

METHOD FOR PURIFYING SMALL MULTI-DOMAIN PROTEINS

FIELD OF THE INVENTION

[0001] The present disclosure provides a method for purifying a T cell engaging peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule produced by a host cell, the method comprising: isolating a supernatant of a host cell culture comprising the TCE-pMHC molecule to form Product (a), subjecting Product (a) to affinity chromatography wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.0 to about 4.0 to form Product (b), neutralizing Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of about 5.0 to about 7.0, subjecting Product (c) to anion exchange chromatography to form Product (d), and subjecting Product (d) to ceramic hydroxyapatite chromatography to obtain Product (e), wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

BACKGROUND

[0002] Protein-based therapeutics, including antibodies and fusion proteins, can be rapidly cleared from the body following administration. Their short circulatory half-life can be attributed to their small size, which allows for effective clearance via renal filtration, and lack of protection from intracellular degradation. To improve dosing, several strategies have been employed to extend circulatory half-life. These strategies include synthesis of multi-domain proteins exploiting recycling via the neonatal Fc receptor (FcRn), through attachment of antibody Fc domains or serum albumin (Konnteman, Curr Opin Biotechnol. 2011 Dec;22(6):868-76).

[0003] Multi-domain proteins can have a propensity to aggregate even after standard isolation methods, including Protein A chromatography, due to electrostatic interactions and self-association between domains. Furthermore, these proteins can include product-related impurities in the form of fragment adduct impurities that are difficult to resolve from the product due to similar physiochemical properties. The small size of these molecules relative to monoclonal antibodies (mAbs) can produce an increased relative host cell impurity burden. A downstream process capable of robust aggregate and impurity removal to improve titer levels can be necessary in some instances to meet regulatory expectations. MAb purification was previously performed using bind and elute

chromatography (B/E) or flow-through (F/T) chromatography. A limitation of B/E chromatography is the restriction of the load density to the actual resin binding capacity. In some instances, F/T chromatography can allow high load density for standard mAbs but may not be implementable for non-platform mAbs. In some instances, the solution conditions that enable F/T operation for these non-platform MAb may be such that they are not implementable in existing manufacturing plants.

SUMMARY OF THE INVENTION

[0004] In some aspects, the disclosure provides a method for purifying a T cell engaging peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule produced by a host cell, wherein the method comprises: (a) isolating a supernatant of a host cell culture comprising the TCE-pMHC molecule to form Product (a), (b) subjecting Product (a) to affinity chromatography, wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.0 to about 4.0 to form Product (b), (c) neutralizing Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of about 5.0 to about 7.0, and (d) subjecting Product (c) to anion exchange chromatography to form Product (d), wherein the TCE-pMHC molecule comprises: (i) a peptide-major histocompatibility complex (pMHC) binding domain including a first variable region linked to a constant region (VC1) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain, (ii) a T cell engaging immune effector domain including an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH), and (iii) a half-life extending domain including a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain, wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region, and wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0005] In some aspects, the TCE-pMHC subjected to the affinity chromatography in (b) is eluted with sodium acetate. In some aspects, the sodium acetate has a concentration of less than 100 mM. In some aspects, the sodium acetate has a concentration of about 25 mM to about 75 mM. In some aspects, the Product (b) has a pH of about 3 to about 4.

[0006] In some aspects, the MES buffer added to neutralize Product (b) is less than 5M. In some aspects, the MES buffer added to neutralize Product (b) is about 0.5M to about 1.5M.

[0007] In some aspects, the Product (c) has a pH of about 5.5 to about 6.5. In some aspects, the Product (c) is diluted at least 2-fold after neutralization to form a Diluted Product (c).

[0008] In some aspects, the Product (c) is diluted to form a Diluted Product (c), and the MES buffer in the Diluted Product (c) has a concentration of less than 20 mM. In some aspects, the MES buffer in the diluted Product (d) has a concentration of about 5mM to about 25 mM.

[0009] In some aspects, the conductivity of Product (c) is 5 mS/cm or less. In some aspects, the conductivity of Diluted Product (c) is 5 mS/cm or less.

[0010] In some aspects, the flow through buffer used with the anion exchange chromatography is a MES buffer. In some aspects, the flow through buffer is less than 100mM. In some aspects, the flow through buffer is about 25 mM to about 75 mM.

[0011] In some aspects, the Product (d) has a pH of about 6 to about 7.

[0012] In some aspects, the affinity chromatography includes a protein A affinity chromatography. In some aspects, the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.2 to about 4.0.

[0013] In some aspects, the anion exchange chromatography includes a salt-tolerant anion-exchange flow through chromatography. In some aspects, the anion exchange chromatography comprises a cross-linked cellulose. In some aspects, the cross-linked cellulose comprises a primary amine.

[0014] In some aspects, the methods as described herein further comprising, (e) subjecting Product (d) to hydroxyapatite chromatography to form Product (e).

[0015] In some aspects, the methods as described herein further comprises expressing the TCE-pMHC molecule in the host cell, wherein the TCE-pMHC molecule is secreted into the host cell culture supernatant before the isolating in (a). In some aspects, the host cell is a mammalian cell. In some aspects, the TCE-pMHC molecule is isolated from the host cell by filtration, centrifugation or other methods known by one of ordinary skill in the art.

[0016] In some aspects, the Product (c) has high molecular weight aggregates of TCE-pMHC molecule (HMW-TCE-pMHC) less than 10% (wt/wt) of total TCE-pMHC. In some aspects, the Product (c) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC.

[0017] In some aspects, the Product (d) has HMW-TCE-pMHC less than 10% (wt/wt) of total TCE-pMHC. In some aspects, the Product (d) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC.

[0018] In some aspects, the Product (e) has HMW-TCE-pMHC less than 10% (wt/wt) of total TCE-pMHC. In some aspects, the Product (e) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC.

[0019] In some aspects, the HMW-TCE-pMHC is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance or a combination thereof.

[0020] In some aspects, the Product (c) comprises host cell DNA (hcDNA) of less than 30 pg/mg. In some aspects, the Product (c) comprises host cell protein of less than 150 ng/mg. In some aspects, the Product (d) comprises host cell DNA (hcDNA) of less than 30 pg/mg.

[0021] In some aspects, the Product (d) comprises host cell protein of less than 150 ng/mg. In some aspects, the Product (e) comprises host cell DNA (hcDNA) of less than 30 pg/mg. In some aspects, the Product (e) comprises host cell protein of less than 150 ng/mg. In some aspects, the Product (e) comprises product related impurities of less than 1.5% (wt/wt). In some aspects, the Product (e) comprises product related impurities of less than 0.5% (wt/wt).

[0022] In some aspects, the TCE-pMHC molecule is greater than 99% (wt/wt) of total protein in Product (e). In some aspects, the TCE-pMHC molecule is greater than 99.5% (wt/wt) of total protein in Product (e).

[0023] In some aspects, the pI of the TCE-pMHC molecule is about 6.5 to about 7.0. In some aspects, the pI of the TCE-pMHC molecule is about 6.8.

[0024] In some aspects, the Product (a) comprises total cell protein of about 0.1 mg/mL to about 25 mg/mL. In some aspects, the Product (a) comprises total cell protein of about 2 mg/mL to about 10 mg/mL.

[0025] In some aspects, the pMHC binding domain binds to SLLQHLIGL (SEQ ID NO: 1) HLA-A*02 complex.

[0026] In some aspects, the disclosure provides a method as described herein, wherein the TCR α variable region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 2 and the TCR β variable region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 8. In some aspects, the TCR α constant region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 15 and the TCR β constant region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 19.

[0027] In some aspects, the disclosure provides a method as described herein, wherein the FC1 and FC2 regions of the half-life extending domains of the TCE-pMHC molecule are IgG1 Fc regions. In some aspects, the FC1 region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 42 and the FC2 region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 43.

[0028] In some aspects, the disclosure provides a method as described herein, wherein the TCE-pMHC molecule comprises the following amino acid sequences, in the following order, from N-terminus to C-terminus: (a) an amino acid sequence of an anti-CD3 scFv, optionally followed by a linker sequence provided in SEQ ID NO: 18, (b) an amino acid sequence of a TCR β variable and constant region, (c) a linker sequence provided in SEQ ID NO: 47 followed by an IgG hinge sequence provided in SEQ ID NO: 44, (d) an Fc region having the sequence provided in SEQ ID NO: 42, (e) a linker sequence provided in SEQ ID NO: 47, (f) an amino acid sequence of a TCR α variable and constant region, (g) a linker sequence provided in SEQ ID NO: 47 followed by an IgG hinge sequence provided in SEQ ID NO: 44, and h) an Fc region having the sequence provided in SEQ ID NO: 43.

[0029] In some aspects, the disclosure provides a method for reducing a product-related impurity during purification of a T cell engaging immune effector domain, peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule produced by a host cell, wherein the method comprises: (a) isolating a supernatant of a host cell culture comprising the TCE-pMHC molecule to form Product (a), (b) subjecting Product (a) to affinity chromatography to form Product (b), (c) subjecting Product (b) to anion exchange chromatography to form Product (d*), (d) subjecting Product (d*) to hydroxyapatite chromatography, wherein the TCE-pMHC molecule is eluted with an elution buffer including an MES buffer, wherein the elution buffer has a pH of about 5.0 to about 7.0, to form Product (e), wherein the TCE-pMHC molecule includes: (i) a peptide-major histocompatibility complex (pMHC) binding domain including a first variable region linked

to a constant region (VCs) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain, (ii) a T cell engaging immune effector domain including an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH), and (iii) a half-life extending domain including a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain, wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region, and wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0030] In some aspects, the hydroxyapatite chromatography comprises a ceramic hydroxyapatite.

[0031] In some aspects, the elution buffer comprises about 50 mM to about 200 mM MES. In some aspects, the elution buffer comprises about 100 mM MES. In some aspects, the elution buffer further comprises about 100 mM to about 1 M of a salt. In some aspects, the elution buffer comprises about 300 mM to about 500 mM of a salt. In some aspects, the salt is a sodium, lithium or potassium salt. In some aspects, the salt is sodium chloride. In some aspects, the elution buffer comprises a phosphate buffer. In some aspects, the phosphate buffer is about 5 mM to about 50 mM. In some aspects, the phosphate buffer is about 10 mM.

[0032] In some aspects, in the methods of reducing a product-related impurity, the product-related impurity includes a fragment adduct impurity. In some aspects, the product-related impurity includes physiochemical properties similar to TCE-pMHC molecule. In some aspects, the product related impurity is assessed before and after purification. In some aspects, the product related impurity is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance, mass spectrophotometry or a combination thereof.

[0033] In some aspects, the Product (d*) has a pH of about 6 to about 7. In some aspects, the Product (d*) includes less than 2% (wt/wt) of the product related impurity. In some aspects, the Product (d*) includes less than 1% (wt/wt) of the product related impurity.

[0034] In some aspects, the disclosure provides a method of manufacturing a T cell engaging, peptide-major histocompatibility complex binding domain (TCE-pMHC)

molecule produced by a host cell, wherein the TCE-pMHC molecule is secreted into the host cell culture supernatant, wherein the method comprises: (a) isolating the supernatant comprising the TCE-pMHC molecule to form Product (a), (b) subjecting Product (a) to affinity chromatography, wherein Product (a) is less than 10 mg/mL, wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.0 to about 4.0 to form Product (b), (c) adjusting the pH of Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of about 5.0 to about 7.0, (d) subjecting Product (c) to anion exchange chromatography to form Product (d), and (e) subjecting Product (d) to hydroxyapatite chromatography, wherein the TCE-pMHC molecule is eluted with an elution buffer including an MES buffer, wherein the elution buffer has a pH of less than 7.0 to form Product (e), wherein Product (e) has a pH of less than 7.0, wherein the TCE-pMHC molecule includes: (i) a peptide-major histocompatibility complex (pMHC) binding domain including a first variable region linked to a constant region (VCs) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain, (ii) a T cell engaging immune effector domain including an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH), and (iii) a half-life extending domain including a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain, wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region, and wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0035] In some aspects, the disclosure provides a method for purifying a T cell engaging, peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule, wherein the method comprises the following steps: (a) expressing the TCE-pMHC molecule in a mammalian host cell, wherein the TCE-pMHC molecule is secreted by the host cell into a host cell culture supernatant, (b) isolating the supernatant comprising the TCE-pMHC molecule to form Product (a), (c) subjecting Product (a) to Protein A affinity chromatography, wherein Product (a) is less than 10 mg/mL; wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of 3.2 to 4.0 to form Product (b), (d) adjusting the pH of Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of 5.5 to 7.0, and optionally diluting

Product (c) at least 2-fold to obtain Diluted Product (c), (e) subjecting Product (c), or Diluted Product (c) if applicable, to Sartobind STIC-PA chromatography to form Product (d), (f) subjecting Product (d) to ceramic hydroxyapatite chromatography to obtain Product (e), and (g) optionally, subjecting Product (e) to one or more polishing steps, wherein Product (c), or Diluted Product (c) if present, has a conductivity of <5 mS/cm, wherein the TCE-pMHC molecule includes: (i) a peptide-major histocompatibility complex (pMHC) binding domain including a first variable region linked to a constant region (VC1) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerize to form the pMHC binding domain, (ii) a T cell engaging immune effector domain including an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH), and (iii) a half-life extending domain including a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerize to form an Fc domain, wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region, and wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0036] In some aspects, the method further comprises viral reduction filtration. In some aspects, the method further comprises diafiltration. In some aspects, the method further comprises adding polysorbate 80 to Product (e) or Product (e).

[0037] In some aspects, the disclosure provides a method for treating cancer in a subject, the method comprises administering the purified TCE-pMHC molecule made by the methods provided herein. In some aspects, the cancer is associated with PRAME expression.

[0038] In some embodiments, the disclosure provides a TCE-pMHC molecule produced by the methods as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] The following drawings form part of the present specification and are included to further demonstrate exemplary embodiments of certain aspects of the present disclosure.

[0040] FIG 1. is a schematic overview of the downstream process of purifying a TCE-

pMHC molecule as described in the examples.

[0041] FIG 2. is a SE-UPLC trace of a TCE-pMHC molecule purified by Protein A affinity chromatography as described in Example 2 under the standard process conditions: 100 mM Sodium Citrate, pH 3 elution, and 2 M Tris pH 6.8 neutralization. Main product peak was measured at retention time 12.063 minutes.

[0042] FIG 3. is a SE UPLC trace of a TCE-pMHC molecule purified by Protein A affinity chromatography as described in Example 2 under the developed process conditions: 50 mM Sodium Acetate, pH 3.6 elution, and 1 M MES pH 6.4 neutralization. Main product peak measured at retention time 13.365 minutes, and product related impurities (HMW1) was measured at 12.707 minutes.

[0043] FIG 4. is a SE-UPLC trace of a TCE-pMHC molecule purified by Sartobind STIC-PA flow through chromatography as described in Example 3. Main product peak was measured at retention time 13.417 minutes and product related impurities (HMW1) was measured at 12.857 minutes.

[0044] FIG 5. is a SE-UPLC trace of a TCE-pMHC molecule purified by CHT Ceramic Hydroxyapatite Type II chromatography as described in Example 4. Main product peak was measured at retention time 13.337 minutes.

DETAILED DESCRIPTION

[0045] It should be understood that any embodiment described herein, including those described only in the examples, can be combined with any one or more other embodiments, unless such combination is expressly disclaimed or is improper. Thus, the term “embodiment”, as used herein, is not to be considered as excluding features recited in other embodiments.

[0046] Unless otherwise defined herein, scientific and technical terms used in the present disclosure shall have meanings that are commonly understood by one of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0047] The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0048] The use of the term “or” in the claims is used to mean “and/or,” unless explicitly indicated to refer only to alternatives or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

[0049] As used herein, the terms “comprising” (and any variant or form of comprising, such as “comprise” and “comprises”), “having” (and any variant or form of having, such as “have” and “has”), “including” (and any variant or form of including, such as “includes” and “include”) or “containing” (and any variant or form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited, elements or method steps.

[0050] The use of the term “for example” and its corresponding abbreviation “e.g.” means that the specific terms recited are representative examples and embodiments of the disclosure that are not intended to be limited to the specific examples referenced or cited unless explicitly stated otherwise.

[0051] As used herein, “about” can mean plus or minus 10% of the provided value. Where ranges are provided, they are inclusive of the boundary values. “About” can additionally or alternately mean either within 10% of the stated value, or within 5% of the stated value, or in some cases within 2.5% of the stated value; or “about” can mean rounded to the nearest significant digit.

[0052] As used herein, “between” is a range inclusive of the ends of the range. For example, a number between x and y explicitly includes the numbers x and y and any numbers that fall within x and y.

[0053] The term “phenotypically silent variants” is understood to refer to a variant which incorporates one or more further amino acid changes, including substitutions, insertions and deletions, in addition to those set out above, which variant has a similar phenotype to the corresponding molecule without said change(s). For the purposes of this application, phenotype comprises binding affinity (K_D and/or binding half-life) and specificity. Preferably, the phenotype for a soluble multi-domain binding molecule includes potency of immune activation and purification yield, in addition to binding affinity and specificity.

[0054] Phenotypically silent variants may contain one or more conservative substitutions and/or one or more tolerated substitutions. By tolerated substitutions it is meant those substitutions which do not fall under the definition of conservative as provided below but

are nonetheless phenotypically silent. The skilled person is aware that various amino acids have similar properties and thus are ‘conservative’. One or more such amino acids of a protein, polypeptide or peptide can often be substituted by one or more other such amino acids without eliminating a desired activity of that protein, polypeptide or peptide.

[0055] Thus, the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulfur containing side chains). It should be appreciated that amino acid substitutions within the scope of the present disclosure can be made using naturally occurring or non-naturally occurring amino acids. For example, it is contemplated herein that the methyl group on an alanine may be replaced with an ethyl group, and/or that minor changes may be made to the peptide backbone. Whether or not natural or synthetic amino acids are used, it is preferred that only L- amino acids are present.

[0056] Substitutions of this nature are often referred to as “conservative” or “semi-conservative” amino acid substitutions. The present disclosure therefore extends to use of a molecule comprising any of the amino acid sequence described above but with one or more conservative substitutions and or one or more tolerated substitutions in the sequence, such that the amino acid sequence of the TCR has at least 90% identity, such as 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity, to the TCR sequences disclosed herein.

[0057] “Identity” as known in the art is the relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case can be, as determined by the match between strings of such sequences. While there exist a number of methods to measure identity between two polypeptide or two polynucleotide sequences, methods commonly

employed to determine identity are codified in computer programs. Preferred computer programs to determine identity between two sequences include, but are not limited to, GCG program package (Devereux, et al., *Nucleic Acids Research*, 12, 387 (1984), BLASTP, BLASTN, and FASTA (Atschul et al., *J. Molec. Biol.* 215, 403 (1990)).

[0058] One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or similarity (identity plus conservation of amino acid type) for an optimal alignment. A program like BLASTx will align the longest stretch of similar sequences and assign a value to the fit. It is thus possible to obtain a comparison where several regions of similarity are found, each having a different score. Both types of identity analysis are contemplated in the present disclosure.

