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Physicochemical characterization of biopharmaceuticals

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ABSTRACT

Biopharmaceuticals are gaining interest in therapy due to their high target selectivity. Most of the recently approved biopharmaceuticals represent drugs that are produced by biotechnological processes involving recombinant DNA. Thus, this review article mainly focusses on protein therapeutics. However analogous considerations also apply for other large molecule therapeutics. As early approved blockbuster biopharmaceuticals run out of patent protection shortly, a growing interest in biosimilar production results in the need of proper analytical characterization and comparison of inventor and biosimilar. In contrast to small molecule drugs small variations in the production process may strongly impact the final biological. Thus, quality assurance of biopharmaceuticals results in much higher analytical effort compared to small molecules.

This review gives an overview on analytical methods for characterization of protein biologicals. Classical methods such as gel electrophoresis and liquid chromatography are summarized and complemented with state-of-the-art mass spectrometric investigations. Full molecule investigations of native or denaturized proteins as well as methods including digestion (middle-down and bottom-up) are discussed. Furthermore, literature on glycoprotein analysis using glycopeptide, released glycan and monosaccharide analysis is reviewed.

Abbreviations: 2D-LC, two-dimensional liquid chromatography; AA, amino acid; AC, affinity chromatography; ADC, antibody-drug conjugates; ADCC, antibody-dependent cellular cytotoxicity; AF4, asymmetric flow field fractionation; APTS, 1-aminopyrene-3,6,8-trisulfonate; Asn, asparagine; Asp, aspartic acid; ATMP, advanced therapy medicinal products; AUC, analytical ultracentrifugation; BAC, boronate affinity chromatography; CAR, chimeric antigen receptor; CD, circular dichroism; CDC, complement-dependent cytotoxicity; CE, capillary electrophoresis; CE-LIF, capillary electrophoresis-laser induced fluorescence; CEX, cation-exchange chromatography; CID, collision-induced dissociation; ConA, Concanavilin A; CQA, critical quality attributes; dAb, single-domain antibodies; DAR, drug-to-antibody ratio; DNA, desoxyribonucleic acid; DSC, differential scanning calorimetry; ECD, electron-capture dissociation; EMA, European Medicines Agency; EPO, erythropoietins; ERLIC, electrostatic repulsion hydrophilic interaction chromatography; ESI, electrospray ionization; ETD, electron-transfer dissociation; FDA, US Food and Drug Administration; FLD, fluorescence detector; FSH, follicle-stimulating hormone; FTICR, Fourier transform ion cyclotron resonance; FTIR, Fourier transform infrared spectroscopy; G-CSF, granulocyte-colony stimulating factor; GC, gas chromatography; GCC, graphitized carbon column extraction; GMP, good manufacturing practice; HAE, hydrophilic affinity extraction; HC, heavy chain; HDX-MS, hydrogen deuterium exchange mass spectrometry; HIC, hydrophobic interaction chromatography; HILIC, hydrophilic interaction liquid chromatography; HOS, higher order structure; HPAEC, high-pH anion-exchange chromatography; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; ICH, International Conference on Harmonisation; IdeS, immunoglobulin-degrading enzyme of Streptococcus pyogenes; IEF, and isoelectric focusing; IEX, ion-exchange chromatography; IMAC, immobilized metal affinity chromatography; ISD, in-source decay; isoAsp, iso-aspartic acid; ITC, isothermal calorimetry; LC, light chain; LCA, lens culinaris agglutinin; LIT, linear ion trap; *m/z*, mass-per-charge; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NDA, new drug application; nESI, nanoelectrospray; nLC, nanoflow liquid chromatography; NMR, nuclear magnetic resonance; NOTA, triazacyclononane triacetic acid; NPLC, normal phase chromatography; PAD, pulsed amperometric detection; PCR, polymerase chain reaction; PGC, porous graphitic carbon; Ph.Eur., European Pharmacopoeia; PNGase, peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase; PTM, post-translational modification; QbD, quality by design; QQQ, triple quadrupole; QTOF, quadrupole time-of-flight; RNA, ribonucleic acid; RPLC, reversed phase high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PEG, polyethlyenglycol; PET, positron emission tomography; SAXS, small-angel X-ray scattering; SCX, Strong cation exchange chromatogroaphy; SEC, size-exclusion chromatography; SPR, surface plasmon resonance; TLC, thin layer chromatography; TMS, trimethylsilyl ether; TOF, time-of-flight; UHPLC, ultrahigh performance liquid chromatography; USP, US Pharmacopoeia; UV, ultraviolet detector; WAX, weak anion exchange; WGA, wheat germ agglutinin.

