

ACTIVITY BASED HOST CELL PROTEIN PROFILING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 63/073,125, filed September 1, 2020, U.S. Provisional Patent Application No. 62/982,346, filed February 27, 2020 and U.S. Provisional Patent Application No. 63/021,181, filed May 7, 2020, the contents of which are incorporated herein by reference in their entirety.

FIELD

[0001] The present invention generally pertains to methods and systems for identifying host cell protein impurities which have enzymatic activities. These methods and systems can be applied in biopharmaceutical products and in samples during manufacturing processes to identify and monitor the host cell protein impurities.

BACKGROUND

[0002] During manufacturing of biopharmaceutical products, such as the purification of monoclonal antibodies, it is required to remove impurities to obtain biopharmaceutical products having high purities. In particular, since DNA technology has been used widely for producing biopharmaceutical products in host cells, residual host cell proteins (HCPs) can present potential safety risks to patients due to compromised product quality and stability. For producing recombinant therapeutic antibodies, several purification processes are needed to ensure that the antibody product is highly purified. Any residual impurities after conducting the bioprocesses should be present at an acceptable low level prior to conducting clinical studies. In particular, HCPs derived from mammalian expression systems, for example, Chinese hamster ovary (CHO) cells, should be monitored and controlled in the final drug substance. Sometimes, trace amounts of particular HCPs may cause an immune response or toxic biologic activities after drug injection, even though the total HCPs may be present at very low levels. Therefore, there are unmet needs to identify and monitor specific HCPs for risk assessment.

[0003] The presence of residual HCPs can cause potential safety risks or problems in drug

stability, especially HCPs which have enzymatic activities, such as active proteases. It will be appreciated that a need exists for methods and systems to identify, profile, characterize, and quantify HCP impurities which have enzymatic activities. These methods and systems should provide robust, reliable and sensitive detection of enzymatic HCP impurities in biopharmaceutical products and in samples during manufacturing processes.

SUMMARY

[0004] Defining acceptable levels of host cell protein (HCP) impurities has become a critical issue for using biological systems to manufacture therapeutic products. There are a large number of HCP impurities, for example, potentially thousands of components, which have to be controlled and monitored, to ensure the safety and efficacy of biopharmaceutical products.

[0005] The present application provides methods and systems for identifying, profiling, characterizing or quantifying HCP impurities which have enzymatic activities. These methods and systems can be used to identify and monitor the HCP impurities in biopharmaceutical products and in samples during manufacturing processes, including providing various activity-based probes to characterize different enzyme classes of HCP impurities.

[0006] This disclosure provides a method of identifying, profiling, characterizing or quantifying HCP impurities in a sample. In some exemplary embodiments, the method of the present application comprises contacting the sample with at least one probe to provide probe-attached HCP impurities, wherein the probe comprises a warhead and a tag, wherein the warhead is capable of binding to an active site of the probe-attached HCP impurity, and wherein the sample comprises at least one high-abundance protein. In one aspect, the warhead of the probe of the method is capable of binding covalently to a residue in the active site of the probe-attached HCP impurity. In one aspect, the tag of the probe of the method is a conjugation tag, an affinity tag, or a reporter tag.

[0007] In one aspect, the method of the present application further comprises: contacting the sample with a solid support, subsequently washing the solid support using a solution to isolate the probe-attached HCP impurities and to provide an eluent, treating the eluent with an enzymatic digestion reaction to generate components of the isolated probe-attached HCP impurities, and subsequently identifying, profiling, characterizing or quantifying the components

of the isolated probe-attached HCP impurities using a mass spectrometer; wherein the solid support comprises a ligand, and wherein the ligand is capable of binding to the tag.

[0008] In one aspect, the warhead of the probe of the method of the present application comprises an enzymatic inhibitor, an enzymatic substrate-based scaffold or a protein-reactive molecule. In one aspect, the at least one probe of the method of the present application further comprises a linker. In one aspect, the at least one high-abundance protein in the sample of the method is an antibody, a bispecific antibody, an antibody fragment, a Fab region of an antibody, an antibody-drug conjugate, a fusion protein, a protein pharmaceutical product or a drug. In another aspect, an enzyme of the enzymatic digestion reaction of the method is trypsin.

[0009] In one aspect, the mass spectrometer of the method of the present application is an electrospray ionization mass spectrometer, nano-electrospray ionization mass spectrometer, such as an Orbitrap mass spectrometer, a Q-TOF mass spectrometer or a triple quadrupole mass spectrometer, wherein the mass spectrometer is coupled to a liquid chromatography system. In one aspect, the mass spectrometer of the method is capable of performing LC-MS (liquid chromatography-mass spectrometry), nano-LC-MS, LC-MS/MS, nano-LC-MS/MS or LC-MRM-MS (liquid chromatography-multiple reaction monitoring-mass spectrometry) analyses.

[0010] In one aspect, the tag of the probe of the method is detected using Western blot, capillary electrophoresis, SDS-PAGE, fluorescent visualization, or fluorescent gel imaging. In another aspect, the active site of the probe-attached HCP impurity of the method is a cysteine protease active site, a serine protease active site, a serine hydrolase active site, a cathepsin active site, a metalloprotease active site, a cholinesterase active site, an active site of a lipid-binding protein, an active site of a sphingolipid-binding protein, an active site of a ceramide-binding protein, a lipase active site, a protease active site, a hydrolase active site, an oxidoreductase active site, or an isomerase active site.

[0011] In one aspect, the tag of the probe of the method comprises a fluorophore or a fluorophore conjugation site, such as rhodamine, biotin, phosphine, alkyne, azide, acetylene, cyclooctyne, phenyl azide or omega-terminal azide. In one aspect, the warhead of the probe comprises fluorophosphonate, epoxysuccinate, photo-activatable lipid, photo-activatable sphingosine, N-acetylated amino acid, quinolimine methide coupled amino acid, or p-aminomandelic acid coupled amino acid. In one aspect, the probe of the method of the present

application comprises azido-fluorophosphonate; desthiobiotin-fluorophosphonate; tetramethylrhodamine-fluorophosphonate; ethyl (2S, 3S)-epoxysuccinate-Leu-Tyr-Acp-Lys(Biotin)-NH₂; 1-palmitoyl-2-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-*sn*-glycero-3-phosphocholine; (2S,3R,E)-2-amino-13-(3-(pent-4-yn-1-yl)-3H-diazirin-3-yl)tridec-4-ene-1,3-diol; N-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-D-erythro-sphingosine; 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(5-hexynoyl); or D-galactosyl- β -1,1' N-(6"-azidohexanoyl)-D-erythro-sphingosine.

[0012] This disclosure, at least in part, provides a system for identifying, profiling, characterizing or quantifying HCP impurities in a sample. In some exemplary embodiments, the system of the present application comprises: at least one probe, wherein the at least one probe comprises a warhead and a tag, wherein the at least one probe is capable of binding to HCP impurities to provide probe-attached HCP impurities, wherein the warhead is capable of binding to an active site of the probe-attached HCP impurity, and wherein the sample comprises at least one high-abundance protein; a solid support, wherein the solid support comprises a ligand which is capable of binding to the tag; a solution for washing the solid support to isolate the probe-attached HCP impurities and to provide an eluent; an enzymatic digestion solution capable of generating components of the isolated probe-attached HCP impurities; and a mass spectrometer capable of identifying, profiling, characterizing and/or quantifying the components from the isolated probe-attached HCP impurity.

[0013] In one aspect, the warhead of the probe of the system is capable of binding covalently to a residue in the active site of the probe-attached HCP impurity. In one aspect, the tag of the probe of the system is a conjugation tag, an affinity tag or a reporter tag. In another aspect, the warhead of the probe of the system comprises an enzymatic inhibitor, an enzymatic substrate-based scaffold or a protein-reactive molecule. In another aspect, the at least one probe of the system further comprises a linker. In yet another aspect, the at least one high-abundance protein in the sample of the system is an antibody, a bispecific antibody, an antibody fragment, a Fab region of an antibody, an antibody-drug conjugate, a fusion protein, a protein pharmaceutical product or a drug. In one aspect, an enzyme of the enzymatic digestion solution of the system is trypsin.

[0014] In one aspect, the mass spectrometer of the system is an electrospray ionization

mass spectrometer, nano-electrospray ionization mass spectrometer, such as an Orbitrap mass spectrometer, a Q-TOF mass spectrometer or a triple quadrupole mass spectrometer, wherein the mass spectrometer is coupled to a liquid chromatography system. In another aspect, the mass spectrometer of the system is capable of performing LC-MS (liquid chromatography-mass spectrometry), nano-LC-MS, LC-MS/MS, nano-LC-MS/MS or a LC-MRM-MS (liquid chromatography-multiple reaction monitoring-mass spectrometry) analyses. In one aspect, the tag of the probe of the system is detected using Western blot, capillary electrophoresis, SDS-PAGE, fluorescent visualization, or fluorescent gel imaging.

