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Optimization of microchip-based electrophoresis for monoclonal antibody product quality analysis revealed needs for extra surfactants during denaturation

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ABSTRACT

Microchip-based electrophoresis has gained increasing popularity in biopharmaceutical development and testing laboratories because of its automation and rapid analysis capabilities. One application of microchip-based electrophoresis is the assessment of size-based variants for product purity analysis. However, monoclonal antibodies analyzed by this technique sometimes exhibited different electrophoretic behaviors. In this study, when three IgG1 and five IgG4 were analyzed using microchip-based electrophoresis under reducing conditions, one of the IgG1s, denoted as mAb1, exhibited an atypical profile attributed to its specific heterogeneity resulting in separation of its heavy chain into two main species. During investigation of the atypical profile, several parameters that were critical to optimal resolution were evaluated, and the data pointed toward incomplete denaturation of mAb1 due to lack of sufficient surfactant in the vendor provided sample buffer (0.7% surfactant). Denaturation studies demonstrated that, although typical antibody profiles could be achieved at 0.7% surfactant for most antibodies analyzed, five out of eight antibodies were not fully denatured until the surfactant concentration reached 0.9% or higher, and mAb1 required a surfactant concentration of 1.3% for complete denaturation. Molecular modeling analysis revealed features in surface charge, hydrophobicity, and structure from mAb1 that led to its unique surfactant concentration-dependent electrophoretic behaviors observed. The optimized method was further evaluated for specificity, linearity, precision, and limit of quantitation for mAb1, and compared with that of conventional CE-SDS.

1. Introduction

In the past decades, decade the number of monoclonal antibodies (mAbs) and fusion proteins produced from genetically modified bacterial or mammalian cell culture systems continues to grow. They are the major therapeutic protein candidates in various development phases [1,2]. Expressed protein products are inherently heterogeneous primarily due to enzymatic or non-enzymatic reactions (e.g., deamidation, oxidation, aggregation, N-terminal glutamine cyclization, proteolytic cleavage, glycosylation, sialylation, C-terminal lysine processing, and disulfide bond scrambling) that occur in the cell culture following secretion, as well as during purification and storage [3,4]. Therefore, analytical techniques that can adequately detect heterogeneity present in proteins are required for successful commercialization of biotherapeutic molecules [4].

Due to the inherent heterogeneity of therapeutic proteins, it is often necessary to utilize multiple analytical tools to gain thorough understanding of the complex product. Among these analytical methods, electrophoretic techniques such as SDS-PAGE play an important role for monitoring size-based separation of proteins. However, several disadvantages of SDS-PAGE, including the use of toxic reagents, high mobility variability and staining variability, limited quantitaion abilities, and its labor-intensive nature, have led to its replacement by CE-SDS and microchip-based electrophoresis. Similar to SDS-PAGE, samples for CE-SDS

Abbreviations: BME, 2-mercaptoethanol; CDR, complementary determining regions; CMC, critical micelle concentration; HC, heavy chain; IAM, iodoacetamide; LC, light chain; mAb, monoclonal antibody; NGHC, non-glycosylated heavy chain; PNGase F, peptide *N*-glycosidase F; TCEP, tris 2-carboxyethyl phosphine HCl.

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and microchip-based electrophoresis are prepared via denaturation in the presence of an anionic surfactant (e.g., SDS and LDS), imparting a uniform negative charge on the protein. Due to its advantage over SDS-PAGE on automation, quantitation abilities, and online detection, CE-SDS is prevalent in the biopharmaceutical industry for release testing, stability studies, and characterization of drug product. However, CE-SDS methods often require approximately 50 min per analysis. When many samples need to be processed routinely, analysis by CE-SDS becomes impractical and inefficient. This leads to demand in faster electrophoretic separation techniques such as microchip-based electrophoresis [5–7]. In a microchip-based electrophoresis, samples are analyzed in a micro-sized capillary on a chip, with complete data collection occurring in approximately 45 s per analysis. Denatured proteins are introduced onto the chip directly from a microtiter plate through a sipper by applying vacuum. Sipped protein forms a complex with SDS in the sieving matrix and binds to the fluorescent dye non-covalently within the chip's capillaries. The fluorescent dye molecules yield substantial emission signal only when bound to SDS micelles. The background emission is quenched by the use of interconnected micro-channels containing sieving matrix that join the separation channel at a point prior to detection, diluting the level of SDS to below its critical micelle concentration (CMC). The SDS micelles dissociate as the concentration drops to below CMC releasing free dye molecules that are no longer fluorescent, while the dye-bound protein-SDS complexes remain intact, generating fluorescence signal [6].

The antibodies that have been marketed are mainly of the IgG class, which can be categorized into four subclasses including IgG1, IgG2, IgG3, and IgG4 [8,9]. Each subclass differs in their primary structure and connectivity in the inter-chain disulfide bonds: 4 for IgG1 and IgG4, 6 for IgG2, and 13 for IgG3 [9]. It has been reported previously that IgGs of different subclasses behave differently in capillary electrophoresis, and equivalent resolution is not always achieved. For instance, when IgG1, IgG2, and IgG4 antibodies were evaluated by capillary electrophoresis under identical separation conditions, IgG1 and IgG4 yielded peaks of typical mAbs, whereas a doublet was observed in IgG2 subclasses [10,11]. The structural isoforms observed in IgG2 population were determined to be disulfide bond-related molecular species that are dependent upon the presence of the complete covalent structure of the form (HCLC)2 [10,11].

