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Research Article

Electrophoretic evidence for the presence of structural isoforms specific for the IgG2 isotype

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Received February 1, 2008

Revised February 20, 2008

Accepted February 21, 2008

Recombinant monoclonal antibodies of therapeutic interest were analyzed by a nonreduced CE-SDS (nrCE-SDS) method developed for the evaluation of size-based variants. We found that immunoglobulins analyzed by this technique exhibited different behavior depending on their subclasses. Under nrCE-SDS conditions, IgG1 molecules were separated in a well-resolved, single peak, whereas IgG2 molecules were consistently separated as a doublet. Investigation of these isoforms showed that they were structurally different, and that the difference was not caused by cell culture condition, glycosylation structure, or recombinant expression system. Commercially available IgG2 affinity-purified from human plasma also showed the presence of structural isoforms. The structural isoforms remained present under pH- and temperature-stressed conditions. Application of a mild cysteine/cystine redox potential converted the main peak doublet into a single peak, indicating that these isoforms were disulfide bond-related species. Bioactivity measured before and after application of a redox potential gave similar values, indicating that the structural isoforms have comparable potency. The nrCE-SDS technique described here demonstrated a unique capability to resolve IgGs, leading to the discovery of novel structural isoforms specific to the IgG2 isotype.

Keywords:

CE-SDS / Immunoglobulin IgG2 / Redox / Structural isoforms

DOI 10.1002/elps.200800083

1 Introduction

The application of recombinant mAb's (rmAb's) in human therapy has grown tremendously in recent years [1, 2]. Twenty-one mAb's had been approved by FDA and hundreds are in clinical development [3]. Almost all of the approved mAb's are of the IgG1 isotype. Vectibix (panitumumab, ABX-EGF), approved at the end of 2006 for the treatment of colon cancer [4, 5], is the first fully human IgG2 to reach the market. Interest in exploiting the unique biological properties of IgG2s, notably their lack of Fc-mediated effector functions, has significantly accelerated the clinical development of this subclass [6]. IgG1 and IgG2 are now equally developed as drug vehicles and represent the vast majority of recombinant

proteins currently investigated for potential therapeutic applications [7, 8].

Production of rmAb's from mammalian cells requires highly resolving analytical methods for the development and monitoring of the manufacturing processes as well as characterization of the final drug product [9]. Among those methods, electrophoretic techniques play an important role in the identity and purity testing of these large and complex molecules. Traditionally, SDS-PAGE has been used as the main technique for monitoring size-based separation of proteins [10], but this method is increasingly being replaced by CE-SDS. CE-SDS offers a rapid, sensitive, and quantitative method for the analysis of biomolecules [11, 12]. Other numerous advantages of this technique, including automation, flexibility of detection systems, and reproducibility, make it well suited for the analysis of proteins [13–20]. As a result, the use of CE-SDS has become prevalent in the biopharmaceutical industry as a characterization technique in

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Abbreviations: CHO, Chinese hamster ovary; EGFR, EGF receptor; nrCE-SDS, non-reduced CE-SDS; rmAb, recombinant mAb

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analytical laboratories as well as a purity assay in QC environments [21–27].

We describe in this paper the development and application of a nonreduced CE-SDS (nrCE-SDS) size-based separation method for the analysis of mAb's. nrCE-SDS measures protein hydrodynamic size under denatured and nonreduced conditions, therefore giving information on the backbone structure of IgG molecules. The results we report here demonstrate that IgGs of different isotypes behave differently in this technique. The data point toward the presence of novel variable arrangements of disulfide bonds in IgG2s. Prior to this work, the application of CE-SDS to the study of disulfide related structural features of antibodies has not been reported. The nrCE-SDS method described here achieves unique resolution providing clear evidence for the presence of structural isoforms specific to the IgG2 subclass.

2 Materials and methods

2.1 CE-SDS sample preparation

Samples were prepared by combining 150 µg of mAb (Amgen, Seattle, WA), 3 µL of internal standard (IS) (BioRad Laboratories, Hercules, CA), and either 10 µL of iodoacetamide (IAM, Sigma–Aldrich, St. Louis, MO) for nrCE-SDS or 10 µL β-mercaptoethanol (β-ME, Sigma–Aldrich) for reduced CE-SDS. SDS sample buffer (BioRad Laboratories) was then added to bring the total volume to 150 µL. The mixture was vortexed, centrifuged briefly, and heated using a water bath at 75°C for 10 min. The solution was then cooled to room temperature, centrifuged briefly to bring the liquid down to the bottom of the tube and vortexed again to mix the solution. The blank sample was prepared the same way except for the replacement of the antibody sample by the same amount of water or respective buffer.