[0015] In one aspect, the active site of the probe-attached HCP impurity is a cysteine protease active site, a serine protease active site, a serine hydrolase active site, a cathepsin active site, a metalloprotease active site, a cholinesterase active site, an active site of a lipid-binding protein, an active site of a sphingolipid-binding protein, an active site of a ceramide-binding protein, a lipase active site, a protease active site, a hydrolase active site, an oxidoreductase active site, or an isomerase active site. In one aspect, the tag of the probe of the system comprises a fluorophore or a fluorophore conjugation site, such as rhodamine, biotin, phosphine, alkyne, azide, acetylene, cyclooctyne, phenyl azide, or omega-terminal azide.

[0016] In one aspect, the warhead of the probe of the system comprises fluorophosphonate, epoxysuccinate, photo-activatable lipid, photo-activatable sphingosine, N-acetylated amino acid, quinolinine methide coupled amino acid, or p-aminomandelic acid coupled amino acid. In one aspect, the probe of the system comprises azido-fluorophosphonate; desthiobiotin-fluorophosphonate; tetramethylrhodamine-fluorophosphonate; ethyl (2S, 3S)-epoxysuccinate-Leu-Tyr-Acp-Lys(Biotin)-NH₂; 1-palmitoyl-2-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-sn-glycero-3-phosphocholine; (2S,3R,E)-2-amino-13-(3-(pent-4-yn-1-yl)-3H-diazirin-3-yl)tridec-4-ene-1,3-diol; N-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-D-erythro-sphingosine; 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(5-hexynoyl); or D-galactosyl-β-1,1' N-(6"-azidohexanoyl)-D-erythro-sphingosine.

[0017] These, and other, aspects of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. The following description, while indicating various embodiments and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications,

additions, or rearrangements may be made within the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 shows a probe containing a tag and a fluorophosphonate (FP) group which is capable of covalently and specifically attaching to a serine residue in the active site of serine hydrolases, including lipases and proteases, according to an exemplary embodiment.

[0019] FIG. 2 shows chemical structures of serine hydrolase probes including azido-FP, desthiobiotin-FP and TAMRA-FP according to an exemplary embodiment.

[0020] FIG. 3 shows the chemical structure of a cysteine protease probe, DCG-04, *e.g.*, Ethyl (2S, 3S)-epoxysuccinate-Leu-Tyr-Acp-Lys(Biotin)-NH₂, CD-892 according to an exemplary embodiment.

[0021] FIG. 4 shows the chemical structure of a lipid probe, 16:0-pacFA PC, *e.g.*, 1-palmitoyl-2-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-*sn*-glycero-3-phosphocholine according to an exemplary embodiment.

[0022] FIG. 5 shows the chemical structure of a lipid probe, PhotoClick Sphingosine (900600), *e.g.*, (2S,3R,E)-2-amino-13-(3-(pent-4-yn-1-yl)-3H-diazirin-3-yl)tridec-4-ene-1,3-diol according to an exemplary embodiment.

[0023] FIG. 6 shows the chemical structure of a lipid probe, pacFA ceramide (900404), *e.g.*, N-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-D-erythro-sphingosine according to an exemplary embodiment.

[0024] FIG. 7 shows the chemical structures and mechanisms of copper-catalyzed azide-alkyne cycloaddition reaction according to an exemplary embodiment.

[0025] FIG. 8 shows the chemical structure of a lipid probe, 16:0 hexynoyl PE (870127), *e.g.*, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(5-hexynoyl) (ammonium salt) according to an exemplary embodiment. This probe contains ester linkages similar to those hydrolyzed in PS-20 (polysorbate 20).

[0026] FIG. 9 shows the chemical structure of a lipid probe, C6(6-azido) GalCer (860833), *e.g.*, D-galactosyl- β -1,1' N-(6"-azidohexanoyl)-D-erythro-sphingosine according to an exemplary embodiment.

[0027] FIG. 10 shows the molecular weights and calculated isoelectric points, *e.g.*, pI, of the 586 proteins which were identified in labelled HCPs in a clarified Chinese Hamster Ovary (CHO) cell-supernatant sample according to an exemplary embodiment. The dot size indicates the differences in protein abundance of labelled HCPs in the clarified-supernatant sample with a probe and without a probe according to an exemplary embodiment. The protein abundance was denoted as color scale from red (high abundance) to blue (low abundance) according to an exemplary embodiment.

[0028] FIG. 11 shows the results of gene ontology analysis for the HCP impurities which were enriched using serine hydrolase probes according to an exemplary embodiment.

[0029] FIG. 12 shows the comparisons of identified HCP impurities among an unenriched sample, a sample enriched by 16:0-pacFA PC probe, and a control sample (no UV) according to an exemplary embodiment.

DETAILED DESCRIPTION

[0030] The presence of host cell protein (HCP) impurities in biopharmaceutical products can impact some clinical consequences including drug efficacy and patient safety. Various approaches have been used to detect and identify HCP impurities in biopharmaceutical products and in samples from manufacturing steps. Some HCP impurities have enzymatic activities, for example, enzymatic HCP impurities. The identification of specific HCPs which have enzymatic activities can provide important information to assess the risk of HCPs to product safety.

[0031] The present application provides methods and systems to characterize, profile, identify or quantify HCP impurities which have enzymatic activities, for example, activity-based HCP profiling, including uses of activity-based probes. The present application provides various activity-based probes to characterize different enzyme classes. The activity-based probes of the present application include functionalized small-molecules and a tag, such as a reporter tag or an affinity-based tag, to directly monitor the enzyme activity of a given target or sets of targets. Some activity-based probes of the present application can covalently bind to enzymatic active sites for profiling different classes of enzymes based on the specific catalytic mechanisms. For example, specific lipids having affinities to lipases can be used as activity-based probes to enrich HCP impurities which have lipase activities. Similar approaches can be used for

characterizing, profiling, identifying or quantifying serine hydrolases, cathepsins, lipid binding proteins, and metalloproteases.

[0032] Activity-based protein profiling has been used to investigate the changes of catalytic activity of enzyme classes in complex proteome, rather than quantitating the protein abundance. Activity-based protein profiling can be used to functionally annotate the enzyme function of proteins. Different probes have been used to differentially label active proteins including proteases, hydrolases, oxidoreductases and isomerases (Blais et al., Activity-based proteome profiling of hepatoma cells during Hepatitis C virus replication using protease substrate probes, *Journal of Proteome Research*, 2010 Feb 5;9(2):912-23. doi: 10.1021/pr900788a). Blais et al. utilized a group of probes composed of an N-acetylated amino acid, which mimics the P₁ position in protease peptide substrates bearing distinct amino acids, such as serine, threonine, phenylalanine, glutamic acid and histidine. The activity-based probes can include amino acid coupled quinolimine methide probes and amino acid coupled p-aminomandelic acid probes. Activity-based protein profiling also has been used to monitor the functional status of enzymes by utilizing active site-directed probes, such as detecting and analyzing functional annotation of host cell serine hydrolases for host-virus interaction (Shahiduzzaman et al., Activity based protein profiling to detect serine hydrolase alterations in virus infected cells, *Frontiers in Microbiology*, August 22, 2012, volume 3, article 308, p 1-5). In addition, since activity-based probes can specifically recognize active proteases, they can be used to detect and quantitate proteolytic activity *in situ*, especially in conjunction with positron emission tomography (Ulrich auf dem Keller et al., *Proteomic techniques and activity-based probes for the system-wide study of proteolysis*, *Biochimie*, 92 (2010), page 1705-1714).

[0033] In some exemplary embodiments, the present application provides a method of identifying, profiling, characterizing or quantifying HCP impurities in a sample, the method comprising: contacting the sample with at least one probe to provide probe-attached HCP impurities, wherein the probe comprises a warhead and a tag, wherein the warhead is capable of binding to an active site of the probe-attached HCP impurity, and wherein the sample comprises at least one high-abundance protein. In some aspects, the present application provides a system for identifying, profiling, characterizing or quantifying HCP impurities in a sample, the system comprising: at least one probe, wherein the at least one probe comprises a warhead and a tag,

wherein the at least one probe is capable of binding to HCP impurities to provide probe-attached HCP impurities, wherein the warhead is capable of binding to an active site of the probe-attached HCP impurity, and wherein the sample comprises at least one high-abundance protein; a solid support, wherein the solid support comprises a ligand which is capable of binding to the tag; a solution for washing the solid support to isolate the probe-attached HCP impurities and to provide an eluent; an enzymatic digestion solution capable of generating components of the isolated probe-attached HCP impurities; and a mass spectrometer capable of identifying, profiling, characterizing or quantifying the components from the isolated probe-attached HCP impurity.

