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High resolution reversed phase analysis of recombinant monoclonal antibodies by ultra-high pressure liquid chromatography column coupling

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ABSTRACT

Monoclonal antibodies (mAbs) represent one of the fastest growing areas of new drug development. However, their analytical characterization is complex and generally requires an array of orthogonal analytical techniques. Reversed phase liquid chromatography is a valuable strategy due to its high resolving power and straightforward compatibility to mass spectrometry. The present study demonstrates that high peak capacity can be attained with intact mAb of ~150 kDa, reduced mAb fragments of ~25–50 kDa and also digested mAb generating numerous peptides of ~0.5–3 kDa. Several long columns packed with fully porous wide-pore sub-2 μm particles were coupled in series to evaluate the effect of column length on peak capacity. By using three columns of 150 mm length, a mobile phase temperature of 80 °C and a gradient time of around 20 min, peak capacities of 117 and 128 were obtained for a commercial intact mAb and its reduced mAb fragments, respectively. On the other hand, when peptide mapping was performed at 50 °C, with a gradient time of 270 min and a column length of 450 mm, a peak capacity of more than 700 was achieved.

1. Introduction

Recombinant monoclonal antibodies (mAbs) have become particularly effective for the treatment of autoimmune diseases or cancers [1]. Since 2007, approximately 40 novel mAbs entered into clinical studies each year, and new products are regularly approved by the US FDA (United States Food and Drug Administration) and the EMA (European Medicines Agency) [2]. Out of the 15 top selling drugs in 2012, six are mAbs (Humira, Enbrel, Remicade, Rituxan, Herceptin and Avastin) [3]. Historically, the approval rate of MAbs appeared to be significantly higher than that of small molecule drugs (~20% vs. 5%) [4]. Furthermore, the difficulty in getting approvals for biosimilars (or follow-on biologics) makes mAbs more attractive than small molecule drugs in regard to facing stiff generic challenge after patent expiration [5]. Reasons are the higher cost of manufacturing biologics, smaller price differentials, as well as the mandatory need to conduct clinical trials for biosimilars.

IgGs are large tetrameric glycoproteins with molecular weights of approximately 150 kDa and are structurally composed of four polypeptide chains: two identical heavy chains (HC, ~50 kDa) and two identical light chains (LC, ~25 kDa) connected through several inter-chain disulfide bonds at their hinge region [6]. Functionally, mAbs consist of two regions, the crystallizable fragment (Fc) and the antigen-binding fragment (Fab) [7]. The Fab domain (~50 kDa) is composed of the LC and the variable domain of the HC. Because of their inherent complexity and for safety reasons, there is a need to develop appropriate analytical methods to provide detailed characterization of mAbs [1]. There are several common modifications leading to antibody charge variants (or isoforms) on the peptide chains (e.g., deamidation, C-terminal lysine truncation, *N*-terminal pyroglutamation, methionine oxidation, glycosylation variants...) and size variants (e.g., aggregation or incomplete formation of disulfide bridges). The combination of these micro-heterogeneity sources in the peptide chains significantly increases the overall micro-heterogeneity of an entire IgG.

In reversed phase liquid chromatography (RPLC), there have been a significant number of innovations in terms of instrumentation and column technology during the last decade. The goal of these new technologies was to allow for faster or higher resolution analysis. These innovations work well for small molecules and increasingly so for large biomolecules, such as therapeutic peptides, proteins or monoclonal antibodies. Beside RPLC, other modes of liquid chromatography such as size exclusion (SEC), ion-exchange (IEX) or hydrophobic interaction chromatography (HIC) are also generally applied for protein characterization. Recently, a systematic study compared the different liquid chromatographic modes for protein and mAb analysis in quantitative point of view [8].

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Today, ultra-high pressure liquid chromatography (UHPLC) is gradually becoming the standard HPLC platform. Recent developments in RPLC, such as columns packed with wide-pore sub-2 μm particles, wide-pore core–shell particles, organic monolith columns, non-porous materials, and porous layer open tubular (PLOT) columns, now allow a dramatic increase in the efficiency and resolution on protein separations, even with intact mAbs of ~150 kDa [9–12]. As an example, Krull and Rathore recently highlighted the advantages of UHPLC over HPLC for mAb samples [13]. It was demonstrated that for large proteins and antibodies (less so for peptides), conventional HPLC often suffered from relatively lower performance (lack of efficiencies, peak capacities, poor peak shapes) when compared with UHPLC separations of proteins using 100–150 mm long columns [13]. Although the papers on this topic remain sparse, several examples illustrate that UHPLC and core–shell technologies are promising approaches for the analysis of mAbs [13].

