METHOD FOR PROTEIN PURIFICATION

FIELD OF THE INVENTION

The current invention is in the field of protein purification, more particularly in the field of antibody purification.

BACKGROUND OF THE INVENTION

In the field of therapeutics the use of proteins and antibodies and antibody-derived molecules in particular has been constantly gaining presence and importance, and, consequently, the need for controlled manufacturing processes has developed in parallel. The commercialization of therapeutic proteins, requires they be produced in large amounts. For this purpose the protein is frequently expressed in a host cell and must subsequently be recovered and purified, prior to its preparation into an administrable form.

The most common class of antibody molecule is immunoglobulin G (IgG), a heterotetramer composed of two heavy chains and two light chains. The IgG molecule can be subdivided into two functional subunits: (1) the fragment crystallizable (Fc), which constitutes the tail of the antibody and interacts with cell surface receptors to activate an immune response, and (2) the fragment antigen-binding (Fab), which mediates antigen recognition. The Fc region comprises two pairs of constant domains (CH2 and CH3) from two paired heavy chains, whereas the Fab region consists of a variable domain followed by a constant domain from the heavy chain (VH and CH1, respectively), which pair with a variable and constant domain from the light chain (VL and CL, respectively). The Fc and Fab regions are demarcated by a hinge region, which contains disulfide linkages holding the two chains together. Full-length antibodies of the IgG class have traditionally been purified using methods that include a capture step of affinity chromatography using protein A derived from *Staphylococcus aureus*. The high-specificity of binding between Protein A and the Fc-region of antibodies enables this mode of chromatography to remove more than 98% of the impurities in a single step starting directly from complex solutions such as cell culture harvest media. The large purification factor obtained from this process step helps to simplify the entire downstream purification process. In general, only trace contaminants (high molecular weight aggregates, residual host cell proteins, leached protein A) remain to be removed after this purification step and this can usually be achieved in one to two subsequent chromatographic steps.

Additionally an “alternate binding” site for binding protein A has been described in antibodies that contain the specific framework subgroup 3 of the human heavy chain variable region Sasso et al. J. Immunol 1991, 147:1877-1883, *Human IgA and IgG F(ab’)2 that bind to staphylococcal*

protein A belong to the VHIII subgroup), also referred to as the variable heavy chain domain VH3, and has often been classified as a secondary interaction. While all five domains of Protein A (A, B, C, D, E) bind IgG via their Fc-region, only domains D and E exhibit significant VH3 binding. In the context of IgG purification using protein A affinity chromatography, that is based on the interaction between the Fc domain of the IgG and the protein A, these VH3 domain interactions with Protein A have been considered undesirable given that they affect the elution profile of the antibody to be purified, and alternative resins have been developed and are available on the market that contain only domain B of Protein A such as SuRe® from GE Healthcare.

However, many of the antibodies and antibody-derived molecules currently available and/or in development don't contain Fc regions and require further tailoring of their purification methods. The above described binding of protein A by VH3 regions could enable the use of protein A in the manufacture of such antibodies.

A particular requirement of antibody purification is the recovery of the desired antibody or antibody-derived molecule in monomeric form, or essentially free from higher molecular weight species such as dimers and trimers.

Certain antibody molecules have a higher tendency to form multimers that result from variable domain promiscuous pairing with variable domains in adjacent molecules. This is particularly the case with more complex antibody derived molecules that use linker regions between different domains of interest.

Therefore there remains a need in the art for further methods of manufacturing and purifying antibodies and antibody-derived molecules in monomeric form, particularly where these molecules don't contain an Fc region.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a chromatogram showing the elution profile of A26Fab-645dsFv from a Protein A resin via a pH gradient elution.

Figure 2 shows the amount of total protein, monomeric and multimeric A26Fab-645dsFv present in each of the fractions resulting from the chromatography depicted in figure 1, as analysed by SEC-HPLC.

Figure 3 is a chromatogram showing the elution profile of an Fc construct from a Protein A resin via a pH gradient elution.

Figure 4 shows the amount of total protein, monomeric and multimeric forms present in each of the fractions resulting from the chromatography depicted in figure 3, as analysed by SEC-HPLC.

Figure 5 is a chromatogram showing the elution profile of A26Fab-645dsFv from a Protein A resin via pH step elution.

Figure 6 shows a SEC-HPLC analysis of the fractions resulting from the protein A chromatography depicted in figure 5. The dotted line represents the analysis of the fraction eluted at pH 3.8, whereas the uninterrupted line represents the analysis of the fraction eluted at pH 3.0.

Figure 7 shows a non-reducing SDS-PAGE analysis of fractions recovered from the protein A chromatography of A26Fab-645dsFv via pH step elution depicted in figure 5. Lane 1 shows molecular weight markers, lane 2 shows a sample of clarified cell culture supernatant as loaded on to the protein A chromatography, lane 3 shows the flow-through fraction recovered from the protein A chromatography, lane 4 the fraction recovered after elution at pH 3.8, and lane 5 the fraction recovered after elution at pH 3.0.

Figure 8 shows a reducing SDS-PAGE analysis of fractions recovered from the protein A chromatography of A26Fab-645dsFv via pH step elution depicted in figure 5. Lane 1 shows molecular weight markers, lane 2 shows a sample of clarified cell culture supernatant as loaded on to the protein A chromatography, lane 3 shows the flow-through fraction recovered from the protein A chromatography, lane 4 the fraction recovered after elution at pH 3.8, and lane 5 the fraction recovered after elution at pH 3.0.

Figure 9 shows a SEC-UPLC analysis of the fractions recovered from the protein A chromatography of A26Fab-645dsFv via gradient elution as described in Example 4, showing the amount of total protein, monomeric and multimeric A26Fab-645dsFv.

Figure 10 shows a SEC-UPLC analysis of the fractions recovered from the protein A chromatography of A26Fab-645dsFv via gradient elution as described in Example 5, showing the amount of total protein, monomeric and multimeric A26Fab-645dsFv.

Figure 11 shows a SEC-UPLC analysis of the fractions recovered from the protein A chromatography of A26Fab-645dsFv via gradient elution as described in Example 6, showing the amount of total protein, monomeric and multimeric A26Fab-645dsFv.

Figure 12 shows a schematic of monomeric Fab-dsFv and multimeric versions of Fab-dsFv, and also of its basic components: Fab and dsFv. This diagram illustrates possible monomers, dimers and trimers. However, all the linkers would be the same length in reality where dimers, and trimers form cyclic structures.

Figure 13 to 20 show various antibody molecule sequences and components thereof.

Figure 21 shows a chromatogram showing the elution profile of TrYbe from a Protein A resin via a pH gradient elution.

Figure 22 shows a SEC-HPLC analysis of the fractions resulting from the protein A chromatography depicted in figure 21.

Figure 23 shows a non-reducing (A) and reducing (B) SDS PAGE analysis of fractions recovered from the protein A chromatography of TrYbe via a pH gradient elution depicted in figure 21.

Figure 24 the amount of total protein, monomeric and multimeric TrYbe present in each of the fractions resulting from the chromatography depicted in figure 21, as analysed by SEC-HPLC.

Figure 25 shows a chromatogram showing the elution profile of BYbe from a Protein A resin via a pH gradient elution.

Figure 26 shows a SEC-HPLC analysis of the fractions resulting from the protein A chromatography depicted in figure 25.

Figure 27 shows a non-reducing (A) and reducing (B) SDS PAGE analysis of fractions recovered from the protein A chromatography of BYbe via a pH gradient elution depicted in figure 25.

Figure 28 the amount of total protein, monomeric and multimeric BYbe present in each of the fractions resulting from the chromatography depicted in figure 25, as analysed by SEC-HPLC.

Figure 29 shows a schematic of alternative monomeric Fab-scFv formats susceptible to purification according to the method of the invention.

Figure 30 shows a schematic of alternative monomeric Fab-2x dsscFv (TrYbe®) and Fab-dsscFv-dsFv formats susceptible to purification according to the method of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention solves the above-identified need by providing a new method for recovering a human VH3 domain-containing antibody in monomeric form. An avidity effect has now been observed between the binding of human VH3 domains and protein A. This finding is surprising given that it has not been described for the interaction between Fc regions and protein A and has led to the development of a new method that allows recovery of monomeric human VH3 domain-containing antibodies from a mixture containing monomeric and multimeric forms of the antibody.

In a first embodiment, the present invention refers to a method for obtaining a human VH3 domain-containing antibody in monomeric form, comprising:

a) applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E, under conditions that allow binding of said antibody to protein A, and

b) recovering the human VH3 domain containing-antibody in monomeric form,

wherein the human VH3 domain containing antibody does not contain an Fc region.