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1. Introduction

In a broader sense biologicals are recognized as drugs that are produced in or extracted from biological sources. This includes antibiotics such as penicillin, polypeptide and macrolid antibiotics, antimycotics, alkaloids, therapeutic proteins, antigens, gene or cellular therapies, so called advanced therapy medicinal products (ATMPs as per regulation EC No 1394/2007 [1]) as well as stem cells, blood, or blood components, etc. [2]. A market value of nearly 400 billion US$ is expected by 2019 [3]. Most of the recently approved biopharmaceuticals represent protein drugs that are produced by biotechnological processes involving recombinant DNA. Thus, in the narrower sense the term biopharmaceuticals may be defined as therapeutic or diagnostic proteins, such as hormones, monoclonal antibodies and receptor constructs (fusion proteins). However, RNA-based therapeutics and polysaccharides may be included in this definition as well. As first biopharmaceutical the hormone insulin was produced involving biotechnological processes. Currently the Pharmacopoia Europea (Ph. Eur.) 8.3 [4] lists several biophamaceuticals such as erythropoietin, somatotropin, somatostatin, choriongonadotropin, follitropin, urofollitropin, gonadorelin and analogues, oxytocin, insulins, glucagon, alteplase (recombinant tissue-type plasminogen activator), bleomycine, blood factors (factor VII, VIII, IX and XI, prothrombin complex, von Willebrand factor), interferone solutions, calcitonin, but also heparine, acarbose, chondroitin sulfate, albumin, tuberculin, immunoglobulin Gs, antithrombin-III concentrate, botulinum toxin, chymotrypsin, pepsin, α-1-proteinase inhibitor, hyaluronidase, and urokinase.

Protein drugs may be more than 1000 times larger than small molecule drugs. As they are produced mainly in a biological medium utilizing genetically modified living systems, their synthesis is influenced by multiple parameters during the manufacturing process. This may lead to a change in three-dimensional structure of the biotherapeutic and to an impact on quality, safety, and efficacy with potentially radical clinical consequences [5].

Biopharmaceuticals are gaining more and more interest as new drugs, due to their selectivity for their respective targets and potential to treat diseases that retained from classical small molecule therapy. They represent one of the most promising areas for the development of new drugs with original mechanisms of action [6,7]. Thus, increasing numbers of biopharmaceuticals are approved in the recent years and pharma companies achieve growing revenues from biopharmaceuticals [8,9]. Approximately 250 biopharmaceutical products are on the market in the two major regions, Europe and USA [9], with peptide hormones, therapeutic antibodies (monoclonal (mAbs, structure of predominant IgG1 class in Fig. 1), bispecific mAbs, single-domain antibodies (dAbs)), and antibody-drug conjugates (ADC) as well as receptor constructs (fusion proteins), and RNA therapeutics being the major structure groups. Major therapeutical classes of biopharmaceuticals currently are hormones (22%), mAbs (22%), growth factors (13%), vaccines (10%), cytokines (8%), blood factors (6%), cytokines (8%) blood factors (6%), and other blood related biopharmaceuticals (6%) [9]. Table 1 lists the biopharmaceuticals approved by the US Food and Drug Administration (FDA) in 2015. Out of the RNA-based therapeuticals only two products are currently approved, i.e. fomivirsen (Vitravene®, cytomegalovirus retinitis) and mipomersen (Kynamro®, homozygous familial hypercholesterolemia) [10–13].

