LOW ACIDIC SPECIES COMPOSITIONS AND METHODS FOR PRODUCING THE SAME USING DISPLACEMENT CHROMATOGRAPHY

RELATED APPLICATION INFORMATION

This application claims priority to U.S. Application No. 14/079,076, filed on November 13, 2013, which is a continuation of U.S. Application No. 14/077,576, filed on November 12, 2013, which in turn claims priority to U.S. Provisional Application Serial No. 61/892,833, filed on October 18, 2013; PCT Application No. PCT/US2013/031380, filed on March 14, 2013; and U.S. Application No. 13/803,808, filed on March 14, 2013, the entire contents of each of which are expressly incorporated herein by reference.

BACKGROUND OF THE INVENTION

The production of compositions comprising proteins for biopharmaceutical applications involves the use of upstream process technologies (*e.g.*, cell culture) and downstream process technologies (*e.g.*, protein purification) that are known to produce proteins exhibiting varying levels of protein variants and impurities within the composition. Such protein variants include, but are not limited to, the presence of charge variants (*e.g.*, basic variants and acidic species, including variants) and process-related impurities. For example, in monoclonal antibody (mAb) preparations, charge variants can be detected by various methods, such as ion exchange chromatography, for example, WCX-10 HPLC (a weak cation exchange chromatography) or IEF (isoelectric focusing). Because of their similar chemical characteristics to the antibody product molecules of interest, reduction of charge variants is a challenge in monoclonal antibody production.

Reduction of charge variants and/or product- or process-related impurities is particularly advantageous in the context of commercially produced recombinant biotherapeutics, as they have the potential to impact numerous product characteristics, including, but not limited to, product stability, product safety and product efficacy. Accordingly, there remains a need in the art for low acidic species compositions and high-efficiency methods of producing protein compositions, *e.g.*, antibodies, having low levels of acidic species.

SUMMARY OF THE INVENTION

The present invention is based on the optimization of displacement chromatography process technologies for protein production, *e.g.,* production of antibodies or antigen-binding portions thereof, resulting in the production of compositions comprising proteins that comprise low percentages of acidic species. These low acidic species compositions have improved therapeutic efficacy and improved biological properties, for example, increased cartilage tissue penetration, reduced cartilage destruction, reduced synovial proliferation, reduced bone erosion, increased protection against the development of arthritis as measured by arthritic scores and/or histopathology scores, reduced cell infiltration, reduced proteoglycan loss, reduced chondrocyte death, and/or increased TNFα affinity, as compared to a non-low acidic species composition.

Displacement chromatography is a chromatographic separation technology that involves the use of a displacer molecule to aid in the separation of a mixture, *e.g.,* an antibody-containing solution derived from cell culture harvest. The displacer molecule is conventionally selected to have a higher affinity for the stationary phase (*i.e.,* the chromatographic support) as compared to the components present in the material to be separated. Due to its higher affinity, the displacer molecule competes with protein mixture components for the binding sites on the stationary phase. Under appropriate conditions, the displacer induces the components of the mixture to develop into consecutive zones of concentrated and purified species in the order of decreasing binding affinity ahead of the displacer front. This ordered displacement of the components of the mixture results in the formation of a so-called "displacement train." In contrast to traditional elution mode chromatography, the displacement process takes advantage of the nonlinearity of the adsorption isotherm, allowing for higher column loading levels without compromising the purity and recovery of the component of interest. Finally, washing of the displacement train with the displacing buffer from the column allows for the component of interest to be isolated by collecting (and pooling if necessary) the proper fraction(s) of the displaced eluate.

Accordingly, in one aspect, the invention provides a method for producing a low acidic species composition comprising an antibody, or antigen-binding portion thereof, by contacting a first sample comprising the antibody, or antigen-binding portion thereof, with a chromatography media, wherein the antibody, or antigen-binding portion thereof, binds to the chromatography media; displacing the antibody, or antigen-binding portion thereof, bound to

the chromatography media with a displacing buffer comprising at least one displacer molecule; and collecting a chromatography sample, wherein the chromatography sample comprises a composition of the antibody, or antigen-binding portion thereof, which contains less than about 10% acidic species, thereby producing a low acidic species composition comprising an antibody, or antigen-binding portion thereof.

In one embodiment, the chromatography media is selected from the group consisting of anion exchange adsorbent material, cation exchange adsorbent material and mixed mode media. In another embodiment, the cation exchange (CEX) adsorbent material is selected from the group consisting of a CEX resin and a CEX membrane adsorber. In another embodiment, the CEX resin is Poros XS resin.

In another embodiment, the chromatography media is a mixed mode media comprising cation exchange (CEX) and hydrophobic interaction functional groups. In one embodiment, the mixed mode media is Capto MMC resin. In another embodiment, the mixed mode media is selected from the group consisting of a CEX-based mixed mode resin and a CEX-based mixed mode membrane adsorber. In another embodiment, the mixed mode media is selected from the group consisting of CaptoMMC ImpRes, Nuvia cPrime, and Toyopearl Trp-650M resins.

In one embodiment, the pH of the displacing buffer is lower than the isoelectric point of the antibody, or antigen-binding portion thereof. In another embodiment, the pH of the displacing buffer is in the range of about 6.0 to about 8.0.

In one embodiment, the displacing buffer carries positive charge and wherein the concentration of the displacer in the displacing buffer is at least about 0.1 mM. In another embodiment, the displacer is a quaternary ammonium salt and the concentration of the displacer in the displacing buffer about 0.1 mM to about 10 mM. In another embodiment, the displacer is protamine sulfate and the concentration of the protamine sulfate in the displacing buffer is about 0.1 mM to 5 about mM.

In one embodiment, the conductivity of the displacing buffer is about 2 mS/cm to about 20 mS/cm. In another embodiment, the chromatography media is in a column, wherein the column length is in the range of about 10 cm to about 30 cm, and wherein flow residence time is in the range of about 5 min to about 25 min.

In one embodiment, one displacing buffer is used.

In another embodiment, a first displacing buffer and a second displacing buffer are used, and wherein the first and second displacing buffers comprise different concentrations of

displacer. In one embodiment, the first displacing buffer comprises a lower displacer concentration of displacer than the second displacing buffer. In one embodiment, the first displacing buffer comprises about 0.5 mM Expell SP1™. In another embodiment, the first displacing buffer comprises about 0.25 mM protamine sulfate.

In one embodiment, the method is run in a two-step displacement chromatography mode.

In another embodiment, the method is run in a multiple-step displacement chromatography mode or a linear displacement chromatography mode.

In one embodiment, the chromatography sample comprises a reduced level of host cell proteins as compared to the first sample. In another embodiment, the chromatography sample comprises a reduced level of one or more of charge variants, structure variants or fragmentation variants as compared to the first sample. In one embodiment, the chromatography sample comprises a reduced level of acidic species region AR1, and wherein the charge variants comprise deamidation variants, glycation variants, afucosylation variants, MGO variants or citric acid variants. In another embodiment, the chromatography sample comprises a reduced level of the acidic species region AR1, and wherein the structure variants comprise glycosylation variants or acetonation variants. In one embodiment, the chromatography sample comprises a reduced level of the acidic species region AR1, and wherein the fragmentation variants comprise Fab fragment variants, C-terminal truncation variants or variants missing a heavy chain variable domain. In another embodiment, the chromatography sample comprises a reduced level of the acidic species region AR2, and wherein the charge variants comprise deamidation variants or glycation variants.

In another embodiment, the chromatography sample comprises a reduced level of basic species as compared to the first sample. In one embodiment, the reduced level of basic species comprise a reduced level of a lysine species Lys 0 as compared to the first sample.

In another embodiment, the chromatography sample comprises a reduced level of aggregates as compared to the first sample. In another embodiment, the chromatography sample comprises a reduced level of antibody fragments as compared to the first sample.

In one embodiment, the antibody, or antigen-binding portion thereof, is an anti-TNFα antibody, or antigen-binding portion thereof. In one embodiment, the antibody, or antigen-binding portion thereof, has dissociates from human TNFα with a K_d of about 1 x 10^{-8} M or less and a K_{off} rate constant of 1x10^{-3} S^{-1} or less. In another embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, comprises a light chain variable region (LCVR)

having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3; and a heavy chain variable region (HCVR) having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4. In another embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, comprises a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 1 and a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 2. In another embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, is adalimumab, or an antigen binding-portion thereof.

In another aspect, the present invention provides a low acidic species (low AR) composition comprising an antibody, or antigen-binding portion thereof, where the composition comprises about 15% or less AR. In one aspect of this embodiment, the low AR composition comprises about 14% or less AR, 13% or less AR, 12% or less AR, 11% or less AR, 10% or less AR, 9% or less AR, 8% or less AR, 7% or less AR, 6% or less AR, 5% or less AR, 4.5% or less AR, 4% or less AR, 3.5% or less AR, 3% or less AR, 2.5% or less AR, 2% or less AR, 1.9% or less AR, 1.8% or less AR, 1.7% or less AR, 1.6% or less AR, 1.5% or less AR, 1.4% or less AR, 1.3% or less AR, 1.2% or less AR, 1.1% or less AR, 1% or less AR, 0.9% or less AR, 0.8% or less AR, 0.7% or less AR, 0.6% or less AR, 0.5% or less AR, 0.4% or less AR, 0.3% or less AR, 0.2% or less AR, 0.1% or less AR, or 0.0% AR, and ranges within one or more of the preceding. In one aspect of this embodiment, the present invention provides a low AR composition comprising an antibody, or antigen-binding portion thereof, where the composition comprises about 0.0% to about 10% AR, about 0.0% to about 5% AR, about 0.0% to about 4% AR, about 0.0% to about 3% AR, about 0.0% to about 2% AR, about 3% to about 5% AR, about 5% to about 8% AR, or about 8% to about 10% AR, or about 10% to about 15% AR, and ranges within one or more of the preceding.

In one embodiment, the low AR composition comprises a first acidic species region (AR1) and a second acidic species region (AR2). In one aspect of this embodiment, the low AR composition comprises about 0.1% or less AR1 and about 3% or less AR2, or about 0.0% AR1 and about 1.4% or less AR2. In a related embodiment, the low AR composition comprises about 15% or less AR1, 14% or less AR1, 13% or less AR1, 12% or less AR1, 11% or less AR1, 10% or less AR1, 9% or less AR1, 8% or less AR1, 7% or less AR1, 6% or

less AR1, 5% or less AR1, 4.5% or less AR1, 4% or less AR1, 3.5% or less AR1, 3% or less AR1, 2.5% or less AR1, 2% or less AR1, 1.9% or less AR1, 1.8% or less AR1, 1.7% or less AR1, 1.6% or less AR1, 1.5% or less AR1, 1.4% or less AR1, 1.3% or less AR1, 1.2% or less AR1, 1.1% or less AR1, 1% or less AR1, 0.9% or less AR1, 0.8% or less AR1, 0.7% or less AR1, 0.6% or less AR1, 0.5% or less AR1, 0.4% or less AR1, 0.3% or less AR1, 0.2% or less AR1, 0.1% or less AR1, or 0.0% AR1, and ranges within one or more of the preceding. In one aspect of this embodiment, the present invention provides a low AR composition comprising an antibody, or antigen-binding portion thereof, where the composition comprises about 0.0% to about 10% AR1, about 0.0% to about 5% AR1, about 0.0% to about 4% AR1, about 0.0% to about 3% AR1, about 0.0% to about 2% AR1, about 3% to about 5% AR1, about 5% to about 8% AR1, or about 8% to about 10% AR1, or about 10% to about 15% AR1, and ranges within one or more of the preceding.

In one aspect of this embodiment, the low AR composition comprises about 15% or less 3.5% or less AR2, 14% or less AR2, 13% or less AR2, 12% or less AR2, 11% or less AR2, 10% or less AR2, 9% or less AR2, 8% or less AR2, 7% or less AR2, 6% or less AR2, 5% or less AR2, 4.5% or less AR2, 4% or less AR2, 3.5% or less AR2, 3% or less AR2, 2.5% or less AR2, 2% or less AR2,about 1.9%, 1.8%, 1.7%, 1.6%, 1.5%, 1.4%, 1.3%, 1.2%, 1.1%, 1.9% or less AR2, 1.8% or less AR2, 1.7% or less AR2, 1.6% or less AR2, 1.5% or less AR2, 1.4% or less AR2, 1.3% or less AR2, 1.2% or less AR2, 1.1% or less AR2, 1% or less AR2, 0.9% or less AR2, 0.8% or less AR2, 0.7% or less AR2, 0.6% or less AR2, 0.5% or less AR2, 0.4% or less AR2, 0.3% or less AR2, 0.2% or less AR2, 0.1% or less AR2, or 0.0% AR2, and ranges within one or more of the preceding. In one aspect of this embodiment, the present invention provides a low AR composition comprising an antibody, or antigen-binding portion thereof, where the composition comprises about 0.0% to about 10% AR2, about 0.0% to about 5% AR2, about 0.0% to about 4% AR2, about 0.0% to about 3% AR2, about 0.0% to about 2% AR2, about 3% to about 5% AR2, about 5% to about 8% AR2, or about 8% to about 10% AR2, or about 10% to about 15% AR2, and ranges within one or more of the preceding0%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2% or 0.1% AR2. In another embodiment, the low AR composition comprises about 0% AR2.

In another embodiment, the low AR composition, *e.g.,* a low AR composition of adalimumab, comprises about 1.4% or less AR. For example, in one aspect of this embodiment, the low AR composition, *e.g.,* a low AR composition of adalimumab

comprising about 1.4% or less AR can comprise about 0.0% AR1 and about 1.4% or less AR2.

In one embodiment, the acidic species in the low AR composition comprise one or more variants selected from the group consisting of charge variants, structure variants, aggretation variants and fragmentation variants. For example, in one embodiment, the charge variants in the low AR composition are AR1 species and comprise, for example, deamidation variants, glycation variants, afucosylation variants, MGO variants or citric acid variants. In another embodiment, the structure variants in the low AR composition are AR1 species and comprise, for example, glycosylation variants or acetonation variants. In still another embodiment, the fragmentation variants in the low AR composition are AR1 and comprise, for example, Fab fragment variants, C-terminal truncation variants or variants missing a heavy chain variable domain.

In another embodiment, the acidic species in the low AR composition are AR2 variants, and comprise, for example, charge variants such as deamidation variants or glycation variants.

In another aspect, the present invention provides compositions comprising an antibody, or antigen-binding portion thereof, wherein the composition is substantially free of acidic species such as process-related impurities, including, for example, host cell proteins (HCPs), host cell nucleic acids, chromatographic materials, and/or media components, as well as product related impurities such as aggregates etc.

In one embodiment, the antibody, or antigen-binding portion thereof, of the compositions disclosed herein is an anti-TNFα antibody, or antigen-binding portion thereof. For example, in one aspect of this embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_d of about 1 x 10^{-8} M or less and a K_{off} rate constant of 1x10^{-3} S^{-1} or less. In another aspect of this embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, comprises a light chain variable region (LCVR) having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3; and a heavy chain variable region (HCVR) having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4.

In still another aspect of this embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, comprises a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 1 and a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 2. In yet another aspect of this embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, is adalimumab, or an antigen binding-portion thereof.

In one embodiment, the low AR composition of the invention comprises adalimumab, and has a percentage of AR that is not the same as the percentage of AR present in adalimumab formulated as HUMIRA® as currently approved and described in the "Highlights of HUMIRA® Prescribing Information" for HUMIRA® (adalimumab) Injection (Revised Jan. 2008), the contents of which are hereby incorporated herein by reference.

In another embodiment, the low AR composition of the invention comprises adalimumab, and has a percentage of AR that is lower than the percentage of AR present in adalimumab formulated as HUMIRA® as currently approved and described in the "Highlights of HUMIRA® Prescribing Information" for HUMIRA® (adalimumab) Injection (Revised Jan. 2008), the contents of which are hereby incorporated herein by reference.

In another embodiment, the present invention provides low AR compositions comprising an anti-TNFα antibody, or antigen-binding portion thereof, comprising a light chain variable region (LCVR) having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3; and a heavy chain variable region (HCVR) having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, wherein the composition comprises less than about 10% AR. In one aspect of this embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, comprises a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 1 and a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 2, wherein the composition comprises less than about 10% AR. In another aspect of this embodiment, the anti-TNFα antibody, or antigen-binding portion thereof, is adalimumab, or an antigen binding-portion thereof, and the composition comprises less than about 10% AR. In one aspect of this embodiment, the low AR composition comprising an anti-TNFα antibody, or

antigen-binding portion thereof, comprises about 0.1% or less AR1 and about 3% or less AR2, or about 0.0% AR1 and about 1.4% or less AR2.

In one embodiment, the acidic species in the low AR composition comprising an antibody, or antigen-binding portion thereof (*e.g.,* an anti-TNFα antibody, or antigen binding portion thereof, such as adalimumab) comprise one or more variants selected from the group consisting of charge variants, structure variants and fragmentation variants. For example, in one aspect of this embodiment, the charge variants in the low AR composition are AR1 species and comprise, for example, deamidation variants, glycation variants, afucosylation variants, methylglyoxal (MGO) variants or citric acid variants. For example, when the low AR composition comprises adalimumab, the deamidation variants can result from deamidation occurring at asparagine residues comprising Asn393 and Asn329 of adalimumab and at glutamine residues comprising Gln3 and Gln6. In another aspect of this embodiment, when the low AR composition comprises adalimumab, the glycation variants can result from glycation occurring at Lys98 and Lys151 of adalimumab.

In another aspect of this embodiment, the structure variants in the low AR composition comprising an antibody, or antigen-binding portion thereof (*e.g.,* an anti-TNFα antibody, or antigen binding portion thereof, such as adalimumab) are AR1 species and comprise, for example, glycosylation variants or acetonation variants.

In still another aspect of this embodiment, the fragmentation variants in the low AR composition comprising an antibody, or antigen-binding portion thereof (*e.g.,* an anti-TNFα antibody, or antigen binding portion thereof, such as adalimumab), are AR1 species and comprise, for example, Fab fragment variants, C-terminal truncation variants or variants missing a heavy chain variable domain.

In another embodiment, the acidic species in the low AR composition comprising an antibody, or antigen-binding portion thereof (*e.g.,* an anti-TNFα antibody, or antigen binding portion thereof, such as adalimumab), are AR2 species, and comprise charge variants, such as deamidation variants or glycation variants. For example, when the low AR composition comprises adalimumab, the deamidation variants can result from deamidation occurring at asparagine residues comprising Asn393 and Asn329 of adalimumab and at glutamine residues comprising Gln3 and Gln6. In another aspect of this embodiment, when the low AR composition comprises adalimumab, the glycation variants result from glycation occurring at Lys98 and Lys151 of adalimumab.

In one embodiment, the percent of acidic species in a low AR composition is determined using ion exchange chromatography, for example WCX-10 HPLC. In another aspect of this embodiment, the percent acidic species in a low AR composition is determined using isoelectric focusing (IEF).

In one embodiment, the low AR compositions of the invention comprise product preparation-derived acidic species. For example, in one aspect of this embodiment, the acidic species are cell culture-derived acidic species. In another aspect of this embodiment, the

acidic species of the low AR compositions are storage-derived acidic species which are primarily generated when stored under process, intermediate or shelf storage conditions prior to use.

In another embodiment, the reduced AR compositions of the invention can be stored 3-fold longer before reaching the same AR level as compared to a non-low acidic species composition. In another embodiment, the reduced AR compositions of the invention can be stored 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, 5-fold, 5.5-fold or 6 fold longer before reaching the same AR level as compared to a non-low acidic species composition.

In still another embodiment, the invention provides low AR compositions that further comprise a pharmaceutically acceptable carrier.

In another aspect, the present invention provides methods for treating a subject having a disorder in which TNFα is detrimental, by administering to the subject a low AR composition of the invention, *e.g.,* a low AR adalimumab composition, thereby treating the subject having a disorder in which TNFα is detrimental. In one aspect of this embodiment, the disorder in which TNFα is detrimental is selected from the group consisting of rheumatoid arthritis (RA), psoriasis, psoriatic arthritis, ankylosing spondylitis, juvenile idiopathic arthritis (JIA), ulcerative colitis, Crohn's Disease, active axial spondyloarthritis (active axSpA) and non-radiographic axial spondyloarthritis (nr-axSpA).

The present invention is further illustrated by the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts (a) comparison of a desired and an undesired displacement chromatogram for Adalimumab on Poros XS resin using Expell SP1™; (b) charge variants

distribution in eluate fractions derived from the undesired displacement chromatography process for Adalimumab (Poros XS resin & Expell SP1™).

Figure 2 depicts CEX-HPLC chromatograms of Expell SP1™-displaced Adalimumab sample fractions.

Figure 3 depicts the separation of Adalimumab charge variants by Poros XS displacement chromatography using Expell SP1™.

Figure 4 depicts the reduction of acidic species level in Adalimumab by Poros XS displacement chromatography using Expell SP1™.

Figure 5 depicts the effect of Expell SP1™ concentration on acidic species reduction in Adalimumab by Poros XS displacement chromatography.

Figure 6 depicts the effect of pH on acidic species reduction in Adalimumab by Poros XS displacement chromatography using Expell SP1™.

Figure 7 depicts the reduction of acidic species level in Adalimumab by Poros XS two-step displacement chromatography using Expell SP1™.

Figure 8 depicts the separation of Adalimumab size variants by Poros XS displacement chromatography using Expell SP1™.

Figure 9 depicts the separation of HCP in Adalimumab by Poros XS displacement chromatography using Expell SP1™.

Figure 10 depicts the separation of Adalimumab charge variants by Poros XS displacement chromatography using protamine sulfate.

Figure 11 depicts the reduction of acidic species in Adalimumab by Poros XS displacement chromatography using protamine sulfate.

Figure 12 depicts the effect of protamine sulfate concentration on acidic species reduction in Adalimumab by Poros XS displacement chromatography.

Figure 13 depicts the effect of pH on acidic species reduction in Adalimumab by Poros XS displacement chromatography using protamine sulfate.

Figure 14 depicts the reduction of acidic species in Adalimumab by Poros XS two-step displacement chromatography using protamine sulfate.

Figure 15 depicts the reduction of acidic species in Adalimumab on Poros XS using protamine sulfate linear gradient displacement chromatography.

Figure 16 depicts the separation of Adalimumab size variants by Poros XS displacement chromatography using protamine sulfate.

Figure 17 depicts the separation of mAb X charge variants by Poros XS displacement chromatography using Expell SP1™.

Figure 18 depicts the reduction of acidic species in mAb X by Poros XS displacement chromatography using Expell SP1™.

Figure 19 depicts the effect of Expell SP1™ concentration on acidic species reduction in mAb X by Poros XS displacement chromatography.

Figure 20 depicts the reduction of acidic species in mAb X by Poros XS two-step displacement chromatography using Expell SP1™.

Figure 21 depicts the separation of mAb X charge variants by Poros XS displacement chromatography using protamine sulfate.

Figure 22 depicts the reduction of acidic species in mAb X by Poros XS displacement chromatography using protamine sulfate.

Figure 23 depicts the effect of protamine sulfate concentration on acidic species reduction in mAb X by Poros XS displacement chromatography.

Figure 24 depicts the reduction of acidic species in mAb X by Poros XS two-step displacement chromatography using protamine sulfate.

Figure 25 depicts the separation of mAb X size variants by Poros XS displacement chromatography using protamine sulfate.

Figure 26 depicts the separation of mAb Y charge variants by Poros XS displacement chromatography using Expell SP1™.

Figure 27 depicts the reduction of acidic species in mAb Y by Poros XS displacement chromatography using Expell SP1™.

Figure 28 depicts the separation of Adalimumab charge variants by Capto MMC displacement chromatography using protamine sulfate.

Figure 29 depicts the reduction of acidic species in Adalimumab by Capto MMC displacement chromatography using protamine sulfate.

Figure 30 depicts the separation of mAb X charge variants by Capto MMC displacement chromatography using protamine sulfate.

Figure 31 depicts the reduction of acidic species in mAb X by Capto MMC displacement chromatography using protamine sulfate.

Figure 32 depicts the effect of pH on acidic species reduction in mAb X by Capto MMC displacement chromatography.

Figure 33 depicts the AR Growth at 25°C of low and high AR containing samples.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the identification and optimization of displacement chromatography technologies for protein production, *e.g.,* production of proteins, *e.g.,* antibodies or antigen-binding portions thereof, *e.g.,* adalimumab, resulting in compositions that comprise low percentages of protein charge variants, *e.g.,* acidic species (AR), and basic species, and/or low levels of product- and process-related impurities (*e.g.*, aggregates, fragments, host cell proteins and media components). In one embodiment, the displacement chromatography methods of the invention can surprisingly be used in large-scale protein purification processes due to superior resolution of closely related species using practically relevant chromatography resins and conditions. In one embodiment, the displacement chromatography methods of the invention can surprisingly be used in large-scale protein purification processes due to reduced buffer volume for a given separation, thereby providing improved process efficiency.

The compositions of the present invention exhibit increased therapeutic efficacy when administered to a subject. For example, compositions comprising anti-TNFα antibodies, or antigen binding portions thereof, comprising low AR are capable of increased therapeutic efficacy in the treatment and prevention of a disorder in which TNFα is detrimental, *e.g.,* rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), psoriasis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, and ulcerative colitis. Accordingly, the instant invention provides compositions comprising proteins that comprise low AR and/or low levels of product- and process-related impurities, and methods for producing and using the same.

In one embodiment, the low AR compositions of the invention comprise about 15% or less AR, 14% or less AR, 13% or less AR, 12% or less AR, 11% or less AR, 10% or less AR, 9% or less AR, 8% or less AR, 7% or less AR, 6% or less AR, 5% or less AR, 4.5% or less AR, 4% or less AR, 3.5% or less AR, 3% or less AR, 2.5% or less AR, 2% or less AR, 1.9% or less AR, 1.8% or less AR, 1.7% or less AR, 1.6% or less AR, 1.5% or less AR, 1.4% or less AR, 1.3% or less AR, 1.2% or less AR, 1.1% or less AR, 1% or less AR, 0.9% or less AR, 0.8% or less AR, 0.7% or less AR, 0.6% or less AR, 0.5% or less AR, 0.4% or less AR, 0.3% or less AR, 0.2% or less AR, 0.1% or less AR, or 0.0% AR, and ranges within one or more of the preceding. In one aspect of this embodiment, the low AR compositions of the invention comprise about 0.0% to about 10% AR, about 0.0% to about 5% AR, about 0.0% to about 4% AR, about 0.0% to about 3% AR, about 0.0% to about 2% AR, about 3% to about

5% AR, about 5% to about 8% AR, or about 8% to about 10% AR, or about 10% to about 15% AR, and ranges within one or more of the preceding.

In another embodiment, the low AR composition comprises a first acidic species region (AR1) and a second acidic species region (AR2). In one aspect of this embodiment, the low AR composition comprises about 0.1% or less AR1 and about 3% or less AR2. In another aspect of this embodiment, the low AR composition comprises about 0.0% AR1 and about 1.4% or less AR2.

In another aspect of this embodiment, the low AR composition comprises about 15% or less AR1, 14% or less AR1, 13% or less AR1, 12% or less AR1, 11% or less AR1, 10% or less AR1, 9% or less AR1, 8% or less AR1, 7% or less AR1, 6% or less AR1, 5% or less AR1, 4.5% or less AR1, 4% or less AR1, 3.5% or less AR1, 3% or less AR1, 2.5% or less AR1, 2% or less AR1, 1.9% or less AR1, 1.8% or less AR1, 1.7% or less AR1, 1.6% or less AR1, 1.5% or less AR1, 1.4% or less AR1, 1.3% or less AR1, 1.2% or less AR1, 1.1% or less AR1, 1% or less AR1, 0.9% or less AR1, 0.8% or less AR1, 0.7% or less AR1, 0.6% or less AR1, 0.5% or less AR1, 0.4% or less AR1 or less, 0.3% or less AR1 or less, 0.2% or less AR1 or less, 0.1% or less AR1, or 0.0% AR1, and ranges within one or more of the preceding. In one aspect of this embodiment, the low AR compositions of the invention comprise about 0.0% to about 10% AR1, about 0.0% to about 5% AR1, about 0.0% to about 4% AR1, about 0.0% to about 3% AR1, about 0.0% to about 2% AR1, about 3% to about 5% AR1, about 5% to about 8% AR1, or about 8% to about 10% AR1, or about 10% to about 15% AR1, and ranges within one or more of the preceding.

In yet another aspect of this embodiment, the low AR composition comprises about 15% or less AR2, 14% or less AR2, 13% or less AR2, 12% or less AR2, 11% or less AR2, 10% or less AR2, 9% or less AR2, 8% or less AR2, 7% or less AR2, 6% or less AR2, 5% or less AR2, 4.5% or less AR2, 4% or less AR2, 3.5% or less AR2, 3% or less AR2, 2.5% or less AR2, 2% or less AR2, 1.9% or less AR2, 1.8% or less AR2, 1.7% or less AR2, 1.6% or less AR2, 1.5% or less AR2, 1.4% or less AR2, 1.3% or less AR2, 1.2% or less AR2, 1.1% or less AR2, 1% or less AR2, 0.9% or less AR2, 0.8% or less AR2, 0.7% or less AR2, 0.6% or less AR2, 0.5% or less AR2, 0.4% or less AR2, 0.3% or less AR2, 0.2% or less AR2, 0.1% or less AR2, or 0.0% AR2, and ranges within one or more of the preceding. In one aspect of this embodiment, the low AR compositions of the invention comprise about 0.0% to about 10% AR2, about 0.0% to about 5% AR2, about 0.0% to about 4% AR2, about 0.0% to about 3% AR2, about 0.0% to about 2% AR2, about 3% to about 5% AR2, about 5% to about 8%

AR2, or about 8% to about 10% AR2, or about 10% to about 15% AR2, and ranges within one or more of the preceding.

In another embodiment, the low AR composition, *e.g.,* a low AR composition of adalimumab, comprises about 1.4% or less AR. For example, in one aspect of this embodiment, the low AR composition, *e.g.,* a low AR composition of adalimumab comprising about 1.4% or less AR comprises about 0.0% AR1 and about 1.4% or less AR2.

In one embodiment, the protein is an antibody or antigen binding portion thereof, such as the adalimumab antibody, or an antigen binding portion thereof.

I. Definitions

In order that the present invention may be more readily understood, certain terms are first defined.

As used herein, the terms "acidic species", "acidic region", and "AR," refer to the variants of a protein, *e.g.,* an antibody or antigen-binding portion thereof, which are characterized by an overall acidic charge. For example, in monoclonal antibody (mAb) preparations, such acidic species can be detected by various methods, such as ion exchange, for example, WCX-10 HPLC (a weak cation exchange chromatography), or IEF (isoelectric focusing). Acidic species of an antibody may include charge variants, structure variants, and/or fragmentation variants. Exemplary charge variants include, but are not limited to, deamidation variants, afucosylation variants, methylglyoxal (MGO) variants, glycation variants, and citric acid variants. Exemplary structure variants include, but are not limited to, glycosylation variants and acetonation variants. Exemplary fragmentation variants include any truncated protein species from the target molecule due to dissociation of peptide chain, enzymatic and/or chemical modifications, including, but not limited to, Fc and Fab fragments, fragments missing a Fab, fragments missing a heavy chain variable domain, C-terminal truncation variants, variants with excision of N-terminal Asp in the light chain, and variants having N-terminal truncation of the light chain. Other acidic species variants include variants containing unpaired disulfides, host cell proteins, and host cell nucleic acids, chromatographic materials, and media components.

In certain embodiments, a protein composition can comprise more than one type of acidic species variant. For example, but not by way of limitation, the total acidic species can be divided based on chromatographic retention time of the peaks appearing, for example, in a WCX-10 Weak Cation Exchange HPLC of the protein preparation. Figure 2 depicts a non-

limiting example of such a division wherein the total acidic species associated with the expression of adalimumab is divided into a first acidic species region (AR1) and a second acidic species region (AR2).