In a second alternative embodiment, the present invention refers to a method for manufacturing a human VH3 domain-containing antibody comprising:

a) expressing the antibody in a host cell,

b) recovering a mixture containing the antibody, host cells and other contaminants,

c) purifying the antibody using at least a protein A chromatography step wherein said protein A comprises domain D and/or E, and

d) recovering the human VH3 domain-containing antibody ,

wherein the human VH3 domain containing antibody does not contain an Fc region.

In a third alternative embodiment, the invention refers to a method of separating a human VH3 domain-containing antibody in monomeric form from the antibody in multimeric form comprising:

a) applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E,

b) allowing binding of said antibody to protein A,

c) applying an elution buffer that selectively disrupts binding of the antibody in monomeric form,

d) recovering the resulting eluate, and optionally

e) applying a second elution buffer that disrupts binding of the antibody in multimeric form and recovering this second eluate,

wherein the human VH3 domain-containing antibody does not contain an Fc region.

In a fourth alternative embodiment, the invention refers to a method of separating a human VH3 domain-containing antibody in monomeric form from the antibody in multimeric form comprising:

a) applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E,

b) allowing binding of the antibody in multimeric form,

c) recovering the antibody in monomeric form in the flow-through, and optionally

d) applying an elution buffer that selectively disrupts binding of the antibody in multimeric form, and

e) recovering the eluate resulting from d);

wherein the human VH3 domain-containing antibody does not contain an Fc region.

In a further embodiment the method of the invention will additionally comprise another one or more chromatography steps to remove remaining impurities. Generally such steps will employ a non-affinity chromatography step using a solid phase with appropriate functionality for use in gel filtration chromatography, cation chromatography, anion chromatography, mixed mode chromatography, hydrophobic chromatography and hydrophobic charge induction chromatography. These may be operated in bind and elute mode or in flow through mode. In flow-through mode, the impurities bind or have reduced mobility in the solid phase whereas the target protein is recovered in the eluate or flow through fraction. Appropriate solid phases for use in chromatography such as beaded resins or membranes with the appropriate functionality are readily available to the skilled artisan. In a particular embodiment according to the method of the invention, the method additionally comprises a step of anion exchange chromatography operated in the flow through mode.

In a further particular embodiment the method of the invention comprises a protein A chromatography step followed by a first chromatography step that is an anion exchange chromatography producing a flow-through containing the protein and a second chromatography step that is a cation exchange chromatography from where an eluate containing the protein is recovered.

Alternatively, the method of the invention comprises a protein A chromatography followed by a first chromatography step that is a cation exchange chromatography from where an eluate containing the protein is recovered, and a second chromatography step that is an anion exchange chromatography to produce a flow-through containing the protein.

Typically, protein A chromatography is performed in bind and elute mode, wherein binding of the protein of interest to the solid phase allows the impurities such as contaminating proteins to flow through the chromatographic medium while the protein of interest remains bound to the solid phase. The bound protein of interest is then recovered from the solid phase with an elution buffer that disrupts the mechanism by which the protein of interest is bound to said solid phase.

In a further embodiment the method of the invention comprises a first solution is added to the protein A chromatography material after applying the mixture comprising the human VH3

domain-containing antibody in monomeric and multimeric form, such that unbound material is removed in the solution.

In a further embodiment of the method according to the invention an elution buffer is applied to the protein A chromatography material such that the bound antibody is released.

In a further embodiment of the method according to the invention the eluate recovered from the protein A chromatography is enriched in monomeric antibody over multimeric antibody with respect to the applied mixture.

As a skilled artisan would understand, in the present context the eluate recovered from the protein A chromatography has a protein content that contains a higher percentage of antibody in monomeric form with respect to the mixture before the protein A chromatography step.

In a particular embodiment, the eluate recovered from the protein A chromatography comprises at least 50%, at least 60%, at least 70%, 75%, 80%, 85%, or at least 90% human VH3 domain-containing antibody in monomeric form.

In a further alternative embodiment, where the antibody in multimeric form is to be recovered, the eluate from the protein A chromatography has a protein content that contains a higher percentage of antibody in multimeric form with respect to the mixture before the protein A chromatography step. In a particular embodiment the eluate recovered from the protein A chromatography comprises at least 50%, at least 60%, at least 70%, 75%, 80%, 85%, or at least 90% human VH3 domain-containing antibody in multimeric form.

In a further embodiment of the method according to the invention, said protein A is native recombinant protein A.

There are many chromatography materials available to the skilled artisan containing said native recombinant protein A, such as for example MabSelect® (GE Healthcare), Absolute® (Novasep), Captiv A® (Repligen), or Amsphere® (JSR).

In a particular embodiment of the method of the invention the bound antibody is released from the protein A chromatography material by applying an elution buffer with a pH suitable to disrupt antibody binding. Said pH is dependent on the specific molecule and generally determined empirically by the skilled artisan and adjusted to achieve the desired endpoint, i.e it may be desired to recover the largest amount of monomer possible from the applied mixture, or it may be desirable to obtain the monomer at the highest possible purity. In a specific embodiment of the method of the invention the elution buffer has pH 3.0 to pH 4.5, preferably, pH 3.2 to pH 4.3, pH 3.5 to pH 4, preferably pH 3.6 to pH 3.9 or pH 3.8.

Buffers suitable for use as wash and elution buffers in protein A chromatography are readily available in the art, and may be chosen by way of non-limiting examples from among phosphate buffered saline (PBS), Tris, histidine, acetate, citrate buffers, or MES (2-(N-morpholino)ethanesulphonic acid Imidazole), BES (N,N-(bis-2-hydroxyethyl)-2-aminoethanesulphonic acid), MOPS (3-(N-morpholino)-propanesulphonic acid), or HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffers.

In a particular embodiment, the method of the invention comprises applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E, under conditions that allow binding of said antibody to protein A, applying a first solution or wash buffer such that unbound material is removed in the solution, applying an elution buffer to the protein A chromatography material such that the bound antibody is released, and recovering the human VH3 domain containing-antibody in monomeric form, wherein the recovered solution is enriched in VH3 domain-containing antibody in monomeric form with respect to the applied mixture and wherein the human VH3 domain containing antibody does not contain an Fc region.

In a further particular embodiment, the method of the invention comprises applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E, under conditions that allow binding of said antibody to protein A, applying a first solution or wash buffer such that unbound material is removed in the solution, applying an elution buffer to the protein A chromatography material such that the bound antibody in monomeric form is released, and recovering the human VH3 domain containing-antibody in monomeric form, wherein the recovered solution is enriched in VH3 domain-containing antibody in monomeric form with respect to the applied mixture and wherein the human VH3 domain containing antibody does not contain an Fc region.

In a further embodiment according to the invention, the VH3 domain containing antibody is selected from Fab', $F(ab')_2$, scFv, Fab-Fv, Fab-scFv, Fab-$(scFv)_2$, Fab-$(Fv)_2$, diabodies, triabodies, and tetrabodies.

In a further embodiment of the method according to the invention, the VH3 domain-containing antibody comprises at least 2 human VH3 domains.

In a further embodiment of the method according to the invention the human VH3 domain-containing antibody specifically binds OX40.

In one embodiment of the method of the invention the antibody, is a FabFv or disulfide stabilised form thereof as disclosed in PCT/EP2014/074409, incorporated herein by reference.

In one embodiment the antibody comprises a binding domain specific to human serum albumin, in particular with CDRs or variable regions as disclosed in WO 2013/068563, incorporated herein by reference.

In a further embodiment of the method of the invention, said human VH3 domain containing antibody comprises:

- heavy chain CDR1, CDR2 and CDR3 as defined in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively; and

- light chain CDR1, CDR2, and CDR3 as defined in SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

In a further particular embodiment of the method according to the invention the antibody is A26Fab-645dsFv that comprises a Fab portion that specifically binds to OX40 and a Fv portion that specifically binds serum albumin, both portions being stabilized via a disulfide bond, as defined in WO 2013/068563, incorporated herein by reference.

In a further particular embodiment, the method of the invention comprises applying a mixture comprising A26Fab-645dsFv in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E, under conditions that allow binding of said antibody to protein A, applying a first solution or wash buffer such that unbound material is removed in the solution, applying an elution buffer to the protein A chromatography material such that the bound antibody in monomeric form is released, and recovering the A26Fab-645dsFv in monomeric form, wherein the recovered solution is enriched in A26Fab-645dsFv in monomeric form with respect to the applied mixture and wherein the elution buffer has pH 3.5 to pH 4.2, preferably, pH 3.6 to pH 4.1, pH 3.7 to pH 4.0, preferably pH 3.8 to pH 3.9 or pH 3.8.