2.2 CE-SDS method

The analyses were performed on an HP^{3D} CE system (Agilent Technologies, Palo Alto, CA) equipped with a DAD. Separations were performed using a bare fused-silica capillary with a total length of 33.0 cm, effective length of 24.5 cm to the detector, with an id of 50 µm, and od of 375 µm (Agilent Technologies). Antibody separation was monitored at a wavelength of 220 nm. The capillary was preconditioned with 0.1 N NaOH at 6 bar for 2 min followed by 0.1 N HCl at 6 bar for 1.5 min. The capillary was then filled with BioRad CE-SDS run buffer (BioRad Laboratories) at 6 bar for 3 min. Samples were injected at –10 kV for 20 s and separated at –15 kV for 30 min.

2.3 CE-SDS data analysis

The nrCE-SDS electropherogram of IgG1 and IgG2 is shown in Fig. 1B. The corrected peak area is defined as [28]:

$$\text{Corrected peak area} = \frac{L_d \times \text{Peak Area}}{t}$$

where L_d is the effective length from capillary inlet to the detector and t is the migration time.

The structural isoform ratio for an IgG2 was calculated by dividing the corrected peak area of the isoform of interest (isoform 1) by the corrected peak area of the total main peaks (isoforms 1 and 2). For example:

$$\% \text{ Isoform 1} = \frac{\text{Corrected Area(Isoform1)}}{\text{Corrected Area(Total Main Peaks)}} \times 100\%$$

2.4 Redox (cysteine/cystine) treatment

Antibody samples at 3 mg/mL were treated with 6 mM cysteine (Sigma–Aldrich) and 0.6 mM cystine (Sigma–Aldrich) in 0.2 M Tris-HCl (Sigma–Aldrich) at pH 8.6 for 96 h at 2–8°C. Samples were then dialyzed into water, respective formulation buffer and prepared as described above for CE-SDS analysis.

2.5 Mass analysis

Samples were introduced into the mass spectrometer by isocratic separation on a polyhydroxyethylaspartamide column flowing at 0.1 mL/min in 0.1% v/v formic acid [29]. Samples eluted as a single peak separated from salt and buffer components. A solution of 1% v/v formic acid in ACN was mixed into the column eluate at 25 µL/min using a syringe pump and tee. The column eluate was directed into an ESI-TOF mass spectrometer (Agilent Technologies) with divert valve settings selected to direct salt and buffer components to waste. The instrument was tuned and calibrated from 50 to 4000 m/z using Agilent supplied tuning and calibration solutions. Optimized source and ion transmission system conditions were empirically determined and included a capillary voltage of 5 kV, nitrogen gas rate of 9.0 L/min, and a fragmentor voltage of 415 V.

2.6 ELISA binding assay

Soluble EGF receptor (EGFr) (Amgen) was added to 96-well plates. The plates were washed with 1 × PBS buffer containing 0.05% Tween 20 v/v and 200 µL of 5 g/L BSA was added. Serial dilutions of antibody reference standard (Amgen) test sample and positive control (different lot from the reference standard, Amgen) were added to the plate. The plates were incubated at room temperature, then washed with 1 × PBS buffer containing 0.05% Tween 20 v/v to remove unbound antibody. Diluted mouse anti-human IgG2 (Southern Bio-

technology, Birmingham, AL) conjugated to horseradish peroxidase (HRP) was added to the plates followed by incubation at room temperature. The plates were washed and a substrate solution, *e.g.*, Enhanced K-Blue (Neogen, Lansing, MI) was added to detect bound antibody. Color development was stopped by addition of 2 M sulfuric acid. Absorbance at 450 nm was measured and the response curves of the reference standard, samples, and positive control were analyzed using a data analysis SOFTmax Pro program (Molecular Devices, Union City, CA) software.

2.7 Cell-based bioassay

Antibody binds to EGFr on the cell surface, inhibiting EGF binding to the EGFr. Such cells were incubated in microtiter plates with a fixed amount of EGF and a varying amount (three-fold differ in concentration) of antibody. The cells were lysed and soluble EGFr was captured on a second microtiter ELISA plate coated with an anti-EGFr antibody (Amgen). Phosphorylated EGFr was detected using an anti-phosphotyrosine PY20 antibody (Zymed Laboratories, South San Francisco, CA) conjugated to HRP, followed by the addition of a chromogenic substrate, *e.g.*, TMB Single Solution Peroxidase Substrate (Zymed Laboratories). Color development was measured at 450 nm using an absorbance plate reader (Molecular Devices). Potency relative to a reference standard (Amgen) was determined by parallel line analysis.