AR1 can comprise, for example, charge variants such as deamidation variants, MGO modified species, glycation variants, and citric acid variants, structural variants such as glycosylation variants and acetonation variants, and/or fragmentation variants. In another embodiment, AR2 can comprise, for example, charge variants such as glycation variants and deamidation variants.

With respect, in particular, to adalimumab (and antibodies sharing certain structural characteristics of adalimumab, *e.g.,* one or more CDR and/or heavy and light chain variable regions of adalimumab), AR1 charge variants can comprise, but are not limited to, deamidation variants, glycation variants, afucosylation variants, MGO variants or citric acid variants. In one embodiment, deamidation variants result from deamidation occurring at asparagine residues comprising Asn393 and Asn329 and at glutamine residues comprising Gln3 and Gln6. In another embodiment, the glycation variants result from glycation occurring at Lys98 and Lys151. AR1 structure variants can comprise, but are not limited to, glycosylation variants or acetonation variants.

AR1 fragmentation variants can comprise Fc and Fab fragments, fragments missing a Fab, fragments missing a heavy chain variable domain, C-terminal truncation variants, variants with excision of N-terminal Asp in the light chain, and variants having N-terminal truncation of the light chain.

AR2 charge variants can comprise, but are not limited to, deamidation variants or glycation variants, wherein the deamidation variants can result from deamidation occurring at asparagine residues comprising Asn393 and Asn329 and at glutamine residues comprising Gln3 and Gln6, and the glycation variants can result from glycation occurring at Lys98 and Lys151.

The term "acidic species" does not include process-related impurities. The term "process-related impurity," as used herein, refers to impurities that are present in a composition comprising a protein but are not derived from the protein itself. Process-related impurities include, but are not limited to, host cell proteins (HCPs), host cell nucleic acids, chromatographic materials, and media components. A "low process-related impurity composition," as used herein, refers to a composition comprising reduced levels of process-related impurities as compared to a composition wherein the impurities were not reduced.

For example, a low process-related impurity composition may contain about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or less of process-related impurities. In one embodiment, a low process-related impurity composition is free of process-related impurities or is substantially free of process-related impurities.

The acidic species may be the result of product preparation (referred to herein as "preparation-derived acidic species"), or the result of storage (referred to herein as "storage-derived acidic species"). Preparation-derived acidic species are acidic species that are formed during the preparation (upstream and/or downstream processing) of the protein, *e.g.,* the antibody or antigen-binding portion thereof. For example, preparation-derived acidic species can be formed during cell culture ("cell culture-derived acidic species"). Storage-derived acidic species are acidic species that may or may not be present in the population of proteins directly after preparation, but are formed or generated while the sample is being stored. The type and amount of storage-derived acidic species can vary based on the formulation of the sample. Formation of storage-derived acidic species can be partially or completely inhibited when the preparation is stored under particular conditions. For example, an aqueous formulation can be stored at a particular temperature to partially or completely inhibit AR formation. For example, formation or storage-derived AR can be partially inhibited in an aqueous formulation stored at between about 2°C and 8°C, and completely inhibited when stored at -80°C. In addition, a low AR composition can be lyophilized or freeze-dried to partially or completely inhibit the formation of storage-derived AR.

The term "low acidic species composition," or "low AR composition," as used herein, refers to a composition comprising an antibody or antigen-binding portion thereof, wherein the composition contains less than about 15% acidic species. As used herein, the percent AR in the low AR composition refers to the weight of the acidic species in a sample in relation to the weight of the total antibodies contained in the sample. For example, the percent AR can be calculated using weak cation exchange chromatography such as WCX-10, as described herein and also in Example 1 of U.S. Provisional Patent Application 61/893,068, entitled "Low Acidic Species Compositions and Methods for Producing the Same", Attorney Docket Number 117813-73901, filed on October 18, 2013, the entire contents of which are expressly incorporated herein by reference.

In one embodiment, a low AR composition of the invention may comprise about 15% or less AR, 14% or less AR, 13% or less AR, 12% or less AR, 11% or less AR, 10% or less AR, 9% or less AR, 8% or less AR, 7% or less AR, 6% or less AR, 5% or less AR, 4.5% or

less AR, 4% or less AR, 3.5% or less AR, 3% or less AR, 2.5% or less AR, 2% or less AR, 1.9% or less AR, 1.8% or less AR, 1.7% or less AR, 1.6% or less AR, 1.5% or less AR, 1.4% or less AR, 1.3% or less AR, 1.2% or less AR, 1.1% or less AR, 1% or less AR, 0.9% or less AR, 0.8% or less AR, 0.7% or less AR, 0.6% or less AR, 0.5% or less AR, 0.4% or less AR, 0.3% or less AR, 0.2% or less AR, 0.1% or less AR, or 0.0% AR, and ranges within one or more of the preceding. A low AR composition of the invention may also comprise about 0.0% to about 10% AR, about 0.0% to about 5% AR, about 0.0% to about 4% AR, about 0.0% to about 3% AR, about 0.0% to about 2% AR, about 3% to about 5% AR, about 5% to about 8% AR, or about 8% to about 10% AR, or about 10% to about 15% AR, and ranges within one or more of the preceding.

A low AR composition of the invention may comprise about 15% or less AR1, 14% or less AR1, 13% or less AR1, 12% or less AR1, 11% or less AR1, 10% or less AR1, 9% or less AR1, 8% or less AR1, 7% or less AR1, 6% or less AR1, 5% or less AR1, 4.5% or less AR1, 4% or less AR1, 3.5% or less AR1, 3% or less AR1, 2.5% or less AR1, 2% or less AR1, 1.9% or less AR1, 1.8% or less AR1, 1.7% or less AR1, 1.6% or less AR1, 1.5% or less AR1, 1.4% or less AR1, 1.3% or less AR1, 1.2% or less AR1, 1.1% or less AR1, 1% or less AR1, 0.9% or less AR1, 0.8% or less AR1, 0.7% or less AR1, 0.6% or less AR1, 0.5% or less AR1, 0.4% or less AR1, 0.3% or less AR1, 0.2% or less AR1, 0.1% or less AR1, or 0.0% AR1, and ranges within one or more of the preceding. A low AR composition of the invention may also comprise about 0.0% to about 10% AR1, about 0.0% to about 5% AR1, about 0.0% to about 4% AR1, about 0.0% to about 3% AR1, about 0.0% to about 2% AR1, about 3% to about 5% AR1, about 5% to about 8% AR1, or about 8% to about 10% AR1, or about 10% to about 15% AR1, and ranges within one or more of the preceding.

A low AR composition of the invention may also comprise about 15% or less AR2, 14% or less AR2, 13% or less AR2, 12% or less AR2, 11% or less AR2, 10% or less AR2, 9% or less AR2, 8% or less AR2, 7% or less AR2, 6% or less AR2, 5% or less AR2, 4.5% or less AR2, 4% or less AR2, 3.5% or less AR2, 3% or less AR2, 2.5% or less AR2, 2% or less AR2, 1.9% or less AR2, 1.8% or less AR2, 1.7% or less AR2, 1.6% or less AR2, 1.5% or less AR2, 1.4% or less AR2, 1.3% or less AR2, 1.2% or less AR2, 1.1% or less AR2, 1% or less AR2, 0.9% or less AR2, 0.8% or less AR2, 0.7% or less AR2, 0.6% or less AR2, 0.5% or less AR2, 0.4% or less AR2, 0.3% or less AR2, 0.2% or less AR2, 0.1% or less AR2, or 0.0% AR2, and ranges within one or more of the preceding. A low AR composition of the invention may also comprise about 0.0% to about 10% AR2, about 0.0% to about 5% AR2,

about 0.0% to about 4% AR2, about 0.0% to about 3% AR2, about 0.0% to about 2% AR2, about 3% to about 5% AR2, about 5% to about 8% AR2, or about 8% to about 10% AR2, or about 10% to about 15% AR2, and ranges within one or more of the preceding.

In one embodiment, a low AR composition comprises between about 0.0% and about 3% AR1. In another embodiment, a low AR composition comprises about between about 0.0% and about 3% AR2. In a preferred embodiment, a low acidic species composition comprises about 3% or less AR2.

In another embodiment, the low AR composition comprises about 1.4% or less AR. For example, in one embodiment, the composition comprises about 1.4% AR2 and about 0.0% AR1.

In one embodiment, a low AR composition of the invention may comprise about 15% or less, 14% or less, 13% or less, 12% or less, 11% or less, 10% or less, 9% or less, 8% or less, 7% or less, 6% or less, 5% or less, 4.5% or less, 4% or less, 3.5% or less, 3% or less, 2.5% or less, 2% or less, 1.9% or less, 1.8% or less, 1.7% or less, 1.6% or less, 1.5% or less, 1.4% or less, 1.3% or less, 1.2% or less, 1.1% or less, 1% or less, 0.9% or less, 0.8% or less, 0.7% or less, 0.6% or less, 0.5% or less, 0.4% or less, 0.3% or less, 0.2% or less, 0.1% or less, or 0.0% of one or more of a deamidation variant, an afucosylation variant, an MGO variant, a glycation variant, a citric acid variant, a glycosylation variant, an acetonation variant, or a fragmentation variant, and ranges within one or more of the preceding. In one aspect of this embodiment, a low AR composition of the invention may also comprise about 0.0% to about 10%, about 0.0% to about 5%, about 0.0% to about 4%, about 0.0% to about 3%, about 0.0% to about 2% , about 3% to about 5%, about 5% to about 8%, or about 8% to about 10%, or about 10% to about 15%, of one or more of a deamidation variant, an afucosylation variant, an MGO variant, a glycation variant, a citric acid variant, a glycosylation variant, an acetonation variant, or a fragmentation variant, and ranges within one or more of the preceding. For example, a low AR composition of the invention may comprise less than 15% of a deamidation variant, while each of the other acidic variants, alone or in combination, are at a percentage that is greater than 15%.

The term "non-low acidic species composition," as used herein, refers to a composition comprising an antibody or antigen-binding portion thereof, which contains more than about 13% acidic species. For example, a non-low acidic species composition may contain about 16% or more, 17% or more, 18% or more, 19% or more, 20% or more, 21% or more, 22% or more, 23% or more, 24% or more, or 25% or more acidic species. In one

embodiment, a non-low acidic species composition can comprise about 5% or more, 6% or more, 7% or more, 8% or more, 9% or more, 10% or more, 11% or more, 12% or more, 13% or more, 14% or more, 15% or more, 16% or more, 17% or more, 18% or more, 19% or more, 20% or more, 21% or more, 22% or more, 23% or more, 24% or more, or 25% or more of AR1. In another embodiment, a non-low acidic species composition can comprise about 10% or more, 11% or more, 12% or more, 13% or more, 14% or more, 15% or more, 16% or more, 17% or more, 18% or more, 19% or more, 20% or more, 21% or more, 22% or more, 23% or more, 24% or more, or 25% or more of AR2, and ranges within one or more of the preceding.

In one embodiment, a low AR composition has improved biological and functional properties, including increased efficacy in the treatment or prevention of a disorder in a subject, *e.g.,* a disorder in which TNFα activity is detrimental, as compared to a non-low acidic species composition. In one embodiment, the low AR composition comprises an anti-TNFα antibody, or antigen-binding portion thereof, such as adalimumab or a fragment thereof. For example, in one embodiment, a low AR composition comprising an antibody, or antigen-binding portion thereof, exhibits increased cartilage penetration, decreased bone erosion, and/or reduced cartilage destruction, as compared to a non-low acidic species composition comprising the same antibody or antigen binding portion thereof, when administered to a subject suffering from a disorder in which TNFα activity is detrimental.

As used herein, the term "increased cartilage penetration" refers to increased penetration of cartilage *in vivo* by a low AR composition as compared to a non-low AR composition comprising the same antibody or antigen binding portion thereof.

As used herein, the term "reduced cartilage destruction" refers to measurable decrease in destruction of cartilage tissue *in vivo* by a low AR composition as compared to a non-low AR composition comprising the same antibody or antigen binding portion thereof. As used herein, the term "decreased bone erosion" refers to measurable decrease, *in vivo*, of the erosion of bone tissue by a low AR composition as compared to a non-low acidic species composition comprising the same antibody or antigen binding portion thereof. For example, an *in vivo* model of a disease or disorder in which TNFα activity is detrimental, *e.g.,* a mouse model of arthritis, can be used to measure cartilage penetration, bone erosion, and/or cartilage destruction by a composition comprising an anti-TNFα antibody or antigen binding portion thereof. One non-limiting example of an art-recognized mouse model of arthritis is the human TNF transgenic 197 mouse model of arthritis (TNF-Tg197) (see Keffer, J. *et al.*,

EMBO J (1991) 10:4025-4031, the contents of which are expressly incorporated herein by reference, for further description of the TNF-Tg197 model of arthritis).

In another embodiment, a low AR composition comprising an antibody, or antigen-binding portion thereof, exhibits increased protection against the development of arthritis, as measured by arthritic scores, and/or histopathology scores as compared to a non-low acidic species composition when administered to an animal model of arthritis, *e.g.,* the TNF-Tg197 model of arthritis. As used herein, "arthritic scores" refer to signs and symptoms of arthritis in an animal model of arthritis. As used herein, "histopathology scores" refer to radiologic damage involving cartilage and bone as well as local inflammation.

In another embodiment, a low AR composition comprising an antibody, or antigen-binding portion thereof, exhibits reduced synovial proliferation, reduced cell infiltration, reduced chondrocyte death, and/or reduced proteoglycan loss as compared to a non-low acidic species composition. In another embodiment, a low AR composition comprising an anti-TNFα antibody, or antigen-binding portion thereof, exhibits increased TNFα affinity as compared to a non-low acidic species composition.

As used herein, the term "a disorder in which TNFα activity is detrimental" is intended to include diseases and other disorders in which the presence of TNFα in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder. Accordingly, a disorder in which TNFα activity is detrimental is a disorder in which inhibition of TNFα activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNFα in serum, plasma, or synovial fluid of the subject), which can be detected, for example, using an anti-TNFα antibody as described above. There are numerous examples of disorders in which TNFα activity is detrimental. In one embodiment, the disorder in which TNFα activity is detrimental is an autoimmune disorder. In one embodiment, the autoimmune disorder is selected from the group consisting of rheumatoid arthritis, juvenile idiopathic arthritis, rheumatoid spondylitis, ankylosing spondylitis, psoriasis, osteoarthritis, gouty arthritis, an allergy, multiple sclerosis, psoriatic arthritis, autoimmune diabetes, autoimmune uveitis, nephrotic syndrome, juvenile rheumatoid arthritis, Crohn's disease and ulcerative colitis. Disorders in which TNFα activity is detrimental are set forth in U.S. Patent No. 6,090,382 and also in the "Highlights of

HUMIRA® Prescribing Information" for HUMIRA® (adalimumab) Injection (Revised Jan. 2008) the entire contents of which are hereby incorporated herein by reference. The use of TNFα antibodies and antibody portions obtained using methods of the invention for the treatment of specific disorders is discussed in further detail below.

The term "antibody" includes an immunoglobulin molecule comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region (CH). The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). 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, FR4.

The term "antigen-binding portion" of an antibody (or "antibody portion") includes fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, in the case of adalimumab, hTNFα). 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 comprising the VL, VH, CL and CH1 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 comprising the VH and CH1 domains; (iv) a Fv fragment comprising the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) Nature 341:544-546, the entire teaching of which is incorporated herein by reference), which comprises 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, the entire teachings of which are incorporated

herein by reference). 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, the entire teachings of which are incorporated herein by reference). Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecule, formed by covalent or non-covalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S. M., *et al.* (1995) Human Antibodies and Hybridomas 6:93-101, the entire teaching of which is incorporated herein by reference) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S. M., *et al.* (1994) Mol. Immunol. 31:1047-1058, the entire teaching of which is incorporated herein by reference). Antibody portions, such as Fab and F(ab')2 fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein. In one aspect, the antigen binding portions are complete domains or pairs of complete domains.

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, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, the entire teachings of which are incorporated herein by reference). 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.

The term "human antibody" includes antibodies having variable and constant regions corresponding to human germline immunoglobulin sequences as described by Kabat *et al.* (See 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). 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*), *e.g.*, in the CDRs and in particular CDR3. The mutations can be introduced using the "selective mutagenesis approach." The human antibody can have at least one position replaced with an amino acid residue, *e.g.*, an activity enhancing amino acid residue which is not encoded by the human germline immunoglobulin sequence. The human antibody can have up to twenty positions replaced with amino acid residues which are not part of the human germline immunoglobulin sequence. In other embodiments, up to ten, up to five, up to three or up to two positions are replaced. In one embodiment, these replacements are within the CDR regions. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The phrase "recombinant human antibody" includes 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, the entire teaching of which is incorporated herein by reference) 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 (see, Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). 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*. In certain embodiments, however, such recombinant antibodies are the result of selective mutagenesis approach or back-mutation or both.

An "isolated antibody" includes an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNFα is substantially free of antibodies that specifically bind antigens other than hTNFα). An isolated antibody that specifically binds hTNFα may bind TNFα molecules from other species. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. A suitable anti-TNFα antibody is adalimumab.

As used herein, the term "adalimumab," also known by its trade name HUMIRA® (AbbVie) refers to a human IgG_1 antibody that binds human tumor necrosis factor α (TNFα). In general, the heavy chain constant domain 2 (CH2) of the adalimumab IgG-Fc region is glycosylated through covalent attachment of oligosaccharide at asparagine 297 (Asn-297). The HUMIRA® Prescribing Information, the contents of each of which are expressly incorporated by reference herein. The light chain variable region of adalimumab is provided herein as SEQ ID NO:1, and the heavy chain variable region of adalimumab is provided herein as SEQ ID NO:2. The light chain of adalimumab is provided herein as SEQ ID NO:11, and the heavy chain of adalimumab is provided herein as SEQ ID NO:12. Adalimumab comprises a light chain variable region comprising a CDR1 of SEQ ID NO:7, a CDR2 of SEQ ID NO:5, and a CDR3 of SEQ ID NO:3. Adalimumab comprises a heavy chain variable region comprising a CDR1 of SEQ ID NO:8, a CDR2 of SEQ ID NO:6 and CDR3 of SEQ ID NO:4. Adalimumab is described in U.S. Patent Nos. 6,090,382; 6,258,562; 6,509,015; 7,223,394; 7,541,031; 7,588,761; 7,863,426; 7,919,264; 8,197,813; 8,206,714; 8,216,583; 8,420,081; 8,092,998; 8,093,045; 8,187,836; 8,372,400; 8,034,906; 8,436,149; 8,231,876; 8,414,894; 8,372,401, and PCT Publication No. WO2012/065072, the entire contents of each which are expressly incorporated herein by reference in their entireties. Adalimumab is also described in the "Highlights of Prescribing Information" for HUMIRA® (adalimumab) Injection (Revised Jan. 2008) the contents of which are hereby incorporated herein by reference.

In one embodiment, adalimumab dissociates from human TNFα with a Kd of $1x10^{-8}$ M or less and a K_{off} rate constant of $1x10^{-3}$ s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with

an IC50 of 1x10- M or less. In another embodiment, adalimumab dissociates from human TNFα with a K_{off} of $5x10^{-4}$ s^{-1} or less, or with a K_{off} of $1x10^{-4}$ s^{-1} or less. In still another embodiment, adalimumab neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC50 of $1x10^{-8}$ M or less, an IC50 of $1x10^{-9}$ M or less or an IC50 of $1x10^{-10}$ M or less.

Analysis of adalimumab has shown that it has three main basic variants (*i.e.,* Lys 0, Lys 1, and Lys 2), referred to herein as "lysine variant species." As used herein, the term "lysine variant species" refers to an antibody, or antigen-binding portion thereof, comprising heavy chains with either zero, one or two C-terminal lysines. For example, the "Lys 0" variant comprises an antibody, or antigen-binding portion thereof, with heavy chains that do not comprise a C-terminal lysine. The "Lys 1" variant comprises an antibody, or antigen-binding portion thereof, with one heavy chain that comprises a C-terminal lysine. The "Lys 2" variant comprises an antibody with both heavy chains comprising a C-terminal lysine. Lysine variant species can be detected by various methods, such as ion exchange, for example, by weak cation exchange chromatography (such as WCX-10) of the expression product of a host cell expressing the antibody, or antigen-binding portion thereof. For example, but not by way of limitation, Figure 2 depicts analysis of adalimumab wherein the three lysine variants, as well as the two acidic species regions, are resolved from each other.

A composition of the invention may comprise more than one lysine variant species of an antibody, or antigen-binding portion thereof. For example, in one embodiment, the composition may comprise a Lys 2 variant of an antibody, or antigen-binding portion thereof. The composition may comprise a Lys 1 variant of an antibody, or antigen-binding portion thereof. The composition may comprise a Lys 0 variant of an antibody, or antigen-binding portion thereof. In another embodiment, the composition may comprise both Lys 1 and Lys 2, or Lys 1 and Lys 0, or Lys 2 and Lys 0 variants of an antibody, or antigen-binding portion thereof. In another embodiment, the composition may comprise all three lysine variant species, *i.e.*, Lys 0, Lys 1 and Lys 2, of an antibody, or antigen-binding portion thereof.

In one embodiment, the invention comprises a composition comprising an antibody, or antigen-binding portion thereof, wherein the composition comprises less than about 50% lysine variant species that lack a C-terminal lysine (Lys 0). In another embodiment, the composition comprises less than about 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11% 10%, 9%,

8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% lysine variant species that lack a C-terminal lysine ("Lys 0"). In another embodiment, the composition comprises about 50% to about 0%, about 40% to about 10%, about 30% to about 20%, about 40% to about 20%, or about 30% to about 15% lysine variant species that lack a C-terminal lysine (Lys 0). In one embodiment, the composition comprises 0% lysine variant species that lack a C-terminal lysine (Lys 0). As used herein, the percent lysine variant species in the composition refers to the weight of the specific lysine variant species in a sample in relation to the weight of the total lysine variant species sum (*i.e.,* the sum of Lys 0, Lys 1 and Lys 2) contained in the sample or composition. For example, the percent lysine variant species can be calculated using weak cation exchange chromatography such as WCX-10, as described herein.

In another embodiment, the composition comprises less than about 25% lysine variant species that have one C-terminal lysine (Lys 1). In another embodiment, the composition comprises less than about 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11% 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% lysine variant species that have one C-terminal lysine (Lys 1). In another embodiment, the composition comprises about 25% to about 0%, about 20% to about 5%, about 15% to about 10%, about 20% to about 10%, about 15% to about 5%, or about about 25% to about 5% lysine variant species that have one C-terminal lysine (Lys 1). In one embodiment, the composition comprises 0% lysine variant species that have one C-terminal Lysine (Lys 1).

In another embodiment, the composition comprises at least about 70% lysine variant species that have two C-terminal lysines (Lys 2). In another embodiment, the composition comprises at least about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% lysine variant species that have two C-terminal lysines (Lys 2). In one embodiment, the composition comprises about 70% to about 100%, about 70% to about 90%, about 70% to about 80%, about 80% to about 100%, about 85% to about 100%, about 90% to about 100%, about 95% to about 100%, about 80% to about 90%, about 85% to about 95%, about 75% to about 85%, or about 97% to about 100% lysine variant species that have two C-terminal lysines (Lys 2). In one embodiment, the composition comprises 100% lysine variant species that have two C-terminal lysines (Lys 2).

As used herein, the term "upstream process technology," in the context of protein, *e.g.,* antibody, preparation, refers to activities involving the production and collection of proteins (*e.g.* antibodies) from cells (*e.g.*, during cell culture of a protein of interest). As used

herein, the term "cell culture" refers to methods for generating and maintaining a population of host cells capable of producing a recombinant protein of interest, as well as the methods and techniques for optimizing the production and collection of the protein of interest. For example, once an expression vector has been incorporated into an appropriate host, the host can be maintained under conditions suitable for expression of the relevant nucleotide coding sequences, and the collection and purification of the desired recombinant protein.

When using the cell culture techniques of the instant invention, the protein of interest can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. In embodiments where the protein of interest is produced intracellularly, the particulate debris, either host cells or lysed cells (*e.g.*, resulting from homogenization) can be removed by a variety of means, including but not limited to, centrifugation or ultrafiltration. Where the protein of interest is secreted into the medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter, *e.g.*, an Amicon™ or Millipore Pellicon™ ultrafiltration unit.

As used herein, the term "downstream process technology" refers to one or more techniques used after the upstream process technologies to purify the protein, *e.g.,* antibody, of interest. For example, downstream process technology includes purification of the protein product, using, for example, displacement chromatography, affinity chromatography, including Protein A affinity chromatography, ion exchange chromatography, such as anion or cation exchange chromatography, hydrophobic interaction chromatography, or displacement chromatography.

The phrase "isolated nucleic acid molecule," as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3), *e.g.*, those that bind hTNFα, includes a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNFα, which other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, *e.g.*, an isolated nucleic acid of the invention encoding a VH region of an anti-TNFα antibody contains no other sequences encoding other VH regions that bind antigens other than, for example, hTNFα. The phrase "isolated nucleic acid molecule" is also intended to include sequences encoding bivalent, bispecific antibodies, such as diabodies in which VH and VL regions contain no other sequences other than the sequences of the diabody.

The phrase "recombinant host cell" (or simply "host cell") includes a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to 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.

As used herein, the term “recombinant protein” refers to a protein produced as the result of the transcription and translation of a gene carried on a recombinant expression vector that has been introduced into a host cell. In certain embodiments the recombinant protein is an antibody, *e.g.,* a chimeric, humanized, or fully human antibody. In certain embodiments the recombinant protein is an antibody of an isotype selected from group consisting of: IgG (*e.g.*, IgG1, IgG2, IgG3, IgG4), IgM, IgA1, IgA2, IgD, or IgE. In certain embodiments the antibody molecule is a full-length antibody (*e.g.*, an IgG1 or IgG4 immunoglobulin) or alternatively the antibody can be a fragment (*e.g.*, an Fc fragment or a Fab fragment). In one embodiment, the antibody has an isoelectric point (pI) of 8.0, 8.1, 8.2, 8.25, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9 or 9.0. In one embodment, the antibody has an isoelectric point of 8.25. In another embodiment, the antibody has an isoelectric point of 8.0-8.5, 8.5-9.0, 8.0-8.3, 8.1-8.3 or 8.2-8.3.

The term “preparative scale,” as used herein, refers to a scale of purification operation that can be readily scaled-up and implemented at large scale manufacturing while still providing desired separation. For instance, one skilled in the field may develop a process using, *e.g.*, a 0.5 cm (i.d.) x 20 cm (L) column in the lab, and transfer it to large scale production using, *e.g.*, a 30 cm (i.d.) x 20 cm (L) column packed with the same resin and operated with the same set of buffers, same linear flow rates (or residence times) and buffer volumes. In preparative scale separation, column bed height is typically $\leq$ about 30 cm and column pressure drop $\leq$ about 5 bar.

The phrase “displacing buffer”, “displacer buffer”, “displacing wash buffer”, or “displacer wash buffer”, as used herein, refers to a buffer that comprises a displacer molecule. The phrase “displacer molecule”, as used herein, refers to a molecule employed to displace from the chromatographic support components of the mixture to be separated. Selection of a particular displacer molecule will, therefore, be dependent on the chromatographic support employed as well as the protein system. Regardless of which chromatographic support is employed, displacer molecules will generally be selected such

that they have a high affinity for the support. However, in certain embodiments, a displacer molecule may be selected that has a reduced affinity for the support, so long as it retains the ability to induce a displacement train that includes the protein of interest. In certain non-limiting embodiments, the displacer molecule will be employed in the context of protein separations in ion exchange chromatography and can be selected from, but not limited to, the group consisting of: polyelectrolytes; polysaccharides; low-molecular-mass dendrimers; amino acids; peptide; antibiotics; and aminoglycosidepolyamines. In certain embodiments the displacer is selected from, but not limited to, the group consisting of: Expell SP1™ (for CEX and for mixed mode); Expell Q3 (for anion-exchange chromatography (AEX) and for mixed mode); Propel Q2 (for AEX and for mixed mode); and protamine sulfate (for CEX and for mixed mode). Exemplary displacer molecules are described in US7,632,409, WO 99/47574, WO 03074148, WO2007/055896, WO 2007/064809; and US patent 6,881,540.

As used herein, the term"aggregates" means agglomeration or oligomerization of two or more individual molecules, including but not limiting to, protein dimers, trimers, tetramers, oligomers and other high molecular weight species. Protein aggregates can be soluble or insoluble.

II. Low Acidic Species Compositions of the Invention

The present invention provides low AR compositions comprising a protein, *e.g.,* an antibody, or antigen-binding portion thereof, such as adalimumab, where the composition comprises about 15% or less AR, 14% or less AR, 13% or less AR, 12% or less AR, 11% or less AR, 10% or less AR, 9% or less AR, 8% or less AR, 7% or less AR, 6% or less AR, 5% or less AR, 4.5% or less AR, 4% or less AR, 3.5% or less AR, 3% or less AR, 2.5% or less AR, 2% or less AR, 1.9% or less AR, 1.8% or less AR, 1.7% or less AR, 1.6% or less AR, 1.5% or less AR, 1.4% or less AR, 1.3% or less AR, 1.2% or less AR, 1.1% or less AR, 1% or less AR, 0.9% or less AR, 0.8% or less AR, 0.7% or less AR, 0.6% or less AR, 0.5% or less AR, 0.4% or less AR, 0.3% or less AR, 0.2% or less AR, 0.1% or less AR, or 0.0% AR, and ranges within one or more of the preceding. A low AR composition of the invention may also comprise about 0.0% to about 10% AR, about 0.0% to about 5% AR, about 0.0% to about 4% AR, about 0.0% to about 3% AR, about 0.0% to about 2% AR, about 3% to about 5% AR, about 5% to about 8% AR, or about 8% to about 10% AR, or about 10% to about 15% AR, and ranges within one or more of the preceding.

In one embodiment, a low AR composition of the invention may comprise about 15% or less AR1, 14% or less AR1, 13% or less AR1, 12% or less AR1, 11% or less AR1, 10% or less AR1, 9% or less AR1, 8% or less AR1, 7% or less AR1, 6% or less AR1, 5% or less AR1, 4.5% or less AR1, 4% or less AR1, 3.5% or less AR1, 3% or less AR1, 2.5% or less AR1, 2% or less AR1, 1.9% or less AR1, 1.8% or less AR1, 1.7% or less AR1, 1.6% or less AR1, 1.5% or less AR1, 1.4% or less AR1, 1.3% or less AR1, 1.2% or less AR1, 1.1% or less AR1, 1% or less AR1, 0.9% or less AR1, 0.8% or less AR1, 0.7% or less AR1, 0.6% or less AR1, 0.5% or less AR1, 0.4% or less AR1, 0.3% or less AR1, 0.2% or less AR1, 0.1% or less AR1, or 0.0% AR1, and ranges within one or more of the preceding. A low AR composition of the invention may also comprise about 0.0% to about 10% AR1, about 0.0% to about 5% AR1, about 0.0% to about 4% AR1, about 0.0% to about 3% AR1, about 0.0% to about 2% AR1, about 3% to about 5% AR1, about 5% to about 8% AR1, or about 8% to about 10% AR1, or about 10% to about 15% AR1, and ranges within one or more of the preceding.