[0059] The percent identity of two amino acid sequences or of two nucleic acid sequences is determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the first sequence for best alignment with the sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The “best alignment” is an alignment of two sequences which results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (i.e., % identity = number of identical positions/total number of positions x 100).

[0060] The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. The NBLAST and XBLAST programs of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules for use in the disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects

distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the CGC sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti (1994) Comput. Appl. Biosci., 10 :3-5; and FASTA described in Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

[0061] Mutations, including conservation and tolerated substitutions, insertions, and deletions, can be introduced into the sequences provided using any appropriate method including, but not limited to, those based on polymerase chain reaction (PCR), restriction enzyme-based cloning, or ligation independent cloning (LIC) procedures. These methods are detailed in many of the standard molecular biology texts. For further details regarding polymerase chain reaction (PCR) and restriction enzyme-based cloning, see Sambrook & Russell, (2001) Molecular Cloning – A Laboratory Manual (3rd Ed.) CSHL Press. Further information on ligation independent cloning (LIC) procedures can be found in Rashtchian, (1995) 13urrO pin Biotechnol 6(1): 30-6. The TCR sequences provided by the disclosure can be obtained from solid state synthesis, or any other appropriate method known in the art.

T cell engaging peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule

[0062] The methods described herein can be used to purify a T cell engaging peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule from a host cell. As used herein, the TCE-pMHC molecule comprises:

- (i) a peptide-major histocompatibility complex (pMHC) binding domain,
- (ii) a T cell engaging immune effector domain, and
- (iii) a half-life extending domain.

i. Peptide-major histocompatibility complex (pMHC) binding domains

[0063] The term “pMHC binding domain”, as used herein, is a protein domain capable of binding to a peptide-MHC complex. In some embodiments, the pMHC binding domain of

the TCE-pMHC molecule described herein binds to a SLLQHLIGL (SEQ ID NO: 1) HLA-A*02 complex. SLLQHLIGL (SEQ ID NO: 1) is a peptide derived from PRAME, a tumor-associated antigen. A first variable region linked to a constant region (VC1) and a second variable region linked to a constant region (VC2) dimerise to form the pMHC binding domain. In this context, “VC1” refers to a region of the pMHC binding domain sequence that comprises the first variable region linked to a constant region and “VC2” refers to a region that comprises the second variable region linked to a constant region. The pMHC binding site is within the variable regions of VC1 and VC2. Suitable variable and constant region sequences include TCR or antibody variable and constant regions. The terms “MHC” and “HLA” as used herein are used interchangeably.

[0064] In some embodiments, the pMHC binding domain may comprise at least part of a TCR α and a TCR β chain. For example, the variable regions of VC1 and VC2 may be TCR variable regions. VC1 may comprise either a TCR α or a TCR β variable region and VC2 may comprise the other of the TCR α and TCR β variable regions. For example: (i) VC1 may comprise either (a) a TCR α variable and constant region or (b) a TCR β variable and constant region; and (ii) VC2 may comprise the other of (a) or (b). Preferably, VC1 comprises the TCR β variable and constant region and VC2 comprises the TCR α variable and constant region.

[0065] In some embodiments, the pMHC binding domain may be a T cell receptor (TCR), such as a soluble TCR, comprising TCR variable regions and constant regions. The TCR sequences defined herein are described with reference to IMGT nomenclature which is widely known and accessible to those working in the TCR field. For example, see: LeFranc and LeFranc, (2001). “T cell Receptor Factsbook”, Academic Press; Lefranc, (2011), Cold Spring Harb Protoc 2011 (6): 595-603; Lefranc, (2001), Curr Protoc Immunol Appendix 1: Appendix 100; and Lefranc, (2003), Leukemia 17(1): 260-266. Briefly, TCRs consist of two disulfide linked chains. Each chain (alpha and beta) is generally regarded as having two extracellular regions, namely a variable and a constant region. A short joining region connects the variable and constant regions and is typically considered part of the alpha variable region. Additionally, the beta chain usually contains a short diversity region next to the joining region, which is also typically considered part of the beta variable region. The variable region of each chain of a typical TCR is located N-terminally and comprises three Complementarity Determining Regions (CDRs) embedded in a framework sequence. The CDRs comprise the recognition site for peptide-MHC binding.

[0066] In some embodiments, the pMHC binding domain may comprise variable regions of an antibody. The VC1 and VC2 variable regions may be antibody heavy or light chain variable regions. For example, VC1 may comprise either a heavy or a light chain antibody variable region and VC2 may comprise the other of the heavy or a light chain antibody variable region. In this regard, the pMHC binding domain may be a TCR-like antibody, also known as a “TCR mimic antibody” (TCRm-Ab). For example, the pMHC binding domain may comprise variable regions of a TCR-like antibody. Antibodies do not naturally recognize a pMHC complex. However, it is known that antibodies with specificity for pMHC can be engineered, as described in Chang et al., *Expert Opin Biol Ther.* 2016 Aug;16(8):979-87 and Dahan et al., *Expert Rev Mol Med.* 2012 Feb 24;14:e6.

[0067] In some embodiments, the pMHC binding domain may comprise at least one immunoglobulin constant region. In embodiments, the constant regions in VC1 and VC2 may be immunoglobulin constant regions. In embodiments, the constant region may correspond to a constant region from a TCR α chain or a TCR β chain (TRAC or TRBC respectively). In embodiments, the constant regions of the pMHC binding domain may be a constant region from an antibody light or heavy chain (CL, CH1, CH2, CH3 or CH4). In embodiments, the constant region may be full length or may be truncated. In embodiments, TCR constant regions may be truncated to remove the transmembrane domain and cytoplasmic tail. Where the constant region is truncated, preferably only membrane-associated and cytoplasmic portions are removed from the C-terminal end. Where the pMHC binding domain comprises TCR α or TCR β chain sequences, VC1 and VC2 may each comprise a TCR variable region and a TCR constant region. In embodiments, VC1 and VC2 do not comprise a transmembrane or cytoplasmic domain, i.e., preferably the pMHC binding domain is soluble. In embodiments, additional mutations may be introduced into the amino acid sequence of the constant regions relative to natural constant regions. In embodiments, the constant regions may also include residues, either naturally-occurring or introduced, that allow for dimerization by, for example, a disulfide bond between two cysteine residues.

[0068] In some embodiments, TCR portions of the molecules of the disclosure may be $\alpha\beta$ heterodimers. Alpha-beta heterodimeric TCR portions of the molecules of the disclosure may comprise an alpha chain TRAC constant region sequence and/or a beta chain TRBC1 or TRBC2 constant region sequence. As described above, the constant regions may be in soluble format (i.e. having no transmembrane or cytoplasmic domains). One or both of the constant regions may contain mutations, substitutions or deletions relative to the native

TRAC and/or TRBC1/2 sequences. The terms TRAC and TRBC1/2 also encompass natural polymorphic variants, for example N to Kat position 4 of TRAC (Bragado et al International immunology. 1994 Feb;6(2):223-30).

[0069] In some embodiments, alpha and beta chain constant region sequences may be modified by truncation or substitution to delete the native disulfide bond between Cys4 of exon 2 of TRAC and Cys2 of exon 2 of TRBC1 or TRBC2. Alpha and/or beta chain constant region sequence(s) may have an introduced disulfide bond between residues of the respective constant domains, as described, for example, in WO 2003/020763, WO 2004/033685 and WO 2006/000830. Alpha and beta constant regions may be modified by substitution of cysteine residues at position Thr 48 of TRAC and position Ser 57 of TRBC1 or TRBC2, the said cysteines forming a disulfide bond between the alpha and beta constant regions of the TCR. TRBC1 or TRBC2 may additionally include a cysteine to alanine mutation at position 75 of the constant domain and an asparagine to aspartic acid mutation at position 89 of the constant domain. In some embodiments, one or both of the extracellular constant regions present in an $\alpha\beta$ heterodimer may be truncated at the C terminus or C termini, for example by up to 15, or up to 10, or up to 8 or fewer amino acids. The C terminus of an alpha chain extracellular constant region may be truncated by 8 amino acids.

[0070] In some embodiments, the amino acid sequence of the VC1 and VC2 variable and constant regions may correspond to those found in nature, or they may contain one or more mutations relative to a natural protein. Such mutations may be made to increase the affinity of the pMHC binding domain for the SLLQHLIGL (SEQ ID NO: 1) HLA-A*02 complex. In some embodiments, mutations may be incorporated to improve stability and manufacturability. In some embodiments, the VC1 and VC2 sequences may be derived from human sequences.

[0071] In some embodiments, the VC1 and VC2 sequences may comprise one or more engineered cysteine residues in the constant region to form a non-native disulfide bond between VC1 and VC2. Suitable positions for introducing disulfide bond between residues of the respective constant regions, are described in WO 2003/020763 and WO 2004/033685. Single chain TCRs are further described in WO2004/033685; W098/39482; WO01/62908; Weidanz et al. (1998) J Immunol Methods 221 (1 -2): 59-76; Hoo et al. (1992) Proc Natl Acad Sci U SA 89(10): 4 759-4763; Schodin (1996) Mol Immunol 33(9): 819-829).

[0072] In some embodiments, the VC1 may comprise a TCR α or TCR β variable region and VC2 may comprise the other of the TCR α and TCR β variable region. Preferably:

- (i) the TCR α variable region comprises CDRs of SEQ ID NO: 3, 4, and 5 as CDR1, CDR2 and CDR3 respectively; and
- (ii) the TCR β variable region comprises CDRs of SEQ ID NO: 9, 10, and 11 as CDR1, CDR2 and CDR3 respectively.

[0073] In some embodiments, the TCR α and TCR β CDR sequences may each optionally have one, two, three, or four amino acid substitutions relative to the sequences recited above.

[0074] In some embodiments, the TCR α variable region may comprise CDRs that are at least 90%, at least 95%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 3, 4, and 5 as CDR1, CDR2 and CDR3 and/or the TCR β variable region may comprise CDRs that are at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 9, 10, and 11 as CDR1, CDR2 and CDR3 respectively.

[0075] In some embodiments, the TCR α variable region may be at least 80% identical to the sequence of SEQ ID NO: 2 and the TCR β variable region may be at least 80% identical to the sequence of SEQ ID NO: 8. The TCR α variable region may be at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 2 and the TCR β variable region may be at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 8. Preferably, the TCR α variable region has the sequence provided in SEQ ID NO: 2 and the TCR β variable region has the sequence provided in SEQ ID NO: 8.

[0076] In some embodiments, VC1 may comprise a TCR α or TCR β constant region and VC2 may comprise the other of the TCR α and TCR β constant region. The TCR α constant region may be at least 80% identical to the sequence of SEQ ID NO: 15 and the TCR β constant region may be at least 80% identical to the sequence of SEQ ID NO: 19. The TCR α constant region may be at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 15 and the TCR β constant region may be at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 19. Preferably, the TCR α constant region has the sequence provided in SEQ ID NO: 15 and the TCR β constant region has the sequence provided in SEQ ID NO: 19.

[0077] In some embodiments, VC1 may comprise a TCR α variable and constant region or TCR β variable and constant region and VC2 may comprise the other of the TCR α and

TCR β variable and constant regions. In some embodiments, the TCR α variable and constant region may comprise an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 14 and the TCR β variable and constant region may comprise an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 16. The TCR α variable and constant region may comprise an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 14 and the TCR β variable and constant region may comprise, or consist of, an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 16. Preferably, the TCR α variable and constant region comprises, or consists of, the amino acid sequence provided in SEQ ID NO: 14 and the TCR β variable and constant region comprises, or consists of, the amino acid sequence provided in SEQ ID NO: 16.

[0078] The skilled person would appreciate that the format of the multi-domain binding molecule of the disclosure could equally be applied to TCR sequences other than those recited above. For example, other suitable TCR chain amino acid sequences are provided in WO2011001152, WO2017109496, WO2017175006 and WO2018234319 which are incorporated herein by reference in their entirety.

[0079] As is well-known in the art, protein molecules may be subject to post-translational modifications. Glycosylation is one such modification, which comprises the covalent attachment of oligosaccharide moieties to defined amino acids in a TCR or antibody chain. For example, asparagine residues, or serine/threonine residues are well-known locations for oligosaccharide attachment. The glycosylation status of a particular protein depends on a number of factors, including protein sequence, protein conformation and the availability of certain enzymes. Furthermore, glycosylation status (i.e., oligosaccharide type, covalent linkage and total number of attachments) can influence protein function. Therefore, when producing recombinant proteins, controlling glycosylation is often desirable. Controlled glycosylation has been used to improve antibody-based therapeutics. (Jefferis et al., (2009) Nat Rev Drug Discov Mar;8(3):226-34.). Glycosylation may be controlled, by using particular cell lines for example (including but not limited to mammalian cell lines such as Chinese hamster ovary (CHO) cells or human embryonic kidney (HEK) cells), or by chemical modification. Such modifications may be desirable, since glycosylation can improve pharmacokinetics, reduce immunogenicity and more closely mimic a native human protein (Sinclair and Elliott, (2005) Pharm Sci. Aug; 94(8):1626-35). Alternatively, glycosylation can lead to a lack of consistency in manufacturing which is not desirable for a therapeutic molecule.

[0080] In some embodiments, VC1 and/or VC2 may comprise one or more amino acid substitutions which remove one or more glycosylation sites. The substitutions in this context are relative to a native (e.g., wild-type) sequence or unmodified sequence. For example:

(i) VC1 or VC2 may comprise a TCR α variable and constant region comprising one or more amino acid substitutions at positions selected from the group consisting of N24, N148, N182 and N193, numbered according to SEQ ID NO: 14; and/or

(ii) the other of VC 1 and VC2 may comprise a TCR β variable and constant region comprising an amino acid substitution at position N184, numbered according to SEQ ID NO: 16. The substitutions may be Asn to Gln (i.e., N to Q) substitutions. Preferably, the TCR α variable and constant region comprises N24Q, N148Q, N182Q and N193Q substitutions, numbered according to SEQ ID NO: 14, and the TCR β variable and constant region comprises a N184Q substitution, numbered according to SEQ ID NO: 16.

[0081] In some embodiments, the pMHC binding domain may not be fully glycosylated, i.e., the pMHC may retain one or more glycosylation site(s) from its native sequence. For example, the pMHC binding domain may be glycosylated at a single glycosylation site (i.e., the pMHC binding domain may contain only one glycosylation site). In some embodiments, the single glycosylation site may be in the variable region of VC1 or VC2. The single glycosylation site may be at position N18 of a TCR β variable region, numbered according to SEQ ID NO: 16. Advantageously, the present inventors have identified that multi-domain binding proteins with this single glycosylated site have better manufacturability (e.g., protein production yield, resistance to thermal stress and aggregation), as compared to other glycosylated and/or aglycosylated variants, in addition to retaining affinity for peptide-MHC binding and potency of target cell killing.

ii. T cell engaging immune effector domains

[0082] A “T cell engaging immune effector domain,” as used herein, is a protein domain that is capable of binding to a target on a T cell to promote an immune response. The T cell engaging immune effector domain comprises an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH). As used herein, “TCE-VL” and “TCE-VH” refer to the light chain variable region and the heavy chain variable region of the T cell engaging immune effector domain, respectively. “TCE-VL” and “TCE-VH” may also be referred to as “TCEVL” and “TCEVH” herein. Thus, the T cell engaging immune effector domain may comprise an antigen-binding site. For example, the T cell

engaging immune effector domain may bind to a protein expressed on a cell surface of a T cell to promote activation of the T cell. For example, the T cell engaging immune effector domain may be a CD3 effector domain. The T cell engaging immune effector domain may bind to, for example specifically bind to, CD3 (i.e., the T cell engaging immune effector domain may be a CD3-binding protein). The T cell engaging immune effector may be an antibody, or a functional fragment thereof, for example a single-chain variable fragment (scFv), or a similar sized antibody-like scaffold, or any other binding protein that activates a T cell through interaction with CD3 and/or the TCR/CD3 complex.

[0083] In some embodiments, the T cell engaging immune effector domain may be a single-chain variable fragment (scFv). “Single chain Fv” also abbreviated as “sFv” or “scFv” are antibody fragments that comprise the VH and VL antibody domains connected into a single polypeptide chain. In some embodiments, the scFv polypeptide may further comprise a polypeptide linker between the VH and VL domains which enables the scFv to form the 5 desired structure for antigen binding. For a review of scFv’s, see Pluckthun in The pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269- 315 (1994).

[0084] CD3 effectors include but are not limited to anti-CD3 antibodies or antibody fragments, in particular an anti-CD3 scFv or antibody-like scaffolds. The T cell engaging immune effector domain may be an anti-CD3 scFv. Further immune effectors include but are not limited to antibodies, including fragments, derivatives and variants thereof, that bind to antigens on T cells. Such antigens include CD28, 4-1 bb (CD137) or CD16 or any molecules that exert an effect at the immune synapse. A particularly preferred immune effector is an anti-CD3 antibody, or a functional fragment or variant of said anti-CD3 antibody. As used herein, the term “antibody” encompasses such fragments and variants. Examples of anti-CD3 antibodies include but are not limited to OKT3, UCHT-1, BMA-031 and 12F6. Antibody fragments and variants/analogues which are suitable for use in the compositions and methods described herein include minibodies, Fab fragments, F(ab’)2 fragments, dsFv and scFv fragments.

[0085] In some embodiments, the T cell engaging immune effector domain comprises:

- (i) a VL region comprising CDRs of SEQ ID NO: 33, 34, and 35 as CDR1, CDR2 and CDR3 respectively; and
- (ii) a VH region comprising CDRs of SEQ ID NO: 36, 37, and 38 as CDR1, CDR2 and CDR3 respectively.

[0086] In some embodiments, the T cell engaging immune effector domain may comprise:

- (i) a VL region comprising CDRs of SEQ ID NO: 33, 34, and 35 as CDR1, CDR2 and CDR3 respectively; and
- (ii) a VH region comprising CDRs of SEQ ID NO: 48, 37, and 38 as CDR1, CDR2 and CDR3 respectively.

[0087] In some embodiments, the VL and VH CDR sequences above may each optionally have one, two, three, or four amino acid substitutions relative to the sequences recited above.

[0088] In some embodiments, the TCE-VL may comprise CDRs that are at least 90%, at least 95%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 33, 34, and 35 as CDR1, CDR2 and CDR3 and/or the TCEVH may comprise CDRs that are at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 36, 37, and 38 as CDR1, CDR2 and CDR3 respectively.

[0089] In some embodiments, the TCE-VL may comprise CDRs that are at least 90%, at least 95%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 33, 34, and 35 as CDR1, CDR2 and CDR3 and/or the TCE-VH may comprise CDRs that are at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 48, 37, and 38 as CDR1, CDR2 and CDR3 respectively.

[0090] In some embodiments, the TCE-VL may comprise, or consist of, an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 31 and the TCE-VH may comprise, or consist of, an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 32. The TCE-VL may comprise, or consist of, an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 31 and the TCE-VH may comprise, or consist of, an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 32.

