HUMAN ANTIBODIES THAT BIND HUMAN TNF-ALPHA AND METHODS OF PREPARING THE SAME

RELATED APPLICATION

[001] This application claims priority to U.S. Provisional Patent Application No. 61/777,883, filed March 12, 2013, which is incorporated by reference into the present application in its entirety and for all purposes.

SEQUENCE LISTING

[002] This application is accompanied by a sequence listing in a computer readable form that accurately reproduces the sequences described herein.

Field of the Invention

[003] This disclosure relates to antibodies that specifically bind to human TNF-alpha. More particular, Methylglyoxal (MGO)-modified recombinant TNF-alpha antibodies are disclosed. Methods for reducing MGO-modified TNF-alpha antibodies are also provided.

Background

[004] Tumor necrosis factor alpha ("TNF-alpha") is a cytokine produced by many cell types such as monocytes and macrophages. *See e.g.*, Old, L. Science 230:630-632 (1985). TNF-alpha plays an important role in many biological processes and has been implicated in the pathophysiology of a variety of other human diseases and disorders, including sepsis, infections, autoimmune diseases, transplant rejection and graft-versus-host disease. *See e.g.*, Vasilli, P., Annu. Rev. Immunol. 10:411-452 (1992); and Tracey, K. J. and Cerami, A. Annu. Rev. Med. 45:491-503 (1994).

[005] In an effort to treat/prevent these diseases, various therapeutic strategies have been designed to inhibit or counteract TNF-alpha activities. U.S. Patent No. 6,090,382 disclosed human antibodies (e.g., recombinant human antibodies) that specifically bind to human TNF-alpha with high affinity and slow dissociation kinetics. Nucleic acids, vectors and host cells for expressing the recombinant human TNF-alpha antibodies were also disclosed. One example of such recombinant TNF-alpha antibodies is called Adalimumab, which is marketed under the trade name Humira®. The entire

contents of U.S. Patent No. 6,090,382 is hereby incorporated by reference into the present disclosure.

[006] Recombinant biotherapeutics are typically produced by live cells and are inherently more complex as compared to traditional small molecule drug. Various post-translational modifications have been reported as major contributors to heterogeneity in recombinant monoclonal antibodies (References 1-4). Some of these modifications, for example, glycosylation and sialic acid incorporation, may occur during fermentation (References 5-7). Some other modifications, such as oxidation and disulfide bond scrambling, may occur during production, purification and storage.

[007] One example of such modifications is the so-called acidic species (charge variants). Acidic species are observed when recombinant monoclonal antibodies are analyzed by weak-cation exchange chromatography (WCX) (Figure 1). One major contributing factor is the removal of the C-terminal lysine of the heavy chain by cell-derived carboxypeptidease, reducing the overall positive charge (Reference 8). These variants are commonly referred to as Lys0, Lys1 and Lys2 species, respectively.

[008] C-terminal amidation (Reference 9) is another enzymatic process during fermentation. Another type of variant is caused by spontaneous non-enzymatic transformations, which include the formation of pyruglutamate (Pyro-Glu) from an N-terminal glutamine (Gln) that remove the positive charge of the free N-terminus (Reference 10), and the deamidation of asparagine (Asn) to aspartic (Asp) or isoaspartic acid (isoAsp or isoD) that introduces negatively charged carboxylic acids (References 11 and 12).

[009] Some modifications may shift the retention time of antibody on weak cation exchange chromatography even though they do not alter the formal charges of the antibody molecule. These modifications may exert their effects through perturbation of local charge and conformation. For instance, incomplete glycosylation (Reference 13) or the presence of free sulfhydryl (References 14-16) may shift the retention time of antibody on weak cation exchange chromatography. It is worth noting that some modifications are imparted by metabolites, such as glycation by glucose, methionine oxidation by reactive oxygen species (ROS), cysteinylation by cysteine (Reference 17), and S-homocysteinylation and N-homocysteinylation by homocysteine (References 2, 18-

23). Although the mechanisms of many modifications have been reported, these mechanisms cannot fully explained the observed heterogeneity of recombinant monoclonal antibodies on weak cation exchange chromatography.

Summary

[010] This disclosure advances the art by identifying novel species of modified recombinant antibodies that may negatively impact the functionalities of such antibodies. The disclosure also provides methods for reducing the amount of such species without substantially compromising the overall yield of the antibody production.

[011] In one embodiment, two acidic species of the Adalimumab antibody are disclosed which exist when the antibody are expressed in Chinese hamster ovary (CHO) cells cultured in chemically defined media (CDM). Detailed analyses have revealed that several arginine residues in Adalimumab are modified by methylglyoxal (MGO), which is further confirmed by the treatment of native antibody with authentic MGO. The reaction between MGO and arginine result in formation of hydroxylimide and/or hydroimidazolone. The resulting hydroxylimide and hydroimidazolone adducts increase the molecular weight of the antibody by 54 and 72 Daltons, respectively.

[012] In another embodiment, these modifications cause the antibody to elute earlier in the weak cation exchange chromatogram as compared to the elution time of unmodified forms. Consequently, the extent to which an antibody was modified at multiple sites corresponds to the degree of shift in acidity and the elution time. The modification of Adalimumab antibody by MGO is the first reported modification of a recombinant monoclonal antibody by MGO.

[013] In another embodiment, a composition is disclosed which contains a binding protein capable of binding TNF-alpha. In one aspect, the binding protein may contain at least one methylglyoxal (MGO)-susceptible amino acid, and at least a portion of the binding protein may contain one or more MGO-modified amino acids.

[014] In another embodiment, a composition is disclosed which contains a binding protein capable of binding TNF-alpha. In one aspect, the binding protein may contain at least one methylglyoxal (MGO)-susceptible amino acid and the composition may be prepared by substantially removing molecules of the binding protein that contain at least one MGO-modified amino acid. The term "substantially" may mean at least 50%.

In another aspect, the term "substantially" may mean at least 60%, 70%, 80%, 90%, or even 100% removal of the molecules that contain at least one MGO-modified amino acid.

[015] For purpose of this disclosure, the term "methylglyoxal (MGO)-susceptible" refers to groups or residues (e.g., arginine) that may react with MGO under appropriate cell culture conditions. List of MGO-susceptible arginines in Adalimumab is shown in Table 1. Examples of MGO-susceptible peptides in Adalimumab are shown in Table 2.

[016] The term "at least a portion of the binding protein" means that although all molecules of the binding protein in the composition are capable of binding TNF-alpha, at least two populations of these molecules exist in the composition, wherein one population contain one or more amino acids that have been modified by MGO, while the other population does not contain amino acids that have been modified by MGO. In another aspect, all molecules of the binding protein may contain one or more amino acids that have been modified by MGO.

[017] In one aspect, the portion of the binding protein that contains at least one MGO-modified amino acid is less than 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% of the total amount of the binding protein.

[018] In another embodiment, the binding protein is a human antibody or an antigen-binding portion thereof, wherein the binding protein dissociates from human TNF-alpha with a K_d of $1x10^{-8}$ M or less and a K_{off} rate constant of $1x\ 10^{-3}\ s^{-1}$ or less, both as determined by surface plasmon resonance. In one aspect, the binding protein neutralizes human TNF-alpha cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of $1x10^{-7}$ M or less, described in Example 4 of U.S. Patent No. 6,090,382. In another aspect, the binding protein is the D2E7 antibody as described in U.S. Patent No. 6,090,382.

[019] In another embodiment, cell culture parameters may affect the extent of modifications by methylglyoxal (MGO). MGO is a highly reactive metabolite that may be generated from glucose, lipids or other metabolic pathways. In one aspect, cell culture conditions may be modified to decrease the production of MGO thereby reducing modification of the recombinant antibodies by MGO. Taken together, the disclosed findings highlight the impact of cell culture conditions on the critical quality attributes of

recombinantly produced antibodies. These findings provide additional parameters for improving manufacturing processes and may prove useful for the quality by design (QbD) approach.

[020] In another embodiment, methods are disclosed for purifying a target protein product from both process and/or product related impurities. Specifically, method for purifying a composition containing a target protein is disclosed. In one aspect, methods are provided for reducing product related charge variants (i.e. acidic and basic species). In another aspect, the method involves contacting the process mixture with an ion (anion or cation) exchange adsorbent in an aqueous salt solution under loading conditions that permit both the target and non-target proteins to bind to the adsorbent and allowing the excess target molecule to pass through the column and subsequently recovering the bound target protein with a wash at the same aqueous salt solution used in the equilibration (i.e. pre-loading) condition.

