Method for Host Protein Analysis in Recombinant Protein Preparations

Technical Field

The invention relates to an improved sample preparation method for LC-MS/MS analysis that enables an efficient isolation, purification and identification of protein contaminants, such as host cell proteins (HCPs) as process-related impurities generated during the production of biopharmaceuticals, such as monoclonal antibodies (mAbs).

Technological Background

Host cell proteins (HCPs) are process-related impurities generated during the production of biopharmaceuticals that may contaminate the final product unless they are efficiently removed. Due to their potential impact on product safety and efficacy, regulatory authorities require removal of HCPs in final manufactured biopharmaceuticals down to trace amounts. The current standard method for detecting HCPs is enzyme-linked immunosorbent assay (ELISA), which only reveals the total amount of HCPs.

A sensitive and high-throughput LC-MS technique that permits identification and quantification of individual HCPs has been demonstrated to be a potential alternative technique. However, differences in sample preparation methods and MS acquisition techniques have led to discrepancies in detected HCPs between studies, which may compromise patient safety and efficacy. To address this issue, the inventors developed a novel and reproducible method for isolating and digesting of HCPs for ensuing mass spectrometry detection that is applicable to downstream process of recombinant proteins, such as monoclonal antibodies (mAbs) and can be easily implemented in standard laboratories without the need for dedicated instrumentation.

Therapeutic monoclonal antibodies (mAbs) are currently the predominant class of newly approved drugs worldwide, with more than 100 mAbs approved for the treatment of various diseases and approximately 1200 under clinical trials.

The majority of mAbs are produced in engineered cell lines, particularly mammalian cells, which may introduce unwanted process-related impurities known as host cell proteins (HCPs). These

HCPs, if not effectively removed, can induce immunogenic responses in patients and compromise product stability by degrading the formulation excipient (e.g., polysorbate) or the mAbs themselves. Therefore, regulatory authorities, such as the European Medicines Evaluation Agency (EMEA) and the Food and Drug Administration (FDA), have stipulated that HCPs should be extensively removed throughout the manufacturing process. Despite a lack of prescribed specifications, manufacturers typically aim for a range of 1 to 100 parts per million (ppm) of HCPs in the final product. Consequently, various analytical techniques have been developed to routinely assess HCPs at the ppm level during biopharmaceutical manufacturing.

Enzyme-linked immunosorbent assay (ELISA) has been traditionally used to monitor ppm levels of HCPs. However, because it cannot identify individual HCPs, most biopharmaceutical manufacturers implement an orthogonal method for risk mitigation. Recent advancements in liquid chromatography-mass spectrometry (LC-MS) could potentially make bottom-up proteomics a promising tool for HCP assessment. In this method, proteins are enzymatically digested and then identified at the peptide level. Once HCPs have been identified, specific removal procedures and risk assessments can be conducted to support process development. However, given the high dynamic range regarding the relative amount of mAbs to HCPs (five to six orders of magnitude) in a sample from any mAb production process, the success of this strategy is strongly dependent on the sample preparation procedure (e.g., peptide fractionation or mAb depletion) and sensitivity of MS detection. Improved sample preparation approaches are needed to address this challenge and to decrease the detection limit of HCPs.

Owing to differences in the utilized workflows, discrepancies in identified HCPs between studies could pose a risk to patient safety and drug efficacy. For example, in several studies, HCPs identified from National Institute of Standards and Technology (NIST) mAb ranged from 60 to 850 proteins. The diversity of HCPs identified in therapeutic drugs is even more important when such data are used to direct the manufacturing process and assess the safety of the final drug for widespread use within the community.

One of the main sources of variability in HCP identification by bottom-up proteomics is sample preparation, which varies between manufacturers and biopharmaceutical products. Various methods have been used to fractionate peptides or deplete mAbs. Native digestion and molecular weight cut-off filtration have recently been used as alternatives. However, these techniques are not standardized and may only be applicable to specific subclasses of mAbs, making them less suitable for quantifying problematic HCPs. For example, previous studies have shown that phospholipase B-like 2 (PLBL2) and cathepsin D interact with mAbs under protein A chromatography conditions and

appear at higher levels in the eluates. These HCPs have the potential to induce immunogenicity or hydrolysis of mAbs. Consequently, use of protein A chromatography to separate HCPs followed by LC-MS/MS analysis may not provide a complete picture of HCPs with specific levels of risk for use in root cause investigations. Significant progress has been made to overcome this problem by using pre-elution washes to disrupt HCP-mAb interactions and improve HCP removal by protein A chromatography. However, studies have revealed that the types of HCPs co-eluting with the product vary between molecules, posing a significant bottleneck in the biopharmaceutical industry, especially when short turnaround times are essential.

Moreover, the time-consuming sample preparation processes require for HCPs, including reduction, alkylation, desalting and overnight digestion, may hamper high-throughput LC-MS analysis. Therefore, the lack of a rapid and generic method for HCP isolation and digestion remains a major obstacle when time and sample throughput are critical constraints.

To address these challenges, the present application provides an integrated strategy with the objective of ensuring efficient and reliable sample preparation for protein contaminants (isolation and digestion) across different classes of recombinant proteins. The approach aims to reduce the dynamic range of recombinant protein to protein contaminants, providing a reproducible, straightforward and easily implementable method that can be conducted in standard laboratories. The approach was applied to investigate the identification and quantification of high-risk HCPs in a case study for mAb bioprocessing.

Recently, Fc receptor (FcγRIIIa) affinity chromatography has been utilized to improve the analytical characterization of mAbs by monitoring Fc glycan heterogeneity during process development and manufacturing [1, 2]. In the present invention a Fc receptor (FcγRIIIa) affinity column was used to deplete mAbs and isolate an enriched HCP fraction. The fraction was then concentrated using a cut-off filter and proteins were precipitated with acetone. Afterwards, the protein pellets were solubilized, denatured, reduced and digested in a solution containing sodium deoxycholate (SDC) and N-dodecyl-beta-D-maltoside (DDM) as suitable detergents. Comparison of the HCP fractions isolated by FcγRIIIa and protein A chromatography showed a significant improvement in HCP identification when FcγRIIIa was used. The newly developed method streamlines the sample preparation process, reducing the variability and turnaround time. This advancement has the potential to provide valuable insights into problematic or high-risk HCPs, thereby enhancing control strategies during purification assessments in biopharmaceutical companies.

To reduce the variability in protein contaminant identification due to different sample preparation procedures and low concentrations of protein contaminants, a more efficient sample preparation

workflow is needed. In the present application, a new procedure is disclosed that combines protein contaminant isolation on a Fc receptor affinity column with “single tube” protein digestion. The method is shown to significantly improve the number of identified protein contaminants compared to protein A chromatography by reducing the ratio of the amounts of recombinant protein to protein contaminants. The approach is applicable to a wide range of recombinant proteins and provides a reproducible, straightforward and easily implementable method that can be conducted in standard laboratories. The disclosed sample preparation method will be valuable for monitoring protein contaminant levels during manufacturing processes and can be widely used for root cause investigations. Furthermore, it can help assess the biosimilarity of a candidate to a reference product and guide process development to improve overall HCP clearance in the downstream process.

The application provides a rapid and efficient workflow for the isolation and digestion of protein contaminants, such as HCPs. Fc-receptor (FcγRIIIa) affinity chromatography was employed to separate the HCP fraction by mAb depletion. The HCPs in the HCP fraction can be precipitated and digested using a "single-tube" method that improves digestion performance and prevents sample loss of problematic low-abundance protein contaminants. The method of the invention is shown to outperform protein A affinity chromatography for monitoring problematic HCPs.

Summary of the invention

In one aspect, the present invention relates to a method for isolating and analyzing one or more protein contaminants in a sample comprising a recombinant protein and one or more protein contaminants, the method comprising (a) fractionation of the sample comprising the recombinant protein and the one or more protein contaminants, wherein the fractionation comprises contacting the sample with an Fc receptor affinity resin and collecting the flow-through (FT) to obtain a fraction comprising the one or more protein contaminants; and (b) analysis of the one or more protein contaminants in the fraction comprising the one or more protein contaminants.

Figures

Figure 1: Experimental workflow. mAbs are depleted using a Fc receptor column (e.g., an FcγRIIIa) and collected HCP fractions are concentrated (e.g., by a 10 kDa cut-off filter). Proteins are precipitated (e.g., by addition of acetone) and dissolved (e.g., in SDC/DDM solution), then reduced and digested (e.g., by trypsin). Peptides were analyzed by LC-MS/MS and raw files were searched against a CHO protein sequence database to identify HCPs.

Figure 2: FcγRIIIa affinity UV chromatograms (at 280 nm) of (A) mAb1 and (B) mAb2 in glycosylated form (dotted trace) and after removal of N-glycan with PNGase F (solid trace).

Figure 3: Analysis of mAb2 fragments after digestion with FabALACTICA. (A) FcγRIIIa affinity chromatogram with UV detection at 280 nm (the FT window of this FcγRIIIa affinity column is from about 5 to about 12 min); RP-LC-MS analysis of (B) FT peak (fraction 1) (the RT window of this RP-LC-column is from 5 to 20 min) and (C) peaks at RT of 30-45 min (fraction 2); deconvoluted mass spectrum of (D) fraction 1 and (E) fraction 2.

Figure 4: Analysis of mAb2 fragments after digestion with IdeS. (A) FcγRIIIa chromatogram with UV detection at 280 nm(the FT window of this FcγRIIIa affinity column is from about 5 to about 12 min); (B) RP-LC-MS analysis of the FT fraction (the RT window of this RP-LC-column is from 5 to 20 min); deconvoluted mass spectrum of the peak at RT of (C) 14.8 min; and (D) 16.8 min.

Figure 5: UPS1 protein identification and peptide intensity of all detected peptides after digestion ranging from 15 min to 60 min at 37 °C. The numeric values above the bars indicate the average sequence coverage of all UPS1 proteins identified. The level of sequence coverage for identified UPS1 proteins is represented by different colors.

Figure 6: Overlay UV chromatograms (at 280 nm) of three subsequent injections of mAb1 obtained by FcγRIIIa affinity chromatography. Fraction 1 was collected and utilized in a bottom-up approach for HCP identification.

Figure 7: Distribution of signal intensities (MS signal) of all detected peptides of protein PLBL2 in each fraction of (A) protein A and (B) FcγRIIIa affinity chromatography.

