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Challenges in liquid chromatographic characterization of proteins

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ABSTRACT

Various liquid chromatographic techniques are considered standard analytical methods in proteins characterization. These methods provide essential information for drug approval, for biological and life sciences. On the other hand, there are some issues and challenges which have to be taken into account when analyzing these biopharmaceuticals. The aim of this review to summarize the most recent knowledge relating to the following topics: i) sample stability and complexity ii) adsorption problems: instrument inertness iii) adsorption problems: recovery from the stationary phase and iv) challenges in method development. This information is supposed to help practicing chromatographers in the emerging field of therapeutic protein chromatography.

1. Introduction

Protein therapeutics, especially monoclonal antibodies and antibody-like products, such as Fc fusion proteins and antibody-drug conjugates, have become a particularly relevant part of the pharmaceutical industry over the past 20 years, and they appear to play an even more significant role in the future of the pharmaceutical inventions [1,2]. In the case of biopharmaceuticals evolving out of patent, the development of biosimilars is rendering enormous possibilities [3–5]. Compared with traditional low molecular weight chemical drugs, which are produced by well-controlled and highly reproducible reactions, protein-based therapeutics meet huge challenges, since biopharmaceuticals are produced by living organisms and manufactured by complex processes. During cellular synthesis post- and co-translational modifications occur, which may affect biological activity and which results in an intrinsic molecular variability. Moreover, these products are sensitive to process conditions, further increasing heterogeneity of the biopharmaceuticals. In the case of biosimilars, exact copies of recombinant proteins cannot be produced due to differences in the cell cloning and in the manufacturing process. Even originator companies experience lot-to-lot variation and often suffer from difficulties in replicating their product after process changes [6,7]. A whale number of modifications presents great challenge to the thorough characterization of the molecules, so the need for suitable analytical techniques has increased. For a biosimilar product the guidelines for quality requirements claim that the active substance in the biosimilar should be similar to the one in the reference product [8]. Demonstration of similarity requires the use of appropriately selected analytical methods that are able to detect slight differences relevant to quality evaluation. Several analytical techniques, such as chromatography, mass spectrometry, electrophoresis, spectroscopy, thermal analysis, immunoassays, and bioassays may be required to completely characterize a protein [9–14].

Significant hardware and software developments in liquid chromatography brought a number of great features including good reproducibility, excellent resolution, ease of selectivity manipulation and high recoveries. Instrument manufacturers presented complete solutions, based on ultrahigh-pressure liquid chromatography (UHPLC) and bio-inert systems, offering possibilities for very fast or high-resolution separations, lower instrument adsorption of analytes and better ability to withstand harsh eluent conditions, commonly applied in biopharmaceutical analysis [15,16]. Column manufacturers have a wide range of stationary phase morphologies in their portfolio, such as sub-2 μm fully porous, superficially porous particles, nonporous materials, as well as organic and inorganic monoliths, with wide range of pore size. These particles offer better peak shape, peak area, selectivity and resolution with

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better pressure, pH and temperature stability. For characterization of therapeutic proteins, the most prevalent chromatographic modes are ion exchange (IEX), size exclusion (SEC), hydrophobic interaction (HIC), and reversed-phase (RPLC) liquid chromatography [17,18]. So far, hydrophilic interaction liquid chromatography (HILIC) has been widely used for the separation of released glycans and peptides. Recently, Zhang et al. and Pedrali et al. reported the HILIC separation of ribonuclease B glyco-variants on commercially available small pore size and custom nonporous amide modified particles [19,20]. In 2015 a new 300 Å pore size HILIC material was launched for the separation of therapeutic glycoproteins. Separation of intact MAb glyco-variants have been presented on this material [21], which may open up new possibilities in the characterization of biopharmaceuticals.

The aim of this review is to gather the possible drawbacks and challenges in the method development of therapeutic protein chromatography. The presented data is supposed to help practicing chromatographers to work out reliable and reproducible chromatographic methods in order to avoid slowing down drug discovery and process development by misleading results. The article builds upon the physicochemical stability of proteins and through these details it intends to represent the possible in-system changes of biopharmaceuticals and the possible ways to minimize or avoid these effects. Last, method development with Quality by Design (QbD) approaches and applications are also reviewed and explained.

2. Sample stability and complexity

Protein stability is a particularly important issue in pharmaceutical field and will continue to gain more significance, since the number of these products in development and registration is increasing. During production, formulation and storage toxic degradants can be generated, which can contribute to side effects, increased immunogenicity and allergic reactions. The intrinsic micro-heterogeneity is of major concern with regard to therapeutic proteins and it should be critically evaluated because differences in impurities and/or degradation products could lead to serious health implications. The presence of heterogeneity at molecular and structural levels and their contributions to biological functionality are being strictly investigated and evaluated using analytical, preclinical and clinical data.