In a further particular embodiment, the method of the invention additionally comprises applying a second elution buffer to the protein A chromatography material to recover the bound A26Fab-645dsFv in multimeric form, wherein said second elution buffer has a pH below 3.5, preferably below pH 3.4, preferably pH 2.8 to pH 3.2, preferably pH 2.9 to pH 3.1, preferably pH 3.0.

Accordingly, the present disclosure provides a bispecific antibody fusion protein which binds human OX40 and human serum albumin comprising:

a heavy chain comprising, in sequence from the N-terminal, a first heavy chain variable domain (VH#1), a CH1 domain and a second heavy chain variable domain (VH#2),

a light chain comprising, in sequence from the N-terminal, a first light chain variable domain (VL#1), a CL domain and a second light chain variable domain (VL#2),

wherein said heavy and light chains are aligned such that VH#1 and VL#1 form a first antigen binding site and VH#2 and VL#2 form a second antigen binding site,

wherein the antigen bound by the first antigen binding site is human OX40 and the antigen bound by the second antigen binding site is human serum albumin, in particular

wherein the first variable domain of the heavy chain (VH#1) comprises the sequence given in SEQ ID NO:1 for CDR-H1, the sequence given in SEQ ID NO:2 for CDR-H2 and the sequence given in SEQ ID NO:3 for CDR-H3 and the first variable domain of the light chain (VL#1) comprises the sequence given in SEQ ID NO:4 for CDR-L1, the sequence given in SEQ ID NO:5 for CDR-L2 and the sequence given in SEQ ID NO:6 for CDR-L3,

wherein the second heavy chain variable domain (VH#2) has the sequence given in SEQ ID NO:11 and the second light chain variable domain (VL#2) has the sequence given in SEQ ID NO: 12 and the second heavy chain variable domain (VH#2) and second light chain variable domain (VL#2) are linked by a disulfide bond.

In one embodiment there is a peptide linker between the CH1 domain and the second heavy chain variable domain (VH#2). In one embodiment there is a peptide linker between the CL domain and the second light chain variable domain (VL#1). In one embodiment the first heavy chain variable domain (VH#1) comprises the sequence given in SEQ ID NO:8. In one embodiment the first light chain variable domain (VL#1) comprises the sequence given in SEQ ID NO:7. In one embodiment the heavy chain comprises or consists of the sequence given in SEQ ID NO:15. In one embodiment the light chain comprises or consists of the sequence given in SEQ ID NO:16.

Thus in one embodiment there is provided a bispecific antibody fusion protein which binds human OX40 and human serum albumin, having a heavy chain comprising the sequence given in SEQ ID NO:15 and a light chain comprising the sequence given in SEQ ID NO:16.

In one embodiment the antibody molecule, such as a Fab-dsFv format is one disclosed in PCT/EP2014/074409 or WO2014/019727, incorporated herein by reference.

In another embodiment the antibody molecule is a Fab-scFv fusion protein format disclosed in WO 2013/068571, incorporated herein by reference.

In another embodiment the antibody molecule is the multi-specific antibody molecule comprising or consisting of:

a) a polypeptide chain of formula (**I**):

VH-CH1-X-V1; and

b) a polypeptide chain of formula (**II**):

VL-CL-Y-V2;

wherein:

VH represents a heavy chain variable domain;

CH1 represents a domain of a heavy chain constant region, for example domain 1 thereof;

X represents a bond or linker;

Y represents a bond or linker;

V1 represents a dsFv, a sdAb, a scFv or a dsscFv;

VL represents a light chain variable domain;

CL represents a domain from a light chain constant region, such as Ckappa;

V2 represents dsFv, a sdAb, a scFv or a dsscFv;

wherein at least one of V1 or V2 is a dsFv or dsscFv, described in WO 2015/197772 incorporated herein by reference.

In one particular embodiment, the antibody molecule is the multispecific antibody molecule of the format Fab-2x dsscFv described in WO 2015/197772, incorporated herein by reference.

In a further particular embodiment, the multispecific antibody molecule of the format Fab-2x dsscFv is a trivalent antibody, i.e. each Fv binds to a different epitope.

In a further particular embodiment the multispecific antibody molecule has a Fab-dsscFv-dsFv format as described in WO2015/197772, incorporated herein by reference.

An antibody that can be manufactured in accordance with the method of the present invention can be produced by culturing eukaryotic host cells transfected with one or more expression vectors encoding the recombinant antibody. The eukaryotic host cells are preferably mammalian cells, more preferably Chinese Hamster Ovary (CHO) cells.

Mammalian cells may be cultured in any medium that will support their growth and expression of the antibody, preferably the medium is a chemically defined medium that is free of animal-derived products such as animal serum and peptone. There are different cell culture mediums available to the person skilled in the art comprising different combinations of vitamins, amino

acids, hormones, growth factors, ions, buffers, nucleosides, glucose or an equivalent energy source, present at appropriate concentrations to enable cell growth and protein production. Additional cell culture media components may be included in the cell culture medium at appropriate concentrations at different times during a cell culture cycle that would be known to those skilled in the art.

Mammalian cell culture can take place in any suitable container such as a shake flask or a bioreactor, which may or may not be operated in a fed-batch mode depending on the scale of production required. These bioreactors may be either stirred-tank or air-lift reactors. Various large scale bioreactors are available with a capacity of more than 1,000 L to 50,000 L, preferably between 5,000 L and 20,000 L, or to 10,000 L. Alternatively, bioreactors of a smaller scale such as between 2 L and 100 L may also be used to manufacture an antibody according to the method of the invention.

An antibody or antigen-binding fragment thereof that can be manufactured in accordance with the methods of the present invention is typically found in the supernatant of a mammalian host cell culture, typically a CHO cell culture. For CHO culture processes wherein the protein of interest such as an antibody or antigen-binding fragment thereof is secreted in the supernatant, said supernatant is collected by methods known in the art, typically by centrifugation.

Therefore in a particular embodiment of the invention, the method comprises a step of centrifugation and supernatant recovery prior to protein purification. In a further particular embodiment said centrifugation is continuous centrifugation. For avoidance of doubt, supernatant denotes the liquid lying above the sedimented cells resulting from the centrifugation of the cell culture.

Alternatively said supernatant may be recovered using clarification techniques known to the skilled artisan such as for example depth filtration. Therefore in a particular embodiment for the invention, the method comprises a step fo depth filtration and supernatant recovery prior to protein purification.

Alternatively, host cells are prokaryotic cells, preferably gram-negative bacteria. More preferably, the host cells are *E. coli* cells. Prokaryotic host cells for protein expression are well known in the art (Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 72, 211-222.). The host cells are recombinant cells which have been genetically engineered to produce the protein of interest such as an antibody fragment. The recombinant *E. coli* host cells may be derived from any suitable *E. coli* strain including from MC4100, TG1, TG2, DHB4, DH5α, DH1, BL21, K12, XL1Blue and JM109. One example is *E.*

coli strain W3110 (ATCC 27,325) a commonly used host strain for recombinant protein fermentations. Antibody fragments can also be produced by culturing modified *E. coli* strains, for example metabolic mutants or protease deficient *E. coli* strains, such as those described in WO 2011/086136, WO 2011/086138 or WI 2011/086139, incorporated herein by reference.

An antibody that can be purified in accordance with the methods of the present invention is typically found in either the periplasm of the *E. coli* host cell or in the host cell culture supernatant, depending on the nature of the protein, the scale of production and the *E. coli* strain used. The methods for targeting proteins to these compartments are well known in the art (Makrides,S.C. (1996). Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol Rev 60, 512-538.). Examples of suitable signal sequences to direct proteins to the periplasm of *E. coli* include the *E. coli* PhoA, OmpA, OmpT, LamB and OmpF signal sequences. Proteins may be targeted to the supernatant by relying on the natural secretory pathways or by the induction of limited leakage of the outer membrane to cause protein secretion examples of which are the use of the pelB leader, the protein A leader, the co-expression of bacteriocin release protein, the mitomycin-induced bacteriocin release protein along with the addition of glycine to the culture medium and the co-expression of the kil gene for membrane permeabilization. Most preferably, in the methods of the invention, the recombinant protein is expressed in the periplasm of the host *E. coli*.

Expression of the recombinant protein in the *E. coli* host cells may also be under the control of an inducible system, whereby the expression of the recombinant antibody in *E. coli* is under the control of an inducible promoter. Many inducible promoters suitable for use in *E. coli* are well known in the art and depending on the promoter expression of the recombinant protein can be induced by varying factors such as temperature or the concentration of a particular substance in the growth medium. Examples of inducible promoters include the *E.coli lac*, *tac*, and *trc* promoters which are inducible with lactose or the non-hydrolyzable lactose analog, isopropyl-b-D-1-thiogalactopyranoside (IPTG) and the *phoA*, *trp* and *araBAD* promoters which are induced by phosphate, tryptophan and L-arabinose respectively. Expression may be induced by, for example, the addition of an inducer or a change in temperature where induction is temperature dependent. Where induction of recombinant protein expression is achieved by the addition of an inducer to the culture the inducer may be added by any suitable method depending on the fermentation system and the inducer, for example, by single or multiple shot additions or by a gradual addition of inducer through a feed. It will be appreciated that there may be a delay between the addition of the inducer and the actual induction of protein expression for example where the inducer is lactose there may be a delay before induction of protein expression occurs while any pre-existing carbon source is utilized before lactose.