Figure 8: Schematic representation of the protein A workflow according to [3]. Briefly, the workflow includes the following steps: (I) Protein A column (HiTrap MabSelect SuRe, 1 mL, Sigma-Aldrich) was equilibrated with 10 mL of 20 mM Na_2HPO_4, pH 7.3. (II) 5 mg of mAb1 is diluted to a final volume of 2 mL (concentration of 5 mg/mL) using 20 mM Na_2HPO_4, pH 7.3, and loaded into the protein A column, and the flow-through was collected (F1-protein A). (III) Load the column with the pre-elution solution and collect the fraction (F2-protein A). (IV) Re-equilibrate the column with 20 mM Na_2HPO_4, pH 7.3 (waste). (V) Elute the mAb1 with 5.0 mL of 100 mM glycine buffer, pH 2.8, and collect the fraction (F3-protein A).

Detailed description of the invention

List of abbreviations used in this application

ABC	ammonium bicarbonate
ACN	acetonitrile
ADCC	antibody-dependent cell-mediated cytotoxicity
AGC	automatic gain control
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DDM	N-dodecyl-beta-D-maltoside
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EMEA	European Medicines Evaluation Agency
FDA	Food and Drug Administration
FDR	false discovery rate
FT	flow through
HC	heavy chain
HCD	higher energy c-trap dissociation
HCP(s)	host cell protein(s)
LC	light chain
LC-MS/MS	liquid-chromatography-(tandem) mass spectrometry
mAb	monoclonal antibody
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NIST	National Institute of Standards and Technology
ppm	parts per million
RP	reverse-phase
RT	retention time
SDC	sodium deoxycholate
SDS	sodium dodecyl sulfate
THPP	tris(3-hydroxypropyl)phosphine
TCEP	tris 2-carboxyethyl phosphine
TFA	trifluoroacetic acid
TIC	total ion currents
UHPLC	ultra-high performance liquid chromatography
UPS-1	universal proteomics standard 1

The fractionation of the sample comprising a recombinant protein and one or more protein contaminants serves to deplete the sample of recombinant protein and thereby achieve a relative

enrichment of the one or more protein contaminants to support an efficient and sensitive detection and analysis, e.g., by mass-spectrometric analysis.

Contacting of a sample with an Fc receptor affinity resin as described herein results in two fractions, a bound fraction and a flow-through fraction. The bound fraction, which can also be called the retained fraction, comprises all proteins comprised in the sample that interact with the employed affinity resin, i.e., that are retained by the Fc receptor affinity resin and therefore elute significantly later from the affinity resin as compared to the flow-through. The flow-through fraction comprises all proteins comprised in the sample that do not interact, or essentially do not interact with the employed affinity resin. As referred to herein “collecting the flow-through” means collecting the eluate, or at least part of the eluate that emerges from the Fc receptor affinity resin under eluent conditions under which the recombinant protein binds to the affinity resin. Accordingly, “collecting the flow-through” may refer to collecting the eluate or at least part of the eluate which exits a chromatography column under eluent conditions under which the recombinant protein binds to the affinity resin comprised in the column. This collected eluate can also be called the collected flow-through. It comprises at least part, preferably all proteins that have not bound to (that were not retained by) the Fc receptor affinity resin. For the ease of reading, the term flow-through is used throughout the specification, but it is to be understood as covering not only the flow-through of a chromatography column, but any solution obtained after contacting of the sample with the Fc receptor affinity resin that comprises at least part, preferably all proteins that have not bound to the Fc receptor affinity resin.

In a preferred embodiment, the one or more protein contaminants are one or more host cell proteins. Host cell proteins are protein contaminants that are derived from the host cells that were used to produce the recombinant protein. Recombinant proteins are commonly produced in cell culture using CHO, NS0, Sp2/0, HEK293 or PER.C6 cells. Thus, in specific embodiments, the one or more protein contaminants are host cell proteins derived from a cell line selected from the group consisting of CHO, NS0, Sp2/0, HEK293, and PER.C6 cells. In a preferred embodiment, the one or more protein contaminants are host cell proteins derived from a CHO cell line.

The method according to the invention comprises fractionation of a sample comprising a recombinant protein and one or more protein contaminants, wherein the fractionation comprises contacting the sample with an Fc receptor affinity resin and collecting the flow-through to obtain the fraction comprising the one or more protein contaminants. In some embodiments, the Fc receptor affinity resin comprises an immobilized Fc receptor that binds to the recombinant protein. That means, the Fc receptor immobilized on the Fc receptor affinity resin is chosen such that the Fc

receptor binds to the recombinant protein. The Fc receptor “binds to” the recombinant protein in the sense of the present application, when a specific interaction occurs between the Fc receptor and the recombinant protein. This results in an association of the recombinant protein to the Fc receptor affinity resin and a reduction of the amount of recombinant protein in the flow-through, as compared to the amount of the recombinant protein in the initial sample (prior to fractionation).

In one embodiment, the Fc receptor affinity resin is a resin onto which a Fc receptor is attached, preferably covalently bonded. The Fc receptor is immobilized on the resin. The resin may be any known resin that is stable under conditions which are conventionally applied for protein affinity purification (e.g. affinity chromatography), a widely used example is polymethacrylate, In a preferred embodiment, the resin is used in form of non-porous or porous polymethacrylate base beads. A typical particle size of the beads is about 2 to 15 micrometers, e.g. 5 or 10 micrometers. In case of a porous resin a typical nominal pore size may be about 100 nm. So, the Fc receptor affinity resin is an Fc receptor immobilized on a stationary phase, the resin, and is usually used in form of a chromatographic column into which the resin with the immobilized Fc receptor is packed.

In one embodiment, the Fc receptor affinity resin is an Fc gamma receptor affinity resin or a neonatal Fc receptor (FcRn) affinity resin, preferably an Fc gamma receptor affinity resin. The Fc gamma receptor affinity resin may be a type I Fc gamma receptor (FcγR) affinity resin, a type II Fc gamma receptor (FcγR) affinity resin or a type III Fc gamma receptor (FcγR) affinity resin; preferably the Fc gamma receptor affinity resin is a type I Fc gamma receptor (FcγR) affinity resin; more preferably, the type I Fc gamma receptor (FcγR) affinity resin is selected from the group of FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb affinity resin, even more preferably, the type I Fc gamma receptor (FcγR) affinity resin is a FcγRIIIa, affinity resin. Accordingly, in a preferred embodiment, the Fc receptor affinity resin is a type I Fc gamma receptor (FcγR) affinity resin. In a further preferred embodiment, the type I Fc gamma receptor (FcγR) affinity resin is selected from the group of FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb affinity resin. In a more preferred embodiment, the type I Fc gamma receptor (FcγR) affinity resin is a FcγRIIIa, affinity resin.

The Fc receptor affinity resin may be used in the form of a chromatography column. The Fc receptor affinity resin in the form of a chromatography column is used for column chromatography. Accordingly, in one embodiment, the fractionation comprises contacting the sample with an Fc receptor affinity resin, wherein the contacting comprises column chromatography.

Affinity chromatography generally involves the use of a stationary phase (e.g., an Fc receptor affinity resin) and a mobile phase. In the context of the present invention, the mobile phase used for

Fc receptor affinity chromatography may be any mobile phase that is suitable for use with the employed stationary phase (i.e., the used Fc receptor affinity chromatography column). Chakrabarti et al. [12] discloses suitable conditions for protein purification using Fc receptor affinity chromatography.

In some embodiments, column chromatography, using an Fc receptor affinity resin as described herein, comprises the use of a mobile phase that is an aqueous buffer having a pH of between about 3 and about 8, preferably of between about 4 and about 8, more preferably of between about 4.5 and about 7.5. In some embodiments, column chromatography comprises the use of a mobile phase that is a citrate buffer (e.g., ammonium citrate) or an acetate buffer (e.g., ammonium acetate). In some embodiments, column chromatography comprises the use of a mobile phase that is an aqueous buffer having a buffer concentration of between about 10 mM and about 200 mM, preferably between about 20 mM and about 150 mM, more preferably between about 30 mM and about 100 mM. In a specific embodiment, the buffer concentration is about 50 mM.

In some embodiments, column chromatography comprises the use of a mobile phase that is a gradient of two aqueous buffers. Typically, elution from an Fc receptor affinity resin is achieved by a gradual decrease of the pH of the mobile phase, e.g., by using a pH gradient, such as a gradient from about pH 7 to about pH 4, preferably from about pH 7.0 to about pH 4.0. Accordingly, in some embodiments, the mobile phase is a gradient of two aqueous buffers, wherein the first buffer has a pH of between about 6 and about 7, preferably of between about 6.0 and about 7.0 and the second buffer has a pH of between about 3.5 and about 5, preferably between about 4 and about 5, more preferably between about 4.0 and about 5.0. In preferred embodiments, the mobile phase is a gradient of two aqueous buffers, wherein the first buffer has a pH of about 6.5 and the second buffer has a pH of about 4.5. In specific embodiments, the mobile phase is a gradient of two aqueous citrate buffers having a buffer concentration of between about 10 mM and about 200 mM, wherein the first buffer has a pH of between about 6 and about 7, preferably of between about 6.0 and about 7.0 and the second buffer has a pH of between about 3.5 and about 5, preferably between about 4 and about 5, more preferably between about 4.0 and about 5.0.

As used herein, the term “recombinant protein” refers to proteins that are recombinantly produced, preferably by a cell line. The recombinant protein may be useful for therapeutic purposes. Accordingly, the recombinant protein may be a therapeutic protein. However, the usefulness of the disclosed method is not limited to recombinant proteins that possess therapeutic activity, but applies to any recombinantly produced protein.

The method according to the invention relies on a retention of the recombinant protein by the Fc receptor affinity resin, while the one or more protein contaminants are not retained by the Fc receptor affinity resin. Accordingly, in some embodiments, the recombinant protein binds to the Fc receptor affinity resin. The recombinant protein “binds to” the Fc receptor affinity resin in the sense of the present application, when a specific interaction occurs between the Fc receptor and the recombinant protein. This results in an association of the recombinant protein to the Fc receptor affinity resin and a reduction of the amount of recombinant protein in the flow-through, as compared to the amount of the recombinant protein in the sample comprising the recombinant protein and the one or more protein contaminants (prior to fractionation).