Due to the delicate balance of stabilizing and destabilizing interactions, proteins are only marginally stable. Degradation of therapeutic proteins and peptides can be divided into two major categories, physical and chemical [22–24]. Physical degradation pathways (denaturation, aggregation, precipitation, adsorption to surfaces) imply disruption of higher-order structures, such as secondary, tertiary, or quaternary structure of a protein. Chemical instability involves damage of the primary structure, covalent modification of the protein through bond formation or cleavage. These reactions are hydrolysis, deamidation, oxidation, disulfide exchange, β-elimination and racemization, etc. Most frequent physical and chemical degradation pathways of proteins are detailed in the following sections. Discussion of sample stability is supposed to provide insight into parameters which can influence sample consistency or affect chromatographic profiles (appearance of artifact peaks and peak distortion) due to molecular changes within the instrument. Most common degradation pathways are illustrated in Fig. 1. We would like to refer interested readers to the work of Bee et al. [25]. This review presents real life examples on the effects of surfaces and leachables on the stability of biopharmaceuticals. Potential sample degradation might be present during downstream processes, formulation, transportation, storage and delivery. Effect of materials interacting solutions of biopharmaceuticals, such as steel and glass surfaces, rubber stoppers or vial septa being present also in the analyitical workflow are discussed in this paper.

2.1. Physical stability of protein samples

Disruption of higher-order structures of proteins through denaturation or unfolding is usually caused by thermal stress, extremes of pH or denaturing chemicals [26]. The term denaturation describes a complex process which involves unfolding of the protein. When proteins easily recover their native state, denaturation process is reversible. If not, it can be considered an irreversible process. If protein chains undergo chemical degradation or modification during denaturation, refolding may be incorrect or failed. Concerning denaturation induced by thermal stress, it is worth discussing the phenomenon of cold denaturation [27]. Disruption of native protein structure upon heating is well-known. Therefore heat denaturation of proteins appeared to be an obvious effect. Under frozen storage conditions, protein destabilization and aggregation can occur, which may arise from cryoconcentration of proteins and co-solutes, protein denaturation on ice-water interface, crystallization of co-solutes, pH shifts associated with buffer crystallization and cold denaturation. The susceptibility of a protein towards unfolding in a given solution is described by the Gibbs-Helmholtz equation. The expression predicts the free energy of unfolding versus temperature profile with a skewed parabola, suggesting equilibrium denaturation transitions occur both at high and low temperature. The higher temperature refers to the melting temperature and the lower one is to the phenomenon of cold denaturation [28,29].

Aggregation of proteins has been thoroughly studied [30–32]. From these results it appears that there are five general mechanisms which are the driving forces of this degradation pathway: reversible association of the native monomers, aggregation of conformationally-altered monomers, aggregation of chemically-modified products, nucleation-controlled aggregation and surface-induced aggregation. The driving force in reversible association of the native proteins is that the monomers are self-complementary; therefore, in higher protein concentrations larger oligomers are formed. These associates often become irreversible aggregates, as a consequence of formation of covalent bonds, such as disulfide linkages. The tendency of different proteins to associate reversibly is highly variable, and the strength of the association varies with solvent conditions, such as pH and ionic strength. Important examples of this mechanism are the aggregation of insulin or Interleukin-1 receptor antagonist (rhIL-1RA) [32]. In the case of conformationally- and chemically-altered monomers, aggregation is driven or catalyzed by damaged forms of protein products. Damage can arise from chemical modification (such as oxidation or deamidation) and from conformationally-altered proteins (arising from thermal stress, shear, or surface-induced denaturation) [32]. This mechanism is the dominant one for many proteins, such as interferon-γ and G-CSF [32]. In nucleation-controlled aggregation native monomers have a low susceptibility to formation of low-sized oligomers. However, if an aggregate of sufficient size manages to form, the growth of this nucleus to a much larger species is strongly favored and becomes very rapid. Two types of nucleation-controlled aggregation can be distinguished, homogeneous and heterogeneous. In homogeneous nucleation the critical nucleus is a protein aggregate itself; contrarily, in heterogeneous nucleation it is a particle of an impurity or contaminant [32]. The surface-induced aggregation starts with binding of native proteins to a surface. The binding is driven by hydrophobic interactions, electrostatic interactions or hydrogen bonding. Through the binding time proteins undergo a conformation change (e.g. to increase the contact with the surface), then altered monomers released back to the solution form oligomers with other monomers [32]. The understanding of the mechanism