[0091] In some embodiments, the TCE-VL comprises, or consists of, the amino acid sequence provided in SEQ ID NO: 31 and the TCE-VH comprises, or consists of, the amino acid sequence provided in SEQ ID NO: 32. In some embodiments, the TCE-VL comprises, or consists of, an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 31 and the TCE-VH comprises, or consists of, an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 41. In some embodiments, the TCE-VL may comprise, or consist of, an amino acid sequence that is at least 90%, at least 95%, or at

least 98% identical to the sequence of SEQ ID NO: 31 and the TCE-VH may comprise, or consist of, an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 41. For example, the TCE-VL may comprise, or consist of, the amino acid sequence provided in SEQ ID NO: 31 and the TCE-VH may comprise, or consist of, the amino acid sequence provided in SEQ ID NO: 41.

[0092] As described above, the T cell engaging immune effector domain may be an scFv. The T cell engaging immune effector domain may be an scFv comprising an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 17 or 40. In some embodiments, the scFv may comprise an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 17 or 40. Preferably, the scFv comprises the 30 amino acid sequence provided in SEQ ID NO: 17. Alternatively, the scFv may comprise the amino acid sequence provided in SEQ ID NO: 40.

iii. Half-life extending domains

[0093] A “half-life extending domain”, as used herein, refers to a protein domain for extending the half-life of the multi-domain binding protein, relative to a multi-domain binding protein lacking the half-life extending domain. The half-life extending domain comprises a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain. As used herein, the term “Fc region” is used to refer to a region of a single polypeptide chain comprising at least a CH2 domain and a CH3 domain sequence, whereas the term “Fc domain” refers to a dimer of two Fc regions (i.e., FC1 and FC2).

[0094] WO 2020/157211 describes an approach for extending the half-life of a TCR-anti-CD3 fusion protein by fusing it to an IgG Fc domain. The present inventors have surprisingly found that the multi-domain binding molecules of the disclosure retain the extended half-life provided by the Fc domain in the format disclosed in WO 2020/157211, but, in addition, have significantly higher potency.

[0095] In some embodiments, the immunoglobulin Fc domain may be any antibody Fc domain. The Fc domain is the tail region of an antibody that interacts with cell surface Fc receptors and some proteins of the complement system. The Fc domain comprises two polypeptide chains (i.e., two Fc “regions”) both having two or three heavy chain constant domains (termed CH2, CH3 and CH4), and optionally a hinge region. The two Fc region chains may be linked by one or more disulfide bonds within the hinge region. Fc domains

from immunoglobulin subclasses IgG1, IgG2 and IgG4 bind to and undergo FcRn mediated recycling, affording a long circulatory half-life (3 – 4 weeks), thus extending the half-life of the multidomain binding molecule of the disclosure. The interaction of IgG with FcRn has been localized in the Fc region covering parts of the CH2 and CH3 domains. Preferred immunoglobulin Fc domains for use in the present disclosure include but are not limited to Fc domains from IgG1 or IgG4. For example, the Fc domain may be an IgG1 Fc domain, i.e., the FC1 and FC2 regions can be IgG1 Fc regions. The Fc domain may be derived from human sequences.

[0096] In some embodiments, the FC1 region may comprise, or consist of, an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 42 and the FC2 region may comprise, or consist of, an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 43. In some embodiments, the FC1 region may comprise, or consist of, an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 42 and the FC2 region may comprise an amino acid sequence that is at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO: 43. Preferably, the FC1 region comprises the amino acid sequence provided in SEQ ID NO: 42 and the FC2 region comprises the amino acid sequence provided in SEQ ID NO: 43. As the skilled person would appreciate, the sequences provided above for FC1 and FC2 are suitable vice versa. For example, the FC1 region may comprise the amino acid sequence provided in SEQ ID NO: 43 and the FC2 region may comprise the amino acid sequence provided in SEQ ID NO: 42.

[0097] In some embodiments, the disclosure provides a method as described herein, wherein the TCE-pMHC comprises the following amino acid sequences, in the following order, from N-terminus to C-terminus: (a) an amino acid sequence of an anti-CD3 scFv, optionally followed by a linker sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 18; (b) an amino acid sequence of a TCR β variable and constant region; (c) a linker sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 47 followed by an IgG hinge sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 44; (d) an Fc region having the sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 42; (e) a linker sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 47; (f) an amino acid sequence of a TCR α variable and constant region; (g) a linker sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 47 followed by an IgG hinge sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 44; and (h) an Fc region having the

sequence at least 80%, at least 90%, or at least 95% identical to SEQ ID NO: 43.

[0098] In some embodiments, the disclosure provides a method as described herein, wherein the TCE-pMHC comprises the following amino acid sequences, in the following order, from N-terminus to C-terminus: (a) an amino acid sequence of an anti-CD3 scFv, optionally followed by a linker sequence provided in SEQ ID NO: 18; (b) an amino acid sequence of a TCR β variable and constant region; (c) a linker sequence provided in SEQ ID NO: 47 followed by an IgG hinge sequence provided in SEQ ID NO: 44; (d) an Fc region having the sequence provided in SEQ ID NO: 42; (e) a linker sequence provided in SEQ ID NO: 47; (f) an amino acid sequence of a TCR α variable and constant region; (g) a linker sequence provided in SEQ ID NO: 47 followed by an IgG hinge sequence provided in SEQ ID NO: 44; and (h) an Fc region having the sequence provided in SEQ ID NO: 43.

[0099] In some embodiments, the Fc regions may comprise mutations relative to a wild-type or unmodified Fc sequence. Mutations include substitutions, insertions and deletions. Such mutations may be made for the purpose of introducing desirable therapeutic properties. For example, to facilitate hetero-dimerization, knobs into holes (KiH) mutations may be engineered into the CH3 domain. Thus, the half-life extending domain may comprise one or more amino acid substitutions which facilitate dimerisation of the FC1 region and the FC2 region. Such substitutions include “Knob-in-hole” substitutions. In this case, one chain (i.e. one of the FC 1 or FC2 regions) is engineered to contain a bulky protruding residue (i.e. the knob), such as Y, and the other chain (i.e., the other of the FC1 and FC2 regions) is engineered to contain a complementary pocket (i.e. the hole). For example, a knob may be constructed by replacing a small amino acid side chain with a larger side chain. A hole may be constructed by replacing a large amino acid side chain with a smaller side chain. Without wishing to be bound to theory, this is thought to stabilize a hetero-dimer of the FC1 and FC2 regions by favouring formation of the hetero-dimer over other species, for example homomultimers of FC1 and FC2, thereby enhancing the stability and manufacturability of the multi-domain binding molecule of the disclosure.

[0100] Suitable positions and substitutions for KiH mutations, and other mutations for facilitating dimerization of Fc regions, are known in the art. For example, the substitutions forming corresponding knobs and holes in two Fc regions may correspond to one or more pairs provided in the following table:

CH3 of one of the FC1 and FC2 regions	CH3 of the other of the FC1 and FC2 regions
T366Y	Y407T
T366W	Y407A
T366W	T366S:L368A:Y407V
F405A	T394W
Y407T	T366Y
T366Y:F405A	T394W:Y407T
T366W:F405W	T394S:Y 407A
F405W:Y407A	T366W:T394S
F405W	T394S

[0101] The substitutions in the table above are denoted by the original residue, followed by the position using the EU numbering system, and then the import residue (all residues are given in single-letter amino acid code). Multiple substitutions are separated by a colon.

[0102] In some embodiments, the FC1 and FC2 regions may comprise one or more substitutions in the table above. For example:

- (i) one of the FC1 region and the FC2 region may comprise one or more amino acid substitutions selected from the group consisting of T366S, L368A, T394S, F405A, Y 407 A, Y 407T and Y407V, according to the EU numbering scheme; and
- (ii) the other of the FC 1 region and the FC2 region may comprise one or more amino acid substitutions selected from the group consisting of T366W, T366Y, T366W, T394W and F405W according to the EU numbering scheme. The substitutions in (i) and (ii) are hole-forming and knob-forming substitutions respectively. The FC1 region may comprise one or more of the substitutions in (i) and the FC2 region may comprise one or more of the substitutions in (ii).

[0103] For example:

- (i) one of the FC1 region and the FC2 region may comprise one or more amino acid substitutions selected from the group consisting of T366S, L368A, and Y 407V, according to the EU numbering scheme; and
- (ii) the other of the FC 1 region and the FC2 region may comprise a T366W amino acid substitution, according to the EU numbering scheme. The FC1 region may comprise

one or more of the substitutions in (i) and the FC2 region may comprise the substitution in (ii).

[0104] In some embodiments, (i) one of the FC1 region and the FC2 region comprises T366S, L368A, and Y407V amino acid substitutions, according to the EU numbering scheme; and (ii) the other of the FC1 region and the FC2 region comprises a T366W amino acid substitution, according to the EU numbering scheme. For example, the FC1 region may comprise T366S, L368A, and Y407V amino acid substitutions, according to the EU numbering scheme; and the FC2 region may comprise a T366W amino acid substitution, according to the EU numbering scheme.

[0105] In some embodiments, the Fc domain may also comprise one or more mutations that attenuate an effector function of the Fc domain. Exemplary effector functions include, without limitation, complement-dependent cytotoxicity (CDC) and/or antibody-dependent cellular cytotoxicity (ADCC). The modification to attenuate effector function may be a modification that alters the glycosylation pattern of the Fc domain, e.g., a modification that results in an aglycosylated Fc domain. Alternatively, the modification to attenuate effector function may be a modification that does not alter the glycosylation pattern of the Fc domain. The modification to attenuate effector function may reduce or eliminate binding to human effector cells, binding to one or more Fc receptors, and/or binding to cells expressing an Fc receptor. For example, the half-life extending domain may comprise one or more amino acid substitutions selected from the group consisting of S228P, E233P, L234A, L235A, L235E, L235P, G236R, G237A, P238S, F241A, V264A, D265A, H268A, D270A, N297A, N297G, N297Q, E318A, K322A, L328R, P329G, P329A, A330S, A330L, P331A and P331S, according to the EU numbering scheme. Particular modifications include a N297G or N297 A substitution in the Fc region of human IgG1 (EU numbering). Other suitable modifications include L234A, L235A and P329G substitutions in the Fc region of human IgG1 (EU numbering), that result in attenuated effector function. In some embodiments, the Fc regions in the multidomain binding molecule of the disclosure may comprise a substitution at residue N297, numbering according to EU index. For example, the substitution may be an N297G or N297 A substitution. Other suitable mutations (e.g., at residue N297) are known to those skilled in the art.

[0106] In some embodiments, Fc variants having reduced effector function refers to Fc variants that reduce effector function (e.g., CDC, ADCC, and/or binding to FcR, etc. activities) by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98%,

99% or more as compared to the effector function achieved by a wildtype Fc region (e.g., an Fc region not having a mutation to reduce effector function, although it may have other mutations). In some embodiments, the Fc variants having reduced effector function may be Fc variants that eliminate all detectable effector function as compared to a wild-type Fc region. Assays for measuring effector function are known in the art and described below.

[0107] In vitro and/or in vivo cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the Fc region or fusion protein lacks FcγR binding (hence likely lacking ADCC activity) but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples of in vitro assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, e.g., Hellstrom, I. et al. *Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I et al., *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)).

[0108] In some embodiments, substitutions may be introduced into the FC1 and FC2 regions that abrogate or reduce binding to Fcγ receptors and/or to increase binding to FcRn, and/or prevent Fab arm exchange, and/or remove protease sites. In this regard, the half-life extending domain may also comprise one or more amino acid substitutions which prevent or reduce binding to activating receptors. In some embodiments, the half-life extending domain may comprise one or more amino acid substitutions which prevent or reduce binding to FcγR. For example, the FC1 region and/or the FC2 region may comprise a N297G amino acid substitution, according to the EU numbering scheme. Both the FC1 region and the FC2 region may comprise the N297G amino acid substitution.

[0109] In some embodiments, the half-life extending domain may comprise one or more amino acid substitutions which promote binding to FcRn. Methods of measuring binding to FcRn are known to the skilled artisan. Binding to FcRn in vivo and serum half-life of human FcRn high-affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides having a variant Fc region are administered.

[0110] In some embodiments, mutations may be made for manufacturing reasons, for

example to remove or replace amino acids that may be subject to post-translational modifications such as glycosylation, as described herein. In some embodiments, the immunoglobulin Fc may be fused to the other domains (i.e., VC1 or VC2) in the molecule of the disclosure via a linker, and/or a hinge sequence as described herein. Alternatively no linker may be used.

[0111] In some embodiments, the two Fc regions in the molecule of the disclosure may comprise CH2 and CH3 constant domains and all or part of a hinge sequence. In some embodiments, the hinge sequence may correspond substantially or partially to a hinge region from IgG1, IgG2, IgG3 or IgG4. The hinge sequence may be an IgG1 hinge sequence, such as the amino acid sequence provided in SEQ ID NO: 44. In some embodiments the hinge may comprise all or part of a core hinge domain and all or part of a lower hinge region.

[0112] In some embodiments, the TCE-pMHC molecule composition is described in US 63/371,863, which is incorporated herein by reference in its entirety.

[0113] In some embodiments, the TCE-pMHC molecule comprises:

i) a peptide-major histocompatibility complex (pMHC) binding domain which binds to a SLLQHLIGL (SEQ ID NO: 1) HLA-A*02 complex, the pMHC binding domain comprising a first variable region linked to a constant region (VC1) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain;

ii) a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and

iii) a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain;

wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and

wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0114] As used herein, the term “antibody” includes, but is not limited to, genetically engineered or otherwise modified forms of immunoglobulins, such as intrabodies, chimeric

antibodies, fully human antibodies, humanized antibodies (e.g. generated by “CDR-grafting”), antibody fragments, and heteroconjugate antibodies (e.g., bispecific antibodies, diabodies, triabodies, tetra-bodies, etc.). The term “antibody” includes cys-diabodies and minibodies. Thus, each and every embodiment provided herein in regard to “antibodies”, or “antibody like constructs” is also envisioned as, bi-specific antibodies, diabodies, scFv fragments, chimeric antibody receptor (CAR) constructs, diabody and/or minibody embodiments, unless explicitly denoted otherwise. The term “antibody” includes a polypeptide of the immunoglobulin family or a polypeptide comprising fragments of an immunoglobulin that is capable of non-covalently, reversibly, and in a specific manner binding a corresponding antigen, as disclosed herein. An exemplary antibody structural unit comprises a tetramer. In some embodiments, a full-length antibody can be composed of two identical pairs of polypeptide chains, each pair having one “light” and one “heavy” chain (connected through a disulfide bond). The term “antibody” also comprises immunoglobulins (Ig’s) of different classes (i.e., IgA, IgG, IgM, IgD and IgE) and subclasses (such as IgG1, IgG2 etc.).

[0115] The terms “anti-CD3 antibody” and “anti-CD3 antibody fragment,” as used herein, mean antibodies or antibody fragments which recognize or bind to CD3. The TCR-anti-CD3 fusion molecule for use in the present disclosure can include one or more conservative substitutions which have a similar amino acid sequence and/or which retain the same function (i.e. are phenotypically silent). The skilled person is aware that various amino acids have similar properties and thus substitutions between them are “conservative”. One or more such amino acids of a protein, polypeptide or peptide can often be substituted by one or more other such amino acids without eliminating a desired activity of that protein, polypeptide, or peptide. A particularly preferred immune effector is an anti-CD3 antibody, or a functional fragment or variant of said anti-CD3 antibody. As used herein, the term “antibody” encompasses such fragments and variants. Examples of anti-CD3 antibodies include but are not limited to OKT3, UCHT-1, BMA-031 and 12F6. Antibody fragments and variants/analogues which are suitable for use in the compositions and methods described herein include minibodies, Fab fragments, F(ab’)z fragments, dsFv and scFv fragments, Nanobodies™ (these constructs, marketed by Ablynx (Belgium), comprise synthetic single immunoglobulin variable heavy domain derived from a camelid (e.g. camel or llama) antibody) and Domain Antibodies (Domantis (Belgium), comprising an affinity matured single immunoglobulin variable heavy domain or immunoglobulin variable light domain) or alternative protein scaffolds that exhibit antibody like binding characteristics

such as Affibodies (Affibody (Sweden), comprising engineered protein A scaffold) or Anticalins (Pieris (Germany)), comprising engineered anticalins) to name but a few the immune effector is linked to the TCR portion of the multi-domain antigen binding polypeptide, where preferably the immune effector is an anti-CD3 antibody.

[0116] In some embodiments, the TCE-pMHC molecule comprises:

i) a soluble TCR comprising a first variable region linked to a constant region (VC 1) and a second variable region linked to a constant region (VC2), wherein VC1 comprises a TCR $\alpha\beta$ variable and constant region having the amino acid sequence provided in SEQ ID NO: 16, or a sequence that is at least 90%, at least 95%, or at least 98% identical thereto, and VC2 comprises a TCR α variable and constant region having the amino acid sequence provided in SEQ ID NO: 14, or a sequence that is at least 90%, at least 95%, or at least 98% identical thereto;

ii) an anti-CD3 scFv comprising an antibody light chain variable region (TCE-VL) having the amino acid sequence provided in SEQ ID NO: 31, or a sequence that is at least 90%, at least 95%, or at least 98% identical thereto, and an antibody heavy chain variable region (TCE-VH) having the amino acid sequence provided in SEQ ID NO: 32, or a sequence that is at least 90%, at least 95%, or at least 98% identical thereto; and

iii) a half-life extending domain comprising a first IgG Fc region (FC1) having the amino acid sequence provided in SEQ ID NO: 42, or a sequence that is at least 90%, at least 95%, or at least 98% identical thereto, and a second IgG Fc region (FC2) having the amino acid sequence provided in SEQ ID NO: 43, or a sequence that is at least 90%, at least 95%, or at least 98% identical thereto, wherein the FC1 region and FC2 region dimerise to form an Fc domain;

wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and

wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0117] In some embodiments, the TCE-pMHC molecule comprises the amino acid sequence provided in SEQ ID NO: 45, also known as IMC-P115C.

[0118] In some embodiments, the method comprises a method of making the TCE-pMHC molecule, comprising maintaining the host cell described above under optimal conditions

for expression of the nucleic acid and isolating the multi-domain binding molecule. In some embodiments, the method comprises culturing a cell comprising a nucleic acid, wherein the nucleic acid encodes the TCE-pMHC molecule. In some embodiments, the method comprises an expression vector comprising the nucleic acid encoding the TCE-pMHC molecule. In some embodiments, the method comprises culturing a host cell comprising the nucleic acid or the vector of this aspect, and then expressing the TCE-pMHC molecule in the cell and secreting it from the cell into the host cell culture supernatant.