Using RPLC, there are different ways to analyze and characterize monoclonal antibodies. The first one – to evaluate the primary structure – is to completely digest these large glycoproteins. This peptide mapping (bottom up approach) is most commonly performed with trypsin as the protease; however other specific proteases can be employed to generate a mixture of peptides [14]. For tryptic digests, the chromatograms are complex and the digestion process is generally time consuming. An alternative approach consists in performing limited proteolysis (LP approach) [2–4]. The principle is to digest the mAb with specific enzymes such as papain, pepsin, lys-C or Fabricator (a modified cysteine protease) to obtain large fragments of ~25–50 or 100 kDa. In this case, the homogeneity or variability of the large antibody fragments can be evaluated, and both the sample preparation and separation are faster. This LP approach generally gives suitable results in ion exchange liquid chromatographic (IEX) conditions and is typically used to resolve Fab and Fc charge variants. Finally, the mAb can also be analyzed in its intact form, to determine the heterogeneity (drug related substances, impurities) of the produced mAb or under its reduced form, to obtain two heavy chain fragments of ~50 kDa and two light chain fragments of ~25 kDa for determining the mAb integrity under denaturing conditions [6]. Fig. 1 shows a schematic diagram of the different approaches commonly used in the chromatographic characterization of mAbs. Both the "bottom up" and "LP" strategies require very high resolving power to separate either the hundreds of peptide peaks or the very closely related large fragments of 25–50 kDa. Therefore, there is a need for very high kinetic efficiency that can primarily be reached by the coupling of very efficient columns in series (e.g. up to 300–450 mm long columns packed with 1.7 μm particles) [11].

The goal of the present study was to investigate the possibilities to perform very high resolution separations by coupling columns in series, and to show the achievable kinetic performance for reversed phase antibody separations. Examples for the high resolution UHPLC separations of intact, fragmented or completely digested mAb samples within reasonable analysis time are presented. The peak capacity was doubled by using 450 mm long columns compared to the generally used 150 mm long columns. Selectivity, efficiency and possible thermal degradations were important considerations when applying these long columns with relatively long gradients.

2. Experimental

2.1. Chemicals, columns

Acetonitrile (gradient grade) was purchased from Sigma–Aldrich (Buchs, Switzerland). Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA).

Commercially available IgG2 monoclonal antibody was purchased from Amgen (Switzerland). Dithiothreitol (DTT), trypsin (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) treated, from bovine pancreas), trifluoroacetic acid (TFA), formic acid (FA) and ammonium hydrogen-carbonate were all obtained from Sigma–Aldrich (Switzerland).

Acquity BEH-300 C18 columns with a particle size of 1.7 μm (150 mm × 2.1 mm, 300 Å) were purchased from Waters (Milford, MA, USA) and coupled in series using 1 μl injection loops.

2.2. Equipment, software

All measurements were performed using a Waters Acquity UPLC™ system equipped with a binary solvent delivery pump, an

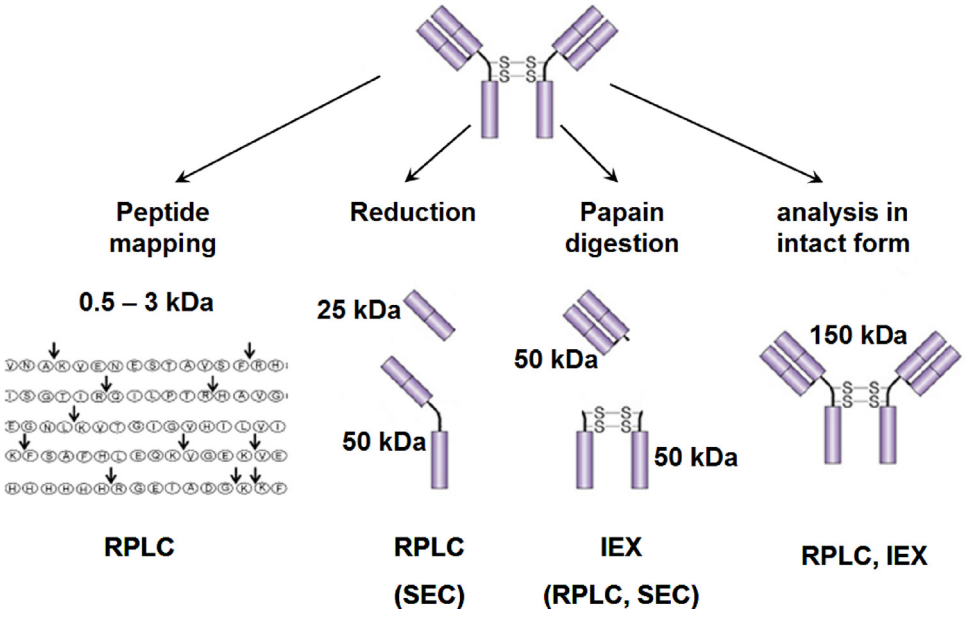

Fig. 1. Schematic presentation of the most common chromatographic mAb analysis approaches. The primarily used techniques are indicated directly below figure while other often used modes are indicated in parenthesis.