In some embodiments, the recombinant protein is an antibody or an antigen-binding fragment thereof or an Fc fusion protein. In a preferred embodiment, the recombinant protein is an antibody or an antigen-binding fragment thereof. Fc fusion proteins are proteins that comprise an Fc domain, which has been recombinantly linked to a peptide or protein of interest. The Fc domain of the Fc fusion protein may for example be derived from an IgG-type constant region as described herein.

Accordingly, in one embodiment, the Fc domain is an IgG-type Fc domain. In a preferred embodiment, the Fc domain is an IgG1-type, an IgG2-type, an IgG3-type or an IgG4-type Fc domain. In a more preferred embodiment, the Fc domain is an IgG1-type, an IgG2-type or an IgG4-type Fc domain. In an even more preferred embodiment, the Fc domain is an IgG1-type or an IgG4-type Fc domain.

In some embodiments, the antibody or antigen-binding fragment thereof is a monoclonal antibody or an antigen-binding fragment thereof. In some embodiments, the antibody or antigen-binding fragment thereof is a chimeric antibody or an antigen-binding fragment thereof. In some embodiments, the antibody or antigen-binding fragment thereof is a humanized antibody or an antigen-binding fragment thereof. In some embodiments, the antibody or antigen-binding fragment thereof is a human antibody or an antigen-binding fragment thereof. In some embodiments, the antibody or antigen-binding fragment thereof is a single-chain antibody or an antigen-binding fragment thereof.

The term monoclonal antibody as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translation modifications (e.g., isomerizations, amidations) that may be present in minor amounts.

The term human antibody includes antibodies having variable and constant regions corresponding substantially to human germline immunoglobulin sequences known in the art, including, for example, those described by Kabat *et al.* [6]. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs, and in particular, CDR3. The human antibody can have at least one, two, three, four, five, or more positions replaced with an amino acid residue that is not encoded by the human germline immunoglobulin sequence.

Furthermore, the term antibody as employed herein also relates to derivatives or variants of the antibodies described herein which display the same specificity as the described antibodies. Examples of antibody variants include humanized variants of non-human antibodies, affinity matured antibodies (see, e.g., Hawkins et al. [7] and Lowman et al. [8]) and antibody mutants with altered effector function(s) (see, e.g., Kontermann and Dübel [9]).

In addition to full-length antibodies, the term antibody as used herein also includes antibody fragments and antibody derivatives, such as scFv-Fc, hclgG or lgNAR antibodies. Further envisaged are (bispecific) single chain antibodies, "Fc DART" antibodies and "lgG DART" antibodies, Triomab antibodies, CrossMab antibodies, otho-Fab lgG antibodies and DVD-lgG (Kontermann 2015 [10]).

In some embodiments, the recombinant protein comprises an Fc domain or a fragment of an Fc domain, preferably a glycosylated Fc domain or a fragment of a glycosylated Fc domain. In certain embodiments, the recombinant protein comprises an Fc domain or a fragment of an Fc domain and is an antibody or an antigen-binding fragment thereof or an Fc fusion protein.

As used herein, the term Fc domain refers to the polypeptides comprising the constant region of an antibody excluding the variable domains(s) and the first constant region immunoglobulin domain. For lgG-type antibodies, the Fc domain comprises immunoglobulin domains CH2 and CH3 and the hinge region between immunoglobulin domains CH2 and CH1.

In some embodiments, the Fc domain is a murine Fc domain or a human Fc domain. In a preferred embodiment, the Fc domain is a human Fc domain. In some embodiments, the Fc domain comprises immunoglobulin domains CH2 and CH3 and the hinge region between immunoglobulin domains CH2 and CH1, wherein the CH2 domain, the CH3 domain and the hinge region are human.

In further embodiments, the recombinant protein comprises an IgG-type constant region. In certain embodiments, the recombinant protein comprises an IgG-type constant region and is an antibody or an antigen-binding fragment thereof or an Fc fusion protein.

As used herein, an IgG-type constant region comprises immunoglobulin domains CH1, CH2 and CH3, together with the hinge region between immunoglobulin domains CH2 and CH1. The IgG-type constant region may be an IgG1-type, an IgG2-type, an IgG3-type or an IgG4-type constant region. Preferably, the IgG-type constant region is an IgG1-type constant region, an IgG2-type constant region or an IgG4-type constant region. More preferably, the IgG-type constant region is an IgG1-type constant region or an IgG4-type constant region.

In some embodiments, the IgG-type constant region is a murine IgG-type constant region or a human IgG-type constant region. In a preferred embodiment, the IgG-type constant region is a human IgG-type constant region.

Accordingly, in some embodiments, the recombinant protein is an IgG-type antibody, preferably an IgG1-type antibody, an IgG2-type antibody, an IgG3-tyspe antibody or an IgG4-type antibody, more preferably an IgG1-type antibody, an IgG2-type antibody or an IgG4-type antibody, even more preferably an IgG1-type antibody or an IgG4-type antibody.

Interactions between Fc receptors and their substrates can depend on the glycosylation profile of the substrates [1, 2, 12]. Accordingly, the degree of retention that can be achieved with an Fc receptor-based resin (e.g., an Fc receptor-based chromatography column) can depend on the substrate recombinant protein being glycosylated, in particular N-glycosylated. This applies in particular to embodiments wherein the recombinant protein is an antibody or an antigen-binding fragment thereof or an Fc fusion protein, or wherein the recombinant protein comprises an Fc domain or an IgG-type constant region. Retention on an Fc receptor-based resin is enhanced for such recombinant proteins that are glycosylated within the Fc domain or within the IgG-type constant region. As described in [1, 2], larger N-glycans generally result in higher affinity of Fc receptors towards the substrate recombinant proteins and therefore longer retention times on an Fc receptor affinity resin (e.g., an FcγRIIIa affinity resin). The number of terminal galactose saccharides within the N-glycan has also been shown to correlate positively with retention times on Fc receptor affinity resin (e.g., an FcγRIIIa affinity resin).

Thus, in certain embodiments, the recombinant protein (e.g., the antibody or antigen-binding fragment thereof or the Fc fusion protein) is glycosylated. In a preferred embodiment, the recombinant protein (e.g., the antibody or antigen-binding fragment thereof or the Fc fusion protein)

comprises an N-glycan, i.e., is N-glycosylated. In a further preferred embodiment, the recombinant protein comprises an N-glycan at position 297 according to EU numbering, or a corresponding amino acid. In specific preferred embodiments, the recombinant protein is an antibody or an antigen-binding fragment thereof (e.g., an IgG-type antibody) or an Fc fusion protein, or comprises an Fc domain or an IgG-type constant region and comprises an N-glycan at position 297 according to EU numbering (according to Edelman et al. [11]), or a corresponding amino acid.

A glycan attached to an asparagine residue is referred to herein as an N-glycan. In the context of the present invention, the structure of the N-glycan and the number of sugar residues comprised therein may have an influence on the retention of the recombinant protein by the Fc receptor affinity resin (an exemplary overview in this regard is provided in [13]). For example, the N-glycan may comprise a core fucose saccharide, or it may not comprise a core fucose saccharide. The N-glycan may further be an oligosaccharide with 3 to 20, preferably 3 to 15, in particular 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 sugar residues.

In a specific embodiment, the recombinant protein comprises an Fc domain (e.g., an IgG-type Fc domain) and the Fc domain is glycosylated. In a preferred embodiment, the recombinant protein comprises an Fc domain (e.g., an IgG-type Fc domain) and the Fc domain comprises an N-glycan (i.e., is N-glycosylated). In a preferred embodiment, the recombinant protein comprises an Fc domain (e.g., an IgG-type Fc domain) and the Fc domain comprises an N-glycan at position 297 according to EU numbering. In some embodiments, the N-glycan comprised in the Fc domain is an oligosaccharide with 3 to 20, preferably 3 to 15, in particular 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 sugar residues.

In a further specific embodiment, the recombinant protein comprises an IgG-type constant region and the IgG-type constant region is glycosylated. In a preferred embodiment, the recombinant protein comprises an IgG-type constant region and the IgG-type constant region comprises an N-glycan. In a preferred embodiment, the recombinant protein comprises an IgG-type constant region and the IgG-type constant region comprises an N-glycan at position 297 according to EU numbering. In some embodiments, the N-glycan within the IgG-type constant region is an oligosaccharide with 3 to 20, preferably 3 to 15, in particular 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 sugar residues.

Given that antibody-dependent cell-mediated cytotoxicity (ADCC activity) requires an interaction between an antibody (or antigen-binding fragment thereof) and Fc receptors in some embodiments, the recombinant protein (e.g., the antibody or antigen-binding fragment thereof) has ADCC activity. ADCC activity can for example be determined as described by Kiyoshi et al. [1].

The fraction comprising the one or more protein contaminants obtained after fractionation of the sample comprising a recombinant protein may be subjected to concentration of the one or more protein contaminants. Accordingly, in one embodiment, the analysis in step b) comprises subjecting the fraction comprising the one or more protein contaminants to concentration of the one or more protein contaminants. In one embodiment, the concentration of the one or more protein contaminants comprises (i.) precipitation of the one or more protein contaminants; and (ii.) dissolution of the precipitated one or more protein contaminants in a suitable solvent. In some embodiments, the concentration of the one or more protein contaminants further comprises reduction of the volume of the fraction comprising the one or more protein contaminants, prior to precipitation of the one or more protein contaminants.

Accordingly, in a specific embodiment, the method comprises subjecting the fraction comprising the one or more protein contaminants to concentration of the one or more protein contaminants, wherein concentration of the one or more protein contaminants comprises precipitation of the one or more protein contaminants; and dissolution of the precipitated one or more protein contaminants in a suitable solvent.

In a further specific embodiment, the method comprises subjecting the fraction comprising the one or more protein contaminants to concentration of the one or more protein contaminants, wherein concentration of the one or more protein contaminants comprises reduction of the volume of the fraction comprising the one or more protein contaminants, followed by precipitation of the one or more protein contaminants; and dissolution of the precipitated one or more protein contaminants in a suitable solvent.