3 Results and discussion

We evaluated the hydrodynamic size of IgG1 and IgG2 antibodies by nrCE-SDS. Typical nrCE-SDS electropherograms of IgG1 and IgG2 molecules are shown in Figs. 1A and B, respectively. nrCE-SDS detected a doublet for IgG2 molecules, whereas a single peak was obtained for IgG1 molecules. As nrCE-SDS separates biomolecules under denaturing and nonreduced conditions, we hypothesized that the IgG2 population, in contrast to IgG1, is composed of molecular species with different covalent structures. We denote these forms, visualized in two peaks, structural isoforms 1 and 2 (Fig. 1B).

In order to understand the nature of the structural isoforms, a series of experiments was performed as detailed below. During development of the nrCE-SDS method for mAb, a progressive increase in incubation temperature with SDS sample buffer was evaluated to determine the optimal conditions for complete denaturation. In the case of IgG2, increase in denaturation temperature resulted in a clear separation of two main species (Fig. 2). At 50°C for 10 min, two populations were observed, indicating the presence of heterogeneous molecular components with incomplete denaturation. A temperature of at least 65°C in the presence of SDS was required to generate fully denaturated IgG2. At such temperature, the fully denaturated molecules still separated as a doublet. The result showed that isoform 1 denatured more readily than isoform 2, and that the isoforms were not an artifact of incomplete denaturation.

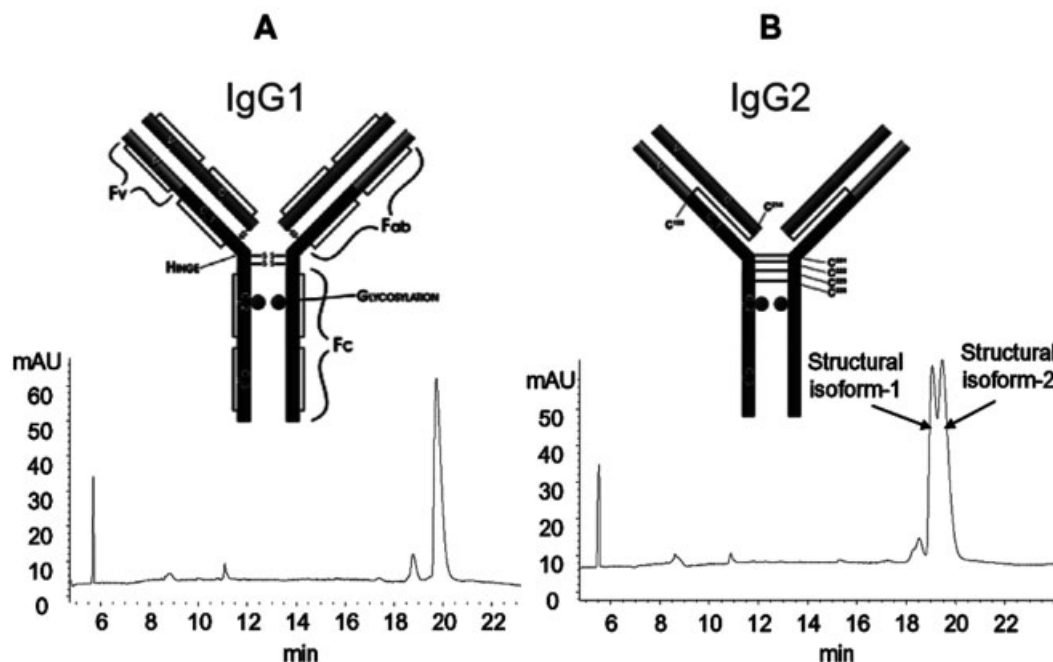


Figure 1. Typical nrCE-SDS electropherogram of IgG1 (A) and IgG2 (B) molecules. IgG1 migrated in a single peak whereas IgG2 was resolved as a doublet.

Separation of the main peak as a doublet was not observed for IgG1. All IgG1 tested were resolved in a single, homogeneous peak by nrCE-SDS, whereas IgG2 exhibited a specific heterogeneity resulting in the separation into two main species (Fig. 3). Commercial IgG2 purified from human plasma of myeloma patients (Sigma–Aldrich) also displayed the doublet profile observed for recombinant IgG2 molecules (Fig. 4). From these results we conclude that the presence of different structural isoforms is an intrinsic property of the IgG2 subclass.