[0034] In one aspect, the activity-based probe of the present application is an enzymatic active site-directed probe comprising a warhead, such as a reactive group for reacting and binding to enzymatic active sites. In one aspect, the activity-based probe of the present application comprises a warhead and a tag, such as a reporter tag. In another aspect, the activity-based probe of the present application comprises a warhead, a tag and a linker. In another aspect, the warhead is a reactive group which can bind to or covalently label the active site of an enzyme. In yet another aspect, the warhead is a reactive group, such as a small molecule inhibitor, a substrate-based scaffold or a protein-reactive molecule. In one aspect, the warhead has been designed to target cysteine or serine proteases by covalently binding to the active site residue. Since proteins in the same enzymatic family have similar functions, the active sites of these proteins commonly have similar structures, therefore an active site-directed probe can be reactive to the active sites of many members of a given enzymatic family. In one aspect, the tag is a reporter tag for detection and identification of the labelled enzyme, such as direct molecular imaging or radiolabeling. For example, the tag can be a fluorophore, such as rhodamine, for visualization. In one aspect, the tag is an affinity tag for enrichment or purification, such as biotin. In another aspect, the tag is a labelling tag, such as azides or acetylenes, for *in vivo* or *in situ* labeling of proteins. In another aspect, the linker serves as a connector for connecting the warhead and the tag. In one aspect, the linker serves as a spacer between the warhead and the tag including a flexible chain with various length and hydrophobicity. In some aspects, the activity-based probes of the present application are active site-directed probes that consist of small molecule inhibitors linked to reporter tags.

[0035] In some exemplary embodiments, serine hydrolase probes are used to label, assay, purify or detect enzymatic HCP impurities which have serine hydrolase activities. Serine hydrolase enzymes are a large class of enzymes that include cholinesterases, hydrolases, lipases, and proteases. In one aspect, the serine hydrolase probe has a fluorophosphonate (FP) group that covalently modifies the active sites of serine hydrolases. Only active serine hydrolases can be modified. The methods and systems of the activity-based HCP profiling of the present application provide the advantages of labelling HCP impurities which have active enzymatic activities, such as active proteases and active lipases. These active proteases and lipases might be problematic for drug integrity in various processing steps.

[0036] In some exemplary embodiments, the methods and systems of the activity-based HCP profiling of the present application in combination with mass spectrometry (MS) can be used to identify protein sequences of enzymatic HCP impurities. In one aspect, the methods and systems of the present application in combination with MS can be used to identify active enzymatic HCP impurities. In one aspect, when a fluorescent probe is used as the activity-based probe in the methods and systems of the present application, active enzymatic HCP impurities can be visualized. In another aspect, the methods and systems of the activity-based HCP profiling of the present application can be used to quantitate active enzymatic HCP impurities.

[0037] The methods and systems of the present application provide the advantages of direct detection and quantification of enzymatic activities of HCP impurities rather than simply quantitating the abundance of the presence of HCP impurities. The methods and systems of the present application also provide the advantages of monitoring the functional state of large numbers of enzymes in HCP impurities.

[0038] The demands of improving the product quality, efficacy and safety of biopharmaceutical products have led to an increasing demand for monitoring HCP impurities which have enzymatic activities. This disclosure provides methods and systems to satisfy the aforementioned demands. Exemplary embodiments disclosed herein satisfy the aforementioned demands by providing methods and systems for identifying, profiling, characterizing, or quantifying HCP impurities which have enzymatic activities in biopharmaceutical products and in samples during manufacturing processes. The present application provides various activity-based probes to characterize different enzyme classes of HCP impurities to satisfy the long felt

needs.

[0039] The term “a” should be understood to mean “at least one”; and the terms “about” and “approximately” should be understood to permit standard variation as would be understood by those of ordinary skill in the art; and where ranges are provided, endpoints are included. As used herein, the terms “include,” “includes,” and “including,” are meant to be non-limiting and are understood to mean “comprise,” “comprises,” and “comprising,” respectively.

[0040] In some exemplary embodiments, the present application provides a system for identifying, profiling, characterizing or quantifying HCP impurities in a sample, the system comprising: at least one probe, wherein the at least one probe comprises a warhead and a tag, wherein the at least one probe is capable of binding to HCP impurities to provide probe-attached HCP impurities, wherein the warhead is capable of binding to an active site of the probe-attached HCP impurity, and wherein the sample comprises at least one high-abundance protein; a solid support; an enzymatic digestion solution capable of generating components of the isolated probe-attached HCP impurities; and a mass spectrometer capable of identifying, profiling, characterizing or quantifying the components from the isolated probe-attached HCP impurity.

[0041] As used herein, the term “protein” includes any amino acid polymer having covalently linked amide bonds. Proteins comprise one or more amino acid polymer chains, generally known in the art as “polypeptides”. A protein may contain one or multiple polypeptides to form a single functioning biomolecule. In some exemplary embodiments, the protein can be an antibody, a monoclonal antibody, a bispecific antibody, a multi-specific antibody, an antibody fragment, a Fab region of an antibody, an antibody-drug conjugate, a fusion protein, a protein pharmaceutical product, a drug or combinations thereof.

[0042] As used herein, a “mass spectrometer” includes a device capable of identifying specific molecular species and measuring their accurate masses. The term is meant to include any molecular detector into which a polypeptide or peptide may be eluted for detection and/or characterization. A mass spectrometer can include three major parts: the ion source, the mass analyzer, and the detector. The role of the ion source is to create gas phase ions. Analyte atoms, molecules, or clusters can be transferred into gas phase and ionized either concurrently (as in electrospray ionization). The choice of ion source depends heavily on the application.

[0043] In one aspect, the at least one high-abundance protein in the sample of the method can be an antibody, a bispecific antibody, an antibody fragment, a Fab region of an antibody, an antibody-drug conjugate, a fusion protein, a protein pharmaceutical product or a drug.

[0044] As used herein, an “antibody” is intended to refer to immunoglobulin molecules consisting of four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. Each heavy chain has a heavy chain variable region (HCVR or VH) and a heavy chain constant region. The heavy chain constant region contains three domains, CH1, CH2 and CH3. Each light chain has of a light chain variable region and a light chain constant region. The light chain constant region consists of one domain (CL). The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL can be composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The term “antibody” includes reference to both glycosylated and non-glycosylated immunoglobulins of any isotype or subclass. The term “antibody” is inclusive of, but not limited to, those that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell transfected to express the antibody. An IgG comprises a subset of antibodies.

[0045] As used herein, a “protein pharmaceutical product” includes an active ingredient which can be fully or partially biological in nature. In one aspect, the protein pharmaceutical product can comprise a peptide, a protein, a fusion protein, an antibody, an antigen, vaccine, a peptide-drug conjugate, an antibody-drug conjugate, a protein-drug conjugate, cells, tissues, or combinations thereof. In another aspect, the protein pharmaceutical product can comprise a recombinant, engineered, modified, mutated, or truncated version of a peptide, a protein, a fusion protein, an antibody, an antigen, vaccine, a peptide-drug conjugate, an antibody-drug conjugate, a protein-drug conjugate, cells, tissues, or combinations thereof.

[0046] In one aspect, the mass spectrometer of the method of the present application can be an electrospray ionization mass spectrometer, nano-electrospray ionization mass spectrometer, such as an Orbitrap mass spectrometer, a Q-TOF mass spectrometer or a triple quadrupole mass spectrometer, wherein the mass spectrometer is coupled to a liquid chromatography system. In

one aspect, the mass spectrometer of the method is capable of performing LC-MS (liquid chromatography-mass spectrometry), nano-LC-MS, LC-MS/MS, nano-LC-MS/MS or a LC-MRM-MS (liquid chromatography-multiple reaction monitoring-mass spectrometry) analyses.

[0047] As used herein, the term “electrospray ionization” or “ESI” refers to the process of spray ionization in which either cations or anions in solution are transferred to the gas phase via formation and desolvation at atmospheric pressure of a stream of highly charged droplets that result from applying a potential difference between the tip of the electrospray needle containing the solution and a counter electrode. There are generally three major steps in the production of gas-phase ions from electrolyte ions in solution. These are: (a) production of charged droplets at the ES infusion tip; (b) shrinkage of charged droplets by solvent evaporation and repeated droplet disintegrations leading to small highly charged droplets capable of producing gas-phase ions; and (c) the mechanism by which gas-phase ions are produced from very small and highly charged droplets. Stages (a)–(c) generally occur in the atmospheric pressure region of the apparatus. In some exemplary embodiments, the electrospray ionization mass spectrometer can be a nano-electrospray ionization mass spectrometer.

[0048] As used herein, the term “triple quadrupole mass spectrometer” refers to a tandem mass spectrometer consisting of two quadrupole mass analyzers in series, with a (non-mass-resolving) radio frequency (RF)-only quadrupole between them to act as a cell for collision-induced dissociation. In a triple quadrupole mass spectrometer, a peptide sample is injected onto an LC coupled with a MS instrument. The first quadrupole can be used as a mass filter to isolate peptides with a targeted m/z . The second quadrupole serves as a collision cell to break the peptide into fragments. The third quadrupole serves as a second mass filter for specified m/z fragments from the initial parent peptide. As used herein, the term “tandem mass spectrometry” includes a technique where structural information on sample molecules can be obtained by using multiple stages of mass selection and mass separation. A prerequisite is that the sample molecules can be transferred into gas phase and ionized intact and that they can be induced to fall apart in some predictable and controllable fashion after the first mass selection step. Tandem MS have been successfully performed with a wide variety of analyzer combinations. What analyzers to combine for a certain application can be determined by many different factors, such as sensitivity, selectivity, and speed, but also size, cost, and availability.