Following the first approval already in 2000, ADCs are rapidly growing in the biopharmaceutical sector. A complex is generated between a mAb and small molecule or a peptide [14,15]. As one example the first approved ADC, Mylotarg, combined an antibody targeting leukemic blast cells with a bacterial toxin (calicheamicin) [9]. The mAb ensures a target specificity of the small drug, while the latter increases the efficacy of the mAb. This combination of two therapeutic approaches is intended to improve the safety and efficacy of the small molecule. Analytical procedures for characterization may be adopted from mAbs, however need to be optimized to address the specific challenges of the ADCs [16]. Currently two ADC biopharmaceuticals are approved within the EU and the US.

Following termination of patent protection follow-up biopharmaceuticals are developed in analogy to the small molecule generics [17]. Out of these products biosimilars represent a subclass of biologicals that were proven to have high structural and clinical similarities compared to the already approved biological (inventor, originator, reference product). Abbreviated procedures for their approval as drugs are possible in analogy to the small molecule generics [18]. However, similarity has to be shown according to European Medicines Agency's biosimilar application pathway criteria or equivalent criteria from other countries. Critical quality attributes (CQA) have to be evaluated that may impact pharmacological response [19]. As many patents of block buster biopharmaceuticals run out of protection shortly, a growing interest in biosimilar production results in the need of proper analytical characterization and comparison [20]. In contrast, biobetters or biosuperiors are considered as follow-up products with improved potency, reduced toxicity or immunogenity, or tailored drug half-life, while therapeutic target or indication remain unchanged [17,21,22]. For example biobetter antibodies are developed to target the same epitope as an originator antibody, but with enhanced attributes, e.g. improved glycosylation pattern to optimize effector function or an engineered fragment crystallizable (Fc) region to prolong the serum half-life [23]. Potential structure modifications are illustrated in Fig. 1 [24]. Glyco-engineered antibodies were achieved from expression systems such as CHO cells and yeast strains. Thereby a fucosylated *N*-glycan in the Fc domain of rituximab and trastuzumab led to an increase of the antibody-dependent cellular cytotoxicity (ADCC) by a factor of 40–100 [25,26]. An alternation of 2–3 amino acids in Fc region of rituximab, trastuzumab, bevacizumab has led to an extended half-life and pharmacokinetics [27]. This may result in lower dosages and lower costs of the product, as well as better compliance due to easier administration schemes.

Within this review we mainly focus on therapeutic proteins and their characterization by (physico-)chemical methods. Characterization of other types of biologicals (esp. polysaccharides or RNA-based therapeutics) may follow analogous workflows.

2. International regulations

2.1. Methods in pharmacopoeias

In current pharmacopoeias a few biopharmaceuticals are monographed. Their monographs usually include the verification of the activity (in addition to identity, purity and assay as in small molecule monographs). In specific cases assay may be replaced by activity testing. For therapeutic proteins mainly gel electrophoresis (SDS-PAGE and isoelectric focusing (IEF) with immunoblotting), capillary electrophoresis with UV detection (CE-UV), reversed phase high performance liquid chromatography (RPLC)-UV after tryptic or endoprotease LysC R digest for peptide mapping (Ph.Eur. 2.2.55), N-terminal sequence analysis, electrospray ionization mass spectrometry (ESI–MS) and HPLC-ESI–MS of the intact proteins, amino acid analysis (Ph.Eur. 2.2.56), glycan analysis after Peptid-*N*-Glycosidase F cleavage (Ph.Eur. 2.2.59), and a determination of neutral sugars are mentioned for identity testing [4,28].

Purity is mainly assessed by RPLC-UV, size exclusion chromatography (SEC)-UV (Ph.Eur. 2.2.30), CE-UV, thin layer chromatography (TLC) on silica detection by *N*-chlorination, total protein content (Ph.Eur. 2.5.33, photometric assay or Kjeldahl method Ph.Eur.