[021] In another embodiment, a method for purifying a composition containing a target protein is disclosed which may include at least the following steps: (a) loading the composition to a cation exchange adsorbent using a loading buffer, wherein the pH of the loading buffer is lower than the pI of the target protein; (b) washing the cation exchange adsorbent with a washing buffer, wherein the pH of the washing buffer is lower than the pI of the target protein; (c) eluting the cation exchange adsorbent with an elution buffer, said elution buffer being capable of reducing the binding between the target protein and the cation exchange adsorbent; and (d) collecting the eluate, wherein the percentage of the target protein is higher in the eluate than the percentage of the target protein in the composition. In one aspect, the washer buffer and the loading buffer are the same. In another aspect, the conductivity of the elution buffer is higher than the conductivity of the washer buffer. In another aspect, the pH of the elution buffer may be between 5.5 and 9.0, between 6 and 8, or between 6.5 and 8. The conductivity of the elution buffer may be raised by increasing the salt concentration of the elution buffer. The salt concentration of the elution buffer may be between 20 mM NaCl and 200 mM NaCl, between 40 mM NaCl and 160 mM NaCl, or between 60 mM NaCl and 120 mM NaCl.

[022] In another embodiment, a method for purifying a composition containing a target protein is disclosed which may include at least the following steps: (a) loading the composition to an anion exchange adsorbent using a loading buffer, wherein the pH of the

loading buffer is lower than the isoelectric point (pI) of the target protein; (b) allowing the majority of the target protein to pass through without binding to the anion exchange adsorbent; (c) collecting the pass-through loading buffer containing said unbound target protein; (d) washing the anion exchange adsorbent with a washing buffer; (e) allowing the target protein bound to the anion exchange adsorbent to disassociate from the anion exchange adsorbent; (f) collecting the washing buffer containing said disassociated target protein. In another aspect, the method may further include a step (g) of pooling the collections from steps (c) and (f) to obtain a purified composition containing the target protein. The percentage of the target protein is higher in the pooled collections than the percentage of the target protein in the original composition.

[023] In one aspect, the loading buffer may contain an anionic agent and a cationic agent, wherein the conductivity and pH of the loading buffer is adjusted by increasing or decreasing the concentration of a cationic agent and maintaining a constant concentration of an anionic agent in the loading buffer. In another aspect, the anionic agent is selected from the group consisting of acetate, citrate, chloride anion, sulphate, phosphate and combinations thereof. In another aspect, the cationic agent is selected from the group consisting of sodium, Tris, tromethalmine, ammonium cation, arginine, and combinations thereof.

[024] In one embodiment, the target protein is a human antibody or an antigen-binding portion thereof that is substantially free from MGO modification. In one aspect, the target protein dissociates from human TNF-alpha with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both as determined by surface plasmon resonance. In another aspect, the target protein neutralizes human TNF-alpha cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less, described in Example 4 of U.S. Patent No. 6,090,382. In another aspect, the target protein is the D2E7 antibody as described in U.S. Patent No. 6,090,382.

Brief Description of the Drawings

[025] Figure 1 shows a typical WCX chromatogram of adalimumab after protein A purification.

[026] Figure 2 shows deconvoluted mass spectra of the light chain and heavy chains in fractions 1 and 2.

[027] Figure 3 shows representative MS/MS mass spectra of peptides containing Arg residues modified by MGO.

[028] Figure 4 shows chemical modification of arginine by MGO.

[029] Figure 5 shows modification of a purified 0 lysine fraction by MGO over a 5-hour time course.

[030] Figure 6 shows the mass spectra of peaks a and b from Figure 5.

[031] Figure 7 shows comparison of peptide MS/MS data between acidic fraction 1 from cell culture and acidic fraction 1 from methylglyoxal incubation.

[032] Figure 8 shows the crystal structure of the adalimumab Fab subunit in complex with TNF-alpha, indicating that modification by MGO may cause conformational change which may impede adalimumab's ability to bind TNF-alpha.

[033] Figure 9 shows Surface Plasmon Resonance (SPR) data for 0 Lys Fraction (Top – 0 Lys) and for the MGO enriched fraction (Bottom - Peak 1).

[034] Figure 10 shows comparison of acidic region affected by methylglyoxal before and after two-step chromatographic separation, wherein the top trace is an expanded view of the acidic region in which the two distinctive MGO peaks are denoted, and the lower trace shows a complete clearance of this acidic region and the MGO variants.

[035] Figure 11 shows the CEX chromatogram when reversible binding mode was performed using Adalimumab with a Tris-acetate buffer system.

[036] Figure 12 shows the removal of acidic species by Poros XS resin with NaCl/Tris-acetate solution.

Detailed Description

[037] The instant disclosure identifies novel species of methylglyoxal (MGO)-modified recombinant antibodies which may have negative impact on the structure and function of the antibodies. The disclosure also provides methods for reducing the percentage of such variant species without substantially compromising the yield of antibody production. More specifically, this disclosure describes methylglyoxal (MGO)-

modified forms of Adalimumab in cell culture when Adalimumab is expressed in CHO cells using chemically defined media (CDM).

[038] In one embodiment, modification of the side chain of certain arginines (e.g., R30 in CDR1 of Adalimumab) by MGO may result in the formation of a five-member ring originating at the guanidinium terminal of the side chain which may further penetrate into the TNF-alpha structure. These MGO modifications may impede Adalimumab's ability to bind TNF-alpha due to steric constraints.

[039] In one embodiment, control of acidic species heterogeneity may be attained by purifying a protein of interest from a mixture comprising the protein with an anion exchange (AEX) adsorbent material and an aqueous salt solution under loading conditions that permit both the protein of interest and non-target proteins to bind to the AEX adsorbent, wherein the bound protein of interest is subsequently recovered with a wash buffer comprising the same aqueous salt solution used in the equilibration (i.e. loading) buffer. In one aspect, the aqueous salt solution used as both the loading and wash buffer has a pH that is greater than the isoelectric point (pI) of the protein of interest.

[040] In another embodiment, the disclosed purification method may include adjusting the conductivity and/or pH of the aqueous salt solution. In one aspect, the adjustments may include decreasing the conductivity of the aqueous salt solution. In another aspect, the adjustment to achieve the desired control over acidic species heterogeneity may involve an increase in the load conductivity of the solution. In another aspect, the adjustment may increase the pH of the aqueous salt solution. In another aspect, the adjustment to achieve the desired control over acidic species heterogeneity may involve a decrease in the pH of the aqueous salt solution. Such increases and/or decreases in the conductivity and/or pH may be of a magnitude of 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%, and ranges within one or more of the preceding, of the conductivity and/or pH of the aqueous salt solution.

[041] In another embodiment, the conductivity and pH of the aqueous salt solution is adjusted by increasing or decreasing the concentration of a cationic agent and maintaining a constant concentration of an anionic agent in the aqueous salt solution. In

one aspect, the anionic agent is maintained at a concentration of between about 0.05 mM and 100 mM, or between about 0.1 mM and 90 mM, or between about 0.5 mM and 80 mM, or between about 1 mM and 70 mM, or between about 1.5 mM and 60 mM, or between about 2 mM and 50 mM, or between about 2.5 mM and 40 mM, or between about 3 mM and 30 mM, or between about 3.5 mM and 25 mM, or between about 4 mM and 20 mM, or between about 4.5 mM and 15 mM, or between about 4.5 mM and 10 mM, or between about 5 mM and 7 mM. In another aspect, the anionic agent is maintained at a concentration of about 5 mM. In another aspect, the anionic agent is maintained at a concentration of about 10 mM. In another aspect, the anionic agent is maintained at a concentration of about 18.5 mM.

[042] In another embodiment, the concentration of the cationic agent in the aqueous salt solution is increased or decreased to achieve a pH of between about 5 and 12, or between about 5.5 and 11.5, or between about 6 and 11, or between about 6.5 and 10.5, or between about 7 and 10, or between about 7.5 and 9.5, or between about 8 and 9, or between about 8.5 and 9. In certain embodiments, the concentration of cationic agent is increased or decreased in the aqueous salt solution to achieve a pH of 8.8. In certain embodiments, the concentration of cationic agent in the aqueous salt solution is increased or decreased to achieve a pH of 9.

[043] In another embodiment, the protein load of the protein mixture is adjusted to a protein load of between about 50 g/L and 500 g/L, or between about 100 g/L and 450 g/L, or between about 120 g/L and 400 g/L, or between about 125 g/L and 350 g/L, or between about 130 g/L and 300 g/L or between about 135 g/L and 250 g/L, or between about 140 g/L and 200 g/L, or between about 145 g/L and 200 g/L, or between about 150 g/L and 200 g/L, or between about 155 g/L and 200 g/L, or between about 160 g/L and 200 g/L. In certain embodiments, the protein load of the protein or antibody mixture is adjusted to a protein load of about 100 g/L. In certain embodiments, the protein load of the protein or antibody mixture is adjusted to a protein load of about 20 g/L. In certain embodiments, the protein load of the protein or antibody mixture is adjusted to a protein load of about 105 g/L. In certain embodiments, the protein load of the protein or antibody mixture is adjusted to a protein load of about 140 g/L. In certain embodiments, the protein load of the protein or antibody mixture is adjusted to a protein

load of about 260 g/L. In certain embodiments, the protein load of the protein or antibody mixture is adjusted to a protein load of about 300 g/L.