The two major categories of tandem MS methods are tandem-in-space and tandem-in-time, but there are also hybrids where tandem-in-time analyzers are coupled in space or with tandem-in-space analyzers. A tandem-in-space mass spectrometer comprises an ion source, a precursor ion activation device, and at least two non-trapping mass analyzers. Specific m/z separation functions can be designed so that in one section of the instrument ions are selected, dissociated in an intermediate region, and the product ions are then transmitted to another analyzer for m/z separation and data acquisition. In tandem-in-time mass spectrometers ions produced in the ion source can be trapped, isolated, fragmented, and m/z separated in the same physical device.

[0049] As used herein, the term “liquid chromatography” refers to a process in which a chemical mixture carried by a liquid or gas can be separated into components as a result of differential distribution of the chemical entities as they flow around or over a stationary liquid or solid phase. Non-limiting examples of chromatography include traditional reversed-phased (RP), ion exchange (IEX), mixed mode chromatography and normal phase chromatography (NP).

Exemplary embodiments

[0050] Embodiments disclosed herein provide methods and systems for identifying, profiling, characterizing or quantifying HCP impurities which have enzymatic activities.

[0051] In some exemplary embodiments, the present application provides a method of identifying, profiling, characterizing or quantifying HCP impurities in a sample, the method comprising contacting the sample with at least one probe to provide probe-attached HCP impurities, wherein the probe comprises a warhead and a tag, wherein the warhead is capable of binding to an active site of the probe-attached HCP impurity, and wherein the sample comprises at least one high-abundance protein.

[0052] In one aspect, the tag of the probe of the method is a conjugation tag, an affinity tag, or a reporter tag, such as a fluorophore or a fluorophore conjugation site, such as rhodamine, biotin, phosphine, alkyne, azide, acetylene, cyclooctyne, phenyl azide or omega-terminal azide. In one aspect, the warhead of the probe contains fluorophosphonate, epoxysuccinate, photo-activatable lipid, photo-activatable sphingosine, N-acetylated amino acid, quinolinine methide coupled amino acid, or p-aminomandelic acid coupled amino acid. In one aspect, the probe of

the method comprises azido-fluorophosphonate; desthiobiotin-fluorophosphonate; tetramethylrhodamine-fluorophosphonate; ethyl (2S, 3S)-epoxysuccinate-Leu-Tyr-Acp-Lys(Biotin)-NH₂; 1-palmitoyl-2-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-*sn*-glycero-3-phosphocholine; (2S,3R,E)-2-amino-13-(3-(pent-4-yn-1-yl)-3H-diazirin-3-yl)tridec-4-ene-1,3-diol; N-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-D-erythro-sphingosine; 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(5-hexynoyl); or D-galactosyl-β-1,1' N-(6"-azidohexanoyl)-D-erythro-sphingosine. In another aspect, the active site of the probe-attached HCP impurity of the method is a cysteine protease active site, a serine protease active site, a serine hydrolase active site, a cathepsin active site, a metalloprotease active site, a cholinesterase active site, an active site of a lipid-binding protein, an active site of a sphingolipid-binding protein, an active site of a ceramide-binding protein, a lipase active site, a protease active site, a hydrolase active site, an oxidoreductase active site, or an isomerase active site.

[0053] It is understood that the system is not limited to any of the aforesaid HCP impurities, activity-based probes, enzymatic active sites, warheads, conjugation tags, affinity tags, reporter tags, solid supports, pharmaceutical products, peptides, proteins, antibodies, anti-drug antibodies, chromatography column, or mass spectrometer.

[0054] The consecutive labeling of method steps as provided herein with numbers and/or letters is not meant to limit the method or any embodiments thereof to the particular indicated order. Various publications, including patents, patent applications, published patent applications, accession numbers, technical articles and scholarly articles are cited throughout the specification. Each of these cited references is incorporated herein by reference, in its entirety and for all purposes. Unless described otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. This disclosure will be more fully understood by reference to the following Examples, which are provided to describe this disclosure in greater detail. They are intended to illustrate and should not be construed as limiting the scope of this disclosure.

EXAMPLES

Material and reagents

1. Biopharmaceutical products containing HCP impurities

[0055] Several biopharmaceutical samples containing HCP impurities, such as samples containing monoclonal antibodies and HCP impurities, were used for characterizing enzymatic HCP impurities. These samples included: (1) clarified cell culture supernatant (5 mg) from CHO cells; (2) LPL (Lipoprotein Lipase) and PLBD2 (Phospholipase B Domain-Containing Protein 2) knockout AEX material (LPL-PLBD2 knockout-AEX, 5 mg) which contained cell culture supernatant from CHO cells derived from LPL-PLBD2 knockout CHO cell line and processed by anion exchange (AEX) chromatography; (3) AEX material (AEX-supernatant, 5 mg) which contained cell culture supernatant processed by anion exchange chromatography; (4) drug substance (DS, 10 mg) which contained purified biopharmaceutical products, such as purified monoclonal antibodies; (5) drug substance which was spiked with lipases at 10 ppm (spiked-DS, 10 mg) including acid ceramidase, PLBD2, lipoprotein lipase, and lysosomal acid lipase.

2. Serine hydrolase probes

[0056] Serine hydrolase probes, *e.g.*, Pierce™ ActivX™ Serine Hydrolase Probes (Thermo Fisher Scientific), were used to label, assay, purify or detect HCP impurities which have serine hydrolase activities as shown in FIG. 1 and FIG. 2. Several serine hydrolase probes containing fluorophosphonate (FP) can be used to modify the serine residue in the active site of serine hydrolases including lipases and proteases, since FP can specifically initiate the covalent modification of the serine nucleophile in the active site. FIG. 1 shows the mechanism and structure of serine hydrolase probes for modifying the active site of serine hydrolases. FIG. 2 shows the chemical structures of serine hydrolase probes including azido-FP, desthiobiotin-FP and TAMRA-FP (tetramethylrhodamine-fluorophosphonate). Azido-FP probes can be used in combination with phosphine- or alkyne-derived tags for detection or enrichment. Desthiobiotin-FP probes can be used for affinity enrichment and detection of serine hydrolases by Western blot or mass spectrometry, such as in combination with streptavidin Dynabeads. TAMRA-FP probe has a fluorescence tag which enables the labelling and detection of serine hydrolase activity in samples through fluorescent gel imaging, capillary electrophoresis or mass spectrometry.

[0057] The workflows for profiling, capturing, and detecting serine hydrolases in HCP samples with ActivX™ FP probes include pre-incubation of samples with probes (*e.g.*, inhibitors) and determination of inhibitor specificity, binding affinity and potency through Western blot, fluorescent SDS-PAGE or mass spectrometry.

3. Cysteine protease probe

[0058] A cysteine protease probe, DCG-04, was used to profile cathepsin activity as shown in FIG. 3. DCG-04, for example, Ethyl (2S, 3S)-epoxysuccinate-Leu-Tyr-Acp-Lys(Biotin)-NH₂, CD-892, an epoxysuccinate-containing probe for clan CA cysteine proteases, is based on the naturally occurring inhibitor E-64 and targets the papain family of cysteine proteases via covalent attachment of the epoxysuccinate group to the active site cysteine. DCG-04 probe can be used for affinity enrichment and detection of cysteine proteases by Western blot or mass spectrometry, such as in combination with streptavidin Dynabeads.

4. Lipid probe, 16:0-pacFA PC

[0059] A lipid probe, 16:0-pacFA PC, *e.g.*, 1-palmitoyl-2-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-*sn*-glycero-3-phosphocholine, was used to purify sphingolipid-binding proteins as shown in FIG. 4. This probe provides photo-activatable linkage of lipid to proteins in close proximity. The lipid-labelled protein can be subsequently conjugated to beads using the alkyne moiety through azide-alkyne Huisgen cycloaddition to azide functionalized beads. The lipid-labelled proteins can be enriched or purified through the isolation of the conjugated-beads.

5. Lipid probe, PhotoClick Sphingosine (900600)

[0060] A lipid probe, PhotoClick Sphingosine (900600), *e.g.*, (2S,3R,E)-2-amino-13-(3-(pent-4-yn-1-yl)-3H-diazirin-3-yl)tridec-4-ene-1,3-diol, was used to purify sphingosine proteins as shown in FIG. 5. This probe provides photo-activatable linkage of lipid to proteins in close proximity. The lipid-labelled protein can be subsequently conjugated to beads using the alkyne moiety through azide-alkyne Huisgen cycloaddition to azide functionalized beads. The lipid-labelled proteins can be enriched or purified through the isolation of the conjugated-beads. This photo-activatable and clickable analog of sphingosine has been used to profile sphingolipid-binding proteins in sphingosine-1-phosphate lyase deficient cells to understand the global cellular interplay between sphingolipids and their interacting proteins (Haberkant et al., Bifunctional Sphingosine for Cell-Based Analysis of Protein-Sphingolipid Interactions, ACS Chem Biol., 2016, Jan 15;11(1):222-30. doi: 10.1021/acscchembio.5b00810. Epub 2015 Nov 25).

