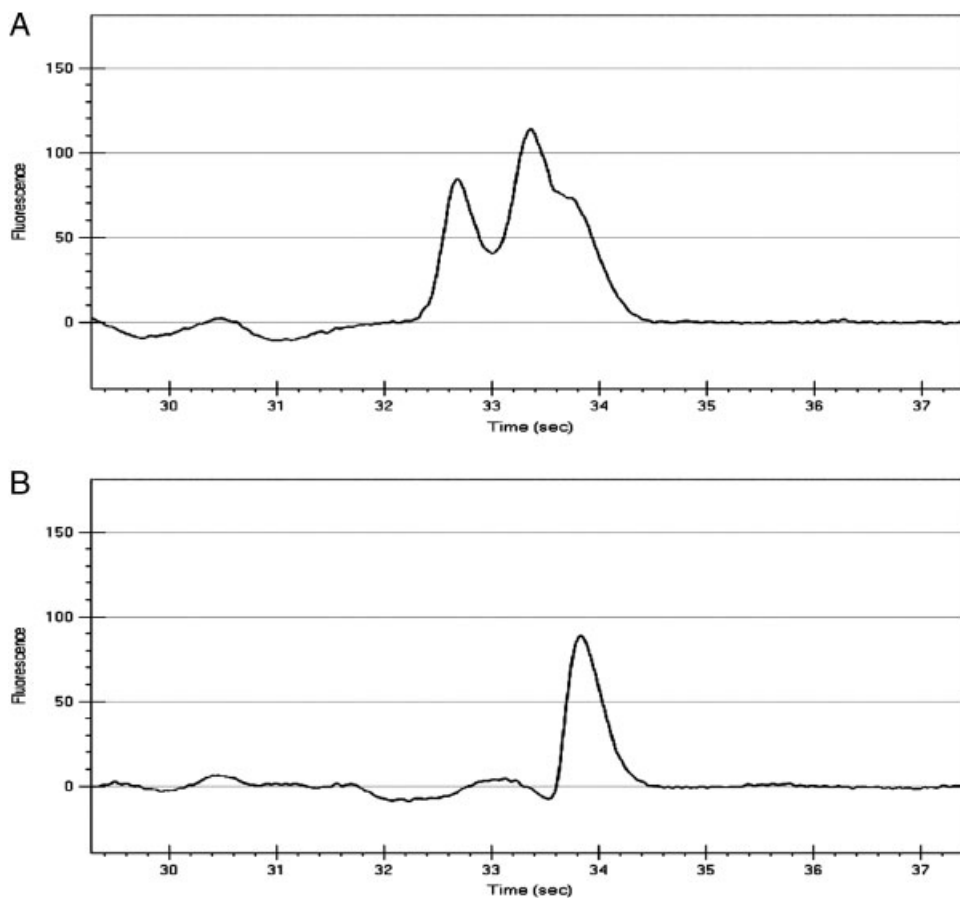
#### 3.4.2 Comparability

In order to have confidence that the process is not changing upon scale-up or across manufacturing sites, it may be necessary to utilize orthogonal methods to ensure disulfide isomer profile consistency. Figure 7 shows data from two orthogonal approaches utilized to probe disulfide isomer distribution as the process was scaled from 30 to 100 to 500 L for clinical supplies. In this case it was expected that the profiles for RP (Fig. 7A) may not necessarily be the same as those generated by CGE (Fig. 7B) due to different modes of separation. Although the isomers are not quite as well resolved with CGE (Fig. 7B), the separation for this antibody is better than previously reported for IgG2s by CGE and does provide complimentary data to that of RP chromatography. Overall, the data presented in Fig. 7 demonstrate very good reproducibility of the process from research scale at 30 L up to a clinical campaign at 500 L.



**Figure 7.** Evaluation of disulfide isomers for monitoring lot consistency at different manufacturing scales, (A) RP chromatography, (B) long capillary CGE.

