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Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products

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ABSTRACT

Hydrophobic interaction chromatography (HIC) is a historical strategy used for the analytical purification and characterization of proteins. Similarly to what can be done in reversed-phase liquid chromatography (RPLC), HIC is able to separate protein species based on their hydrophobicity, but using different conditions. Compared to RPLC, the main benefit of HIC is its ability to perform separations under non denaturing conditions (i.e. physiological pH conditions, ambient mobile phase temperature and no need for organic solvents) and so an orthogonal method. The goal of this review is to provide a general overview of theoretical and practical aspects of modern HIC applied for the characterization of therapeutic protein biopharmaceuticals including monoclonal antibodies (mAbs), antibody drug conjugates (ADCs) and bispecific antibodies (bsAbs). Therefore, method development approaches, state-of-the-art column technology, applications and future perspectives are described and critically discussed.

1. Introduction

Monoclonal antibodies (mAbs) and related products such as antibody-drug conjugates (ADCs) and bispecific antibodies (bsAbs) are the fastest growing class of therapeutics [1]. Therefore, the need for analytical techniques applied for the detailed characterization of such biopharmaceuticals has increased drastically due to the growing number of approved therapeutic proteins and biosimilars (or follow-on-biologics) potentially entering the market. More than 60 IgGs and IgG derivatives have been approved so far for use in various indications such as cancers or inflammatory diseases [2]. ADCs become more and more popular, with two already approved drugs on the market (brentuximab vedotin—Adcetris, and adotrastuzumab emtansine—Kadcyla) and more than 30 others in clinical trials [3,4]. ADCs are biochemotherapeutics constituted of a cytotoxic chemical drug covalently linked to a mAb. It combines the specificity of a mAb and the efficacy of the cytotoxic drug [5].

In general, the identity, heterogeneity, impurity content, and activity of each new batch of therapeutic proteins has to be thoroughly investigated before release. For the detailed characterization of mAbs and ADCs (and other related products), various chromatographic approaches such as reversed-phase (RPLC), size exclusion (SEC), ion exchange (IEX), hydrophobic interaction chromatography (HIC) or affinity chromatography are often applied [6]. Beside chromatographic techniques, 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 also frequently used for the characterization. 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 [7,8].

Among the several liquid chromatographic modes applied for the characterization of mAbs and ADCs, HIC is the preferred technique for determining the relative hydrophobicity of mAbs and to separate the different populations of ADC molecules that differ in their number of drugs *per* antibody which are often known as DAR (drug-to-antibody ratio) species [9,10]. Positional isomers of DARs can also be separated by using high resolution HIC. The main advantage of HIC compared to other LC modes (e.g. RP) is that it is non-denaturating, so the native forms of the proteins are expected to be maintained. Moreover, the separated proteins can be collected for further activity measurements (such as cell based potency, receptor binding, cell proliferation assay, enzyme assay, functional ELISA...). HIC is also often used for protein purification based on the apparent hydrophobicity of impurities and is a valuable tool in downstream purification procedure [11–13].

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Previous reviews and book chapters have already presented earlier HIC works [12–15]. In this review, we focus on the possibilities of modern HIC applied for the characterization of therapeutic proteins (mAbs and related products), both from theoretical and practical points of view. Then, method development approaches, state-of-the-art column technology and applications are also reviewed and discussed.

2. Theoretical aspects of HIC

Since RPLC employs harsh conditions (i.e. mobile phase with acidic additive, organic modifier, and elevated temperature) that denaturate proteins, there was a need for alternative (milder) chromatographic conditions able to differentiate proteins based on their hydrophobicity. In this context, HIC was suggested as alternative [14], and its industrial significance has been growing. The main difference between HIC and RP is that proteins maintain their native structure with intra-molecular forces in HIC while they are denaturated in RP conditions. Fig. 1 shows the chromatographic profiles of a reduced mAb obtained by RP and HIC. As can be seen, the original "Y" shape of the mAb is maintained in HIC conditions even if the disulphide bridges have been reduced. In contrast, the heavy- and light chains (Hc, Lc) are well separated in RP conditions. Despite the publication of many fundamental studies and retention models in the literature, the retention mechanism in HIC is often misunderstood and none of the proposed theories has received general acceptance. Different interpretations and approaches such as hydrophobic interaction, hydrophobic effects, solvophobic theory, salting-out effect, dehydration of proteins or structural rearrangement of proteins are often confused. Here, we will try to clarify the various concepts and briefly summarize the different parameters affecting proteins retention in HIC.