Volume reduction can be achieved by various suitable methods known to the skilled artisan. In one embodiment, reduction of the volume of the fraction comprising the one or more protein contaminants comprises the use of a molecular weight cut-off (MWCO) filter. In specific embodiments, the molecular weight cut-off filter has a molecular weight cut-off of between about 2 kDa and about 30 kDa, between about 3 kDa and about 25 kDa, between about 4 kDa and about 20 kDa or between about 5 kDa and about 15 kDa. In a preferred embodiment, the molecular weight cut-off filter has a molecular weight cut-off of between about 5 kDa and about 15 kDa. In further embodiments, the molecular weight cut-off filter has a molecular weight cut-off of about 5 kDa, about 6 kDa, about 7 kDa, about 8 kDa, about 9 kDa, about 10 kDa, about 11 kDa, about 12 kDa, about 13 kDa, about 14 kDa or about 15 kDa. In a preferred embodiment, the molecular weight cut-off filter has a molecular weight cut-off of about 10 kDa.

Precipitation of the one or more protein contaminants is generally followed by sedimentation of the precipitated one or more protein contaminants (e.g., by centrifugation), removal of the supernatant and discarding of said supernatant. The obtained protein pellet comprises the analyte of interest, i.e., the one or more protein contaminants, and is used for further processing and analysis as described herein. Thus, in some embodiments, precipitation of the one or more protein contaminants is followed by sedimentation of the precipitated one or more protein contaminants, removal of the supernatant. The sedimentation can be followed by drying of the obtained protein pellet. Drying can be performed by common methods known to the skilled artisan, such as air-drying, lyophilization or vacuum-centrifugation.

Precipitation of proteins can be achieved by various means known to the skilled artisan. In some embodiments, precipitation of the one or more protein contaminants comprises the addition of a precipitating agent. Preferably, the precipitating agent is selected from the group consisting of trichloroacetic acid (TCA), acetone, ammonium sulfate, a combination of chloroform and methanol and water, and a combination of chloroform and methanol. In a further preferred embodiment, the precipitating agent is acetone. The precipitating agent may be cooled, i.e., it may have a temperature of between about 2 °C and about 8 °C, e.g., at about 4 °C.

The agent that is added for the purpose of precipitating the one or more protein contaminants (the precipitating agent) is generally added in an amount that is sufficient to result in complete or essentially complete precipitation of protein comprised in the fraction comprising the one or more protein contaminants. In some embodiments, the precipitation of the one or more protein contaminants comprises the addition of the precipitating agent in an amount that is at least about 2:1, at least about 3:1, at least about 4:1 or at least about 5:1 relative to the volume of the fraction comprising the one or more protein contaminants (optionally after reduction of said volume of the fraction and/or addition of an aqueous salt solution). In a preferred embodiment, the precipitation of the one or more protein contaminants comprises the addition of the precipitating agent in an amount that is at least about 2:1 relative to the volume of the fraction comprising the one or more protein contaminants (optionally after reduction of said volume of the fraction and/or addition of an aqueous salt solution). In a further preferred embodiment, the precipitation of the one or more protein contaminants comprises the addition of the precipitating agent in an amount that is at least about 3:1 relative to the volume of the fraction comprising the one or more protein contaminants (optionally after reduction of said volume of the fraction and/or addition of an aqueous salt solution) (e.g., at least 300 µL agent is added to 100 µL of fraction comprising the one or more protein contaminants). In a more preferred embodiment, the precipitation of the one or more protein

contaminants comprises the addition of the precipitating agent in an amount that is at least about 4:1 relative to the volume of the fraction comprising the one or more protein contaminants

Precipitation of the one or more protein contaminants may comprise the addition of an aqueous salt solution. Preferably, the addition of an aqueous salt solution is done prior to the addition of the precipitating agent. More preferably, the aqueous salt solution is an aqueous solution of a salt selected from the group consisting of NaCl. In a preferred embodiment, the aqueous salt solution is an aqueous solution of NaCl. The salt comprised in the aqueous salt solution is not a precipitating agent as disclosed herein, e.g., it is not ammonium sulfate.

In some embodiments, the aqueous salt solution comprises said salt in a concentration of between about 50 mM and about 500 mM, between about 100 mM and about 400 mM or between about 150 mM and about 300 mM. In a preferred embodiment, the aqueous salt solution comprises said salt in a concentration of between about 150 mM and about 300 mM. In further embodiments, the aqueous salt solution comprises said salt in a concentration of about 100 mM, about 200 mM, about 300 mM, about 400 mM or about 500 mM. In a preferred embodiment, the aqueous salt solution comprises said salt in a concentration of about 200 mM.

After addition of the agent that is added for the purpose of precipitating the one or more protein contaminants, the resulting suspension may be kept at a reduced temperature prior to further processing (e.g., prior to sedimentation of the precipitated one or more protein contaminants) to facilitate protein precipitation. In some embodiments, the suspension is kept at a temperature of about -20 °C or less for at least about 5 min. In some embodiments, the suspension is kept at a temperature of about -60 °C or less for at least about 8 min. In some embodiments, the sample is kept at a temperature of about -65 °C or less for at least about 10 min.

Sedimentation of the precipitated one or more protein contaminants is generally achieved by centrifugation. In some embodiments, sedimentation of the precipitated one or more protein contaminants comprises centrifugation for at least about 5 min at a radial force of at least about 10'000 g. In further embodiments, sedimentation of the precipitated one or more protein contaminants comprises centrifugation for at least about 10 min at a radial force of at least about 14'000 g. Centrifugation may be performed at reduced temperature (relative to room temperature), e.g., at between about 2 °C and about 8 °C, e.g., at about 4 °C.

Accordingly, in some embodiments, the method according to the invention comprises

(a) fractionation of a sample comprising a recombinant protein and one or more protein contaminants, wherein the fractionation comprises contacting the sample with an Fc receptor affinity resin and collecting the flow-through to obtain a fraction comprising the one or more protein contaminants;

(b) analysis of the one or more protein contaminants in the fraction comprising the one or more protein contaminants, wherein the analysis comprises

subjecting the fraction comprising the one or more protein contaminants to concentration of the one or more protein contaminants, wherein concentration of the one or more protein contaminants comprises reduction of the volume of the fraction comprising the one or more protein contaminants, followed by addition of an aqueous salt solution, followed by precipitation of the one or more protein contaminants, comprising addition of a precipitating agent selected from the group consisting of trichloroacetic acid (TCA), acetone, ammonium sulfate, a combination of chloroform and methanol and water, and a combination of chloroform and methanol, followed by keeping the resulting suspension at a reduced temperature, followed by sedimentation of the precipitated one or more protein contaminants (e.g., by centrifugation), removal of the supernatant and discarding of said supernatant; and dissolution of the precipitated one or more protein contaminants in a suitable solvent.

The precipitated one or more protein contaminants is/are dissolved in a suitable solvent. The suitable solvent and possible additives should generally be compatible with subsequent chemical reduction and proteolytic digestion, i.e., they should not interfere with either of these reactions.

A suitable solvent is preferable a suitable aqueous buffer. Suitable aqueous buffers are commonly based on ammonium salts. The buffer salt may be selected from the group consisting of ammonium formate, tris-HCl, ammonium acetate and ammonium bicarbonate or carbonate. In a preferred embodiment, the aqueous buffer is an aqueous solution of ammonium bicarbonate. The aqueous buffer concentration may be between about 10 mM and about 100 mM, between about 20 mM and about 90 mM, between about 30 mM and about 70 mM, between about 40 mM and about 60 mM. In a specific embodiment, the aqueous buffer concentration is about 50 mM. In some embodiments, the pH of the aqueous buffer is between about 7 and about 9, preferably between about 7.0 and about 9.0. In a preferred embodiment, the pH of the aqueous buffer is between about 7.4 and about 8.6. In a more preferred embodiment, the pH of the aqueous buffer is between about 7.6 and about 8.4. In a particularly preferred embodiment, the pH of the aqueous buffer is about 8, more preferably about 8.0.

Accordingly, in a preferred embodiment, the aqueous buffer is an aqueous solution of ammonium bicarbonate, wherein the buffer concentration is between about 10 mM and about 100 mM and the pH of the aqueous buffer is between about 7 and about 9, preferably between about 7.0 and about 9.0. In a particularly preferred embodiment, the aqueous buffer is an aqueous solution of ammonium bicarbonate, wherein the buffer concentration is about 50 mM and the pH of the aqueous buffer is about 8, preferably about 8.0.

The suitable solvent may be an aqueous buffer comprising a detergent. The at least one detergent is preferably a detergent that does not interfere with a subsequent analysis by liquid chromatography-mass spectrometry. The at least one detergent may be N-octyl-beta-glucopyranoside, sodium deoxycholate, N-dodecyl-beta-D-maltoside or a combination thereof. In some embodiments, the suitable solvent is an aqueous buffer comprising a detergent, preferably, the detergent is selected from the group of sodium deoxycholate, N-dodecyl-beta-D-maltoside and a combination thereof. In a particularly preferred embodiment, the detergent is a combination of sodium deoxycholate and N-dodecyl-beta-D-maltoside.

The detergent is preferably used in a concentration which ensures that the detergent does not interfere with a subsequent analysis by liquid chromatography-mass spectrometry. In certain embodiments, the aqueous buffer comprises detergent in a concentration of between about 0.05% to about 1.0% (w/v). Sodium deoxycholate (SDC) may be used at concentrations ranging from about 0.05% to about 0.8% (w/v). N-dodecyl-beta-D-maltoside may be used at concentrations ranging from about 0.01% to about 0.5% (w/v). Thus, in specific embodiments, the aqueous buffer comprises sodium deoxycholate in a concentration of between about 0.05% to about 0.8% (w/v) and N-dodecyl-beta-D-maltoside in a concentration of between about 0.01% to about 0.5% (w/v). In a preferred embodiment, the aqueous buffer comprises sodium deoxycholate in a concentration of between about 0.1% to about 0.6% (w/v) and N-dodecyl-beta-D-maltoside in a concentration of between about 0.04% to about 0.5% (w/v). In a more preferred embodiment, the aqueous buffer comprises sodium deoxycholate in a concentration of between about 0.2% to about 0.4% (w/v) and N-dodecyl-beta-D-maltoside in a concentration of between about 0.05% to 0.5%, preferably of between about 0.06% to about 0.2% (w/v).

In some embodiments, the analysis in step b) comprises fragmentation of the one or more protein contaminants. In a preferred embodiment, the fragmentation of the one or more protein contaminants is done by proteolytic digestion. In some embodiments, the method comprises chemical reduction of the one or more protein contaminants prior to or after the fragmentation, preferably prior to the fragmentation.