Method of making the TCE-pMHC molecule

[0119] In some embodiments, the disclosure provides a nucleic acid, and/or a cell comprising a nucleic acid, encoding the TCE-pMHC molecule of the disclosure, which can be used to express the TCE-pMHC, and then the methods as described herein can be used to purify the TCE-pMHC from the host cell. The nucleic acid may be cDNA. The nucleic acid may be mRNA. The nucleic acid may be non-naturally occurring and/or purified and/or engineered. The nucleic acid sequence may be codon optimized, in accordance with the expression system utilized. As is known to those skilled in the art, expression systems may include bacterial cells such as *E. coli*, or yeast cells, or mammalian cells, or insect cells, or they may be cell free expression systems. Thus, in some embodiments, the methods described herein provide for expressing the TCE-pMHC molecule in a host cell, wherein the TCE-pMHC molecule is secreted into a host cell culture supernatant before the isolating the supernatant of the host cell culture comprising the TCE-pMHC molecule. In some embodiments, the host cell for expressing TCE-pMHC is a mammalian cell.

[0120] The present disclosure also provides constructs in the form of plasmids, vectors, transcription or expression cassettes which comprise at least one nucleic acid as described above. The present disclosure also provides a recombinant host cell which comprises one or more constructs as above. As mentioned, a nucleic acid encoding a specific binding molecule of the disclosure forms an aspect of the present disclosure, as does a method of production of the specific binding molecule comprising expression from a nucleic acid encoding a specific binding molecule of the disclosure. Expression may conveniently be achieved by culturing recombinant host cells containing the nucleic acid under appropriate conditions. Following production by expression, a specific binding molecule may be isolated and/or purified using any suitable technique, then used as appropriate.

[0121] Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is *E. coli*. The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Plückthun, *Bio/Technology* 9:545-551 (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a specific binding molecule, see for recent review, for example Reff, *Curr. Opinion Biotech.* 4:573-576 (1993); Trill et al., *Curr. Opinion Biotech.* 6:553-560 (1995).

[0122] Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be any suitable vectors known in the art, including plasmids or viral vectors (e.g. 'phage, or phagemid), as appropriate. For further details see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual: 2nd Edition*, Cold Spring Harbor Laboratory Press (1989). Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Ausubel et al. eds., *Short Protocols in Molecular Biology*, 2nd Edition, John Wiley & Sons (1992).

[0123] The present disclosure also provides a host cell containing a nucleic acid encoding the TCE-pMHC as disclosed herein. Further, the disclosure provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene.

[0124] Suitable host cells for cloning or expression of polynucleotides and/or vectors of the present disclosure are known in the art. Suitable host cells for the expression of

(glycosylated) proteins are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures can also be utilized as hosts. See, e.g., US 5,959,177, US 6,040,498, US 6,420,548, US 7,125,978, and US 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants). Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293T cells as described, e.g., in Graham, F.L. et al., *J. Gen Virol.* 36 (1977) 59-74); baby hamster kidney cells (BHK); mouse Sertoli cells (TM4 cells as described, e.g., in Mather, J.P., *Biol. Reprod.* 23 (1980) 243-252); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MOCK); buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells (as described, e.g., in Mather, J.P. et al., *Annals N.Y. Acad. Sci.* 383 (1982) 44-68); MRC 5 cells; and FS4 cells. other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR- CHO cells (Urlaub, G. et al., *Proc. Natl. Acad. Sci. USA* 77 (1980) 4216-4220); and myeloma cell lines such as YO, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for protein production, see, e.g., Yazaki, P. and Wu, A.M., *Methods in Molecular Biology*, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2004), pp. 255-268. The host cell may be eukaryotic, e.g., a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., YO, NS0, Sp20 cell).

[0125] In some embodiments, the nucleic acid of the disclosure may be integrated into the genome (e.g. chromosome) of the host cell. In some embodiments, integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

[0126] Further provided herein are methods for making the multi-domain binding molecule described herein. The methods comprise maintaining the host cell of the disclosure under optimal conditions for expression of the nucleic acid or expression vector of the disclosure and isolating the multi-domain binding molecule.

[0127] Methods of producing recombinant proteins such as TCE-pMHC molecules are well known in the art. Nucleic acids encoding the protein can be cloned into expression

constructs or vectors, which are then transfected into host cells, such as E.coli cells, yeast cells, insect cells, or mammalian cells, such as simian COS cells, Chinese Hamster Ovary (CHO) cells, human embryonic kidney (HEK) cells, or myeloma cells that do not otherwise produce the protein. Exemplary mammalian cells used for expressing a protein are CHO cells, myeloma cells or HEK cells. Molecular cloning techniques to achieve these ends are known in the art and described, for example in Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) or Sambrook et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989). A wide variety of cloning and in vitro amplification methods are suitable for the construction of recombinant nucleic acids. Methods of producing recombinant antibodies are also known in the art, see, e.g., US4816567 or US5530101.

[0128] In some embodiments, the nucleic acid encoding the TCE-pMHC molecule may be inserted operably linked to a promoter in an expression construct or expression vector for further cloning (amplification of the DNA) or for expression in a cell-free system or in cells. As used herein, the term "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a genomic gene, including the TATA box or initiator element, which is required for accurate transcription initiation, with or without additional regulatory elements (e.g., upstream activating sequences, transcription factor binding sites, enhancers and silencers) that alter expression of a nucleic acid, e.g., in response to a developmental and/or external stimulus, or in a tissue specific manner. In the present context, the term "promoter" is also used to describe a recombinant, synthetic or fusion nucleic acid, or derivative which confers, activates or enhances the expression of a nucleic acid to which it is operably linked. Exemplary promoters can contain additional copies of one or more specific regulatory elements to further enhance expression and/or alter the spatial expression and/or temporal expression of said nucleic acid. As used herein, the term "operably linked to" means positioning a promoter relative to a nucleic acid such that expression of the nucleic acid is controlled by the promoter.

[0129] Many vectors for expression of a protein in cells, e.g., the TCE-pMHC molecules, are commercially available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, a sequence encoding a protein (e.g., derived from the information provided herein), an enhancer element, a promoter, and a transcription termination sequence. The skilled person will be aware of suitable sequences for expression of a protein. Exemplary signal sequences include prokaryotic secretion

signals (e.g., pe1 B, alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II), yeast secretion signals (e.g., invertase leader, a factor leader, or acid phosphatase leader) or mammalian secretion signals (e.g., herpes simplex gD signal).

[0130] Exemplary promoters active in mammalian cells include cytomegalovirus immediate early promoter (CMV-IE), human elongation factor 1- α promoter (EF1), small nuclear RNA promoters (U1a and U1b), α -myosin heavy chain promoter, Simian virus 40 promoter (SV40), Rous sarcoma virus promoter (RSV), Adenovirus major late promoter, β -actin promoter; hybrid regulatory element comprising a CMV enhancer/ β -actin promoter or an immunoglobulin promoter or an active fragment thereof. 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); baby hamster kidney cells (BHK, ATCC CCL 10); or Chinese hamster ovary cells (CHO).

[0131] Typical promoters suitable for expression in yeast cells such as for example a yeast cell selected from the group comprising *Pichia pastoris*, *Saccharomyces cerevisiae* and *S. pombe*, include, but are not limited to, the ADHI promoter, the GAL 1 promoter, the GALA promoter, the CUP1 promoter, the PH0S promoter, the nmt promoter, the RPR 1 promoter, or the TEF1 promoter.

[0132] The host cells used to produce the protein may be cultured in a variety of media, depending on the cell type used. 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 mammalian cells. Media for culturing other cell types discussed herein are known in the art.

Methods of isolating

[0133] The disclosure provides a method for purifying a TCE-pMHC molecule produced by a host cell. In some embodiments, the described methods, and obvious variations thereof, can result in lower than expected impurities (e.g., protein, oligonucleotide, lipid, etc., impurities) from the media and or host cells. In some embodiments, the described methods, and obvious variations thereof, can result in a higher-than-expected yield of TCE-pMHC. In some embodiments, the described methods, and obvious variations thereof, can result in a reduction in high molecular weight aggregates of TCE-pMHC. In some embodiments, the described methods, and obvious variations thereof, provide for

purification of TCE-pMHC using a lower pH, resulting in less degradation and/or aggregation. In some embodiments, the described methods, and obvious variations thereof, the product comprising the TCE-pMHC can be in a lower buffer and/or salt concentration that previously performed, resulting in lower conductivity which can aid in processing of one or more steps, e.g., the anion exchange chromatography. In some embodiments, the described methods, and obvious variations thereof, can result in a more efficient, e.g., time efficient, labor efficient, and/or cost efficient, process which can be especially important when performed on a commercial scale. In some embodiments, the described methods, and obvious variations thereof, can result in reduced concentrations of product-related impurities.

[0134] The methods described herein include a multi-step process, wherein each step can be performed in the order presented, or alternatively, each step can be performed in a different order than is presented. One of skill in the art will understand and recognize that additional steps, e.g., purification steps, can be performed before, between, and after the specified purification steps as outlined herein. Solely for clarity, e.g., the disclosure provides that one or more steps can be included between any of the listed steps, e.g., one or more steps can be included between the “isolating” of step (a) and the “affinity chromatography” of step (b), etc.

[0135] The terms “isolate,” “isolated,” and “isolating” or “purify,” “purified,” and “purifying” or “separate,” “separated” and “separating” as well as “extracted” and “extracting” are used interchangeably and refer to the state of a preparation (e.g., a plurality of known or unknown amount and/or concentration) of the TCE-pMHC molecule, that have undergone one or more processes of purification, e.g., a selection or an enrichment of the desired TCE-pMHC molecule preparation. In some embodiments, isolating or purifying or separating as used herein is the process of removing, partially removing (e.g., a fraction) of the desired protein, e.g., TCE-pMHC molecule, from a sample containing host cells or host cell proteins, host cell lipids, host cells oligonucleotides, etc. In some embodiments, an isolated TCE-pMHC molecule has no detectable undesired activity or, alternatively, the level or amount of the undesired activity is at or below an acceptable level or amount as a result of the host cells or host cell proteins, host cell lipids, host cells oligonucleotides, etc. In some embodiments, an isolated protein composition, e.g., purified TCE-pMHC molecule, has an amount and/or concentration of purified TCE-pMHC molecule at or above an acceptable amount and/or concentration. In some embodiments, the isolated or purified or separated TCE-pMHC molecule is enriched as compared to the starting material (e.g.,

host cell preparations) from which the composition is obtained. This enrichment can be by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, 99.9%, 99.99%, 99.999%, 99.9999%, or greater than 99.9999% as compared to the starting material. In some embodiments, purified TCE-pMHC molecule resulting from the methods described herein are substantially free of residual biological products. In some aspects, the isolated or separated or purified TCE-pMHC molecule preparations are 100% free, 99% free, 98% free, 97% free, 96% free, 95% free, 94% free, 93% free, 92% free, 91% free, or 90% free of any residual biological products. Residual biological products can include media components, abiotic materials (including chemicals) or unwanted nucleic acids, proteins, lipids, or metabolites. Substantially free of residual biological products can also mean that the TCE-pMHC molecule composition contains no detectable impurity and that only the desired TCE-pMHC molecule is detectable.

[0136] General methods for isolating or separating a protein such as TCE-pMHC molecule are known in the art, however, selecting the order of process steps, as well as the specifics of each step, to most efficiently purify the TCE-pMHC molecule at a commercial scale while avoid aggregation, e.g., HMW-TCE-pMHC, was previously unknown. In some embodiments, when a protein is secreted into culture medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. In some embodiments, a protease inhibitor such as PMSF can be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants. In some embodiments, supernatants can be filtered and/or isolated from cells expressing the protein, e.g., using continuous centrifugation.

[0137] In some embodiments, the TCE-pMHC molecule is expressed or produced by host cells in culture where the TCE-pMHC molecule is secreted into the host cell culture supernatant. The secreted TCE-pMHC is isolated from host cells by filtration, or by other methods known by one of ordinary skill in the art. In some embodiments, the secreted TCE-pMHC in the host cell culture supernatant is isolated from host cells by clarification methods known in the art, e.g., centrifugation or depth filtration.

[0138] The secreted TCE-pMHC molecule isolated from the host cell, i.e., "Product (a)" can be further purified using, for example, ion exchange, hydroxyapatite chromatography, hydrophobic interaction chromatography, gel electrophoresis, dialysis, affinity chromatography (e.g., protein A affinity chromatography or protein G chromatography), or

any combination of the foregoing. In one embodiment, Product (a) is further purified by subjecting the affinity chromatography, e.g., Protein A affinity chromatography.

Affinity chromatography

[0139] The isolated proteinaceous Product (a) can contain a complex mixture of modified and unmodified peptides and non-peptide sources, including but not limited to host cell proteins, host cell oligonucleotides (DNA/RNA), high molecular weight aggregates of proteins, product related impurities, or a combination thereof. In some embodiments, product-related impurities comprise fragment adduct impurities. In some embodiments, Product (a) is further purified by affinity chromatography.

[0140] Affinity chromatography methods are known in the art and are described, for example in WO99/57134 or Ed Harlow and David Lane (editors) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, (1988). The skilled person will also be aware that the TCE-pMHC molecule can be modified to include a tag to facilitate purification, e.g., a poly-histidine tag, a hexa-histidine tag, an influenza virus hemagglutinin (HA) tag, a Simian Virus 5 (VS) tag, a LLAG tag, or a glutathione S-transferase (GST) tag. The resulting protein is then purified using methods known in the art, such as, affinity purification. For example, a protein comprising a hexa-his tag is purified by contacting a sample comprising the protein with nickel-nitrilotriacetic acid (Ni-NTA) that specifically binds a hexa-his tag immobilized on a solid or semi-solid support, washing the sample to remove unbound protein, and subsequently eluting the bound protein. Alternatively, or in addition a ligand or antibody that binds to a tag is used in an affinity purification method.

[0141] When the TCE-pMHC molecule has been modified to include a tag, purification can be performed by contacting Product A with an immobilized, tag-specific antibody (e.g. anti-phosphothreonine) in order to isolate many, if not most, peptides containing the tag for which the immobilized antibody is specific. TCE-pMHC molecules with the appropriate tag bind to the immobilized antibody, while unmodified peptides and/or proteins/oligonucleotides/lipids do not. Antibodies may be immobilized by non-covalent attachment to protein A or protein G. When the complex mixture of peptides in Product (a) is contacted with the antibody-resin, in either batch or column format, the antibody-resin selectively binds the tagged TCE-pMHC molecule, even when they are present at low levels (i.e. picomole amounts).

[0142] In some embodiments, the TCE-pMHC molecule is not modified to include a tag to

facilitate purification or detection. In some embodiments, the TCE-pMHC molecule is further purified by using a molecule that preferentially binds to the TCE-pMHC molecule itself, e.g., Protein A and/or Protein G. In some embodiments, the TCE-pMHC molecule is further purified by using Protein A affinity chromatography. Protein A affinity chromatography is known in the art, a summary of which can be found, e.g., in Hober et al, "Protein A chromatography for antibody purification," *J. Chromatography* 848:40-47 (2007), incorporated by reference herein in its entirety. In some embodiments, the affinity chromatography is Protein G affinity chromatography.

[0143] In some embodiment, the affinity chromatography step comprises subjecting Product (a) to a column comprising a suitable affinity chromatographic support. Non-limiting examples of such chromatographic supports include, but are not limited to Protein A resin, Protein G resin, affinity supports comprising the antigen against which the antibody of interest was raised, and affinity supports comprising an Fc binding protein. Protein A resin is useful for affinity purification and isolation of antibodies (IgG). In some embodiments, a Protein A column is equilibrated with a suitable buffer prior to sample loading. An example of a suitable buffer is a Tris/NaCl buffer, pH around 7.2. Following this equilibration, the sample, i.e., homogenized cells, can be loaded onto the column.

[0144] In some embodiments, Product (a) is loaded onto the column at a total protein concentration of less than 10 mg/mL. In some embodiments, the disclosure provides a method wherein lower than normal amounts of total cell protein isolated from host cell supernatant, i.e., Product (a), is subjected to the affinity chromatography. For example, in some embodiments, Product (a) comprises the total cell protein of about 0.1 mg/mL to about 25 mg/mL. In some embodiments, Product (a) comprises the total cell protein of about 2 mg/mL to about 10 mg/mL. In some embodiments, Product (a) is about 1 mg/mL to about 10 mg/mL. In some embodiments, Product (a) is less than 1mg/mL. In some embodiments Product (a) is about 2 mg/mL to about 10 mg/mL, about 3 mg/mL to about 9 mg/mL, about 4 mg/mL to about 5 mg/mL or about 1 to about 3.5 mg/mL. In some embodiments, Product (a) is about 3 mg/mL. In some embodiments, Product (a) is about 5 mg/mL to about 10 mg/mL. In some embodiments, Product (a) is about 10 mg/mL. Product (a) can be 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL. In some embodiments, Product (a) is 3 mg/mL, 5 mg/mL or 10 mg/mL. In some embodiments, Product (a) is 3 mg/mL. In some embodiments, Product (a) is 5 mg/mL.

[0145] Following the loading of the column, the column can be washed one or multiple times using, e.g., the equilibrating buffer. Other washes including washes employing different buffers can be used before eluting the column.

[0146] The TCE-pMHC molecule can be eluted from the Protein A chromatographic column/matrix using an appropriate elution buffer. An example of a suitable elution buffer is sodium acetate, e.g., at a pH around 3.5, but other elution buffers are known in the art. Elution can occur at various pH levels. In some embodiments, the TCE-pMHC molecule is eluted at a pH of about 3.0 to about 4.0 to form Product (b). In some embodiments, the TCE-pMHC molecule is eluted from Protein A at a pH of about 3.0 to about 4.0 to form Product (b). In some embodiments, the TCE-pMHC molecule is eluted at a pH of about 3.1 to 3.9, about 3.2 to about 4.0, about 3.2 to about 3.8 or about 3.3 to about 3.7. In some embodiments, the TCE-pMHC molecule is eluted at a pH of about 3.4, about 3.5, or about 3.6.

[0147] The eluate can be monitored using techniques well known to those skilled in the art. For example, the absorbance at OD280 can be followed. The eluted fraction(s) of interest can then be prepared for further processing.

[0148] In some embodiments, Product (a) is eluted with an elution buffer having a concentration less than 100mM to form Product (b). In some embodiments, the elution buffer has a concentration of about 10 mM and about 100 mM, about 10 mM to about 90 mM, about 20 mM to about 80 mM, about 25 mM to about 75 mM, about 30 mM to about 70 mM, about 40 mM to about 60 mM, or about 50 mM to about 60 mM. In some embodiments, the elution buffer has a concentration of about 45 mM to about 95 mM, about 45 mM to about 80 mM, about 45 mM to about 70 mM, about 45 mM to about 65 mM, or about 50 mM to about 65 mM.

[0149] In some embodiments, Product (a) is eluted with sodium acetate at a concentration less than 100mM to form Product (b). In some embodiments, the sodium acetate has a concentration of about 10 mM and about 100 mM, about 10 mM to about 90 mM, about 20 mM to about 80 mM, about 25 mM to about 75 mM, about 30 mM to about 70 mM, about 40 mM to about 60 mM, or about 50 mM to about 60 mM. In some embodiments, the sodium acetate has at a concentration of about 45 mM to about 95 mM, about 45 mM to about 80 mM, about 45 mM to about 70 mM, about 45 mM to about 65 mM, or about 50 mM to about 65 mM.

i. *Neutralization of Product (b)*

[0150] In some embodiments, Product (b) resulting from the elution of from the affinity chromatography can have a low pH, e.g., a pH below 5, below 4 or below 3.6. In some embodiments, Product (b) resulting from the elution of from the affinity chromatography can have a pH of about 3 to about 4, about 3.2 to about 3.8 or about 3.4 to about 3.6.