[044] In another embodiment, the concentration of cationic agent in the aqueous salt solution is increased or decreased in an amount effective to reduce the amount of acidic species heterogeneity in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%, and ranges within one or more of the preceding, when the aqueous salt solution is used as a load and wash buffer to purify the protein of interest (for example, an antibody) from the sample containing the protein.

[045] In another embodiment, the anionic agent is acetate, citrate, chloride anion, sulphate, phosphate or combinations thereof. In certain embodiments, the cationic agent is sodium, Tris, tromethalmine, ammonium cation, arginine, or combinations thereof.

[046] By way of example but not limitation, as detailed in this disclosure, up to 60% of the acidic species in an antibody preparation was removed when the antibody was purified using chromatography comprising an anion exchange adsorbent material, a protein load of 150 g/L, and a load/wash buffer containing 5 mM Acetate/Arginine at pH 8.8.

[047] In another embodiment of the instant disclosure, control of acidic species heterogeneity can be attained by purifying a protein of interest from a mixture comprising the protein with a cation exchange (CEX) adsorbent material and an aqueous salt solution under loading conditions that permit both the protein of interest and non-target proteins to bind to the CEX adsorbent, washing off the acidic species, charged variants, molecular variants and impurities using the same buffer conditions as the loading buffer, and eluting the bound protein target from the CEX adsorbent with a buffer having a higher conductivity than the loading buffer. In certain embodiments, the aqueous salt solution used as both the loading and wash buffer has a pH that is lower than the isoelectric point (pI) of the protein of interest.

[048] In another embodiment, the purification method may include adjusting the conductivity and/or pH of the aqueous solution. In certain embodiments, such

adjustments will be to decrease the conductivity, while in other embodiments the necessary adjustment to achieve the desired control over acidic species heterogeneity will involve an increase in the load conductivity. In certain embodiments, such adjustments will also be to increase the pH of the aqueous salt solution, while in other embodiments the necessary adjustment to achieve the desired control over acidic species heterogeneity will involve a decrease in the pH of the aqueous salt solution. Such increases and/or decreases in the conductivity and/or pH can be of a magnitude of 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or100%, and ranges within one or more of the preceding, of the conductivity and/or pH of the aqueous salt solution.

[049] In certain embodiments, the conductivity and pH of the aqueous salt solution is adjusted by increasing or decreasing the concentration of a anionic agent and maintaining a constant concentration of a cationic agent in the aqueous salt solution. In certain embodiments, the cationic agent is maintained at a concentration of between about 0.5 mM and 500 mM, or between about 1 mM and 450 mM, or between about 5 mM and 400 mM, or between about 10 mM and 350 mM, or between about 15 mM and 300 mM, or between about 20 mM and 250 mM, or between about 25 mM and 200 mM, or between about 30 mM and 150 mM, or between about 35 mM and 100 mM, or between about 40 mM and 50 mM. In certain embodiments, the anionic agent is maintained at a concentration of about 15 mM, or about 20 mM, or about 25 mM, or about 30 mM, or about 35 mM, or about 40 mM, or about 45 mM, or about 50 mM, or about 60 mM, or about 65 mM, or about 75 mM, or about 90 mM, or about 115 mM, or about 120 mM, or about 125 mM, or about 135 mM, or about 140 mM, or about 145 mM, or about 150 mM, or about 175 mM, or about 250 mM, or about 275 mM, or about 300 mM, or about 350 mM, or about 375 mM, or about 400 mM.

[050] In certain embodiments, the concentration of the anionic agent in aqueous salt solution is increased or decreased to achieve a pH of between about 2 and 12, or between about 2.5 and 11.5, or between about 3 and 11, or between about 3.5 and 10.5, or between about 4 and 10, or between about 4.5 and 9.5, or between about 5 and 9, or between about 5.5 and 8.5, or between about 6 and 8, or between about 6.5 and 7.5. In certain embodiments, the concentration of anionic agent is increased or decreased in the aqueous salt solution to achieve a pH of 5, or 5.5, or 6, or 6.5, or 6.8, or 7.5.

[051] In certain embodiments, the protein load of the protein mixture is adjusted to a protein load of between about 50 and 500 g/L, or between about 100 and 450 g/L, or between about 120 and 400 g/L, or between about 125 and 350 g/L, or between about 130 and 300 g/L or between about 135 and 250 g/L, or between about 140 and 200 g/L, or between about 145 and 150 g/L. In certain embodiments, the protein load of the protein or antibody mixture is adjusted to a protein load of about 40 g/L.

[052] In certain embodiments, the concentration of anionic agent in the aqueous salt solution is increased or decreased in an amount effective to reduce the amount of acidic species heterogeneity in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%, and ranges within one or more of the preceding, when the aqueous salt solution is used as a load and wash buffer to purify the protein of interest (for example, an antibody) from the sample containing the protein.

[053] In certain embodiments, the cationic agent is sodium, Tris, tromethalmine, ammonium cation, arginine, or combinations thereof. In certain embodiments, the anionic agent is acetate, citrate, chloride anion, sulphate, phosphate or combinations thereof.

[054] By way of example but not limitation, as detailed in this disclosure, the presence of acidic species in an antibody preparation was reduced by 6.5% from starting material after purification using a cation exchange adsorbent material, and a load and wash buffer comprising 140 mM Tris at pH 7.5.

[055] Unless otherwise defined herein, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The use of “or” means “and/or” unless stated otherwise. The use of the term “including”, as well as other forms, such as “includes” and “included”, is not limiting.

[056] Generally, nomenclatures used in connection with cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid

chemistry and hybridization described herein are those well known and commonly used in the art. The methods and techniques provided herein are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[057] That the disclosure may be more readily understood, select terms are defined below.

[058] The term "antibody" refers to an immunoglobulin (Ig) molecule, which is generally comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains, or a functional fragment, mutant, variant, or derivative thereof, that retains the epitope binding features of an Ig molecule. Such fragment, mutant, variant, or derivative antibody formats are known in the art. In an embodiment of a full-length antibody, each heavy chain is comprised of a heavy chain variable region (VH) and a heavy chain constant region (CH). The heavy chain variable region (domain) is also designated as VDH in this disclosure. The CH is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (VL) and a light chain constant region (CL). The CL is comprised of a single CL domain. The light chain variable region (domain) is also designated as VDL in this disclosure. The VH and VL can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Generally, each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), or subclass.

[059] The term "antigen-binding portion" of an antibody (or "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., hTNF-alpha). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH I domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426: and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger, P., et al. (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., et al. (1994) Structure 2:1121-1123).

[060] The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. 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.

[061] The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial human antibody library, antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see e.g., Taylor, L. D., et al. (1992) Nucl. Acids Res. 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to in vitro mutagenesis (or, when an animal transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire in vivo.

[062] The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.). For further descriptions, see Example 1 and Jonsson, U., et al. (1993) Ann. Biol. Clin. 51:19-26; Jonsson, U., et al. (1991) Biotechniques 11:620-627; Johnsson, B., et al. (1995) J. Mol. Recognit. 8:125-131; and Johnnson, B., et al. (1991) Anal. Biochem. 198:268-277.

[063] The term “biological activity” refers to any one or more biological properties of a molecule (whether present naturally as found in vivo, or provided or enabled by recombinant means). Biological properties include, but are not limited to, binding a receptor or receptor ligand, inducing cell proliferation, inhibiting cell growth, inducing other cytokines, inducing apoptosis, and enzymatic activity.

[064] The term “neutralizing” refers to counteracting the biological activity of an antigen/ligand when a binding protein specifically binds to the antigen/ligand. In an embodiment, the neutralizing binding protein binds to an antigen/ligand (e.g., a cytokine)

and reduces its biologically activity by at least about 20%, 40%, 60%, 80%, 85% or more.

[065] "Specificity" refers to the ability of a binding protein to selectively bind an antigen/ligand.

[066] "Affinity" is the strength of the interaction between a binding protein and an antigen/ligand, and is determined by the sequence of the binding domain(s) of the binding protein as well as by the nature of the antigen/ligand, such as its size, shape, and/or charge. Binding proteins may be selected for affinities that provide desired therapeutic end-points while minimizing negative side-effects. Affinity may be measured using methods known to one skilled in the art (US 20090311253).

[067] The term "potency" refers to the ability of a binding protein to achieve a desired effect, and is a measurement of its therapeutic efficacy. Potency may be assessed using methods known to one skilled in the art (US 20090311253).

[068] The term "cross-reactivity" refers to the ability of a binding protein to bind a target other than that against which it was raised. Generally, a binding protein will bind its target tissue(s)/antigen(s) with an appropriately high affinity, but will display an appropriately low affinity for non-target normal tissues. Individual binding proteins are generally selected to meet two criteria. (1) Tissue staining appropriate for the known expression of the antibody target. (2) Similar staining pattern between human and tox species (mouse and cynomolgus monkey) tissues from the same organ. These and other methods of assessing cross-reactivity are known to one skilled in the art (US 20090311253).