Accordingly, in some embodiments, the method comprises chemical reduction of the one or more protein contaminants, followed by fragmentation of the one or more protein contaminants by proteolytic digestion.

In specific embodiments, the method comprises (a) fractionation of a sample comprising a recombinant protein and one or more protein contaminants, wherein the fractionation comprises contacting the sample with an Fc receptor affinity resin and collecting the flow-through to obtain a fraction comprising the one or more protein contaminants; (b) analysis of the one or more protein contaminants in the fraction comprising the one or more protein contaminants, wherein said analysis comprises subjecting the fraction comprising the one or more protein contaminants to concentration of the one or more protein contaminants, wherein concentration of the one or more protein contaminants comprises precipitation of the one or more protein contaminants, dissolution of the precipitated one or more protein contaminants in a suitable solvent; chemical reduction of the one or more protein contaminants, followed by fragmentation of the one or more protein contaminants by proteolytic digestion.

Any common agent for chemical reduction prior to mass-spectrometric analysis may be used in the method of the invention. In some embodiments, chemical reduction comprises the use of a reducing agent selected from the group consisting of dithiothreitol (DTT), tris(3-hydroxypropyl)phosphine (THPP) and tris 2-carboxyethyl phosphine (TCEP). In a preferred embodiment, chemical reduction comprises the use of dithiothreitol (DTT) or tris 2-carboxyethyl phosphine (TCEP). In a particularly preferred embodiment, chemical reduction comprises the use of tris 2-carboxyethyl phosphine (TCEP).

The reducing agent is used in a concentration that results in quantitative, or essentially quantitative reduction of peptides within the sample. In some embodiments, the reducing agent is used in a final concentration of between about 1 mM and about 10 mM, between about 2 mM and about 9 mM, between about 3 mM and about 7 mM or between about 4 mM and about 6 mM. In a preferred embodiment, the reducing agent is used in a final concentration of between about 3 mM and about 7 mM. In a more preferred embodiment, the reducing agent is used in a final concentration of between about 4 mM and about 6 mM.

Chemical reduction of proteins or peptides for subsequent mass-spectrometric analysis is usually performed at elevated temperature. Thus, in some embodiments, chemical reduction is performed at between about room temperature and about 60 °C, preferably at between about 40 °C and about 60 °C. In a more preferred embodiment, chemical reduction is performed at between about 45 °C

and about 55 °C. In an even more preferred embodiment, chemical reduction is performed at about 50 °C.

Proteolytic digestion for subsequent mass-spectrometric analysis can generally be achieved with proteases that generate a regular cleavage pattern. In some embodiments, the proteolytic digestion comprises the use of a protease selected from the group consisting of trypsin, chymotrypsin, ArgC, GluC, AspN, LysC, LysN, elastase and proteinase K and a combination thereof. In some embodiments, the proteolytic digestion involves the use of one specific protease (such as trypsin). In some embodiments, the proteolytic digestion involves the use of a combination of two specific proteases (such as trypsin and LysC). In a preferred embodiment, the proteolytic digestion comprises the use of trypsin and LysC. In a further preferred embodiment, the proteolytic digestion comprises the use of trypsin.

Proteases used for proteolytic digestion prior to mass-spectrometric analysis are used at their respective temperature optimum (e.g., about 37 °C). Accordingly, in some embodiments, the proteolytic digestion is performed at a temperature of between about 30°C and about 60°C, preferably of between about 34°C and about 40°C, more preferably between about 35°C and about 39°C and even more preferably between about 36°C and about 38°C. In a particularly preferred embodiment, the proteolytic digestion is performed at a temperature of about 37°C.

The protease can be used in various amounts. In some embodiments, the proteolytic digestion comprises the use of one or more protease(s) in a ratio of between about 1:5 and about 1:100, preferably between about 1:5 and about 1:40, more preferably between about 1:10 and about 1:30 and even more preferably between about 1:15 and about 1:25 (w/w, protease(s) to protein substrate). In a particularly preferred embodiment, the proteolytic digestion comprises the use of one or more protease(s) in a ratio of about 1:20 (w/w, protease(s) to protein substrate).

In some embodiments, the proteolytic digestion is allowed to proceed for at least about 15 minutes. In further embodiments, the proteolytic digestion is allowed to proceed for at least about 30 minutes. In even further embodiments, the proteolytic digestion is allowed to proceed for at least about 45 minutes. In even further embodiments, the proteolytic digestion is allowed to proceed for at least about 60 minutes. As to duration the proteolytic digestion can be allowed to proceed over night. In preferred embodiments, the proteolytic digestion is allowed to proceed for between about 15 minutes and about 90 minutes. In more preferred embodiments, the proteolytic digestion is allowed to proceed for between about 30 minutes and about 60 minutes.

The method may further comprise an identification and/or quantification of one or more individual protein contaminants in the fraction comprising the one or more protein contaminants.

In one embodiment, the analysis in step b) of the one or more protein contaminants comprises identification of one or more individual protein contaminants, preferably by peptide analysis by liquid-chromatography-mass spectrometry (LC-MS). In some embodiments, the liquid-chromatography-mass spectrometry is liquid-chromatography-tandem mass spectrometry (LC-MS/MS).

In some embodiments, the method according to the invention comprises (a) fractionation of a sample comprising a recombinant protein and one or more protein contaminants, wherein the fractionation comprises contacting the sample with an Fc receptor affinity resin and collecting the flow-through to obtain a fraction comprising the one or more protein contaminants; (b) analysis of the one or more protein contaminants in the fraction comprising the one or more protein contaminants, wherein said analysis comprises subjecting the fraction comprising the one or more protein contaminants to concentration of the one or more protein contaminants, wherein concentration of the one or more protein contaminants comprises precipitation of the one or more protein contaminants, dissolution of the precipitated one or more protein contaminants in a suitable solvent; chemical reduction of the one or more protein contaminants, followed by fragmentation of the one or more protein contaminants by proteolytic digestion and identification of one or more individual protein contaminants by peptide analysis by liquid-chromatography-mass spectrometry (LC-MS).

As used herein, mass spectrometry (MS) refers to an analytical technique to filter, detect, identify and/or measure compounds by their mass to charge ratio, of "Da/e" (also commonly denoted by the symbol "m/z"). MS technology generally includes (1) ionizing the compounds and potentially fragmenting the compounds; and (2) detecting the molecular weight of the charged compound and/or fragment ion and calculating a mass-to-charge ratio (m/z). The compound may be ionized and detected by any suitable means. A mass spectrometer generally includes an ionizer and an ion detector.

Tandem mass spectrometry (MS/MS) refers to a method of mass spectrometry wherein parent ions generated from a sample are selected by a first mass filter/analyzer and are then passed to a collision cell wherein they are fragmented by collisions with neutral gas molecules to yield daughter (or "product") ions. The daughter ions are then mass analyzed by a second mass filter/analyzer, and the resulting daughter ion spectra can be used to determine the structure and hence identify

the parent (or "precursor") ion. Tandem mass spectrometry is particularly useful for the analysis of complex mixtures such as biomolecules.

As used herein, liquid chromatography (LC) means a process of selective retention of one or more components of a fluid solution as the fluid uniformly percolates through a column of a finely divided substance, or through capillary passageways. The retention results from the distribution of the components of the mixture between one or more stationary phases and the bulk fluid, (i.e., mobile phase), as this fluid moves relative to the stationary phase(s). Liquid chromatography includes, without limitation, reverse phase liquid chromatography (RPLC), high performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), supercritical fluid chromatography (SFC) and ion chromatography. In some embodiments, the liquid chromatography performed as part of the LC-MS analysis is reverse-phase (RP) liquid chromatography.

RP chromatography is based on non-polar stationary phases and polar mobile phases. Routinely used stationary phases in RP reverse-phase (RP) liquid chromatography include silica resins functionalized with a variety of non-polar functional groups, such as alkyl groups. Alkyl functionalized resin surfaces may include C-4, C-8, or C-18 functionalized resin, preferably C-18 functionalized resin.

Peptide analysis by liquid-chromatography-mass spectrometry (LC-MS) may be performed using a variety of suitable instruments. Suitable LC-MS instruments are known to the skilled person and include, e.g., an Orbitrap mass spectrometer of Thermo Fisher Scientific Inc.

Suitable solvent systems for reverse-phase liquid chromatography include mobiles phases consisting of water and an organic solvent (e.g., methanol, ethanol, propanol or acetonitrile) in varying ratios. The mobile phase may optionally comprise an agent for pH adjustment, such as acetic acid, formic acid, trifluoroacetate, aqueous ammonia, ammonium acetate, ammonium formate or combinations thereof. In a preferred embodiment, the mobile phase comprises water, acetonitrile and formic acid.

After mass spectrometric analysis, peptides are mapped to a database of peptides that corresponds to the source of the one or more protein contaminants, e.g., the cell line that was used to produce the recombinant protein. Protein sequence data is publicly available, for example from uniprot.org. For example, a CHO reference proteome is deposited under the reference number UP000001075. Sequences of common contaminants may be added to the employed reference proteome, e.g., keratins, trypsin (UniProt ID P00761) and the sequence of the recombinant protein. Software performs *in silico* digests on proteins in the database with the same protease(s) that was/were used

for proteolytic digestion of the protein contaminants (e.g., trypsin). The masses of these peptides are calculated and compared to the peak list of measured masses. If a protein sequence in the reference database gives rise to a significant number of predicted masses that match the experimental masses, there is evidence that this protein was present in the original sample. Peptides can be fragmented with MS/MS to provide higher quality data and to enable more definitive peptide identification. The results are statistically analyzed and possible matches are used to identify and optionally quantify one or more protein contaminants in the sample.

Thus, in some embodiments, peptide analysis by liquid-chromatography-mass spectrometry (LC-MS) and identification of individual protein contaminants comprises comparing the mass spectrometric data with a peptide database generated through *in silico* proteolytic digestion of a reference proteome.

It will be obvious for a person skilled in the art that these embodiments and items only depict examples of a plurality of possibilities. Hence, the embodiments shown here should not be understood to form a limitation of these features and configurations. Any possible combination and configuration of the described features can be chosen according to the scope of the invention.

