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Characterization of cation exchanger stationary phases applied for the separations of therapeutic monoclonal antibodies

Szabolcs Fekete[a,*], Alain Beck[b], Davy Guillarme[a]

[a] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland
[b] Center of Immunology Pierre Fabre, 5 Avenue Napoléon III, BP 60497, 74160 Saint-Julien-en-Genevois, France[1]

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ABSTRACT

Cation exchange chromatography (CEX) is a well established strategy for the characterization of monoclonal antibodies (mAbs). The optimization of mobile phase conditions is well described in the literature, but there is a lack of information about CEX stationary phases for the analysis of therapeutic proteins. The aim of this study was to compare five state-of-the-art CEX stationary phases based on the retention, selectivity and resolving power achieved in pH- and salt-gradient modes, with various therapeutic mAbs and their variants. The Sepax Antibodix WCX-NP3, Thermo MAbPac SCX-10 RS, YMC BioPro SP-F, Waters Protein-Pak Hi Res SP and Agilent Bio mAb NP1.7 SS were considered in this study.

In terms of retention, the YMC Bio Pro SP-F material was the less retentive one, while the Agilent Bio mAb NP1.7 SS provides the highest retention. Regarding the selectivity achieved between the main mAbs isoforms and their variants, the Thermo MabPac SCX column generally gave the highest selectivity. Finally, it was hard to rank columns in term of kinetic performance since their performance is strongly solute (mAb) and elution mode (pH or salt gradient) dependent. However, the highest resolution – in most cases – was observed on the strong cation exchanger YMC Bio Pro SP-F material.

1. Introduction

Monoclonal antibodies (mAbs) represent an important class of biopharmaceuticals with a wide range of clinical indications [1]. Similarly to other proteins, mAbs are susceptible to chemical and enzymatic modifications during manufacture, formulation and storage [1]. The intrinsic micro-heterogeneity is of major concern with mAbs and should be critically evaluated because differences in impurities and/or degradation products could lead to serious health implications [2]. Common modifications of the primary sequence include N-glycosylation [3], methionine oxidation [4], proteolytic fragmentation, lysine truncation and deamidation [5,6].

For the detailed characterization of mAbs, a wide range of analytical methods, including ion exchange chromatography (IEX), reversed-phase liquid chromatography (RPLC), hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), capillary isoelectric focusing (cIEF), capillary zone electrophoresis (CZE), circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR), fluorescence spectrophotometry (FL), and mass spectrometry (MS) are often used [7]. The goal of this multi-method strategy is to demonstrate the similarity between production batches of mAbs by precisely characterizing their structure at the primary, secondary, and tertiary levels [8,9].

Among the analytical techniques, IEX can be considered as a reference technique for the separation of charge variants of intact and fragmented mAbs. Chemical modifications of mAbs, such as sialylation and deamidation, introduce additional negative charges to the molecules, leading to acidic species. C-terminal processing of lysine residues introduces one or two additional positive charges to the molecules and generates basic species. Such acidic and basic variants can be separated from the main isoform using ionic interaction based separations [1]. Among the different IEX modes, cation-exchange chromatography (CEX) is the most efficient approaches for mAb purification and characterization [10]. CEX is considered as the gold standard for charge sensitive antibody analysis, but method parameters, such as column type, mobile phase pH, and salt concentration gradient or pH gradient need to be optimized for each individual mAb [11].

Moorhouse et al. were among the first ones to demonstrate the potential of CEX for mAb characterization [12]. MAb fragments and their variants were separated with sufficient resolution and identified with off-line MS. The C-terminal lysine variability

* Corresponding author. Tel.: +41 22 37 963 34; fax: +41 22 379 68 08.
E-mail address: szabolcs.fekete@unige.ch (S. Fekete).
[1] www.cipf.com.

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of the crystallizable fraction (Fc) and the N-terminal glutamine-pyroglutamate variability of the antigen-binding fraction (Fab) were observed. Since then, several study showed the suitability of CEX for the characterization of mAb heterogeneity [7,13–17].

Currently, two modes of CEX are applied for mAb analysis, namely the salt- and pH gradient modes. The theory and practice to optimize mobile phase composition, pH value, salt concentration and gradient program is well described [18]. However, the impact of stationary phase chemistry has not been reported up to now. Regarding the stationary phase, there are two main aspects that need to be considered: (1) the strength of interaction and associated retention (strong or weak ion-exchanger) and the (2) achievable peak widths (efficiency). Weak cation exchangers contain a weak acid that gradually loses its charge as the pH decreases (e.g. carboxymethyl groups), while strong cation exchangers contain a strong acid that is able to sustain its charge over a wide pH range (e.g. sulfopropyl groups). Strong exchangers remain under ionized form in the pH range between 2 and 12. As a rule of thumb, it is preferred to begin the method development with a strong exchanger to enable working over a broad pH range [18]. Strong exchangers are also useful if the maximum resolution occurs at an extreme pH. In the case of proteins, the cation exchange mode is well suited. Weak exchangers are especially useful, if the selectivity of strong ion exchangers is unsatisfactory. However, it is important to keep in mind that the ion exchange capacity of weak ion exchangers may vary with pH.