In another embodiment, a low AR composition of the invention may also comprise about 15% or less AR2, 14% or less AR2, 13% or less AR2, 12% or less AR2, 11% or less AR2, 10% or less AR2, 9% or less AR2, 8% or less AR2, 7% or less AR2, 6% or less AR2, 5% or less AR2, 4.5% or less AR2, 4% or less AR2, 3.5% or less AR2, 3% or less AR2, 2.5% or less AR2, 2% or less AR2, 1.9% or less AR2, 1.8% or less AR2, 1.7% or less AR2, 1.6% or less AR2, 1.5% or less AR2, 1.4% or less AR2, 1.3% or less AR2, 1.2% or less AR2, 1.1% or less AR2, 1% or less AR2, 0.9% or less AR2, 0.8% or less AR2, 0.7% or less AR2, 0.6% or less AR2, 0.5% or less AR2, 0.4% or less AR2, 0.3% or less AR2, 0.2% or less AR2, 0.1% or less AR2, or 0.0% AR2, and ranges within one or more of the preceding. A low AR composition of the invention may also comprise about 0.0% to about 10% AR2, about 0.0% to about 5% AR2, about 0.0% to about 4% AR2, about 0.0% to about 3% AR2, about 0.0% to about 2% AR2, about 3% to about 5% AR2, about 5% to about 8% AR2, or about 8% to about 10% AR2, or about 10% to about 15% AR2, and ranges within one or more of the preceding.

A low AR composition of the invention may further comprise more than one lysine variant species of an antibody, or antigen-binding portion thereof. For example, in a preferred embodiment, the composition may comprise a Lys 2 variant of an antibody, or antigen-binding portion thereof. The composition may comprise a Lys 1 variant of an antibody, or antigen-binding portion thereof. The composition may comprise a Lys 0 variant of an antibody, or antigen-binding portion thereof. In another embodiment, the composition

may comprise both Lys 1 and Lys 2, or Lys 1 and Lys 0, or Lys 2 and Lys 0 variants of an antibody, or antigen-binding portion thereof. In another embodiment, the composition may comprise all three lysine variant species, *i.e.*, Lys 0, Lys 1 and Lys 2, of an antibody, or antigen-binding portion thereof.

In one embodiment, a low AR composition of the invention comprises less than about 50% lysine variant species that lack a C-terminal lysine (Lys 0). In another embodiment, the composition comprises less than about 55%, 54%, 53%, 52%, 51%, 50%, 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11% 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% lysine variant species that lack a C-terminal lysine ("Lys 0"). In another embodiment, the composition comprises about 50% to about 0%, about 40% to about 10%, about 30% to about 20%, about 40% to about 20%, or about 30% to about 15% lysine variant species that lack a C-terminal lysine (Lys 0). In one embodiment, the composition comprises 0% lysine variant species that lack a C-terminal lysine (Lys 0).

In one embodiment, a low AR composition comprises less than about 25% lysine variant species that have one C-terminal lysine (Lys 1). In another embodiment, the composition comprises less than about 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11% 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% lysine variant species that have one C-terminal lysine (Lys 1). In another embodiment, the composition comprises about 25% to about 0%, about 20% to about 5%, about 15% to about 10%, about 20% to about 10%, about 15% to about 5%, or about about 25% to about 5% lysine variant species that have one C-terminal lysine (Lys 1). In one embodiment, the composition comprises 0% lysine variant species that have one C-terminal Lysine (Lys 1).

In another embodiment, a low AR composition of the invention comprises at least about 70% lysine variant species that have two C-terminal lysines (Lys 2). In another embodiment, the composition comprises at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% lysine variant species that have two C-terminal lysines (Lys 2). In one embodiment, the composition comprises about 70% to about 100%, about 70% to about 90%, about 70% to about 80%, about 80% to about 100%, about 85% to about 100%, about 90% to about 100%, about 95% to about 100%, about 80% to about 90%, about 85% to about

95%, about 75% to about 85%, or about 97% to about 100% lysine variant species that have two C-terminal lysines (Lys 2). In one embodiment, the composition comprises 100% lysine variant species that have two C-terminal lysines (Lys 2).

As demonstrated herein, these low AR compositions of the invention have improved biological properties. For example, the low AR compositions of the invention are characterized by increased cartilage tissue penetration, reduced cartilage destruction, reduced synovial proliferation, reduced bone erosion, increased protection against the development of arthritic scores and/or histopathology scores, reduced cell infiltration, reduced proteoglycan loss, reduced chondrocyte death, and/or increased TNFα affinity, as compared to non-low acidic species compositions. In addition, the compositions of the present invention exhibit increased therapeutic efficacy when administered to a subject.

In one embodiment, the protein in the low AR composition of the invention is an antibody or antigen binding portion thereof. For example, the antibody, or antigen binding portion thereof may be an anti-TNFα antibody, or antigen binding portion thereof, such as adalimumab, or an antigen binding portion thereof. In one aspect of this embodiment, the antibody, or antigen binding portion thereof, can comprise a light chain variable region comprising the sequence set forth as SEQ ID NO:1, and a heavy chain variable region comprising the sequence set forth as SEQ ID NO:2. In another aspect of this embodiment, the antibody can comprise a light chain variable region comprising a CDR1 having the sequence set forth as SEQ ID NO:7, a CDR2 having the sequence set forth as SEQ ID NO:5, and a CDR3 having the sequence set forth as SEQ ID NO:3. In another aspect of this embodiment, the antibody can comprise a heavy chain variable region comprising a CDR1 having the sequence set forth as SEQ ID NO:8, a CDR2 having the sequence set forth as SEQ ID NO:6 and a CDR3 having the sequence set forth as SEQ ID NO:4.

The antibody, or antigen binding portion thereof, used in the low AR compositions of the invention, may be a human, humanized, or chimeric antibody.

The antibodies that can be used in the low AR compositions of the present disclosure can be generated by a variety of techniques, including immunization of an animal with the antigen of interest followed by conventional monoclonal antibody methodologies *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein (1975) Nature 256: 495. Somatic cell hybridization procedures can be used. In principle, other techniques for producing monoclonal antibody can be employed as well, including viral or oncogenic transformation of B lymphocytes.

One exemplary animal system for preparing hybridomas is the murine system. Hybridoma production is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known.

An antibody used in the low AR compositions of the invention can be a human, a chimeric, or a humanized antibody. Chimeric or humanized antibodies used in the low AR compositions of the invention can be prepared based on the sequence of a non-human monoclonal antibody prepared as described above. DNA encoding the heavy and light chain immunoglobulins can be obtained from the non-human hybridoma of interest and engineered to contain non-murine (*e.g.*, human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, murine variable regions can be linked to human constant regions using methods known in the art (see *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). To create a humanized antibody, murine CDR regions can be inserted into a human framework using methods known in the art (see *e.g.*, U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*).

In one non-limiting embodiment, the antibodies to be used in the low AR compositions of the invention are human monoclonal antibodies. Such human monoclonal antibodies can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomic mice include mice referred to herein as the HuMAb Mouse® (Medarex, Inc.), KM Mouse® (Medarex, Inc.), and XenoMouse® (Amgen). The antibodies, or antigen-binding portions thereof, used in the low AR compositions of the invention can also be produced using the methods described in U.S. Patent No. 6,090,382, the entire contents of which is expressly incorporated herein by reference.

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise antibodies of the disclosure. For example, mice carrying both a human heavy chain transchromosome and a human light chain transchromosome, referred to as "TC mice" can be used; such mice are described in Tomizuka *et al.* (2000) Proc. Natl. Acad. Sci. USA 97:722-727. Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art (*e.g.*, Kuroiwa *et al.* (2002) Nature Biotechnology 20:889-894 and PCT application No. WO 2002/092812) and can be used to raise antibodies of this disclosure.

Recombinant human antibodies to be used in the low AR compositions of the invention can be isolated by screening of a recombinant combinatorial antibody library, *e.g.*, a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (*e.g.*, the Pharmacia Recombinant Phage Antibody System, catalog no. 27-9400-01; and the Stratagene SurfZAP^{TM} phage display kit, catalog no. 240612, the entire teachings of which are incorporated herein), examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, *e.g.*, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT Publication No. WO 92/18619; Dower *et al.* PCT Publication No. WO 91/17271; Winter *et al.* PCT Publication No. WO 92/20791; Markland *et al.* PCT Publication No. WO 92/15679; Breitling *et al.* PCT Publication No. WO 93/01288; McCafferty *et al.* PCT Publication No. WO 92/01047; Garrard *et al.* PCT Publication No. WO 92/09690; Fuchs *et al.* (1991) Bio/Technology 9:1370-1372; Hay *et al.* (1992) Hum Antibody Hybridomas 3:81-85; Huse *et al.* (1989) Science 246:1275-1281; McCafferty *et al.*, Nature (1990) 348:552-554; Griffiths *et al.* (1993) EMBO J 12:725-734; Hawkins *et al.* (1992) J Mol Biol 226:889-896; Clackson *et al.* (1991) Nature 352:624-628; Gram *et al.* (1992) PNAS 89:3576-3580; Garrard *et al.* (1991) Bio/Technology 9:1373-1377; Hoogenboom *et al.* (1991) Nuc Acid Res 19:4133-4137; and Barbas *et al.* (1991) PNAS 88:7978-7982; the entire teachings of which are incorporated herein.

Human monoclonal antibodies to be used in the low AR compositions of the invention can also be prepared using SCID mice into which human immune cells have been reconstituted such that a human antibody response can be generated upon immunization. Such mice are described in, for example, U.S. Patent Nos. 5,476,996 and 5,698,767 to Wilson *et al.*

In certain embodiments, the human antibodies to be used in the low AR compositions of the invention are anti-TNFα antibodies and antibody portions thereof, anti-TNFα-related antibodies and antibody portions, and human antibodies and antibody portions with equivalent properties to anti-TNFα antibodies, such as high affinity binding to hTNFα with low dissociation kinetics and high neutralizing capacity. In one aspect, the invention provides low AR compositions containing an isolated human antibody, or an antigen-binding portion thereof, that dissociates from hTNFα with a Kd of about 1 x 10^{-8} M or less and a Koff

rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance. In specific non-limiting embodiments, an anti-TNFα antibody to be used in the low AR compositions of the invention competitively inhibits binding of adalimumab to TNFα under physiological conditions. In one embodiment, the low AR compositions of the invention comprise adalimumab, or an antigen binding fragment thereof.

Antibodies or fragments thereof to be used in the low AR compositions of the invention can be altered wherein the constant region of the antibody is modified to reduce at least one constant region-mediated biological effector function relative to an unmodified antibody. To modify an antibody of the invention such that it exhibits reduced binding to the Fc receptor, the immunoglobulin constant region segment of the antibody can be mutated at particular regions necessary for Fc receptor (FcR) interactions (see, *e.g.*, Canfield and Morrison (1991) J. Exp. Med. 173:1483-1491; and Lund *et al.* (1991) J. of Immunol. 147:2657-2662, the entire teachings of which are incorporated herein). Reduction in FcR binding ability of the antibody may also reduce other effector functions which rely on FcR interactions, such as opsonization and phagocytosis and antigen-dependent cellular cytotoxicity.

To express an antibody or antigen-binding fragment thereof to be used in the low AR compositions of the invention, DNAs encoding the protein, such as DNAs encoding partial or full-length light and heavy chains in the case of antibodies, are inserted into one or more expression vector such that the genes are operatively linked to transcriptional and translational control sequences. (See, *e.g.*, U.S. Pat. No. 6,090,382, the entire teaching of which is incorporated herein by reference.) In this context, the term "operatively linked" is intended to mean that a gene encoding the protein of interest is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. In certain embodiments, the protein of interest will comprising multiple polypeptides, such as the heavy and light chains of an antibody. Thus, in certain embodiments, genes encoding multiple polypeptides, such as antibody light chain genes and antibody heavy chain genes, can be inserted into a separate vector or, more typically, the genes are inserted into the same expression vector. Genes are inserted into expression vectors by standard methods (*e.g.*, ligation of complementary restriction sites on the gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the gene or genes, the

expression vector may already carry additional polypeptide sequences, such as, but not limited to, antibody constant region sequences. For example, one approach to converting the anti-TNFα antibody or anti-TNFα antibody-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the protein from a host cell. The gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.,* a signal peptide from a non-immunoglobulin protein).

In addition to protein coding genes, a recombinant expression vector can carry one or more regulatory sequence that controls the expression of the protein coding genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the protein coding genes. Such regulatory sequences are described, *e.g.*, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990), the entire teaching of which is incorporated herein by reference. It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Suitable regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see, *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.,* the entire teachings of which are incorporated herein by reference.

A recombinant expression vector may also carry one or more additional sequences, such as a sequence that regulates replication of the vector in host cells (*e.g.*, origins of replication) and/or a selectable marker gene. The selectable marker gene facilitates selection

of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*, the entire teachings of which are incorporated herein by reference). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Suitable selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr- host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

An antibody, or antibody portion, to be used in the low AR compositions of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), Molecular Cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), Ausubel *et al.* (eds.) Current Protocols in Molecular Biology, Greene Publishing Associates, (1989) and in U.S. Patent Nos. 4,816,397 & 6,914,128, the entire teachings of which are incorporated herein.

For expression of protein, for example, the light and heavy chains of an antibody, the expression vector(s) encoding the protein is (are) transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the proteins of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, such as mammalian host cells, is suitable because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active protein. Prokaryotic expression of protein genes has been reported to be ineffective for production of high yields of active protein (Boss and Wood (1985) Immunology Today 6:12-13, the entire teaching of which is incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, *e.g.*, Enterobacteriaceae such as Escherichia, *e.g.*, E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, *e.g.*, Salmonella typhimurium, Serratia, *e.g.*, Serratia marcescans, and Shigella, as well as Bacilli such as B. subtilis and B. licheniformis (*e.g.*, B. licheniformis 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as P. aeruginosa, and Streptomyces. One suitable E. coli cloning host is E. coli 294 (ATCC 31,446), although other strains such as E. coli B, E. coli X1776 (ATCC 31,537), and E. coli W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as Schizosaccharomyces pombe; Kluyveromyces hosts such as, *e.g.*, K. lactis, K. fragilis (ATCC 12,424), K. bulgaricus (ATCC 16,045), K. wickeramii (ATCC 24,178), K. waltii (ATCC 56,500), K. drosophilarum (ATCC 36,906), K. thermotolerans, and K. marxianus; yarrowia (EP 402,226); Pichia pastoris (EP 183,070); Candida; Trichoderma reesia (EP 244,234); Neurospora crassa; Schwanniomyces such as Schwanniomyces occidentalis; and filamentous fungi such as, *e.g.*, Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as A. nidulans and A. niger.

Suitable host cells for the expression of glycosylated proteins, for example, glycosylated antibodies, are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mori have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of Spodoptera frugiperda cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

Mammalian cells can be used for expression and production of the recombinant protein used in the low AR compositions of the invention, however other eukaryotic cell types can also be employed in the context of the instant invention. See, *e.g.*, Winnacker, From Genes to Clones, VCH Publishers, N.Y., N.Y. (1987). Suitable mammalian host cells for expressing recombinant proteins according to the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) PNAS USA 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in Kaufman and Sharp (1982) Mol. Biol. 159:601-621, the entire teachings of which are incorporated herein by reference), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding protein genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or secretion of the antibody into the culture medium in which the host cells are grown. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2), the entire teachings of which are incorporated herein by reference.

Host cells are transformed with the above-described expression or cloning vectors for protein production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce a protein may be cultured in a variety of media. Commercially available media such as Ham's F10™ (Sigma), Minimal Essential Medium™ (MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium™ (DMEM), (Sigma) are suitable for culturing the host cells. In addition, any of the media

described in Ham *et al.*, Meth. Enz. 58:44 (1979), Barnes *et al.*, Anal. Biochem. 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells, the entire teachings of which are incorporated herein by reference. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as gentamycin drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Host cells can also be used to produce portions of intact proteins, for example, antibodies, including Fab fragments or scFv molecules. It is understood that variations on the above procedure are within the scope of the present invention. For example, in certain embodiments it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to an antigen. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than the target antibody, depending on the specificity of the antibody of the invention, by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a suitable system for recombinant expression of a protein, for example, an antibody, or antigen-binding portion thereof, a recombinant expression vector encoding the protein, for example, both an antibody heavy chain and an antibody light chain, is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the protein gene(s) are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the gene(s). The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO

cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the protein, for example, the antibody heavy and light chains, and intact protein, for example, an antibody, is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the protein from the culture medium.

When using recombinant techniques, the protein, for example, antibodies or antigen binding fragments thereof, can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. In one aspect, if the protein is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (*e.g.*, resulting from homogenization), can be removed, *e.g.*, by centrifugation or ultrafiltration. Where the protein is secreted into the medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter, *e.g.*, an Amicon™ or Millipore Pellicon™ ultrafiltration unit.

Some antibodies can be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For antibodies made intracellularly, the first step of a purification process typically involves: lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. Where the antibody is secreted, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, *e.g.*, an Amicon™ or Millipore Pellicon™ ultrafiltration unit. Where the antibody is secreted into the medium, the recombinant host cells can also be separated from the cell culture medium, *e.g.*, by tangential flow filtration. Antibodies can be further recovered from the culture medium using the antibody purification methods of the invention.

III. Preparation of Low AR Compositions Using Displacement Chromatography

In certain embodiments, the low AR compositions of the present invention may be produced using downstream process technologies (*e.g.,* displacement chromatography), following cell culture of a protein.

The methods described herein for the production of compositions comprising low AR and/or low process-related impurities comprise the purification of a protein, such as an antibody or antigen-binding portion thereof, using displacement chromatography.

The methods described herein for the production of compositions comprising low AR and/or low process-related impurities also comprise the purification of a protein, such as an antibody or antigen-binding portion thereof, using displacement chromatography in combination with, for example, other types of chromatography, such as multimodal (MM) chromatography, wherein the MM media comprises both ion exchange and hydrophobic interaction functional groups, and an aqueous salt solution. In one embodiment, the same or substantially the same aqueous salt solution is used as a loading buffer and a wash buffer.

In further embodiments, the methods described herein for the production of compositions comprising low AR and/or low process-related impurities comprise the purification of a protein, such as an antibody or antigen-binding portion thereof, using displacement chromatography in combination with chromatography comprising an anion exchange (AEX) resin and an aqueous salt solution. In one embodiment, the same or substantially the same aqueous salt solution is used as a loading buffer and a wash buffer.

In yet further embodiments, the methods described herein for the production of compositions comprising low AR and/or low process-related impurities comprise the purification of a protein, such as an antibody or antigen-binding portion thereof, using displacement chromatography in combination with chromatography comprising a cation exchange (CEX) adsorbent resin and an aqueous salt solution. In one embodiment, the same or substantially the same aqueous salt solution is used as a loading buffer and a wash buffer, and the target protein bound to the CEX adsorbent resin is eluted with a buffer having a higher conductivity and/or pH than the loading/wash buffer.

In still further embodiments, the methods described herein for production of compositions comprising low AR and/or low process-related impurities comprise the purification of a protein, such as an antibody or antigen-binding portion thereof, using displacement chromatography in combination with several media, for example by using an anion exchange (AEX) resin, and chromatography using a cation exchange (CEX) adsorbent resin, in a suitable buffer, such as, for example, a Tris/Formate buffer system. In one embodiment, the sample is purified affinity chromatography media, *e.g.,* Protein A, prior to the ion chromatography resins. For example, in one embodiment, the methods described herein for production of compositions comprising low AR comprise the exemplary process

reflected in Figure 190 of U.S. Provisional Application No. 61/893,068, entitled "Low Acidic Species Compositions and Methods for Producing and Using the Same", filed on October 18, 2013, the entire contents of which are expressly incorporated herein by reference.

In one embodiment, the method for producing a low AR composition comprising an antibody, or antigen binding portion thereof, comprises contacting a first sample comprising the antibody, or antigen binding portion thereof, to affinity chromatography media in a load buffer (for example a low concentration Tris/Formate buffer), and eluting the sample from the affinity chromatography media as a first eluted sample, contacting the first eluted sample to a first chromatography media, such as an AEX chromatography resin, in a load buffer, and eluting the sample from the AEX chromatography resin as a second eluted sample. The second eluted sample is then contacted with a second chromatography media, such as a CEX chromatography resin, in a load buffer, and the sample is eluted from the CEX chromatography resin as a third eluted sample. In one embodiment, the CEX chromatography resin is eluted one, two, three or more times. In one embodiment, the process optionally includes one or more intermediate filtration steps, pH adjustment steps and inactivation steps.

In one embodiment, the displacement chromatography steps, alone or in combination with other purification steps, produce a low AR composition comprising an antibody, or antigen binding portion thereof, which contains 15% or less AR, 14% or less AR, 13% or less AR, 12% or less AR, 11% or less AR, 10% or less AR, 9% or less AR, 8% or less AR, 7% or less AR, 6% or less AR, 5% or less AR, 4.5% or less AR, 4% or less AR, 3.5% or less AR, 3% or less AR, 2.5% or less AR, 2% or less AR, 1.9% or less AR, 1.8% or less AR, 1.7% or less AR, 1.6% or less AR, 1.5% or less AR, 1.4% or less AR, 1.3% or less AR, 1.2% or less AR, 1.1% or less AR, 1% or less AR, 0.9% or less AR, 0.8% or less AR, 0.7% or less AR, 0.6% or less AR, 0.5% or less AR, 0.4% AR, 0.3% or less AR, 0.2% or less AR, 0.1% or less AR, or 0.0% AR, and ranges within one or more of the preceding. In one aspect of this embodiment, the low AR composition of the invention comprises about 0.0% to about 10% AR, about 0.0% to about 5% AR, about 0.0% to about 4% AR, about 0.0% to about 3% AR, about 0.0% to about 2% AR, about 3% to about 5% AR, about 5% to about 8% AR, or about 8% to about 10% AR, or about 10% to about 15% AR, and ranges within one or more of the preceding.

In one embodiment, the displacement chromatography steps, alone or in combination with other purification steps, produce a low AR composition comprising an antibody, or

antigen binding portion thereof, which contains 15% or less AR1, 14% or less AR1, 13% or less AR1, 12% or less AR1, 11% or less AR1, 10% or less AR1, 9% or less AR1, 8% or less AR1, 7% or less AR1, 6% or less AR1, 5% or less AR1, 4.5% or less AR1, 4% or less AR1, 3.5% or less AR1, 3% or less AR1, 2.5% or less AR1, 2% or less AR1, 1.9% or less AR1, 1.8% or less AR1, 1.7% or less AR1, 1.6% or less AR1, 1.5% or less AR1, 1.4% or less AR1, 1.3% or less AR1, 1.2% or less AR1, 1.1% or less AR1, 1% or less AR1, 0.9% or less AR1, 0.8% or less AR1, 0.7% or less AR1, 0.6% or less AR1, 0.5% or less AR1, 0.4% or less AR1, 0.3% or less AR1, 0.2% or less AR1, 0.1% or less AR1 , or 0.0% AR1, and ranges within one or more of the preceding. In one aspect of this embodiment, the low AR composition of the invention comprises about 0.0% to about 10% AR1, about 0.0% to about 5% AR1, about 0.0% to about 4% AR1, about 0.0% to about 3% AR1, about 0.0% to about 2% AR1, about 3% to about 5% AR1, about 5% to about 8% AR1, or about 8% to about 10% AR1, or about 10% to about 15% AR1, and ranges within one or more of the preceding.

In one embodiment, the displacement chromatography steps, alone or in combination with other purification steps, produce a low AR composition comprising an antibody, or antigen binding portion thereof, which contains 15% or less AR2, 14% or less AR2, 13% or less AR2, 12% or less AR2, 11% or less AR2, 10% or less AR2, 9% or less AR2, 8% or less AR2, 7% or less AR2, 6% or less AR2, 5% or less AR2, 4.5% or less AR2, 4% or less AR2, 3.5% or less AR2, 3% or less AR2, 2.5% or less AR2, 2% or less AR2, 1.9% or less AR2, 1.8% or less AR2, 1.7% or less AR2, 1.6% or less AR2, 1.5% or less AR2, 1.4% or less AR2, 1.3% or less AR2, 1.2% or less AR2, 1.1% or less AR2, 1% or less AR2, 0.9% or less AR2, 0.8% or less AR2, 0.7% or less AR2, 0.6% or less AR2, 0.5% or less AR2, 0.4% or less AR2, 0.3% or less AR2, 0.2% or less AR2, 0.1% or less AR2, or 0.0% AR2, and ranges within one or more of the preceding. In one aspect of this embodiment, the low AR composition of the invention comprises about 0.0% to about 10% AR2, about 0.0% to about 5% AR2, about 0.0% to about 4% AR2, about 0.0% to about 3% AR2, about 0.0% to about 2% AR2, about 3% to about 5% AR2, about 5% to about 8% AR2, or about 8% to about 10% AR2, or about 10% to about 15% AR2, and ranges within one or more of the preceding.

Protein Purification Generally

Following upstream processing of a protein of interest, downstream process technologies can be used to purify the protein. For example, but not by way of limitation, once a clarified solution or mixture comprising the protein of interest, for example, an

antibody or antigen binding fragment thereof, has been obtained, separation of the protein of interest from the process-related impurities, aggregates, fragments, and/or charge variant species (*e.g.,* acidic species and basic species) can be effected using displacement chromatography, either alone or in combination with different purification techniques, including, but not limited to, affinity separation steps, ion exchange separation steps, mixed mode separation steps, and hydrophobic interaction separation steps, singularly or in combination. The separation steps separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size, or any combination thereof, depending on the particular form of separation, including chromatographic separation. In one aspect of the invention, separation is performed using chromatography, including cationic, anionic, and hydrophobic interaction. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. Each of the separation methods result in the protein traversing at different rates through a column, to achieve a physical separation that increases as they pass further through the column, or adhere selectively to the separation medium. The proteins are then differentially eluted by different solvents. In some cases, the antibody is separated from impurities when the impurities preferentially adhere to the column and the antibody less so, *i.e.,* the desired antibody variant is present in the Flow Through.

In certain embodiments, a low AR composition is produced using displacement chromatography to identify the particular conditions, *e.g.*, displacers, displacer concentration, salt concentration, pH, temperature, load amount and conditions, and washing conditions, sufficient to elicit the desired fractionation profile, *e.g.*, fractionation of acidic species and lysine variants, of a sample comprising the protein of interest and at least one process-related impurity. In certain embodiments, the method further comprises pooling the resulting fractions comprising the desired low AR composition. In another embodiment, the method does not comprise pooling fractions.

The purification process may begin at the separation step after the antibody has been produced using upstream production methods described herein and in U.S. Provisional Application No. 61/893,068, entitled "Low Acidic Species Compositions and Methods for Producing and Using the Same", filed on October 18, 2013, the entire contents of which are incorporated herein by reference, and/or by alternative production methods conventional in the art. Once a clarified solution or mixture comprising the protein of interest, *e.g.*, an antibody, has been obtained, separation of the protein of interest from process-related

impurities, such as the other proteins produced by the cell, as well as product-related substances, such as acidic or basic variants, is performed.

In certain non-limiting embodiments, such separation is performed using cation exchange (CEX), anion exchange (AEX), and/or mixed mode (MM) chromatography. In certain embodiments, a combination of one or more different purification techniques, including affinity separation step(s), ion exchange separation step(s), mixed-mode step(s), and/or hydrophobic interaction separation step(s) can also be employed. Such additional purification steps separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, and/or size. In one aspect of the invention, such additional separation steps are performed using chromatography, including hydrophobic, anionic or cationic interaction (or a combination thereof). Numerous chromatography resins are commercially available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. Each of the separation methods allow proteins to either traverse at different rates through a column, achieving a physical separation that increases as they pass further through the column, or to adhere selectively to a separation resin (or medium). The proteins are then differentially eluted using different eluents. In some cases, the protein of interest is separated from impurities when the impurities specifically adhere to the column's resin and the protein of interest does not, *i.e.,* the protein of interest is contained in the effluent, while in other cases the protein of interest will adhere to the column's resin, while the impurities and/or product-related substances are extruded from the column's resin during a wash cycle.

Primary Recovery and Virus Inactivation

In certain embodiments, the initial steps of the purification methods of the present invention involve the clarification and primary recovery of antibody from a sample matrix. In certain embodiments, the primary recovery will include one or more centrifugation steps to separate the antibody product from the cells and cell debris. Centrifugation of the sample can be performed at, for example, but not by way of limitation, 7,000 x g to approximately 12,750 x g. In the context of large scale purification, such centrifugation can occur on-line with a flow rate set to achieve, for example, but not by way of limitation, a turbidity level of 150 NTU in the resulting supernatant. Such supernatant can then be collected for further purification, or in-line filtered through one or more depth filters for further clarification of the sample.

In certain embodiments, the primary recovery will include the use of one or more depth filtration steps to clarify the sample matrix and thereby aid in purifying the antibodies of interest in the present invention. In other embodiments, the primary recovery will include the use of one or more depth filtration steps post centrifugation to further clarify the sample matrix. Non-limiting examples of depth filters that can be used in the context of the instant invention include the Millistak+ X0HC, F0HC, D0HC, A1HC, B1HC depth filters (EMD Millipore), Cuno™ model 30/60ZA, 60/90 ZA, VR05, VR07, delipid depth filters (3M Corp.). A 0.2 μm filter such as Sartorius's 0.45/0.2μm Sartopore™ bi-layer or Millipore's Express SHR or SHC filter cartridges typically follows the depth filters.

In certain embodiments, the primary recovery process can also be a point at which to reduce or inactivate viruses that can be present in the sample matrix. For example, any one or more of a variety of methods of viral reduction/inactivation can be used during the primary recovery phase of purification including heat inactivation (pasteurization), pH inactivation, buffer/detergent treatment, UV and γ-ray irradiation and the addition of certain chemical inactivating agents such as β-propiolactone or *e.g.*, copper phenanthroline as described in U.S. Pat. No. 4,534,972. In certain embodiments of the present invention, the sample matrix is exposed to detergent viral inactivation during the primary recovery phase. In other embodiments, the sample matrix may be exposed to low pH inactivation during the primary recovery phase.

In those embodiments where viral reduction/inactivation is employed, the sample mixture can be adjusted, as needed, for further purification steps. For example, following low pH viral inactivation, the pH of the sample mixture is typically adjusted to a more neutral pH, *e.g.*, from about 4.5 to about 8.5, prior to continuing the purification process. Additionally, the mixture may be diluted with water for injection (WFI) to obtain a desired conductivity.

Affinity Chromatography

In certain embodiments, it will be advantageous to subject a sample produced by the techniques of the instant invention to affinity chromatography to further purify the protein of interest away from acidic species before a displacement chromatography step. In certain embodiments the chromatographic material is capable of selectively or specifically binding to the protein of interest ("capture"). Non-limiting examples of such chromatographic material include: Protein A, Protein G, chromatographic material comprising, for example, an antigen

bound by an antibody of interest, and chromatographic material comprising an Fc binding protein. In specific embodiments, the affinity chromatography step involves subjecting the primary recovery sample to a column comprising a suitable Protein A resin. In certain embodiments, Protein A resin is useful for affinity purification and isolation of a variety of antibody isotypes, particularly IgG1, IgG2, and IgG4. Protein A is a bacterial cell wall protein that binds to mammalian IgGs primarily through their Fc regions. In its native state, Protein A has five IgG binding domains as well as other domains of unknown function.

There are several commercial sources for Protein A resin. One suitable resin is MabSelect™ from GE Healthcare. Suitable resins include, but not limited to, MabSelect SuRe™, MabSelect SuRe LX, MabSelect, MabSelect Xtra, rProtein A Sepharose from GE Healthcare, ProSep HC, ProSep Ultra, and ProSep Ultra Plus from EMD Millipore, MapCapture from Life Technologies. A non-limiting example of a suitable column packed with MabSelect™ is an about 1.0 cm diameter x about 21.6 cm long column (~17 mL bed volume). This size column can be used for small scale purifications and can be compared with other columns used for scale ups. For example, a 20 cm x 21 cm column whose bed volume is about 6.6 L can be used for larger purifications. Regardless of the column, the column can be packed using a suitable resin such as MabSelect™.