Examples

Materials and Methods

Chemicals

Tris 2-carboxyethyl phosphine (TCEP), ammonium bicarbonate (ABC), ultrapure formic acid, acetic acid, guanidine-HCl, chloroform, methanol, acetonitrile (ACN), water, trifluoroacetic acid (TFA), sodium deoxycholate (SDC) and universal proteomics standard (UPS-1) were purchased from Sigma-Aldrich (St. Louis, MO). Vivaspin 500 (10 kDa MWCO) was purchased from Cytiva (Marlborough, MA). Sequencing grade trypsin/Lys-C mix and N-dodecyl-beta-D-maltoside (DDM) were purchased from Promega (Milwaukee, WI) and Thermo Fisher Scientific (Waltham, MA), respectively. IdeS and FabALACTICA were purchased from Genovis. PNGaseF was purchased from New England BioLabs.

Sample preparation for Affinity Chromatography

The mAb1 sample was produced in-house at Lonza using recombinant expression technology with CHO cell lines and standard purification procedures. The mAb2 sample (8670 NIST mAb) was purchased from the NIST. PNGase F was used to remove N-glycans from the samples after

incubation at 37 °C for 3 h. Fc and Fc/2 fragments of mAb2 were prepared by digestion with FabALACTICA and IdeS, respectively, according to the manufacturer's recommendations.

Affinity Chromatography

A FcR-IIIA-5PW 7.8 mm x 7.5 cm column (P/N 0023532, TOSOH Bioscience, King of Prussia, PA) was used to collect flow-through (FT) fractions. Mobile phase A was 50 mM citrate at pH 6.5 and mobile phase B was 50 mM citrate at pH 4.5. All samples were buffer exchanged (3 times) with mobile phase A prior to injection. Samples were injected at a flow rate of 0.3 mL/min with a column temperature of 15 °C and UV detection at 280 nm.

The gradient elution step was modified to a trap-and-elute method to shorten the HPLC run time as follows: after 15 min of loading with 100% mobile phase A, the gradient was switched to 100% B during 1 min and then the flow rate was maintained at 0.4 mL/min for 30 min. Before starting a new injection, the column was equilibrated with 100% mobile phase A for 27 min.

Protein A chromatography was carried out using a HiTrap MabSelect SuRe column in accordance with the previously reported procedure [3]. Fig. 8 shows the steps in more detail.

Sample Preparation for Peptide Mapping Analysis

Protein Precipitation by Acetone

FT fractions (approximately 3.0 mL) collected during affinity chromatography were concentrated to approximately 100 µL concentrates using Vivaspin filters with 10 kDa cut-off membranes and centrifugation at 10'000 g and 20 °C for approximately 20 min. Proteins in the concentrates were precipitated with acetone using a procedure similar to the one described by Jiang et al. [4]. Briefly, the sample was diluted (1:1) with 200 mM NaCl, then 800 µL of cold acetone (4 °C) was added to the sample and the solution was incubated in a freezer at ≤ - 65 °C for 10 min. After centrifugation for 10 min (at 14'000 g and 4 °C), the liquid was carefully removed and discarded, and the remaining protein pellet was used for proteomic sample preparation.

Tryptic Digestion

The protein pellets were dissolved in 15 µL of 50 mM ammonium bicarbonate solution containing SDC (2.3% w/v) and DDM (0.66% w/v). After vortex mixing at room temperature, the mixture was diluted by adding an additional 74 µL of 50 mM ammonium bicarbonate solution. Proteins were reduced by adding 1 µL of 500 mM TCEP and incubating the solutions at 50 °C for 10 min. Samples

were digested for 1 h at 37 °C with a trypsin/LysC mix (1:20 enzyme to protein ratio; the trypsin : LysC ratio can be 1:1, w/w), then transferred directly to a HPLC vial for LC-MS/MS analysis, as described below. The final concentrations of SDC and DDM prior to injection were 0.35% w/v and 0.1% w/v, respectively. The amount of protease required for digestion was calculated based on an assumption of 200 ppm HCPs in the loaded material in the FcγRIIIa column.

Liquid Chromatography-Mass Spectrometry

A system consisting of Vanquish Neo UHPLC instrument coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used for LC-MS/MS analysis. For peptide mapping analysis, a NanoEase *m/z* Peptide CSH C18 column (130 Å, 1.7 µm, 0.3 mm x 150 mm, Waters Corporation) was used to separate peptides in the digested samples with a mobile phase consisting of 0.1% formic acid in water (A) and acetonitrile (B). Peptides were separated and eluted at a flow rate of 5 µL/min using a linear gradient from 1% B to 40% B over 75 min, followed by 98% B over 5 min. The column was then washed with 98% B for 3 min and conditioned with 1% B for 10 min before the next injection.

The mass spectrometer was operated in a data dependent mode with a 200-2000 *m/z* range, spray voltage of 3500 kV and heated capillary temperature of 325 °C. Full scan spectra were recorded at a resolution of 120 000 (full width at half maximum resolution at 400 *m/z*) using an automatic gain control (AGC) target value of 2.0 $x10^5$ with a maximum injection time of 100 ms. Up to 20 of the most intense ions with 2-8 charge states were selected for higher energy c-trap dissociation (HCD) with a normalized collision energy of 30%. Fragment spectra were recorded with an isolation width of 2.5 Da and resolution of 15000 using an AGC target value of 5.0 x 10^4 and maximum injection time of 200 ms. Dynamic exclusion was activated for 20 s within a 10 ppm window for precursor selection. Fragment ions were recorded on the Orbitrap analyzer.

Data Analysis

For peptide mapping, tolerances of 5 and 20 ppm were applied in a database search for peptides identified in the MS and MS/MS analyses, respectively. The search was performed against a CHO protein sequence database obtained from UniProt (reference proteome UP000001075, last modified on 2019-01-24, downloaded on 2022-10-10 containing 23,886 sequences), to which was added sequences of common contaminants such as keratins and trypsin (UniProt ID P00761) and the analyzed mAbs. The searches included methionine and tryptophan oxidation, and asparagine deamidation as variable modifications. The false discovery rate (FDR) was set at 2%.

Example 1: mAb Depletion Using FcγRIIIa Affinity Chromatography

In the present application, mAb1 (an IgG4) and mAb2 (an IgG1) were used as proof-of-concept samples. Hence, their ability to be retained on the FcγRIIIa affinity column with and without Fc domain glycosylation was evaluated. After buffer exchange with mobile phase A, samples were loaded onto the FcγRIIIa affinity column. The gradient elution step was modified to a trap-and-elute approach, as described in the Materials and Methods section. **Error! Reference source not found.**A and 2B show the retention of mAb1 and mAb2 in their native (dotted trace) and deglycosylated (solid trace) forms by the FcγRIIIa affinity column. The deglycosylated mAbs showed only low affinity for binding to the column and eluting in the FT fraction.

The suitability of the current method for analyzing HCPs based on mAb therapeutic fragments was also evaluated. To generate smaller fragments, mAb2 was digested using enzymes IdeS and FabALACTICA, which cleave mAbs above and below the hinge region. Afterwards, the ability of the FcγRIIIa resin to retain smaller fragments with Fc domain glycosylation was assessed (Figs. 3 and 4).

As shown in **Error! Reference source not found.**A, FabALACTICA can generate both Fab and Fc fragments. The FcγRIIIa affinity chromatogram of this digest revealed two peaks: one eluted as the FT fraction at a retention time (RT) of 8.3 min (collected as fraction 1), whereas the other eluted as a multiple peak at RT of 30-45 min (collected as fraction 2) (**Error! Reference source not found.**A). The two collected fractions were concentrated using a cut-off filter (10 kDa) and analyzed by RP-LC-MS. The total ion currents (TIC) of each fraction are shown in **Error! Reference source not found.**B and **Error! Reference source not found.**C. Deconvoluted mass spectra of the TIC showed the expected molecular weights of the detected fragments (**Error! Reference source not found.**D and 3E). The results confirmed that the Fab fragment showed no affinity for the FcγRIIIa affinity column and was subsequently eluted in the FT fraction.

IdeS cleaves mAbs at a single site below the hinge region, resulting in F(ab')2 and Fc/2 fragments, as shown in **Error! Reference source not found.**A. Surprisingly, the FcγRIIIa chromatogram of mAb1 digested with IdeS showed a single peak eluted as the FT fraction at RT of 8.0 min. RP-LC-MS analysis of this fraction revealed two well resolved peaks (RT of 14.8 and 16.8 min). Deconvoluted MS spectra of each peak confirmed the presence of Fc/2 and F(ab)'2 fragments, as shown in **Error! Reference source not found.**C and 4D, respectively.

The presence of a single peak in the FcγRIIIa chromatogram, followed by RP-LC-MS identification of the Fc/2 fragment of the collected fraction, indicated that the Fc/2 fragment had no or at least low binding affinity to the FcγRIIIa resin and was eluted in the FT fraction.

These results show that the Fc receptor immobilized on the FcγRIIIa column had a lower ability to bind to smaller mAb fragments with glycosylated Fc domain fragments (e.g., Fc/2). Best results were obtained with antibodies comprising full-length Fc domains (e.g., IgG-isotype antibodies). These results also demonstrate that and how the Fc affinity resin, that is the Fc receptor, can be identified and chosen so that it matches the chosen and given recombinant protein, such that the chosen and given recombinant protein is effectively bound onto and retained by the Fc resin while any protein contaminants are separated from the recombinant protein bound on the resin and can be collected in the flow through for analysis. The interaction profile of different types of Fc receptors is known to the skilled artisan. For example, Russell et al. discusses binding profiles of various types of Fc receptors [13]. The vast majority of approved mAb therapies currently belong to the IgG1 and 4 subclasses. Accordingly, the disclosed method could have broad utility for HCP characterization in commercially relevant antibody preparations.

Example 2: Rapid Digestion for High-Throughput HCP analysis

The digestion procedure of this invention has the advantage of a short processing time, efficient and high through-put. Sample loss can be a major problem in proteomic analyses of HCPs, as only trace amounts are typically present in the final products. Thus, it is challenging to apply general classical sample processing workflows, given that at least 2 to 3 unique peptides are required for HCP identification and quantification. To assess the quality of the digestion of the current approach for identifying low level HCPs, UPS1 was reduced and digested for up to 60 min with measurements taken at 15 min intervals according to the procedure described in the Materials and Methods. UPS1 contains a mixture of 48 proteins with equimolar concentration and molecular weight (MW) ranging from 6.3 to 82.9 kDa. To further investigate the quality of the digestion, the average protein sequence coverage, number of identified proteins and sum of the signal intensity of the proteins were compared for each timepoint (Fig. 5).