Commercially available IEX columns are based on silica or polymer particles. Both porous and non-porous particles are available, but for large molecules which possess low diffusivity, non-porous materials are clearly preferred to avoid the unwanted band broadening effects of the transparticle mass transfer resistance [18]. Highly cross-linked non-porous poly(styrene–divinylbenzene) (PS/DVB) particles are most frequently used in protein separations due to their extended pH stability ($2 \leq pH \leq 12$). Some of those materials can withstand pressure drop of up to a 500–600 bar and can be routinely used beyond 400 bar. Columns packed with 10, 5 or 3 μm non-porous particles are often used, but sub-2 μm materials are also available since recently.

The aim of this work was to study the impact of the CEX stationary phase nature on the retention, selectivity and resolution power for therapeutic mAb separations. Therefore, five different commercially available columns were systematically compared in terms of retentivity, selectivity and separation power when applied for the separation of intact mAbs and their variants. In addition, the goal of the present study was to investigate the possibilities to perform very high resolution separations by coupling columns in series and extending gradient times. Some examples for the high resolution CEX separations of heterogeneous intact mAb samples within reasonable analysis time are presented.

2. Experimental

2.1. Chemicals and columns

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). CX-1 pH gradient buffer A (pH = 5.6) and CX-1 pH gradient buffer B (pH = 10.2) were purchased from Thermo Fisher Scientific AG (Reinach, Switzerland). 1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) solution (BioReagent), 1 M sodium hydroxide (NaOH) solution and sodium chloride (NaCl) (BioChemika) were purchased from Sigma–Aldrich (Buchs, Switzerland).

FDA and EMA approved therapeutic IgG monoclonal antibodies including panitumumab, natalizumab, cetuximab, trastuzumab, palivizumab, adalimumab, denosumab and ofatumumab were kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France).

YMC BioPro SP-F 100 × 4.6 mm, 5 μm strong cation exchange column was purchased from Stacroma (Reinach, Switzerland). Waters Protein-Pak Hi Res SP 100 × 4.6 mm, 7 μm strong cation exchange column was a generous gift from Waters (Milford, MA, USA). Sepax Antibodix WCX-NP3 150 × 4.6 mm, 3 μm weak cation exchange column was purchased from Supelco (Sigma–Aldrich). Thermo MAbPac SCX-10 RS 150 × 4.6 mm, 5 μm strong cation exchange column was purchased from Thermo Fisher Scientific AG. Agilent Bio MAb NP1.7 SS 50 × 4.6 mm, 1.7 μm weak cation exchange column was purchased from Agilent (Waldbronn, Germany).

2.2. Equipment and software

All the experiments were performed using a Waters Acquity UPLC™ system equipped with a binary solvent delivery pump, an autosampler and fluorescence detector (FL). The Waters Acquity system included a 5 μL sample loop and a 2 μL FL flow-cell. The loop is directly connected to the injection switching valve (no needle seat capillary). The connection tube between the injector and column inlet was 0.13 mm I.D. and 250 mm long (passive preheating included), and the capillary located between the column and detector was 0.10 mm I.D. and 150 mm long. The overall extra-column volume (V_{ext}) was about 14 μL as measured from the injection seat of the auto-sampler to the detector cell. The measured dwell volume was around 100 μL. Data acquisition and instrument control were performed by Empower Pro 2 Software (Waters). Calculation and data transferring were achieved by using Excel templates.

The mobile phase pH was adjusted using a SevenMulti S40 pH meter (Mettler Toledo, Greifensee, Switzerland).

2.3. Apparatus and methodology

2.3.1. Mobile phase composition and sample preparation

For the pH gradient CEX separation of mAbs and their variants, the mobile phase "A" was a ten times diluted CX-1 pH gradient buffer A (pH = 5.6), while the mobile phase "B" was a ten times diluted CX-1 pH gradient buffer B (pH = 10.2) – as described in the protocol provided by the vendor (Thermo Fisher Scientific).

For the salt gradient separations, the mobile phase "A" consisted of 10 mM MES in water, while the mobile phase "B" was 10 mM MES in water containing 1 M NaCl. The pH of both mobile phases was adjusted by adding 1 M NaOH solution to set pH = 5.7.

The intact mAb samples were diluted with water to 100 μg/ml, and directly injected using low volume insert vials.

2.3.2. Investigation of retention properties and selectivity

In both pH and salt gradient modes, generic conditions were applied enabling the elution of all mAbs from all columns within reasonable analysis time and maintaining similar apparent retention in both elution modes. The conditions were adapted from previous studies, and adjusted accordingly taking the column dimensions into account [7,17]. All the columns applied in this study possess 4.6 mm i.d. but different lengths, therefore the gradient program was adjusted proportionally with the column length (10, 20 and 30 min gradients on the 50, 100 and 150 mm long columns, respectively at flow rate of 0.6 mL/min), to maintain a similar gradient steepness.

For studying the retention properties of intact mAbs and the selectivity between their charge variants, eight therapeutic mAbs were selected based on their type (IgG class and isotype), theoretical p*I*, and natural heterogeneity, namely panitumumab (huIgG2, p*I* = 6.7), natalizumab (hzIgG4, p*I* = 7.3), cetuximab (chIgG1, p*I* = 7.9), adalimumab (huIgG1, p*I* = 8.4), trastuzumab