2.1. Salting-out effect

The concept of protein chromatography based on hydrophobic interactions was first described by Tiselius [16]. He first used the term "salting-out chromatography" since salt solutions were applied as mobile phases. Salting-out effect is based on electrolyte/nonelectrolyte interaction (corresponding to mobile phase—protein interaction in HIC), in which the nonelectrolyte becomes less soluble at high salt concentrations. In aqueous solutions, proteins fold and the hydrophobic amino acids usually form some protected hydrophobic areas, while hydrophilic amino acids form hydrogen bonds with the surrounding water. If the hydrophilic surface of the protein is large enough, then the protein can be dissolved in water. When adding salts, water molecules will solvate predominantly salt ions. Therefore, the number of water molecules available to interact with the hydrophilic part (charged) of the protein will decrease. Under these conditions, the protein–protein intermolecular interactions become stronger due to the decreased amount of surrounding water molecules. At the end, the protein molecules can associate by forming hydrophobic interactions with each other (aggregation).

Later on, this separation mode was called as "hydrophobic chromatography" or "hydrophobic affinity chromatography" [17]. The name "hydrophobic interaction chromatography" was introduced by Hjertén et al. [45], but he also named this mode as "salt mediated separation of proteins". In 1986, Porath suggested "salt-promoted adsorption" or "salt-promoted adsorption chromatography" (SPAC) as alternative expressions for HIC [18].

2.2. Hydrophobic effects, formation of cavity

The hydrophobic effect is generally defined as an interaction of nonpolar substances or moieties of the molecules with water that is responsible for their low solubility [19,20]. On the other hand, the term "hydrophobic interactions" has also been used to describe the forces resulting in the association of nonpolar molecules or the binding of hydrophobic moieties in aqueous solutions [21]. Hydrophobicity generally means the repulsion between a nonpolar moiety of the protein and of the polar aqueous environment of water [12]. The structure of water is highly ordered and stabilized by dipole–dipole interactions over a 3-dimensional structure, characterized by the high surface tension of water (~72 mN/m at 25 °C) [22]. Each oxygen atom has four hydrogens as neighbors in a tetraeder configuration, and each hydrogen atom forms a bridge between two oxygen atoms (through covalent or hydrogen bonds). When dissolving hydrophobic moieties of a protein in an aqueous system, the neighboring water molecules have to be separated from each other in order to form a cavity for the protein [22]. This requires the investment of energy, which corresponds to the surface of the cavity multiplied by the surface tension. If two or more partners are associated, their hydrophobic contact surface area is reduced, and energy is released. The amount of energy is proportional to the size of the hydrophobic contact surface area of the protein. In other words, the interaction between two or more hydrophobic molecules in aqueous solutions takes place spontaneously and is mainly driven by the entropy change [23–25]. Frank and Evans reported that the large entropic effect arises from an orientation of the water molecules at the cavity around the nonpolar solute molecule (protein) [26,27]. Further studies indeed showed that the heat capacity change in such transfer-into-water processes is determined by the change in the water-accessible nonpolar surface area of the molecule [28,29]. At first, it was assumed that hydrophobic interactions involve only entropic effects. However, Baldwin has shown that hydrophobic interactions are entropy driven at low temperatures, but enthalpy driven at elevated temperatures, when the heat capacity change remains constant in the range of experimental temperature [30]. Such model experiments provided the basis of a more detailed understanding of the influence of temperature on hydrophobic interactions and hydrophobic effect.

2.3. Solvophobic theory

In general, the solvophobic theory explains the interactions between polar solvent (aqueous mobile phase) and less polar solute (protein). Due to H-bonding and other polar interactions, strong cohesive forces exist between the solvent molecules and provide a strongly structured order for the solvent. Therefore, less polar solutes tend to be insoluble due to the strong solvent–solvent binding interactions. The retention in RPLC is often explained by the solvophobic theory. According to this, the solute molecules stick to the surface of the stationary phase due to their rejection form the solvent and their affinity for the hydrophobic stationary phase. So, the retention is partly explained by the interactions between the solute and stationary phase and partly by the rejection of solute from the mobile phase solvent.

Horváth et al. developed the basis for describing retention mechanisms in RPLC, employing the framework of the solvophobic theory [31]. A simplified mass balance equation was introduced and led to an expression of the different free energy contributions to the overall retention process. It was shown that the structural forces of H-bond interlinked water molecules represent an energetically low state of the water structure. In contrast, in the neighborhood of the stationary phase alkyl-chains, the water (or aqueous solvent) is under an energetically "excited" state as it has no contact with neighboring water molecules. One way to return to an energetically low state is an enforced association among the alkyl-chains, and another way is to combine the alkyl-ligand and hydrophobic solute to form an association complex. Horváth called the latter one

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