Another aspect of comparability is to ensure that major product changes are not occurring over the shelf life of the antibody. Drug substance and drug product generally undergo a rigorous stability protocol to ensure that important product attributes remain at an acceptable level over time to guarantee product quality/consistency for clinical and commercial use. There is currently no reported evidence that the disulfide isomer distribution changes occur in purified and formulated



**Figure 8.** Use of microchip CGE for monitoring disulfide isomer heterogeneity for bioprocess development. (A) IgG2, (B) IgG1.

materials although the disulfide isomer distribution has been reported to change *in vivo* [14]. The separation methodology will continue to be probed in the future to ensure that other changes (chemical and physical degradation) that an antibody undergoes during accelerated conditions are not detrimental to the disulfide-mediated isomer separation.

### 3.4.3 High throughput analysis – microchip CE

A proof of concept experiment was performed to demonstrate the applicability of the analysis of disulfide-mediated isomers using high-throughput microchip CE (Caliper GXII) instrumentation. This technology has the potential to be used in conjunction with high-throughput cell culture systems for clone selection, media optimization, and bioprocess optimization that can potentially generate up to ~700 samples with greatly reduced sample volume (~500  $\mu$ L) every 2 weeks. The microchip CE technology has already been utilized for screening monoclonal antibody product quality including CE-SDS for purity and high mannose glycan analysis [34]. A new application using this technology to monitor disulfide heterogeneity during process development would be beneficial as many of the processing optimization conditions can directly impact the disulfide isomer distribution.

The microchip CE systems have been designed and optimized to give comparable performance to that of conventional CGE in a much shorter time (45 s *versus* 30 min). The buffers included in microchip reagent kits generally only give a single peak for intact antibody similar to that shown in Fig. 1A with the Beckman ProteomeLab SDS-MW sample buffer. In order to achieve a separation of disulfide isomers using microchip CE technology, the Protein Express run buffer (Caliper) was thoroughly mixed 50/50 with Bio-Rad CE-SDS buffer prior to loading the separation buffer and analyzing the samples on the GXII system. The standard Protein Express script was utilized to draw sample on to the chip, inject the sample, separate the sample, destain the sample, and detect the sample after it had traversed a 1.4 cm in length separation channel. The electropherograms generated by microchip CE are shown in Fig. 8. The separation of disulfide-mediated isomers in an IgG2 antibody is included in Fig. 8A while a separation of an IgG1 antibody devoid of disulfide isomers is shown in Fig. 8B. It is noted that the script should be modified in the future to further enhance the injection/separation as the mixed buffer matrix has reduced sample loading capacity as evidenced by lower peak area compared with the buffer included in the Protein Express kit. It is noteworthy that the disulfide isomers are separated

on a microchip channel with a separation length of only 1.4 cm. Further optimization of the assay should allow high-throughput analysis with data quality comparable to conventional CGE.

#### 4 Concluding remarks

CGE is an orthogonal analytical technique that can be used for profiling disulfide isomer distribution within the IgG2 subclasses. The CGE method provides a generic methodology that can be used across an antibody platform to study disulfide isomer distribution, which may not be the case with chromatographic methods. Optimization of the NR CGE method has allowed for an improved separation of disulfide isomers with resolution in some cases enhanced to the point where it is almost equivalent to the other orthogonal techniques. CGE can also be used as a tool to look at purity of isomer fractions obtained by orthogonal chromatographic methods prior to further use for isomer identification and potency determination.

It will continue to be important to utilize multiple orthogonal methods to study disulfide isoforms as there currently is no single method available with sufficient acuity for studying all of the chemical modifications that may exist in an antibody product. There are advantages to having multiple techniques based on different modes of selectivity depending on the matrices of the sample which may be a complex buffer or possibly even biological in nature. The orthogonal separation mechanisms of RP, IEX, and CGE provide slightly different results but have been useful for showing consistency in disulfide isomer profiles for a given antibody. These assays will allow for process optimization, lot comparability, and formulation development. It will be necessary to continue to monitor disulfide isomer distribution across the lifecycle for a given antibody.

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