[069] The term "biological function" refers the specific *in vitro* or *in vivo* actions of a binding protein. Binding proteins may target several classes of antigens/ligands and achieve desired therapeutic outcomes through multiple mechanisms of action. Binding proteins may target soluble proteins, cell surface antigens, as well as extracellular protein deposits. Binding proteins may agonize, antagonize, or neutralize the activity of their targets. Binding proteins may assist in the clearance of the targets to which they bind, or may result in cytotoxicity when bound to cells. Portions of two or more antibodies may be incorporated into a multivalent format to achieve distinct

functions in a single binding protein molecule. The *in vitro* assays and *in vivo* models used to assess biological function are known to one skilled in the art (US 20090311253).

[070] The term "solubility" refers to the ability of a protein to remain dispersed within an aqueous solution. The solubility of a protein in an aqueous formulation depends upon the proper distribution of hydrophobic and hydrophilic amino acid residues, and therefore, solubility can correlate with the production of correctly folded proteins. A person skilled in the art will be able to detect an increase or decrease in solubility of a binding protein using routine HPLC techniques and methods known to one skilled in the art (US 20090311253).

[071] Binding proteins may be produced using a variety of host cells or may be produced in vitro, and the relative yield per effort determines the "production efficiency." Factors influencing production efficiency include, but are not limited to, host cell type (prokaryotic or eukaryotic), choice of expression vector, choice of nucleotide sequence, and methods employed. The materials and methods used in binding protein production, as well as the measurement of production efficiency, are known to one skilled in the art (US 20090311253).

[072] The term "conjugate" refers to a binding protein, such as an antibody, that is chemically linked to a second chemical moiety, such as a therapeutic or cytotoxic agent. The term "agent" includes a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials. In an embodiment, the therapeutic or cytotoxic agents include, but are not limited to, pertussis toxin, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. When employed in the context of an immunoassay, the conjugate antibody may be a detectably labeled antibody used as the detection antibody.

[073] The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional

DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Other vectors include RNA vectors. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, other forms of expression vectors are also included, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions. A group of pHybE vectors (US Patent Application Serial No. 61/021,282) were used for parental binding protein cloning.

[074] The terms "recombinant host cell" or "host cell" refer to a cell into which exogenous DNA has been introduced. Such terms refer not only to the particular subject cell, but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. In an embodiment, host cells include prokaryotic and eukaryotic cells. In an embodiment, eukaryotic cells include protist, fungal, plant and animal cells. In another embodiment, host cells include but are not limited to the prokaryotic cell line *E.Coli*; mammalian cell lines CHO, HEK293, COS, NS0, SP2 and PER.C6; the insect cell line Sf9; and the fungal cell *Saccharomyces cerevisiae*.

[075] The term "transfection" encompasses a variety of techniques commonly used for the introduction of exogenous nucleic acid (e.g., DNA) into a host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like.

[076] The term "cytokine" refers to a protein released by one cell population that acts on another cell population as an intercellular mediator. The term "cytokine"

includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

[077] The term "biological sample" means a quantity of a substance from a living thing or formerly living thing. Such substances include, but are not limited to, blood, (e.g., whole blood), plasma, serum, urine, amniotic fluid, synovial fluid, endothelial cells, leukocytes, monocytes, other cells, organs, tissues, bone marrow, lymph nodes and spleen.

[078] The term "component" refers to an element of a composition. In relation to a diagnostic kit, for example, a component may be a capture antibody, a detection or conjugate antibody, a control, a calibrator, a series of calibrators, a sensitivity panel, a container, a buffer, a diluent, a salt, an enzyme, a co-factor for an enzyme, a detection reagent, a pretreatment reagent/solution, a substrate (e.g., as a solution), a stop solution, and the like that can be included in a kit for assay of a test sample. Thus, a "component" can include a polypeptide or other analyte as above, that is immobilized on a solid support, such as by binding to an anti-analyte (e.g., anti-polypeptide) antibody. Some components can be in solution or lyophilized for reconstitution for use in an assay.

[079] "Control" refers to a composition known to not analyte ("negative control") or to contain analyte ("positive control"). A positive control can comprise a known concentration of analyte. "Control," "positive control," and "calibrator" may be used interchangeably herein to refer to a composition comprising a known concentration of analyte. A "positive control" can be used to establish assay performance characteristics and is a useful indicator of the integrity of reagents (e.g., analytes).

[080] The term "Fc region" defines the C-terminal region of an immunoglobulin heavy chain, which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain. Replacements of amino acid residues in the Fc portion to alter antibody effector function are known in the art (e.g., US Patent Nos. 5,648,260 and 5,624,821). The Fc region mediates several important effector functions, e.g., cytokine induction, antibody dependent cell mediated cytotoxicity (ADCC), phagocytosis, complement dependent cytotoxicity (CDC), and half-

life/ clearance rate of antibody and antigen-antibody complexes. In some cases these effector functions are desirable for a therapeutic immunoglobulin but in other cases might be unnecessary or even deleterious, depending on the therapeutic objectives.

[081] The terms "Kabat numbering", "Kabat definitions" and "Kabat labeling" are used interchangeably herein. These terms, which are recognized in the art, refer to a system of numbering amino acid residues which are more variable (i.e., hypervariable) than other amino acid residues in the heavy and light chain variable regions of an antibody, or an antigen binding portion thereof (Kabat et al. (1971) Ann. NY Acad. Sci. 190:382-391 and, Kabat et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). For the heavy chain variable region, the hypervariable region ranges from amino acid positions 31 to 35 for CDR1, amino acid positions 50 to 65 for CDR2, and amino acid positions 95 to 102 for CDR3. For the light chain variable region, the hypervariable region ranges from amino acid positions 24 to 34 for CDR1, amino acid positions 50 to 56 for CDR2, and amino acid positions 89 to 97 for CDR3.

[082] The term "CDR" means a complementarity determining region within an immunoglobulin variable region sequence. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the heavy and light chain variable regions. The term "CDR set" refers to a group of three CDRs that occur in a single variable region capable of binding the antigen. The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat et al. (1987) and (1991)) not only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also provides precise residue boundaries defining the three CDRs. These CDRs may be referred to as Kabat CDRs. Chothia and coworkers (Chothia and Lesk (1987) J. Mol. Biol. 196:901-917; Chothia et al. (1989) Nature 342:877-883) found that certain sub- portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. These sub-portions were designated as L1, L2 and L3 or H1, H2 and H3 where the "L" and the "H" designates the light chain and the heavy chain regions, respectively. These regions may be referred to as Chothia CDRs, which have boundaries that overlap with Kabat CDRs. Other boundaries defining CDRs overlapping with the Kabat CDRs have been

described by Padlan (1995) FASEB J. 9:133-139 and MacCallum (1996) J. Mol. Biol. 262(5):732-45). Still other CDR boundary definitions may not strictly follow one of the herein systems, but will nonetheless overlap with the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. The methods used herein may utilize CDRs defined according to any of these systems, although certain embodiments use Kabat or Chothia defined CDRs.

[083] The term "epitope" means a region of an antigen that is bound by a binding protein, e.g., a polypeptide and/or other determinant capable of specific binding to an immunoglobulin or T-cell receptor. In certain embodiments, epitope determinants include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain embodiments, may have specific three dimensional structural characteristics, and/or specific charge characteristics. In an embodiment, an epitope comprises the amino acid residues of a region of an antigen (or fragment thereof) known to bind to the complementary site on the specific binding partner. An antigenic fragment can contain more than one epitope. In certain embodiments, a binding protein specifically binds an antigen when it recognizes its target antigen in a complex mixture of proteins and/or macromolecules. Binding proteins "bind to the same epitope" if the antibodies cross-compete (one prevents the binding or modulating effect of the other). In addition, structural definitions of epitopes (overlapping, similar, identical) are informative; and functional definitions encompass structural (binding) and functional (modulation, competition) parameters. Different regions of proteins may perform different functions. For example specific regions of a cytokine interact with its cytokine receptor to bring about receptor activation whereas other regions of the protein may be required for stabilizing the cytokine. To abrogate the negative effects of cytokine signaling, the cytokine may be targeted with a binding protein that binds specifically to the receptor interacting region(s), thereby preventing the binding of its receptor. Alternatively, a binding protein may target the regions responsible for cytokine stabilization, thereby designating the protein for degradation. The methods of visualizing and modeling epitope recognition are known to one skilled in the art (US 20090311253).

[084] "Pharmacokinetics" refers to the process by which a drug is absorbed, distributed, metabolized, and excreted by an organism. To generate a multivalent binding protein molecule with a desired pharmacokinetic profile, parent binding proteins with similarly desired pharmacokinetic profiles are selected. The PK profiles of the selected parental binding proteins can be easily determined in rodents using methods known to one skilled in the art (US 20090311253).