All the peaks observed in the electropherogram were correlated to their relative migration on SDS-PAGE (Fig. 5B). The resolution achieved by the non-reducing SDS-PAGE technique was not sufficient to separate the isoforms in the main band. The minor fragments, characterized by Western blot and N-terminal sequencing, were identified as free light (L) and heavy (H) chains in various combinations of L, HL, HH, and HHL. Some of these free fragments could be present in the cell culture medium and co-purified through protein A affinity purification. In addition, if assay conditions were not controlled properly, free fragments artifacts could be generated due to reduction of disulfide linkages by free thiol groups present at low level in the molecule [30, 31]. Alkylation of the potential free cysteines, with iodoacetamide (IAM) or N-ethylmaleimide (NEM), prior to exposure to SDS and heat, significantly reduced these side reactions [25, 27, 32].

The structural isoforms were shown to be very stable under various stress conditions. Exposure at 90°C for up to 30 min did not influence the doublet separation (Fig. 6A). Incubation at pH 4 for 2 months at 37°C led to partial degradation of the antibody and generated different fragments and chemical modifications (data not shown), but did not affect the resolution of the main peak doublet (Fig. 6B).

Another important aspect of the chemical structure of antibodies that can influence their behavior in CE-SDS

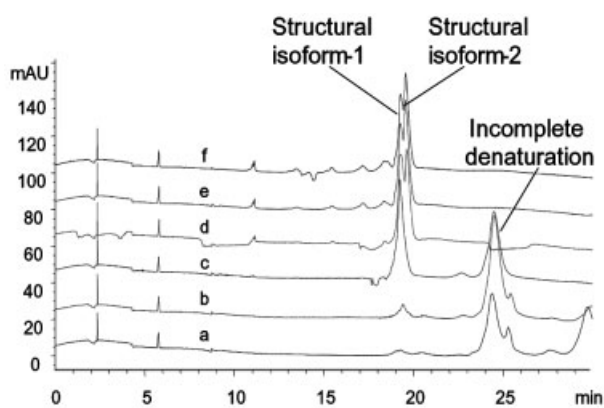


Figure 2. Influence of denaturing temperature. Increasing the temperature resulted in the complete denaturation of two molecular species. Isoform-1 denatured more readily in SDS sample buffer as temperature increased. The heating temperature studied were room temperature (a), 37°C (b), 50°C (c), 65°C (d), 85°C (e), and 95°C (f) for 10 min.

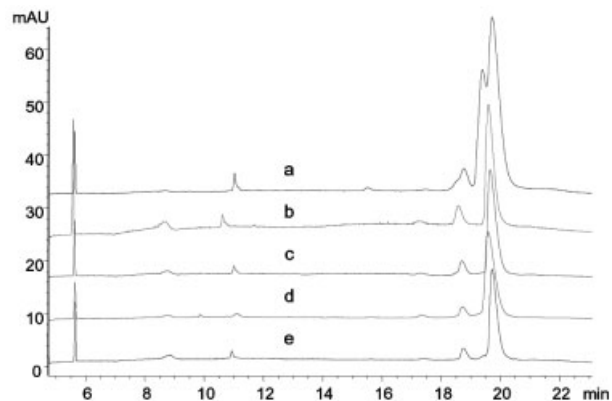


Figure 3. Comparison of IgG2 with several commercial IgG1 mAb's. The structural isoforms are IgG2 subclass specific. The nrCE-SDS profile of IgG2 from Amgen (a) showed the structural isoform doublet, whereas all IgG1s tested did not (Rituxan IgG1 (b), Synagis IgG1 (c), Herceptin IgG1 (d), and Remicade IgG1 (e)).

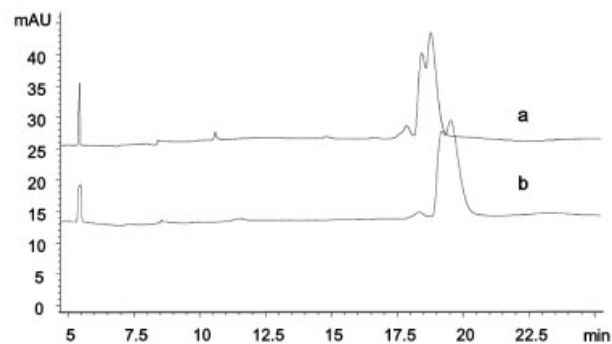


Figure 4. Both recombinant IgG2 (a) and purified from human plasma (b) exhibited structural isoforms.