Protein A Affinity Chromatography

In certain embodiments, particularly where the protein of interest is an antibody, the composition, *e.g.,* a primary recovery sample, is subjected to Protein A affinity chromatography before displacement chromatography to produce the low AR compositions of the invention. There are a variety of commercial sources for Protein A resin. Suitable resins include, but not limited to, MabSelect SuRe™, MabSelect SuRe LX, MabSelect, MabSelect Xtra, rProtein A Sepharose from GE Healthcare, ProSep HC, ProSep Ultra, and ProSep Ultra Plus from EMD Millipore, MapCapture from Life Technologies.

The Protein A column can be equilibrated with a suitable buffer prior to sample loading. Following the loading of the column, the column can be washed one or multiple times using a suitable sets of buffers. The Protein A column can then be eluted using an appropriate elution buffer. For example, glycine-HCL or citric acid can be used as an elution buffer. The eluate can be monitored using techniques well known to those skilled in the art. The eluate fractions of interest can be collected and then prepared for further processing.

The Protein A eluate may subject to a viral inactivation step either by detergent or low pH, provided this step is not performed prior to the Protein A capture operation. A proper detergent concentration or pH and time can be selected to obtain desired viral inactivation results. After viral inactivation, the Protein A eluate is usually pH and/or conductivity adjusted for subsequent purification steps.

The Protein A eluate may be subjected to filtration through a depth filter to remove turbidity and/or various impurities from the antibody of interest prior to additional chromatographic polishing steps. Examples of depth filters include, but not limited to, Millistak+ X0HC, F0HC, D0HC, A1HC, and B1HC Pod filters (EMD Millipore), or Zeta Plus 30ZA/60ZA, 60ZA/90ZA, delipid, VR07, and VR05 filters (3M). The Protein A eluate pool may need to be conditioned to proper pH and conductivity to obtain desired impurity removal and product recovery from the depth filtration step.

The invention is not limited to capture of the protein of interest using Protein A chromatography. A non-Protein A chromatography capture step can also be carried out. For example, cation exchange capture and non-chromatographic methods, such as aqueous two phase extraction or precipitation, or other methods known in the art, can be used,

Displacement Chromatography

In certain embodiments of the present invention, a protein sample, *e.g.,* a primary recovery sample from cell culture, a Protein A eluate sample, or a sample having undergone one or more of the other purification strategies outlined herein, is subjected to displacement chromatography to produce the low AR compositions of the invention. In certain embodiments, the displacer molecule is selected to have a higher affinity for the stationary phase (*i.e.,* the chromatographic support) than the components present in the material to be separated. In certain embodiments, the displacer induces the components of the mixture to develop into consecutive zones of concentrated and purified species in the order of decreasing binding affinity ahead of the displacer front (a "displacement train"). In certain embodiments, the displacement process allows for higher column loading levels (as compared to conventional high-resolution chromatographic separations such as bind and linear gradient elution mode) without compromising the purity and recovery of the component of interest. In certain embodiments, washing of the displacement train from the column using the displacer solution allows for the component of interest to be isolated by collecting (and pooling if necessary) the proper fraction(s) of the displaced eluate. Along

with acidic species, other product-related substances, such as basic species, product aggregates, and/or product fragments, and process-related impurities, such as HCPs, can be selectively collected or reduced using displacement chromatography.

Displacement chromatography in described, in general, in Brgles *et al.,* Journal of Chromatography A, 1218 (2011) 2389-2395; Gajdosik *et al.,* Journal of Chromatography A, 1239 (2012) 1-9; Gerstner *et al.,* Biotechnol. Prog., (1992), 8, 540-545; Kundu *et al.,* Analytical Biochemistry, 248, 111-116, (1997); and Vogt *et al.,* Journal of Chromatography A, 760 (1997) 125-137.

In certain embodiments, the displacer will be employed in the context of an ion exchange, *e.g.,* anion exchange or cation exchange, or mixed mode chromatographic separation. A detailed description of ion exchange chromatography and a listing of exemplary chromatographic supports which can be employed in the context of displacement chromatography are presented below. A detailed description of mixed mode chromatography and a listing of exemplary chromatographic supports which can be employed in the context of displacement chromatography are also presented below. In certain non-limiting embodiments, a cation exchange, an anion exchange, or a mixed mode displacement chromatography step is employed to effectively reduce product-related substances (*e.g.,* acidic species and/or basic species such as lysine variant species) from, *e.g.,* a monoclonal antibody feed stream. In specific embodiments, conventional (or relatively weak) binding conditions can be employed and cationic molecules having high affinity for a CEX, AEX, or multimodal ligand (such as Expell SP1™ and protamine sulfate) can be employed to induce the formation of a product-related substances displacement train. In certain of such embodiments, the acidic species variant population is enriched in the front followed by the main isoform, and, thereafter, the basic population. Thus, in certain embodiments, exclusion of those earlier fractions from the remainder eluate results in an AR-reduced product. Alternatively, exclusion of the fractions following the main isoform results in a lysine species variant-reduced product. In certain embodiments, the fragments and aggregates are reduced in an AR-reduced product. In certain embodiments, the HCPs are reduced in an AR-reduced product.

In certain embodiments, the displacer concentration will be selected from the range of about 0.1 mM to about 10 mM, or about 0.25 mM to about 10 mM. In certain embodiments, the displacer concentration will be selected from a range of about 0.1 mM to about 5 mM, or about 0.25 mM to about 3 mM. In certain embodiments, the displacer concentration will be

selected from a range of about 0.1 mM to about 5 mM, or about 0.25 mM to about 2 mM. In certain embodiments, the displacer concentration will be selected from a range of about 0.1 mM to about 2 mM, or about 0.25 mM to about 1 mM. In certain embodiments, the displacer concentration will be selected from a concentration of about 0.1 mM to about 1 mM, or about 0.25 mM to about 0.5 mM.

In certain embodiments the displacer is Expell SP1™ and the displacer concentration will be selected from the range of about 0.1 mM to about 10 mM, or about 0.25 mM to about 10 mM. In certain embodiments, the displacer is protamine sulfate and the displacer concentration will be selected from the range of about 0.1 mM to about 5 mM, or about 0.25 mM to about 5 mM.

In certain embodiments, a displacing buffer is used in one-step displacement process. In certain embodiments, the total volume of the one-step displacing buffer is in the range of about 20 CVs to about 50 CVs, or about 25 CVs to about 40 CVs, or about 30 CVs. In another embodiment, the total volume of the one-step displacing buffer is about 15 CVs, about 20 CVs, about 25 CVs, about 30 CVs, about 35 CVs, about 40 CVs, about 45 CVs, or about 50 CVs.

Although displacement chromatography conventionally employs a displacer at a fixed concentration to achieve component separation, an improved method using multiple displacing buffers is also disclosed herein. For example, a two-step displacement method is employed where a first displacer concentration is employed for a certain initial number of column volumes (CVs) and a second, higher, displacer concentration is employed for a subsequent number of CVs. The total volume of the displacing buffers needed to complete the displacement process is significantly (*e.g.,* 25-45%) less than that needed when using one displacing buffer in the one-step displacement process in order to achieve comparable separation performances. In certain embodiments, the first displacer concentration is about 0.25 mM, about 0.3 mM, about 0.35 mM, about 0.4 mM, about 0.45 mM, or about 0.5 mM. In certain embodiments, the second displacer concentration is about 0.5 mM, about 1 mM, about 1.5 mM, about 2 mM, about 2.5 mM, about 3 mM, about 3.5 mM, about 4 mM, about 4.5 mM, or about 5 mM.

In certain embodiments, a two-step displacement method is employed where the first displacer concentration is employed for up to about 10 CVs. In certain embodiments, the first displacer concentration is employed for up to about 25 CVs. In another embodiments,

the first displacer concentration is employed for up to about 10, 11 12, 13, 14, 15, 16, 17, 18, 19, 20 , 21, 22, 23, 24 or 25 CVs.

In certain embodiments, the second displacer concentration is employed for up to about 10 CVs. In certain embodiments, the second displacer concentration is employed for up to about 25 CVs. In another embodiment, the second displacer concentration is employed for up to about 10, 11 12, 13, 14, 15, 16, 17, 18, 19, 20 , 21, 22, 23, 24 or 25 CVs.

In certain embodiments, the total required displacing buffer volume is about 13 CVs for a two-step displacement process. In certain embodiments, the total required displacer buffer volume is about 15 CVs for a two-step displacement process. In certain embodiments, the total required displacer buffer volume is about 33 CVs for a two-step displacement process. In another embodiment, the total required displacing buffer volune is about 10, 11, 12, 13, 14, 115, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 CVs for a two-step displacement process.

One of skill in the art would understand that further reduction in required buffer volumes for each displacement step is expected. In certain embodiments, multiple steps of increasing displacer concentration are employed. As outlined in the Examples section, below, incorporation of additional displacement concentration steps into the purification strategy can allow for unexpectedly efficient charge variant, product aggregate, product fragment, and/or HCP clearance. For example, in one embodiment, three, four, five, six, seven, eight, nine or ten displacement concentration steps are used.

In certain embodiments, a linear gradient displacement method is employed where an initial, low, displacer concentration is followed by the addition of displacer at increasing concentrations in accordance with a linear gradient. For example, but not by way of limitation, the displacer concentration can range from about 0 mM to about 1 mM over the course of about 40 CVs. Again, as outlined in the Examples section, below, incorporation of a linear displacer concentration gradient into the purification strategy can allow for unexpectedly efficient charge variant, product aggregate, product fragment, and/or HCP clearance.

In certain embodiments, a displacement buffer consisting of two or more displacers is used. In certain embodiments, different displacers are used in the multi-step displacement process. For example, a displacement buffer consisting of two, three, four, five, six, seven, eight, nine or ten displacers may be used. Alternatively, two, three, four five, six, seven, eight, nine or ten displacers may be used in a multi-step displacement process.

In certain embodiments of the present invention, the pH of the displacing wash buffer is below the pI of the protein of interest. In certain embodiments, the pH of the displacing wash buffer is in the range of about 5.0 to about 9.0, about 6.0 to about 8.0, about 7.0 to about 7.7, or about 7.5 to about 7.7. In another embodiment, the pH of the displacing wash buffer is about 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, or 9.0.

In certain embodiments of the present invention, the conductivity of the displacing wash buffer is between about 1 to about 86 mS/cm, about 2 to about 20 mS/cm, about 2 to about 7 mS/cm, or about 5 to about 6.6 mS/cm. In another embodiment, the conductivity of the displacing wash buffer is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 or 86 mS/cm.

In certain embodiments of the present invention, the column bed height is between about 10 cm to about 30 cm, about 15 cm to about 25 cm, about 20 cm to about 30 cm, or about 25 cm. In another embodiment, the column bed height is about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 cm.

In certain embodiments of the present invention, the flow residence time is between about 2 minutes to about 25 minutes, about 5 minutes to about 20 minutes, about 10 minutes to about 20 minutes, or about 15 minutes to about 20 minutes. In another embodiment, the flow residence time is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 minutes.

In certain embodiments, the displacer buffer pH and displacer concentration can affect the displacement profile and, as a result, impact clearance of process-related impurities and/or product-related substances, such as charge variants, in unexpected ways. Thus, effective operating regimes with regard to the reduction of process-related impurities and/or product-related substances depend on the specific protein-resin-displacer system. For example, but not by way of limitation, when a feed stream containing Adalimumab is separated using displacement chromatography, significant AR reduction (ΔAR%) can be achieved using a displacing buffer with pH in the range of 6-8 with displacer concentration as low as 0.25-0.5 mM. In fact, as described in Example 2 below, the extent of Adalimumab AR reduction increases significantly as pH varies from 6.5 to 7.5, for example, over a 6% decrease in AR level can be achieved at pH 7.5 with a product yield ~75%. In certain embodiments, as outlined in the Examples presented below, the total AR level (%) in Adalimumab product

pool can be reduced by over 10% with an acceptable processing yield (≥ 75%) from a CEX displacement chromatography process, or 4-7% from a mixed mode displacement chromatography process. Similarly, for mAb X, Figure 32 indicates that ΔAR% surprisingly increases from 3.3 to 6.5% as pH varies from 7 to 7.7 in a mixed mode displacement chromatography process.

In one embodiment, the product yield is greater than or equal to 70%. In another embodiment, the product yield is greater than or equal to 75%. In another embodiment, the product yield is greater than or equal to 65%, 66%, 67, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. In one embodiment, the product yield is about 70%. In another embodiment, the product yield is about 75%. In another embodiment, the product yield is about 65%, 66%, 67, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%.

In certain embodiments, conditions selected for reducing AR are also capable of reducing process-related impurities and/or other product-related substances. For example, but not by way of limitation, conditions selected for AR reduction are also capable of reducing process-related impurities, such as HCPs. In additional, non-limiting examples, conditions selected for AR reduction are also capable of reducing product-related impurities, such as aggregates and/or fragments.

In certain embodiments, displacement chromatography can be used as the sole method of purification of the protein of interest. In certain embodiments, displacement chromatography can be used in combination with other purification strategies, such as, but not limited to, the alternative purification techniques described herein, to reduce process-related impurities and/or other product-related substances. In one embodiment, displacement chromatography is used following Protein A affinity steps.

In certain embodiments, fractions are collected during the displacement step and are combined (pooled) after appropriate analysis to provide a protein preparation, which is also referred to herein as a purified or partially-purified sample, that contains a desired level of the protein of interest and which can include one or more process-related impurities and/or other product-related substances. In certain embodiments, one or more process monitoring tools can be used in connection with the techniques described herein to facilitate the identification of an effective product pooling strategy. In certain embodiments, such monitoring can

include on-line or in-line process monitoring. For example, but not by way of limitation, spectroscopy methods such as UV, NIR, FTIR, Fluorescence, and Raman may be used to monitor levels of product-related species, *e.g.,* acidic species and lysine variants, in an on-line, at line or in-line mode. These methods allow for the production of data that can then be used to control the level of product-related species in the pooled material collected. In certain embodiments, specific signals arising from the chemical modification of the proteins such as glycation, MGO modification, deamidation, glycosylation may be specifically measurable by spectroscopic methods through such in-line, on-line or at-line methods, enabling real time or near-real time control of product quality of the resulting product.

In certain embodiments, the purification and/or pooling techniques described herein allow for the reduction of process-related impurities and/or other product-related substances. In certain embodiments, the purification and/or pooling techniques described herein allow for reduction of process-related impurities and the selective inclusion of particular product-related substances. For example, but not by way of limitation, the purification and/or pooling techniques described herein allow for modulation of the concentration of product-related substances in the purified sample, *e.g.,* increasing or decreasing the amount of AR and/or basic species. In certain embodiments, the concentration of particular AR and/or basic species, *e.g.,* Lys 0, Lys 1, and/or Lys 2, are modulated (increased or decreased) in the purified sample. In certain embodiments, such techniques can be used to ensure product uniformity over the course of multiple production runs.

Anion Exchange (AEX) Chromatography

In certain embodiments, the low AR compositions of the invention are produced by subjecting a primary protein recovery sample to at least one anion exchange separation step after the above-described displacement chromatography step. In another embodiment, the anion exchange step will occur before the above-described displacement chromatography step. In one embodiment, the anion exchange chromatography step will occur after the above-described Protein A affinity and displacement chromatography steps.

The use of an anionic exchange material versus a cationic exchange material, such as those cation exchange materials discussed in detail below, is based on the local charges of the protein of interest in a given solution. 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 an anionic exchange step, only an cationic exchange step, or any serial combination of the two (including serial combinations of one or both ion exchange steps with the other chromatographic separation technologies described herein).

In performing the separation, the initial protein composition can be contacted with the anion exchange material by using any of a variety of techniques, *e.g.*, using a batch purification technique or a chromatographic technique.

For example, in the context of batch purification, anion exchange material is prepared in, or equilibrated to, the desired starting buffer. Upon preparation, or equilibration, a slurry of the anion exchange material is obtained. The protein of interest, *e.g.*, antibody, solution is contacted with the slurry to allow for protein adsorption to the anion exchange material. The solution comprising the acidic species that do not bind to the AEX material is separated from the slurry, *e.g.*, by allowing the slurry to settle and removing the supernatant. The slurry can be subjected to one or more washing steps and/or elution steps.

In the context of chromatographic separation, a chromatographic apparatus, commonly cylindrical in shape, is employed to contain the chromatographic support material (*e.g.*, AEX material) prepared in an appropriate buffer solution. The chromatographic apparatus, if cylindrical, can have a diameter of about 5 mm to about 2 meters, and a height of 5 cm to 50 cm, and in certain embodiments, particularly for large scale processing, a height of $\leq$ 30 cm is employed. Once the chromatographic material is added to the chromatographic apparatus, a sample containing the protein of interest, *e.g.*, an antibody, is contacted to the chromatographic material to induce the separation. Any portion of the solution that does not bind to the chromatographic material, *e.g.*, which may comprise, depending on the AEX material being employed, the protein of interest, acidic species, is separated from the chromatographic material by washing the material and collecting fractions from column. The chromatographic material can be subjected to one or more wash steps. If desired, the chromatographic material can then be contacted with a solution designed to desorb any components of the solution that have bound to the chromatographic material.

In certain embodiments, a wash step can be performed in the context of AEX chromatography using conditions similar to the load conditions or alternatively by decreasing the pH and/or increasing the ionic strength/conductivity of the wash in a step wise or linear gradient manner. The resulting Flow Through and wash fractions can be analyzed and appropriate fractions pooled to achieve the desired reduction in charged variant species. In certain embodiments, the aqueous salt solution used as both the loading and wash buffer has a

pH that at or near the isoelectric point (pI) of the protein of interest. In certain embodiments the pH is about 0 to 2 units higher or lower than the pI of the protein of interest. In certain embodiments, it will be in the range of 0 to 0.5 units higher or lower. In certain embodiments, it will be at the pI of the antibody.

In certain non-limiting embodiments, the anionic agent is selected from the group consisting of acetate, formate, or combinations thereof. In certain non-limiting embodiments, the cationic agent is selected from the group consisting of Tris, arginine, or combinations thereof. In one embodiment, the buffer solution is a Tris/formate buffer. In another embodiment, the buffer is selected from the group consisting of pyridine, piperazine, L-histidine, Bis-tris, Bis-tris propane, imidazole, N-Ethylmorpholine, TEA (triethanolamine), Tris, Morpholine, N-Methyldiethanolamine, AMPD (2-amino-2-methyl-1,3-propanediol), diethanolamine, ethanolamine, AMP (2-amino-2-methyl-1-propaol), piperazine, 1,3-Diaminopropane and piperidine.

A packed anion-exchange chromatography column, anion-exchange membrane device, anion-exchange monolithic device, or depth filter media can be operated either in bind-elute mode, flow-through mode, or a hybrid mode wherein the product exhibits binding to the chromatographic material, yet can be washed from the column using a buffer that is the same or substantially similar to the loading buffer. In the bind-elute mode, the column or the membrane device is first conditioned with a buffer with appropriate ionic strength and pH under conditions where certain proteins will be immobilized on the resin based matrix. For example, in certain embodiments, during the feed load, the protein of interest will be adsorbed to the resin due to electrostatic attraction. After washing the column or the membrane device with the equilibration buffer or another buffer with different pH and/or conductivity, the product recovery is achieved by increasing the ionic strength (*i.e.*, conductivity) of the elution buffer to compete with the solute for the charged sites of the anion 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 or pH may be gradual (gradient elution) or stepwise (step elution). In the flow-through mode, the column or the membrane device is operated at selected pH and conductivity such that the protein of interest does not bind to the resin or the membrane while the acidic species will either be retained on the column or will have a distinct elution profile as compared to the protein of interest. In the context of this hybrid strategy, acidic species will bind to the chromatographic material (or Flow Through) in a manner distinct from the protein of interest,

e.g., while the protein of interest and certain aggregates and/or fragments of the protein of interest may bind the chromatographic material, washes that preferentially remove the protein of interest can be applied. The column is then regenerated before next use.

Non-limiting examples of anionic exchange substituents include diethylaminoethyl (DEAE), quaternary aminoethyl (QAE) and quaternary amine (Q) groups. Additional non-limiting examples include: Poros 50PI and Poros 50HQ, which are a rigid polymeric bead with a backbone consisting of cross-linked poly[styrene-divinylbenzene]; Capto Q Impres and Capto DEAE, which are a high flow agarose bead; Toyopearl QAE-550, Toyopearl DEAE-650, and Toyopearl GigaCap Q-650, which are a polymeric base bead; Fractogel® EMD TMAE Hicap, which is a synthetic polymeric resin with a tentacle ion exchanger; Sartobind STIC® PA nano, which is a salt-tolerant chromatographic membrane with a primary amine ligand; Sartobind Q nano; which is a strong anion exchange chromatographic membrane; CUNO BioCap; which is a zeta-plus depth filter media constructed from inorganic filter aids, refined cellulose, and an ion exchange resin; and X0HC, which is a depth-filter media constructed from inorganic filter aid, cellulose, and mixed cellulose esters. The detailed information is listed in Table A.

Table A: List of AEX Adsorbent Properties

AEX Adsorbent	Vendor	Media Type	Ligand Type	Particle/ Pore Size	Catalog Number
Poros PI	Applied Biosystems	Resin	Weak	~50µm	1-2459-11
Poros HQ			Strong	~50µm	1-2559-11
Capto DEAE	GE		Weak	~90µm	17-5443-10
CaptoQ Impres			Strong	~90µm	17-5316-10
QAE-550	Tosoh		Strong	~100µm	43271
DEAE-650			Weak	~65µm	43201
GigaCap Q-650			Strong	~75µm	21854
TMAE HiCap	EMD/Millipore		Strong	~40-90µm	1.16881.0013
Sartobind STIC® PA Nano	Sartorius	Membrane	Weak	3-5µm	92STPA42DN-11—A
Sartobind Q Nano			Strong	3-5µm	92IEXQ42DN-11
CUNO BioCap 25	3M	Depth Filter	NA	NA	BC0025L60ZA05A
X0HC	Millipore		NA	NA	MX0HC23CL3

In certain embodiments, the protein load of the mixture comprising protein of interest is adjusted to a total protein load to the column of between about 50 and 500 g/L, or between about 75 and 350 g/L, or between about 200 and 300 g/L. In certain embodiments, the protein concentration of the load protein mixture is adjusted to a protein concentration of the material loaded to the column of about 0.5 and 50 g/L, between about 1 and 20 g/L, or between 3 and 10 g/L. In certain embodiments, the protein concentration of the load protein mixture is adjusted to a protein centration of the material to the column of about 37 g/L.

In certain embodiments, additives such as poly ethylene glycol, detergents, amino acids, sugars, chaotropic agents can be added to enhance the performance of the separation, so as to achieve better recovery or product quality.

In certain embodiments, including, but not limited to those relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR in the Flow Through and wash fractions while enriching for

the same in the flow elution fraction, thereby producing protein compositions that have reduced AR or are free of AR. In certain embodiments relating to the purification of adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR1 charge variants in the Flow Through and wash fractions while enriching for the same in the flow elution fraction, thereby producing protein compositions that have reduced AR1 or are free of AR1 variants. In certain embodiments relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR2 charge variants in the flow-through and wash fractions while enriching for the same in the flow elution fraction, thereby producing protein compositions that have reduced AR2 or are free of AR2 variants.

In certain embodiments, including but not limited to those relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of the MGO variants in the Flow Through and wash fractions while enriching for the same in the elution fraction, thereby producing protein compositions that have reduced MGO or are free of MGO variants (for example, see U.S. Patent Application Serial No. 61/777,883, filed on March 12, 2013). In certain embodiments, including, but not limited to those relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of the glycated variants (Schiff's base and permanently glycated forms) in the Flow Through and wash fractions while enriching for the same in the elution fraction, thereby producing protein preparations with reduced or free of glycated variants.

In certain embodiments, the loading, pH, conductivity of the AEX chromatography step, as well as elution pH conductivity, can be modified to achieve a desired distribution of product-relates substances (AR or lysine variants) For example, but not by way of limitation, certain embodiments are directed to the modulation of the lysine distribution of purified sample of a protein of interest, *e.g.*, increasing Lys 0 and decreasing Lys 1 and Lys 2. In certain embodiments, the methods of the present invention allow for the preparation of samples wherein the amount of Lys 0 is decreased, while the amount of Lys 1 and/or Lys 2 is increased.

In certain embodiments, an AEX chromatographic separation can be performed and combinations of fractions can be pooled to achieve a combination of desired process-related impurity and/or product-relates substance levels, in addition to, or in place of merely modulating charge variant concentration.

Spectroscopy methods such as UV, NIR, FTIR, Fluorescence, and Raman may be used to monitor levels of AR species in an on-line, at-line or in-line mode, which can then be used to control the level of charge variants, *e.g.*, acidic species, in the pooled material collected from the AEX effluent.

In certain embodiments, specific signals arising from the chemical modification of the proteins such as glycation, MGO modification, deamidation, glycosylation may be specifically measurable by spectroscopic methods through such in-line, on-line or at-line methods, enabling realtime or near-real time control of product quality of the resulting product. In certain embodiments, on-line, at-line or in-line monitoring methods can be used either on the effluent line of the chromatography step or in the collection vessel, to enable achievement of the desired product quality/recovery. In certain embodiments, the UV signal can be used as a surrogate to achieve an appropriate product quality/recovery, wherein the UV signal can be processed appropriately, including, but not limited to, such processing techniques as integration, differentiation, moving average, such that normal process variability can be addressed and the target product quality can be achieved. In certain embodiments, such measurements can be combined with in-line dilution methods such that ion concentration/conductivity of the load/wash can be controlled by feedback and hence facilitate product quality control.

In certain embodiments, a combination of AEX and CEX and MM methods can be used to prepare product-related substance-modulated materials, including certain embodiments where one technology is used in a complementary/supplementary manner with another technology. In certain embodiments, such a combination can be performed such that certain sub-species are removed predominantly by one technology, such that the combination provides the desired final composition/product quality. In certain embodiments, such combinations include the use of additional intervening chromatography, filtration, pH adjustment, and/or UF/DF steps so as to achieve the desired AR, product quality, ion concentration, and/or viral reduction.

AEX chromatography can be used in conjunction with recycle chromatography modes and continuous chromatography modes.

Cation Exchange (CEX) Chromatography

The low AR compositions of the invention can be produced by subjecting the composition, *e.g.,* a primary recovery sample, to at least one cation exchange separation step

after the above-described displacement chromatography step. In another embodiment, the cation exchange step will occur before the above-described displacement chromatography step. In one embodiment, the cation exchange chromatography step will occur after the above-described Protein A affinity and displacement chromatography steps.

The use of a cationic exchange material versus an anionic exchange material, such as those anionic exchange materials discussed in detail above, is based on the local charges of the protein of interest in a given solution. Therefore, it is within the scope of this invention to employ a cationic exchange step prior to the use of an anionic exchange step, or an anionic exchange step prior to the use of a cationic 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 (including serial combinations of one or both ion exchange steps with the other chromatographic separation technologies described herein).

In performing the separation, the initial protein mixture can be contacted with the cation exchange material by using any of a variety of techniques, *e.g.*, using a batch purification technique or a chromatographic technique, as described above in connection with Protein A or AEX.

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. In certain embodiments, the pH is about 0 to 5 units lower than the pI of the protein. In certain embodiments, it is in the range of 1 to 2 units lower. In certain embodiments, it is in the range of 1 to 1.5 units lower.

In certain embodiments, the concentration of the anionic agent in aqueous salt solution is increased or decreased to achieve a pH of 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. Buffer systems suitable for use in the CEX methods include, but are not limited to, tris formate, tris acetate, ammonium sulfate, sodium chloride and sodium sulfate.

In certain embodiments, the conductivity and pH of the aqueous salt solution is adjusted by increasing or decreasing the concentration of a cationic agent. In certain embodiments, the cationic agent is maintained at a concentration of between about range of 20mM to 500mM, or between about 50 to 350mM or between about 100 to 300mM or

between about 100 to 200mM. In certain non-limiting embodiments, the cationic agent is selected from the group consisting of sodium, Tris, tromethalmine, ammonium, arginine, or combinations thereof. In certain non-limiting embodiments, the anionic agent is selected from the group consisting of formate, acetate, citrate, chloride anion, sulphate, phosphate or combinations thereof.

A packed cation-exchange chromatography column or a cation-exchange membrane device can be operated either in bind-elute mode, flow-through mode, or a hybrid mode wherein the product exhibits binding to the chromatographic material, yet can be washed from the column using a buffer that is the same or substantially similar to the loading buffer. The details of these modes are outlined above.

Cationic substituents include carboxymethyl (CM), sulfoethyl (SE), sulfopropyl (SP), phosphate (P) and sulfonate (S). Additional cationic materials include, but are not limited to: Capto SP ImpRes, which is a high flow agarose bead; CM Hyper D grade F; which is a ceramic bead coated and permeated with a functionalized hydrogel, 250 – 400 ionic groups μeq/mL; Eshmuno S, which is a hydrophilic polyvinyl ether base matrix with 50-100 μeq/mL ionic capacity; Nuvia C Prime, which is a hydrophobic cation exchange media composed of a macroporous highly crosslinked hydrophilic polymer matrix 55-75 μeq/mL; Nuvia S, which has a UNOsphere base matrix with 90 -150μeq/mL ionic groups; Poros HS; which is a rigid polymetic bead with a backbone consisting of cross-linked poly[styrene-divinylbenzene]; Poros XS; which is a rigid polymetic bead with a backbone consisting of cross-linked poly[styrene-divinylbenzene]; Toyo Pearl Giga Cap CM 650M, which is a polymeric base bead with 0.225meq/mL ionic capacity; Toyo Pearl Giga Cap S 650M which is a polymeric base bead; Toyo Pearl MX TRP, which is a polymeric base bead. Detailed information concerning the aforementioned materials is listed in Table B. It is noted that CEX chromatography can be used with MM resins, described herein.

Table B: Cationic Materials

Resin	Vendor	Type	Particle Size	Catalog Number
Capto SP ImpRes	GE	Strong	~40µm	17-5468-10
CM Hyper D	Pall	Weak	~50µm	20050-027
Eshmuno S	Millipore	Strong	~85µm	1.20078
Nuvia C Prime	Biorad	Mix Mode	~70µm	156-3401
Nuvia S	Biorad	Strong	~85µm	156-0315
Poros HS	Applied Biosystems	Weak	~50µm	13359-06
Poros XS	Applied Biosystems	Strong	~50µm	4404337
Toyo Pearl Giga Cap CM 650M	Tosoh	Weak	~75µm	21946
Toyo Pearl Giga Cap S 650M	Tosoh	Strong	~75µm	21833
Toyo Pearl MX Trp 650M	Tosoh	Mix Mode	~75µm	22817

In certain embodiments, the protein load of the mixture comprising protein of interest is adjusted to a total protein load to the column of between about 5 and 150 g/L, or between about 10 and 100 g/L, between about 20 and 80 g/L, between about 30 and 50 g/L, or between about 40 and 50 g/L. In certain embodiments, the protein concentration of the load protein mixture is adjusted to a protein concentration of the material loaded to the column of about 0.5 and 50 g/L, or between about 1 and 20 g/L.

In certain embodiments, additives such as poly ethylene glycol, detergents, amino acids, sugars, chaotropic agents can be added to enhance the performance of the separation, so as to achieve better recovery or product quality.

In certain embodiments, including, but not limited to those relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR in the Flow Through and wash fractions while enriching for the same in the elution fraction, thereby producing protein compositions that have reduced

AR or are free of AR. In certain embodiments relating to the purification of adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR1 charge variants in the Flow Through and wash fractions while enriching for the same in the flow elution fraction, thereby producing protein compositions that have reduced AR1 or are free of AR1 variants. In certain embodiments relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR2 charge variants in the flow-through and wash fractions while enriching for the same in the flow elution fraction, thereby producing protein compositions that have reduced AR2 or are free of AR2 variants.

In certain embodiments, including, but not limited to those relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of the MGO variants in the elution fractions while enriching for the same in the Flow Through and wash fractions, thereby producing protein preparations with reduced or free of MGO variants. In certain embodiments, including, but not limited to those relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of the glycated variants (Schiff's base and permanently glycated forms) in the elution fractions while enriching for the same in the Flow Through and wash fractions, thereby producing protein preparations with reduced or free of glycated variants.