[085] "Bioavailability" refers to the amount of active drug that reaches its target following administration. Bioavailability is function of several of the previously described properties, including stability, solubility, immunogenicity and pharmacokinetics, and can be assessed using methods known to one skilled in the art (US 20090311253).

[086] The term "K_{on}" means the on rate constant for association of a binding protein (e.g., an antibody) to the antigen to form the, antibody/antigen complex. The term "K_{on}" also means "association rate constant", or "ka", as is used interchangeably herein. This value indicating the binding rate of a binding protein to its target antigen or the rate of complex formation between a binding protein, e.g., an antibody, and antigen also is shown by the equation below:

$$\text{Antibody ("Ab")} + \text{Antigen ("Ag")} \rightarrow \text{Ab-Ag}$$

[087] The term "K_{off}" means the off rate constant for dissociation, or "dissociation rate constant", of a binding protein (e.g., an antibody) from the, antibody/antigen complex as is known in the art. This value indicates the dissociation rate of a binding protein, e.g., an antibody, from its target antigen or separation of Ab-Ag complex over time into free antibody and antigen as shown by the equation below:

$$\text{Ab} + \text{Ag} \leftarrow \text{Ab-Ag}$$

[088] The terms "K_d" and "equilibrium dissociation constant" means the value obtained in a titration measurement at equilibrium, or by dividing the dissociation rate constant (K_{off}) by the association rate constant (K_{on}). The association rate constant, the dissociation rate constant and the equilibrium dissociation constant, are used to represent the binding affinity of a binding protein (e.g., an antibody) to an antigen. Methods for determining association and dissociation rate constants are well known in the art. Using fluorescence based techniques offers high sensitivity and the ability to examine samples in

physiological buffers at equilibrium. Other experimental approaches and instruments such as a BIAcore® (biomolecular interaction analysis) assay, can be used (e.g., instrument available from BIAcore International AB, a GE Healthcare company, Uppsala, Sweden). Additionally, a KinExA® (Kinetic Exclusion Assay) assay, available from Sapidyne Instruments (Boise, Idaho), can also be used.

[089] The term "variant" means a polypeptide that differs from a given polypeptide in amino acid sequence or in post-translational modification. The difference in amino acid sequence may be caused by the addition (e.g., insertion), deletion, or conservative substitution of amino acids, but that retains the biological activity of the given polypeptide (e.g., a variant TNF-alpha antibody can compete with anti- TNF-alpha antibody for binding to TNF-alpha). A conservative substitution of an amino acid, i.e., replacing an amino acid with a different amino acid of similar properties (e.g., hydrophilicity and degree and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art (see, e.g., Kyte et al. (1982) J. Mol. Biol. 157: 105-132). The hydropathic index of an amino acid is based on a consideration of its hydrophobicity and charge. It is known in the art that amino acids of similar hydropathic indexes in a protein can be substituted and the protein still retains protein function. In one aspect, amino acids having hydropathic indexes of ± 2 are substituted. The hydrophilicity of amino acids also can be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity (see, e.g., US Patent No. 4,554,101). Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. In one aspect, substitutions are performed with amino acids having hydrophilicity values within ± 2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity,

hydrophilicity, charge, size, and other properties. The term "variant" also includes polypeptide or fragment thereof that has been differentially processed, such as by proteolysis, phosphorylation, or other post-translational modification, yet retains its biological activity or antigen reactivity, e.g., the ability to bind to TNF-alpha. The term "variant" encompasses fragments of a variant unless otherwise defined. A variant may be 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%,85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, or 75% identical to the wild-type sequence.

[090] The difference in post-translational modification may be effected by addition of one or more chemical groups to the amino acids of the modified molecule, or removal of one or more such groups from the molecule. Examples of modification may include but are not limited to, phosphorylation, glysosylation, or MGO modification.

[091] It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods described herein are obvious and may be made using suitable equivalents without departing from the scope of the embodiments disclosed herein. Having now described certain embodiments in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to be limiting.

EXAMPLES

Example 1: Identification of Different Forms of MGO-mAb

[092] In a traditional process for making Adalimumab, antibody expression typically takes place by using Hydrolysate and Phytone as raw materials. When adalimumab was expressed with CHO cells using chemically defined media (CDM), the percentage of acidic species as defined by the weak cation exchange chromatography method increased as compared to the percentage of acidic species generated by the traditional production process. Specifically, two distinct early eluting chromatographic peaks were observed as shown in Figure 1. The peaks labeled as Lys 0, Lys 1 and Lys 2 are antibody without C-terminal Lys, with one C-terminal Lys and with two C-terminal Lys on the heavy chains, respectively. The top trace is from adalimumab produced using chemically defined media (CDM) and the bottom trace is from adalimumab produced using yeastolate. Two peaks were observed in antibody expressed in cell culture using

CDM and are denoted by Fractions 1 and 2, respectively. These peaks are unique to adalimumab production with CDM. The peaks were subsequently isolated using weak cation exchange fractionation.

[093] Analysis of the isolated peaks by reduced LC/MS revealed mass spectra of the expected values for the adalimumab heavy chain and light chain but with additional peak corresponding to mass increases of +54 Da and +72 Da with additional lower intensity peaks which are likely due to additional modifications at multiple sites of the respective chains (Figure 2). As shown in Fig. 2 left panel, three major peaks corresponding to the theoretical molecular weight of the light chain at 23408 Da plus masses of 23462 and 23480 were observed. The two peaks that shift from the theoretical molecular weight diverge from the expected mass by increases of 54 and 72 daltons, respectively. As shown in Fig. 2 Right Panel, three peaks corresponding to the theoretical molecular weight of the heavy chain at 50637 Da plus an additional ladder of masses corresponding to 54 and 72 Da increases were observed. Peaks with these molecular weight increases were observed for both the light chain and heavy chain from fractions 1 and 2 but were noticeably absent from the Lys-0 controls (bottom spectra of Fig. 2).

[094] The peaks were subsequently analyzed by peptide mapping with LC/MS/MS detection. Modifications that resulted in the molecular weight increases of both 54 Da and 72 Da were localized to a particular Arg for this peptide and has resulted in a tryptic mis-cleavage (Fig. 3). This observation supports the hypothesis of hydroxylimidine conversion to a hydroimadazolone after loss of water. The results suggest that the modifications are localized to miscleaved tryptic peptides where the adduction is on the arginine side chain.

[095] Based on these observations, it is likely that the adduction of the antibody was due to methylglyoxal (MGO) accumulation in cell cultures grown in the presence of chemically defined media (CDM). The reaction scheme for methylglyoxal modification of arginine residues is shown in Fig. 4. The initial adduction of MGO with an arginine side chain results in the formation of a hydroxylimidine with an observed mass increase of + 72Da. Following a dehydration to a hydroimadazolone, the resulting product has a + 54Da mass increase.

[096] In order to confirm that an accumulation of methylglyoxal is the cause of the +54 Da and +72 Da mass increases associated with the early eluting acidic peaks, antibody was incubated with synthetic methylglyoxal and analyzed over a time course. WCX-10 fractionation was used to isolate zero lysine species, which is the adalimumab antibody with only the dominant main peak of the weak cation exchange chromatogram present. The 0 Lys species was incubated in the presence of 2.7 mM MGO over the course of five hours at 37 C.

[097] As shown in Fig. 5, over the time course, nearly all of the 0 Lys was converted to the two distinct acidic peaks found in the initial material analyzed from the CDM expressions. The lysine 0 after incubation under the same condition without exposure to MGO is also shown as a control. Peaks a and b from the sample treated with MGO for 120 minutes were subsequently collected and analyzed by LC/MS to assess the level of chemical modifications which have resulted.

[098] Subsequent analysis of 0 Lys material incubated with MGO showed the previously observed ladder of +54 Da and +72 Da mass heterogeneity as a prevalent pattern in the mass spectra of both the adalimumab light chain and heavy chain (Fig. 6). More specifically, peaks a and b from the 0 Lys recombinant antibody species treated with MGO were fractionated and analyzed by reduced LC/MS. The top pane shows the corresponding light chain mass spectra of the two peaks and the bottom pane depicts the heavy chain for the fractionated peaks. Mass heterogeneity of the chains corresponding to +54 Da and +72 Da were observed for both fractions. The resulting modifications are in agreement with the observations found in the cell culture acidic peaks supporting the previous data that the modification is due to methylglyoxal. Thus, fractionation of the acidic-shifted 0 Lys material followed by LC/MS/MS tryptic mapping confirmed that MGO modification of arginine residues was the cause of the observed adductions.

[099] In addition, acid species from both cell culture and the MGO spike were compared to each other by LC/MS/MS. The resulting MS/MS spectra showed fragmentation profiles that were highly comparable for mis-cleavages at arginine residues with the MGO adduction characteristic + 54 Da and + 72 Da mass increases (Figure 7). The data provide a confirmation that the acidic peaks resulting from the use of chemically defined media are due to modifications of the expressed adalimumab recombinant antibody by methylglyoxal which has accumulated in the cell culture bioreactor.