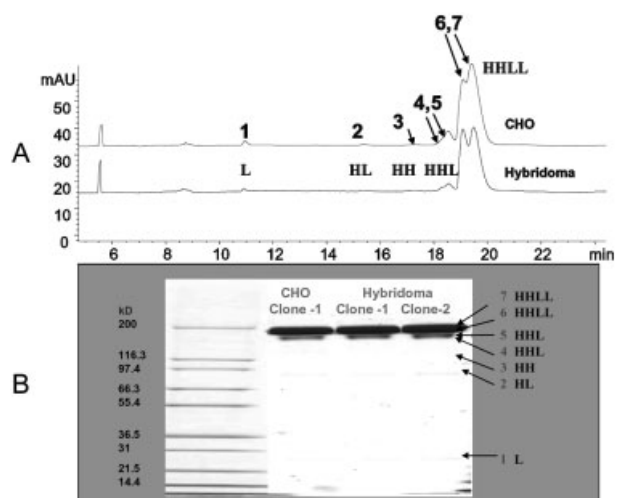


Figure 5. nrCE-SDS profile (A) and nrSDS-PAGE (B) of IgG2s. Subfragments resulting from different arrangements of light (L) and heavy (H) chains were visible in the two techniques, but the isoform doublet was not visualized by SDS-PAGE.

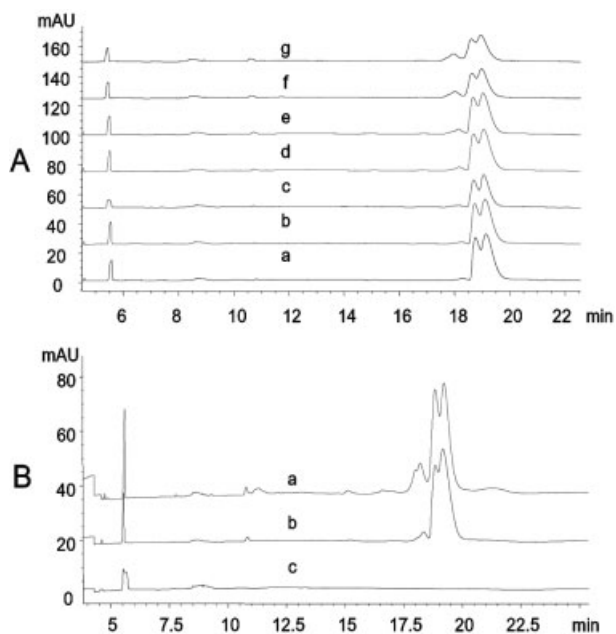


Figure 6. The structural isoforms were stable under stressed conditions: (A) Temperature stressed at 90°C for 1 min (a), 2 min (b), 3 min (c), 5 min (d), 10 min (e), 20 min (f), and 30 min (g); (B) pH 4 and 37°C stressed for 2 months (a), unstressed control (b), blank (c).

separation is the fact that they are glycoproteins. IgGs have a consensus *N*-linked site located on Asn-297 of the CH2 domain of heavy chains [33]. Although other glycosylation sites have been identified on IgGs (such as an *O*-linked site on light chain [34] or an additional *N*-linked site on the variable domain of the heavy chain [35]) most therapeutic mAb's are singly modified on the consensus site at Asn-297. Chinese hamster ovary (CHO) cells glycosylate appropriately human IgGs [36] and in both cases, the major glycoforms are biantennary structures with a distribution of 0, 1, or 2 terminal galactose residues (G0F, G1F, and G2F glycoforms). HPLC and CE analyses of purified glycan structures isolated from CHO-expressed antibodies show that both IgG1 and IgG2 have similar *N*-linked glycan structures. CHO cells glycosylate recombinant antibodies almost to completion. Among the various IgG1s and IgG2s we have examined, an average of only 1% of the molecules were found non-occupied. This minor component is well separated from the major glycosylated molecules by nrCE-SDS, and is identified as peaks 4 and 5 (also comigrating with HHL fragment) in nrCE-SDS electropherograms (Fig. 5A). To assess whether the structural isoforms were related to the glycosylation state of the molecule, the *N*-linked glycan structures present on the IgG2 mAb were removed by enzymatic treatment using PNGase F, and the resulting de-glycosylated molecule was separated by nrCE-SDS (Fig. 7). The result showed that when the *N*-linked glycan structures were removed, the doublet remained, indicating that the structural difference between these isoforms was not glycosylation-dependent.