E. coli host cell cultures (fermentations) may be cultured in any medium that will support the growth of *E. coli* and expression of the recombinant protein. The medium may be any chemically defined medium such as e.g. described in Durany O, et al. (2004). Studies on the expression of recombinant fuculose-1-phosphate aldolase in Escherichia coli. Process Biochem 39, 1677-1684.

Culturing of the *E. coli* host cells can take place in any suitable container such as a shake flask or a fermenter depending on the scale of production required. Various large scale fermenters are available with a capacity of more than 1,000 liters up to about 100,000 liters. Preferably, fermenters of 1,000 to 50,000 liters are used, more preferably 1,000 to 25,000, 20,000, 15,000, 12,000 or 10,000 liters. Smaller scale fermenters may also be used with a capacity of between 0.5 and 1,000 liters.

Fermentation of *E. coli* may be performed in any suitable system, for example continuous, batch or fed-batch mode depending on the protein and the yields required. Batch mode may be used with shot additions of nutrients or inducers where required. Alternatively, a fed-batch culture may be used and the cultures grown in batch mode pre-induction at the maximum specific growth rate that can be sustained using the nutrients initially present in the fermenter and one or more nutrient feed regimes used to control the growth rate until fermentation is complete. Fed-batch mode may also be used pre-induction to control the metabolism of the *E. coli* host cells and to allow higher cell densities to be reached.

If desired, the host cells may be subject to collection from the fermentation medium, e.g. host cells may be collected from the sample by centrifugation, filtration or by concentration.

In one embodiment the process according to the present invention comprises a step of centrifugation and cell recovery prior to extracting the protein.

For *E. coli* fermentation processes wherein the protein of interest such as an antibody fragment is found in the periplasmic space of the host cell it is required to release the protein from the host cell. The release may be achieved by any suitable method such as cell lysis by mechanical or pressure treatment, freeze-thaw treatment, osmotic shock, extraction agents or heat treatment. Such extraction methods for protein release are well known in the art. Therefore in a particular embodiment, the method of the invention comprises an additional protein extraction step prior to protein purification.

In a further embodiment the method according to the invention further comprises recovering the host cells from the cell culture medium, harvesting the protein using a protein extraction step, recovering the protein containing mixture resulting from the protein extraction step and purifying

said protein from the mixture wherein said purification comprises at least one protein A chromatography step.

In a specific embodiment the extraction step comprises adding an extraction buffer to the sample and recovering the resulting protein. Preferably the extraction step is performed during a suitable time and at a suitable temperature to allow recovery of the protein in its native conformation and is optimized empirically for each particular protein. In a particular embodiment of the present invention said extraction step is performed at 25ºC to 35ºC, 27ºC to 33ºC, preferably 29ºC to 31ºC. The protein extraction step is performed over a period of time that is also optimized empirically depending on the particular protein and temperature to be used. In a particular embodiment said extraction step is performed for 4 to 20 hours, for 6 to18 hours, preferably from 8 to 12 hours. In a particular embodiment the protein extraction step is performed from 8 to 12 hours at 29 ºC to 31ºC, preferably for 11 hours at 30ºC.

In an alternative embodiment an extraction buffer is added to the sample and the sample is then subjected to a heat treatment step. The heat treatment step is preferably as described in detail in US 5,665,866, incorporated herein by reference.

Following the step of extraction the mixture containing the protein of interest such as an antibody may be subjected to a step of centrifugation and/or filtration.

In a further particular embodiment, the method of the invention may comprise a step of adjusting the pH of the mixture containing the protein of interest following the extraction step and prior to purification of the protein from said mixture.

The term "antibody" or "antibodies" as used herein refers to monoclonal or polyclonal antibodies. The term "antibody" or "antibodies" as used herein includes but is not limited to recombinant antibodies that are generated by recombinant technologies as known in the art. "Antibody" or "antibodies" include antibodies' of any species, in particular of mammalian species, including antibodies having two essentially complete heavy and two essentially complete light chains, human antibodies of any isotype, including IgD, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgG_4, IgE and antibodies that are produced as dimers of this basic structure including IgA_1, IgA_2, or pentamers such as IgM and modified variants thereof, non-human primate antibodies, e.g. from chimpanzee, baboon, rhesus or cynomolgus monkey, rodent antibodies, e.g. from mouse, or rat; rabbit antibodies, goat or horse antibodies, and camelid antibodies (e.g. from camels or llamas such as Nanobodies™) and derivatives thereof, or of bird species such as chicken antibodies or of fish species such as shark antibodies. The term "antibody" or "antibodies" also refers to "chimeric" antibodies in which a first portion of at least one heavy and/or light chain

antibody sequence is from a first species and a second portion of the heavy and/or light chain antibody sequence is from a second species. Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences. "Humanized" antibodies are chimeric antibodies that contain a sequence derived from non-human antibodies. For the most part, humanized antibodies are human antibodies (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region [or complementarity determining region (CDR)] of a non-human species (donor antibody) such as mouse, rat, rabbit, chicken or non-human primate, having the desired specificity, affinity, and activity. In most instances residues of the human (recipient) antibody outside of the CDR; i.e. in the framework region (FR), are additionally replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. Humanization reduces the immunogenicity of non-human antibodies in humans, thus facilitating the application of antibodies to the treatment of human disease. Humanized antibodies and several different technologies to generate them are well known in the art. The term "antibody" or "antibodies" also refers to human antibodies, which can be generated as an alternative to humanization. For example, it is possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of production of endogenous murine antibodies. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies with specificity against a particular antigen upon immunization of the transgenic animal carrying the human germ-line immunoglobulin genes with said antigen. Technologies for producing such transgenic animals and technologies for isolating and producing the human antibodies from such transgenic animals are known in the art. Alternatively, in the transgenic animal; e.g. mouse, only the immunoglobulin genes coding for the variable regions of the mouse antibody are replaced with corresponding human variable immunoglobulin gene sequences. The mouse germline immunoglobulin genes coding for the antibody constant regions remain unchanged. In this way, the antibody effector functions in the immune system of the transgenic mouse and consequently the B cell development are essentially unchanged, which may lead to an improved antibody response upon antigenic challenge *in vivo*. Once the genes coding for a particular antibody of interest have been isolated from such transgenic animals the genes coding for the constant regions can be replaced with

human constant region genes in order to obtain a fully human antibody. Other methods for obtaining human antibodies *in vitro* are based on display technologies such as phage display or ribosome display technology, wherein recombinant DNA libraries are used that are either generated at least in part artificially or from immunoglobulin variable (V) domain gene repertoires of donors. Phage and ribosome display technologies for generating human antibodies are well known in the art. Human antibodies may also be generated from isolated human B cells that are ex vivo immunized with an antigen of interest and subsequently fused to generate hybridomas which can then be screened for the optimal human antibody. The term "antibody" or "antibodies" as used herein, also refers to an aglycosylated antibody.

Antibody molecules to be used in any of the embodiments of the invention include antibody fragments such as Fab, Fab', F(ab')2, and Fv and scFv fragments; as well as diabodies, including formats such as BiTEs® (Bi-specific T-cell Engagers) and DARTs™ (Dual Affinity Re-Targeting technology), triabodies, tetrabodies, minibodies, domain antibodies(dAbs), such as sdAbs, VHH and VNAR fragments, single-chain antibodies, bispecific, trispecific, tetraspecific or multispecific antibodies formed from antibody fragments or antibodies, including but not limited to Fab-Fv, Fab-scFv, Fab$(Fv)_2$ or Fab-$(scFv)_2$ constructs. Antibody fragments as defined above are known in the art. For the purpose of clarity Fab-Fv should be understood to refer to a construct containing one Fv region and one Fab region joined in any order, i.e. Fab-Fv, or Fv-Fab, wherein the last amino acids in one region are followed by the first amino acids in the next region or vice versa. Similarly Fab-scFv should be understood to refer to a construct containing one scFv region and one Fab region joined in any order and in the case of the Fab to either polypeptide chain, i.e. Fab-scFv, or scFv-Fab, wherein the last amino acid in one region is followed by the first amino acid in the next region or vice versa. In the same manner Fab-$(Fv)_2$ should be understood to refer to a construct containing two Fv regions and one Fab region joined in any order, i.e. Fab-Fv-Fv, Fv-Fab-Fv, or Fv-Fv-Fab, wherein the last amino acids in one region are followed by the first amino acids in the next region or vice versa. Similarly Fab-$(scFv)_2$ should be understood to refer to a construct containing two scFv regions and one Fab region joined in any order and in the case of the Fab to either polypeptide chain, resulting in 20 possible permutations.