In certain embodiments, the loading, pH, conductivity of the CEX chromatography step, as well as elution pH conductivity, can be modified to achieve a desired distribution of acidic species. For example, but not by way of limitation, certain embodiments are directed to the modulation of the lysine distribution of a purified sample of a protein of interest, *e.g.*, increasing Lys 0 and decreasing Lys 1 and Lys 2. In certain embodiments, the methods of the present invention allow for the preparation of samples wherein the amount of Lys 0 is decreased, while the amount of Lys 1 and/or Lys 2 is increased.

In certain embodiments, a CEX chromatographic separation can be performed and combinations of fractions can be pooled to achieve a combination of desired process-related impurity and/or product-relates substance levels, in addition to, or in place of merely modulating charge variant concentration.

In certain embodiments, spectroscopy methods such as UV, NIR, FTIR, Fluorescence, Raman may be used to monitor levels of product-related charge variants, aggregates, low molecular weight variants (*e.g.*, fragments of the protein of interest) in an on-line, at-line or

in-line mode, which can then be used to control the level of charge variants, *e.g.*, acidic species, in the pooled material collected from the CEX effluent. In certain embodiments, specific signals arising from the chemical modification of the proteins such as glycation, MGO modification, deamidation, glycosylation may be specifically measurable by spectroscopic methods through such in-line, on-line or at-line methods, enabling realtime or near-real time control of product quality of the resulting product. In certain embodiments, on-line, at-line or in-line monitoring methods can be used either on the effluent line of the chromatography step or in the collection vessel, to enable achievement of the desired product quality/recovery. In certain embodiments, the UV signal can be used as a surrogate to achieve an appropriate product quality/recovery, wherein the UV signal can be processed appropriately, including, but not limited to, such processing techniques as integration, differentiation, moving average, such that normal process variability can be addressed and the target product quality can be achieved. In certain embodiments, such measurements can be combined with in-line dilution methods such that ion concentration/conductivity of the load/wash can be controlled by feedback and hence facilitate product quality control.

In certain embodiments, a combination of CEX and AEX and/or MM methods can be used to prepare product-related substance-modulated materials, including certain embodiments where one technology is used in a complementary/supplementary manner with another technology. In certain embodiments, such a combination can be performed such that certain sub-species are removed predominantly by one technology, such that the combination provides the desired final composition/product quality. In certain embodiments, such combinations include the use of additional chromatography, filtration, pH adjustment, UF/DF steps so as to achieve the desired product quality, AR, ion concentration, and/or viral reduction.

CEX chromatography can be used in conjunction with recycle chromatography and continuous chromatography modes.

Mixed Mode Chromatography

Mixed mode ("MM") chromatography may also be used to prepare the low AR compositions of the invention. MM chromatography, also referred to herein as "multimodal chromatography", is a chromatographic strategy that utilizes a support comprising a ligand that is capable of providing at least two different, and in certain embodiments co-operative, sites that interact with the substance to be bound. In certain embodiments, one of these sites

gives an attractive type of charge-charge interaction between the ligand and the substance of interest and the other site provides for electron acceptor-donor interaction and/or hydrophobic and/or hydrophilic interactions. Electron donor-acceptor interactions include interactions such as hydrogen-bonding, π-π, cation- π, charge transfer, dipole-dipole, induced dipole etc.

In certain embodiments, the resin employed for a mixed mode separation is Capto Adhere. Capto Adhere is a strong anion exchanger with multimodal functionality. Its base matrix is a highly cross-linked agarose with a ligand (N-Benzyl-N-methyl ethanol amine) that exhibits many functionalities for interaction, such as ionic interaction, hydrogen bonding and hydrophobic interaction. In certain embodiments, the resin employed for a mixed mode separation is selected from PPA-HyperCel and HEA-HyperCel. The base matrices of PPA-HyperCel and HEA-HyperCel are high porosity cross-linked cellulose. Their ligands are Phenylpropylamine and Hexylamine, respectively. Phenylpropylamine and Hexylamine offer different selectivity and hydrophobicity options for protein separations. Additional mixed mode chromatographic supports include, but are not limited to, Nuvia C Prime, Toyo Pearl MX Trp 650M, and Eshmuno® HCX.

In certain embodiments, the mixed mode chromatography resin is comprised of ligands coupled to an organic or inorganic support, sometimes denoted a base matrix, directly or via a spacer. The support may be in the form of particles, such as essentially spherical particles, a monolith, filter, membrane, surface, capillaries, etc. In certain embodiments, the support is prepared from a native polymer, such as cross-linked carbohydrate material, such as agarose, agar, cellulose, dextran, chitosan, konjac, carrageenan, gellan, alginate etc. To obtain high adsorption capacities, the support can be porous, and ligands are then coupled to the external surfaces as well as to the pore surfaces. Such native polymer supports can be prepared according to standard methods, such as inverse suspension gelation (S Hjerten: Biochim Biophys Acta 79(2), 393-398 (1964). Alternatively, the support can be prepared from a synthetic polymer, such as cross-linked synthetic polymers, *e.g.* styrene or styrene derivatives, divinylbenzene, acrylamides, acrylate esters, methacrylate esters, vinyl esters, vinyl amides etc. Such synthetic polymers can be produced according to standard methods, *see e.g.* "Styrene based polymer supports developed by suspension polymerization" (R Arshady: Chimica e L'Industria 70(9), 70-75 (1988)). Porous native or synthetic polymer supports are also available from commercial sources, such as Amersham Biosciences, Uppsala, Sweden.

In certain embodiments, the protein load of the mixture comprising protein of interest is adjusted to a total protein load to the column of between about 50 and 750 g/L, or between about 75 and 500 g/L, or between about 100 and 300 g/L. In certain embodiments, the protein concentration of the load protein mixture is adjusted to a protein concentration of the material loaded to the column of about 1 and 50 g/L, or between about 9 and 25 g/L.

In certain embodiments, additives such as poly ethylene glycol, detergents, amino acids, sugars, chaotropic agents can be added to enhance the performance of the separation, so as to achieve better recovery or product quality.

In certain embodiments, including, but not limited to those relating to adalimumab, the MM methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR in the Flow Through and wash fractions while enriching for the same in the flow elution fraction, thereby producing protein compositions that have reduced AR or are free of AR. In certain embodiments relating to the purification of adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR1 charge variants in the Flow Through and wash fractions while enriching for the same in the flow elution fraction, thereby producing protein compositions that have reduced AR1 or are free of AR1 variants. In certain embodiments relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of AR2 charge variants in the flow-through and wash fractions while enriching for the same in the flow elution fraction, thereby producing protein compositions that have reduced AR2 or are free of AR2 variants.

In certain embodiments, including, but not limited to those relating to adalimumab, the MM methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of the MGO variants in the Flow Through and wash fractions while enriching for the same in the elution fraction, thereby producing protein preparations with reduced or free of MGO variants. In certain embodiments, including, but not limited to those relating to adalimumab, the methods of the instant invention can be used to selectively remove, significantly reduce, or essentially remove all of the glycated variants (Schiff's base and permanently glycated forms) in the Flow Through and wash fractions while enriching for the same in the elution fraction, thereby producing protein preparations with reduced or free of glycated variants.

In certain embodiments, the loading, pH, conductivity of the MM chromatography step, wash pH and conductivity, as well as elution pH conductivity, can be modified to

achieve a desired distribution of acidic species. For example, but not by way of limitation, certain embodiments are directed to the modulation of the lysine distribution of a purified sample of a protein of interest, *e.g.*, increasing Lys 0 and decreasing Lys 1 and Lys 2. In certain embodiments, the methods of the present invention allow for the preparation of samples wherein the amount of Lys 0 is decreased, while the amount of Lys 1 and/or Lys 2 is increased.

In certain embodiments, a MM chromatographic separation can be performed and combinations of fractions can be pooled to achieve a combination of desired process-related impurity and/or product-relates substance levels, in addition to, or in place of merely modulating charge variant concentration.

In certain embodiments, spectroscopy methods such as UV, NIR, FTIR, Fluorescence, Raman may be used to monitor levels of AR species in an on-line, at-line or in-line mode, which can then be used to control the level of charge variants, *e.g.*, acidic species, in the pooled material collected from the MM effluent. In certain embodiments, specific signals arising from the chemical modification of the proteins such as glycation, MGO modification, deamidation, glycosylation may be specifically measurable by spectroscopic methods through such in-line, on-line or at-line methods, enabling real time or near-real time control of product quality of the resulting product. In certain embodiments, on-line, at-line or in-line monitoring methods can be used either on the effluent line of the chromatography step or in the collection vessel, to enable achievement of the desired product quality/recovery. In certain embodiments, the UV signal can be used as a surrogate to achieve an appropriate product quality/recovery, wherein the UV signal can be processed appropriately, including, but not limited to, such processing techniques as integration, differentiation, moving average, such that normal process variability can be addressed and the target product quality can be achieved. In certain embodiments, such measurements can be combined with in-line dilution methods such that ion concentration/conductivity of the load/wash can be controlled by feedback and hence facilitate product quality control.

In certain embodiments, a combination of mixed mode and AEX and CEX methods can be used to prepare the low AR compositions of the invention, including certain embodiments where one technology is used in a complementary/supplementary manner with another technology. In certain embodiments, such a combination can be performed such that certain sub-species are removed predominantly by one technology, such that the combination provides the desired final composition/product quality. In certain embodiments, such

combinations include the use of additional intervening chromatography, filtration, pH adjustment, UF/DF steps so as to achieve the desired product quality, AR, ion concentration, and/or viral reduction.

MM chromatography can be used in conjunction with recycle chromatography and continuous chromatography modes.

Continuous and Recycle Chromatography

Continuous and recycle chromatography modes can be used to produce the low AR compositions of the invention, and are described below. These methods result in significant improvements in recovery of the protein, *e.g.,* antibody, of interest while maintaining the AR reduction levels. These continuous and recycle chromatography modes are applicable to chromatography methods where (a) the low acidic species component of interest is collected in the unbound fraction during the chromatography (Flow Through/wash chromatography) or (b) where the low acidic species component of interest is first bound to the media and subsequently recovered by washing the media with conditions that elute the bound component.

Continuous and Recycle Chromatography -- Flow Through/Wash Chromatography

In the case where the low acidic species component of interest is collected in the unbound fraction, the following approach is employed which prevents loss of the material loaded on the column.

In one embodiment, a recycle chromatography mode is used wherein the column is loaded and the unbound fractions that results in the target AR level are collected. Subsequently, instead of regenerating the column and losing the product, the column is washed under conditions that result in recovery of the product remaining bound to the column. This product recovered under these conditions contains significantly higher AR levels than the original feed material. This wash fraction is adjusted to the appropriate conditions to achieve the separation desired on subsequent processing (typically similar conditions to the initial preparation) and combined with the original feed material and loaded on the column again (after preparing the column appropriately for the next cycle). The amount of material prepared for the next cycle, combining the wash fraction from the first

cycle and the fresh material is adjusted to the target loading capacity for the column to achieve the desired separation (typically similar to the capacity targeted for the first cycle).

In performing the second cycle, a similar strategy is employed, collecting the unbound fraction so as to achieve the target AR level and then subsequently washing the column under conditions to recover the product remaining on the column.

In one embodiment, this recycle chromatography mode is continued until all the load materials are used. The number of cycles can be controlled by designing the column size appropriately.

In employing the recycle chromatography mode, the recovery of the product loaded on the column is significantly improved while achieving the target AR levels.

Several variations of the recycle chromatography mode can be employed. In one embodiment, the fractions that are collected targeting a certain AR level can be determined based on predetermined criteria or based on at-line, off-line or on-line analysis of the effluent of the column or the collected pool.

In another embodiment, the wash conditions used for the first cycle can be adjusted to recover the desired amount of product at the desired product quality, only limited by the feasibility of preparing an appropriate load mixture for the subsequent step. In one aspect of this embodiment, the wash condition may be similar to the load condition. In another aspect of this embodiment, the wash condition can be stringent to recover all of the product species (desired and undesired) remaining on the column.

In still another embodiment, the loading amount, the loading conditions and the washing conditions used for the subsequent cycles can be modified to achieve the desired purity, given that that loading material for the subsequent cycles are likely to contain higher levels of AR.

In another embodiment, the last cycle of the operation can be performed under different conditions such that the target purity and target recovery can be achieved to optimize overall recovery and purity.

The methods for producing the low AR composition of the invention can also be implemented in a continuous chromatography mode. In this mode, at least two columns are employed (referred to as a "first" column and a "second" column). In one embodiment, the feed material is loaded onto the first column, and the unbound fraction from the first column is collected such that the pool material contains the target AR level. The column is then washed under conditions that recover the remaining product. This material is then

dynamically diluted with appropriate solutions to achieve the desired loading conditions, mixed with fresh feed material and directed to the second column. The unbound fraction from the second column is collected to achieve the target AR level. The second column is then washed under conditions to recover the product and diluted with appropriate solutions, mixed with fresh materials dynamically and directed to the first column (which is prepared to receive the load after regeneration/cleaning). In one embodiment, this cycling is continued until all the load material is used. The last cycle can be operated in a "typical" mode, with appropriate adjustments to the load and wash conditions as necessary.

In certain embodiments this continuous chromatography mode can be carried out such that the wash material containing the higher AR levels can be directed back into the load tank after appropriate dilution. This material can then be loaded subsequently or concurrently onto the second column, such that the operation of the two columns are not in tandem, reducing complexity of the operation.

This continuous chromatography mode, while similar to the recycle chromatography mode, can be carried out more efficiently, and therefore has a reduced processing time.

For this continuous chromatography mode, several variations can be employed. In one embodiment, the fractions that are collected targeting a certain AR level can be determined based on predetermined criteria or based on at-line, off-line or on-line analysis of the effluent of the column or the collected pool.

In another embodiment, the wash conditions used for the first cycle can be adjusted to recover the desired amount of product at the desired product quality, only limited by the feasibility of preparing an appropriate load mixture for the subsequent step. In one aspect of this embodiment, the wash conditions may be similar to the load conditions. In another aspect of the embodiment, the wash conditions can be stringent to recover all of the product species (desired and undesired) remaining on the column.

In still another embodiment, the loading amount, the loading conditions and the washing conditions used for the subsequent cycles can be modified to achieve the desired purity, given that that loading material for the subsequent cycles are likely to contain higher levels of AR.

In another embodiment, the last cycle of the operation can be performed under different conditions such that the target purity/recovery can be achieved to optimize overall recovery and and/or purity.

In one embodiment, the media choice for the recycle or continuous modes can be one of many chromatographic resins with pendant hydrophobic and anion exchange functional groups, monolithic media, membrane adsorbent media or depth filtration media.

In certain embodiments, membrane or depth filter based media ("convective media") can be used in the recycle or continuous chromatography modes because selectivity of separation is not required to be high given the fact that the less enriched portions of the product are "recycled" while the pure fractions are selectively pooled.

Continuous and Recycle Chromatography -- Elution Chromatography

In the elution mode of chromatography or separation, as exemplified by the CEX technology for AR reduction, the conditions are chosen for the load and wash steps such that the AR enriched material is collected in the Flow Through and/or wash fractions, while the AR reduced material is collected in the elution fraction. In the typical implementation of the CEX technology, about 10 to 40% of the product (the desired charge variant) may be lost in the Flow Through/Wash fractions. Two modes of operation, namely the recycle chromatography mode and the continuous chromatography mode provide improved recovery, while maintaining the target AR levels.

In the recycle chromatography mode, the load material is, in general, processed over multiple cycles. In implementing the recycle chromatography mode, the load material is prepared such that the eluate contains the target product purity or AR level. Under these conditions, the AR enriched material is collected in the Flow Through/wash fractions. This material is pooled and additional fresh load material is added to achieve the appropriate loading capacity for the next cycle of chromatography on the same column. In particular, in one embodiment, the column is eluted under conditions where the bound product (having low AR levels) is recovered, and subsequently regenerated and equilibrated to prepare for the next cycle.

In the next cycle, the combined load (Flow Through/wash from cycle 1 above, as well as fresh material) is loaded to the target capacity. The Flow Through/wash fractions are collected and pooled. The column is eluted to obtain the second eluate, again containing the target low AR composition. In one embodiment, this sequence is continued until all the load materials are processed.

In another embodiment, by implementing the recycle chromatography mode, the material that would otherwise be discarded as AR enriched material is further purified to

"recover" pure protein product, thereby improving the overall recovery of the protein. In one embodiment, the level of recovery depends on the number of cycles employed.

For the recycle chromatography mode, several variations can be employed. In one embodiment, the entire pool of the Flow Through/wash fractions are preferably combined with fresh materials to maximize recovery of the entire operation. However, a portion of the flow through wash can be discarded to achieve higher purity or efficiency. For example, in one embodiment, certain fractions containing very high levels of AR species can be discarded. To enable such selective pooling, off-line, in-line or at line methods can be used to directly or indirectly measure the levels of AR.

In another embodiment, the loading amount and the conditions for loading, washing and eluting can be modified for the second and subsequent cycles to accommodate the higher levels of AR that will be present in the loading pool.

In still another embodiment, the last cycle of the method can be performed under conditions such that the target purity and recovery can be achieved to optimize overall recovery and purity.

A continuous chromatography mode provides additional advantages in terms of time efficiency. In this mode of operation, two or more columns are used. Specifically, as with the recycle mode, an appropriate condition for the load capacity, load, wash and elution conditions are chosen for the operation. The Flow Through and wash fractions (or a portion thereof) is directed to the load tank containing the fresh material. After completion of the load and wash steps, the first column is eluted and subsequently regenerated. Meanwhile, the second column is loaded with the material that is a mix of fresh material and the wash and Flow Through from the previous cycle. The wash and Flow Through from the second column is again directed back to the load tank. The second column is then eluted and regenerated. The first column is then ready to be loaded and the cycle continues. This continuous chromatography mode is efficient as the product is processed continuously and the purified product is obtained in a semi-continuous manner.

Several variations of the continuous chromatography mode can be employed. In one embodiment, the entire pool of the Flow Through/wash fractions is combined with fresh materials to maximize recovery of the entire operation. However, a portion of the Flow Through wash can be discarded to achieve higher purity or efficiency. For example, certain fractions containing very high levels of AR species can be discarded. To enable such

selective pooling, off-line, in-line or at line methods can be used to measure directly or indirectly the levels of acidic species.

In another embodiment, the loading amount, conditions for loading, washing and eluting can be modified for the second and subsequent cycles to accommodate the higher levels of AR that will be present in the loading pool.

In still another embodiment, the last cycle of the operation can be performed under different conditions to optimize overall recover and purity.

The recycle chromatography mode and the continuous chromatography mode are not limited to use with any particular chromatography resin. The media used for the recycle or continuous modes can be one of many chromatographic resins with pendant hydrophobic and anion exchange functional groups, monolithic media, membrane adsorber media or depth filtration media.

In certain embodiments, membrane depth filter-based media ("convective media") can be used with the recycle or continuous modes as the selectivity of separation is not required to be high given the fact that the less enriched portions of the product are "recycled" while the pure fractions are selectively pooled.

Recycle chromatography mode and the continuous chromatography mode can be used inconjunction with AEX, CEX, or MM chromatography methods, as described herein, to produce the low AR compositions of the invention.

Hydrophobic Interaction Chromatography

The low AR compositions of the invention may also be prepared using a hydrophobic interaction chromatography (HIC) step in addition to the displacement chromatography step.

In performing the separation, the sample mixture is contacted with the HIC material, *e.g.*, using a batch purification technique or using a column or membrane chromatography. Prior to HIC purification it may be desirable to adjust the concentration of the salt buffer to achieve desired protein binding to the resin or the membrane.

Whereas ion exchange chromatography relies on the local charge of the protein of interest for selective separation, hydrophobic interaction chromatography employs the hydrophobic properties of the proteins to achieve selective separation. Hydrophobic groups on the protein interact with hydrophobic groups of the resin or the membrane. The more hydrophobic a protein is the stronger it will interact with the column or the membrane. Thus

the HIC step removes process-related impurities (*e.g.*, HCPs) as well as product-related substances (*e.g.*, aggregates and fragments).

Like ion exchange chromatography, a HIC column or membrane device can also be operated in product a bind-elute mode, a flow-through, or a hybrid mode wherein the product exhibits binding to the chromatographic material, yet can be washed from the column using a buffer that is the same or substantially similar to the loading buffer. The details of these modes are outlined above in connection with AEX purification.

As hydrophobic interactions are strongest at high ionic strength, this form of separation is conveniently performed following salt elution step, such as those that are typically used in connection with ion exchange chromatography. Alternatively, salts can be added into a low salt level feed stream before this step. Adsorption of the antibody to a HIC column is favored by high salt concentrations, but the actual concentrations can vary over a wide range depending on the nature of the protein of interest, salt type and the particular HIC ligand chosen. Various ions can be arranged in a so-called soluphobic series depending on whether they promote hydrophobic interactions (salting-out effects) or disrupt the structure of water (chaotropic effect) and lead to the weakening of the hydrophobic interaction. Cations are ranked in terms of increasing salting out effect as Ba^{2+}; Ca^{2+}; Mg^{2+}; Li^{+}; Cs^{+}; Na^{+} ; K^{+}; Rb^{+}; NH_4^{+}, while anions may be ranked in terms of increasing chaotropic effect as PO_4^{3-}; SO_4^{2-}; $CH_3CO_3^{-}$; Cl^{-}; Br^{-}; NO_3^{-}; ClO_4^{-}; I^{-}; SCN^{-}.

In general, Na^{+}, K^{+} or NH_4^{+} sulfates effectively promote ligand-protein interaction in HIC. Salts may be formulated that influence the strength of the interaction as given by the following relationship: $(NH_4)_2SO_4 > Na_2SO_4 > NaCl > NH_4C1 > NaBr > NaSCN$. In general, salt concentrations of between about 0.75 M and about 2 M ammonium sulfate or between about 1 and 4 M NaCl are useful.

HIC media normally comprise a base matrix (*e.g.*, cross-linked agarose or synthetic copolymer material) to which hydrophobic ligands (*e.g.*, alkyl or aryl groups) are coupled. A suitable HIC media comprises an agarose resin or a membrane functionalized with phenyl groups (*e.g.*, a Phenyl Sepharose™ from GE Healthcare or a Phenyl Membrane from Sartorius). Many HIC resins are available commercially. Examples include, but are not limited to, Capto Phenyl, Phenyl Sepharose™ 6 Fast Flow with low or high substitution, Phenyl Sepharose™ High Performance, Octyl Sepharose™ High Performance (GE Healthcare); Fractogel™ EMD Propyl or Fractogel™ EMD Phenyl (E. Merck, Germany); Macro-Prep™ Methyl or Macro-Prep™ t-Butyl columns (Bio-Rad, California); WP HI-

Propyl (C3)™ (J. T. Baker, New Jersey); and Toyopearl™ ether, phenyl or butyl (TosoHaas, PA).

Viral Filtration

Viral filtration is a dedicated viral reduction step in the entire purification process. This step is usually performed post chromatographic polishing steps. Viral reduction can be achieved via the use of suitable filters including, but not limited to, Planova 20N™, 50 N or BioEx from Asahi Kasei Pharma, Viresolve™ filters from EMD Millipore, ViroSart CPV from Sartorius, or Ultipor DV20 or DV50™ filter from Pall Corporation. It will be apparent to one of ordinary skill in the art to select a suitable filter to obtain desired filtration performance.

Ultrafiltration/Diafiltration

Certain embodiments of the present invention employ ultrafiltration and diafiltration steps to further concentrate and formulate the protein of interest, *e.g.*, an antibody product, in addition to the displacement chromatography steps. Ultrafiltration is described in detail in: Microfiltration and Ultrafiltration: Principles and Applications, L. Zeman and A. Zydney (Marcel Dekker, Inc., New York, N.Y., 1996); and in: Ultrafiltration Handbook, Munir Cheryan (Technomic Publishing, 1986; ISBN No. 87762-456-9). One filtration process is Tangential Flow Filtration as described in the Millipore catalogue entitled "Pharmaceutical Process Filtration Catalogue" pp. 177-202 (Bedford, Mass., 1995/96). Ultrafiltration is generally considered to mean filtration using filters with a pore size of smaller than 0.1 μm. By employing filters having such small pore size, the volume of the sample can be reduced through permeation of the sample buffer through the filter membrane pores while proteins, such as antibodies, are retained above the membrane surface.

Diafiltration is a method of using membrane filters to remove and exchange salts, sugars, and non-aqueous solvents, to separate free from bound species, to remove low molecular-weight species, and/or to cause the rapid change of ionic and/or pH environments. Microsolutes are removed most efficiently by adding solvent to the solution being diafiltered at a rate approximately equal to the permeate flow rate. This washes away microspecies from the solution at a constant volume, effectively purifying the retained protein of interest. In certain embodiments of the present invention, a diafiltration step is employed to exchange the various buffers used in connection with the instant invention, optionally prior to further

chromatography or other purification steps, as well as to remove impurities from the protein preparations.

One of ordinary skill in the art can select appropriate membrane filter device for the UF/DF operation. Examples of membrane cassettes suitable for the present invention include, but not limited to, Pellicon 2 or Pellicon 3 cassettes with 10 kD, 30kD or 50 kD membranes from EMD Millipore, Kvick 10 kD, 30 kD or 50 kD membrane cassettes from GE Healthcare, and Centramate or Centrasette 10 kD, 30 kD or 50 kD cassettes from Pall Corporation.

Exemplary Purification Strategies

In certain embodiments, primary recovery can proceed by sequentially employing pH reduction, centrifugation, and filtration steps to remove cells and cell debris (including HCPs) from the production bioreactor harvest. In certain embodiments, the present invention is directed to subjecting a sample mixture from said primary recovery to Protein A affinity followed by displacement chromatography. Certain embodiments of the present invention will include further purification steps. Examples of additional purification procedures which can be performed prior to, during, or following the displacement chromatography method include ethanol precipitation, isoelectric focusing, reverse phase HPLC, chromatography on silica, chromatography on heparin Sepharose™, further anion exchange chromatography and/or further cation exchange chromatography, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (*e.g.*, using protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

Specific examples of such combinations of strategies is presented below, with specific data relating to particular combinations useful in the context of the instant invention included in Examples 1-8 and in Tables 80-87 and 76-78 of U.S. Provisional Application No. 61/893,068, entitled "Low Acidic Species Compositions and Methods for Producing and Using the Same", filed on October 18, 2013, the entire contents of which are expressly incorporated herein by reference.

In certain embodiments, the unbound Flow Through and wash fractions can be further fractionated and a combination of fractions providing a target product purity can be pooled.

In certain embodiments, the protein concentration can be adjusted to achieve a differential partitioning behavior between the antibody product and the product-related

substances, such that the purity and/or yield can be further improved. In certain embodiments, the loading can be performed at different protein concentrations during the loading operation to improve the product quality/yield of any particular purification step.

In certain embodiments, the column temperature can be independently varied to improve the separation efficiency and/or yield of any particular purification step.

In certain embodiments, the loading and washing buffer matrices can be different or composed of mixtures of chemicals, while achieving similar "resin interaction" behavior such that the above novel separation can be effected. For example, but not by way of limitation, the loading and washing buffers can be different, in terms of ionic strength or pH, while remaining substantially similar in function in terms of the washout of the product achieved during the wash step. In certain embodiments, additives such as amino acids, sugars, PEG, etc can be added to the load or wash steps to modulate the partitioning behavior to achieve the separation efficiency and/or yield.

In certain embodiments, the loading & washing steps can be controlled by in-line, at-line or off-line measurement of the product related impurity/substance levels, either in the column effluent, or the collected pool or both, so as to achieve the target product quality and/or yield. In certain embodiments, the loading concentration can be dynamically controlled by in-line or batch or continuous dilutions with buffers or other solutions to achieve the partitioning necessary to improve the separation efficiency and/or yield.

IV. Methods of Assaying Sample Purity

Assaying Host Cell Protein

The present invention also provides methods for determining the residual levels of host cell protein (HCP) concentration in the low AR compositions of the invention. As described above, HCPs are desirably excluded from the final target substance product. Exemplary HCPs include proteins originating from the source of the antibody production. Failure to identify and sufficiently remove HCPs from the target antibody may lead to reduced efficacy and/or adverse reactions in a subject.

As used herein, the term "HCP ELISA" refers to an ELISA where the second antibody used in the assay is specific to the HCPs produced from cells, *e.g.*, CHO cells, used to generate the antibody of interest. The second antibody may be produced according to conventional methods known to those of skill in the art. For example, the second antibody may be produced using HCPs obtained by sham production and purification runs, *i.e.*, the

same cell line used to produce the antibody of interest is used, but the cell line is not transfected with antibody DNA. In an exemplary embodiment, the second antibody is produced using HCPs similar to those expressed in the cell expression system of choice, *i.e.*, the cell expression system used to produce the target antibody.

Generally, HCP ELISA comprises sandwiching a liquid sample comprising HCPs between two layers of antibodies, *i.e.*, a first antibody and a second antibody. The sample is incubated during which time the HCPs in the sample are captured by the first antibody, for example, but not limited to goat anti-CHO, affinity purified (Cygnus). A labeled second antibody, or blend of antibodies, specific to the HCPs produced from the cells used to generate the antibody, *e.g.*, anti-CHO HCP Biotinylated, is added, and binds to the HCPs within the sample. In certain embodiments the first and second antibodies are polyclonal antibodies. In certain aspects the first and second antibodies are blends of polyclonal antibodies raised against HCPs. The amount of HCP contained in the sample is determined using the appropriate test based on the label of the second antibody.

HCP ELISA may be used for determining the level of HCPs in an antibody composition, such as an eluate or flow-through obtained using the process described above. The present invention also provides a composition comprising an antibody, wherein the composition has no detectable level of HCPs as determined by an HCP Enzyme Linked Immunosorbent Assay ("ELISA").

Assaying Acidic Species (AR)

The levels of acidic species in the chromatographic samples produced using the techniques described herein may be analyzed as described in the Examples section. In certain embodiments a CEX-HPLC method is employed. For example, but not by way of limitation, cation exchange chromatography can be performed on a Dionex ProPac WCX-10, Analytical column 4 mm x 250 mm (Dionex, CA). An Agilent 1200 HPLC system can then be used as the HPLC. In certain embodiments, mobile phases such as 10mM Sodium Phosphate dibasic pH 7.5 (Mobile phase A) and 10mM Sodium Phosphate dibasic, 500 mM Sodium Chloride pH 5.5 (Mobile phase B) can be used. In certain embodiments, a binary gradient (94% A, 6% B: 0-20 min; 84% A, 16% B: 20-22 min; 0% A, 100%B: 22-28 min; 94% A, 6% B: 28-34 min) can be used with detection at 280 nm. In certain embodiments, quantitation is based on the relative area percent of detected peaks. In certain embodiments, the peaks that elute at relative residence time less than a certain time are together represented as the acidic peaks.

Assaying Size Variants

In certain embodiments, the levels of aggregates, monomer, and fragments in the chromatographic samples produced using the techniques described herein are analyzed. In certain embodiments, the aggregates, monomer, and fragments are measured using a size exclusion chromatographic (SEC) method for each molecule. For example, but not by way of limitation, a TSK-gel G3000SWxL, 5 μm, 125 Å, 7.8 X 300 mm column (Tosoh Bioscience) can be used in connection with certain embodiments, while a TSK-gel Super SW3000, 4 μm, 250 Å, 4.6 X 300 mm column (Tosoh Bioscience) can be used in alternative embodiments. In certain embodiments, the aforementioned columns are used along with an Agilent or a Shimazhu HPLC system. In certain embodiments, sample injections are made under isocratic elution conditions using a mobile phase consisting of, for example, 100 mM sodium sulfate and 100 mM sodium phosphate at pH 6.8, and detected with UV absorbance at 214 nm. In certain embodiments, the mobile phase will consist of 1X PBS at pH 7.4, and elution profile detected with UV absorbance at 280 nm. In certain embodiments, quantification is based on the relative area of detected peaks.

