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Review

Ion-exchange chromatography for the characterization of biopharmaceuticals

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ABSTRACT

Ion-exchange chromatography (IEX) is a historical technique widely used for the detailed characterization of therapeutic proteins and can be considered as a reference and powerful technique for the qualitative and quantitative evaluation of charge heterogeneity. The goal of this review is to provide an overview of theoretical and practical aspects of modern IEX applied for the characterization of therapeutic proteins including monoclonal antibodies (Mabs) and antibody drug conjugates (ADCs). The section on method development describes how to select a suitable stationary phase chemistry and dimensions, the mobile phase conditions (pH, nature and concentration of salt), as well as the temperature and flow rate, considering proteins isoelectric point (p*I*). In addition, both salt-gradient and pH-gradient approaches were critically reviewed and benefits as well as limitations of these two strategies were provided. Finally, several applications, mostly from pharmaceutical industries, illustrate the potential of IEX for the characterization of charge variants of various types of biopharmaceutical products.

Contents

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1. Introduction

Proteins and monoclonal antibodies (mAbs) are an emerging class of therapeutic agents currently being developed by many pharmaceutical companies [1]. Due to the increasing number of approved therapeutic proteins in the pharmaceutical area and the number of biosimilars (or follow-on-biologics) potentially entering the market, the need for analytical techniques for their detailed characterization has increased. Several characteristics of protein-based therapy contribute to its success by improving the risk–benefit ratio. These characteristics include improved tolerance, good efficacy, high specificity, and limited side effects. However, the intrinsic micro-heterogeneity is of major concern with biomolecules and should be critically evaluated because differences in impurities and/or degradation products could lead to health implications [2]. Furthermore, producing biosimilars is more challenging than manufacturing generic small molecule based pharmaceuticals [3].

In general, the identity, heterogeneity, impurity content, and activity of each new batch of therapeutic proteins has to be thoroughly investigated before release. This examination is achieved using 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). The goal of this multi-method strategy is to demonstrate the similarity between production batches by precisely characterizing the primary, secondary, and tertiary structure of the proteins [4,5].

IEX is a historical and non-denaturing technique widely used for the characterization of charge variants of therapeutic proteins and is considered as a reference technique for the qualitative and quantitative evaluation of charge heterogeneity of therapeutic proteins [1]. The history and continuous evolution of IEX was reviewed by Lucy [6]. Among the different IEX modes, cation-exchange chromatography (CEX) is the most widely used for protein purification and characterization [7]. CEX is considered as the gold standard for charge sensitive analysis, but method parameters, such as column type, mobile phase pH, and salt concentration gradient, often need to be optimized for each individual protein [8]. IEX separates charge variants by differential interactions on a charged support. The number of possible charge variants increases with the molecular weight of the analyzed sample. In addition, changes in charge may be additive or subtractive, depending on any modifications. Thus, IEX profiles become more complex, and the overall resolution of individual variants may be lost [1]. This property is particularly apparent for large biomolecules. Therefore, not only the intact but also the reduced or digested forms (limited proteolysis or peptide mapping) of therapeutic proteins are commonly characterized by IEX.

In this review, we focus on the possibilities of IEX chromatography for the characterization of therapeutic proteins. Moreover, the aim of this review is to detail the theoretical and practical aspects of modern IEX. Last, method development approaches and applications are also reviewed and explained.

2. Theoretical aspects of IEX

2.1. Salt-gradient based separations

IEX separates proteins based on differences in the surface charge of the molecules, with separation being dictated by the protein interactions with the stationary phase [9]. As a classical mode of IEX, a linear salt-gradient is regularly applied for the elution. Several models for chromatographic retention of ion-exchange adsorbents have been proposed in the past years [10]. The retention models can be divided into stoichiometric and non-stoichiometric models. Stoichiometric models describe the multi-faceted binding of the protein molecules to the stationary phase as a stoichiometric exchange of mobile phase protein and bound counter-ions [11]. This stoichiometric displacement model (SDM) predicts that the retention of a protein under isocratic, linear conditions is related to counter-ion concentration. This model was extended to describe protein retention under linear gradient elution conditions (LGE model) [12], as well as under non-linear protein adsorption conditions (steric mass action (SMA) model) [13,14] for isocratic and gradient elution mode. Another extension of the stoichiometric model for the ion-exchange adsorption which accounts for charge regulation was developed recently [15,16].

Even if stoichiometric models are capable of describing the behavior of ion-exchange chromatographic systems, they assume that the individual charges on the protein molecules interact with discrete charges on the ion-exchange surface. In reality, retention through ion-exchange is more complex and primarily due to the interaction of the electrical fields of the protein molecules and the chromatographic surface [11]. Therefore, several non-stoichiometric models for describing protein retention as a function of the salt concentration in the mobile phase have also been proposed [17–20]. Quantitative structure–property relationship (QSPR) models have been derived for protein retention modeling in IEX by means of different numerical approaches that attempt to correlate retention to functions of descriptors derived from the three-dimensional structure of the proteins [21–23]. More recently, theories used in colloid and surface chemistry to describe electrostatic and other interactions have also been applied to describe retention properties of proteins in IEX [24–28].

The work of Snyder and co-workers showed that IEX systems follow non-linear solvent strength (LSS) type retention mechanism [29,30]. Consequently, solute-specific correction factors are required to use LSS model for retention predictions, thereby limiting the applicability of the LSS model. The non-linearity of LSS model was assessed by comparing the elution data to the stoichiometric displacement model (SDM) commonly used in IEX. The retention factor (k) can be written in the following way according to the SDM model:

$$\log\ k = \log K - z \log C \quad (1)$$

where K is the distribution constant, z is associated with the protein net charge or number of binding sites (effective charge) and C is the salt concentration (that determines the ionic strength). This model is probably the most accepted one and is useful from a practical point of view. The non-linearity of Eq. (1) is most pronounced for small values of z [30]. If $z > 6$ (which is very often the case of therapeutic proteins), an LSS type model may provide reliable data for retention factor (retention time) [31]. Fig. 1A shows experimentally observed $\log k$ versus C plot for $z = 1$, while Fig. 1B shows some calculated $\log k$–C plots for various z values.

Proteins are eluted in order of increasing binding charge (correlates more or less with the isoelectric point (pI)) and equilibrium constant. The retention of large proteins in salt-gradient mode is strongly dependent on the salt concentration (gradient steepness or gradient time) – due to the relatively high z value – and a small change could lead to significant shift in retention. Therefore, isocratic conditions are impractical, and gradient elution is preferred in real-life proteins separations. For linear salt-gradient in IEX, the