Typically these constructs include a peptide linker between the first region (e.g. Fab) and the second region (e.g. Fv). Such linkers are well known in the art, and can be one or more amino acids, typically optimized in length and composition by a skilled artisan. Alternatively said regions may be linked directly, i.e. without a peptide linker.

Examples of suitable linker regions for linking a variable domain to a Fab or Fab' are described in WO 2013/068571 and WO 2014/096390 incorporated herein by reference, and include, but

are not limited to, flexible linker sequences and rigid linker sequences. Flexible linker sequences include those disclosed in Huston et al., 1988, 10 PNAS 85:5879-5883; Wright & Deonarain, Mol. Immunol., 2007, 44(11):2860-2869; Alfthan et al., Prot. Eng., 1995, 8(7):725-731; Luo et al., J. Biochem., 1995, 118(4):825-831; Tang et al., 1996, J. Biol. Chern. 271(26): 15682-15686; and Turner et al., 1997, JIMM 205, 42-54.

The term "VH3 domain" as used herein refers to the framework subgroup 3 of the human heavy chain variable region of an immunoglobulin. The heavy chain variable domains of antibodies are classified into distinct subfamilies (VH1 to VH6) on the basis of DNA sequence and protein homologies (Walter et al. Am. J. Hum. Genet. 42:446-451, 1988, *Analysis for genetic variation reveals human immunoglobulin VH-region gene organization*; Schroeder et al. Int Immunol. 1990; 2(1):41-50, *Structure and evolution of mammalian VH families*.

The term "Fc region" as used herein refers to the Fc region of a native antibody, this is a constant region dimer lacking constant heavy domain 1 (CH1). As is known in the art the Fc region of an antibody is the Fragment crystallizable (Fc) recovered after digestion of the native antibody with either pepsin or papain.

An antibody that "does not contain an Fc region" as used herein refers to an antibody that does not contain a native constant heavy domain 2 (CH2), a native constant heavy domain 3 (CH3) nor a native constant heavy domain 4 (CH4) region.

The residues within the Fc region responsible for binding to protein A have been previously described in the art (Nagaoka et al. *Single amino acid substitution in the mouse IgG1 Fc region induces drastic enhancement of the affinity to protein A,* PEDS Vol. 16, Issue 4, pages 243-245). Consequently it is possible for a skilled artisan to develop an antibody having an Fc region that has lost the ability to bind to protein A, and such antibody would be suitable for use in the method according to the present invention.

The term "multimer" or "multimeric form" as used herein refers to antibody forms consisting of the domains from two or more monomers in which all of the domains are correctly folded and paired. Examples of multimers are provided in figure 12, where the different antibody molecules are correctly folded and each VH domain is paired with a complementary VL domain. For the purposes of clarity, it should be understood that a complementary VH-VL pair binds the same antigen cooperatively.

The term "Protein A" or "Staphylococcal Protein A" as used herein, is a type I membrane protein covalently linked to the cell wall of most strains of the Gram-positive bacterium *Staphylococcus*

aureus. It has high affinity to IgG from various species, for instance human, rabbit and guinea pig but only weak interaction with bovine and mouse. Protein A interacts with antibodies through two distinct binding events: the "classical" binding site on the Fc portion of human IgG_1, IgG_2, and IgG_4, and the "alternate" binding site found on the Fab portion of human IgG, IgM, IgA, and IgE that contain heavy chains of the VH3 subfamily. The most reported molecular weight of protein A from *Staphylococcus aureus* is about 42,000 Da. The recombinant Streptococci protein A consists of 299 amino acids and has a predicted molecular mass of 33.8 kDa as estimated by SDS-PAGE.

Protein A consists of three regions: S, being the signal sequence that is processed during secretion; five homologous IgG binding domains E, D, A, B and C and a cell-wall anchoring region XM. The truncated protein lacking region X has a molecular weight of about 31kD. The domains are independently capable to bind to the Fc-part of IgG_1, IgG_2 and IgG_4, but show only weak interaction with IgG_3. In addition, all native protein A domains show comparable Fab binding, that has been described to be mediated by regions D and E.

The term "OX40" as used herein refers to a molecule also known as CD134, TNFRSF4, ACT35 or TXGP1L, is a member of the TNF receptor superfamily, that acts as a costimulatory receptor with sequential engagement of CD28 and OX40 being required for optimal T cell proliferation and survival.

The term "specifically binds to", "specifically binding to" a given molecule, and equivalents as used herein when referring to an antibody means the antibody will bind to said given molecule with sufficient affinity and specificity to achieve a biologically meaningful effect. The antibody selected will normally have a binding affinity for the given molecule, for example , the antibody may bind the given molecule with a Kd value of between 100 nM and 1 pM. Antibody affinities may be determined by a surface plasmon resonance bases assay, such as the BIAcore assay; enzyme-linked immunoabsorbent assay (ELISA); and competition assays (e.g. RIA's), for example. Within the meaning of the present invention an antibody specifically binding to said given molecule, may also bind to another molecule; such as by way of a non-limiting example in the case of a bispecific antibody.

EXAMPLES

EXAMPLE 1: Protein-A Purification of A26Fab-645dsFv via a pH gradient elution

E.coli expression, extraction and clarification of A26Fab-645dsFv

A26Fab-645dsFv (an antibody fragment that binds human OX40 and serum albumin) was expressed as a heterologous protein in *E. coli* W3110 host cells upon induction by IPTG (isopropyl-b-D-1-thiogalactopyranoside) and the heterologous protein was released from the periplasmic space of the host cells by the addition of 100 mM Tris/10 mM EDTA buffer adjusted to pH 7.4 and protein extraction step at 30°C. Cellular material was removed through centrifugation and the cell extract containing the heterologous protein was then clarified using a combination of centrifugation and 0.22 µm filtration.

Protein-A Purification of A26Fab-645dsFv via a pH gradient elution

The clarified *E. coli* extract was applied to a native protein A chromatography column, 5ml HiTrap MabSelect (GE Healthcare), equilibrated in Delbeccos Phosphate Buffered Saline (PBS) pH 7.4. The column was first washed with PBS to remove unbound material, and bound material was subsequently eluted with a pH gradient, pH 7.4 to pH 2.1. Eluted material was fractionated and analysed via SEC-HPLC (size exclusion chromatography - high performance liquid chromatography) using TSK gel G3000SWXL SEC-HPLC (Tosoh Corporation). SEC-HPLC analysis was used to determine the % monomer and multimer present in each fraction. A26Fab-645dsFv monomer had a retention time around 9 minutes. Dimer, trimer, tetramer, and higher order structures all showed retention times below 9 minutes and were collectively termed multimeric species or higher molecular weight species (HMWS).

During elution from the protein A chromatography column, two peaks were observed across the pH gradient, see figure 1. Elevated levels of monomer were observed in the first peak compared to elevated levels of multimeric species in the second peak, see table 1 and figure 2.

A26Fab-645dsFv lacks an Fc therefore binding to Protein-A was due to the human VH3 variable framework subclass of the V-regions. It is proposed that the increased binding of the multimeric species was due to the increased avidity of these molecules for protein-A. Multimeric species have more VH3 regions and therefore bind stronger to the Protein-A resin requiring a lower pH for elution.

Table 1 SEC G3000 Analysis of fractions from Protein-A Purification of A26Fab-645dsFv via a pH gradient elution

Fraction	Volume (ml)	Conc (mg/ml)	Protein (mg)	HMWS (%)	Monomer (%)
A1	3.3	0.3	0.9	4.0	96.1
A2	3.1	0.6	1.9	6.7	93.3
A3	3.2	1.6	5.0	15.0	85.0
A4	3.2	1.6	5.1	22.7	77.3
A5	3.2	1.8	5.8	44.2	55.8

A6	3.3	1.4	4.6	53.8	46.2
A7	3.3	0.5	1.5	54.7	45.3
A8	1.0	0.2	0.2	50.6	49.4

EXAMPLE 2: Protein-A Purification of an Fc construct via a pH gradient elution

CHO expression and clarification of Multimeric Fc

An Fc construct containing an Fc domain from human IgG1 fused to the human IgM tail piece that caused the Fc to assemble into multimers, was expressed in a stable dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary cell line (CHO DG44). Cells were transfected using a Nuclefector (Lonza) following the manufacturer's instructions with a plasmid vector containing both the gene for DHFR as a selectable marker and the genes encoding the product. Transfected cells were selected in medium lacking hypoxanthine and thymidine, and in the presence of the DHFR inhibitor methotrexate. Cultures were maintained in shaken flasks culture in batch mode and harvested after 14 days.