6. Lipid probe, pacFA ceramide (900404)

[0061] A lipid probe, pacFA ceramide (900404), for example, N-(9-(3-pent-4-ynyl-3-H-

diazirin-3-yl)-nonanoyl)-D-erythro-sphingosine, was used to purify ceramide-binding proteins as shown in FIG. 6. This probe provides linkage of lipid to proteins in close proximity. The lipid-labelled protein can be subsequently conjugated to beads using the alkyne moiety through azide-alkyne Huisgen cycloaddition to azide functionalized beads. The lipid-labelled proteins can be enriched or purified through the isolation of the conjugated-beads.

7. Agarose and beads with azide-alkyne cycloaddition reaction

[0062] Agarose and magnetic beads were used to pull down the labelled proteins. Magnetic or agarose beads containing azide can be used to capture proteins which were cross-linked to alkyne-containing lipid through copper-catalyzed azide-alkyne cycloaddition reaction. FIG. 7 shows the chemical structures and mechanisms of copper-catalyzed azide-alkyne cycloaddition reaction.

8. Lipid probe, 16:0 hexynoyl PE (870127)

[0063] A lipid probe, 16:0 hexynoyl PE (870127), for example, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(5-hexynoyl) (ammonium salt), was used to purify lipid-binding proteins as shown in FIG. 8. This probe was used to purify labelled proteins using the alkyne moiety through azide-alkyne Huisgen cycloaddition to azide functionalized beads or between cyclooctyne and phenyl azide-functionalized beads. The labelled proteins can be enriched or purified through the isolation of the conjugated-beads. This probe contains ester linkages similar to those hydrolyzed in PS-20 (polysorbate 20). However, the photo-activatable linkage is not available.

9. Lipid probe, C6(6-azido) GalCer (860833)

[0064] A lipid probe, C6(6-azido) GalCer (860833), *e.g.*, D-galactosyl- β -1,1' N-(6"-azidohexanoyl)-D-erythro-sphingosine, which is an azide-functionalized sphingolipid with sugar group as shown in FIG. 9, was used to purify lipid-binding proteins. This probe is a modified lipid containing an omega-terminal azide. The terminal azide group can be used in a highly specific linking reaction with alkyne-containing reagents, *e.g.*, click chemistry, in the presence of a copper (Cu)-containing catalyst.

10. Reagent and sample preparation for labelling HCP impurities with serine hydrolase probes

[0065] Labelling buffer (10X TBS (Tris buffered saline), MgCl_2 , CaCl_2 , NP-40): 500 mM potassium phosphate buffer, pH 7.5, containing 20 mM MgCl_2 , 5 mM CaCl_2 and 1% NP-40. 0.2 g of MgCl_2 , 0.1 g of CaCl_2 and 1 mL of NP-40 added to 100 mL of 10X TBS.

[0066] 10 M urea lysis buffer: 0.9 g of urea dissolved with 1.5 mL of Lysis Buffer for each labeling reaction.

[0067] 1.5 M urea in 1X TBS: 1.35 g of urea dissolved with 10 mL 1X TBS.

[0068] Lipase mixture: (LPL: Lipoprotein Lipase; LIPA: Lipase A lysosomal acid type; ASAH: N-Acylsphingosine Amidohydrolase, Acid Ceramidase; PLBD2: Phospholipase B Domain Containing 2). 10 ppm lipases were spiked in samples for conducting the experiments.

Table 1. Preparation of lipase mixture.

	Sample Lot	Concentration of lipase	Volume for master mix (mL)	Concentration in master mix	μg to give 10 ppm in 10 mg
LPL	MAB2-L8	0.491	10.18	0.05	0.1
LIPA	MAB3-L1	0.7	7.14	0.05	0.1
ASAH	MAB4-L1	0.43	11.63	0.05	0.1
PLBD2	MAB5-L4	1.66	3.01	0.05	0.1
PBS buffer			68.03		

[0069] 500 mM Iodoacetamide: 60 μL of water added to one pre-weighed vial (56 mg) of iodoacetamide.

[0070] 5 M urea lysis buffer: 1 mL of 10 M Urea/IP Lysis Buffer diluted with 1 mL of Lysis Buffer for each labeling reaction.

[0071] 50 mM biotin in 1X TBS: 40.9 mL of 1X TBS and 1 mL of ammonium hydroxide added to 500 mg of biotin. Vortex the solution and store it at 4 °C.

[0072] 5 mM biotin in 1X TBS: 900 μL of 1X TBS added to 100 μL of 50 mM biotin.

[0073] Digestion buffer containing trypsin: one vial of 20 µg trypsin dissolved in 1000 µL of 5 mM biotin elution buffer. 20 µL of 1 mU/µL PNGase F and 100 µL of 1% protease max added.

11. Reagent and sample preparation for labelling HCP impurities with lipid probes.

[0074] 10 M urea lysis buffer: 0.9 g of urea dissolved with 1.5 mL of Lysis Buffer for each labeling reaction.

[0075] Digestion buffer (2M urea/20 mM Tris, pH 8.0): 2.4 g of urea dissolved with 0.4 mL 1M Tris, pH 8.0 and 19.6 mL of LC-MS grade water.

[0076] 10 ppm lipases were spiked in samples for conducting the experiments. The lipases included LPL, LIPA, ASAH, and PLBD2. (LPL: Lipoprotein Lipase; LIPA: Lipase A lysosomal acid type; ASAH: N-Acylsphingosine Amidohydrolase, Acid Ceramidase; PLBD2: Phospholipase B Domain Containing 2). The samples, MAB1-C3-DS, contained monoclonal antibody (MAB1) and HCP impurities.

[0077] Buffer S (PBS, 1% SDS): 1 mL of 10% SDS added to 9 mL of PBS and vortexed to mix.

[0078] 500 mM DTT: 7.7 mg of DTT dissolved with 0.1 mL water.

[0079] 1 M Iodoacetamide: 18.4 mg of iodoacetamide dissolved with 0.1 mL water.

[0080] 16:0-pacFA PC solution (molecular weight: 741.978 g/mol): 674 µL ethanol added to 16:0-pacFA PC to make a 2 mM stock solution.

Methods

1. Label, enrich and identify HCP impurities with serine hydrolase probes:

[0081] Samples containing HCP impurities were labelled with serine hydrolase probes, such as desthiobiotin-FP. Subsequently, labelled HCP impurities were enriched and identified. Samples containing HCP impurities were prepared according to Table 2 for labelling HCP impurities with serine hydrolase probes. Lysate samples were diluted with lysis buffer and transferred to a micro-centrifuge tube according to Table 2. CCF in Table 2 indicates cell culture fluid, *e.g.*, supernatant sample, clarified cell culture supernatant sample or clarified cell culture fluid sample. AEX in Table 2 indicates anion exchange chromatography. DS in Table 2

indicates drug substance. Standards in Table 2 indicates the mixture of recombinant lipase proteins as shown in Table 1. "LPL and PLBD2 KO AEX Pool Material" indicates cell culture material collected from cells from a LPL (Lipoprotein Lipase)-PLBD2 (Phospholipase B Domain-Containing Protein 2) double knockout CHO cell line. Conc. indicates concentration.

Table 2. Sample preparations for labelling HCP impurities with serine hydrolase probes.

	Conc. (mg/mL)	Sample (μ L)	Labeling Buffer (μ L)	Lipases (μ L)	Water (μ L)	Sample (mg)	Final Volume (mL)	Probe (μ L)
Bioreactor Fluid	27.4	182.5	100	0	217.5	5	0.5	20
AEX Pool	14.8	337.8	100	0	62.2	5	0.5	20
DS	214	46.7	100	0	353.3	10	0.5	20
DS Plus Standards	214	46.7	100	2	351.3	10	0.5	20
LPL and PLBD2 KO AEX Pool Material	5.6	892.9	200	0	0.0	5	1	20
Bioreactor Fluid (no probe)	27.4	182.5	100	0	217.5	5	0.5	0

[0082] The serine hydrolase probe containing fluorophosphonate (FP), serine hydrolase FP probe, was equilibrated to room temperature in a pouch with desiccants. Subsequently, serine hydrolase FP probe was dissolved in 100 μ L DMSO to make a 0.1 mM stock solution. The stock solution of serine hydrolase FP probe was added to each sample to reach a final mix concentration of 4 μ M. The samples were incubated for 60 minutes at room temperature.