We then investigated the covalent structure involved in IgG dimerization. IgGs are present in solution as a complex (HC-LC)₂ of approximately 150 kDa. The intrachain disulfide structures of the constant C_{H1}, C_{H2}, C_{H3}, C_L and variable domains, V_H and V_L, are essentially the same between the IgG1 and IgG2 subtypes. A notable difference between these subtypes resides in the number of interchain disulfides, four for IgG1 and six for IgG2, involved in dimerization. Based on the published literature, the covalent structures of IgG1 and IgG2 antibodies are represented schematically in Fig. 1. The antibodies were reduced by treatment with β-mercaptoethanol (β-ME) and the resulting light chain and heavy chain were separated by rCE-SDS (Fig. 8). This result showed that reducing conditions eliminated the structural isoforms, demonstrating that the structural isoforms are disulfide bond related molecular species that are dependent upon the presence of the complete covalent structure of the form (HC-LC)₂.

The impact of the cell type and culture media were evaluated. IgG2s expressed in hybridoma or CHO cell lines were analyzed by CE-SDS (Fig. 5A). The characteristic doublet was visible in the two expression systems, a result showing that the structural isoforms were not related to the cell type used for antibody production. The effect of cell culture on structural isoforms was studied using a variety of media conditions. PFCHO media (Sigma–Aldrich) with animal-derived peptone (PP3) and plant derived peptone (HyPep) in the presence or absence of amino acid (AA) showed similar CE-SDS profile (Fig. 9). The results demonstrated that the structural isoforms were not dependent on the cell culture process.

Additionally, we investigated the influence of disulfide structure on the ratio of the structural isoforms. Application of a redox potential modified the structural isoforms, resulting in a significant decrease of structural isoform 1 (39 to 11%) concomitant with an increase of structural isoform 2 (61 to 89%) (Fig. 10). This result suggests that the structural isoforms are based on disulfide bond arrangements.

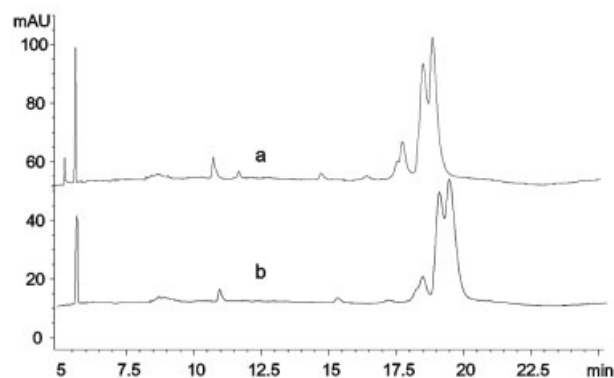


Figure 7. Glycosylation of IgG2 molecules does not affect the presence of the two structural isoforms. (a) PNGaseF treated; (b) reference.

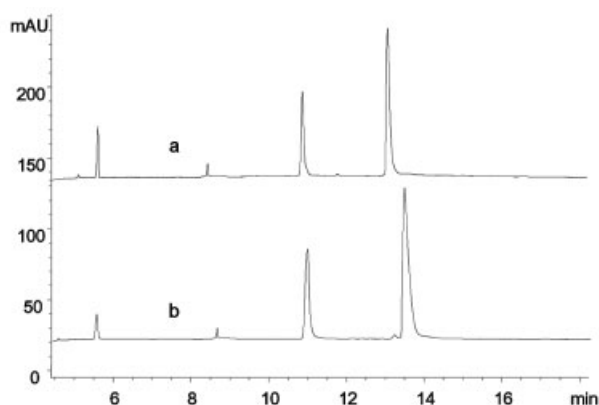


Figure 8. Comparison of nrCE-SDS profiles (a) deglycosylated and (b) glycosylated. Reduction into heavy and light chain components using β -ME abolished the structural isoforms. Deglycosylation did not change the profile.

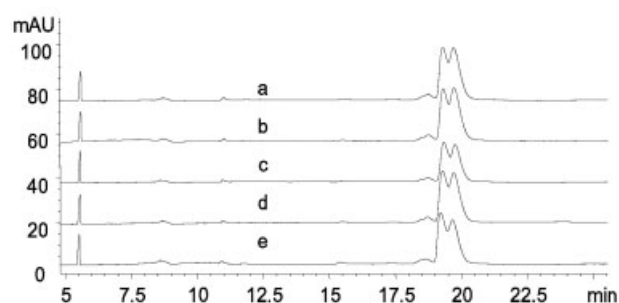


Figure 9. The recombinant IgG2s prepared using different fermentation media conditions were all resolved as a main peak doublet: CHO medium (PFCHO) with plant-derived peptone (HyPep) (a), PFCHO with animal-derived peptone (PP3) and amino acid (AA) fed at day 0 (b), PFCHO with HyPep using different fermentation conditions (c), PFCHO with PP3 and AA fed using a different schedule (d), and PFCHO with PP3 (e).