In this study, when the hydrodynamic size of three IgG1 and five IgG4 monoclonal antibodies were evaluated using microchip-based electrophoresis under reducing conditions, one of the IgG1s, denoted as mAb1, exhibited an atypical profile attributed to its specific heterogeneity resulting in the separation of its heavy chain (HC) into two main species. To optimize the method conditions and gain comprehensive understanding of the underlying mechanisms of the different electrophoretic behaviors observed, we (i) evaluated parameters that were critical to optimal resolution for microchip-based electrophoresis; (ii) performed denaturation studies to understand the surfactant concentration-dependent denaturation profiles for each mAb; (iii) performed molecular modeling analysis to reveal features in surface charge, hydrophobicity, and structure contributed to unique electrophoretic behavior observed; (iv) qualified the optimized method on specificity, linearity, precision, and limit of quantitation (LOQ) for mAb1; and (iv) compared the data from microchip-based electrophoresis with that of a conventional CE-SDS method. This study reports, for what we believe the first time, the need for extra surfactant in vendor provided sample buffer for analysis of monoclonal antibodies. The study also sheds insights on the underlying mechanisms for the different electrophoretic profiles observed by microchip-based electrophoresis.

2. Materials and methods

2.1. Materials

HT Protein Express LabChip® Kits and HT Protein Express Chips were purchased from Perkin Elmer (Waltham, MA). Dithiothreitol (DTT), 2-Mercaptoethanol (BME), and tris 2-carboxyethyl phosphine HCl (TCEP) were purchased from Sigma–Aldrich (St. Louis, MO). PNGase F and reaction buffer were purchased from New England Biolabs (Ipswich, MA). SDS-MW Analysis Kit, which includes separation capillary (57 cm x 50 μm ID bare fused-silica), SDS gel separation buffer, SDS sample buffer (100 mM Tris–HCl, pH 9.0, 1% SDS), SDS Protein Sizing Standard (10–225 kDa, 16 mg/mL), internal standard (10 kDa protein, 5 mg/mL), acidic wash solution (0.1 N HCl), and basic wash solution (0.1 N NaOH) were purchased from Beckman Coulter (Jersey City, NJ). The monoclonal antibodies evaluated were produced in-house at Bristol-Myers Squibb.

2.2. PNGase F digestion

Deglycosylated mAb was prepared by incubating mAb with PNGase F overnight at 37 °C in 100 mM Tris, pH 8.0, following manufacturer's instructions.

2.3. Microchip-based electrophoresis

High-throughput analyses of monoclonal antibodies were performed on the Caliper LabChip® GXII instrument (Perkin Elmer, Waltham, MA). The regular microchip-based electrophoresis has been described in details elsewhere [6] with minor modifications. Unless otherwise noted, 2 μL of antibody at 2 mg/mL was mixed with 14 μL of sample buffer. The sample buffer was prepared by mixing 700 μL of HT Protein Express sample buffer with either 24.5 μL of BME (for reducing assay) or 35 μL of 0.5 M iodoacetamide (IAM, for the non-reducing assay). The samples were incubated at 100 °C for 5 min (before optimization), 90 °C for 1 min (after optimization), or other incubation conditions as described in the study during method optimization. After cooling to room temperature, 70 μL of water was added to each sample before loading onto the instrument. The samples were analyzed using the HT Protein Express 200 script. The chip was prepared according to the manufacturer's instruction.

2.4. Conventional CE-SDS

To prepare samples for CE-SDS, 90 μg of mAb1 was combined with 5 μL of 14.3 M BME in a microcentrifuge tube. Sample buffer (100 mM Tris–HCl pH 9.0, 1% SDS) was added to a final volume of 100 μL. The solution was mixed, centrifuged, and incubated at 70 °C for 10 min. The mixture was cooled at room temperature, centrifuged briefly to degas, and transferred to sample vials for injection. The electrophoresis was carried out in a bare-fused silica capillary with a total length of 30.2 cm, effective length of 20 cm, with an inner diameter (I.D.) of 50 μm and outer diameter (O. D.) of 375 μm (Beckman Coulter, Brea, CA).

Prior to sample injection and separation, the capillary was rinsed with basic wash 3 min at 70 psi, acidic wash 1 min at 70 psi, water 1 min at 70 psi, and gel buffer 10 min at 70 psi. Following the rinses, the capillary and electrode ends were dipped twice in separate water vials prior to sample injection at 5 kV. Another water dip was performed followed by separation at 15 kV. During separation, pressure was applied to both ends of the capillary at 20 psi. Separation was detected with a PDA detector at 220 nm using the Beckman Coulter PA800 plus system (Brea, CA).