[0083] The samples were filtered using micro-biospin 6 desalting columns or equivalent to remove unbound probes, including the steps of: (1) invert the column sharply several times to re-suspend the settled gel and remove any bubbles; snap off the tip and place the column in a 2.0 mL micro-centrifuge tube; remove the top cap; if the column does not begin to flow, push the

cap back on the column and then remove it again to start the flow; allow the excess packing buffer to drain by gravity to the top of the gel bed (about two minutes); discard the drained buffer then place the column back into the 2.0 mL tube; (2) centrifuge for 2 minutes in a micro-centrifuge at 1,000 xg to remove the remaining packing buffer; discard the buffer; (3) apply 500 μ L of 5 M Urea/Lysis Buffer; after each application of new buffer, let the buffer drain out by gravity, or centrifuge the column for 1 minute to remove the buffer; discard buffer from collection tube; repeat 3 times; (4) place the column in a clean 1.5 or 2.0 mL micro-centrifuge tube; carefully apply the sample (20-75 μ L) directly to the center of the column (application of more or less than the recommended sample volume may decrease column performance); (5) after loading the sample, centrifuge the column for 4 minutes at 1,000 xg; and (6) following centrifugation, the purified sample is in 5 M urea buffer.

[0084] The labelled HCP impurities were captured, digested and analyzed including the steps of: (1) add 500 μ L of 5 M urea/Lysis Buffer to each reaction for a total volume of 1 mL; (2) add 2 mg of streptavidin dynabeads (purchased from Thermo Fisher Scientific) to each sample to capture labelled HCP impurities and incubate for 1 hour at room temperature with constant mixing on a rotator; (3) collect dynabeads using a magnetic rack for 3 minutes; remove supernatant; (4) add 500 μ L of 5 M urea/Lysis Buffer and vortex briefly to mix; collect beads using a magnet and remove the supernatant; repeat this step two additional times; discard buffer after each wash; (5) add 1.5 M urea solution with 5 mM TCEP and incubate at 55 °C for 30 minutes; (6) add iodoacetamide to a final concentration of 10 mM and shake the beads in the dark at room temperature for 30 minutes; (7) wash once with 1000 μ L 1X TBS; (8) for eluting labelled HCP impurities, add 75 μ L 5 mM biotin elution solution and shake for 10 minutes at 37 °C, then collect the supernatant; (9) add trypsin elution solution to the beads and shake for 10 minutes at 37 °C, then combine the supernatant with the previous elution step; (10) digest overnight at 37 °C; (11) add 4 μ L of 20% TFA, vortex and incubate at room temperature for 10 minutes; (12) centrifuge for 5 minutes at 14,000 xg; and (13) load 18 μ L treated eluted labeled HCP impurities on the nano-LC and analyze by nano-LC-MS/MS.

2. Label, enrich and identify HCP impurities with lipid probes:

[0085] Samples containing HCP impurities were labelled with lipid probes, such as 16:0-pacFA PC. Subsequently, labelled HCP impurities were enriched and identified. Samples

containing HCP impurities were prepared according to Table 3 for labelling HCP impurities with lipid probes. Lysate samples were diluted with PBS (phosphate buffer saline) and transferred to a micro-centrifuge tube according to Table 3. AEX in Table 3 indicates anion exchange chromatography. DS in Table 3 indicates drug substance. MAB1-DS indicates drug substance containing monoclonal antibody, MAB1. Standards in Table 3 indicates the mixture of recombinant lipase proteins as shown in Table 1. “LPL and PLBD2 KO AEX Pool Material” indicates cell culture material collected from cells from a LPL (Lipoprotein Lipase)-PLBD2 (Phospholipase B Domain-Containing Protein 2) double knockout CHO cell line.

Table 3. Sample preparations for labelling HCP impurities with 16:0-pacFA PC.

	Conc. (mg/mL)	Sample (μ L)	Labeling Buffer (μ L)	Lipases (μ L)	Sample (mg)	Final Volume (mL)	Probe (μ L)
Bioreactor Fluid	15	166.7	100	0	5	0.5	10
Bioreactor Fluid (no UV control)	15	166.7	100	0	5	0.5	10
AEX Pool	14.8	337.8	100	0	5	0.5	10
DS	214	46.7	100	0	10	0.5	10
MAB1-DS Plus Standards	214	46.7	100	2	10	0.5	10
LPL and PLBD2 KO AEX Pool Material	5.6	892.9	200	0	5	1	20
AEX Pool (no probe)	14.8	337.8	100	0	5	0.5	0

[0086] The lipid probe, 16:0-pacFA PC, was equilibrated to room temperature in a pouch. Subsequently, the lipid probe was dissolved in 674 μ L ethanol to make a 2 mM stock solution. The unused probe can be stored in glass vial for up to 6 months at -80 °C. 10 μ L of 16:0-pacFA PC stock solution was added to each sample for a final mix concentration of 20 μ M. The reaction mixtures were protected from light, when the photo-activatable probe was added. The sample was incubated for 60 minutes at room temperature. The samples were exposed to greater

than 345 nm UV light from a UV trans-illuminator at 365 nm for 15 min at a distance of 5 cm from the light source on ice.

[0087] The labelled HCP impurities were captured, digested and analyzed. Subsequently unreacted lipid probe was removed by TCA (trichloroacetic acid) precipitation. Cell lysate, ice-cold acetone, and TCA solution (100% TCA, w/v) were mixed in a 1:8:1 ratio, such as mixing 1 mL cell lysate, 8 mL 100% ice-cold acetone and 1 mL 100% TCA. TCA precipitation was performed at -20 °C for 1 hr followed by centrifugation at 11,500 rpm (18,000 xg) for 15 min at 4 °C in a micro-centrifuge. Supernatant was discarded. Precipitated pellets were washed with 1 mL ice-cold acetone to re-suspend pellets completely followed by centrifugation at 11,500 rpm for 15 min at 4 °C. Washing and centrifugation steps were repeated twice to remove all of the TCA. All acetone was removed. The pellet was dried at room temperature to remove residual acetone. The protein pellet was dissolved in 800 µL of buffer S and was transferred to a 1.5 mL eppendorf tube. The protein solution was kept in a shaker at 37 °C for 30 minutes including vortexing approximately every 10 min.

[0088] Picolyl azide-agarose resin was prepared by: washing and mixing the picolyl azide-agarose resin in 50% resin slurry for complete re-suspension, transferring 200 µL of the well-mixed resin with a 1 mL pipet into a clean 2 mL centrifuge tube, adding 1.3 mL water, pelleting the resin using centrifugation for 2 minutes at 1000 xg, and aspirating the supernatant by leaving approximately 200 µL of the resin. A catalyst solution was prepared by: using 4730 µL water, 550 µL Additive 1, 110 µL of Copper (II) sulfate solution and 110 µL Additive 2 to prepare 5.5 mL of 2X copper catalyst solution for enrichment. A sample mixture was prepared for conjugating the labelled HCP impurities to agarose resin (beads) by combining 200 µL washed picolyl azide-agarose resin, 800 µL sample and 1000 µL of 2X copper solution. The sample solution was rotated for mixing overnight.

[0089] The agarose wash buffer containing SDS was warmed up to room temperature. The resin was centrifuged. Subsequently, the supernatant was aspirated. 1.8 mL water was added to the resin. Subsequently, the resin was centrifuged. Subsequently, the supernatant was aspirated. 1 mL agarose wash buffer containing SDS and 10 µL of 1 M DTT were added to the resin to re-suspend the resin. The resin was heated at 70 °C on a heating block for 15 minutes, followed by cooling to room temperature for 15 minutes. Subsequently the resin was centrifuged for 5

minutes at 1000 xg. The supernatant was aspirated. 6 mL of 40 mM iodoacetamide was prepared by dissolving 44.4 mg of iodoacetamide in 6 mL of agarose wash buffer containing SDS. 1 mL of 40 mM iodoacetamide was added to the resin followed by vortexing the resin for re-suspending. Subsequently, the resin was incubated in the dark for 30 minutes at room temperature. The resin was re-suspended and transferred to a spin column. 2 mL of wash buffer containing SDS was added to the spin column followed by centrifuging at 1000 xg for 1 minute with three repeating processes. 2 mL of 8 M urea/100 mM Tris pH 8 was added to the spin column followed by centrifuging at 1000 xg for 1 minute with five repeating processes. 2 mL of 20% acetonitrile was added to the spin column followed by centrifuging at 1000 xg for 1 min with five repeating processes. A cap was added to the bottom of the spin column followed by adding 500 μ L of digestion buffer (100 mM Tris, 2 mM CaCl_2 , 10% acetonitrile). A 1 mL pipet was used to transfer the resin to a new tube. The spin column was rinsed with another 500 μ L of digestion buffer by combining the resin in the new tube. 20 μ L of 0.1 μ g/ μ L trypsin and protease max was added to the samples (final concentration of protease max = 0.05%). 2 μ L of 1 mU/ μ L PNGase F was added, followed by incubating with rotation overnight. The resin was pelleted by centrifuging for 5 min at 1000 xg. The supernatant was retained. 500 μ L of water was added to wash the beads two times. All of the supernatants were combined. 2 μ L of TFA was added. The digestion mixture was desalted and concentrated using a C-18 cartridge. The eluent was dried down from desalted sample and analyzed by nano-LC-MS/MS.

