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Monoclonal antibody heterogeneity analysis and deamidation monitoring with high-performance cation-exchange chromatofocusing using simple, two component buffer systems

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ABSTRACT

The use of either a polyampholyte buffer or a simple buffer system for the high-performance cation-exchange chromatofocusing of monoclonal antibodies is demonstrated for the case where the pH gradient is produced entirely inside the column and with no external mixing of buffers. The simple buffer system used was composed of two buffering species, one which becomes adsorbed onto the column packing and one which does not adsorb, together with an adsorbed ion that does not participate in acid–base equilibrium. The method which employs the simple buffer system is capable of producing a gradual pH gradient in the neutral to acidic pH range that can be adjusted by proper selection of the starting and ending pH values for the gradient as well as the buffering species concentration, pK_a, and molecular size. By using this approach, variants of representative monoclonal antibodies with isoelectric points of 7.0 or less were separated with high resolution so that the approach can serve as a complementary alternative to isoelectric focusing for characterizing a monoclonal antibody based on differences in the isoelectric points of the variants present. Because the simple buffer system used eliminates the use of polyampholytes, the method is suitable for antibody heterogeneity analysis coupled with mass spectrometry. The method can also be used at the preparative scale to collect highly purified isoelectric variants of an antibody for further study. To illustrate this, a single isoelectric point variant of a monoclonal antibody was collected and used for a stability study under forced deamidation conditions.

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1. Introduction

Monoclonal antibodies (mAbs) have become important therapeutic agents used in the treatment of various illnesses such as autoimmune disease and cancer. In 2008 there were 24 monoclonal antibodies approved by the US Food and Drug Administration (FDA) for clinical use [1]. Total global mAb sales reached $45 billion in 2011, and it is expected that mAb sales will increase to $58 billion by 2016 [2].

A variety of structural variants of monoclonal antibodies can be produced during the manufacturing process due to glycosylation, deamidation, phosphorylation, hinge-region fragmentation, methionine oxidation, N-terminal pyroglutamate formation, and truncation of C-terminal lysine or arginine [3]. The existence of certain variants may affect the immunogenicity, half-life, bioactivity and stability of the therapeutic antibody [4]. Regulatory agencies such as the US Food and Drug Administration (FDA) demand increasingly sophisticated heterogeneity analysis of therapeutic antibodies to assure lot-to-lot consistency, safety and efficacy. However, characterization of monoclonal antibody structural variants can be very challenging since the variants are often similar in their physical and chemical characteristics. Isoelectric focusing (IEF) and capillary isoelectric focusing (cIEF) are currently the preferred techniques to separate protein variants based on charge heterogeneity. However, only a very limited amount of separated protein is obtainable from these methods due to the fact that they are not easily scalable. A number of chromatographic techniques such as reversed-phase chromatography [5] and ion-exchange chromatography using a salt gradient [6] have been employed to separate the charge variants of antibodies. Generally, these methods can provide good resolution, although some of the methods may result in denatured antibodies or have other undesirable features. In some situations, higher resolution is required than can be achieved by these existing techniques, such as when the antibody variants to be separated have very similar isoelectric points (p*I*s).

In this work, chromatofocusing methods are developed which separate monoclonal antibody charge variants having p*I*s in the

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neutral to acidic range using a cation-exchange column and either a polyampholyte elution buffer or a simple elution buffer containing a limited number of buffering species. Chromatofocusing is a relatively new type of ion-exchange chromatography (IEC) developed by Sluyterman and co-workers in the late 1970 s [7–10]. Instead of using a salt gradient as in traditional IEC, chromatofocusing takes advantage of the focusing effect of a pH gradient that is dynamically formed inside the column when an appropriate buffer is introduced isocratically into an ion-exchange column. Due to the interactions between a protein and the retained pH gradient employed in the technique, proteins become focused at a certain position on the pH gradient, termed the apparent isoelectric point (pI_{app}), which is often close to the true p*I* [11,12]. Chromatofocusing therefore combines elements of both IEC and IEF and at the same time eliminates many shortcomings of these two techniques, such as the difficulty of scaling up IEF and the tendency of proteins to denature when pH gradients produced externally to the column are used in IEC [7–10].

Traditionally, the column packings used in chromatofocusing are weak-base anion exchangers, such as the various Polybuffer Exchangers available from GE Healthcare [13]. However, it was demonstrated by Kang and Frey [14] that weak-acid cation-exchange column packings can also be used for chromatofocusing. In spite of the fact that chromatofocusing is well suited for separating proteins with similar p*I*s, relatively few previous studies have reported separating the charge variants of monoclonal antibodies using the anion-exchange version of this technique [15–17], and no studies have been reported where the cation-exchange version of this technique has been used to separate these types of variants with high resolution. Ng et al. [18], however, used cation-exchange chromatofocusing to separate monoclonal antibodies from host cell proteins. In addition, a few previous studies have separated monoclonal antibody variants using a cation-exchange column and a pH gradient formed partly or entirely by mixing buffers external to the column. For example, Roshkova [19] employed a weak-acid cation exchanger in the pH range from 6.8 to 7.4 where the column packing used was nearly fully ionized along with a gradient pump to introduce a pH gradient into the column. Although Roshkova achieved useful separations of monoclonal antibody variants, the shape of the pH gradient measured at the column exit by Roshkova suggests that the gradients used were formed entirely by the gradient pump and passed though the column in an unretained manner, so that the method does not fully exploit the characteristics of an internally produced, retained pH gradient.