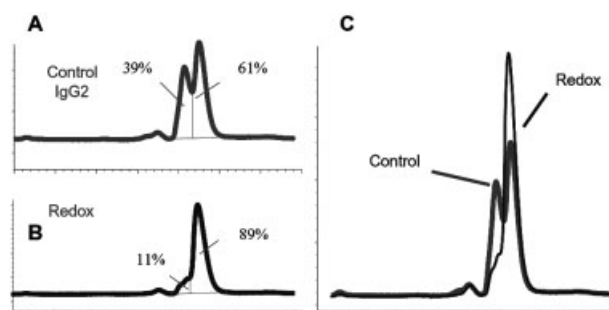


Figure 10. The application of a redox potential converted isoform-1 to isoform-2. nrCE-SDS of the starting material (A), after Redox exposure (B), and an overlay of the e-grams (C).

Mass analysis of the intact IgG2 molecule before and after redox treatment showed comparable molecular mass (Table 1), indicating that separation as a doublet does not result from differences in primary sequences or from the presence of adducts. These data point to intrinsic structural

difference within the IgG2 molecules. Deconvolution of the mass data resulted in the clear visualization of four glycoforms interpreted as the antibody modified by *N*-linked bi-antennary structures capped by a variable number of terminal galactose residues. These CHO-expressed glycoforms carry well-characterized *N*-linked glycan structures also present on natural IgGs isolated from human serum [36]. As shown in Table 1, the experimental masses (147 kDa) corroborate the expected sequence from translation of the DNA sequence and confirm the presence of structures of the form (HC-LC)₂ in solution. Based on the mass accuracy, no evidence of adducts was found.

Finally, the relative potency of the isoforms was explored. The original material with a 39–61% isoform 1/isoform 2 ratio was compared to the material exposed to redox conditions using ELISA binding and cell-based bioassays. The potency of these materials was comparable (Table 2), indicating that the distribution of structural isoforms has no influence on the ability of the antibody to recognize its antigen.

4 Concluding remarks

We report that the separation of antibodies by CE results in a clear distinction between IgG subclasses 1 and 2. Development of an nrCE-SDS method allowed us to provide evidence for the presence of novel structural isoforms specific to IgG2s. Analysis by CE of the structural isoforms under a variety of conditions shows that the presence of isoforms is independent of expression system, clone, fermentation media, and glycosylation pattern. Observation of the structural isoforms in human IgG2 purified from the serum of myeloma patients suggests that their presence is a naturally occurring phenomenon. The molecular mass and potency of the structural isoforms are comparable. Under mild redox conditions, structural isoform-1 is converted to structural isoform-2. Combined, these results suggest that the isoforms detected by nrCE-SDS differ in their disulfide bond arrangement. Understanding the complex nature of these structural isoforms has required further investigations, in which we will report in the near future (Martinez *et al.*, Wypych *et al.*, Dillon *et al.*, in preparation). The nrCE-SDS method has proved to be a powerful tool for protein analysis that opens intriguing questions on structural features of IgGs.

The authors would like to thank Alison Wallace, Bob Bailey, Ann Potter, Ralph Klinke, Michael Mulkerrin, and Dean Pettit for helpful discussions. Special thanks to Sihong Deng and Lisa Taylor for providing some of the samples used in this study, to Lowell Brady and Sean Macneil for their help with whole mass analysis, and to Michael Mulkerrin for the potency analysis.

The authors have declared no conflict of interest.

Table 1. Intact IgG2 mass analysis before and after Redox treatment

Sample	Expected mass (Da)	Experimental mass (Da)	Species	Delta (ppm)
A. Reference material	146 924.8	146 931.3	G0F/G0F	44.2
	147 086.9	147 093.6	G1F/G0F	45.6
	147 249.0	147 256.6	G1F/G1F	51.6
	147 411.2	147 415.1	G1F/G2F	26.5
B. After Redox treatment	146 924.8	146 935.0	G0F/G0F	69.4
	147 086.9	147 095.8	G1F/G0F	60.5
	147 249.0	147 258.5	G1F/G1F	64.5
	147 411.2	147 417.7	G1F/G2F	44.1

Table 2. Potency before and after Redox treatment

Samples	ELISA-binding assay % potency	Cell-based bioassay % potency
Before redox	103	89
After redox	112	93