3. Label, enrich and identify HCP impurities with cysteine protease probes:

[0090] Samples containing HCP impurities were labelled using cysteine protease probes, such as DCG-04. Subsequently, labelled HCP impurities were enriched and identified. Samples containing HCP impurities were prepared according to Table 4 for labelling HCP impurities with cysteine protease probes. The 10X reaction buffer contained 500 mM sodium acetate (pH 6), 10 mM EDTA, and 25 mM DTT.

[0091] The cysteine protease probe, DCG-04, was equilibrated to room temperature in a pouch. Subsequently, the probe was dissolved in 2000 μ L DMSO to make a 0.5 mM stock solution. 5 μ L of DCG-04 stock solution was added to each sample for a final mix concentration of 2.5 μ M. The samples were incubated for 60 minutes at room temperature.

[0092] The samples were filtered using Amicon 3 kDa molecular weight desalting filters to

remove unbound probes, including the steps of: (1) apply each sample to the top of the desalting filters; (2) apply 500 μ L of 8 M guanidine to the top of each filter; (3) apply 500 μ L of 5 M Urea/Lysis Buffer to the top of the filter; (4) at the end of each step centrifuge at 14,000 $\times$ g for 20 minutes, then discard the volume from the collection tube. Finally, place the column in a clean collection tube, invert the filter and centrifuge for 2 minutes at 2,000 $\times$ g to collect the buffer-exchanged and labelled samples.

[0093] The labelled HCP impurities were captured, digested and analyzed including the steps of: (1) add 500 μ L of 5 M urea/Lysis Buffer to each reaction; (2) add 1 mg of streptavidin dynabeads (purchased from Thermo Fisher Scientific) to each sample to capture labelled HCP impurities and incubate for 1 hour at room temperature with constant mixing on a rotator; (3) collect dynabeads using a magnetic rack for 3 minutes; remove supernatant; (4) add 500 μ L of 5 M urea/Lysis Buffer and vortex briefly to mix; collect beads using a magnet and remove the supernatant; repeat this step two additional times; discard buffer after each wash; (5) add 1.5 M urea solution with 5 mM TCEP and incubate at 55 °C for 30 minutes; (6) add iodoacetamide to a final concentration of 10 mM and shake the beads in the dark at room temperature for 30 minutes; (7) wash once with 1000 μ L 1X TBS; (8) add trypsin elution solution to the beads and shake for 4 hours at 37 °C to completely digest and elute proteins from the beads; (10) add 4 μ L of 20% TFA, vortex and incubate at room temperature for 10 minutes; (11) move the supernatant from the beads containing the digested HCP into a clean tube; (12) centrifuge for 5 minutes at 14,000 $\times$ g; and (13) load 18 μ L treated eluted labeled HCP impurities on the nano-LC and analyze by nano-LC-MS/MS.

Table 4. Sample preparations for labelling HCP impurities with cysteine protease probes.

Sample Name	Concentration (mg/mL)	Volume Sample (μ L)	Volume 10x reaction buffer (μ L)	Volume 10x HCP standards (μ L)	Volume MilliQ water	Amount of sample (mg)	Final volume (mL)	Volume probe (μ L)
MAB2-DS + HCP (no probe)	221.4	45	100	5.3	850	10	1	0

MAB2-DS + spiked HCP	221.4	45	100	5.3	845	10	1	5
MAB2-DS	221.4	45	100	0	850	10	1	5
Spiked HCP	NA	NA	100	5.3	890	NA	1	5

4. Direct digestion of starting material

[0094] Four volumes of ice-cold acetone were added to each sample and precipitated for 1 hour in a -20 °C freezer followed by washing once with ice-cold 80% acetone. Acetone was carefully decanted and dried for 2 minutes on a paper towel. Samples were re-suspended in 20 µL urea denaturing and reducing solution followed by shaking at 800 rpm at 56 °C on the thermomixer for 30 minutes and cooling to room temperature. 6 µL of 50 mM iodoacetamide was added followed by vortexing briefly. The mixture was kept at room temperature in the dark for 30 minutes. Sequencing grade modified trypsin (20 ug/vial) was prepared in 1.95 mL of 50 mM Tris, pH 7.5 to give a final concentration of 10 ng/µL. 100 µL of 100 ng/µL of trypsin and 4.5 µL of protease max were added to the samples (final concentration of protease max = 0.05%). 2 µL of 1 mU/µL PNGase F were added. The samples were vortexed for 3-5 seconds, followed by spinning down the sample(s). The final volume of the solution was 125 uL. The final concentration of urea was 1.25 M. The HCP samples were incubated for 5 hours at 37 °C in the dark with shaking at 750 rpm in a thermomixer. The digestion mixtures were acidified with 5 µL of glacial acetic acid followed by vortexing the samples for 3-5 seconds. Subsequently, the samples were centrifuged at 14,000 xg for 5 minutes. The pH of the samples was below pH 3. 15 µL of each sample was loaded onto a mass spectrometer and analyzed using the data dependent method (150 minutes, top 20).

Example 1. Enrichment of HCP impurities having serine hydrolase activity

[0095] A serine hydrolase probe, desthiobiotin-FP, was used to enrich, identify, characterize or profile HCP impurities which had serine hydrolase activities. Several samples containing biopharmaceutical products, such as monoclonal antibodies, and HCP impurities from

CHO cells were used for the enrichments. These samples included clarified-supernatant, LPL-PLBD2 knockout-AEX (designated as AEX KO in Table 5), AEX-supernatant (designated as AEX in Table 5), DS and spiked-DS (designated as DS+lipase in Table 5). The experiments were conducted by incubating samples with desthiobiotin-FP at a final probe concentration of 4 μ M for 1 hour at room temperature. Excess probes were removed by buffer exchanges. Some samples were used as controls for monitoring non-specific binding in the absence of desthiobiotin-FP during incubation. For protein enrichments, HCP impurities which were labeled with desthiobiotin-FP were captured using magnetic beads, such as streptavidin-beads. Subsequently, the beads were washed using stringent 5 M urea. Labelled HCPs were eluted using 5 mM biotin. The eluted labelled HCPs were digested overnight at 37 °C and subsequently loaded directly onto nano-LC (liquid chromatography) using trap. Protein identifications were conducted using nano-LC-MS/MS. The experimental results are shown in Table 5. MAB1 HC indicates the heavy chain of monoclonal antibody MAB1. MAB1 LC indicates the light chain of monoclonal antibody MAB1,

Table 5. Identifications of HCP impurities.

Protein Name	Protein Activity/Function	AEX	AEX KO	DS	DS + Lipase
MAB1 HC		High	High	High	High
MAB1 LC		High	High	High	High
ScFV	Non-CHO protein fusion of HC and LC	High	High	High	High
Protein unc-93-like B1	Protein transport	High	High	High	High
Complement C1r-A subcomponent	Serine protease, complement system	High	Not Found	High	Not Found
Lipoprotein lipase	Serine hydrolase, lipase	Not Found	Not Found	Not Found	High
Histone H2A type 1	Histone	Peak Found	Peak Found	Peak Found	High
Annexin	Lipid binding protein	High	High	High	High

Protein Name	Protein Activity/Function	AEX	AEX KO	DS	DS + Lipase
Peroxisome proliferator-activated receptor delta	Fatty acid binding protein	High	High	High	High
Elongation factor 1-alpha	Protein transport	High	Peak Found	High	Peak Found
Histone H3.1t	Histone	Not Found	Not Found	Not Found	High
Maltase-glucoamylase, intestinal-like protein	Alpha-glucosidase	High	High	High	High
Actin, cytoplasmic 1	Structural protein	Not Found	High	High	Peak Found
Calcium-dependent serine proteinase	Serine protease	High	Not Found	Not Found	Not Found
Putative phospholipase B-like 2	Serine hydrolase, lipase	High	Not Found	Not Found	Not Found
Sucrase-isomaltase, intestinal-like protein	Alpha-glucosidase	Not Found	High	High	High
Putative inactive serine protease 58-like protein	Serine protease (similar to trypsin)	High	High	High	High
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis enzyme	High	Peak Found	High	Peak Found
Peroxiredoxin-1	Hydrogen peroxide reduction	High	Peak Found	Peak Found	Not Found
Peptidyl-prolyl cis-trans isomerase	Proline isomerase, protein binding	Peak Found	Not Found	Peak Found	High

Protein Name	Protein Activity/Function	AEX	AEX KO	DS	DS + Lipase
L-lactate dehydrogenase	Lactate to pyruvate conversion	High	Not Found	High	Not Found
Serine protease HTRA1	Serine protease	High	Not Found	Not Found	Not Found
Lamin-A/C	Structural protein	Peak Found	High	High	Not Found
Peroxiredoxin-2	Hydrogen peroxide reduction	High	Not Found	High	Not Found
Sal-like protein 2	Nucleic acid binding	Peak Found	Peak Found	High	High
Lipase	Serine hydrolase, lipase	Not Found	Not Found	Not Found	High
Transient receptor potential cation channel subfamily V member 5	Structural protein	Not Found	Not Found	High	Not Found
Glutathione S-transferase Mu 6	Catalyzes glutathione addition to substrates with electrophilic groups	Not Found	Not Found	High	Not Found
14-3-3 protein sigma	Protein binding, signaling	Not Found	Peak Found	High	Not Found
Annexin	Lipid binding	Not Found	Not Found	High	Peak Found

[0096] Various proteins were identified in HCP impurities in clarified-supernatant samples (e.g., clarified cell culture supernatant sample or cell culture fluid sample). There were 586 proteins identified in eluted labelled HCPs in clarified Chinese Hamster Ovary (CHO) cell-supernatant samples. Within these 586 proteins, 359 proteins were not found in non-specific

binding control samples (*e.g.*, in the absence of desthiobiotin-FP during incubation). According to gene ontology analysis, 68% of these 586 proteins had catalytic activities. The molecular weights and calculated isoelectric points (pI) of these 586 proteins were analyzed as shown in FIG. 10. The dot size in FIG. 10 indicates the differences in protein abundance of eluted labelled HCPs in clarified-supernatant samples with a probe and without a probe. The clarified-supernatant sample without a probe was a control for non-specific binding. The protein abundance was denoted as color scale from red (high abundance) to blue (low abundance). Three abundant proteins, *e.g.*, calcium-dependent serine proteinase, complement C1r-A subcomponent, and serine protease HTRA1, were marked as shown in FIG. 10.