Farnan and Moreno [20] employed a technique similar to that used by Roshkova [19] to separate monoclonal antibody variants, but in the broader pH range of 5.5–9 where the cation-exchange column packing employed exhibited a buffering capacity so that the pH gradient was retained to some extent. Although Farnan and Moreno also achieved useful separations of antibody variants, their technique differs in several respects from traditional chromatofocusing. For example, in their method the pI_{app} value observed for an antibody variant depended strongly on the ionic strength employed and varied considerably from the true p*I* value. Related earlier studies of the use of externally produced pH gradients to separate antibody variants and fragments have been reported by several other workers [21–23].

The methods developed here are a useful alternative to the previous methods described in this section since they employ a cation-exchange column packing instead of the more commonly used anion-exchange column packings, in which case variants with positively charged structural differences may be more resolvable. In addition, the methods developed here exploit the chromatographic behavior of pH gradients that are dynamically formed entirely inside the column.

2. Experimental

The buffering species used in this work are diethanolamine (DEA), ethanolamine (EA), triethanolamine (TEA), and citric acid, all purchased from Sigma–Aldrich (St. Louis, MO, USA). To produce the presaturation buffer (i.e., the equilibration buffer) and the elution buffer for chromatofocusing when simple buffers were employed, mixtures of these species were titrated with NaOH to the desired pH. Polybuffer 74 was used as the polyampholyte buffer and was purchased from GE Healthcare (Piscataway, NJ, USA). For chromatofocusing with Polybuffer 74, the presaturation buffer was 5 mM citric acid titrated to pH 5 with sodium hydroxide and the elution buffer was 0.5% (w/v) Polybuffer 74 titrated to appropriate pH with sodium hydroxide. All buffers were vacuum filtered through a 0.22 μm membrane bottle-top filter composed of polyethersulfone from Corning (Corning, NY, USA). The feed sample used for chromatofocusing was 10 μg of purified Antibody A or B, which are proprietary products of Genzyme. The columns used were a 25 × 0.4 cm I.D. ProPac WCX-10 analytical column and a 25 × 0.9 cm I.D. ProPac WCX-10 semi-preparative column obtained from Dionex (Sunnyvale, CA, USA). The two antibodies used (Antibodies A and B) are of the IgG4 subclass and were expressed in a GS NS0 mammalian expression system (Lonza, Basel, Switzerland).

The chromatography equipment used was either an HP 1100 HPLC system from Agilent Technologies (Santa Clara, CA, USA) or an AKTA purifier from GE Healthcare equipped with a P-900 pump, a pH/C-900 meter for monitoring pH and conductivity, a low-dead-volume, in-line pH/conductivity sampling cell for pH/conductivity monitoring, and a UV-900 detector. The concentration of collected fractions from chromatography was accomplished using a Vivaspin 2 Centrifugal Concentrator equipped with a 30 kDa MWCO membrane (Vivaproducts, Littleton, MA, USA).

Carboxypeptidase B (CPB, Sigma–Aldrich) treatment of the antibody was performed by incubating a mixture of 700 μl of 5 mg/ml antibody and 10 μl of 5 mg/ml CPB at 37 °C for 2 h. Reagents used in the accelerated deamidation of antibody are ammonium bicarbonate, sodium azide, and Protein Inhibitor Cocktail, all from Sigma–Aldrich. 10 ml of D.I. water was added into one vial of the Protein Inhibitor Cocktail to make a 10× stock solution. The antibody sample was deamidated by adding 1 ml of sample into 4 ml of a mixture of 1% ammonium bicarbonate, 0.05% sodium azide and 1× Protein Cocktail Inhibitor, and then incubating the final mixture at 37 °C.

HPLC–MS was performed on an Agilent 1100 HPLC coupled to a Finnigan LCQ Deca XP Plus ion trap mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA). The sample protein to be analyzed was first reduced with dithiolthreitol (DTT, Sigma–Aldrich), alkylated by iodoacetic acid (Sigma–Aldrich), then buffer exchanged to 25 mM Tris buffer at pH 8.5. One vial of sequencing grade trypsin (25 μg, Roche Applied Science, Indianapolis, IN, USA) was dissolved with 0.01% trifluoroacetic acid (TFA, J.T. Baker, Phillipsburg, NJ, USA). Peptide digestion was conducted by mixing the proper amount of trypsin stock solution with the sample protein (1:50 enzyme to substrate ratio) and incubating at 37 °C for 18–20 h. The reaction was quenched with 1% TFA after the incubation. Reversed-phase HPLC was performed using a 100 × 2.1 mm I.D. Biosuite PA-B C18 column from Waters (Milford, MA, USA). Buffer A for reversed-phase chromatography was composed of HPLC grade water and 0.1% TFA and buffer B was acetonitrile (Honeywell Burdick and Jackson, Morristown, NJ, USA) with 0.08% TFA. A flow rate of 0.25 ml/min was used. The gradient began with a 3-minute hold at 2% buffer B, followed by an increase to 45% organic component over 160 min. Mass spectral data were acquired to determine the identity of the peptides resulting from the trypsin digestion.

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