Clarification of the cell culture supernatant was carried out via centrifugation (4000 x g for 60 minutes at room temperature) followed by depth and sterile filtration.

Clarified cell culture supernatant was concentrated and all Fc containing constructs were purified using a protein A chromatography column, a 5ml HiTrap MabSelect SuRe (GE Healthcare), equilibrated in PBS pH 7.4. The column was washed with PBS and bound material was eluted with 0.1M Citrate pH 3.4. Eluted material was buffer exchanged into PBS pH 7.4.

The purified Fc constructs was applied to a native protein A chromatography column, 5ml HiTrap MabSelect (GE Healthcare), equilibrated in PBS pH 7.4. The column was first washed with PBS to remove unbound material, and bound material was subsequently eluted with a pH gradient, pH7.4 to pH 2.1, see figure 3. Eluted material was fractionated and analysed via SEC-HPLC (G3000 SEC-HPLC, Tosoh Corporation). SEC-HPLC analysis was used to determine the % monomeric and multimeric Fc present in each fraction. Fc monomer has a retention time around 9.3 minutes. Trimer, hexamer, and higher order structures all have retention times below 9.4 minutes and were collectively termed multimeric species or higher molecular weight species (HMWS), see table 2 and figure 4.

Table 2 G3000 SEC Analysis of fractions from Protein-A Purification of Multimeric Fc via a pH gradient elution

Fraction	Total Protein (mg)	% Fc Monomer	% Fc Multimer

A1	0.89	2.3	97.6
A2	3.13	4.7	95.3
A3	3.14	9.5	90.4
A4	0.94	7.8	92.3

The Fc construct eluted as a single peak with a slight shoulder on the upward and downward inflection. However analysis of the fractions by SEC-HPLC revealed that monomeric and multimeric forms of the molecule elute in parallel, see table 2 and figure 4. No separation between the different species was observed across the gradient elution.

The multimers of Fc construct contain multiple Fc regions but lack variable regions and so lack the possibility of binding Protein-A through the human VH3 domain. The monomeric and multimeric Fc constructs co-elute from the Protein-A column in a gradient elution, demonstrating that Fc region avidity for the resin was not a factor for elution. Therefore monomeric and multimeric Fc region containing molecules cannot be efficiently separated by this technique. This is in contrast to examples 1 and 2 where the elution from Protein-A of VH3-domain containing antibodies that do not have an Fc region using a pH gradient was able to separate monomers from multimers.

EXAMPLE 3: Protein-A Purification of A26Fab-645dsFv via pH Step Elution

CHO expression and clarification of A26Fab-645dsFv

The construct which binds human OX40 and serum albumin was expressed in a stable dihyrofolate reductase (DHFR) deficient Chinese Hamster Ovary cell line (CHO DG44). Cells were transfected by electroporation using a Nuclefector (Lonza) following the manufacturer's instructions with a plasmid vector containing both the gene for DHFR as a selectable marker and the genes encoding the product. Transfected cells were selected in medium lacking hypoxanthine and thymidine, and in the presence of the DHFR inhibitor methotrexate. Cultures were maintained in shaken flasks culture in batch mode and harvested after 14 days.

Clarification of the cell culture supernatant was carried out via centrifugation (4000 x g for 60 minutes at room temperature) followed by depth and sterile filtration.

Protein-A Purification of A26Fab-645dsFv via pH Step Elution

Clarified cell culture supernatant was applied to a native protein A chromatography column, 5ml HiTrap MabSelect (GE Healthcare), equilibrated in Delbeccos Phosphate Buffered Saline (PBS) pH 7.4. The column was first washed with PBS to remove unbound material and bound material

was subsequently eluted first at pH3.8 and then a second elution step was carried out at pH 3.0, see figure 5. Eluted material was fractionated and analysed via SEC-HPLC (G3000 SEC-HPLC, Tosoh Corporation) and 4-20% Tris/Glycine SDS-PAGE performed under both reducing and non-reducing conditions.

SEC-HPLC analysis was used to determine the % monomer and multimer present in each fraction. A26Fab-645dsFv monomer has a retention time around 9 minutes. Dimer, trimer, tetramer, and higher order structures all have retention times below 9 minutes and were collectively termed multimeric species or higher molecular weight species (HMWS), see figure 6 and table 3.

Table 3 G3000 SEC Analysis of Fractions from Protein-A Purification of A26Fab-645dsFv via pH Step Elution

	Volume (ml)	Conc (mg/ml)	Total Protein (mg)	HMWS (%)	Monomer (%)
pH3.8	13.8	0.67	9.2	21.3	78.7
pH3.0	7.5	1.04	7.8	96.8	3.2

Non-reducing SDS-PAGE confirmed the above monomer and multimer levels in the elution peaks. A26Fab-645dsFv monomer migrates between the 97-66 kDa and is the major band in lane 4, corresponding to elution at pH 3.8, A26Fab-645dsFv multimers migrate as multiple bands above 120 kDa and are the majority of bands in lane 5, corresponding to the fraction recovered from elution at pH 3.0, see figure 7.

Reducing SDS-PAGE confirmed that all bands on the non-reduced SDS-PAGE were related to A26Fab-645dsFv, see figure 8.

The elution at pH 3.8 eluted a single peak in 2.8 column volumes with a slight tail on the downward inflection. SEC-HPLC analysis demonstrated that this peak contained 79% A26Fab-645dsFv in monomeric form, the high amount of monomer was also confirmed by non-reducing SDS-PAGE analysis. The elution at pH 3.0 eluted a single peak. HPLC-SEC analysis demonstrated that this peak was 97% A26Fab-645dsFv in multimeric form, the high amount of multimer was also confirmed by non-reducing SDS-PAGE analysis.

The above results demonstrate that efficient separation of monomer from multimer species via VH3 binding to protein A is possible. This is in contrast to Fc binding to Protein-A as shown in the previous example.

EXAMPLE 4: Protein-A (Amsphere) Purification of A26Fab-645dsFv via a gradient elution

CHO expression and clarification of A26Fab-645dsFv

The construct which binds human OX40 and serum albumin was expressed in a stable dihyrofolate reductase (DHFR) deficient Chinese Hamster Ovary cell line (CHO DG44). Cells were transfected by electroporation using a Nuclefector (Lonza) following the manufactures instructions with a plasmid vector containing both the gene for DHFR as a selectable marker and the genes encoding the product. Transfected cells were selected in medium lacking hypoxanthine and thymidine, and in the presence of the DHFR inhibitor methotrexate. Cultures were maintained in shaken flasks culture in batch mode and harvested after 14 days.

Clarification of the cell culture supernatant was carried out via centrifugation (4000 x g for 60 minutes at room temperature) followed by depth and sterile filtration.

Protein-A Purification (Amsphere) of A26Fab-645dsFv via a pH gradient elution

The clarified supernatant was applied to the Amsphere protein A chromatography column, (5ml column volume with 10cm bed height) and was equilibrated in Delbeccos Phosphate Buffered Saline (PBS) pH 7.4. The column was then washed with PBS to remove unbound material, and bound material was subsequently eluted with a pH gradient, pH 6.0 to pH 2.1. Eluted material was fractionated and analysed via SEC-UPLC (size exclusion chromatography - ultra performance liquid chromatography) using Acquity UPLC BEH450 SEC 2.5µm column. SEC-UPLC analysis was used to determine the % monomer and multimer present in each fraction. A26Fab-645dsFv monomer had a retention time around 2.2 minutes. Dimer, trimer, tetramer, and higher order structures all showed retention times below 2.2 minutes and were collectively termed multimeric species or higher molecular weight species (HMWS).

During elution from the Amsphere protein A chromatography column, two peaks were observed across the pH gradient, see figure 9. Elevated levels of monomer were observed in the first peak compared to elevated levels of multimeric species in the second peak, see table 5 and figure 9.

A26Fab-645dsFv lacks an Fc therefore binding to Amsphere Protein-A was due to the human VH3 variable framework subclass of the V-regions. It is proposed that the increased binding of the multimeric species was due to the increased avidity of these molecules for protein-A. Multimeric species have more VH3 regions and therefore bind stronger to the Protein-A resin requiring a lower pH for elution.