Any additional technique, such as mass spectroscopy, can be used for assaying size variants.

V. Methods of Treatment Using the Low AR Compositions of the Invention

The low AR compositions of the invention may be used to treat any disorder in a subject for which the therapeutic protein comprised in the composition is appropriate for treating. \

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the subject to the disorder in question. In the case of an anti-TNFα antibody, or antigen binding portion thereof, such as adalimumab, a therapeutically effective amount of the low AR composition may be administered to treat a disorder in which TNFα activity is detrimental.

A disorder in which TNFα activity is detrimental includes a disorder in which inhibition of TNFα activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the

concentration of TNFα in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an anti-TNFα antibody.

TNFα has been implicated in the pathophysiology of a wide variety of a TNFα-related disorders including sepsis, infections, autoimmune diseases, transplant rejection and graft-versus-host disease (see *e.g.*, Moeller, A., *et al.* (1990) *Cytokine* 2:162-169; U.S. Patent No. 5,231,024 to Moeller *et al.*; European Patent Publication No. 260 610 B1 by Moeller, A., *et al.*Vasilli, P. (1992) *Annu. Rev. Immunol.* 10:411-452; Tracey, K.J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491-503). Accordingly, the low AR compositions or a low process-related impurity compositions of the invention may be used to treat an autoimmune disease, such as rheumatoid arthritis, juvenile idiopathic arthritis, or psoriatic arthritis, an intestinal disorder, such as Crohn's disease or ulcerative colitis, a spondyloarthropathy, such as ankylosing spondylitis, or a skin disorder, such as psoriasis.

Disorders in which TNFα activity is detrimental are well known in the art and described in detail in U.S. Patent No. 8,231,876 and U.S. Patent No. 6,090,382, the entire contents of each of which are expressly incorporated herein by reference. In one embodiment, "a disorder in which TNFα activity is detrimental" includes sepsis (including septic shock, endotoxic shock, gram negative sepsis and toxic shock syndrome), autoimmune diseases (including rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis and gouty arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis, nephrotic syndrome, multisystem autoimmune diseases, lupus (including systemic lupus, lupus nephritis and lupus cerebritis), Crohn's disease and autoimmune hearing loss), infectious diseases (including malaria, meningitis, acquired immune deficiency syndrome (AIDS), influenza and cachexia secondary to infection), allograft rejection and graft versus host disease, malignancy, pulmonary disorders (including adult respiratory distress syndrome (ARDS), shock lung, chronic pulmonary inflammatory disease, pulmonary sarcoidosis, pulmonary fibrosis, silicosis, idiopathic interstitial lung disease and chronic obstructive airway disorders (COPD), such as asthma), intestinal disorders (including inflammatory bowel disorders, idiopathic inflammatory bowel disease, Crohn's disease and Crohn's disease-related disorders (including fistulas in the bladder, vagina, and skin; bowel obstructions; abscesses; nutritional deficiencies; complications from corticosteroid use; inflammation of the joints; erythem nodosum; pyoderma gangrenosum; lesions of the eye, Crohn's related arthralgias, fistulizing Crohn's indeterminant colitis and pouchitis), cardiac disorders (including ischemia of the heart, heart insufficiency, restenosis, congestive heart

failure, coronary artery disease, angina pectoris, myocardial infarction, cardiovascular tissue damage caused by cardiac arrest, cardiovascular tissue damage caused by cardiac bypass, cardiogenic shock, and hypertension, atherosclerosis, cardiomyopathy, coronary artery spasm, coronary artery disease, valvular disease, arrhythmias, and cardiomyopathies), spondyloarthropathies (including ankylosing spondylitis, psoriatic arthritis/spondylitis, enteropathic arthritis, reactive arthritis or Reiter's syndrome, and undifferentiated spondyloarthropathies), metabolic disorders (including obesity and diabetes, including type 1 diabetes mellitus, type 2 diabetes mellitus, diabetic neuropathy, peripheral neuropathy, diabetic retinopathy, diabetic ulcerations, retinopathy ulcerations and diabetic macrovasculopathy), anemia, pain (including acute and chronic pains, such as neuropathic pain and post-operative pain, chronic lower back pain, cluster headaches, herpes neuralgia, phantom limb pain, central pain, dental pain, opioid-resistant pain, visceral pain, surgical pain, bone injury pain, pain during labor and delivery, pain resulting from burns, including sunburn, post partum pain, migraine, angina pain, and genitourinary tract-related pain including cystitis), hepatic disorders (including hepatitis, alcoholic hepatitis, viral hepatitis, alcoholic cirrhosis, a1 antitypsin deficiency, autoimmune cirrhosis, cryptogenic cirrhosis, fulminant hepatitis, hepatitis B and C, and steatohepatitis, cystic fibrosis, primary biliary cirrhosis, sclerosing cholangitis and biliary obstruction), skin and nail disorders (including psoriasis (including chronic plaque psoriasis, guttate psoriasis, inverse psoriasis, pustular psoriasis and other psoriasis disorders), pemphigus vulgaris, scleroderma, atopic dermatitis (eczema), sarcoidosis, erythema nodosum, hidradenitis suppurative, lichen planus, Sweet's syndrome, scleroderma and vitiligo), vasculitides (including Behcet's disease), and other disorders, such as juvenile rheumatoid arthritis (JRA), endometriosis, prostatitis, choroidal neovascularization, sciatica, Sjogren's syndrome, uveitis, wet macular degeneration, osteoporosis, osteoarthritis, active axial spondyloarthritis (active axSpA) and non-radiographic axial spondyloarthritis (nr-axSpA).

As used herein, the term "subject" is intended to include living organisms, *e.g.*, prokaryotes and eukaryotes. Examples of subjects include mammals, *e.g.*, humans, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. In specific embodiments of the invention, the subject is a human.

As used herein, the term "treatment" or "treat" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder, as well as those in which the disorder is to be prevented.

In one embodiment, the invention provides a method of administering a low AR composition comprising an anti-TNFα antibody, or antigen binding portion thereof, to a subject such that TNFα activity is inhibited or a disorder in which TNFα activity is detrimental is treated. In one embodiment, the TNFα is human TNFα and the subject is a human subject. In one embodiment, the anti-TNFα antibody is adalimumab, also referred to as HUMIRA®.

The low AR compositions can be administered by a variety of methods known in the art. Exemplary routes/modes of administration include subcutaneous injection, intravenous injection or infusion. In certain aspects, a low AR compositions may be orally administered. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. In certain embodiments it is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit comprising a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of a low AR composition of the invention is 0.01-20 mg/kg, or 1-10 mg/kg, or 0.3-1 mg/kg. With respect to low AR compositions comprising an anti-TNFα antibody, or antigen-binding portion thereof, such as adalimumab, an exemplary dose is 40 mg every other week. In some embodiments, in particular for treatment of ulcerative colitis or Crohn's disease, an exemplary dose includes an initial dose (Day 1) of 160 mg (*e.g.,* four 40 mg injections in one day or two 40 mg injections per day for two consecutive days), a second dose two weeks later of 80 mg, and a maintenance dose of 40 mg every other week beginning two weeks later.

Alternatively, for psoriasis for example, a dosage can include an 80 mg initial dose followed by 40 mg every other week starting one week after the initial dose.

It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

VI. Pharmaceutical Formulations Containing the Low AR Compositions of the Invention

The present invention further provides preparations and formulations comprising the low AR compositions of the invention. It should be understood that any of the antibodies and antibody fragments described herein, including antibodies and antibody fragments having any one or more of the structural and functional features described in detail throughout the application, may be formulated or prepared as described below. When various formulations are described in this section as including an antibody, it is understood that such an antibody may be an antibody or an antibody fragment having any one or more of the characteristics of the antibodies and antibody fragments described herein. In one embodiment, the antibody is an anti-TNFα antibody, or antigen-binding portion thereof.

In certain embodiments, the low AR compositions of the invention may be formulated with a pharmaceutically acceptable carrier as pharmaceutical (therapeutic) compositions, and may be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The term "pharmaceutically acceptable carrier" means one or more non-toxic materials that do not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. Such pharmaceutically acceptable preparations may also routinely contain compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the antibodies of the

present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The low AR compositions of the invention are present in a form known in the art and acceptable for therapeutic uses. In one embodiment, a formulation of the low AR compositions of the invention is a liquid formulation. In another embodiment, a formulation of the low AR compositions of the invention is a lyophilized formulation. In a further embodiment, a formulation of the low AR compositions of the invention is a reconstituted liquid formulation. In one embodiment, a formulation of the low AR compositions of the invention is a stable liquid formulation. In one embodiment, a liquid formulation of the low AR compositions of the invention is an aqueous formulation. In another embodiment, the liquid formulation is non-aqueous. In a specific embodiment, a liquid formulation of the low AR compositions of the invention is an aqueous formulation wherein the aqueous carrier is distilled water.

The formulations of the low AR compositions of the invention comprise an antibody in a concentration resulting in a w/v appropriate for a desired dose. The antibody may be present in the formulation at a concentration of about 1mg/ml to about 500mg/ml, *e.g.,* at a concentration of at least 1 mg/ml, at least 5 mg/ml, at least 10 mg/ml, at least 15 mg/ml, at least 20 mg/ml, at least 25 mg/ml, at least 30 mg/ml, at least 35 mg/ml, at least 40 mg/ml, at least 45 mg/ml, at least 50 mg/ml, at least 55 mg/ml, at least 60 mg/ml, at least 65 mg/ml, at least 70 mg/ml, at least 75 mg/ml, at least 80 mg/ml, at least 85 mg/ml, at least 90 mg/ml, at least 95 mg/ml, at least 100 mg/ml, at least 105 mg/ml, at least 110 mg/ml, at least 115 mg/ml, at least 120 mg/ml, at least 125 mg/ml, at least 130 mg/ml, at least 135 mg/ml, at least 140 mg/ml, at least 150 mg/ml, at least 200 mg/ml, at least 250 mg/ml, or at least 300 mg/ml.

In a specific embodiment, a formulation of the low AR compositions of the invention comprises at least about 100 mg/ml, at least about 125 mg/ml, at least 130 mg/ml, or at least about 150 mg/ml of an antibody of the invention.

In one embodiment, the concentration of antibody, which is included in the formulation of the invention, is between about 1 mg/ml and about 25 mg/ml, between about 1 mg/ml and about 200 mg/ml, between about 25 mg/ml and about 200 mg/ml, between about 50 mg/ml and about 200 mg/ml, between about 75 mg/ml and about 200 mg/ml, between about 100 mg/ml and about 200 mg/ml, between about 125 mg/ml and about 200 mg/ml, between about 150 mg/ml and about 200 mg/ml, between about 25 mg/ml and about 150 mg/ml, between about 50 mg/ml and about 150 mg/ml, between about 75 mg/ml and about

150 mg/ml, between about 100 mg/ml and about 150 mg/ml, between about 125 mg/ml and about 150 mg/ml, between about 25 mg/ml and about 125 mg/ml, between about 50 mg/ml and about 125 mg/ml, between about 75 mg/ml and about 125 mg/ml, between about 100 mg/ml and about 125 mg/ml, between about 25 mg/ml and about 100 mg/ml, between about 50 mg/ml and about 100 mg/ml, between about 75 mg/ml and about 100 mg/ml, between about 25 mg/ml and about 75 mg/ml, between about 50 mg/ml and about 75 mg/ml, or between about 25 mg/ml and about 50 mg/ml.

In a specific embodiment, a formulation of the low AR compositions of the invention comprises between about 90 mg/ml and about 110 mg/ml or between about 100 mg/ml and about 210 mg/ml of an antibody.

The formulations of the low AR compositions of the invention comprising an antibody may further comprise one or more active compounds as necessary for the particular indication being treated, typically those with complementary activities that do not adversely affect each other. Such additional active compound/s is/are suitably present in combination in amounts that are effective for the purpose intended.

The formulations of the low AR compositions of the invention may be prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers, including, but not limited to buffering agents, saccharides, salts, surfactants, solubilizers, polyols, diluents, binders, stabilizers, salts, lipophilic solvents, amino acids, chelators, preservatives, or the like (Goodman and Gilman's The Pharmacological Basis of Therapeutics, 12th edition, L. Brunton, *et al.* and *Remington's Pharmaceutical Sciences*, 16th edition, Osol, A. Ed. (1999)), in the form of lyophilized formulations or aqueous solutions at a desired final concentration. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as histidine, phosphate, citrate, glycine, acetate and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including trehalose,

glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.,* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN, polysorbate 80, PLURONICS™ or polyethylene glycol (PEG).

The buffering agent may be histidine, citrate, phosphate, glycine, or acetate. The saccharide excipient may be trehalose, sucrose, mannitol, maltose or raffinose. The surfactant may be polysorbate 20, polysorbate 40, polysorbate 80, or Pluronic F68. The salt may be NaCl, KCl, $MgCl_2$, or $CaCl_2$

The formulations of the low AR compositions of the invention may include a buffering or pH adjusting agent to provide improved pH control. A formulation of the invention may have a pH of between about 3.0 and about 9.0, between about 4.0 and about 8.0, between about 5.0 and about 8.0, between about 5.0 and about 7.0, between about 5.0 and about 6.5, between about 5.5 and about 8.0, between about 5.5 and about 7.0, or between about 5.5 and about 6.5. In a further embodiment, a formulation of the invention has a pH of about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0. In a specific embodiment, a formulation of the invention has a pH of about 6.0. One of skill in the art understands that the pH of a formulation generally should not be equal to the isoelectric point of the particular antibody to be used in the formulation.

Typically, the buffering agent is a salt prepared from an organic or inorganic acid or base. Representative buffering agents include, but are not limited to, organic acid salts such as salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid; Tris, tromethamine hydrochloride, or phosphate buffers. In addition, amino acid components can also function in a buffering capacity. Representative amino acid components which may be utilized in the formulations of the invention as buffering agents include, but are not limited to, glycine and histidine. In certain embodiments, the buffering agent is chosen from histidine, citrate, phosphate, glycine, and acetate. In a specific embodiment, the buffering agent is histidine. In another specific embodiment, the buffering agent is citrate. In yet another specific embodiment, the buffering agent is glycine. The purity of the buffering agent should be at least 98%, or at least 99%, or at least 99.5%. As used herein, the term "purity" in the context of histidine and glycine refers

to chemical purity of histidine or glycine as understood in the art, *e.g.,* as described in The Merck Index, 13th ed., O'Neil *et al.* ed. (Merck & Co., 2001).

Buffering agents are typically used at concentrations between about 1 mM and about 200 mM or any range or value therein, depending on the desired ionic strength and the buffering capacity required. The usual concentrations of conventional buffering agents employed in parenteral formulations can be found in: Pharmaceutical Dosage Form: Parenteral Medications, Volume 1, 2nd Edition, Chapter 5, p. 194, De Luca and Boylan, "Formulation of Small Volume Parenterals", Table 5: Commonly used additives in Parenteral Products. In one embodiment, the buffering agent is at a concentration of about 1 mM, or of about 5 mM, or of about 10 mM, or of about 15 mM, or of about 20 mM, or of about 25 mM, or of about 30 mM, or of about 35 mM, or of about 40 mM, or of about 45 mM, or of about 50 mM, or of about 60 mM, or of about 70 mM, or of about 80 mM, or of about 90 mM, or of about 100 mM. In one embodiment, the buffering agent is at a concentration of 1 mM, or of 5 mM, or of 10 mM, or of 15 mM, or of 20 mM, or of 25 mM, or of 30 mM, or of 35 mM, or of 40 mM, or of 45 mM, or of 50 mM, or of 60 mM, or of 70 mM, or of 80 mM, or of 90 mM, or of 100 mM. In a specific embodiment, the buffering agent is at a concentration of between about 5 mM and about 50 mM. In another specific embodiment, the buffering agent is at a concentration of between 5 mM and 20 mM.

In certain embodiments, the formulation of the low AR compositions of the invention comprises histidine as a buffering agent. In one embodiment the histidine is present in the formulation of the invention at a concentration of at least about 1 mM, at least about 5 mM, at least about 10 mM, at least about 20 mM, at least about 30 mM, at least about 40 mM, at least about 50 mM, at least about 75 mM, at least about 100 mM, at least about 150 mM, or at least about 200 mM histidine. In another embodiment, a formulation of the invention comprises between about 1 mM and about 200 mM, between about 1 mM and about 150 mM, between about 1 mM and about 100 mM, between about 1 mM and about 75 mM, between about 10 mM and about 200 mM, between about 10 mM and about 150 mM, between about 10 mM and about 100 mM, between about 10 mM and about 75 mM, between about 10 mM and about 50 mM, between about 10 mM and about 40 mM, between about 10 mM and about 30 mM, between about 20 mM and about 75 mM, between about 20 mM and about 50 mM, between about 20 mM and about 40 mM, or between about 20 mM and about 30 mM histidine. In a further embodiment, the formulation comprises about 1 mM, about 5 mM, about 10 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about

45 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM, about 150 mM, or about 200 mM histidine. In a specific embodiment, a formulation may comprise about 10 mM, about 25 mM, or no histidine.

The formulations of the low AR compositions of the invention may comprise a carbohydrate excipient. Carbohydrate excipients can act, *e.g.,* as viscosity enhancing agents, stabilizers, bulking agents, solubilizing agents, and/or the like. Carbohydrate excipients are generally present at between about 1% to about 99% by weight or volume, *e.g.,* between about 0.1% to about 20%, between about 0.1% to about 15%, between about 0.1% to about 5%, , between about 1% to about 20%, between about 5% to about 15%, between about 8% to about 10%, between about 10% and about 15%, between about 15% and about 20%, between 0.1% to 20%, between 5% to 15%, between 8% to 10%, between 10% and 15%, between 15% and 20%, between about 0.1% to about 5%, between about 5% to about 10%, or between about 15% to about 20%. In still other specific embodiments, the carbohydrate excipient is present at 1%, or at 1.5%, or at 2%, or at 2.5%, or at 3%, or at 4%, or at 5%, or at 10%, or at 15%, or at 20%.

Carbohydrate excipients suitable for use in the formulations of the invention include, but are not limited to, monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol) and the like. In one embodiment, the carbohydrate excipients for use in the present invention are chosen from, sucrose, trehalose, lactose, mannitol, and raffinose. In a specific embodiment, the carbohydrate excipient is trehalose. In another specific embodiment, the carbohydrate excipient is mannitol. In yet another specific embodiment, the carbohydrate excipient is sucrose. In still another specific embodiment, the carbohydrate excipient is raffinose. The purity of the carbohydrate excipient should be at least 98%, or at least 99%, or at least 99.5%.

In a specific embodiment, the formulations of the low AR compositions of the invention may comprise trehalose. In one embodiment, a formulation of the invention comprises at least about 1%, at least about 2%, at least about 4%, at least about 8%, at least about 20%, at least about 30%, or at least about 40% trehalose. In another embodiment, a formulation of the invention comprises between about 1% and about 40%, between about 1% and about 30%, between about 1% and about 20%, between about 2% and about 40%,

between about 2% and about 30%, between about 2% and about 20%, between about 4% and about 40%, between about 4% and about 30%, or between about 4% and about 20% trehalose. In a further embodiment, a formulation of the invention comprises about 1%, about 2%, about 4%, about 6%, about 8%, about 15%, about 20%, about 30%, or about 40% trehalose. In a specific embodiment, a formulation of the invention comprises about 4%, about 6% or about 15% trehalose.

In certain embodiments, a formulation of the low AR compositions of the invention comprises an excipient. In a specific embodiment, a formulation of the invention comprises at least one excipient chosen from: sugar, salt, surfactant, amino acid, polyol, chelating agent, emulsifier and preservative. In one embodiment, a formulation of the invention comprises a salt, *e.g.,* a salt selected from: NaCl, KCl, $CaCl_2$, and $MgCl_2$. In a specific embodiment, the formulation comprises NaCl.

A formulation of the low AR compositions of the invention may comprise at least about 10 mM, at least about 25 mM, at least about 50 mM, at least about 75 mM, at least about 80 mM, at least about 100 mM, at least about 125 mM, at least about 150 mM, at least about 175 mM, at least about 200 mM, or at least about 300 mM sodium chloride (NaCl). In a further embodiment, the formulation may comprise between about 10 mM and about 300 mM, between about 10 mM and about 200 mM, between about 10 mM and about 175 mM, between about 10 mM and about 150 mM, between about 25 mM and about 300 mM, between about 25 mM and about 200 mM, between about 25 mM and about 175 mM, between about 25 mM and about 150 mM, between about 50 mM and about 300 mM, between about 50 mM and about 200 mM, between about 50 mM and about 175 mM, between about 50 mM and about 150 mM, between about 75 mM and about 300 mM, between about 75 mM and about 200 mM, between about 75 mM and about 175 mM, between about 75 mM and about 150 mM, between about 100 mM and about 300 mM, between about 100 mM and about 200 mM, between about 100 mM and about 175 mM, or between about 100 mM and about 150 mM sodium chloride. In a further embodiment, the formulation may comprise about 10 mM, about 25 mM, about 50 mM, about 75 mM, about 80 mM, about 100 mM, about 125 mM, about 150 mM, about 175 mM, about 200 mM, or about 300 mM sodium chloride.

A formulation of the low AR compositions of the invention may also comprise an amino acid, *e.g.,* lysine, arginine, glycine, histidine or an amino acid salt. The formulation may comprise at least about 1mM, at least about 10mM, at least about 25 mM, at least about

50 mM, at least about 100 mM, at least about 150 mM, at least about 200 mM, at least about 250 mM, at least about 300 mM, at least about 350 mM, or at least about 400 mM of an amino acid. In another embodiment, the formulation may comprise between about 1 mM and about 100 mM, between about 10 mM and about 150 mM, between about 25 mM and about 250 mM, between about 25 mM and about 300 mM, between about 25 mM and about 350 mM, between about 25 mM and about 400 mM, between about 50 mM and about 250 mM, between about 50 mM and about 300 mM, between about 50 mM and about 350 mM, between about 50 mM and about 400 mM, between about 100 mM and about 250 mM, between about 100 mM and about 300 mM, between about 100 mM and about 400 mM, between about 150 mM and about 250 mM, between about 150 mM and about 300 mM, or between about 150 mM and about 400 mM of an amino acid. In a further embodiment, a formulation of the invention comprises about 1 mM, 1.6 mM, 25 mM, about 50 mM, about 100 mM, about 150 mM, about 200 mM, about 250 mM, about 300 mM, about 350 mM, or about 400 mM of an amino acid.

The formulations of the low AR compositions of the invention may further comprise a surfactant. The term "surfactant" as used herein refers to organic substances having amphipathic structures; namely, they are composed of groups of opposing solubility tendencies, typically an oil-soluble hydrocarbon chain and a water-soluble ionic group. Surfactants can be classified, depending on the charge of the surface-active moiety, into anionic, cationic, and nonionic surfactants. Surfactants are often used as wetting, emulsifying, solubilizing, and dispersing agents for various pharmaceutical compositions and preparations of biological materials. Pharmaceutically acceptable surfactants like polysorbates (*e.g.,* polysorbates 20 or 80); polyoxamers (*e.g.,* poloxamer 188); Triton; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (*e.g.,* lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUA™ series (Mona Industries, Inc., Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (*e.g.,* PLURONICS™, PF68, etc.), can optionally be added to the formulations of the invention to reduce aggregation. In one embodiment, a formulation of the invention comprises Polysorbate 20, Polysorbate 40, Polysorbate 60, or Polysorbate 80. Surfactants are

particularly useful if a pump or plastic container is used to administer the formulation. The presence of a pharmaceutically acceptable surfactant mitigates the propensity for the protein to aggregate. The formulations may comprise a polysorbate which is at a concentration ranging from between about 0.001% to about 1%, or about 0.001% to about 0.1%, or about 0.01% to about 0.1%. In other specific embodiments, the formulations of the invention comprise a polysorbate which is at a concentration of 0.001%, or 0.002%, or 0.003%, or 0.004%, or 0.005%, or 0.006%, or 0.007%, or 0.008%, or 0.009%, or 0.01%, or 0.015%, or 0.02%.

The formulations of the low AR compositions of the invention may optionally further comprise other common excipients and/or additives including, but not limited to, diluents, binders, stabilizers, lipophilic solvents, preservatives, adjuvants, or the like. Pharmaceutically acceptable excipients and/or additives may be used in the formulations of the invention. Commonly used excipients/additives, such as pharmaceutically acceptable chelators (for example, but not limited to, EDTA, DTPA or EGTA) can optionally be added to the formulations of the invention to reduce aggregation. These additives are particularly useful if a pump or plastic container is used to administer the formulation.

Preservatives, such as phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, phenylmercuric nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride (for example, but not limited to, hexahydrate), alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof can optionally be added to the formulations of the invention at any suitable concentration such as between about 0.001% to about 5%, or any range or value therein. The concentration of preservative used in the formulations of the invention is a concentration sufficient to yield a microbial effect. Such concentrations are dependent on the preservative selected and are readily determined by the skilled artisan.

Other contemplated excipients/additives, which may be utilized in the formulations of the invention include, for example, flavoring agents, antimicrobial agents, sweeteners, antioxidants, antistatic agents, lipids such as phospholipids or fatty acids, steroids such as cholesterol, protein excipients such as serum albumin (human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, salt-forming counterions such as sodium and the like. These and additional known pharmaceutical excipients and/or additives suitable for use in the formulations of the invention are known in the art, *e.g.,* as listed in "Remington: The Science & Practice of Pharmacy", 21st ed., Lippincott Williams & Wilkins, (2005), and

in the "Physician's Desk Reference", 60th ed., Medical Economics, Montvale, N.J. (2005). Pharmaceutically acceptable carriers can be routinely selected that are suitable for the mode of administration, solubility and/or stability of an antibody, as well known those in the art or as described herein.

In one embodiment, the low AR compositions of the invention are formulated with the same or similar excipients and buffers as are present in the commercial adalimumab (HUMIRA®) formulation, as described in the "Highlights of HUMIRA® Prescribing Information" for HUMIRA® (adalimumab) Injection (Revised Jan. 2008) the entire contents of which are expressly incorporated herein by reference. For example, each prefilled syringe of HUMIRA®, which is administered subcutaneously, delivers 0.8 mL (40 mg) of drug product to the subject. Each 0.8 mL of HUMIRA® contains 40 mg adalimumab, 4.93 mg sodium chloride, 0.69 mg monobasic sodium phosphate dihydrate, 1.22 mg dibasic sodium phosphate dihydrate, 0.24 mg sodium citrate, 1.04 mg citric acid monohydrate, 9.6 mg mannitol, 0.8 mg polysorbate 80, and water for Injection, USP. Sodium hydroxide is added as necessary to adjust pH.

It will be understood by one skilled in the art that the formulations of the low AR compositions of the invention may be isotonic with human blood, wherein the formulations of the invention have essentially the same osmotic pressure as human blood. Such isotonic formulations will generally have an osmotic pressure from about 250 mOSm to about 350 mOSm. Isotonicity can be measured by, for example, using a vapor pressure or ice-freezing type osmometer. Tonicity of a formulation is adjusted by the use of tonicity modifiers. "Tonicity modifiers" are those pharmaceutically acceptable inert substances that can be added to the formulation to provide an isotonity of the formulation. Tonicity modifiers suitable for this invention include, but are not limited to, saccharides, salts and amino acids.

In certain embodiments, the formulations of the low AR compositions of the invention have an osmotic pressure from about 100 mOSm to about 1200 mOSm, or from about 200 mOSm to about 1000 mOSm, or from about 200 mOSm to about 800 mOSm, or from about 200 mOSm to about 600 mOSm, or from about 250 mOSm to about 500 mOSm, or from about 250 mOSm to about 400 mOSm, or from about 250 mOSm to about 350 mOSm.

The concentration of any one component or any combination of various components, of the formulations of the low AR compositions of the invention is adjusted to achieve the desired tonicity of the final formulation. For example, the ratio of the carbohydrate excipient

to antibody may be adjusted according to methods known in the art (*e.g.,* U.S. Patent No. 6,685,940). In certain embodiments, the molar ratio of the carbohydrate excipient to antibody may be from about 100 moles to about 1000 moles of carbohydrate excipient to about 1 mole of antibody, or from about 200 moles to about 6000 moles of carbohydrate excipient to about 1 mole of antibody, or from about 100 moles to about 510 moles of carbohydrate excipient to about 1 mole of antibody, or from about 100 moles to about 600 moles of carbohydrate excipient to about 1 mole of antibody.

The desired isotonicity of the final formulation may also be achieved by adjusting the salt concentration of the formulations. Pharmaceutically acceptable salts and those suitable for this invention as tonicity modifiers include, but are not limited to, sodium chloride, sodium succinate, sodium sulfate, potassuim chloride, magnesium chloride, magnesium sulfate, and calcium chloride. In specific embodiments, formulations of the invention comprise NaCl, $MgCl_2$, and/or $CaCl_2$. In one embodiment, concentration of NaCl is between about 75 mM and about 150 mM. In another embodiment, concentration of $MgCl_2$ is between about 1 mM and about 100 mM. Pharmaceutically acceptable amino acids including those suitable for this invention as tonicity modifiers include, but are not limited to, proline, alanine, L-arginine, asparagine, L-aspartic acid, glycine, serine, lysine, and histidine.

In one embodiment the formulations of the low AR compositions of the invention are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside a microorganism and are released only when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, even low amounts of endotoxins must be removed from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with antibodies, even trace amounts of harmful and dangerous endotoxin must be removed. In certain specific embodiments, the endotoxin and pyrogen levels in the composition are less then 10 EU/mg,

or less then 5 EU/mg, or less then 1 EU/mg, or less then 0.1 EU/mg, or less then 0.01 EU/mg, or less then 0.001 EU/mg.

When used for *in vivo* administration, the formulations of the low AR compositions of the invention should be sterile. The formulations of the invention may be sterilized by various sterilization methods, including sterile filtration, radiation, etc. In one embodiment, the antibody formulation is filter-sterilized with a presterilized 0.22-micron filter. Sterile compositions for injection can be formulated according to conventional pharmaceutical practice as described in "Remington: The Science & Practice of Pharmacy", 21st ed., Lippincott Williams & Wilkins, (2005). Formulations comprising antibodies, such as those disclosed herein, ordinarily will be stored in lyophilized form or in solution. It is contemplated that sterile compositions comprising antibodies are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having an adapter that allows retrieval of the formulation, such as a stopper pierceable by a hypodermic injection needle. In one embodiment, a composition of the invention is provided as a pre-filled syringe.

In one embodiment, a formulation of the low AR compositions of the invention is a lyophilized formulation. The term "lyophilized" or "freeze-dried" includes a state of a substance that has been subjected to a drying procedure such as lyophilization, where at least 50% of moisture has been removed.

The phrase "bulking agent" includes a compound that is pharmaceutically acceptable and that adds bulk to a lyo cake. Bulking agents known to the art include, for example, carbohydrates, including simple sugars such as dextrose, ribose, fructose and the like, alcohol sugars such as mannitol, inositol and sorbitol, disaccharides including trehalose, sucrose and lactose, naturally occurring polymers such as starch, dextrans, chitosan, hyaluronate, proteins (*e.g.,* gelatin and serum albumin), glycogen, and synthetic monomers and polymers.

A "lyoprotectant" is a molecule which, when combined with a protein of interest (such as an antibody of the invention), significantly prevents or reduces chemical and/or physical instability of the protein upon lyophilization and subsequent storage. Lyoprotectants include, but are not limited to, sugars and their corresponding sugar alcohols; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher molecular weight sugar alcohols, *e.g.,* glycerin, dextran, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; PLURONICS™; and combinations thereof.