[0151] The pH of Product (b) can be adjusted to a more neutral or basic pH. In some embodiments, the Product (b) is further subjected to subsequent chromatographic steps such as ion exchange and hydroxyapatite chromatography.

[0152] In some embodiments, Product (b) resulting from affinity chromatography is prepared or pre-treated for ion exchange by adjusting the pH and ionic strength, or conductivity, of the sample buffer to form Product (c). For example, Product (b) can be adjusted to a pH of about 5.0 to about 7.0 using an MES buffer to form Product (c). In some embodiments, the MES buffer used to form Product (c) is less than 5 M MES. In some embodiments, Product (b) is neutralized with a buffer comprising about 0.1 M to about 5M, about 0.5 M to about 3 M, about 0.5 M to about 2 M, or about 0.5 M to about 1.5 M MES to form Product (c). In some embodiments, Product (b) is neutralized with a buffer comprising about 0.5 M, about 0.8 M, about 1 M, about 1.3 M, or about 1.5 M MES to form Product (c). In some embodiments, the MES buffer has a pH of less than 7.0, less than 6.8, less than 6.5 or less than 6.3. In some embodiments, the MES buffer has a pH of about 5.0 to about 7.0, about 5.5 to about 6.8, or about 6 to about 6.5.

[0153] In some embodiments, Product (b) is neutralized to form Product (c), wherein Product (c) has a pH of about 5.0 to about 7.0, about 5.5 to about 6.8, about 5.5 to about 6.5, about 5.8 to about 6.8 about 6.0 to about 6.7, or about 6.2 to about 6.6. In some embodiments, Product (c) has a pH of about 6.4.

[0154] In some embodiments, Product (c) has a conductivity of 5 mS/cm or less. In some embodiments, Product (c) has a conductivity of about 0.1 mS/cm to about 5 mS/cm. In some embodiments, Product (c) has a conductivity of less than 1 mS/cm. In some embodiments Product (c) has a conductivity of about 2 mS/cm to about 5 mS/cm, about 3 mS/cm to about 4 mS/cm, about 1 mS/cm to about 2 mS/cm or about 1 to about 3.5 mS/cm. In some embodiments, Product (c) has a conductivity of about 3 mS/cm.

[0155] In some embodiments, Product (c) is diluted to form Diluted Product (c). In some

embodiments, Product (c) is diluted at least 2-fold after neutralization to form a Diluted Product (c), i.e., the volume of Product (c) is doubled. In some embodiments, Product (c) is diluted at least 1.5-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold or more after neutralization to form a Diluted Product (c). In some embodiments, Product (c) is about 1.5-fold to about 5-fold, about 2-fold to about 4 fold, or about 3-fold after neutralization to form a Diluted Product (c).

[0156] In some embodiments, Diluted Product (c) comprises MES buffer at a concentration of less than 20 mM, less than 15 mM, or less than 10 mM. In some embodiments, Diluted Product (c) comprises MES buffer at a concentration of about 2 mM to about 30 mM, about 5 mM to about 25 mM, or about 10 mM to about 20 mM.

[0157] In some embodiments, Diluted Product (c) has a conductivity of 5 mS/cm or less. In some embodiments, Diluted Product (c) has a conductivity of about 0.1 mS/cm to about 5 mS/cm. In some embodiments, Diluted Product (c) has a conductivity of less than 1 mS/cm. In some embodiments, Diluted Product (c) has a conductivity of about 2 mS/cm to about 5 mS/cm, about 3 mS/cm to about 4 mS/cm, about 1 mS/cm to about 2 mS/cm or about 1 to about 3.5 mS/cm. In some embodiments, Diluted Product (c) has a conductivity of about 3 mS/cm.

[0158] In some embodiments, the quantity and/or purity of the TCE-pMHC molecule in Product (a), Product (b), Product (c), or Diluted Product (c) can be analyzed using methods well known to those skilled in the art, e.g., size-exclusion chromatography, Poros™ A HPLC Assay, HCP ELISA, Protein A ELISA, and western blot analysis, or a combination thereof. In some embodiments, product purity is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance, mass spectrophotometry or a combination thereof.

ii. Anion exchange chromatography

[0159] In some embodiments, the disclosure provides subjecting Product (b), Product (c), or Diluted Product (c) to anion exchange chromatography. This step can be a single anion exchange procedure or can include multiple anion exchange steps. In some embodiments, the anion exchange step is a one-step procedure, e.g., a single anion exchange column.

[0160] Anion exchange chromatography can be used to further reduce impurities such as host cell proteins and oligonucleotides, e.g., host DNA and/or host RNA, from the TCE-

pMHC molecule using a positively charged matrix, e.g., a positively charged resin. Anion exchangers can be ion exchange resins (functionalized porous or gel polymer), zeolites, montmorillonite, clay, or even soil humus. Anion exchange chromatography can facilitate the isolation of TCE-pMHC molecule from impurities in Product (b), Product (c), or Diluted Product (c) according to its surface charge. This separation is dependent on the pI of the TCE-pMHC molecule, the pH and salt concentration of the buffer, and on the charge of the stationary anion exchange matrix. In some embodiments, the pI of the TCE-pMHC molecule is about 6.5 to about 7.0. In some embodiments, the pI of the TCE-pMHC molecule is about 6.7, 6.8, or 6.9. In some embodiments, TCE-pMHC molecule can be reversibly bound to a charged matrix of beaded cellulose, agarose, dextran, or polystyrene.

[0161] In some embodiments, the disclosure provides a method for purifying a TCE-pMHC molecule and/or methods for producing a process-related impurity and/or product-related substance-reduced protein preparation from a mixture comprising a protein of interest (i.e., TCE-pMHC molecule) and at least one process-related impurity and/or product-related substance by subjecting the mixture to at least one anion exchange separation step. In some embodiments, the anion exchange step will occur after the above-described Protein A affinity step.

[0162] In performing the separation, the initial protein mixture 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.

[0163] For example, in the context of batch purification, the anion exchange matrix is prepared in, or equilibrated to, the desired starting buffer. Upon preparation, or equilibration, a slurry of the anion exchange matrix is obtained. A composition comprising the protein of interest, e.g., TCE-pMHC molecule, is contacted with the slurry to allow for protein adsorption to the anion exchange material. The solution comprising the process-related impurities and/or product-related substances 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.

[0164] In the context of chromatographic separation, a chromatographic apparatus, commonly cylindrical in shape, is employed to contain the chromatographic support material (e.g., anion exchange matrix) 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 ≤ 30 cm is employed. Once the chromatographic material is added to the chromatographic apparatus, a sample containing the TCE-pMHC molecule, e.g., Product (b), Product (c), or Diluted Product (c), 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 anion exchange matrix being employed, the protein of interest, process-related impurities, and/or product-related substances, is separated from the chromatographic material by washing the matrix and collecting fractions from column. The anion exchange matrix can be subjected to one or more wash steps. If desired, the anion exchange matrix can then be contacted with a solution designed to desorb any components of the solution that have bound to the anion exchange matrix.

[0165] In some embodiments, a wash step can be performed in the context of anion exchange 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 some 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 TCE-pMHC molecule. In some embodiments the pH is about 0 to 2 units higher or lower than the pI of the TCE-pMHC molecule. In some embodiments, the pH will be in the range of 0 to 0.5 units higher or lower. In some embodiments, pH will be at the pI of the TCE-pMHC molecule.

[0166] A packed anion-exchange chromatography column, anion-exchange membrane device, anion-exchange monolithic device, or depth filter anion exchange media can be operated either in bind-elute mode, flow-through mode, or a hybrid mode wherein the TCE-pMHC molecule 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, e.g., TCE-pMHC molecule, will be immobilized on the resin-based matrix. For example, in some embodiments, during the feed load, the TCE-pMHC molecule 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 TCE-

pMHC molecule 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 TCE-pMHC molecule. 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 process-related impurities and product-related substances 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, process-related impurities and product-related substances will bind to the chromatographic material (or flow through) in a manner distinct from the TCE-pMHC molecule, while the TCE-pMHC molecule and certain aggregates and/or fragments of the protein of interest may bind the chromatographic material, washes that preferentially remove the TCE-pMHC molecule can be applied. The column is then regenerated before next use.

[0167] A suitable anion exchange matrix can include a matrix whose stationary phase comprises cationic groups. In some embodiments, the anion exchange chromatography comprises salt-tolerant anion exchange flowthrough chromatography. In some embodiments, the membrane comprises a cross-linked cellulose comprising a cationic group. In some embodiments, the cross-linked cellulose comprises a primary amine.

[0168] 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. In some embodiments, an example of such a column is a Q Sepharose™ column, or a Sartobind STIC-PA column.

[0169] In some embodiments, Product (b), Product (c) or Diluted Product (c) as disclosed herein, is further subjected to an anion exchange chromatography to form Product (d). In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to a QFF matrix. In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to a DEAE-Sepharose matrix. In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to a Sartobind STIC-PA matrix. In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to a Sartobind Q matrix. In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to a Cellex D matrix. In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to a DEAE-Sepharose matrix. In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to a quaternary aminoethyl (QAE) matrix. Numerous additional anion exchange matrices are known in the art. Thus, in some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to an anion exchange matrix not explicitly recited within this application, but is known to those of ordinary skill in the art.

[0170] In some embodiments, Product (b), Product (c) or Diluted Product (c) is subjected to anion exchange chromatography by using a Sartobind STIC-PA exchanger with a Sartobind STIC-PA membrane. In some embodiments, the membrane comprises a cross-linked cellulose. In some embodiments, the cross-linked cellulose comprises a primary amine. Salt-tolerant interaction chromatography (STIC) with primary amine (PA) ligand is based on anion-exchange chromatography (AEX) principles. AEX is the established method for removing process-derived contaminants such as host-cell proteins/DNA. Sartobind STIC PA membrane is composed of cross-linked, regenerated macroporous cellulose. The primary amine ligand is attached to the matrix at approximately six-fold higher ligand density than that of Sartobind Q membrane. In some aspects, both the free amine and the high ligand density are crucial for developing its high salt tolerance.

[0171] In some embodiments, the disclosure provides a method of intermediate polishing of the TCE-pMHC molecule before loading onto the Sartobind STIC PA membrane. In some embodiments, loading occurs at low conductivity instead of high conductivity. In some embodiments, pH adjustments and dilutions improve the binding capacity of impurities relative to pH adjustment alone. In some embodiments, the intermediate polishing steps increase the purity level of the flow-through product, Product (d).

[0172] In some embodiments, the flow through buffer used with the anion exchange chromatography comprises Tris, Piperazine, Diethylamine, Pyridine, L-Histidine, Bis-tris,

Bis-tris propane, Imidazole, N-Ethylmorpholine, Triethanol-amine (TEA), Morpholine, N-Methyl-diethanolamine, 2-amino-2-methyl-1,3-propanediol (AMPD), Diethanolamine, Ethanolamine, 2-amino-2-methyl-1-propanol, Piperazine, 1,3-Diaminopropane, Piperidine, MES buffer or a combination thereof. In some embodiments, the flow through buffer used with the anion exchange chromatography is an MES buffer.

[0173] Various concentrations of buffer can be used in the flow-through buffer. In some embodiments, the flow through buffer is less than 100mM. In some embodiments, the flow through buffer is about 25 mM to about 75 mM. In some embodiments, the flow through buffer is about 10 mM and about 100 mM, about 10 mM to about 90 mM, about 20 mM to about 80 mM, about 30 mM to about 100 mM, about 40 mM to about 100 mM, about 50 mM to about 90 mM, about 60 mM to about 80 mM, about 70 mM to about 80 mM, about 45 mM to about 95 mM, about 45 mM to about 80 mM, about 45 mM to about 70 mM, about 45 mM to about 65 mM, about 50 mM to about 65 mM, about 50 mM to about 60 mM, about 50 mM to about 55 mM, about 50 mM to about 55 mM, or about 51 mM to about 54 mM. In some embodiments, the flow through buffer is between about 50 mM to about 65 mM. In some embodiments, the flow through buffer is between about 50 mM to about 60 mM. In some embodiments, the flow through buffer is between about 50 mM to about 55 mM. In some embodiments, the flow through buffer is between about 50 mM to about 55 mM.

[0174] In some embodiments, the flow through buffer is about 10 mM and about 100 mM, about 10 mM to about 90 mM, about 20 mM to about 80 mM, about 30 mM to about 100 mM, about 40 mM to about 100 mM, about 50 mM to about 90 mM, about 60 mM to about 80 mM, about 70 mM to about 80 mM, about 45 mM to about 95 mM, about 45 mM to about 80 mM, about 45 mM to about 70 mM, about 45 mM to about 65 mM, about 50 mM to about 65 mM, about 50 mM to about 60 mM, about 50 mM to about 55 mM, about 50 mM to about 55 mM, or about 51 mM to about 54 mM of a MES buffer.

[0175] In some embodiments, the flow through from the anion exchange chromatography, Product (d), has a pH of about 6 to about 7. In some embodiments, Product (d) has a pH of about 6.1 to about 6.9, about 6.2 to about 6.8, or about 6.3 to about 6.7.

[0176] In some embodiments, the purity of the TCE-pMHC molecule in Product (d) can be analyzed using methods well known to those skilled in the art, e.g., size-exclusion chromatography, Poros™ A HPLC Assay, HCP ELISA, Protein A ELISA, and western blot

analysis, or a combination thereof. In some embodiments, the TCE-pMHC molecule purity is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance, mass spectrophotometry or a combination thereof.

v. Hydroxyapatite chromatography

[0177] In some embodiments, Product (d) as provided in the disclosure is further subjected to a second chromatography process to form Product (e). In some embodiments, Product (d) is subjected to a hydroxyapatite (HA) chromatography. HA is a calcium-phosphate complex $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, composed of two sites, C-site due to Ca and P-site due to PO_4 . The P-site works as a cation-exchange ligand whereas C-site has a metal affinity as well as an anion-exchange ligand function. At neutral pH regions basic proteins are retained on HAC based on the electrostatic interaction with P-site and eluted with NaCl as well as sodium phosphate. This is similar to cation exchange chromatography (CIEC). Acidic proteins are retained due to a strong interaction (metal affinity) with C-site, and are not eluted with NaCl, requiring sodium phosphate for their elution. Therefore, the elution or retention behavior is different from that for anion-exchange chromatography (AIEC).

[0178] In some embodiments, the hydroxyapatite can include hydrated hydroxyapatite gels such as Bio-Gel HT gel (suspended in sodium phosphate buffer), Bio-Gel HTP gel (a dried form of Bio-Gel HT), and DNA-grade Bio-Gel HTP (a dried form of Bio-Gel HT with a smaller particle size than Bio-Gel HTP), as well as ceramic hydroxyapatite (CHT). Ceramic hydroxyapatite (CHT), which is utilized in the examples herein, is a chemically pure form of hydroxyapatite that has been sintered at high temperatures. Ceramic hydroxyapatite is spherical in shape, with particle diameters ranging from about 10 microns to about 100 microns, and is typically available at nominal diameters of 20 microns, 40 microns, and 80 microns. Ceramic hydroxyapatite is macroporous, and is available in two types: Type I, with a medium porosity and a relatively high binding capacity, and Type II, with a larger porosity and a lower binding capacity. Either porosity can be used, and the optimal porosity for any particular protein separation or purification will vary with the proteins or the composition of the source mixture. Any of the forms of hydroxyapatite can be used alone, rather than in admixture with another separation medium or support, and can be used in a non-functionalized form, whether naturally-occurring or hydrated. In some embodiments, the hydroxyapatite chromatography comprises a ceramic hydroxyapatite. In some embodiments, the hydroxyapatite chromatography is a non-ceramic hydroxyapatite chromatography. In some embodiments, Product (d) as disclosed herein is subjected to

ceramic hydroxyapatite type II 40 μ m chromatography to form Product (c).

[0179] In some embodiments, calcium ion for inclusion in the elution buffers for the hydroxyapatite matrix described herein can be supplied by any calcium salt that is soluble in the elution buffer, which is typically an aqueous solution, and that is inert to the other components of the elution buffer, the hydroxyapatite resin, and the proteins retained on the resin, and in many cases also the remaining components of the source solution from which the proteins are sought to be extracted. Calcium halide salts are convenient to use, and calcium chloride is particularly convenient.

[0180] Phosphate ion for inclusion in the elution buffers for the hydroxyapatite matrix described herein can likewise be supplied from any phosphate salt that is soluble in the elution buffer, which is again typically aqueous, and that is inert to the other components of the buffer, the resin, the proteins, and the remaining components of the source solution. Alkali metal or alkaline earth metal phosphates are convenient, with sodium phosphate as an example.

[0181] In some embodiments, Product (d) (or Product d*) is normally loaded in low ionic strength phosphate buffer (1–10 mM sodium or potassium phosphate) at or near neutral pH. Higher loading concentrations can be advantageous (Figure 2). Elution is normally done with a gradient of phosphate buffer (100–400 mM sodium phosphate) of the same pH, but many different combinations are possible. Binding of basic proteins becomes stronger with reducing pH, due to increasing positive charge on the protein. In some embodiments, the lower the pH of the buffer the stronger the binding to the support is, and the higher the molarity of the sodium phosphate buffer required to desorb the protein will be. This can reflect the dominant cation exchange component of the interaction, but the selectivity is distinct from classical cation exchange. Concurrent repellence of amines by C-sites, and the geometric distribution of charges, impart a unique stereochemical element that sometimes endows hydroxyapatite chromatography with the ability to discriminate among closely related protein variants. Examples include fractionation of light chain idiotypes from monoclonal mixtures with common heavy chains and fractionation of bifunctional antibodies from complex parent/sibling mixtures.

[0182] In some embodiments, various TCE-pMHC molecules with various charges can be purified with hydroxyapatite chromatography, whether they are acidic, neutral, or basic. TCE-pMHC molecules that bind predominantly as acidic proteins can be applied to the

column in a sufficient concentration of sodium chloride to maintain their solubility during loading (Josic et al. 1991). Tolerance of high sodium chloride can allow dissociation of ionic complexes between TCE-pMHC molecules and acidic contaminants like DNA, thereby increasing purification performance and product binding capacity. High sodium chloride tolerance can allow TCE-pMHC molecules to be loaded with no equilibration other than pH titration. In some embodiments, binding of weakly interacting basic TCE-pMHC molecules can be strengthened by inclusion of 1 mM phosphate in the buffer. Free phosphate ions can pair with C-sites and suppress their ability to repel amines. Low concentration does not interfere with ionic binding between amines and P-sites. Basic proteins can be eluted with chloride or phosphate ions in a gradient from 50–500 mM. In some embodiments, elution can require displacers with stronger affinity for C-sites, such as phosphate, citrate, or fluoride ions. This has important ramifications, for example for TCE-pMHC molecules that behave as basic proteins. It means that elution can be achieved with sodium chloride, completely avoiding the risk of contamination from the bulk of acidic sample components. In some embodiments, solubility of a protein in a weak phosphate solution can be an issue, especially for some TCE-pMHC molecules. In some embodiments, a 10 mM phosphate loading buffer can be used.