Table 5 SEC Analysis of fractions from Amsphere Protein-A Purification of A26Fab-645dsFv via a pH gradient elution

Fraction	Volume (ml)	Conc (mg/ml)	Protein (mg)	HMWS (%)	Monomer (%)
B15	5	0.54	2.68	0.0	85.2
B14	5	1.01	5.05	1.8	95.7
B13	5	1.14	5.7	48.2	49.7
B12	5	3.32	16.61	96.1	3.9
B11	5	1.85	9.235	96.1	2.8
B10	5	0.51	2.535	79.9	7.0

EXAMPLE 5: Protein-A (NovaSep Absolute) Purification of A26Fab-645dsFv via a gradient elution

CHO expression and clarification of A26Fab-645dsFv

The construct which binds human OX40 and serum albumin was expressed in a stable dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary cell line (CHO DG44). Cells were transfected by electroporation using a Nuclefector (Lonza) following the manufactures instructions with a plasmid vector containing both the gene for DHFR as a selectable marker and the genes encoding the product. Transfected cells were selected in medium lacking hypoxanthine and thymidine, and in the presence of the DHFR inhibitor methotrexate. Cultures were maintained in shaken flasks culture in batch mode and harvested after 14 days.

Clarification of the cell culture supernatant was carried out via centrifugation (4000 x g for 60 minutes at room temperature) followed by depth and sterile filtration.

Protein-A Purification (NovaSep Absolute) of A26Fab-645dsFv via a pH gradient elution

The clarified supernatant was applied to the NovaSep Absolute protein A chromatography column, (5ml column volume with 10cm bed height) and was equilibrated in Delbeccos Phosphate Buffered Saline (PBS) pH 7.4. The column was first washed with PBS to remove unbound material, and bound material was subsequently eluted with a pH gradient, pH 6.0 to pH 3.0. Eluted material was fractionated and analysed via SEC-UPLC using Acquity UPLC BEH450 SEC 2.5µm column. SEC-UPLC analysis was used to determine the % monomer and multimer present in each fraction. A26Fab-645dsFv monomer had a retention time around 2.2 minutes. Dimer, trimer, tetramer, and higher order structures all showed retention times below 2.2 minutes and were collectively termed multimeric species or higher molecular weight species (HMWS).

During elution from the NovaSep Absolute protein A chromatography column, two peaks were observed across the pH gradient, see figure 10. Elevated levels of monomer were observed in the first peak compared to elevated levels of multimeric species in the second peak, see table 6 and figure 10.

A26Fab-645dsFv lacks an Fc therefore binding to NovaSep Absolute Protein-A was due to the human VH3 variable framework subclass of the V-regions. It is proposed that the increased binding of the multimeric species was due to the increased avidity of these molecules for protein-A. Multimeric species have more VH3 regions and therefore bind stronger to the Protein-A resin requiring a lower pH for elution.

Table 6 SEC Analysis of fractions from NovaSep Absolute Protein-A Purification of A26Fab-645dsFv via a pH gradient elution

Fraction	Volume (ml)	Conc (mg/ml)	Protein (mg)	HMWS (%)	Monomer (%)
B15	2	0.36	0.72	0	99.7
B14	2	1.21	2.42	0	99.7
B13	2	0.46	0.92	4.6	94.8
B12	2	0.64	1.28	87.3	12.2
B11	2	2.05	4.1	98.4	1.4
B10	2	2.68	5.36	99	0.8
B9	2	0.67	1.34	97.2	2.3
B8	2	0.37	0.74	94.8	4.3
B6	2	0.03	0.06	94.7	4

EXAMPLE 6: Protein-A (AcroSep) Purification of A26Fab-645dsFv via a gradient elution

CHO expression and clarification of A26Fab-645dsFv

The construct which binds human OX40 and serum albumin was expressed in a stable dihyrofolate reductase (DHFR) deficient Chinese Hamster Ovary cell line (CHO DG44). Cells were transfected by electroporation using a Nuclefector (Lonza) following the manufactures instructions with a plasmid vector containing both the gene for DHFR as a selectable marker and the genes encoding the product. Transfected cells were selected in medium lacking hypoxanthine and thymidine, and in the presence of the DHFR inhibitor methotrexate. Cultures were maintained in shaken flasks culture in batch mode and harvested after 14 days.

Clarification of the cell culture supernatant was carried out via centrifugation (4000 x g for 60 minutes at room temperature) followed by depth and sterile filtration.

Protein-A Purification (AcroSep) of A26Fab-645dsFv via a pH gradient elution

The clarified supernatant was applied to the AcroSep Protein A chromatography column, (1ml column volume with 1.5cm bed height) was equilibrated in Delbeccos Phosphate Buffered Saline (PBS) pH 7.4. The column was first washed with PBS to remove unbound material, and bound material was subsequently eluted with a pH gradient, pH 6.0 to pH 3.0. Eluted material was fractionated and analysed via SEC-UPLC using Acquity UPLC BEH450 SEC 2.5µm column. SEC-HPLC analysis was used to determine the % monomer and multimer present in each fraction. A26Fab-645dsFv monomer had a retention time around 2.2 minutes. Dimer, trimer, tetramer, and higher order structures all showed retention times below 2.2 minutes and were collectively termed multimeric species or higher molecular weight species (HMWS).

During elution from the AcroSep Protein A chromatography column, a single broad elution peak was observed across the pH gradient, see figure 11. The reduced resolution maybe due to the reduced bed height however elevated levels of monomer were still observed during the upward inflection of the peak compared to elevated levels of multimeric species during the downward inflection of the elution peak, see table 7 and figure 11.

A26Fab-645dsFv lacks an Fc therefore binding to AcroSep Absolute Protein-A was due to the human VH3 variable framework subclass of the V-regions. It is proposed that the increased binding of the multimeric species was due to the increased avidity of these molecules for protein-A. Multimeric species have more VH3 regions and therefore bind stronger to the Protein-A resin requiring a lower pH for elution.

Table 7 SEC Analysis of fractions from AcroSep Absolute Protein-A Purification of A26Fab-645dsFv via a pH gradient elution

Fraction	Volume (ml)	Conc (mg/ml)	Protein (mg)	HMWS (%)	Monomer (%)
B13	1	0.17	0.17	28.9	71.1
B12	1	0.22	0.22	44.6	55
B11	1	0.35	0.35	67.1	32.8
B10	1	0.52	0.52	87.4	12.4
B9	1	0.73	0.73	96.2	3.6
B8	1	1.00	1.00	98.6	1.2
B7	1	1.19	1.19	99.1	0.7
B6	1	1.05	1.05	99.2	0.6
B5	1	0.54	0.54	98.6	0.9
B4	1	0.22	0.22	97.5	1.4

EXAMPLE 7: Protein-A Purification of TrYbe® via a pH gradient elution

Protein-A Purification of TrYbe® via a pH gradient elution

CHO expression and clarification of TrYbe®

A multispecific trivalent antibody molecule of the format Fab-2x dsscFv as described in WO 2015/197772 (TrYbe®) was expressed in a stable dihyrofolate reductase (DHFR) deficient Chinese Hamster Ovary cell line (CHO DG44). Cells were transfected using a Nuclefector (Lonza) following the manufactures instructions with a plasmid vector containing both the gene for DHFR as a selectable marker and the genes encoding the product. Transfected cells were selected in medium lacking hypoxanthine and thymidine, and in the presence of the DHFR inhibitor methotrexate. An expression was carried out in an in house proprietary fed batch process yielding a high cell number.

Clarification of the cell culture supernatant was carried out via centrifugation (4000 x g for 60 minutes at room temperature) followed by depth and sterile filtration. Clarified cell culture supernatant was applied to a 5ml HiTrap MabSelect (GE Healthcare) equilibrated in Delbeccos Phosphate Buffered Saline (PBS) pH7.4. The column was washed with PBS and bound material was eluted with a pH gradient, pH7.4 to pH 2.1, see figure 21. Eluted material was fractionated and analysed via G3000 SEC-HPLC and 4-20% Tris/Glycine SDS-PAGE (reduced & non-reduced). SEC-HPLC analysis was used to determine the % monomer and multimer. TrYbe® monomer has a retention time around 9.4 minutes. Dimer, trimer, tetramer, and higher order structures all have retention times <9.4 minutes and were collectively termed multimeric species or HMWS. For SEC-HPLC analysis chromatograms of fractions see figure 22.

Across the pH gradient elution two peaks were observed along with a shoulder on the downward inflection, see figure 21. These 3 fractions were analysed by SDS-PAGE, see figure 23. Across the elution profile several bands were observed via non reducing SDS-PAGE. Monomer migrates between the 116-200kDa molecular weight bands. All the bands which migrate above the monomer have been collectively termed multimeric or HMWS. A light chain related impurity lacking the inter chain disulphide species and non-disulphide bonded heavy and light chain migrate between the 37-55kDa molecular weight markers. Fraction 1 (lane 2) was predominantly made up of monomer and light chain related species with little or no HMWS visible. The monomer is the main band in fraction 2 (lane 3). Although HMWS bands are visible the levels are significantly reduced to that seen in fraction 3 (lane 4). In the reduced gel all

product related species are reduced down to heavy and light chain with a minor band of non-reducible material visible in lanes 3 and 4. Lane 2 confirms that fraction 1 was heavily enriched for the light chain related species.