Moreover, the modification of the arginine may influence the fragmentation of the peptide backbone. The strong similarities between the two mass spectra further support the notion that the arginine has undergone a modification which may result in destabilization of the peptide backbone.

Example 2 Functional Liabilities Associated with Methylglyoxal Modifications to Adalimumab Antibodies

[0100] Methylglyoxal modifications of arginine residues lead to miscleavages due to the steric constraints imparted by the adducted MGO to the active site of trypsin. In order to better quantitate and determine all susceptible arginine residues in the adalimumab primary structure, an endoprotease Lys-C digestion was performed where arginine residues were no longer recognized as target substrates in the peptide mapping protocol. All Lys-C peptides were evaluated using the Sequest algorithm against the FASTA sequence for adalimumab. Several sites were identified as potential susceptible sites but one site of particular susceptibility was identified at R30 of the light chain. The sequences of the light chain and heavy chain of the Adalimumab D2E7 are designated as SEQ ID No. 1 and SEQ ID No. 2, respectively. A list of all potential susceptible arginine residues is shown in Table 1. Different sites may have different level of susceptibility to MGO modification. Not all sites have to be modified by MGO in a single molecule. Table 2 lists peptide fragments on Adalimumab that are susceptible to modification by methylglyoxal.

Table 1 Potential Sites of MGO modification in Adalimumab

Ab Chain Type	Adalimumab Light Chain (SEQ ID No. 1)	Adalimumab Heavy Chain (SEQ ID No. 2)
Arginine Sites	Arginine 30 Arginine 93 Arginine 108	Arginine 16 Arginine 259 Arginine 359 Arginine 420

Table 2. List of peptides susceptible to modification by methylglyoxal

Sequence	Activation Type	Modifications	Charge	m/z [Da]	MH+ [Da]	RT [min]	MS Order
EPQVYTLPPSrDELTK	HCD	R11(MGO (R) 72)	2	972.9988	1944.99	27.71	MS2
EPQVYTLPPSrDELTK	CID	R11(MGO (R) 72)	3	649.0014	1944.99	27.72	MS2
EPQVYTLPPSrDELTK	CID	R11(MGO)	3	642.9988	1926.982	27.81	MS2
EPQVYTLPPSrDELTK	HCD	R11(MGO)	3	642.9988	1926.982	27.82	MS2
EPQVYTLPPSrDELTK	CID	R11(MGO)	2	963.9942	1926.981	27.88	MS2
EPQVYTLPPSrDELTK	HCD	R11(MGO)	2	963.9942	1926.981	27.89	MS2
EVQLVESGGGLVQPGrSLR	CID	R16(MGO (R) 72)	2	1027.055	2053.103	32	MS2
EVQLVESGGGLVQPGrSLR	HCD	R16(MGO (R) 72)	2	1027.055	2053.103	32.01	MS2
EVQLVESGGGLVQPGrSLR	CID	R16(MGO)	3	679.0353	2035.091	32.11	MS2
EVQLVESGGGLVQPGrSLR	CID	R16(MGO)	2	1018.05	2035.092	32.13	MS2
EVQLVESGGGLVQPGrSLR	HCD	R16(MGO)	2	1018.05	2035.092	32.15	MS2
DIQMTQSPSSLSASVGDrVTITcR	HCD	R18(MGO), C23(Carboxymethyl)	3	888.7587	2664.261	35.6	MS2
DIQMTQSPSSLSASVGDrVTITcR	HCD	R18(MGO), C23(Carboxymethyl)	3	888.7583	2664.26	36.63	MS2
YNrAPYTFGQGTK	CID	R3(MGO (R) 72)	2	787.8835	1574.76	17.61	MS2
YNrAPYTFGQGTK	HCD	R3(MGO (R) 72)	2	787.8835	1574.76	17.62	MS2
YNrAPYTFGQGTK	CID	R3(MGO (R) 72)	3	525.5911	1574.759	17.63	MS2
YNrAPYTFGQGTK	HCD	R3(MGO (R) 72)	3	525.5911	1574.759	17.64	MS2
YNrAPYTFGQGTKVEIK	CID	R3(MGO (R) 72)	2	1022.461	2043.916	46.16	MS2
SLrLScAASGFTFDDYAMHWVR	CID	R3(MGO (R) 72), C6(Carboxymethyl)	3	888.4062	2663.204	49.36	MS2
SLrLScAASGFTFDDYAMHWVR	HCD	R3(MGO (R) 72), C6(Carboxymethyl)	3	888.4062	2663.204	49.38	MS2
YNrAPYTFGQGTK	CID	R3(MGO)	2	778.8782	1556.749	17.49	MS2
YNrAPYTFGQGTK	HCD	R3(MGO)	2	778.8782	1556.749	17.5	MS2
YNrAPYTFGQGTK	CID	R3(MGO)	3	519.5878	1556.749	17.56	MS2
YNrAPYTFGQGTK	HCD	R3(MGO)	3	519.5878	1556.749	17.57	MS2
SFNrGEc	HCD	R4(MGO), C7(Carboxymethyl)	2	462.8614	924.7156	5.29	MS2
ASQGlrNYLAWYQQKPGK	CID	R6(MGO (R) 72)	3	727.3791	2180.123	32.15	MS2
ASQGlrNYLAWYQQKPGK	HCD	R6(MGO (R) 72)	3	727.3791	2180.123	32.16	MS2

ASQGIrNYLAWYQQKPGK	CID	R6(MGO (R) 72)	2	1090.566	2180.125	32.2	MS2
ASQGIrNYLAWYQQKPGK	HCD	R6(MGO (R) 72)	2	1090.566	2180.125	32.21	MS2
ASQGIrNYLAWYQQKPGK	CID	R6(MGO)	3	721.3756	2162.112	31.52	MS2
ASQGIrNYLAWYQQKPGK	HCD	R6(MGO)	3	721.3756	2162.112	31.53	MS2
ASQGIrNYLAWYQQKPGK	CID	R6(MGO)	2	1081.561	2162.115	31.55	MS2
ASQGIrNYLAWYQQKPGK	HCD	R6(MGO)	2	1081.561	2162.115	31.56	MS2
DTLMISrTPEVTcVVVDVSHEDPEVK	CID	R7(MGO (R) 72), C13(Carboxymethyl)	3	1010.155	3028.451	44.42	MS2
DTLMISrTPEVTcVVVDVSHEDPEVK	HCD	R7(MGO (R) 72), C13(Carboxymethyl)	3	1010.155	3028.451	44.43	MS2
DTLMISrTPEVTcVVVDVSHEDPEVK	CID	R7(MGO), C13(Carboxymethyl)	3	1004.152	3010.442	44.14	MS2
DTLMISrTPEVTcVVVDVSHEDPEVK	HCD	R7(MGO), C13(Carboxymethyl)	3	1004.152	3010.442	44.15	MS2

[0101] The crystal structure of the adalimumab Fab unit in complex with its cognate binding partner TNF-alpha shows that R30 is intimately involved in the contact surface between CDR1 and the antigen surface (Fig. 8). The figure shows the side chain of arginine 30 (indicated by arrow) protruding into the TNF-alpha structure (indicated by arrow). A modification of this side chain by MGO would result in the formation of a five-member ring originating at the guanidinium terminal of the side chain and further penetrating into the TNF-alpha structure. The MGO modification is therefore likely to impede adalimumab's ability to bind TNF-alpha due to steric constraints.

[0102] In order to further elucidate any functional liabilities associated with adalimumab and chemical modifications due to an accumulation of MGO in a cell culture expression using chemically defined media, an enriched MGO-modified fraction was isolated using weak cation exchange chromatography. A control fraction of a pure 0 Lys fraction was also obtained. The two fraction were analyzed by surface plasmon resonance to calculate the association and dissociation rates of TNF-alpha to the immobilized antibody. A three-fold reduction was observed for the MGO modified adalimumab as compared to the 0 Lys control (Fig. 9). Thus, it appears that the methylglyoxal modification of Arginine 30 (R30) of the light chain does impart a functional liability to the affected population of adalimumab drug substance. These data support the hypothesis that a chemical modification on the side chain of Arginine 30 of the light chain induces steric interference with the CDR1 and the TNF-alpha binding

surface which may lead to a significant drop in adalimumab potency. It is therefore desirable to reduce the amount of this modified form of antibody in adalimumab drug substance or drug product.

Example 3 Removal of Methylglyoxal-modified Adalimumab Using an AEX and/or CEX Strategy

[0103] A chromatographic strategy was employed to remove the early eluting acidic region on the WCX-10 chromatogram. After the removal process is performed, adalimumab drug substance devoid of this region was generated. As disclosed herein, expression of adalimumab in chemically defined media may cause an increase of species eluting in this acidic region as a result of the accumulating MGO adducting to the positively charged guanidinium groups of the affected arginine residues. The disclosed chromatographic strategy helps clear this functional liability of the adalimumab preparation. The resulting adalimumab BDS is free of or substantially free of the negative impact from the methylglyoxal modification and has normal binding to its target, TNF-alpha.