Additional examples of lyoprotectants include, but are not limited to, glycerin and gelatin, and the sugars mellibiose, melezitose, raffinose, mannotriose and stachyose. Examples of reducing sugars include, but are not limited to, glucose, maltose, lactose, maltulose, iso-maltulose and lactulose. Examples of non-reducing sugars include, but are not limited to, non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols. Examples of sugar alcohols include, but are not limited to, monoglycosides, compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose. The glycosidic side group can be either glucosidic or galactosidic. Additional examples of sugar alcohols include, but are not limited to, glucitol, maltitol, lactitol and iso-maltulose. In specific embodiments, trehalose or sucrose is used as a lyoprotectant.

The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the protein in the presence of the lyoprotecting amount of the lyoprotectant, the protein essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

In one embodiment, the molar ratio of a lyoprotectant (*e.g.,* trehalose) and antibody molecules of a formulation of the invention is at least about 10, at least about 50, at least about 100, at least about 200, or at least about 300. In another embodiment, the molar ratio of a lyoprotectant (*e.g.,* trehalose) and antibody molecules of a formulation of the invention is about 1, is about 2, is about 5, is about 10, about 50, about 100, about 200, or about 300.

A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized antibody formulation in a diluent such that the antibody is dispersed in the reconstituted formulation. The reconstituted formulation is suitable for administration (*e.g.,* parenteral administration) to a patient to be treated with the antibody and, in certain embodiments of the invention, may be one which is suitable for intravenous administration.

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation, such as a formulation reconstituted after lyophilization. In some embodiments, diluents include, but are not limited to, sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (*e.g.,* phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. In an alternative embodiment, diluents can include aqueous solutions of salts and/or buffers.

In certain embodiments, a formulation of the low AR compositions of the invention is a lyophilized formulation comprising an antibody of the invention, wherein at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of said antibody may be recovered from a vial upon shaking said vial for 4 hours at a speed of 400 shakes per minute wherein the vial is filled to half of its volume with the formulation. In another embodiment, a formulation of the invention is a lyophilized formulation comprising an antibody of the invention, wherein at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the antibody may be recovered from a vial upon subjecting the formulation to three freeze/thaw cycles wherein the vial is filled to half of its volume with said formulation. In a further embodiment, a formulation of the invention is a lyophilized formulation comprising an antibody of the invention, wherein at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% of the antibody may be recovered by reconstituting a lyophilized cake generated from said formulation.

In one embodiment, a reconstituted liquid formulation may comprise an antibody at the same concentration as the pre-lyophilized liquid formulation.

In another embodiment, a reconstituted liquid formulation may comprise an antibody at a higher concentration than the pre-lyophilized liquid formulation, *e.g.,* .about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, or about 10 fold higher concentration of an antibody than the pre-lyophilized liquid formulation.

In yet another embodiment, a reconstituted liquid formulation may comprise an antibody of the invention at a lower concentration than the pre-lyophilized liquid formulation, *e.g.,* about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold or about 10 fold lower concentration of an antibody than the pre-lyophilized liquid formulation.

The pharmaceutical formulations of the low AR compositions of the invention are typically stable formulations, *e.g.,* stable at room temperature.

The terms "stability" and "stable" as used herein in the context of a formulation comprising an antibody of the invention refer to the resistance of the antibody in the formulation to aggregation, degradation or fragmentation under given manufacture, preparation, transportation and storage conditions. The "stable" formulations of the invention retain biological activity under given manufacture, preparation, transportation and storage conditions. The stability of the antibody can be assessed by degrees of aggregation,

degradation or fragmentation, as measured by HPSEC, static light scattering (SLS), Fourier Transform Infrared Spectroscopy (FTIR), circular dichroism (CD), urea unfolding techniques, intrinsic tryptophan fluorescence, differential scanning calorimetry, and/or ANS binding techniques, compared to a reference formulation. For example, a reference formulation may be a reference standard frozen at -70°C consisting of 10 mg/ml of an antibody of the invention in PBS.

Therapeutic formulations of the low AR compositions of the invention may be formulated for a particular dosage. Dosage regimens may be adjusted to provide the optimum desired response (*e.g.,* a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the antibody and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an antibody for the treatment of sensitivity in individuals.

Therapeutic compositions of the low AR compositions of the invention can be formulated for particular routes of administration, such as oral, nasal, pulmonary, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. By way of example, in certain embodiments, the antibodies (including antibody fragments) are formulated for intravenous administration. In certain other embodiments, the antibodies (including antibody fragments) are formulated for local delivery to the cardiovascular system,

for example, via catheter, stent, wire, intramyocardial delivery, intrapericardial delivery, or intraendocardial delivery.

Formulations of the low AR compositions of the invention which are suitable for topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required (US Patent No. 7,378,110; 7,258,873; 7,135,180; 7,923,029; and US Publication No. 2004-0042972 and 2004-0042971).

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the low AR compositions of the invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

In certain embodiments, antibodies of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention can cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.,* U.S. Pat. Nos. 4,522,811; 5,374,548; 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, *e.g.,* V. V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or

biotin (see, *e.g.,* U.S. Pat. No. 5,416,016); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P. G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant Protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the invention, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinanen; M. L. Laukkanen (1994) *FEBS Lett.* 346:123; J. J. Killion; I. J. Fidler (1994) *Immunomethods* 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in another embodiment, the liposomes include a targeting moiety. In another embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the desired area. When administered in this manner, the composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms such as bacteria and fungi. Additionally or alternatively, the antibodies of the invention may be delivered locally to the brain to mitigate the risk that the blood brain barrier slows effective delivery.

In certain embodiments, the low AR compositions of the invention may be administered with medical devices known in the art. For example, in certain embodiments an antibody or antibody fragment is administered locally via a catheter, stent, wire, or the like. For example, in one embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Pat. Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824; 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Pat. No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Pat. No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Pat. No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Pat. No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Pat. No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Pat. No. 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known to those skilled in the art.

The efficient dosages and the dosage regimens for the low AR compositions of the invention depend on the disease or condition to be treated and can be determined by the persons skilled in the art. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

VII. Alternative Formulations Containing the Low AR Compositions of the Invention

a. Aqueous Formulations

The invention also provides a low AR composition formulated as an aqueous formulation comprising a protein and water, as described in U.S. Patent No. 8,420,081 and PCT Publication No. WO2012/065072, the contents of which are hereby incorporated by reference. In these aqueous formulations, the protein is stable without the need for additional agents. This aqueous formulation has a number of advantages over conventional formulations in the art, including stability of the protein in water without the requirement for additional excipients, increased concentrations of protein without the need for additional excipients to maintain solubility of the protein, and low osmolality. These also have advantageous storage properties, as the proteins in the formulation remain stable during storage, *e.g.*, stored as a liquid form for more than 3 months at 7°C or freeze/thaw conditions, even at high protein concentrations and repeated freeze/thaw processing steps. In one embodiment, formulations described herein include high concentrations of proteins such that the aqueous formulation does not show significant opalescence, aggregation, or precipitation.

In one embodiment, an aqueous low AR composition comprising a protein, *e.g.,* an antibody, *e.g.,* an anti-TNFα antibody or antigen biding portion thereof, and water is provided, wherein the formulation has certain characteristics, such as, but not limited to, low conductivity, *e.g.*, a conductivity of less than about 2.5 mS/cm, a protein concentration of at least about 10 μg/mL, an osmolality of no more than about 30 mOsmol/kg, and/or the protein has a molecular weight (Mw) greater than about 47 kDa. In one embodiment, the formulation has improved stability, such as, but not limited to, stability in a liquid form for an extended time (*e.g.*, at least about 3 months or at least about 12 months) or stability through at least one freeze/thaw cycle (if not more freeze/thaw cycles). In one embodiment, the formulation is stable for at least about 3 months in a form selected from the group consisting of frozen, lyophilized, or spray-dried.

In one embodiment, the formulation has a low conductivity, including, for example, a conductivity of less than about 2.5 mS/cm, a conductivity of less than about 2 mS/cm, a conductivity of less than about 1.5 mS/cm, a conductivity of less than about 1 mS/cm, or a conductivity of less than about 0.5 mS/cm.

In another embodiment, low AR compositions included in the formulation have a given concentration, including, for example, a concentration of at least about 1 mg/mL, at least about 10 mg/mL, at least about 50 mg/mL, at least about 100 mg/mL, at least about 150 mg/mL, at least about 200 mg/mL, or greater than about 200 mg/mL. In another embodiment, the formulation of the invention has an osmolality of no more than about 15 mOsmol/kg.

The aqueous formulations described herein do not rely on standard excipients, *e.g.*, a tonicity modifier, a stabilizing agent, a surfactant, an anti-oxidant, a cryoprotectant, a bulking agent, a lyroprotectant, a basic component, and an acidic component. In other embodiments of the invention, the formulation contains water, one or more proteins, and no ionic excipients (*e.g.*, salts, free amino acids).

In certain embodiments, the aqueous formulation as described herein comprise a low AR composition comprising a protein concentration of at least 50 mg/mL and water, wherein the formulation has an osmolality of no more than 30 mOsmol/kg. Lower limits of osmolality of the aqueous formulation are also encompassed by the invention. In one embodiment the osmolality of the aqueous formulation is no more than 15 mOsmol/kg. The aqueous formulation of the invention may have an osmolality of less than 30 mOsmol/kg, and also have a high protein concentration, *e.g.*, the concentration of the protein is at least 100 mg/mL, and may be as much as 200 mg/mL or greater. Ranges intermediate to the above recited concentrations and osmolality units are also intended to be part of this invention. In addition, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

The concentration of the aqueous formulation as described herein is not limited by the protein size and the formulation may include any size range of proteins. Included within the scope of the invention is an aqueous formulation comprising at least 40 mg/mL and as much as 200 mg/mL or more of a protein, for example, 40 mg/mL, 65 mg/mL, 130 mg/mL, or 195 mg/ml, which may range in size from 5 kDa to 150 kDa or more. In one embodiment, the protein in the formulation of the invention is at least about 15 kD in size, at least about 20 kD in size; at least about 47 kD in size; at least about 60 kD in size; at least about 80 kD in size; at least about 100 kD in size; at least about 120 kD in size; at least about 140 kD in size; at

least about 160 kD in size; or greater than about 160 kD in size. Ranges intermediate to the above recited sizes are also intended to be part of this invention. In addition, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

The aqueous formulation as described herein may be characterized by the hydrodynamic diameter (D_h) of the proteins in solution. The hydrodynamic diameter of the protein in solution may be measured using dynamic light scattering (DLS), which is an established analytical method for determining the D_h of proteins. Typical values for monoclonal antibodies, *e.g.*, IgG, are about 10 nm. Low-ionic formulations may be characterized in that the D_h of the proteins are notably lower than protein formulations comprising ionic excipients. It has been discovered that the D_h values of antibodies in aqueous formulations made using the disfiltration/ultrafilteration (DF/UF) process, as described in U.S. Patent No. 8,420,081 and PCT Publication No. WO2012/065072, using pure water as an exchange medium, are notably lower than the D_h of antibodies in conventional formulations independent of protein concentration. In one embodiment, antibodies in the aqueous formulation as described herein have a D_h of less than 4 nm, or less than 3 nm.

In one embodiment, the D_h of the protein in the aqueous formulation is smaller relative to the D_h of the same protein in a buffered solution, irrespective of protein concentration. Thus, in certain embodiments, protein in an aqueous formulation made in accordance with the methods described herein, will have a D_h which is at least 25% less than the D_h of the protein in a buffered solution at the same given concentration. Examples of buffered solutions include, but are not limited to phosphate buffered saline (PBS). In certain embodiments, proteins in the aqueous formulation of the invention have a D_h that is at least 50% less than the D_h of the protein in PBS in at the given concentration; at least 60% less than the D_h of the protein in PBS at the given concentration; at least 70% less than the D_h of the protein in PBS at the given concentration; or more than 70% less than the D_h of the protein in PBS at the given concentration. Ranges intermediate to the above recited percentages are also intended to be part of this invention, *e.g.*, about 55%, 56%, 57%, 64%, 68%, and so forth. In addition, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included, *e.g.*, about 50% to about 80%.

In one aspect, the aqueous formulation includes the protein at a dosage of about 0.01 mg/kg-10 mg/kg. In another aspect, the dosages of the protein include approximately 1 mg/kg administered every other week, or approximately 0.3 mg/kg administered weekly. A skilled practitioner can ascertain the proper dosage and regime for administering to a subject.

b. "Solid Unit" Formulations

The present invention also provides a low AR composition of the invention formulated as a stable composition of a protein, *e.g.,* particularly a therapeutic protein such as an antibody, or antigen binding portion thereof, and a stabilizer, referred to herein as solid units. These formulations are described, for example, in U.S. Provisional Patent Application 61/893,123, entitled "Stable Solid Protein Compositions and Methods of Making Same", Attorney Docket Number 117813-31001, filed on October 18, 2013, the entire contents of which are expressly incorporated herein by reference.

Specifically, it has been discovered that despite having a high proportion of sugar relative to the protein, the solid units of the invention maintain structural rigidity and resist changes in shape and/or volume when stored under ambient conditions, *e.g.,* room temperature and humidity, for extended periods of time. The solid units of the invention remain free-flowing and are able to maintain long-term physical and chemical stability of the protein without significant degradation and/or aggregate formation. The solid units of the invention have many advantages over the art, including that they can be formulated for oral delivery and are easily reconstituted in a diluent, such as water. Because the solid units are readily dissolved, they may be used in dual chamber delivery devices and may be prepared directly in a device for patient use.

As used herein, the term "solid unit," refers to a composition which is suitable for pharmaceutical administration and comprises a protein, *e.g.,* an antibody or peptide, and a stabilizer, *e.g.,* a sugar. The solid unit has a structural rigidity and resistance to changes in shape and/or volume. In a preferred embodiment, the solid unit is obtained by lyophilizing a pharmaceutical formulation of a therapeutic protein. The solid unit may be any shape, *e.g.,* geometric shape, including, but not limited to, a sphere, a cube, a pyramid, a hemisphere, a cylinder, a teardrop, and so forth, including irregularly shaped units. In one embodiment, the solid unit has a volume ranging from about 1 ml to about 20 ml. In one embodiment, the solid unit is not obtained using spray drying techniques, *e.g.,* the solid unit is not a powder or granule.

As used herein, the phrase "a plurality of solid units" refers to a collection or population of solid units, wherein the collection comprises two or more solid units having a substantially uniform shape, *e.g.,* sphere, and/or volume distribution. In one embodiment, the plurality of solid units is free-flowing.

VIII. Kits and Articles of Manufacture Comprising the Low AR Compositions of the Invention

Also within the scope of the present invention are kits comprising the low AR compositions of the invention and instructions for use. The term "kit" as used herein refers to a packaged product comprising components with which to administer the antibody, or antigen-binding portion thereof, of the invention for treatment of a disease or disorder. The kit may comprise a box or container that holds the components of the kit. The box or container is affixed with a label or a Food and Drug Administration approved protocol. The box or container holds components of the invention which may be contained within plastic, polyethylene, polypropylene, ethylene, or propylene vessels. The vessels can be capped-tubes or bottles. The kit can also include instructions for administering an antibody of the invention.

The kit can further contain one more additional reagents, such as an immunosuppressive reagent, a cytotoxic agent or a radiotoxic agent or one or more additional antibodies of the invention (*e.g.,* an antibody having a complementary activity which binds to an epitope in the TNFα antigen distinct from a first anti-TNFα antibody). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with a liquid formulation or lyophilized formulation of an antibody or antibody fragment thereof of the invention. In one embodiment, a container filled with a liquid formulation of the invention is a pre-filled syringe. In a specific embodiment, the formulations of the invention are formulated in single dose vials as a sterile liquid. For example, the formulations may be supplied in 3 cc USP Type I borosilicate amber vials (West Pharmaceutical Services - Part No. 6800-0675) with a target volume of 1.2 mL. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products,

which notice reflects approval by the agency of manufacture, use or sale for human administration.

In one embodiment, a container filled with a liquid formulation of the invention is a pre-filled syringe. Any pre-filled syringe known to one of skill in the art may be used in combination with a liquid formulation of the invention. Pre-filled syringes that may be used are described in, for example, but not limited to, PCT Publications WO05032627, WO08094984, WO9945985, WO03077976, US Patents US6792743, US5607400, US5893842, US7081107, US7041087, US5989227, US6807797, US6142976, US5899889, US7699811, US7540382, US7998120, US7645267, and US Patent Publication No. US20050075611. Pre-filled syringes may be made of various materials. In one embodiment a pre-filled syringe is a glass syringe. In another embodiment a pre-filled syringe is a plastic syringe. One of skill in the art understands that the nature and/or quality of the materials used for manufacturing the syringe may influence the stability of a protein formulation stored in the syringe. For example, it is understood that silicon based lubricants deposited on the inside surface of the syringe chamber may affect particle formation in the protein formulation. In one embodiment, a pre-filled syringe comprises a silicone based lubricant. In one embodiment, a pre-filled syringe comprises baked on silicone. In another embodiment, a pre-filled syringe is free from silicone based lubricants. One of skill in the art also understands that small amounts of contaminating elements leaching into the formulation from the syringe barrel, syringe tip cap, plunger or stopper may also influence stability of the formulation. For example, it is understood that tungsten introduced during the manufacturing process may adversely affect formulation stability. In one embodiment, a pre-filled syringe may comprise tungsten at a level above 500 ppb. In another embodiment, a pre-filled syringe is a low tungsten syringe. In another embodiment, a pre-filled syringe may comprise tungsten at a level between about 500 ppb and about 10 ppb, between about 400 ppb and about 10 ppb, between about 300 ppb and about 10 ppb, between about 200 ppb and about 10 ppb, between about 100 ppb and about 10 ppb, between about 50 ppb and about 10 ppb, between about 25 ppb and about 10 ppb.

In certain embodiments, kits comprising antibodies of the invention are also provided that are useful for various purposes, *e.g.,* research and diagnostic including for purification or immunoprecipitation of protein of interest from cells, detection of the protein of interest *in vitro* or *in vivo*. For isolation and purification of a protein of interest, the kit may contain an antibody coupled to beads (*e.g.,* sepharose beads). Kits may be provided which contain the

antibodies for detection and quantitation of a protein of interest *in vitro*, *e.g.,* in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a container and a label or package insert on or associated with the container. The container holds a composition comprising at least one antibody of the invention. Additional containers may be included that contain, *e.g.,* diluents and buffers, control antibodies. The label or package insert may provide a description of the composition as well as instructions for the intended *in vitro* or diagnostic use.

The present invention also encompasses a finished packaged and labeled pharmaceutical product. This article of manufacture includes the appropriate unit dosage form in an appropriate vessel or container such as a glass vial, pre-filled syringe or other container that is hermetically sealed. In one embodiment, the unit dosage form is provided as a sterile particulate free solution comprising an antibody that is suitable for parenteral administration. In another embodiment, the unit dosage form is provided as a sterile lyophilized powder comprising an antibody that is suitable for reconstitution.

In one embodiment, the unit dosage form is suitable for intravenous, intramuscular, intranasal, oral, topical or subcutaneous delivery. Thus, the invention encompasses sterile solutions suitable for each delivery route. The invention further encompasses sterile lyophilized powders that are suitable for reconstitution.

As with any pharmaceutical product, the packaging material and container are designed to protect the stability of the product during storage and shipment. Further, the products of the invention include instructions for use or other informational material that advise the physician, technician or patient on how to appropriately prevent or treat the disease or disorder in question, as well as how and how frequently to administer the pharmaceutical. In other words, the article of manufacture includes instruction means indicating or suggesting a dosing regimen including, but not limited to, actual doses, monitoring procedures, and other monitoring information.

Specifically, the invention provides an article of manufacture comprising packaging material, such as a box, bottle, tube, vial, container, pre-filled syringe, sprayer, insufflator, intravenous (i.v.) bag, envelope and the like; and at least one unit dosage form of a pharmaceutical agent contained within said packaging material, wherein said pharmaceutical agent comprises a liquid formulation containing an antibody. The packaging material includes instruction means which indicate how that said antibody can be used to prevent, treat and/or manage one or more symptoms associated with a disease or disorder.

The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references, including literature references, issued patents, and published patent applications, as cited throughout this application are hereby expressly incorporated herein by reference. It should further be understood that the contents of all the figures and tables attached hereto are expressly incorporated herein by reference.

The entire contents of the following applications are also expressly incorporated herein by reference: U.S. Provisional Patent Application 61/893,123, entitled "STABLE SOLID PROTEIN COMPOSITIONS AND METHODS OF MAKING SAME", Attorney Docket Number 117813-31001, filed on October 18, 2013; U.S. Provisional Application Serial No. 61/892,833, entitled "LOW ACIDIC SPECIES COMPOSITIONS AND METHODS FOR PRODUCING THE SAME USING DISPLACEMENT CHROMATOGRAPHY", Attorney Docket Number 117813-73602, filed on October 18, 2013; U.S. Provisional Patent Application 61/892,710, entitled "MUTATED ANTI-TNFa ANTIBODIES AND METHODS OF THEIR USE", Attorney Docket Number 117813-73802, filed on October 18, 2013; U.S. Provisional Patent Application 61/893,068, entitled "LOW ACIDIC SPECIES COMPOSITIONS AND METHODS FOR PRODUCING THE SAME", Attorney Docket Number 117813-73901, filed on October 18, 2013; U.S. Provisional Patent Application 61/893,088, entitled "MODULATED LYSINE VARIANT SPECIES AND METHODS FOR PRODUCING AND USING THE SAME", Attorney Docket Number 117813-74101, filed on October 18, 2013; and U.S. Provisional Patent Application 61/893,131, entitled "PURIFICATION OF PROTEINS USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY", Attorney Docket Number 117813-74301, filed on October 18, 2013.

IX. EXAMPLES

Three antibodies were used in connection with the studies outlined below (Examples 1-8). Adalimumab antibody was generated from cell culture processed using chemical defined medium (CDM) and purified by a 4.4 cm (id.) x ~20 cm (L) MabSelect SuRe Protein A column. mAb X bulk drug substance was obtained from a three-step large scale purification process. mAb Y antibody was generated from a large scale manufacturing process and purified by a MabSelect SuRe Protein A column. Adalimumab Protein A eluate was in a buffer of ~20 mM acetic acid at pH ~4.2. The mAb X was in a buffer containing

~15 mM histidine, pH ~6. The mAb Y was in a buffer containing ~10 mM sodium formate, pH ~4.2. Each mAb feed was conditioned to the targeted pH, conductivity and concentration prior to the displacement chromatography experiment.

The cationic displacers, Expell SP1™ and protamine sulfate (from salmon sperm), were purchased from SACHEM Chemical Company and Sigma Aldrich, respectively.

Poros XS CEX resin (Life Technologies) was packed in a 0.66 cm x ~25 cm column. The column was equilibrated with a 140 mM Tris/Acetate buffer or a 30 mM MES, 10 mM NaCl buffer at the targeted pH and conductivity (Table 1). After equilibration, the column was loaded with each pre-conditioned feed at a resin loading level of ~40 g/L followed by a 2 CV of equilibration buffer wash. The displacing buffer, which consists of defined concentration of Expell SP1™ or protamine sulfate in the equilibration buffer, was flowed through the column to initiate the displacement process. In standard one-step displacement wash process, this step was continued for at least 30 CV at a flow rate corresponding to 15 to 22 min residence time (RT) before column regeneration and cleaning with a caustic solution consisting of 0.5 N NaOH and 0.5 M KCl. Alternatively, the displacement wash step comprised two displacement buffers each flowing for defined volumes, or a linear gradient flow from low to high concentration displacer buffer. Sample fractions were collected at every 0.5 or 1 CV for protein concentration and quality analysis. The specific processing conditions are detailed in Tables 1 and 2.

Capto MMC resin (GE Healthcare) was packed in a 0.66 cm x ~30 cm column. The column was equilibrated with a 140 mM Tris/Acetate buffer at the targeted pH and conductivity (Table 3). After equilibration, the column was loaded with each pre-conditioned feed at a resin loading level about 34 to 40 g/L followed by a 2 CV equilibration buffer wash. The displacing buffer, which consists of defined concentration of protamine sulfate in the equilibration buffer, was flowed through the column to initiate the displacement process. This step was continued for 30 CV at a flow rate corresponding to ~22 min RT before column regeneration and cleaning. Sample fractions were collected at every 0.5 or 1 CV for protein concentration and quality analysis. The specific processing conditions are detailed in Table 3.

The levels of acidic species and other charge variants in the Adalimumab, mAb X and mAb Y samples were quantified using the respective qualified CEX-HPLC method. For Adalimumab, a 4 mm x 250 mm analytical Dionex ProPac WCX-10 column (Dionex, CA) was used along with a Shimazhu HPLC system. The mobile phases were 10 mM Sodium

Phosphate dibasic pH 7.5 buffer (Mobile phase A) and 10 mM Sodium Phosphate dibasic, 500 mM Sodium Chloride pH 5.5 buffer (Mobile phase B). A binary gradient (6% B: 0 min; 6-16% B: 0-20 min; 16-100%B: 20-22 min; 100% B: 22-26 min; 100-6% B: 26-28 min; 6% B: 28-35 min) was used with detection at 280 nm. Quantitation was based on the relative area percentage of detected peaks. The peaks that elute at residence time less than ~7 min were together represented as the acidic peaks or AR region.

For mAb X, a 4 mm x 250 mm analytical Dionex ProPac WCX-10 column (Dionex, CA) was used along with a Shimazhu HPLC system. The mobile phases were 20 mM MES, pH 6.5 buffer (Mobile phase A) and 20 mM MES, 500 mM NaCl, pH 6.5 buffer (Mobile phase B). A binary gradient (10% B: 0 min; 10-28% B: 1-46 min; 28-100% B: 46-47 min; 100% B: 47-52 min; 100-10% B: 52-53 min; 10% B: 53-58 min) was used with detection at 280 nm. Quantitation was based on the relative area percentage of detected peaks. All peaks eluting prior to the Main Isoform peak were summed as the acidic region, and all peaks eluting after the Main peak were summed as the basic region.

For mAb Y, a 4 mm x 250 mm Dionex ProPac analytical WCX-10 column (Dionex, CA) was used on a Shimazhu HPLC system. The mobile phases were 20 mM MES, pH 6.2 (Mobile phase A) and 20 mM MES, 250 mM NaCl, pH 6.2 (Mobile phase B). A binary gradient (1% B: 0-1 min; 1-25% B: 1-46 min; 25-100% B: 46-47 min; 100% B: 47-52 min; 100-1% B: 52-53 min; 1% B: 53-60 min) was used with detection at 280 nm. Column temperature was set at 35 °C. Quantitation was based on the relative area percentage of detected peaks. All peaks eluting prior to the Main Isoform peak (but after 2 min retention time) were summed as the acidic region, and all peaks eluting after the Main peak were summed as the basic region.

The levels of aggregates, monomer and fragments in eluate samples were measured using a SEC method for each molecule. For Adalimumab and mAb Y, a TSK-gel G3000SWxL, 5 μm, 125 Å, 7.8 X 300 mm column (Tosoh Bioscience) was used while a TSK-gel Super SW3000, 4 μm, 250 Å, 4.6 X 300 mm column (Tosoh Bioscience) was used for mAb X along with an Agilent or a Shimazhu HPLC system. For Adalimumab and mAb X, injections were made under isocratic elution conditions using a mobile phase consisting of 100 mM sodium sulfate and 100 mM sodium phosphate at pH 6.8, and detected with UV absorbance at 214 nm. For mAb Y, the mobile phase consists of 1X PBS at pH 7.4, and elution profile detected with UV absorbance at 280 nm. Quantification is based on the relative area of detected peaks.

An HCP ELISA assay was used to determine the HCP levels in various samples and feeds for all three mAbs.

Table 1. Processing conditions for Poros XS one-step displacement chromatography

Molecule	Displacer	Displacer Conc. (mM)	Equilibration/ Wash/ Displacing buffer			Loading Conditions	Regeneration
			Buffer System	pH	Conductivity (mS/cm)		
Adalimumab	Expell SP1	0.5-3	Tris/Acetate	6.7-7.8	5.4-6.6	pH ~7.5, ~6 mS/cm	2M NaCl
		2-5	MES/NaCl	6.1	2.1	pH 6.1, ~2 mS/cm	0.2M acetic acid & 1M KCl
	Protamine Sulfate	0.25-2	Tris/Acetate	6.5-7.5	5.6-6.6	pH 7.5, 5.4-6.3 mS/cm	2M NaCl, 6M Guanidine HCl
mAb X	Expell SP1	0.5-2	Tris/Acetate	6	6.2-6.5	pH 6, ~6 mS/cm	2M NaCl
	Protamine Sulfate	0.25-0.5	Tris/Acetate	6	6.0-6.5	pH 6, 5.6-6.5 mS/cm	2M NaCl, 6M Guanidine HCl
mAb Y	Expell SP1	0.5-1	Tris/Acetate	5	~6	pH 5, 6.2 mS/cm	2M NaCl

Table 2. Processing conditions for Poros XS two-step or linear gradient displacement chromatography

Molecule	Displacer	Displacement Method	Displacer Concentration (mM)	Equilibration/ Wash/ Displacing buffer			Loading Conditions	Regeneration
				Buffer System	pH	Conductivity (mS/cm)		
Adalimumab	Expell SP1	Two-step	(1): 0.5mM, 25CV; (2): 2mM, 20CV	Tris / Acetate	7	~6	pH 7.5, 6.1 mS/cm	2M NaCl
	Protamine Sulfate	Two-step	(1): 0.25mM, 10CV; (2): 2mM, 10CV	Tris / Acetate	7.5	5.5	pH 7.5, 6.1 mS/cm	2M NaCl, 6M Guanidine HCl
	Expell SP1	Linear Gradient	0-1 mM over 40 CV	Tris / Acetate	7	~6	pH 7.5, 6.0 mS/cm	2M NaCl
	Protamine Sulfate	Linear Gradient	0-1 mM over 40 CV	Tris / Acetate	7.5	~6	pH 7.5, 5.9 mS/cm	2M NaCl, 6M Guanidine HCl
mAb X	Expell SP1	Two-step	(1): 0.5mM, 22CV; (2): 2mM, 12CV	Tris / Acetate	6	6.1	pH 6, 6.3 mS/cm	2M NaCl
	Protamine Sulfate	Two-step	(1): 0.35mM, 10CV; (2): 0.5mM, 10CV	Tris / Acetate	6	~6	pH 6, 6.3 mS/cm	2M NaCl

Table 3. Processing conditions for Capto MMC one-step displacement chromatography

Molecule	Displacer	Displacer Concentration (mM)	Equilibration/ Wash/ Displacing buffer			Loading Conditions	Regeneration	CIP
			Buffer System	pH	Conductivity (mS/cm)			
Adalimumab	Protamine Sulfate	0.25-0.5	Tris / Acetate	7-7.5	~6	pH 7.5, 5.3-6.1 mS/cm	2M NaCl, 6M Guanidine HCl	0.5N NaOH + 0.5M KCl
mAb X	Protamine Sulfate	0.25-0.5	Tris / Acetate	7-7.7	~6	pH 7-7.7, 5.9-6.5 mS/cm	2M NaCl, 6M Guanidine HCl	
mAb Y	Protamine Sulfate	0.25-0.5	Tris / Acetate	5-5.5	~6.5	pH 5.5, 5.2-5.6 mS/cm	2M NaCl, 6M Guanidine HCl	

Example 1. Displacement chromatography performances of Expell SP1™ for Adalimumab on Poros XS resin

Expell SP1™ is a low molecular weight quaternary ammonium salt that exhibited pronounced displacement effect for Adalimumab on Poros XS resin under selected sets of operating conditions. The feed material used for this set of experiments contained about 20-25% total AR, of which 2-5% was AR1 and 18-20% AR2. The results for this system are shown in the following sections.