[0183] As noted above, the optimal composition of the elution buffer may vary with the type of interaction by which the TCE-pMHC molecules bind to the hydroxyapatite. In some embodiments where the interaction is one of cation exchange, for example, the inclusion of sodium chloride, particularly at a high concentration such as one within the range of about 30 mM to about 2000 mM, can be beneficial. In some embodiments where the interaction is one involving the formation of a calcium coordination complex such as by chelation chemistry, a buffer with a low sodium chloride concentration, or in certain cases a buffer that is devoid of sodium chloride, can be used.

[0184] Elution buffers for use in purifying TCE-pMHC from high-molecular-weight aggregates in a HA chromatography matrix can include calcium ion at a concentration of about 50 ppm to about 225 ppm, phosphate ion at a concentration of about 5 mM to about 40 mM, and an alkali metal salt at a concentration of about 0.3M to about 1.5M. In some embodiments, the HA elution buffer can comprise about 50 ppm to about 100 ppm calcium ion, or about 0.4M to about 0.8M alkali metal salt. In some embodiments, the alkali metal salts are sodium and potassium salts, or alkali metal halides and nitrates. In some embodiments, sodium and potassium chloride are particularly preferred.

[0185] In some embodiments, the HA elution buffer comprises ethylenediamine tetraacetic acid (EDTA), succinate, citrate, aspartic acid, glutamic acid, maleate, cacodylate, 2-(N-morpholino)-ethanesulfonic acid (MES), N-(2-acetamido)-2-aminoethanesulfonic acid (ACES), piperazine-N,N'-2-ethanesulfonic acid (PIPES), 2-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO), N,N-bis-(hydroxyethyl)-2-aminoethanesulfonic acid (BES), 3-(N-morpholino)-propanesulfonic acid (MOPS), N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES), 3-(N-tris-(hydroxymethyl)methylamino)-2-hydroxypropanesulfonic acid (TAPSO), 3-(N,N-bis[2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid (DIPSO), N-(2-hydroxyethyl)piperazine-N'-(2-hydroxypropanesulfonic acid) (HEPPSO), 4-(2-hydroxyethyl)-1-piperazine propanesulfonic acid (EPPS), N-[tris(hydroxymethyl)-methyl]glycine (Tricine), N,N-bis(2-hydroxyethyl)glycine (Bicine), [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid (TAPS), N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO), tris(hydroxymethyl)aminomethane (Tris), and bis[2-hydroxyethyl]iminotris-[hydroxymethyl]methane (Bis-Tris) or a combination thereof. Other buffers known in the art may be used as well.

[0186] In some embodiments, the HA elution buffer comprises MES buffer. In some embodiments, the MES buffer is about 50 mM to about 200 mM. In some embodiments, the MES buffer is about 100 mM. In some embodiments, the MES buffer is about 50 mM to about 75 mM, about 60 mM to about 80 mM, about 75 mM to about 100 mM, about 90 mM to about 110 mM, about 100 mM to about 125 mM, about 120 mM to about 150 mM, about 130 to about 160 mM, about 150 mM to about 175 mM, about 170 mM to about 190 mM, about 160 mM to about 200 mM.

[0187] In some embodiments, the HA elution buffer comprises a salt. In some embodiments, the salt is a sodium, lithium or potassium salt. In some embodiments, the salt is sodium chloride. In some embodiments, the sodium chloride is about 100 mM to about 1M. In some embodiments, the sodium chloride is about 300 mM to about 500 mM, about 100 mM to 200 mM, about 150 mM to about 300 mM, about 200 mM to about 400 mM, about 250 mM to about 500 mM, about 300 mM to about 600 mM, about 350 mM to about 700 mM, about 400 mM to about 800 mM, about 450 mM to about 900 mM, about 500 mM to 1M. In some embodiments, the sodium chloride is about 400 mM.

[0188] In some embodiments, the HA elution buffer comprises a phosphate buffer. In some embodiments, the phosphate buffer is sodium or potassium phosphate. In some

embodiments, the phosphate buffer is about 5 mM to about 50 mM. In some embodiments, the phosphate buffer is about 10 mM. In some embodiments, the phosphate buffer is about 5 mM. In some embodiments, the phosphate buffer is about 15 mM. In some embodiments, the phosphate buffer is about 20 mM. In some embodiments, the phosphate buffer is about 25 mM. In some embodiments, the phosphate buffer is about 30 mM. In some embodiments, the phosphate buffer is about 35 mM. In some embodiments, the phosphate buffer is about 40 mM. In some embodiments, the phosphate buffer is about 45 mM. In some embodiments, the phosphate buffer is about 50 mM. In some embodiments, the phosphate buffer is about 5 mM. to about 40 mM, about 10 mM to about 30 mM, about 15 mM to about 25 mM.

[0189] When hydroxyapatite resins are used in successive protein separations with the elution buffers disclosed herein, the resins can be regenerated after each separation by conventional means to clean the resins of residual proteins and contaminants and to equilibrate the resins to the conditions to be used for protein retention and elution. Regeneration in many cases will thus include, for example, neutralization of the resin with an appropriate basic solution, followed by regeneration to a neutral pH, followed in turn by equilibration to a slightly acidic pH within the range best suited for protein retention and to a salt concentration when a salt is included. In general, hydroxyapatite resins can be used for ten or more, often 25 or more, and often 50 or more protein separations and elutions without loss of resin integrity and function.

[0190] In some embodiments, the eluted product from the HA chromatography, Product (e), has a pH of about 6 to about 7. In some embodiments, Product (e) has a pH of about 6.5. In some embodiments, Product (e) has a pH of about 6.1. In some embodiments, Product (e) has a pH of about 6.2. In some embodiments, Product (e) has a pH of about 6.3. In some embodiments, Product (e) has a pH of about 6.4. In some embodiments, Product (e) has a pH of about 6.6. In some embodiments, Product (e) has a pH of about 6.7. In some embodiments, Product (e) has a pH of about 6.8. In some embodiments, Product (e) has a pH of about 6.9. In some embodiments, Product (e) has a pH of about 7.0. In some embodiments, Product (e) has a pH of about 6.0-7.0, 6.0-6.9, 6.0- 6.8, 6.0-6.7, 6.0-6.6, 6.0-6.5, 6.0-6.4, 6.0-6.3, 6.0-6.2, or 6.0-6.1.

[0191] In some embodiments, the purity of the antibodies of interest in Product (e) can be analyzed using methods well known to those skilled in the art, e.g., size-exclusion chromatography, Poros™ A HPLC Assay, HCP ELISA, Protein A ELISA, and western blot

analysis, or a combination thereof. In some embodiments, the product purity is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance, mass spectrophotometry or a combination thereof.

Additional purification/Polishing

[0192] In some embodiments, one or more of the purified products as disclosed herein, e.g., Product (a), Product (b), Product (c), Diluted Product (c), Product (d), or Product (e), or any additional product made during the purification process, can be further treated with viral reduction filtration and/or diafiltration. In some embodiments, Product (e), can be further treated with viral reduction filtration and/or diafiltration.

[0193] Virus reduction filtration can feature membranes with pores small enough to retain viruses while still allowing passage of the TCE-pMHC molecule. This size-exclusion retention mechanism can be a virus removal step which is complementary to other removal steps, such as the described chromatography, or to viral inactivation steps, such as a low pH hold. In some embodiments, viral reduction filtration can be used together with other steps to achieve an overall level of safety which meets regulatory guidance by a government organization, (e.g. one viral particle or less per 1,000,000 doses).

[0194] In some embodiments, the viral reduction filter features a filter with a poer size of about 20 nM to about 400 nM, about 40 nM to about 300 nM, about 50 nM to about 250 nM, or about 75 nM to about 200 nM, In some embodiments, the Viresolve® Pro and Viresolve® NFP filters (MilliporeSigma) can be used, which are highly asymmetric membranes with a thick microporous support layer (with pore size ≥ 200 nm) and a thin virus-retentive skin layer. In some embodiments, the Ultipor DV20 and Pegasus™ SV4 filters (Pall) are used, which are relatively homogeneous, with minimal variation in pore size through the depth of the membrane. These flat sheet membranes are used in two- or three-layer configurations to obtain a high degree of virus removal. In some embodiments, Planova™ 20 N and BioEX virus filters (Asahi Kasei) can be used, which include hollow fiber membranes, used as just a single “layer”, with less variation in pore size than found in the highly asymmetric flat sheet virus filters. In some embodiments, the viral filter can be a depth filter. In some embodiments, the viral filter can be a tangential flow filter.

[0195] In some embodiments, diafiltraton can be used in the purification of the TCE-pMHC molecule. Diafiltration (DF) can be used in combination with the chromatography and/or other processing steps described herein to get the TCE-pMHC to further

purification, concentration, buffer exchange, and desalting. DF enables buffer exchange by adding new buffer to the retentate. In some embodiments, the buffer of Product (c) is replaced, while retaining the original volume. In some embodiments, the DF can be repeated until the composition comprising the TCE-pMHC molecule reaches a desired concentration.

[0196] In some embodiments, a detergent is added to Product (d) or Product (e), or after viral filtration and/or diafiltration of Product (d) or Product (e). In some embodiments, the detergent is polysorbate 80. In some embodiments, polysorbate 80 can be used to protect protein against agitation-induced aggregation in the final purified TCE-pMHC composition. Polysorbate 80 can be used to prevent agitation-induced aggregation because of its effectiveness at low concentrations, relative low toxicity, and ability to not only inhibit protein surface adsorption and aggregation under various processing conditions but also act as a stabilizer against protein aggregation.

Methods for reducing product related impurities

[0197] In some embodiments, the disclosure provides a method for reducing a product-related impurity during purification of a T cell engaging immune effector domain, peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule produced by a host cell, the method comprising: (a) isolating supernatant of a host cell culture comprising the TCE-pMHC molecule to form Product (a), (b) subjecting Product (a) to affinity chromatography to form Product (b), (c) subjecting Product (b) to anion exchange chromatography to form Product (d*), (d) subjecting Product (d*) to hydroxyapatite chromatography, wherein the TCE-pMHC molecule is eluted with an elution buffer comprising an MES buffer, wherein the elution buffer has a pH of about 5.0 to about 7.0, to form Product (e); wherein the TCE-pMHC molecule comprises: (i) a peptide-major histocompatibility complex (pMHC) binding domain comprising a first variable region linked to a constant region (VCs) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain; (ii) a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and (iii) a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain; wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C

terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0198] Product (d*) is similar to Product (d), except that it may or may not be subjected to a neutralization reaction prior to the anion exchange chromatography process. In some embodiments, Product (d*) has not been subjected to a neutralization reaction prior to the anion exchange chromatography process. In some embodiments, Product (d*) has been subjected to a neutralization reaction prior to the anion exchange chromatography process.

[0199] The disclosure provides for a method of reducing “product-related impurity.” A product-related impurity can include, e.g., a TCE-pMHC degradation product, or a partial-TCE-pMHC protein, e.g., wherein the TCE-pMHC is not fully formed, e.g., fully translated. In some embodiments, a product-related impurity can include a fragment adduct impurity, i.e., degradants or reaction products of the TCE-pMHC molecule. In some embodiments, a product-related impurity can include a multimer of TCE-pMHC, e.g., aggregated TCE-pMHC. In some embodiments, the product-related impurity comprises a High Molecular Weight TCE-pMHC, i.e., HMW-TCE-pMHC. A HMW-TCE-pMHC includes 2 or more TCE-pMHC molecules aggregated together. The methods described herein reduce the formation of, and the presence of, product-related impurities in the TCE-pMHC product. In some embodiments, the methods described herein, e.g., the HA chromatography process, greatly reduces product-related impurities associated with TCE-pMHC.

[0200] In some embodiments, the disclosure provides a method for reducing a product-related impurity during purification of a TCE-pMHC molecule in a host cell, wherein the product-related impurity comprises a fragment adduct impurity. In some embodiments, the disclosure provides a method for reducing a product-related impurity during purification of a TCE-pMHC molecule in a host cell, wherein the product-related impurity comprises physiochemical properties similar to TCE-pMHC molecule. In some embodiments, the product related impurity is assessed before and after purification. In some embodiments, the product related impurity is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance, mass spectrophotometry or a combination thereof.

[0201] In some embodiments, the disclosure provides a method of manufacturing a T cell engaging, peptide-major histocompatibility complex binding domain (TCE-pMHC)

molecule produced by a host cell, wherein the method comprises: (a) isolating a supernatant of a host cell culture comprising the TCE-pMHC molecule to form Product (a), (b) subjecting Product (a) to affinity chromatography, wherein Product (a) is less than 10 mg/mL; wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.0 to about 4.0 to form Product (b); (c) adjusting the pH of Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of about 5.0 to about 7.0; (d) subjecting Product (c) to anion exchange chromatography to form Product (d); and (e) subjecting Product (d) to hydroxyapatite chromatography wherein the TCE-pMHC molecule is eluted with an elution buffer comprising an MES buffer, wherein the elution buffer has a pH of less than 7.0 to form Product (e), wherein Product (e) has a pH of less than 7.0; wherein the TCE-pMHC molecule comprises: (i) a peptide-major histocompatibility complex (pMHC) binding domain comprising a first variable region linked to a constant region (VCs) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain; (ii) a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and (iii) a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain; wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

[0202] In some embodiments, the disclosure provides a method for purifying a T cell engaging, peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule, the method comprising the following steps: (a) expressing the TCE-pMHC molecule in a mammalian host cell, wherein the TCE-pMHC molecule is secreted by the host cell into the host cell culture supernatant; (b) isolating the supernatant comprising the TCE-pMHC molecule from the host cell to form Product (a), (c) subjecting Product (a) to Protein A affinity chromatography, wherein Product (a) is less than 10 mg/mL; wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of 3.2 to 4.0 to form Product (b); (d) adjusting the pH of Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of 5.5 to 7.0, and optionally

diluting Product (c) at least 2-fold to obtain Diluted Product (c); (e) subjecting Product (c), or Diluted Product (c) if applicable, to Sartobind STIC-PA chromatography to form Product (d); and (f) subjecting Product (d) to ceramic hydroxyapatite chromatography to obtain Product (e); and (g) optionally, subjecting Product (e) to one or more polishing steps; wherein Product (c), or Diluted Product (c) if present, has a conductivity of <5 mS/cm; wherein the TCE-pMHC molecule comprises: (i) a peptide-major histocompatibility complex (pMHC) binding domain comprising a first variable region linked to a constant region (VC1) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerize to form the pMHC binding domain; (ii) a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and (iii) a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerize to form an Fc domain; wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

Purity levels

[0203] In some embodiments, the disclosure provided herein describes a 3-step, 4-step, or more chromatography processes to obtain purified TCE-pMHC. In some embodiments, the process described herein reduces the high molecular weight aggregates of TCE-pMHC molecule (HMW-TCE-pMHC) in the purified composition during various steps in the purification process as described herein. In some embodiments, Product (c) has high molecular weight aggregates of TCE-pMHC molecule (HMW-TCE-pMHC) less than 10% (wt/wt) of total TCE-pMHC. In some embodiments, Product (c) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC. In some embodiments, Product (c) has HMW-TCE-pMHC of about 0.1% to about 10%, about 0.1% to about 8%, about 0.1% to about 6%, about 0.5% to about 5%, or about 1% to about 5% (wt/wt) of total TCE-pMHC.

[0204] In some embodiments, Product (d) (or Product (d*)) has high molecular weight aggregates of HMW-TCE-pMHC less than 10% (wt/wt) of total TCE-pMHC. In some embodiments, Product (d) (or Product (d*)) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC. In some embodiments, Product (d) (or Product (d*)) has HMW-TCE-

pMHC of about 0.1% to about 10%, about 0.1% to about 8%, about 0.1% to about 6%, about 0.5% to about 5%, or about 1% to about 5% (wt/wt) of total TCE-pMHC.

[0205] In some embodiments, Product (e) has high molecular weight aggregates of HMW-TCE-pMHC less than 10% (wt/wt) of total TCE-pMHC. In some embodiments, Product (e) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC. In some embodiments, Product (e) has HMW-TCE-pMHC of about 0.1% to about 10%, about 0.1% to about 8%, about 0.1% to about 6%, about 0.5% to about 5%, or about 1% to about 5% (wt/wt) of total TCE-pMHC.

[0206] In some embodiments, the HMW-TCE-pMHC of Product (c), Product (d) (or Product (d*)), or Product (e) is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance or a combination thereof.

[0207] In some embodiments, the disclosure provided herein describes a 3-step, 4-step, or more chromatography processes to reduce the host cell oligonucleotides, e.g., host cell DNA (hcDNA) in the purified composition during various steps in the purification process as described herein. In some embodiments, Product (c) comprises host cell DNA (hcDNA) of less than 30 pg/mg, less than 25 pg/mg, less than 20 pg/mg, less than 15 pg/mg, less than 10 pg/mg, or less than 5 pg/mg. In some embodiments, Product (d) (or Product (d*)) comprises host cell DNA (hcDNA) of less than 30 pg/mg, less than 25 pg/mg, less than 20 pg/mg, less than 15 pg/mg, less than 10 pg/mg, or less than 5 pg/mg. In some embodiments, Product (e) comprises host cell DNA (hcDNA) of less than 30 pg/mg, less than 25 pg/mg, less than 20 pg/mg, less than 15 pg/mg, less than 10 pg/mg, or less than 5 pg/mg.

[0208] In some embodiments, the disclosure provided herein describes a 3-step, 4-step, or more chromatography processes to reduce the host cell proteins in the purified composition during various steps in the purification process as described herein. In some embodiments, Product (c) comprises host cell protein of less than 150 ng/mg, less than 125 ng/mg, less than 100 ng/mg, less than 75 ng/mg, less than 50 ng/mg, less than 25 ng/mg, or less than 10 ng/mg. In some embodiments, Product (d) comprises host cell protein of less than 150 ng/mg, less than 125 ng/mg, less than 100 ng/mg, less than 75 ng/mg, less than 50 ng/mg, less than 25 ng/mg, or less than 10 ng/mg. In some embodiments, Product (e) comprises host cell protein of less than 150 ng/mg, less than 125 ng/mg, less than 100 ng/mg, less than 75 ng/mg, less than 50 ng/mg, less than 25 ng/mg, or less than 10 ng/mg.

[0209] In some embodiments, the disclosure provides a method to reduce the amount of

product-related impurities in the purified composition during various steps in the purification process as described herein. In some aspects, the Product (d*) includes less than 2%, less than 1.5%, less than 1%, less than 0.8%, or less than 0.5% (wt/wt) of the product related impurity. In some aspects, the Product (d*) includes less than 1% (wt/wt) of the product related impurity. In some embodiments, Product (e) comprises product related impurities of less than 1.5% (wt/wt), less than 1.0% (wt/wt), less than 0.75% (wt/wt) or less than 0.5% (wt/wt).