Error! Reference source not found. summarizes the total signal intensity, shown as "Total peptide MS intensity", and average sequence coverage (shown as the percent figures on top of each of the four columns) for each of the digestion conditions investigated. A 15 min digestion time resulted in identification of 47 of 48 UPS1 proteins (the number of identification is the height of the respective column, the scaling is shown on the left side with "Number of proteins detected"). The four different shadings of each column show the percentage of sequence coverage: Proteins were mostly

identified with 45% average sequence coverage when digested for 15 min. Increasing the digestion time to up to 45 min increased the average sequence coverage from 42 to 64%, as well as signal intensity identification (**Error! Reference source not found.**).

The slightly reduced sequence coverage and peptide MS intensity after 60 min digestion compared to 30 and 45 min digestion indicates that the percentage of fully cleaved peptides (in case that a peptide has more than one cleaving site) increased with longer digestion times, thereby generating on one hand very short peptides that are not retained on the RP column, but on the other hand respectively fully cleaved peptide fragments which are not only well retained by the RP column but also provide better and more reliable identification and quantification. So 60 min digestion enhanced the overall throughput with respect to digestion completion while contributing more intensity for peptides without missing cleavages. The current methodology is expected to be robust, low-cost and improve the throughput of HCP analysis.

Example 3: HCP Analysis of Isolated Fraction of FcγRIIIa Affinity Chromatography

A semi-preparative FcγRIIIa affinity column was used due to its larger loading capacity, which allowed a higher amount of sample to be injected onto the column and required only one run to collect sufficient material for proteomic analysis. Following the steps outlined in the Materials and Methods, 5 mg of mAb1 sample was buffer exchanged with FcγRIIIa loading buffer (mobile phase A) and loaded onto the FcγRIIIa affinity column (**Error! Reference source not found.**). The FT fraction was collected (fraction 1), concentrated and the proteins were precipitated with acetone. The proteins were then digested using a trypsin/Lys-C mixture and analyzed using LC-MS/MS, as described in the Materials and Methods.

The protein/enzyme ratio was calculated assuming the samples loaded onto the FcγRIIIa column contained 200 ppm of HCPs. The latter value can be adjusted to be applicable to samples from different purification processes. Identification of the HCPs was based on identification of at least one unique peptide having at least five amino acid residues.

For data analysis, a strategy similar to one recently reported was employed [3], with minor adjustments, as described in the following. Peptide identification was only expanded to the full scan MS level when the peptides were detected by MS/MS in other fractions of the same mAb. The following criteria were used to re-evaluate these "in silico peptides": retention time difference of less than 30 s in the same sequence, 5 ppm mass accuracy for peptide masses, MS1 correlation of 0.90, signal intensity ≥ 5 x10^4, and experimental isotope pattern of the identified peptide adequately matched to the theoretical average distribution.

In total, 63 HCPs were identified with at least one unique peptide. A summary of HCPs found by the FcγRIIIa affinity column is presented in Table 1.

The main identified HCPs with at least 15% sequence coverage were phospholipase B-like (PLBL2), Protein S100, C-C motif chemokine, Glutathione S-transferase P and heat shock cognate 71 kDa protein. The maximum signal intensity of the identified HCPs corresponded to PLBL2 with 72.5% sequence coverage.

In addition to PLBL2, light chain (LC) and heavy chain (HC) were identified with more than 90% amino acid sequence coverage. Although the FcγRIIIa column efficiently depletes mAb1 product-related impurities, such as fragments (e.g., LC or HC without the Fc domain), do not bind to the column and are washed off into the FT fraction (HCP-rich fraction).

Table 1: Identified HCPs in mAb 1 after depletion by protein A or FcγRIIIa affinity column.

Uniprot Accession Number	CHO protein	MW (kDa)	pI	Number of amino acids	Protein A, Fractions	FcγRIIIa, Fraction 1
G3I6T1	Phospholipase B-like	65.5	5.6	585	+	+
G3HUU6	Protein S100	11.2	6.6	100	-	+
G3IIK9	Cornifin-A	12.5	8.3	113	+	+
G3IGQ2	Heat shock cognate 71 kDa protein	7.4	6.5	70	-	+
G3GTT2	C-C motif chemokine	15.9	9.4	143	+	+
G3I3Y6	Glutathione S-transferase P	25.0	8.2	221	+	+
G3H2K2	Cytochrome c, somatic	11.7	9.5	105	+	-
G3HL18	Anionic trypsin-2	26.7	7.6	252	+	+
G3II73	Olfactory receptor	35.0	8.8	318	+	-
G3HNJ3	Clusterin	51.8	5.5	447	-	+
G3H5Z4	Synaptotagmin-like protein 4	17.4	9.5	150	-	+
G3H4L1	3-oxoacyl-[acyl-carrier-protein] synthase, mitochondrial	44.1	6.1	421	+	+
G3IIL9	Phosphotidylinositol phosphatase PTPRQ	40.0	5.9	360	+	-
G3HTW3	Uncharacterized protein FLJ39785	15.6	5.3	138	+	+
G3H262	Interleukin-31	14.1	5.5	123	+	+
G3HWE0	Ectodysplasin-A receptor-associated adapter protein	23.7	4.9	207	+	+
G3IIZ3	Ig-like domain-containing protein	15.9	6.3	146	+	+
G3H7X4	Natural killer cell receptor 2B4 (Fragment)	32.0		280	-	+

G3HK46	Serine/threonine-protein kinase 3	30.1	4.8	260	+	+
G3GV63	Secreted frizzled-related protein 4	40.0	9.2	348	+	+
G3I405	Cardiotrophin-like cytokine factor 1	25.2	9.1	225	+	+
G3IGS6	Receptor protein-tyrosine kinase	71.4	5.9	650	+	+
G3H9N4	Polypeptide N-acetylgalactosaminyltransferase 1	47.5	5.9	413	+	+
G3GZP7	Kelch-like protein 29	84.7	6.4	800	+	+
G3HZP0	DCC-interacting protein 13-beta	70.5	5.3	634	+	+
G3I2H3	Neuronal PAS domain-containing protein 4	103.2	4.8	953	+	+
G3H2P3	Beta-galactosidase (Fragment)	73.7		650	+	+
G3HMV2	60S ribosomal protein L11	20.3	9.6	178	+	+
G3HX80	Cyclic AMP-dependent transcription factor ATF-1	29.3	8.4	269	-	+
G3GRL8	DNA-directed RNA polymerase II subunit GRINL1A	41.2	5.9	366	+	+
G3I7L6	Desmocollin-1	60.8	4.7	544	+	+
G3HBF6	Tyrosine-protein phosphatase non-receptor type	134.2	7.3	1178	+	+
G3GVK4	Nuclear receptor corepressor 1	173.5	9.0	1591	+	-
G3ICR2	Olfactory receptor	36.0	9.3	321	+	+
G3GWC5	Transcriptional adapter 2-beta	38.1	9.2	328	-	+
G3GSM5	Transferrin receptor protein 1	85.1	6.3	757	-	+
G3I912	Activity-regulated cytoskeleton-associated protein	138.8	7.9	1214	+	-
G3HWU1	C-C chemokine receptor 1-like protein 1	37.8	9.7	329	+	+
G3HWK8	Alanine aminotransferase 1	53.6	5.9	481	+	+
G3GU05	NKG2D ligand 4	76.4	6.5	671	+	-
G3HCT4	Putative ATP-dependent RNA helicase DDX5	69.2	9.1	615	+	+
G3H322	Phosphodiesterase	88.5	5.9	786	+	+
G3HMM0	protein-tyrosine-phosphatase	251.4	4.6	2289	+	+
G3HGT4	Uncharacterized protein KIAA0564-like	213.5	6.3	1905	+	+
G3IFV4	Ubiquitin carboxyl-terminal hydrolase 34	90.3	5.2	800	+	+
G3HII3	Centrosomal protein of 290 kDa	253.9	6.1	2172	+	+
G3GXJ4	BEN domain-containing protein 3	94.3	5.5	824	-	+
G3HPQ3	Voltage-dependent calcium channel subunit alpha-2/delta-1	93.2	5.0	823	-	+
G3HL08	Phosphoinositide phospholipase C	98.5	5.3	867	+	+

G3H907	Tripartite motif-containing protein 45	64.1	8.2	580	+	+
G3I6F8	non-specific serine/threonine protein kinase	156.1	5.7	1397	+	+
G3HZP1	UPF0681 protein KIAA1033	76.2	6.4	645	+	+
G3GV43	Pleckstrin-likey domain-containing family H member 2 (Fragment)	85.5		772	+	+
G3I951	Forkhead-associated domain-containing protein 1	136.1	8.6	1178	+	+
G3HD94	non-specific serine/threonine protein kinase	328.4	6.5	2836	+	+
G3IBT1	Pre-mRNA-processing factor 6	61.3	8.4	535	-	+
G3GZB3	Pericentriolar material 1 protein	221.0	4.8	1960	+	+
G3HGN5	Cytosolic 5'-nucleotidase 1B	71.8	8.8	632	+	+
G3H685	Anoctamin	80.1	7.9	683	+	+
G3HQH3	DENN domain-containing protein 2C	97.4	8.9	855	-	+
G3GWC9	Uncharacterized protein KIAA0232	135.6	4.8	1225	+	+
G3H8R0	Sacsin	497.3	6.5	4371	+	+
G3IFP5	Uncharacterized protein KIAA1211	139.3	5.5	1251	-	+
G3HG75	Nestin	199.6	4.2	1800	+	+
G3IFR8	Rho guanine nucleotide exchange factor 17	186.9	5.6	1723	+	-
G3GT01	E3 ubiquitin-protein ligase listerin	199.9	5.9	1771	+	+
G3H098	Spindle and centriole-associated protein 1	256.0	6.3	2231	+	+
G3HZ26	Ankyrin repeat domain-containing protein 12	232.5	6.8	2042	+	+
G3H7S6	Trophinin	158.5	9.2	1618	+	+
G3HAC6	Titin	4007.1	5.8	36026	+	+

Example 4: HCP Analysis of an Isolated Fraction from Protein A Affinity Chromatography: Comparison with FcγRIIIa Affinity Chromatography

Protein A affinity chromatography has been frequently employed as a generic approach for mAb depletion prior to proteomic investigation of HCPs [3]. Protein A-based separation relies on the strong affinity of mAb Fc regions to bind to a protein A resin under neutral pH conditions, whereas the majority of HCPs and other impurities are washed off in the FT fraction. Despite the excellent ability of protein A to deplete mAbs, non-specific interactions of the chromatographic resin and mAbs with certain HCPs reduce the usefulness of this approach in a proteomics workflow. HCP-mAb interactions may involve a variety of forces, including electrostatic interactions, hydrogen

bonding, hydrophobic interactions and/or van der Waal forces. These interactions may prevent the full removal of several types of HCPs by protein A chromatography, since said HCPs elute with mAbs. To disrupt these interactions, a pre-elution wash is often applied prior to elution of mAbs in protein A chromatography. The effectiveness of pre-elution washes should be assessed for each mAb to improve HCP recovery prior to proteomic analysis and prevent certain risk levels for use in root cause investigations [3]. However, product-specific pre-elution wash optimization is usually a major bottleneck in biopharmaceutical companies when rapid turnaround is required.