A representative, desired displacement chromatographic profile is shown in Figure 1a (solid line). In this experiment, the column was equilibrated with a pH 7 Tris/acetate buffer (6.4 mS/cm), loaded with a pre-adjusted protein A eluate feed (pH 7.5, 6.3 mS/cm, ~3.4 g/L) to ~40 g/L resin loading level, followed by EQ buffer wash and then displacement process using 1 mM Expell SP1™ in the pH 7 EQ buffer. The extended, square shape UV280 "elution" profile indicated establishing a proper displacement train and thus a degree of separation of the feed components can be realized.

Figure 2 illustrates the CEX-HPLC chromatograms for several samples taken along this well-established displacement UV trace. Clearly, the variant species were rearranged during the displacement process according to their respective binding affinity to the resin:

AR1 was enriched in the foremost of the displacement train followed by AR2, Lys 0, Lys 1 and Lys 2 in order.

Figure 3 shows the distribution of each variant species in all the collected sample fractions. The acidic species were enriched in the earlier fractions compared to the Lys variants. By excluding those earlier fractions the product pool AR level will be reduced relative to that in the feed. This is reflected in Figure 4 which plots the reduction of total AR (*i.e.,* AR1+AR2) and AR1 level versus cumulative product yield. At a yield of ~75%, the total AR% was reduced by 11.7% and AR1% by 4.2% under this set of condition. Along with the removal of AR species, the product pool lysine variant species distribution profile was also modulated. As shown in Table 4, below, the ratio of Lys 0 species to the lysine variant sum decreased from 0.67 to 0.62; the ratio of Lys 1 species to the lysine variant sum increased from 0.24 to 0.28; and the ratio of Lys 2 species to the lysine variant sum increased from 0.08 to 0.1. The lysine prolife can be further altered by pooling different fractions from the displacement chromatography process.

Varying the processing conditions such as the buffer pH and displacer concentration can modulate the shape of the displacement chromatogram and hence the separation performance. In an extreme case, the chromatogram more or less resembles the typical elution "peak" profile without incurring the separation of variant species (Figures 1A and 1B). Interestingly, this occurs at stronger binding conditions; for instance, the conditions corresponding to Figure 1B is pH 6.1 and ~2 mS/cm for equilibration, loading, wash, and displacement. Without being bond by theory, the lack of variant separation under such conditions may be due to the diminishing difference in binding affinity of each species and thus the selectivity by the displacer.

The effect of Expell SP1™ concentration on Adalimumab AR reduction was measured in pH 7.5 Tris/Acetate buffer, as shown in Figure 5. The same equilibration/wash and feed loading conditions were used for all the runs here. Increasing Expell SP1™ concentration from 0.5 to 3 mM decreased ΔAR% from 8.9% to 3.5% at similar product yield ~75%. Controlling the Expell SP1™ concentration within 2 mM will consistently achieve ≥6% AR% reduction.

The effect of displacing buffer pH on AR reduction for Adalimumab was measured at 1 mM Expell SP1™ concentration in the Tris/Acetate buffer, as shown in Figure 6. In this set of experiments, the column was conditioned with an EQ buffer at the respective displacing buffer pH, and then loaded with protein feed at pH 7.5 and ~6 mS/cm followed by

a brief EQ buffer wash before starting the displacement step. The buffer pH significantly impacts AR clearance in pH range of 6-8. At similar yield (~75%), the maximal reduction in AR level (~12%) is seen at pH 7. Despite such pH-dependency, the majority of the conditions here (pH 6.5 to 7.8) gave at least 5% AR removal in final product pool.

In the aforementioned experiments, one displacing buffer was used to achieve the protein variant separation. It was observed that, relatively lower displacer concentration gives better separation but tends to elongate the process due to substantial increase in the required displacing buffer volume. For instance, when using 0.5 mM Expell SP1™ in a pH 7 displacing buffer (Table 1), the displacement phase requires 44 column volumes (CV) of this buffer for completion. To accelerate the operation without affecting the acidic species separation, a two-step displacement process was explored at this pH condition. In the example provided here, the displacement process was started with 0.5 mM Expell SP1™ at pH 7 and continued for 25 CV, followed by 20 CV of 2 mM Expell SP1™ solution at the same pH. Under such conditions, the protein displacement profile was completed in a total of 33 CV which is 25% less than that required for one-step displacement process, thus significantly shortening the process.

Figure 7 shows the reduction of AR% versus product yield for the aforementioned two-step displacement run. The net total AR level in product pool was reduced by 6.6% at ~75% yield. In contrast to the conventional use of a single displacing solution consisting of a single displacer at a defined concentration, herein the AR clearance was achieved by excluding the AR-enriched early fractions as induced by 0.5 mM Expell SP1™ displacement, while the higher Expell SP1™ concentration was used to accelerate the displacement of the remainder proteins off the solid phase. In light of this unexpected similar product quality and yield results, step-gradient displacement schemes are considered to be advantageous over conventional strategies.

Besides the two-step displacement scheme, a linear gradient displacement method was also tested for the Adalimumab charge variant separation. As detailed in Table 2, after the feed loading at pH 7.5 (~6 mS/cm), the column was briefly washed with the equilibration buffer (pH 7, ~6 mS/cm) and then started with a 40 CV linear gradient from the EQ buffer to a 1 mM Expell SP1™ displacing buffer (which was made from the EQ buffer). Under such condition, the displacement profile matured within this 40 CV gradient. The product eluate was pooled by excluding the first a few fractions. In this case, the net AR% decreased by 6.8% at a product recovery of 72%.

Apart from acidic species, other product- or process-related impurities can be effectively separated by Poros XS displacement chromatography using Expell SP1™ as the displacer. Figure 8 shows the separation of aggregates, monomer and fragments in Adalimumab sample fractions obtained from a one-step displacement experiment using 1 mM Expell SP1™, pH 7 buffer. It should be noted that the last two fractions from this run were not collected, therefore the increased aggregate levels at the end of the displacement train was not fully exemplified here. Interestingly, the early fractions which contained elevated acidic species also showed enriched aggregates, indicating that this population of aggregates may consist of more acidic species, or the acidic species has higher propensity to form aggregates. As summarized in Table 4, the aggregate level in the product pool (at ~75% yield) was reduced from the feed level 1.16% to 0.11% and the fragment level down to 0.04% along with significant reduction in the AR concentration. In addition to the standard method, the linear gradient displacement run also showed aggregate reduction from the feed level of 0.9% to about 0.2% in final product.

Figure 9 shows the distribution of HCP in the Adalimumab displacement train coming off the Poros XS column. Relatively higher level of HCP was observed at both ends of the train, due to their diverse charge characteristics and associated binding strength. The final product pool HCP level was reduced to 5 ng/mg from the starting feed, representing approximately 50-fold reduction.

Table 4. Step yield & product quality in Adalimumab before and after Poros XS displacement chromatography using Expell SP1™ (pH 7, 1 mM Expell SP1™)

	Yield %	AR1 %	AR2 %	Lys Sum %	Lys 0 %	Lys 1 %	Lys 2 %	HMW %	Monomer %	LMW %	HCP (ng / mg)
Feed	--	4.3	17.8	77.9	52.5	19.0	6.5	1.16	98.57	0.27	267
Product pool	74	0.1	10.3	89.6	55.5	24.8	9.3	0.11	99.85	0.04	5

Example 2. Displacement chromatography performance of protamine sulfate for Adalimumab on Poros XS resin

Protamine sulfate, a cationic peptide with molecule weight ~5.1 kD, was also evaluated as a cation exchange displacer for Adalimumab on Poros XS resin under various

operating conditions. The feed material used for this set of experiments contained about 17-24% total AR, of which 3-6% was AR1 and 14-19% AR2. The results for this system are illustrated in the following sections.

Figure 10 shows the distribution of charge variant species in sample fractions collected from a well established displacement process induced by protamine sulfate. In this experiment, the column was equilibrated with a pH 7.5 Tris/acetate buffer (5.6 mS/cm), loaded with a pre-adjusted protein A eluate feed (pH 7.5, 5.4 mS/cm, 5.2 g/L) to 39 g/L resin loading level, followed by a brief EQ buffer wash and then displacement process using 0.5 mM protamine sulfate dissolved in the pH 7.5 EQ buffer. Similar to the Expell SP1™ displacement profile (Figure 3), the charge variants were enriched at different locations of the displacement train and were peaked in the order of AR1, AR2, Lys0, Lys1 and Lys2. The cumulative AR% reduction as a function of product yield is illustrated in Figure 11. A 6-8% decrease in the total AR level can be obtained at a yield of 75-85% under this set of condition. The actual levels of AR1, AR2 and total lysine variant species (*i.e.,* Lys 0 + Lys 1+ Lys 2) for the feed and the final product pool are shown in Table 5.

The effect of protamine sulfate concentration on Adalimumab AR reduction was measured in pH 7.5 Tris/Acetate buffer, as shown in Figure 12. The same equilibration/wash and feed loading conditions as described above were used for all the runs here. At similar yield (~75%), the total AR% was reduced by approximately 7-8% when using 0.25 to 2 mM protamine sulfate. This broad concentration range reflects the robustness of charge variant separation by protamine sulfate displacement process.

The effect of displacing buffer pH on AR clearance for Adalimumab was measured at 0.5 mM protamine sulfate concentration in Tris/Acetate buffer. In this set of experiments, the column was conditioned with an EQ buffer at the respective displacing buffer pH, loaded with protein feed at pH 7.5 and ~6 mS/cm followed by a brief EQ buffer wash before starting the displacement phase. As shown in Figure 13, the extent of AR reduction increases significantly as pH varies from 6.5 to 7.5. Over 6% decrease in AR level can be achieved at pH 7.5 with a product yield ~75%.

The two-step displacement scheme was also tested with protamine sulfate. In one experiment, the displacement process consists of 10 CV of 0.25 mM protamine and 10 CV of 2 mM protamine at pH 7.5 (Table 2). The protein displacement profile was completed in a total of 13 CV, which is about 11 CV or almost 2 fold shorter than that in the one-step displacement process with 0.25 mM protamine sulfate. The reduction of AR% versus

product yield is shown in Figure 14. The total AR level in product pool were reduced by ~8% at ~75% yield, which is comparable to that achieved by the one-step displacement process using 0.25 mM protamine sulfate.

The linear gradient displacement scheme was also evaluated with protamine sulfate on Poros XS resin for Adalimumab charge variant separation. As summarized in Table 2, after the feed loading at pH 7.5 (5.9 mS/cm), the column was briefly washed with the equilibration buffer (pH 7.5, ~6 mS/cm) and then started with a 40 CV linear gradient from the EQ buffer to a 1 mM protamine sulfate displacing buffer (which was made from the EQ buffer). Figure 15 shows the cumulative ΔAR% versus yield from this run. At a product yield of 75.6%, the total AR% was reduced from the feed level of 21.3% to 12.1%.

Protamine sulfate displacement chromatography also demonstrated significant clearance of aggregates, fragments and HCP. Figure 16 exemplifies the size variant profiles of Adalimumab from the same experiment described above (i.e., 0.5 mM protamine sulfate, pH 7.5, one-step displacement run). As expected, the fragments were mostly enriched at the front while the aggregates primarily resided at the back of the train. Similar to that shown in Figure 8, a subpopulation of the aggregates was also observed in the displacement front; in addition, a portion of fragments was noticed at the tail. Table 5 compares the levels of aggregates, fragments and HCP in final product pool (at ~75% yield) relative to the feed.

Table 5. Step yield & product quality in Adalimumab before and after Poros XS displacement chromatography using protamine sulfate

	Yield %	AR1 %	AR2 %	Lys Sum%	HMW %	Monomer %	LMW %	HCP (ng/mg)
Feed	--	4.1	16.9	79.0	0.8	98.0	1.2	153
Product pool	73	1.3	13.1	84.7	0.3	99.6	0.1	14

Example 3. Displacement chromatography performance of Expell SP1™ for mAb X on Poros XS resin

The displacement separation performance of Expell SP1™ was assessed for mAb X on the Poros XS resin. A purified mAb X drug substance was used in this study, which contained about 16-17% acidic species and 12-14% basic species.

A representative set of mAb X charge variant separation profiles are shown in Figures 17 and 18. In this experiment, the Poros XS column was loaded with 40 g/L of mAb X at pH 6, 6 mS/cm Tris/Acetate binding condition, and was displaced using 1 mM Expell SP1™ in a pH 6, ~ 6 mS/cm buffer. The specific conditions are detailed in Table 1. Pronounced enrichment and separation of acidic, main and basic species were achieved, with AR% reduced by 9.4% at 76% yield.

The effect of Expell SP1™ concentration on AR reduction for mAb X was measured in the pH 6 Tris/Acetate buffer. As shown in Figure 19, increasing the Expell SP1™ concentration from 0.5 to 2 mM decreased the ΔAR% for mAb X from 9.8% at 81% yield to 7.9% at 69% yield.

The two-step displacement scheme was evaluated for mAb X. As shown in Table 2, the displacement process comprised of 22 CV of 0.5 mM Expell SP1™ and 12 CV of 2 mM Expell SP1™ at pH 6. The protein displacement profile was completed within 30 CV of total displacing buffer volume, which was 30% less than that required for one-step displacement separation. The reduction of AR% versus product yield is shown in Figure 20. The total AR% in product pool was reduced by ~9% at ~75% yield, again comparable to that obtained with one-step displacement process using 0.5 mM Expell SP1™ buffer.

Example 4. Displacement chromatography performance of protamine sulfate for mAb X on Poros XS Resin

Protamine sulfate was also evaluated for separating acidic species for mAb X on Poros XS resin. The feed material for this set of experiments contained about 12-16% acidic and 12-13% basic species. The results for this system are shown in the following sections.

A representative set of variant separation profiles are shown in Figures 21 and 22. In this experiment, the Poros XS column was loaded with ~36 g/L of mAb X at pH 6, 6.5 mS/cm binding condition, and was displaced using 0.25 mM protamine sulfate in a pH 6, 6.5 mS/cm Tris/Acetate buffer. The specific conditions are detailed in Table 1. Pronounced enrichment and separation of acidic, main and basic species were achieved, with AR level reduced by 6% at 75% yield.

The effect of protamine sulfate concentration on mAb X AR reduction was measured in a pH 6 Tris/Acetate buffer, as shown in Figure 23. In this case, the protamine sulfate concentration strongly affects the AR clearance in a relatively small protamine concentration range (*i.e.,* from 0.35 to 0.5 mM). Nevertheless, over 8% of AR reduction can be achieved at pH 6 for mAb X at acceptable yield (≥ 70%).

The two-step displacement scheme was evaluated for mAb X with protamine sulfate. In this experiment, the displacement process comprised of 10 CV of 0.35 mM Expell and 10 CV of 0.5 mM Expell at pH 6 (see Table 2). The protein displacement profile was completed in ~15 CV of total displacing buffer volume, representing ~26% reduction of buffer volume relative to the one-step displacement operation. The reduction of AR% versus product yield is shown in Figure 24. The product pool AR level was reduced by ~6% at ~75% yield.

The mAb X BDS has about 0.74% of aggregates, which can be further reduced during the protamine sulfate displacement process. Figure 25 shows the size variant profiles of mAb X as displaced by a 0.25 mM protamine sulfate, pH 6 buffer (i.e., the one-step displacement run shown in Figures 21 and 22). The aggregates were all enriched at the end of the displacement train, which differs from the observations with Adalimumab. Using the same product pooling strategy based on AR reduction, the monomer level was enhanced to 99.9% (Table 6).

Table 6. Step yield & product quality in mAb X before and after Poros XS displacement chromatography using protamine sulfate

	Yield%	Acidic%	Main%	HMW%	Monomer%
Feed	--	12.3	75.7	0.7	99.3
Product pool	79	4.7	80.8	0.1	99.9

Example 5. Displacement chromatography performance of Expell SP1™ for mAb Y on Poros XS Resin

The displacement separation performance of Expell SP1™ was further assessed for mAb Y on Poros XS resin. The mAb Y has a pI of 7-7.5, much lower than Adalimumab and mAb X. An mAb Y protein A eluate was used in this study, which contained about 22% acidic species and 15% basic species.

An appropriate set of displacement conditions for mAb Y is shown in Table 1. The equilibration, wash and displacement buffers are all at pH 5 with conductivity around 6 mS/cm. The 0.5 mM Expell SP1™ buffer generated the desired displacement profile. The sample fractions from this run were analyzed by cation exchange HPLC. Figures 26 and 27 indicate the distribution of charge variant species and the cumulative ΔAR% versus product

yield, respectively. A 6.6% decrease in AR% was observed at 74% yield under such condition.

Example 6. Displacement chromatography performance of protamine sulfate for Adalimumab on Capto MMC (Multimodal) Resin

Capto MMC™ is a mixed mode resin based on weak cation-exchange and hydrophobic interaction mechanism. Its capability for acidic species and aggregates removal by displacement chromatography was assessed here. The Adalimumab feed material for this set of experiments contained about 20-21% total AR. The results for protamine sulfate system are shown in the following sections.

A representative set of variant separation profiles are shown in Figures 28 and 29. In this experiment, the Capto MMC column was equilibrated with a 140 mM Tris/acetate, pH 7 buffer (~5.7 mS/cm), loaded with ~34 g/L of Adalimumab at pH 7.5 and 5.3 mS/cm binding condition, briefly washed with EQ buffer and then displaced with 0.35 mM protamine sulfate in the pH 7 EQ buffer. A typical displacement chromatogram was generated under such experimental condition. As shown in Figure 10, Capto MMC also showed enrichment of each variant in the train, yielding total AR reduction of ~4% at ~75% yield. The shape of the ΔAR% versus yield curve (Figure 29) differs from that given by the Poros XS resin, possibly due to stronger binding of each protein species (related to secondary mode of interaction) by this mixed mode ligand.

The buffer pH and protamine sulfate concentrations were varied to assess the overall AR clearance by Capto MMC displacement chromatography. Table 7 summarized the results for three runs. Overall, 3-5% of AR reduction can be achieved for Adalimumab when using protamine sulfate as a displacer for Capto MMC resin.

Table 7. AR removal for Adalimumab by Capto MMC displacement chromatography using protamine sulfate

Run No.	EQ/ Displacing buffer pH	Load pH	Protamine Conc. (mM)	Yield (%)	ΔAR%
1	7	7	0.5	75	3.1
2	7	7.5	0.35	75	4.0
3	7.25	7.5	0.25	78	5.2

The clearance of aggregates by Capto MMC displacement chromatography is illustrated in Table 8. The same operating conditions as described for obtaining the results in Table 7 were used here. The product pool monomer level was enhanced from 98.8% in the feed to 99.4% with aggregates reduced from 1.0% to 0.5%.

Table 8. Step yield & product quality in Adalimumab before and after Capto MMC displacement chromatography using protamine sulfate

	Yield%	AR1%	AR2%	Lys Sum%	HMW%	Monomer%	LMW%
Feed	--	4.4	16.5	79.1	1.0	98.8	0.2
Product pool	75	2.5	14.4	83.1	0.5	99.4	0.1

Example 7. Displacement chromatography separation of protamine sulfate for mAb X on Capto MMC Resin

Protamine sulfate was evaluated for removing mAb X acidic species on Capto MMC resin. The same feed material as shown in Example 4 was used for this set of experiments. As detailed in Table 3, the pH and protamine concentrations were varied in order to generate desired displacement profile. One representative set of working condition is to load Capto MMC column with 40 g/L of mAb X at pH 7.5 and ~6 mS/cm, and to use 0.25 mM protamine sulfate in the pH 7.5, ~ 6 mS/cm EQ buffer for displacement (Table 3). The separation of charge variants and AR reduction as a function of product recovery are shown

in Figures 30 and 31, respectively. In this case, 3-5% of AR reduction was resulted at product yield of 70-90%.

The effect of protamine sulfate concentration on mAb X AR reduction by Capto MMC resin was measured in a pH 7, 6 mS/cm Tris/Acetate buffer, as shown in Table 9. Varying the protamine concentration from 0.25 mM to 0.5 mM had very little effect on ΔAR% and product yield, which is quite different from the observations with the Poros XS resin (Figure 23). The mixed mode MMC resin gave over 3% AR clearance under such selected conditions.

Table 9. Reduction of AR level by Capto MMC displacement chromatography for mAb X at different protamine sulfate concentrations

Expell concentration (mM)	Yield (%)	ΔAR (%)
0.25	75	3.4
0.5	77	3.3

The effect of displacing buffer pH on AR clearance for mAb X was measured at 0.25 mM protamine sulfate concentration in Tris/Acetate buffer. In this set of experiments, the column was conditioned with an EQ buffer at the respective displacing buffer pH, loaded with protein feed at the same pH and ~6 mS/cm followed by a brief EQ buffer wash before starting the displacement phase. As shown in Figure 32, ΔAR% increases from 3.3 to 6.5% as pH varies from 7 to 7.7.

The Capto MMC resin also removes aggregates during protamine sulfate displacement chromatography. Table 10 shows the level of charge and size variants of mAb X in product eluate when using 0.5 mM protamine sulfate, pH 7.5 (~6 mS/cm) displacing buffer for separation. The product pool monomer level was enhanced from 98.7% in the feed to 99.2% with aggregate and fragment levels reduced by 50% and 28%, respectively.

Table 10. Step yield & product quality for mAb X before and after Capto MMC displacement chromatography using protamine sulfate

	Yield%	Acidic%	Main%	HMW%	Monomer%	LMW%
Feed	--	16.8	74.0	0.6	98.7	0.7
Product pool	77	12.4	71.8	0.3	99.2	0.5

Example 8. Displacement chromatography separation of protamine sulfate for mAb Y on Capto MMC Resin

Protamine sulfate was also evaluated for removing mAb Y acidic species on Capto MMC resin. The same feed material as shown in Example 5 was used for the experiments here. Two sets of conditions were evaluated here. In one experiment, the equilibration, wash and displacement buffers were adjusted to pH 5.5 and ~ 6.5 mS/cm, and the displacement buffer contained 0.25 mM protamine sulfate. In the other experiment, all those buffers were adjusted to pH 5, ~ 6.5 mS/cm and the protamine sulfate concentration was 0.5 mM. In both runs the feed was adjusted to pH 5.5, 5.2-5.5 mS/cm and loaded to the Capto MMC column at ~ 40 g/L loading level. As shown in Table 11, both sets of conditions resulted in AR% decrease by 5-7% in product pool. In addition, significant aggregates and fragments reduction was achieved with the same product pooling strategy.

Table 11. Step yield & product quality for mAb Y before and after Capto MMC displacement chromatography using protamine sulfate

Conditions	Sample	Yield %	Acidic%	Main%	HMW%	Monomer %	LMW%
pH 5.5, 0.25 mM Protamine	Feed	--	16.4	75.8	8.5	91.0	0.5
	Product pool	76	9.8	79.4	2.0	97.6	0.4
pH 5, 0.5 mM Protamine	Feed	--	20.9	72.8	6.3	93.2	0.5
	Product pool	75	15.9	81.1	1.6	98.2	0.2

Summary of Examples 1-8

Examples 1-8 above, demonstrate the use of cation exchange and mixed mode displacement chromatography for effectively reducing acidic species along with various other impurities from different monoclonal antibody feed streams. Under appropriate (or relatively weak) binding conditions, cationic molecules with high affinity for a CEX or multimodal ligand (such as Expell SP1™ and protamine sulfate) can induce the formation of charge variant displacement train, wherein the acidic population is enriched in the front followed by the main isoform, and, thereafter, the basic population. Thus, in certain embodiments, exclusion of those earlier fractions from the remainder eluate results in an AR-reduced product. Alternatively, exclusion of the fractions following the main isoform results in a Lys variant- or basic species-reduced product.

Also demonstrated in the preceding experiments is the fact that the operating pH and displacer concentration can strongly affect the displacement profile and as a result the charge variant, product aggregate, product fragment, and HCP clearance profile. The selection of a particular operating regime with regard to charge variant reduction depends, in general, on the specific protein-resin-displacer system. For example, for Adalimumab, significant AR reduction can be achieved using a displacing buffer with pH in the range of 6-8 with displacer concentration as low as 0.25-0.5 mM. The total AR level (%) in Adalimumab product pool can be reduced by over 10% with an acceptable processing yield (≥75%) from a CEX displacement chromatography process, or 4-7% from a mixed mode displacement chromatography process. Along with acidic species, other product variants or process impurities such as basic species, aggregates, fragments and HCP can be selectively collected or reduced to meet the quality requirements. In addition to the surprisingly effective preparative scale standard one-step displacement operation, unconventional displacement separation schemes are shown above to have unexpected properties, including two-step displacement chromatography and linear gradient displacement chromatography, which can significantly reduce buffer volumes and shorten the processing time without compromising the charge variant, product aggregate, product fragment, and HCP clearance at a given yield target.

The instant invention provides a method for reducing acidic species for a given protein of interest at high recovery yields. For example, adalimumab was prepared using a combination of AEX and CEX technologies to produce a Low-AR and High-AR sample with a final AR of 2.5% and 6.9%, respectively. Both samples were incubated in a controlled

environment at 25°C and 65% relative humidity for 10 weeks, and the AR measured every two weeks. Figure 23 shows the growth of AR for each sample over the 10 week incubation. It is evident from Figure 23 the growth rate of AR is linear and similar between both the Low-AR and High-AR samples. Based on these results, the reduced AR compositions of the invention can be stored 3-fold longer before reaching the same AR level as the High –AR sample. These surprising results are very beneficial for storage, handling, and use of an antibody or other protein for therapeutic use.

Example 9.

An adalimumab drug substance sample generated from a large scale manufacturing process was used as a feed for a Poros XS displacement purification using Expell SP1™. This material containing ~2% AR1 and ~12% AR2 was diluted to ~ 5 g/L with the equilibration buffer (140 mM Tris/acetate, pH 6.2 mS/cm), pH titrated to 7.5 with 1M Tris pH 9.5 and conductivity adjusted to 6 mS/cm. The adjusted feed was loaded to a pre-equilibrated Poros XS column (~41 mL packed bed volume) at 28.7 or 40 g/L loading level. The column was then washed with 2 CV of EQ buffer followed by 30 CV of displacement buffer consisting of 140 mM Tris/acetate, 1 mM Expell SP1 at pH 7. The displacement sample fractions were collected and analyzed for the charge variant profiles along with the starting material.

Table 12 below summarizes the change of acidic species level in the product pool for adalimumab. Approximately 5-6% AR reduction was observed at the target yield of ~75%.

Table 12. Step yield & acidic species levels in Adalimumab before and after Poros XS displacement chromatography using Expell SP1™ (pH 7, 1 mM Expell SP1™)

Run #	Load (g/L)	Yield (%)	AR% (load)	AR% (product pool)	ΔAR%
1	28.7	73.7	14.1	9.4	4.7
2	40	75.2	13.5	7.8	5.7

* * *

The present invention 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 the appended claims.

CLAIMS

What is claimed is:

1. A method for producing a low acidic species composition comprising an antibody, or antigen-binding portion thereof, the method comprising:

(a) contacting a first sample comprising the antibody, or antigen-binding portion thereof, with a chromatography media, wherein the antibody, or antigen-binding portion thereof, binds to the chromatography media;

(b) displacing the antibody, or antigen-binding portion thereof, bound to the chromatography media with a displacing buffer comprising at least one displacer molecule; and

(c) collecting a chromatography sample,

wherein the chromatography sample comprises a composition of the antibody, or antigen-binding portion thereof, which contains less than about 10% acidic species, thereby producing a low acidic species composition comprising an antibody, or antigen-binding portion thereof.

2. The method of claim 1, wherein the pH of the displacing buffer is lower than the isoelectric point of the antibody, or antigen-binding portion thereof.

3. The method of claim 1, wherein the displacing buffer carries positive charge and wherein the concentration of the displacer in the displacing buffer is at least about 0.1 mM.

4. The method of claim 1, wherein the conductivity of the displacing buffer is about 2 mS/cm to about 20 mS/cm.

5. The method of claim 1, wherein one displacing buffer is used.

6. The method of claim 1, wherein a first displacing buffer and a second displacing buffer are used, and wherein the first and second displacing buffers comprise different concentrations of displacer.

7. The method of claim 1, wherein the method is run in a linear displacement chromatography mode, a two-step displacement chromatography mode, or a multiple-step displacement chromatography mode.

8. The method of claim 1, wherein the chromatography sample comprises a reduced level of host cell proteins as compared to the first sample.

9. The method of claim 1, wherein the chromatography sample comprises a reduced level of one or more of charge variants, structure variants or fragmentation variants as compared to the first sample.

10. The method of claim 9, wherein the chromatography sample comprises a reduced level of basic species as compared to the first sample.

11. The method of claim 10, wherein the reduced level of basic species comprise a reduced level of a lysine species Lys 0 as compared to the first sample.

12. The method of claim 1, wherein the chromatography sample comprises a reduced level of aggregates as compared to the first sample.

13. The method of claim 1, wherein the chromatography sample comprises a reduced level of antibody fragments as compared to the first sample.

14. The method of claim 1, wherein the antibody, or antigen-binding portion thereof, is an anti-TNFα antibody, or antigen-binding portion thereof.

15. A low acidic species composition comprising an antibody, or antigen-binding portion thereof, wherein the composition comprises less than about 15% acidic species.

16. The composition of claim 15, wherein the acidic species comprise a first acidic region (AR1) and a second acidic region (AR2).

17. The composition of claim 15, wherein the composition comprises about 5% or less acidic species (AR).

18. The composition of claim 15, wherein the composition comprises about 3% or less acidic species (AR).

19. The composition of claim 16, wherein the composition comprises about 0.1% or less AR1 and about 3% or less AR2.

20. The composition of claim 17, wherein the composition comprises about 1.4% or less acidic species (AR).

21. The composition of claim 16, wherein the composition comprises about 1.4% AR2 and about 0.0% AR1.

22. The composition of claim 15, wherein the composition exhibits increased cartilage tissue penetration, increased TNF affinity, reduced cartilage destruction, reduced bone erosion, reduced synovial proliferation, reduced cell infiltration, reduced chondrocyte death, or reduced proteoglycan loss as compared to a non-low acidic species composition, or

wherein the composition exhibits increased protection against the development of arthritic scores or histopathology scores as compared to a non-low acidic species composition when administered to an animal model of arthritis.

23. A low acidic species composition comprising an anti-TNFα antibody, or antigen-binding portion thereof, comprising a light chain variable region (LCVR) having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3; and a heavy chain variable region (HCVR) having a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, wherein the composition comprises less than about 10% acidic species (AR).

24. The composition of claim 23, wherein the anti-TNFα antibody, or antigen-binding portion thereof, comprises a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 1 and a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 2.

25. The composition of claim 23, wherein the anti-TNFα antibody, or antigen-binding portion thereof, is adalimumab, or an antigen binding-portion thereof.

26. The composition of claim 23, wherein the acidic species comprise a first acidic region (AR1) and a second acidic region (AR2), and wherein the composition comprises about 0.1% or less AR1 and about 3% or less AR2.

27. The composition of claim 23, wherein the composition exhibits increased cartilage tissue penetration, increased TNF affinity, reduced cartilage destruction, reduced bone erosion, reduced synovial proliferation, reduced cell infiltration, reduced chondrocyte death, or reduced proteoglycan loss as compared to a non-low acidic species composition, or

wherein the composition exhibits increased protection against the development of arthritic scores or histopathology scores as compared to a non-low acidic species composition when administered to an animal model of arthritis.

28. The composition of claim 23, wherein the acidic species are product preparation-derived acidic species, cell-culture-derived acidic species or storage-derived acidic species.

29. A method for treating a subject having a disorder in which TNFα is detrimental, comprising administering to the subject the composition of claim 15, thereby treating the subject having a disorder in which TNFα is detrimental.

30. The method of claim 29, wherein the disorder in which TNFα is detrimental is rheumatoid arthritis (RA), psoriasis, psoriatic arthritis, ankylosing spondylitis, Crohn's Disease, Ulcerative Colitis, juvenile idiopathic arthritis, active axial spondyloarthritis or non-radiographic axial spondyloarthritis.