The first peak (1) was identified as a light chain related impurity by reducing SDS-PAGE and when analysed by SEC-HPLC contained 2% HMWS. The second peak (2) was identified as TrYbe® and contained 72% monomer and 22% HMWS, see table 8 and figure 24. The downward inflection on peak 2, fraction 3, was identified as TrYbe® and contained 6% monomer and 92% HMWS.

TrYbe® lacks an Fc therefore binding to Protein-A was due to the human VH3 variable framework subclass of the v-regions. It is proposed that the increased binding of the multimeric species was due to the increased avidity of these molecules for protein-A. Multimeric species have more VH3 regions and therefore bind stronger to the Protein-A resin requiring a lower pH for elution.

Table 8 G3000 SEC Analysis of Fractions from Protein-A Purification of TrYbe® via a pH gradient elution

Fraction	Protein (mg)	HMWS (%)	Monomer (%)
1	3.9	2.0	97.3
2	33.9	22.1	72.0
3	0.4	91.5	6.4

EXAMPLE 8: Protein-A Purification of BYbe via a pH gradient elution

CHO expression and clarification of BYbe

A Fab-scFv fusion protein (Bybe) was constructed essentially as described in Example 4 of WO2013/068571, using different variable region sequences. The construct was expressed in a stable dihyrofolate reductase (DHFR) deficient Chinese Hamster Ovary cell line (CHO DG44). Cells were transfected using a Nuclefector (Lonza) following the manufacturer instructions with a plasmid vector containing both the gene for DHFR as a selectable marker and the genes encoding the product. Transfected cells were selected in medium lacking hypoxanthine and thymidine, and in the presence of the DHFR inhibitor methotrexate. After culture up to shaker flask stage, growth and productivity were assessed and the 24 highest expressing clones were chosen for evaluation in a fed-batch shake flask process. A 3L shake flask was inoculated with 1L of culture at a starting density of 0.3×10^6 viable cells/mL and controlled at 36.8°C, in a 5% CO_2 atmosphere. Nutrient feeds were added from day 3 to 12 and glucose was added as a

bolus addition when the concentration dropped below 5.8g/L. The culture was harvested on day 14, via centrifugation at 4000 x g for 60 min followed by 0.2µm filtration.

Protein-A Purification of BYbe via a pH gradient elution

Clarified cell culture supernatant was applied to a 4.7ml HiScreen MabSelect (GE Healthcare) column equilibrated in Sigma Phosphate Buffered Saline (PBS) pH7.4. The column was washed with PBS followed by 90% 0.2M Sodium Phosphate/10% citric acid, pH7.4 and bound material was eluted with a pH gradient of pH7.4 to pH 2.1, see figure 25. Eluted material was fractionated and analysed via G3000 SEC-HPLC and 4-20% Tris/Glycine SDS-PAGE (reduced & non-reduced). SEC-HPLC analysis was used to determine the % monomer and multimer. BYbe monomer has a retention time of around 9.6 minutes. Dimer, trimer, tetramer, and higher order structures all have retention times <9.6 minutes and were collectively termed multimeric species or HMWS. For SEC-HPLC analysis chromatograms of fractions see figure 26.

Across the pH gradient elution a single peak was observed, see figure 25. Elution peak fractions were analysed by SDS-PAGE, see figure 27. Across the elution profile several bands were observed via non reducing SDS-PAGE. Monomer migrates close to the 98kDa molecular weight marker. All the bands which migrate above the monomer (between the 250kDa and 98kDa molecular weight markers) have been collectively termed multimeric or HMWS. Non-disulphide bonded heavy and light chain migrate between the 50-64kDa and at the 30kDa molecular weight markers respectively. Fractions B9-B6 (lanes 6-9) were predominantly made up of monomer with little or no HMWS visible. HMWS bands increase/become more intense in fractions B5-B3 (lanes 10-12) as the pH gradient becomes more acidic. In the reduced gel all product related species are reduced down to heavy and light chain.

The elution peak was identified as BYbe and overall contained 84% monomer and 16% HMWS, see table 9 and figure 28.

BYbe lacks an Fc therefore binding to Protein-A was due to the human VH3 variable framework subclass of the v-regions. It is proposed that the increased binding of the multimeric species was due to the increased avidity of these molecules for Protein-A. Multimeric species have more VH3 regions and therefore bind more strongly to the Protein-A resin requiring a lower pH for elution.

Table 5 G3000 SEC Analysis of Fractions from Protein-A Purification of BYbe via a pH gradient elution

Fraction	Volume (ml)	Concentration (mg/ml)	Protein (mg)	HMWS (%)	Monomer (%)
B9	2.12	0.026	0.055	0.0	100.0
B8	2.14	0.103	0.220	0.0	100.0
B7	2.16	0.527	1.138	0.0	100.0
B6	2.18	2.395	5.221	0.7	99.3
B5	2.20	4.290	9.438	5.4	94.6
B4	2.22	1.886	4.187	28.8	71.2
B3	2.24	0.308	0.690	52.2	47.8
B2	2.26	0.047	0.106	40.8	59.2
			Average:	16.0	84.0

CLAIMS

1. A method for obtaining a human VH3 domain-containing antibody in monomeric form comprising:

a) applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E, under conditions that allow binding of said antibody to protein A, and

b) recovering the human VH3 domain-containing antibody in monomeric form,

wherein the eluate recovered from the protein A chromatography is enriched in monomeric antibody over multimeric antibody with respect to the applied mixture, and

wherein the human VH3 domain-containing antibody does not contain an Fc region.

2. A method for manufacturing a human VH3 domain-containing antibody comprising:

a) expressing the antibody in a host cell,

b) recovering a mixture containing the antibody in monomeric and multimeric form, host cells and other contaminants,

c) purifying the antibody by applying the mixture to at least a protein A chromatography material wherein said protein A comprises domain D and/or E, and

d) recovering the human VH3 containing antibody

wherein the eluate recovered from the protein A chromatography is enriched in monomeric antibody over multimeric antibody with respect to the mixture, and

wherein the human VH3 domain containing antibody does not contain an Fc region.

3. A method of separating a human VH3 domain-containing antibody in monomeric form from the antibody in multimeric form comprising:

a) applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E,

b) allowing binding of said antibody to protein A,

c) applying an elution buffer that selectively disrupts binding of the antibody in monomeric form,

d) recovering the resulting eluate, and optionally

e) applying a second elution buffer that disrupts binding of the antibody in multimeric form and recovering this second eluate,

wherein the human VH3 domain-containing antibody does not contain an Fc region.

4. A method of separating a human VH3 domain-containing antibody in monomeric form from the antibody in multimeric form comprising:

a) applying a mixture comprising a human VH3 domain-containing antibody in monomeric and multimeric form to a protein A chromatography material wherein said protein A comprises domain D and/or E,

b) allowing binding of the antibody in multimeric form,

c) recovering the antibody in monomeric form in the flow-through, and optionally

d) applying an elution buffer that selectively disrupts binding of the antibody in multimeric form, and

e) recovering the eluate resulting from d);

wherein the human VH3 domain-containing antibody does not contain an Fc region.

5. A method according to any preceding claim wherein a first solution is added to the protein A chromatography material after applying the mixture comprising the human VH3 domain-containing antibody in monomeric and multimeric form such that unbound material is removed in the solution.

6. A method according to claims 1, 2 or 5, wherein an elution buffer is applied to the protein A chromatography material such that the bound antibody is released.

7. A method according to any preceding claim wherein said protein A is native recombinant protein A.

8. A method according to any preceding claim wherein the VH3 domain containing antibody is selected from Fab', $F(ab')_2$, scFv, Fab-Fv, Fab-scFv, Fab-$(scFv)_2$, Fab-$(Fv)_2$, diabodies, triabodies, and tetrabodies.

9. A method according to any preceding claim wherein the VH3 domain-containing antibody comprises at least 2 human VH3 domains

10. A method according to any preceding claim wherein the VH3 domain-containing antibody specifically binds OX40.

11. A method according to claim 10 wherein said VH3 domain-containing antibody comprises:

- heavy chain CDR1, CDR2 and CDR3 as defined in SEQ ID NO: 1, SEQ ID NO:2 and SEQ ID NO:3, respectively; and

- light chain CDR1, CDR2, and CDR3 as defined in SEQ ID NO: 4, SEQ ID NO:5 and SEQ ID NO: 6, respectively.