FcγRIIIa and protein A resins may both be used in affinity chromatography, however, non-specific interactions that may potentially be equivalent to protein A-based purification methods have not been studied for FcγRIIIa chromatography. Accordingly, the inventors investigated how efficiently HCPs are isolated using FcγRIIIa versus protein A chromatography.

To this end, 5 mg of mAb1 was injected into a protein A affinity column according to a procedure recently described [3]. The FT fraction (referred to as “F1-Protein A”) was collected, and the column was rinsed with water. Pre-elution was carried out in the presence of a high concentration of salt and at basic pH (referred to as “F2-Protein A”) [3]. Finally, mAb1 was eluted from the column using glycine buffer at pH 2.80 (referred to as “F3-Protein A”). Fig. 8 shows a schematic of the protein A workflow.

The FT and pre-elution fractions (the F1-Protein A and the and F2-Protein A) collected during protein A affinity chromatography were concentrated, precipitated and digested as described in Materials and Methods, that is in analogy to the treatment of the FT from the FcγRIIIa chromatography.

Comparing the protein identifications for each approach, the protocol using protein A yielded 57 identified HCPs, whereas the Fc receptor-based method of this invention yielded 63 proteins in Example 3.

Similar to the Fc receptor approach, PLBL2 was detected in protein A fractions (F1- and F2-Protein A) with more than 70% sequence coverage. PLBL2 is known as a "Hitchhiker HCP" because it is difficult to remove even after several chromatography steps and may trigger an immune response in patients. To better understand HCP-mAb interactions, we evaluated levels of PLBL2 in the FcγRIIIa and protein A affinity chromatography fractions.

First, the MS peak areas of all the PLBL2 peptides detected in the protein A fractions (F1-, F2-, and F3-protein A) were calculated. The data revealed that F2- and F3-protein A had the highest and

lowest signal intensities of 93.9% (sum of MS signals: 3.8 x 10^9) and 0.6% (sum of MS signals: 2.6 x 10^7), respectively (Fig. 7A). The relevance of the pre-elution step was demonstrated by detection of 5.5% (sum of MS signals: 2.2 x 10^8) of the total PLBL2 signal in F1-protein A. This would be particularly critical if just F1-protein A fractions were employed for proteomics studies since the majority of HCPs are present in trace quantities and insufficient elution could result in concentrations below the MS method's detection limit. Furthermore, the level of PLBL2 in protein A elutes (F3-protein A) depends on several parameters, including the mAb subclass (IgG4 has higher PLBL2 levels in eluates than IgG1), PLBL2 concentration in the chromatographic load, resin load density and pre-elution wash conditions.

A corresponding study was carried out on the PLBL2 level in fractions collected from FcγRIIIa chromatography. The main measured signal intensities were in F1 (99.6%, MS signal: 5.6 x 10^9), indicating the efficiency of PLBL2 isolation using FcγRIIIa chromatography in only a single step. **Error! Reference source not found.**A and 7B show details of the detected signal intensities of PLBL2 for protein A and FcγRIIIa chromatography, respectively.

Based on this invention's findings, the new FcγRIIIa-based approach for mAb depletion was found to be more efficient than protein A chromatography. The majority of approved therapeutic mAbs are antibodies comprising IgG1 or IgG4-type constant regions, which can be readily depleted using Fc receptor-based chromatography (e.g., FcγRIIIa chromatography) without any significant optimization. Due to the isolation of HCPs in a single fraction, the time needed for sample preparation and data analysis is reduced. Therefore, process optimization is accelerated. The comprehensive information acquired using the workflow disclosed herein provides highly valuable insights into HCP identification of approved therapeutic mAbs to assist the development of biosimilars.

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Claims

1. A method for isolating and analyzing one or more protein contaminants in a sample comprising a recombinant protein and one or more protein contaminants, the method comprising

   i. fractionation of the sample comprising the recombinant protein and the one or more protein contaminants, wherein the fractionation comprises contacting the sample with an Fc receptor affinity resin and collecting the flow-through to obtain a fraction comprising the one or more protein contaminants;

   ii. analysis of the one or more protein contaminants in the fraction comprising the one or more protein contaminants.

2. The method according to claim 1, wherein the analysis in step b) comprises subjecting the fraction comprising the one or more protein contaminants to concentration of the one or more protein contaminants.

3. The method according to claim 2, wherein the concentration of the one or more protein contaminants comprises

   i. precipitation of the one or more protein contaminants; and

   ii. dissolution of the precipitated one or more protein contaminants in a suitable solvent.

4. The method according to any one of claims 1 to 3, wherein the analysis in step b) comprises fragmentation of the one or more protein contaminants.

5. The method according to claim 4, comprising chemical reduction of the one or more protein contaminants prior to or after the fragmentation, preferably prior to the fragmentation.

6. The method according to claims 1 to 5, wherein the analysis in step b) of the one or more protein contaminants comprises identification of one or more individual protein contaminants, preferably by peptide analysis by liquid-chromatography-mass spectrometry (LC-MS).

7. The method according to any one of claims 1 to 6, wherein the Fc receptor affinity resin comprises an immobilized Fc receptor that binds to the recombinant protein.

8. The method according to any one of claims 1 to 7, wherein the Fc receptor affinity resin is an Fc gamma receptor affinity resin or a neonatal Fc receptor (FcRn) affinity resin, preferably an Fc gamma receptor affinity resin.

9. The method according to claim 8, wherein the Fc gamma receptor affinity resin is a type I Fc gamma receptor (FcγR) affinity resin, a type II Fc gamma receptor (FcγR) affinity resin or a type III Fc gamma receptor (FcγR) affinity resin; preferably the Fc gamma receptor affinity resin is a type I Fc gamma receptor (FcγR) affinity resin; more preferably, the type I Fc gamma receptor (FcγR) affinity resin is selected from the group of FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb affinity resin, even more preferably, the type I Fc gamma receptor (FcγR) affinity resin is a FcγRIIIa, affinity resin.

10. The method according to any one of claims 1 to 9, wherein the recombinant protein binds to the Fc receptor affinity resin.

11. The method according to any one of claims 1 to 10, wherein the recombinant protein comprises an Fc domain or a fragment of an Fc domain, preferably a glycosylated Fc domain or a fragment of a glycosylated Fc domain.

12. The method according to any one of claims 1 to 11, wherein the recombinant protein is an antibody or an antigen-binding fragment thereof or an Fc fusion protein.

13. The method according to any one of claims 1 to 12, wherein the recombinant protein comprises an IgG-type constant region; preferably the IgG-type constant region is an IgG1-type constant region, an IgG2-type constant region, an IgG3-type constant region or an IgG4-type constant region; more preferably the IgG-type constant region is an IgG1-type constant region, an IgG2-type constant region or an IgG4-type constant region; even more preferably the IgG-type constant region is an IgG1-type constant region or an IgG4-type constant region.

14. The method according to any one of claims 1 to 13, wherein the recombinant protein is an IgG-type antibody, preferably an IgG1-type antibody, an IgG2-type antibody, an IgG3-type antibody or an IgG4-type antibody, more preferably an IgG1-type antibody, an IgG2-type antibody or an IgG4-type antibody, even more preferably an IgG1-type antibody or an IgG4-type antibody.

15. The method according to any one of claims 1 to 14, wherein the recombinant protein is glycosylated, preferably wherein the recombinant protein comprises an N-glycan.

16. The method according to claim 15, wherein the N-glycan is an oligosaccharide with 3 to 20, preferably 3 to 15, in particular 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 sugar residues.

17. The method according to claim 3, wherein the precipitation of the one or more protein contaminants comprises the addition of a precipitating agent; preferably the precipitating agent is selected from the group consisting of trichloroacetic acid (TCA), acetone, ammonium sulfate, a combination of chloroform and methanol and water, and a combination of chloroform and methanol, preferably acetone.

18. The method according to claim 17, wherein the precipitation of the one or more protein contaminants comprises the addition of the precipitating agent in an amount that is at least about 2:1 relative to the volume of the fraction comprising the one or more protein contaminants; and/or the precipitation of the one or more protein contaminants comprises the addition of an aqueous salt solution; preferably the addition of an aqueous salt solution is done prior to the addition of the precipitating agent; more preferably, the aqueous salt solution is an aqueous solution of a salt selected from the group consisting of NaCl.

19. The method according to any one of claims 3, 17 or 18, wherein the suitable solvent is an aqueous buffer comprising a detergent, preferably the detergent is selected from the group of sodium deoxycholate, N-dodecyl-beta-D-maltoside and a combination thereof.

20. The method according to claim 4, wherein the fragmentation of the one or more protein contaminants is done by proteolytic digestion; preferably the proteolytic digestion comprises the use of a protease selected from the group consisting of trypsin, chymotrypsin, ArgC, GluC, AspN, LysC, LysN, elastase and proteinase K and a combination thereof; more preferably of trypsin and LysC.

21. The method according to any one of claims 4 or 20, wherein the proteolytic digestion is performed at a temperature of between about 34°C and about 40°C; and/or wherein the proteolytic digestion comprises the use of one or more protease(s) in a ratio of between about 1:5 and 1:40 (w/w, protease(s) to protein substrate); and/or the proteolytic digestion is allowed to proceed for at least about 15 minutes; and/or the proteolytic digestion is allowed to proceed for between about 30 minutes and about 60 minutes.