[0210] In some embodiments, the disclosure provides a method to provide a purified TCE-pMHC molecule, wherein the TCE-pMHC molecule is greater than 95% (wt/wt), greater than 99% (wt/wt), greater than 99% (wt/wt), greater than 99.5% (wt/wt), or greater than 99.9% (wt/wt) of total protein in Product (e).

Methods of Treatment

[0211] The TCE-pMHC molecule purified by the methods described herein can be administer to a subject for treating diseases such as cancer, particularly cancers which are associated with expression of a tumor-associated antigen. For example, the cancer may be associated with expression of GP100, NYESO, MAGEA4, or PRAME as described in WO2011001152, WO2017109496, WO2017175006 and WO2018234319.

[0212] The cancer to be treated may be a cancer associated with PRAME expression. By "associated with PRAME expression" it is meant that the cancer comprises cancer cells that express PRAME. In this regard, the cancer may be a PRAME-positive cancer. The cancer may be known to be associated with expression of PRAME, and thus PRAME expression may not be assessed. Alternatively, PRAME expression can be assessed using any method known in the art, including, for example, histological methods. However, the disclosure is not intended to be limited to the treatment of cancers for which PRAME expression can be detected by histological methods. Cancers associated with PRAME expression include, but are not limited to, melanoma, lung cancer, breast cancer, ovarian cancer, endometrial cancer, oesophageal cancer, bladder cancer, head and neck cancer, uterine cancer, Acute myeloid leukemia, chronic myeloid leukemia, and Hodgkin's lymphoma. For example, the cancer associated with PRAME expression may be melanoma. The melanoma may be uveal melanoma or cutaneous melanoma. The lung cancer may be non-small cell lung carcinoma (NSCLC) or small cell lung cancer (SCLC). The breast cancer may be triple-negative breast cancer (TNBC) The bladder cancer may be urothelial carcinoma. The oesophageal cancer

may be gastroesophageal junction (GEJ) adenocarcinoma. The ovarian cancer may be epithelial ovarian cancer, such as high grade serous ovarian cancer.

[0213] In some embodiments, the TCE-pMHC molecule purified by the methods described herein can be used in the treatment of PRAME positive cancers. The term "PRAME positive cancer" refers to a PReferentially expressed Antigen in MELanoma (i.e., PRAME) cancer in which at least some of the cancer cells express PRAME. PRAME was first identified as an antigen that is over expressed in melanoma (Ikeda et al Immunity. 1997 Feb;6(2):199-208); it is also known as CT130, MAPE, OIP-4 and has Uniprot accession number P78395. The protein functions as a repressor of retinoic acid receptor signaling (Epping et al., Cell. 2005 Sep 23; 122(6): 835-47). PRAME belongs to the family of germline-encoded antigens known as cancer testis antigens. Cancer testis antigens are attractive targets for immunotherapeutic intervention since they typically have limited or no expression in normal adult tissues. PRAME is expressed in a number of solid tumors as well as in leukemias and lymphomas. PRAME targeting therapies of the disclosure may be particularly suitable for treatment of cancers including, but not limited to, melanoma, lung cancer, breast cancer, ovarian cancer, endometrial cancer, esophageal cancer, bladder cancer, head and neck cancer, uterine cancer, Acute myeloid leukemia, chronic myeloid leukemia, and Hodgkin's lymphoma.

[0214] The peptide SLLQHLIGL (SEQ ID NO: 1) corresponds to amino acids 425-433 of the full length PRAME protein and is presented on the cell surface in complex with HLA-A*02 (Kessler et al., J Exp Med. 2001 Jan 1 ;193(1):73-88). This peptide-HLA complex provides a useful target for TCR-based immunotherapeutic intervention.

[0215] US 63/371,863, and WO2020157211, describe TCRs that bind to the SLLQHLIGL-HLA-A*02 complex, each of which is incorporated by reference herein in their entirety. The TCRs are mutated relative to a native PRAME TCR alpha and/or beta variable domains to have improved binding affinities for, and/or binding half-lives, for the complex, and can be associated (covalently or otherwise) with a therapeutic agent. One such therapeutic agent is an anti-CD3 antibody, or a functional fragment or variant of said anti-CD3 antibody such as a single chain variable fragment (scFv). The anti-CD3 antibody or fragment may be covalently linked to the C- or N- terminus of the alpha or beta chain of the TCR. The resulting molecule is a TCR bispecific. The methods described herein can be used for the purification of TCE-pMHC which specifically binds to SLLQHLIGL (SEQ IS NO: 1) HLA-A*02 complex.

[0216] In some embodiments, the disclosure provides for a pharmaceutical composition comprising the TCE-pMHC molecule prepared by the methods described herein.

[0217] The term “subject” means any subject, particularly a mammalian subject, in need of treatment, e.g., with a composition comprising a compound of formula (I). In some embodiments, the term “subject” refers to a human subject. In some embodiments, the term “subject” refers to an adult human subject. In some embodiments, the term “subject” refers to a male human subject. In some embodiments, the term “subject” refers to a female human subject. In some embodiments, the term “subject” refers to administration to a subject in need thereof, i.e., a subject having cutaneous melanoma and/or a subject having a PRAME-positive cancer. As used herein, a “subject in need thereof” can refer to the subject for whom it is desirable to treat, e.g., a subject being diagnosed with cutaneous melanoma and/or a PRAME-positive cancer as described herein. In some embodiments, the term “subject in need thereof” can refer to a subject having one or more symptoms associated with cutaneous melanoma and/or a PRAME-positive cancer, e.g., a subject having a significant change in an existing mole, a subject having developed a new pigmented or unusual-looking growth on subject’s skin, etc. In some embodiments, the term “subject in need thereof” can refer to a subject at high risk for suffering from a cutaneous melanoma and/or a PRAME-positive cancer suitable to treatment with TCE-pMHC molecule as described herein, independently of whether the subject has physical manifestations of such condition. In some embodiments, the subject is an adult, i.e., at least 18 years old. In some embodiments, the subject is 12-18 years old. In some embodiments, the subject is less than 12 years old.

Description of the sequences

[0218] SEQ ID NO: 1 HLA-A*02 restricted peptide: SLLQHLIGL

[0219] SEQ ID NO: 2 Amino acid sequence of the alpha chain variable domain of an exemplary TCR. CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 3, 4 and 5 respectively, framework regions (FR1, FR2, FR3 and FR4) are in italics and are designated SEQ ID NO: 27, 6, 7 and 28 respectively. This sequence contains a N24Q mutation (double underlined), which removes an N-linked glycosylation site.

GDAKTTQPNSMESNEEEPVHLPCQHSTISGTDYIHWYRQLPSQGPEYVIHGLTSNVNNR
MASLAIAEDRKSSSTLILHRATLRDAAVYYCILILGHSRLGNYIATFGKGTKLSVIP

[0220] SEQ ID NO: 8 Amino acid sequence of the TCR β chain variable domain of an exemplary TCR. CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 9, 10 and 11 respectively, framework regions (FR1, FR2, FR3 and FR4) are in italics and are designated SEQ ID NO: 29, 12, 13 and 30 respectively.

DGGITQSPKYLFRKEGQNVTLSCEQMLNHDAMYWYRQDPGQGLRLIYYSQIMGDEQKG
DIAEGYSVSREKKESFPLTVTSAQKNPTAFYLCASSWWTGGASPIRFGPGTRLTVT

[0221] SEQ ID NO: 14 Amino acid sequence of the TCR α chain of an exemplary TCR. CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 3, 4 and 5 respectively, framework regions (FR1, FR2, FR3 and FR4) are in italics and are designated SEQ ID NO: 27, 6, 7 and 28 respectively. The constant region is shown in bold and is designated SEQ ID NO: 15. Within the constant region, a non-native cysteine residue is double underlined (at position 48 of the constant region) which was introduced to create an inter-chain disulfide bond. The sequence also contains N24Q, N148Q, N182Q and N193Q substitutions (double underlined), which each remove an N-linked glycosylation site.

GDAKTTQPNSMESNEEEPVHLPCQHSTISGTDYIHWYRQLPSQGPEYVIHGLTSNVNNR
MASLAIAEDRKSSTLILHRATLRDAAVYYCILILGHSRLGNYIATFGKGTKLSPINIQNP
PAVYQLRDSKSSDKSVCLFTDFDSQTQVSQSKSDVYITDKCVLDMRSMDFKSN
SAVAWSQKSDFACANAFQNSIIPEDT

[0222] SEQ ID NO: 16 Amino acid sequence of the TCR β chain of an exemplary TCR. CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 9, 10 and 11 respectively, framework regions (FR1, FR2, FR3 and FR4) are in italics and are designated SEQ ID NO: 29, 12, 13 and 30 respectively. Constant region is shown in bold (no underline) and is designated SEQ ID NO: 19. Within the constant region, a non-native cysteine residue is shaded (at position 57 of the constant region) which was introduced to create an inter-chain disulfide bond. The sequence also contains an N184Q substitution (double underlined), which removes an N-linked glycosylation site.

DGGITQSPKYLFRKEGQNVTLSCEQMLNHDAMYWYRQDPGQGLRLIYYSQIMGDEQKG
DIAEGYSVSREKKESFPLTVTSAQKNPTAFYLCASSWWTGGASPIRFGPGTRLTVTEDLK
NVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCT
DPQPLKEQPALQDSRYALSSRLRVSATFWQDPRNHFRQVQFYGLSENDEWTQ
DRAKPVTQIVSAEAWGRAD

[0223] **SEQ ID NO: 17** An exemplary anti-CD3 scFv (T cell engaging immune effector domain) referred to herein as "U0". The light chain variable domain (VL) is in italics and is designated SEQ ID NO: 31. The light chain CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 33, 34 and 35. The heavy chain variable domain (VH) is shown in bold and is designated SEQ ID NO: 32. The heavy chain CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 36, 37 and 38. A glycine-serine linker, linking the VL and VH, is shown in plain text and is designated SEQ ID NO: 39.

AIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFS
GSGSGTDYTLTISSLPEDFATYYCQOQNTLPWTFGQGTKVEIKGGGGSGGGGSGGGGS
GGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQAPGKG
LEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCA
RSGYYGDSDWYFDVWGQGLTVTVSS

[0224] **SEQ ID NO: 40** Another exemplary anti-CD3 scFv (T cell engaging immune effector domain) referred to herein as "U28". This sequence is the same as SEQ ID NO: 17 above, except for two substitutions that are double underlined (T164A and I201F). The light chain variable domain (VL) is in italics and is designated SEQ ID NO: 31. The light chain CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 33, 34 and 35. The heavy chain variable domain (VH) is shown in bold and is designated SEQ ID NO: 41. The heavy chain CDRs (CDR1, CDR2 and CDR3) are underlined and are designated SEQ ID NO: 48, 37 and 38. A glycine-serine linker, linking the VL and VH, is shown in plain text and is designated SEQ ID NO: 39.

AIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFS
GSGSGTDYTLTISSLPEDFATYYCQOQNTLPWTFGQGTKVEIKGGGGSGGGGSGGGGS
GGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYAMNWVRQAPGK
GLEWVALINPYKGVSTYNQKFKDRFTESVDKSKNTAYLQMNSLRAEDTAVYYC
ARSGYYGDSDWYFDVWGQGLTVTVSS

[0225] **SEQ ID NO: 54** Human IgG1 Fc region (CH2 and CH3 domains), unmodified

APELLGGPSVFLFPPKPKDTLMISRTPEVTCVWDVSHEDPEVKFNWYVDGVEVHNA
 KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTKAKGGQP
 REPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
 DGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

[0226] SEQ ID NO: 42 An exemplary IgG1 Fc region sequence. This sequence has four substitutions, double underlined, relative to the above unmodified IgG1 Fc sequence (SEQ ID NO: 54). These are an N297G substitution for inhibiting binding to FcγR as well as T366S, L368A, and Y407V substitutions (hole-forming substitutions) for enhancing dimerization with another Fc region (e.g., SEQ ID NO: 43) containing a T366W substitution (knob-forming substitution). The numbering of the substitutions in this sequence is according to the EU numbering scheme.

APELLGGPSVFLFPPKPKDTLMISRTPEVTCVWDVSHEDPEVKFNWYVDGVEVHNA
KTKPREEQYGSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS
DGSFFLYYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[0227] SEQ ID NO: 43 Another exemplary IgG1 Fc region sequence. This sequence has two substitutions, double underlined, relative to the above unmodified IgG1 Fc sequence (SEQ ID NO: 54). These are an N297G substitution for inhibiting binding to FcγR as well as a T366W substitution (knob-forming substitution) for enhancing dimerization with another Fc region (e.g., SEQ ID NO: 42) containing T366S, L368A, and Y407V substitutions (hole-forming substitutions). The numbering of the substitutions in this sequence is according to the EU numbering scheme.

APELLGGPSVFLFPPKPKDTLMISRTPEVTCVWDVSHEDPEVKFNWYVDGVEVHNA
KTKPREEQYGSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[0228] SEQ ID NO: 44 An exemplary IgG1 hinge sequence (containing a C to S substitution at position 5, numbered according to SEQ ID NO: 44, relative to the native human IgG1 sequence):

EPKSSDKTHTCPPCP

[0229] SEQ ID NO: 52 A truncated IgG1 hinge sequence:

DKTHTCPPCP

[0230] SEQ ID NO: 53 An IgG4 hinge sequence:

ESKYGPPCPSCP

[0231] SEQ ID NO: 45 A complete amino acid sequence of an exemplary multi-domain, single-chain binding molecule. The T cell engaging immune effector domain (underlined) is the anti-CD3 scFv sequence provided in SEQ ID NO: 17 ("U0"). The pMHC binding domain is double underlined and comprises the TCR β chain sequence (which in this case is "VC1 ") provided in SEQ ID NO: 16 (double underlined, plain text) and the TCR α chain sequence (which in this case is "VC2") provided in SEQ ID NO: 14 (double underlined, bold text}. The half-life extending domain is an Fc domain which is a dimer formed between the Fc region sequence provided in SEQ ID NO: 42 (italics), which in this case is the FC1 region, and the Fc region sequence provided in SEQ ID NO: 43 (italics and bold), which in this case is the FC2 region.

AIQMTQSPSSLSASVGDRVITTCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGV
PSRFSGSGSGTDYTLTISSLOPEDFATYYCQOGNTLPWTFGQGTKVEIKGGGGSGGG
GSGGGGSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSTGYTMNWVRQ
APGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYY
CARSGYYGDSDWYFDVWGQGLTVTVSSGGGSDGGITQSPKYLFRKEGQNVTLSC
EQNLNHDAMYWYRQDPGQGLRLIYYSQIMGDEQKGDAEGYSVSREKKESFPLTVT
SAQKNPTAFYLCASSWWTGGASPIRFGPGTRLTVTEDLKNVFPPEVAVFEPSEAEISH
TQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCTDPOPLKEQPALQDSRYALSSR
LRVSATFWQDPRNHFRQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADGGGS
GGGGEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYGSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPGKGGGS
GGGGDAKTTQPNMESNEEPEVHLPCQHSTISGTDYIHWYRQLPSQGPEYVIH
GLTSNVNRMASLAIAEDRKSSSTLILHRAHLRDAAVYYCILILGHSRLGNVIATF
GKGTKLSVIPNIQNPDPAVYQLRDSKSSDKSVCLFTEDEDSQTOVSQSKSDSVYIT
DKCYLDMRSMDFKSNSAVAWSQKSDFACANAFQNSIIPEDTGGGSGGGGEPKSS
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
YVDGVEVHNAKTKPREEQYGSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENN
YKTTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPGK

[0232] SEQ ID NO: 46 A complete amino acid sequence of an exemplary multi-domain, single-chain binding molecule. The T cell engaging immune effector domain (underlined) is the anti-CD3 scFv sequence provided in SEQ ID NO: 40 ("U28"). The pMHC binding

domain is double underlined and comprises the TCR β chain sequence (which in this case is "VC1 ") provided in SEQ ID NO: 16 (double underlined, plain text) and the TCR α chain sequence (which in this case is "VC2") provided in SEQ ID NO: 14 (double underlined, bold text). The half-life extending domain is an Fe domain which is a dimer formed between the Fe region sequence provided in SEQ ID NO: 42 (italics), which in this case is the FC1 region, and the Fe region sequence provided in SEQ ID NO: 43 (italics and bold), which in this case is the FC2 region.

AIQMTQSPSSLSASVGDRVITTCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGV
PSRFSGSGSGTDYTLTISSLOPEDFATYYCQQGNTLPWTFGQGTKVEIKGGGGSGGG
GSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYAMNWVRQ
APGKGLEWVALINPYKGVSTYNQKFKDRFTFSVDKSKNTAYLQMNSLRAEDTAVY
YCARGYYGDSDWYFDVWGQGLTVTVSSGGGSDGGITQSPKYLFRKEGQNVTL
CEQNLNHDAMYWYRQDPGQGLRLIYYSQIMGDEQKGDIAEGYSVSREKKESFPLTV
TSAQKNPTAFYLCASSWWTGGASPIREFPGTRLTVTEDLKNVFPPEVAVFEPSEAEIS
HTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCTDPQPLKEQPALQDSRYALS
SRLRVSATFWQDPRNHFRQCQVQFYGLSENDEWTQDRAKPVTOIVSAEAWGRADGG
 GSGGGGEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV
 VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYGSTYRVVSVLTVLHQDWLNGKEYKCK
 VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESN
 GPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLS
 PGKGGGSGGGGG**GDAKTTQPNMESNEEEPVHLPCQHSTISGTDYIHWYROLPSQ**
GPEYVIHGLTSNYYNNRMASLAIAEDRKSSTLILHRATLRDAAVYYCILILGHSRL
GNYIATFGKGTKLSVIPNIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTQVSQSK
DSDVYITDKCYLDMRSMDEKSNSA VAWSQKSDFACANAFQNSIIPEDTGGGSGG
 GGEKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
 EVKFNWYVDGVEVHNAKTKPREEQYGSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
 ALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLWCLVKGFYPSDIAVEWESN
 GPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKS
 LSLSPGK

[0233] Additional linker sequences:

GGGGS (SEQ ID NO: 18), GGGSG (SEQ ID NO: 20), GGSGG (SEQ ID NO: 21), GSGGG
 (SEQ ID NO: 22), GSGGGP (SEQ ID NO: 23), GGEPS (SEQ ID NO: 24), GGEGGGP
 (SEQ ID NO: 25), GGEGGGSEGGGS (SEQ ID NO: 26), GGGSGGGG (SEQ ID NO: 47),

GGGSGGGSGGGSGGGSGGGSGGG (SEQ ID NO: 39), GGGSGGGSGGGSGGGSGGGSGGG (SEQ ID NO: 49), EAAAK (SEQ ID NO: 50) and EAAAKEAAAKEAAAK (SEQ ID NO: 51).

EXAMPLES

[0234] The present disclosure has been described with respect to representative examples that are to be considered illustrative embodiments that do not limit the scope of the disclosure which is defined solely by the claims. All references to publications, including scientific publications, treatises, textbooks, patent applications and issued patents are hereby incorporated by reference for all purposes.