5 References

- [1] Carter, P., *Nat. Rev. Immunol.* 2006, 6, 343–357.
- [2] Adams, G. P., Weiner, L. M., *Nat. Biotechnol.* 2005, 23, 1147–1157.
- [3] Hagemeyer, C. E., Schwarz, M., Peter, K., *Seminars in Thrombosis and Hemostasis. Platelets in Inflammation and Atherothrombosis* 2007, 33, 185–195.
- [4] Yang, X. D., Jia, X. C., Corvalan, J. R. F., Wang, P., Davis, C. G., *Crit. Rev. Oncol. Hematol.* 2001, 38, 17–23.
- [5] Easley, C., Kirkpatrick, P., *Nat. Rev. Drug Discov.* 2006, 5, 987–988.
- [6] Penichet, M. L., Morrison, S. L., *Drug Dev. Res.* 2004, 61, 121–136.
- [7] Tabrizi, M. A., Tseng, C. M. L., Roskos, L. K., *Drug Discov. Today* 2006, 11, 81–88.
- [8] Reichert, J. M., Valge-Archer, V., *Nat. Rev.* 2007, 6, 349–356.
- [9] Flatman, S., Alam, I., Gerard, J., Mussa, N., *J. Chromatogr. B* 2007, 848, 79–87.
- [10] Deyl, Z., *Electrophoresis: A Survey of Techniques and Applications. Part A Techniques*, Elsevier, Amsterdam 1979.
- [11] Altria, K. D., *J. Chromatogr. A* 1999, 856, 443–463.
- [12] Quigley, W. W. C., Dovichi, N. J., *Anal. Chem.* 2004, 76, 4645–4658.
- [13] Hunt, G., Moorhouse, K. G., Chen, A. B., *J. Chromatogr. A* 1996, 744, 295–301.
- [14] Guzman, N. A., Park, S. S., Schaufelberger, D., Hernández, L. *et al.*, *J. Chromatogr. B* 1997, 697, 37–66.
- [15] Ma, S., Nashabeh, W., *Chromatogr. Suppl.* 2001, 53, S-75–S-89.
- [16] Patrick, J. S., Lagu, A. L., *Electrophoresis* 2001, 22, 4179–4196.
- [17] Hutterer, K., Dolnick, V., *Electrophoresis* 2003, 24, 3998–4012.
- [18] Wehr, T., *LCGC* 2005, 23, 676–681.
- [19] Han, M., Guo, A., Jochheim, C., Zhang, Y. *et al.*, *Chromatographia* 2007, 66, 969–976.
- [20] Guo, A., Camblin, G., Han, M., Meert, C., Park, S., in: Ahuja, S., Jimidar, M. I. (Eds.), *Capillary Electrophoresis Methods for Pharmaceutical Analysis, Vol. 9*, Elsevier, Amsterdam 2008, Chapter 14, 357–399.
- [21] Moorhouse, K. G., Eusebio, C. A., Hunt, G., Chen, A. B., *J. Chromatogr. A* 1995, 717, 61–69.
- [22] Bowen, S. H., Schenerman, M. A., *BioPharm* 1998, 11 42–50.
- [23] Hunt, G., Nashabeh, W., *Anal. Chem.* 1999, 71, 2390–2397.
- [24] Schenerman, M. A., Bowen, S. H., *Chromatogr. Suppl.* 2001, 53, S-66–S-74.
- [25] Salas-Solano, O., Tomlinson, B., Du, S., Parker, M. *et al.*, *Anal. Chem.* 2006, 78, 6583–6594.
- [26] Han, M., Phan, D., Nightlinger, N., Taylor, L. *et al.*, *Chromatographia* 2006, 64, 335–342.
- [27] Michels, D. A., Brady, L. J., Guo, A., Bolland, A., *Anal. Chem.* 2007, 79, 5963–5971.
- [28] *Beckman PA 800 Operation Manual*, Beckman-Coulter Inc, Fullerton, CA 2003.
- [29] Brady, L. J., Valliere-Douglass, J., Martinez, T., Bolland, A., *J. Am. Soc. Mass. Spectrom.* 2008, 19, 502–509.
- [30] Gray, W., *Protein Sci.* 1993, 2, 1732–1748.
- [31] Li, L., Sun, M., Gao, Q., Sudhir, P., *Mol. Immunol.* 1996, 33, 593–600.
- [32] Gilbert, H. F., *Methods Enzymol.* 1995, 251, 8–28.
- [33] Edelman, Cunningham, B. A., Gall, W. E., Gottlieb, P. D. *et al.*, *Proc. Natl. Acad. Sci USA* 1969, 63, 78–85.
- [34] Martinez, T., Pace, D., Brady, L., Gerhart, M., Bolland, A., *J. Chromatogr. A* 2007, 1156, 183–187.
- [35] Leibiger, H., Wustner, D., Stigler, R. D., Marx, U., *Biochem. J.* 1999, 338, 529–538.
- [36] Jefferis, R., *Biotechnol. Prog.* 2005, 21, 11–16.