[0097] Active lipases and esterases which were identified HCP impurities in clarified-supernatant samples after enrichment with the serine hydrolase probe are shown in Table 6. CCF in Table 6 indicates cell culture fluid, for example, clarified-supernatant sample, clarified cell culture supernatant sample or clarified cell culture fluid sample. Calc. pI indicates calculated isoelectric points. PSMs indicates the number of peptide spectrum matches. The number of PSMs is the total number of identified peptide spectra matched for the protein. The PSM value may be higher than the number of peptides identified for high-scoring proteins because peptides may be identified repeatedly. PEP indicates posterior error probability which is the probability that the observed PSM is incorrect. Sum PEP score is calculated on the basis of the PEP values of the PSMs.

Table 6. Active lipases and esterases identified in clarified-supernatant sample treated with serine hydrolase probe.

Accession	Protein Name	Found in CCF	Sum PEP Score	Coverage [%]	# PSMs	MW [kDa]	Calc. pI
A0A061IKA1	Lipoprotein lipase	High	100.363	46	39	54.5	7.85
G3HQY6	Lipase	High	89.381	39	45	45.6	7.68
G3I6T1	Putative phospholipase B-like 2	High	43.073	28	22	65.5	6.28
G3HH63	Phospholipase A-2-activating protein	High	2.527	2	1	87.1	6.16
G3GZB2	Acid ceramidase	High	14.094	11	6	44.7	7.9

Accession	Protein Name	Found in CCF	Sum PEP Score	Coverage [%]	# PSMs	MW [kDa]	Calc. pI
A0A061I883	Liver carboxylesterase 22-like protein (Fragment)	High	7.876	16	6	27.9	6.09
G3I7X5	Liver carboxylesterase 4	High	61.579	83	38	11.9	5.48
G3IHH9	Isoamyl acetate-hydrolyzing esterase 1-like	High	33.533	44	14	31.3	5.53
A0A061IFE2	Liver carboxylesterase 1-like protein	High	134.525	32	77	97.5	6.35
G3IP80	Acyl-protein thioesterase 2	High	13.903	37	4	17.7	7.42
G3HNG2	Acyl-coenzyme A thioesterase 2, mitochondrial	High	60.516	35	20	49.8	8.22
A0A061I6Q8	Liver carboxylesterase B-1-like protein (Fragment)	High	124.983	20	66	90.2	7.34
G3I7X4	Carboxylic ester hydrolase (Fragment)	High	16.692	14	8	56.7	7.31
G3I5K6	Carboxylic ester hydrolase	High	16.206	13	8	56.8	6.48
G3I1Y9	Sulfated glycoprotein 1	High	1.173	3	1	27.4	5.49

[0098] There were 586 proteins identified in eluted labelled HCPs in clarified-supernatant samples. Within these 586 proteins, 359 proteins were not found in non-specific binding control samples (*e.g.*, in the absence of desthiobiotin-FP during incubation). The results of gene ontology analysis of these 359 proteins are shown in FIG. 11. The results indicated that the HCP impurity proteins having catalytic activities were significantly enriched using serine hydrolase probe, *e.g.*, desthiobiotin-FP, in comparison with control samples.

Example 2. Enrichment of HCP impurities having lipid-binding activities

[0099] Many hydrophobic proteins, such as lipases, can bind to fatty acids in cells. Fatty

acid-based probes, such as a clickable lipid probe, were used to enrich, identify, characterize or profile hydrophobic HCP impurities, such as lipases or proteins having lipid-binding activities, for example, lipid binding protein. These lipid probes were tested for activity-based profiling of HCP impurities. In addition, these lipid probes were tested for identifying HCP impurities which can bind to polysorbate-like molecules in the cell culture supernatant or in process samples.

[0100] A lipid probe, 16:0-pacFA PC, *e.g.*, 1-palmitoyl-2-(9-(3-pent-4-ynyl-3-H-diazirin-3-yl)-nonanoyl)-*sn*-glycero-3-phosphocholine, was used to purify sphingolipid-binding HCP impurities. This probe had a photo-activatable diazine ring that can react with proteins in close proximity when it is exposed to UV light. This probe also contained an alkyne for click-chemistry based purification. The lipid-labelled HCP impurities were subsequently conjugated to beads using the alkyne moiety through azide-alkyne Huisgen cycloaddition to azide functionalized beads. The lipid-labelled HCP impurities were subsequently enriched or purified through the isolation of the conjugated-beads.

[0101] The HCP impurities in the cell supernatant sample which were specifically enriched using 16:0-pacFA PC included malate dehydrogenase (fragment), beta-galactosidase (fragment), glutathione synthetase, insulin-like growth factor-binding protein 4, leukocyte elastase inhibitor A, prostaglandin reductase 1-like protein, cysteine-rich with EGF-like domain protein 2, pigment epithelium-derived factor, bleomycin hydrolase, citrate synthase, angiopoietin-related protein 4, N-acetylglucosamine-1-phosphotransferase subunit gamma, proteasome subunit beta type-1, 40S ribosomal protein S28, cysteine and glycine-rich protein 1, tripeptidyl-peptidase 1, and splicing factor (proline-and glutamine-rich). Among these identified HCP impurities, beta-galactosidase, insulin-like growth factor-binding protein 4, prostaglandin reductase 1-like protein and angiopoietin-related protein 4 were considered to have potential sphingolipid binding based on the literature. The comparisons of identified HCP impurities among unenriched sample, sample enriched by 16:0-pacFA PC probe, and control sample (no UV) are shown in FIG. 12.

[0102] The lipid probe, 16:0-pacFA PC, was used to enrich proteins in DS (drug substance) sample containing spiked recombinant lipases. The specifically enriched proteins included vomeronasal type-2 receptor 26, centriolin, NFX1-type zinc finger-containing protein 1, desmoglein-4-like protein, glyceraldehyde-3-phosphate dehydrogenase, tubulin alpha chain, acid ceramidase, putative non-specific cytotoxic cell receptor protein 1 like protein (fragment),

putative inactive serine protease 58-like protein, fatty acid-binding protein (adipocyte), histone acetyltransferase, proteasome endopeptidase complex (fragment), proteasome subunit alpha type, and putative phospholipase B-like 2. Among these identified proteins, acid ceramidase and putative phospholipase B-like 2 were spiked recombinant lipase proteins.

Example 3. Enrichment of proteins with cysteine protease activity

[0103] A cysteine protease probe, DCG-04, was used to enrich proteins in the DS sample containing a spiked recombinant host cell protein mixture (HCP mix) at 100 ppm. The mixture consisted of three cysteine proteases (Cathepsin L1, Cathepsin Z, Cathepsin B) and two other commonly detected host cell proteins that should not have any cysteine protease activity (Beta-hexosaminidase and Cathepsin D). All the spiked recombinant host cell proteins were identified in the sample incubated with the probe, but not in a sample without the probe added, as shown in Table 7. The peak area for the top 3 peptides from each identified protein was averaged and then divided by the peak areas for the top 3 peptides from trypsin to normalize protein abundances across samples. Two of the recombinant cysteine proteases (Cathepsin Z and Cathepsin B) were enriched to levels ~11 fold higher than the other spiked recombinant proteins.

Table 7. Active proteases identified in DS sample spiked with a mixture of host cell proteins and treated with cysteine protease probe.

Protein Description	Protein Accession	DS w/ HCP mix (no probe)	DS w/ HCP mix	DS	HCP mix
Beta-hexosaminidase	G3HYN7	0.004	0.011	0.000	0.087
Cathepsin D	G3I4W7	0.000	0.003	0.000	0.014
Cathepsin B	G3H0L9	0.000	0.102	0.001	0.236
Cathepsin L1	G3INC5	0.000	0.004	0.000	0.143
Cathepsin Z	Q9EPP7	0.000	0.058	0.005	0.142
Trypsin	TRYP_PIG	1	1	1	1