Example 1. Expression of TCE-pMHC molecule

[0235] A TCE-pMHC molecule wherein the pMHC molecule binds to SEQ ID NO: 1 HLA-A *02 complex, was stably expressed in suspension-adapted Chinese Hamster Ovary (CHO) cells according to the Apollo™ X (Fuji) Advanced Mammalian Expression system. Briefly, cultured cells were diluted to a concentration of 6×10^6 prior to transfection. Cells were transfected using mammalian expression plasmids containing the relevant TCR chains fused to Ig Fc domains. Feed additions were performed on day 1 and day 5 post transfection. Cells were harvested on day 14 post transfection, with temperature shift to 32°C at day 1 post-transfection. Clarification was performed with depth filtration and two successive centrifugation steps, at 300 x g and 17,500 x g. The resulting supernatant was passed through 0.45 µm and 0.2 µm membrane filters and collected for further purification as outlined in FIG. 1.

Example 2. Purification of TCE-pMHC molecule using Protein A affinity chromatography

[0236] The clarified and filtered supernatant from Example 1 was purified by Protein A chromatography. A 20 cm bed height MabSelect SuRe LX Protein A resin column was prepared. The column was loaded with 5 g/L of supernatant and eluted using either a standard process method comprising 100 mM sodium citrate buffer at pH 3, or a developed method of 50 mM sodium acetate buffer at pH 3.6. In the standard method, the eluate was neutralized with the addition of 2M Tris at pH 6.8. Elution was assessed by size-exclusion Ultra Performance Liquid Chromatography (SE-UPLC) and the trace of the eluate is shown in FIG. 2. The main product peak was measured at a retention time of 12.063 minutes and concentration of 39.98% based on peak area. High molecular weight (HMW) aggregate peaks comprised 54.34% by area. In the developed method, the eluate was neutralized with the addition of 1M MES at pH 6.4. The SE-UPLC trace is shown in FIG. 3. The main product peak in the developed method was detected at a retention time of 13.365 minutes with a peak area of 80%, and 20% for HMW aggregates. The developed method showed superior purification of the product with reduced aggregation compared to the standard method. A summary of the Protein A eluate composition in the developed method is presented in Table 1. The Protein A eluate from the developed protocol was further diluted with 10 mM MES buffer until conductivity of the eluate was reduced to ≤ 5 mS/cm.

Table 1

	Protein A Eluate
HCP (ng/mg)	5000
hcDNA (pg/mg)	2800
HMW aggregates (%)	20
Product related impurities (%)	1.6
Product purity (%)	80

Example 3. Purification of TCE-pMHC molecule using Salt Tolerant anion exchange flow through (F/T) chromatography

[0237] To further reduce HMW aggregates, and host cell proteins and DNA in the product,

the conditioned Protein A eluate from Example 2 was passed at a high throughput of 10 membrane volumes per minute through a Sartobind STIC PA cellulose membrane in 50 mM MES buffer at pH 6.4. The flow through product was assessed by SE-UPLC and the trace is presented in FIG. 4. The main product peak was measured at retention time 13.417 minutes with peak area of 95%, while HMW aggregate peak areas comprised 5%. A summary of the flow through composition is presented in Table 2, showing significant decreases in host cell impurities.

Table 2

	Protein A Eluate	Sartobind STIC FT
HCP (ng/mg)	5000	89
hcDNA (pg/mg)	2800	<24
HMW aggregates (%)	20	5
Product related impurities (%)	1.6	1.6
Product purity (%)	80	95

Example 4. Purification of TCE-pMHC molecule using ceramic hydroxyapatite (B/E) chromatography

[0238] For further removal of product related impurities, the flow through from Example 3 was loaded onto a CHT ceramic hydroxyapatite (size: 40 μM) type II resin. Briefly, the resin was equilibrated with 100 mM MES, 10 mM phosphate, 0.18 mM calcium chloride, at pH 6.5. The flow through product was loaded and then washed with 100 mM MES, 10 mM phosphate, 0.18 mM calcium chloride, at pH 6.5, followed by a second wash with 25 mM Tris, 25 mM NaCl, 5 mM phosphate, at pH 7.5. The TCE-pMHC molecule was eluted with 100 mM MES, 10 mM phosphate, 0.18mM calcium chloride, 400 mM NaCl, at pH 6.5. The eluate was assessed by SE-UPLC, shown in FIG. 5. The main product peak measured 99.7% by area with no HMW aggregate peak detected. A summary of the eluate composition is presented in Table 3, highlighting reduced quantities of HCP and hcDNA, and undetectable levels of HMW aggregates and product related impurities.

Table 3

	Protein A Eluate	Sartobind STIC FT	Ceramic Hydroxyapatite
HCP (ng/mg)	5000	89	13
hcDNA (pg/mg)	2800	<24	<7
HMW aggregates (%)	20	5	0
Product related impurities (%)	1.6	1.6	0
Product purity (%)	80	95	99.7

CLAIMS

What is claimed is:

1. A method for purifying a T cell engaging peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule produced by a host cell, the method comprising:
 - a) isolating a supernatant of a host cell culture comprising the TCE-pMHC molecule to form Product (a),
 - b) subjecting Product (a) to affinity chromatography wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.0 to about 4.0 to form Product (b);
 - c) neutralizing Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of about 5.0 to about 7.0; and
 - d) subjecting Product (c) to anion exchange chromatography to form Product (d);wherein the TCE-pMHC molecule comprises:
 - i. a peptide-major histocompatibility complex (pMHC) binding domain comprising a first variable region linked to a constant region (VC1) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain;
 - ii. a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and
 - iii. a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form Fc domain;wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and
wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.
2. The method of claim 1, wherein the TCE-pMHC subjected to the affinity chromatography in (b) is eluted with sodium acetate.

3. The method of claim 2, wherein the sodium acetate has a concentration of less than 100 mM.
4. The method of claim 2, wherein the sodium acetate has a concentration of about 25 mM to about 75 mM.
5. The method of any one of claims 1 to 4, wherein Product (b) has a pH of about 3 to about 4.
6. The method of any one of claims 1 to 5, wherein the MES buffer added to neutralize Product (b) is less than 5 M.
7. The method of any one of claims 1 to 6, wherein the MES buffer added to neutralize Product (b) is about 0.5 M to about 1.5 M.
8. The method of any one of claims 1 to 7, wherein Product (c) has a pH of about 5.5 to about 6.5.
9. The method of any one of claims 1 to 8, wherein Product (c) is diluted at least 2-fold after neutralization to form a Diluted Product (c).
10. The method of any one of claims 1 to 9, wherein Product (c) is diluted to form a Diluted Product (c), and the MES buffer in the Diluted Product (c) has a concentration of less than 20 mM.
11. The method of any one of claims 1 to 10, wherein the MES buffer in the diluted Product (d) has a concentration of about 5mM to about 25 mM.
12. The method of any one of claims 1 to 11, wherein the conductivity of Product (c) is 5 mS/cm or less.
13. The method of any one of claims 1 to 12, wherein the conductivity of Diluted Product (c) is 5 mS/cm or less.
14. The method of any one of claims 1 to 13, wherein flow through buffer used with the anion exchange chromatography is a MES buffer.
15. The method of claim 14, wherein the flow through buffer is less than 100mM.

16. The method of claim 14, wherein the flow through buffer is about 25 mM to about 75 mM.
17. The method of any one of claims 1 to 16, wherein Product (d) has a pH of about 6 to about 7.
18. The method of any one of claims 1 to 17, wherein the affinity chromatography comprises a protein A affinity chromatography.
19. The method of any one of claims 1 to 18, wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.2 to about 4.0.
20. The method of any one of claims 1 to 19, wherein the anion exchange chromatography comprises a salt-tolerant anion-exchange flow through chromatography.
21. The method of any one of claims 1 to 20, wherein the anion exchange chromatography comprises a cross-linked cellulose.
22. The method of any one of claims 1 to 21, wherein the cross-linked cellulose comprises a primary amine.
23. The method of any one of claims 1 to 22, further comprising: (e) subjecting Product (d) to hydroxyapatite chromatography to form Product (e).
24. The method of any one of claims 1 to 23, further comprising expressing the TCE-pMHC in the host cell, wherein the TCE-pMHC molecule is secreted by the host cell into the host cell culture supernatant before the isolating in (a).
25. The method of any one of claims 1 to 24, wherein the host cell comprises a mammalian cell.
26. The method of any one of claims 1 to 25, wherein the supernatant comprising the TCE-pMHC molecule is isolated by filtration or centrifugation.
27. The method of any one of claims 1 to 26, wherein Product (c) has high molecular weight aggregates of TCE-pMHC molecule (HMW-TCE-pMHC) less than 10% (wt/wt) of total TCE-pMHC.
28. The method of any one of claims 1 to 27, wherein Product (c) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC.

29. The method of any one of claims 1 to 28, wherein Product (d) has HMW-TCE-pMHC less than 10% (wt/wt) of total TCE-pMHC.
30. The method of any one of claims 1 to 29, wherein Product (d) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC.
31. The method of any one of claims 23 to 30, wherein Product (e) has HMW-TCE-pMHC less than 10% (wt/wt) of total TCE-pMHC.
32. The method of any one of claims 23 to 30, wherein Product (e) has HMW-TCE-pMHC less than 6% (wt/wt) of total TCE-pMHC.
33. The method of any one of claims 1 to 32, wherein the HMW-TCE-pMHC is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance or a combination thereof.
34. The method of any one of claims 1 to 33, wherein Product (c) comprises host cell DNA (hcDNA) of less than 30 pg/mg.
35. The method of any one of claims 1 to 34, wherein Product (c) comprises host cell protein of less than 150 ng/mg.
36. The method of any one of claims 1 to 35, wherein Product (d) comprises host cell DNA (hcDNA) of less than 30 pg/mg.
37. The method of any one of claims 1 to 36, wherein Product (d) comprises host cell protein of less than 150 ng/mg.
38. The method of any one of claims 23 to 37, wherein Product (e) comprises host cell DNA (hcDNA) of less than 30 pg/mg.
39. The method of any one of claims 23 to 38, wherein Product (e) comprises host cell protein of less than 150 ng/mg.
40. The method of any one of claims 23 to 39, wherein Product (e) comprises product related impurities of less than 1.5% (wt/wt).
41. The method of any one of claims 23 to 40, wherein Product (e) comprises product related impurities of less than 0.5% (wt/wt).

42. The method of any one of claims 23 to 41, wherein the TCE-pMHC molecule is greater than 99% (wt/wt) of total protein in Product (e).
43. The method of any one of claims 23 to 42, wherein the TCE-pMHC molecule is greater than 99.5% (wt/wt) of total protein in Product (e).
44. The method of any one of claims 1 to 43, wherein the pI of the TCE-pMHC molecule is about 6.5 to about 7.0.
45. The method of any one of claims 1 to 44, wherein the pI of the TCE-pMHC molecule is about 6.8.
46. The method of any one of claims 1 to 45, wherein Product (a) comprises the total cell protein of about 0.1 mg/mL to about 25 mg/mL.
47. The method of any one of claims 1 to 46, wherein Product (a) comprises the total cell protein of about 2 mg/mL to about 10 mg/mL.
48. The method of any one of claims 1 to 47, wherein the pMHC binding domain binds to SLLQHLIGL (SEQ ID NO: 1) HLA-A*02 complex.
49. The method of any one of claims 1 to 48, wherein the TCR α variable region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 2 and the TCR β variable region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 8.
50. The method of any one of claims 1 to 48, wherein the TCR α constant region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 15 and the TCR β constant region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 19.
51. The method of any one of claims 1 to 50, wherein the FC1 and FC2 regions are IgG1 Fc regions.
52. The method of any one of claims 1 to 51, wherein the FC1 region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 42 and the FC2 region comprises an amino acid sequence that is at least 80% identical to the sequence of SEQ ID NO: 43.

53. The method of any one of claims 1 to 52, wherein the TCE-pMHC comprises the following amino acid sequences, in the following order, from N-terminus to C-terminus:
- a) an amino acid sequence of an anti-CD3 scFv, optionally followed by a linker sequence provided in SEQ ID NO: 18;
 - b) an amino acid sequence of a TCR β variable and constant region;
 - c) a linker sequence provided in SEQ ID NO: 47 followed by an IgG hinge sequence provided in SEQ ID NO: 44;
 - d) an Fc region having the sequence provided in SEQ ID NO: 42;
 - e) a linker sequence provided in SEQ ID NO: 47;
 - f) an amino acid sequence of a TCR α variable and constant region;
 - g) a linker sequence provided in SEQ ID NO: 47 followed by an IgG hinge sequence provided in SEQ ID NO: 44; and
 - h) an Fc region having the sequence provided in SEQ ID NO: 43.
54. A method for reducing a product-related impurity during purification of a T cell engaging immune effector domain, peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule produced by a host cell, the method comprising:
- a) isolating a supernatant of a host cell culture comprising the TCE-pMHC molecule to form Product (a),
 - b) subjecting Product (a) to affinity chromatography to form Product (b),
 - c) subjecting Product (b) to anion exchange chromatography to form Product (d*),
 - d) subjecting Product (d*) to hydroxyapatite chromatography, wherein the TCE-pMHC molecule is eluted with an elution buffer comprising an MES buffer, wherein the elution buffer has a pH of about 5.0 to about 7.0, to form Product (e);
- wherein the TCE-pMHC molecule comprises:
- i. a peptide-major histocompatibility complex (pMHC) binding domain comprising a first variable region linked to a constant region (VCs) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain;
 - ii. a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and
 - iii. a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain;

wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and

wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

55. The method of claim 54, wherein the hydroxyapatite chromatography comprises a ceramic hydroxyapatite.
56. The method of any one of claims 54 to 55, wherein with the elution buffer comprises about 50 mM to about 200 mM MES.
57. The method of any one of claims 54 to 56, wherein with the elution buffer comprises about 100 mM MES.
58. The method of any one of claims 54 to 57, wherein the elution buffer further comprises about 100 mM to about 1 M of a salt.
59. The method of any one of claims 54 to 58, wherein elution buffer comprises about 300 mM to about 500 mM of a salt.
60. The method of claim 59, wherein the salt is a sodium, lithium or potassium salt.
61. The method of claim 60, wherein the salt is sodium chloride.
62. The method of any one of claims 54 to 61, wherein with the elution buffer comprises a phosphate buffer.
63. The method of claim 62, wherein the phosphate buffer is about 5 mM to about 50 mM.
64. The method of claim 63, wherein the phosphate buffer is about 10 mM.
65. The method of any one of claims 54 to 64, wherein the product-related impurity comprises a fragment adduct impurity.
66. The method of any one of claims 54 to 65, wherein the product-related impurity comprises physiochemical properties similar to TCE-pMHC molecule.

67. The method of any one of claims 54 to 66, wherein the product related impurity is assessed before and after purification.
68. The method of any one of claims 54 to 67, wherein the product related impurity is assessed by SE-UPLC, HPLC, AUC, light scattering, UV absorbance, mass spectrophotometry or a combination thereof.
69. The method of any one of claims 54 to 68, wherein Product (d*) has a pH of about 6 to about 7.
70. The method of any one of claims 54 to 69, wherein Product (d*) comprises less than 2% (wt/wt) of the product related impurity.
71. The method of any one of claims 54 to 70, wherein Product (d*) comprises less than 1% (wt/wt) of the product related impurity.
72. A method of manufacturing a T cell engaging, peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule produced by a host cell, wherein the TCE-pMHC molecule is secreted by the host cell into a host cell culture supernatant, wherein the method comprises:
- isolating the supernatant comprising the TCE-pMHC molecule to form Product (a),
 - subjecting Product (a) to affinity chromatography, wherein Product (a) is less than 10 mg/mL; wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of about 3.0 to about 4.0 to form Product (b);
 - adjusting the pH of Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of about 5.0 to about 7.0;
 - subjecting Product (c) to anion exchange chromatography to form Product (d); and
 - subjecting Product (d) to hydroxyapatite chromatography wherein the TCE-pMHC molecule is eluted with an elution buffer comprising an MES buffer, wherein the elution buffer has a pH of less than 7.0 to form Product (e), wherein Product (e) has a pH of less than 7.0;
- wherein the TCE-pMHC molecule comprises:
- a peptide-major histocompatibility complex (pMHC) binding domain comprising a first variable region linked to a constant region (VCs) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerise to form the pMHC binding domain;

- ii. a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and
- iii. a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerise to form an Fc domain;

wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and

wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

73. A method for purifying a T cell engaging, peptide-major histocompatibility complex binding domain (TCE-pMHC) molecule, the method comprising the following steps:

- a) expressing the TCE-pMHC molecule in a mammalian host cell, wherein the TCE-pMHC molecule is secreted by the host cell into the host cell culture supernatant;
- b) isolating the supernatant comprising the TCE-pMHC molecule to form Product (a),
- c) subjecting Product (a) to Protein A affinity chromatography, wherein Product (a) is less than 10 mg/mL; wherein the TCE-pMHC molecule is eluted from the affinity chromatography at a pH of 3.2 to 4.0 to form Product (b);
- d) adjusting the pH of Product (b) by adding MES buffer with a pH of less than 7.0 to form Product (c), wherein Product (c) has a pH of 5.5 to 7.0, and optionally diluting Product (c) at least 2-fold to obtain Diluted Product (c);
- e) subjecting Product (c), or Diluted Product (c) if applicable, to Sartobind STIC-PA chromatography to form Product (d); and
- f) subjecting Product (d) to ceramic hydroxyapatite chromatography to obtain Product (e); and
- g) optionally, subjecting Product (e) to one or more polishing steps;

wherein Product (c), or Diluted Product (c) if present, has a conductivity of <5 mS/cm;

wherein the TCE-pMHC molecule comprises:

- i. a peptide-major histocompatibility complex (pMHC) binding domain comprising a first variable region linked to a constant region (VC1) and a second variable region linked to a constant region (VC2), wherein VC1 and VC2 dimerize to form the pMHC binding domain;

- ii. a T cell engaging immune effector domain comprising an antibody light chain variable region (TCE-VL) and an antibody heavy chain variable region (TCE-VH); and
- iii. a half-life extending domain comprising a first IgG Fc region (FC1) and a second IgG Fc region (FC2), wherein the FC1 region and FC2 region dimerize to form an Fc domain;

wherein the T cell engaging immune effector domain is linked to the N terminus of VC1, VC1 is linked via its C terminus to the N terminus of the FC1 region, the FC1 region is linked via its C terminus to the N terminus of VC2, and VC2 is linked via its C terminus to the N terminus of the FC2 region; and

wherein the pMHC binding domain and the T cell engaging immune effector domain are capable of binding to a pMHC complex and a T cell respectively.

74. The method of any one of claims 1 to 73, wherein the method further comprises viral reduction filtration.

75. The method of any one of claims 1 to 74, wherein the method further comprises diafiltration.

76. The method of any one of claims 23 to 75, wherein the method further comprises adding polysorbate 80 to Product (c) or Product (e).

77. A method for treating cancer in a subject, the method comprises administering the TCE-pMHC molecule purified by any of one of the methods of claims 1 to 76.

78. The method of claim 77, wherein the cancer is associated with PRAME expression.

79. A TCE-pMHC molecule produced by the method of any one of claims 1 to 76.