[0104] The decision whether to use cationic exchange chromatography (CEX), anionic exchange chromatography (AEX), or both, to purify a protein is primarily based on the overall charge of the protein. Therefore, it is within the scope of this invention to employ an anionic exchange step prior to the use of a cationic exchange step, or a cationic exchange step prior to the use of an anionic exchange step. Furthermore, it is within the scope of this invention to employ only a cationic exchange step, only an anionic exchange step, or any serial combination of the two.

[0105] In performing the separation, the initial protein mixture can be contacted with the ion exchange material by using any of a variety of techniques, e.g., using a batch purification technique or a chromatographic technique.

[0106] For example, ion exchange chromatography is used as a purification technique to separate the MGO-modified forms from the non-MGO-modified forms. Ion exchange chromatography separates molecules based on differences between the overall charge of the molecules. In the case of an antibody, the antibody has a charge opposite to that of the functional group attached to the ion exchange material, e.g., resin, in order to bind. For example, antibodies, which generally have an overall positive charge in a buffer

having a pH below its pI, will bind well to cation exchange material, which contain negatively charged functional groups.

[0107] In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e., conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity and/or pH may be gradual (gradient elution) or stepwise.

Example 3.1 Removal of Methylglyoxal-modified Adalimumab Using AEX

[0108] A process is described here for purifying a target protein product from both process and product related impurities. Specifically, a method is provided for reducing product related charge variants (i.e. acidic and basic species). The method involves contacting the process mixture with an anion exchange (AEX) adsorbent in an aqueous salt solution under loading conditions that permit both the target and non-target proteins to bind to the AEX adsorbent and allowing the excess target molecule to pass through the column and subsequently recovering the bound target protein with a wash at the same aqueous salt solution used in the equilibration (i.e. pre-loading) condition.

[0109] **Source Material**-The antibody used in this study was derived from cell culture conditions employing both chemically defined media (CDM) and hydrolysate media. The antibody was captured from the clarified harvest through affinity chromatography (Protein-A, GE MabSuRe) where the eluate is in a buffer system of about 20 mM acetic acid at a pH of about 4.2.

[0110] **Induced pH Gradient Anion Exchange Chromatography**-POROS 50PI (Applied Biosystems) resin was packed in 1.0 cm x 10.0 cm (OmniFit) column. The column was equilibrated in a two-component buffer containing acetate as the anion and either tromethalmine (Tris) or arginine as the cation. In these experiments, the anion (i.e. acetate) concentration was held constant and the cation (Tris/Arginine) was added to achieve the desired pH. Induced pH gradients were initially performed, without protein, by equilibrating the column with an Acetate/Tris or Acetate/Arginine buffer at pH 9.0 followed by a step change of the equivalent buffer at pH 7.0. Induced pH gradients

without protein were run at controlled acetate concentrations of 5 mM, 10 mM, 20 mM, and 30 mM.

[0111] The POROS 50PI column was then loaded with 20 g/L of D2E7 in 5mM Acetate/Tris (or Arginine) pH 9.0, followed by a 10 column volume (CV) isocratic wash, and then an induced pH gradient elution with a step change in the running buffer to 5 mM Acetate/Tris (or Arginine) pH 7.0. The column was then regenerated (5 CVs of 100 mM acetate + 1 M NaCl), cleaned in place (3 CVs 1M NaOH, 60 min hold), and stored (5 CVs 20% ethanol). During elution, the column effluent was fractionated into 0.5 x CV and analyzed for UV280, WCX-10, and SEC (described below). The D2E7 AEX-load was prepared by diluting the source material described above with Milli-Q water to 5 mM acetate and titrating with arginine to the desired pH.

[0112] **Flow-Through Anion Exchange Chromatography-**Using the induced pH gradient results, an operational pH was selected to operate the POROS 50PI column in flow-through mode. The pH was selected (e.g. pH 8.8) to optimize the resolution between the acidic species and Lysine variants. The first run was performed by loading 150 g/L in a 5 mM Acetate/Arginine pH 8.8 buffer system, followed with a 20 CV isocratic wash. A FTW fraction was collected from 50-150 mAU and analyzed for UV280, WCX-10, and SEC. The results from this run are shown in Table 3. This run was able to reduce acidic species by 60% and remove almost all detectable high molecular weight species (i.e. aggregates) with about 68% recovery.

Table 3 Acidic species and aggregates reduction by AEX

AEX Poros 50PI, 150 g/L FT, 5 mM Acetate/Arginine pH 8.8	Acidic Species		SEC		
	AR1+2	LysSum	HMW	Mono	LMW
AEX Load (t=0)	17.805	81.685	1.704	97.947	0.348
AEX Load (t=10 days, 4°C)	19.711	79.746	1.975	97.831	0.194
AEX FTW (t=0)	7.085	92.108	0.019	99.889	0.092
AEX FTW (t=10 days, 4°C)	8.069	91.773	0.04	99.853	0.107

[0113] The data presented here demonstrates a method for the fine purification of D2E7 from both product related (i.e. charge variants and molecular weight variants) impurities by loading the process stream to an anion exchange adsorbent under aqueous

salt conditions (i.e. low conductivity and high pH) that permit both the target and non-target proteins to bind to the AEX adsorbent and allowing the excess target molecule to pass through the column and subsequently recovering the bound target protein with a wash at the same aqueous salt solution used in the equilibration (i.e. pre-loading) condition.

Example 3.2 Removal of Methylglyoxal-modified Adalimumab Using CEX

[0114] This Example describes a process for purifying a target protein product from both process and product related impurities by using a cation exchange (CEX) technique. Specifically, a reversible binding method is disclosed for reducing product related charge variants (i.e. acidic species) of the target molecule. By way of example, the method may involve some or all of the following steps.

[0115] In one step, the process mixture is caused to be in contact with a cation exchange (CEX) adsorbent in an controlled aqueous buffer solution with pH and conductivity under loading conditions that permit both the target and non-target proteins to bind to the CEX adsorbent. The pH of the loading buffer is below the pI of the antibody molecule.

[0116] In another step, the charged variants, molecular variants and impurities are washed off using the same buffer conditions as the loading buffer. The product may then be eluted with a buffer having higher conductivity than that of the loading buffer.

[0117] In this Example, three antibody molecules were used. Adalimumab antibody was obtained from concentrated fractogel eluate in AY04 manufacturing process and CDM 300 L scale up run Protein A eluate. They were buffer exchanged into 29 mM Tris-acetate buffer pH 7.5 as CEX loading material.

[0118] Poros XS, (Applied Biosystems) strong CEX resin, CM Hyper D (Pall), weak CEX resin, Nuvia S (Bio-Rad) strong resin and GigaCap S 650 (Tosoh Biosciences) strong resin were packed in 1.0 cm x 10.0 cm (OmniFit) columns. The column was equilibrated in a buffer system with appropriate pH and conductivity. The column load was prepared in the equilibration buffer and loaded on the column at 40 g protein/L resin followed by washing with the equilibration buffer for 20 CV. The antibody product was eluted out with 150 mM sodium chloride and 30 mM Tris-acetate buffer

solution. 1M of NaCl was used for column regeneration and 1M of NaOH solution was used for column cleaning.

[0119] Four buffer/salt systems, sodium chloride/Tris-acetate, Tris-acetate, Ammonium sulfate/Tris-acetate and arginine/Tris-acetate at different pH and conductivity were evaluated. The buffer conditions are listed in Table 4.

Table 4. Buffer conditions

Resin	Buffer	pH	Conductivity
Poros XS (strong)	Tris-acetate	7.5, 6.5, 5.5	3 conductivity for each pH
	Sodium chloride	7.5, 6.5, 5.5	3 conductivity for each pH
	Ammonium sulfate	7.5	3 conductivity for each pH
CM Hyper D (weak)	Tris-acetate	7.5	3 conductivity
	Sodium chloride	7.5, 6.8, 6.0	3 conductivity for each pH
	Ammonium sulfate	7.5	3 conductivity
Nuvia S (strong)	Tris-acetate	7.5	3 conductivity
	Sodium chloride		3 conductivity
	Ammonium sulfate		3 conductivity
GigaCap S 650	Tris-acetate	7.5	3 conductivity

[0120] A reversible binding mode was performed using Adalimumab with Tris-acetate buffer system. The loading utilized buffer at pH 7.5 and Tris concentration at 145 mM with 40 g protein /L resin. The column wash was fractionated. The wash fractions and elute pool were analyzed by UV280, WCX-10 and SEC assays. The chromatogram is shown in Fig. 11.

Example 4 Charge variants reduction in Adalimumab by Poros XS resin

[0121] In this Example, different resins and buffer conditions were evaluated. The starting material contained 14% total AR and 3% AR1. Experiments were performed

by varying resins and buffer conditions for acidic species removal. The results are described in the following sections.

[0122] Experiments were performed on Poros XS resin using NaCl to vary the conductivity with a fixed 29 mM Tris-acetate buffer for pH control. Three pH levels were tested, pH 7.5, 6.8 and 6.0. Each pH was studied at conductivities wherein the amount of NaCl was varied. As shown in Fig. 12, acidic species can be removed by 3% with 90% yield. For further reduction in acidic species, the yields achieved vary under different buffer conditions. At pH 7.5 and 45 mM NaCl, the amount of acidic species was reduced by 6.8%, with 75% yield of Adalimumab. AR1 was significantly reduced to about zero percent, with a yield of 72% of Adalimumab, and to less than 0.5% with over 80% yield of Adalimumab, as shown in Table 5. The column wash was fractionated and specified as Fraction 1 to Fraction 5 by the order of adjacent to the eluate. The AR1, AR2, Lys sum versus yield was calculated based on the results of each fraction.

Table 5. AR1 removal versus yield by CEX

Wash fractions	%AR1	%AR2	%Lys Sum	Yield (%)
Load	2.9	12.1	84.3	n/a
Eluate	0	7.8	92.2	72
Eluate + Fraction 1	0.3	8.8	91.0	79
Eluate + Fraction 1+ Fraction 2	0.6	9.6	89.8	83
Eluate + Fraction 1+ Fraction 2 + Fraction 3	1.6	10	88.4	88
Eluate + Fraction 1+ Fraction 2 + Fraction 3 +_Fraction 4	2.2	10.9	86.8	92
Eluate + Fraction 1+ Fraction 2 + Fraction 3 +_Fraction 4 + Fraction 5	2.9	11	86.1	93

[0123] In summary, methods for the purification of Adalimumab from product related impurities (i.e. charge variants and molecular weight variants) are disclosed. More particularly, the process stream may be loaded to a cation exchange adsorbent under appropriate aqueous conditions, wherein the pH and conductivity of the loading and wash buffer is below the pI of the target protein that permit both the target protein and impurities to bind to the CEX adsorbent. The acidic species and other impurities may then be washed off by using wash buffer which is the same as the loading buffer. Lastly, the bound target protein may be recovered by using a high conductivity aqueous solution.

[0124] The present disclosure is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of this disclosure and the claims.

References

[0125] The contents of all cited references (including literature references, patents, patent applications, and websites) that may be cited throughout this application or listed below are hereby expressly incorporated by reference in their entirety for any purpose into the present disclosure. The disclosure may employ, unless otherwise indicated, conventional techniques of immunology, molecular biology and cell biology, which are well known in the art.

[0126] The present disclosure also incorporates by reference in their entirety techniques well known in the field of molecular biology and drug delivery. These techniques include, but are not limited to, techniques described in the following publications:

1. Awdeh, Z.L., A.R. Williamson, and B.A. Askonas, *One cell-one immunoglobulin. Origin of limited heterogeneity of myeloma proteins.* Biochem J, 1970. **116**(2): p. 241-8.

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19. Chumsae, C., et al., Comparison of methionine oxidation in thermal stability and chemically stressed samples of a fully human monoclonal antibody. Journal of Chromatography B, 2007. **850**(1-2): p. 285-294.

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22. Cordoba, A.J., et al., *Non-enzymatic hinge region fragmentation of antibodies in solution.* Journal of Chromatography B, 2005. **818**(2): p. 115-121.

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24. U.S. Patent No. 6,090,382.

Equivalents

[0127] The disclosure may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting of the disclosure. Scope of the disclosure is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are therefore intended to be embraced herein.

Claims

We claim

1. A composition comprising a binding protein capable of binding TNF-alpha, wherein said binding protein comprises at least one methylglyoxal (MGO)-susceptible amino acid, and wherein at least a portion of said binding protein comprises one or more MGO-modified amino acids.

2. The composition of Claim 1, wherein the portion of the binding protein that comprises at least one MGO-modified amino acid is less than 12%.

3. The composition of Claim 1, wherein the portion of the binding protein that comprises at least one MGO-modified amino acid is less than 6%.

4. The composition of any one of Claims 1-3, wherein the MGO-susceptible amino acid is an arginine.

5. The composition of any one of Claims 1-4, wherein the binding protein is a human antibody or an antigen-binding portion thereof, wherein the binding protein dissociates from human TNF-alpha with a K_d of $1x10^{-8}$ M or less and a K_{off} rate constant of $1x\ 10^{-3}\ s^{-1}$ or less, both as determined by surface plasmon resonance, and wherein the binding protein neutralizes human TNF-alpha cytotoxicity in a standard in vitro L929 assay with an IC_{50} of $1x10^{-7}$ M or less.

6. A composition comprising a binding protein capable of binding TNF-alpha, said binding protein comprising a methylglyoxal (MGO)-susceptible amino acid, wherein said composition is prepared by substantially removing molecules of said binding protein that comprise at least one MGO-modified amino acid.

7. The composition of Claim 6, wherein more than 70% of said molecules that comprise at least one MGO-modified amino acid is removed.

8. The composition of Claim 6, wherein more than 90% of said molecules that comprise at least one MGO-modified amino acid is removed.

9. The composition of any one of Claims 6-8, wherein the MGO-susceptible amino acid is an arginine.

10. The composition of any one of Claims 6-9, wherein the binding protein is a human antibody or an antigen-binding portion thereof, wherein the binding protein dissociates from human TNF-alpha with a K_d of $1x10^{-8}$ M or less and a K_{off} rate constant of $1x\ 10^{-3}\ s^{-}$

$^{-1}$ or less, both determined by surface plasmon resonance, and wherein the binding protein neutralizes human TNF-alpha cytotoxicity in a standard in vitro L929 assay with an IC_{50} of $1x10^{-7}$ M or less.

11. A method for purifying a composition comprising a target protein, said method comprising:

(a) loading the composition to a cation exchange adsorbent using a loading buffer, wherein the pH of the loading buffer is lower than the pI of the target protein;

(b) washing the cation exchange adsorbent with a washing buffer, wherein the pH of the washing buffer is lower than the pI of the target protein;

(c) eluting the cation exchange adsorbent with an elution buffer, said elution buffer being capable of reducing the binding between the target protein and the cation exchange adsorbent; and

(d) collecting the eluate, wherein the percentage of the target protein is higher in the eluate than the percentage of the target protein in the composition.

12. The method of Claim 11, wherein the conductivity of the elution buffer is higher than the conductivity of the washer buffer.

13. The method of any one of Claims 11-12, wherein the conductivity of the elution buffer is raised by increasing the salt concentration of the elution buffer.

14. The method of any one of Claims 11-13, wherein the pH of the elution buffer is between 5.5 and 9.0.

15. The method of Claim 13, wherein the salt concentration of the elution buffer is between 20 mM NaCl and 200 mM NaCl.

16. The method of any one of Claims 11-15, wherein the target protein is a human antibody or an antigen-binding portion thereof, wherein the target protein dissociates from human TNF-alpha with a K_d of $1x10^{-8}$ M or less and a K_{off} rate constant of $1x\ 10^{-3}\ s^{-1}$ or less, both determined by surface plasmon resonance, and wherein the target protein neutralizes human TNF-alpha cytotoxicity in a standard in vitro L929 assay with an IC_{50} of $1x10^{-7}$ M or less.

17. A method for purifying a composition comprising a target protein, said method comprising:

(a) loading the composition to an anion exchange adsorbent using a loading buffer, wherein the pH of the loading buffer is lower than the isoelectric point (pI) of the target protein;

(b) allowing the majority of the target protein to pass through without binding to the anion exchange adsorbent;
(c) collecting the pass-through loading buffer containing said unbound target protein;
(d) washing the anion exchange adsorbent with a washing buffer;
(e) allowing the target protein bound to the anion exchange adsorbent to disassociate from the anion exchange adsorbent; and
(f) collecting the washing buffer containing said disassociated target protein.

18. The method of Claim 17, wherein the loading buffer comprises an anionic agent and a cationic agent, wherein the conductivity and pH of the loading buffer is adjusted by increasing or decreasing the concentration of a cationic agent and maintaining a constant concentration of an anionic agent in the loading buffer.

19. The method of Claim 18, wherein the anionic agent is selected from the group consisting of acetate, citrate, chloride anion, sulphate, phosphate and combinations thereof.

20. The method of any one of Claims 18-19, wherein the cationic agent is selected from the group consisting of sodium, Tris, tromethalmine, ammonium cation, arginine, and combinations thereof.

21. The method of any one of Claims 17-20, wherein the target protein is a human antibody or an antigen-binding portion thereof, wherein the target protein dissociates from human TNF-alpha with a K_d of $1x10^{-8}$ M or less and a K_{off} rate constant of $1x\ 10^{-3}\ s^{-1}$ or less, both determined by surface plasmon resonance, and wherein the target protein neutralizes human TNF-alpha cytotoxicity in a standard in vitro L929 assay with an IC_{50} of $1x10^{-7}$ M or less.