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(54) **Title:** AFFINITY AGENTS

(57) **Abstract:**

AFFINITY AGENTS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to and the benefit of U.S. Provisional Patent Application No. 63/405,301, filed September 9, 2022, the disclosure of which is incorporated by referenced herein in its entirety.

BACKGROUND

[0002] The purity of biologically produced therapeutics is tightly scrutinized and regulated by authorities to ensure safety and efficacy. Thus, there remains a need for means to efficiently purify biologically produced therapeutics to a high degree of purity and in sufficient quantities.

SUMMARY

[0003] To support clinical efforts for therapeutic proteins, compositions and methods to efficiently purify proteins from recombinant sources are needed. Affinity purification is a means to isolate and/or achieve desired purity of a protein in few steps, or a single step. However, the development of affinity agents (e.g., comprising an affinity ligand) can be a resource intensive and time-consuming task. This has resulted in the development of affinity agents for a limited number of proteins. In the absence of an affinity agent, purification typically involves inefficient, labor intensive, and expensive processes (e.g., a multi-column process).

[0004] Exemplary therapeutic proteins include, but are not limited to, bioactive polypeptides/proteins, fusion proteins, enzymes, hormones, antibodies, antibody fragments and recombinant vaccines. Certain proteins, such as fusion proteins, present additional challenges for purification due to product inhomogeneity, or the existence of product related impurities. Some impurities may arise from incorrectly assembled fusion proteins and/or proteolytic cleavage which can be especially difficult to remove because they are closely related to the desired product.

[0005] Affinity agents that bind proteins and are useful for isolation and/or affinity purification are described herein. In some embodiments, an affinity agent comprises a ligand and a solid support.

[0006] The COVID-19 pandemic has highlighted the benefits of leveraging platform production processes for rapid development of both therapeutics and vaccines. For example, monoclonal antibody therapies were developed and achieved emergency authorization in unprecedented timelines since they were able to leverage a platform production process, principally the protein A affinity purification platform. In the case of mRNA vaccines, the speed of development was also aided by the use of pre-existing affinity purification technologies (e.g., Oligo-dT and cellulose chromatography) for purification of mRNA moieties. Thus, clearly future pandemic preparedness as well as facilitating the production of vaccines for current global initiatives would benefit from the expansion of affinity purification tools that provide platform processes, *i.e.*, are applicable across multiple vaccines. Affinity agents that bind a trimerization domain and are useful for isolation and/or affinity purification are described herein. In some embodiments, an affinity agent comprises a ligand and a solid support.

[0007] Some subunit vaccines are based upon viral proteins that form a trimeric structure. To facilitate efficient production of the correctly folded trimeric form, a trimerization domain can be fused to the viral protein. The most commonly used trimerization domains are the T4 phage fibritin trimerization domain (foldon) [Tao Y, Strelkov SV, Mesyanzhinov VV, Rossmann MG, 1997. Structure of bacteriophage T4 fibritin: a segmented coiled coil and the role of the C-terminal domain. Structure 5, 789-798] and the yeast GCN4 trimerization domain [Harbury PB, Zhang T, Kim PS, Alber T, A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science, 1993, 262, 1401-1407]. More recently, another trimerization domain (referred to as 'molecular clamp') has been developed and demonstrated to confer stabilizing properties on the conformation of enveloped virus fusion protein-based vaccine constructs (see, e.g., WO 2018/176103 which is incorporated herein by reference in its entirety). Different viral proteins or variants of a viral protein may be fused with the same trimerization domain to make different vaccines. Moreover, said trimerization domain may also be utilized for effecting a trimerization of other (i.e., non-vaccine) polypeptides/proteins, in particular therapeutic proteins, for example, without intending to be limiting, any of those referred to in paragraphs [0395]-[0400] of WO 2018/176103. An affinity agent that binds this trimerization domain enables a facile purification of related subunit vaccines independent of the antigen portion and thus provides a platform technology for vaccine production and/or purification.

[0008] In some embodiments, an affinity agent (or the ligand comprised in said affinity agent) comprises a 3-helical bundle protein (alternatively referred to as "three-helix bundle protein"), preferably an anti-parallel 3-helical bundle protein. In some embodiments, the structure of a 3-helical bundle protein can be envisaged as a triangular prism, with each triangle vertex representing a helix, for example, as shown in Figure 1. In some embodiments, any 2 combinations of helices define a rectangular face of a 3 helical bundle protein. For example, in some embodiments the 3 faces of a 3 helical bundle protein are defined by:

1) helix 1 and 2 (Face 1,2 in Figure 1);
2) helix 2 and 3 (Face 2,3 in Figure 1);
3) helix 1 and 3 (Face 1,3 in Figure 1); and

combinations thereof.

[0009] In some embodiments, an affinity agent (or the ligand comprised in said affinity agent) comprises a face formed from helix 2 and 3 of a 3-helical bundle protein. In some embodiments, the primary function of helix 1 of a 3-helical bundle protein is to complete and stabilize the 3-helical bundle. In some embodiments, variations of helix 1 can be made that maintain the structure of a 3 helical bundle protein.

[0010] In some embodiments, provided herein are affinity agents comprising SEQ ID NO: 1, EQRRNFIENLRWDPSKSARLLARAKRFNDW.

[0011] In some embodiments, provided herein the affinity agents comprising SEQ ID NO: 1 are contained within helix 2 and 3 of a 3 helical bundle protein.

[0012] In some embodiments, provided herein are affinity agents comprising SEQ ID NO: 2, VDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQAPK.

[0013] In some embodiments, provided herein are affinity agents that comprise multimer polypeptides comprising at least two subunits, each subunit being a polypeptide according to the aforementioned embodiments.

[0014] In some embodiments, provided herein are affinity agents that comprise multimer polypeptides where the subunits are not all the same.

[0015] In some embodiments, provided herein are affinity agents comprising SEQ ID NOs: 3 – 10.

[0016] In some embodiments, provided herein are affinity agents that bind to a trimerization domain.

[0017] In some embodiments, provided herein are affinity agents used for the purification of proteins containing a trimerization domain.

[0018] In some embodiments, provided herein are affinity agents used for the purification of vaccines containing a trimerization domain.

DEFINITIONS

[0019] In order for the present disclosure to be more readily understood, certain terms are defined below. Unless defined otherwise herein, technical and scientific terms have the same meaning as commonly understood by one of ordinary skill in the art.

[0020] *Approximately or about*: As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within, with increasing preference, 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0021] *Biologically active*: As used herein, the term "biologically active" refers to a characteristic of any agent that has activity in a biological system, and particularly in an organism. For instance, an agent that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active.

[0022] *Conservative and non-conservative substitution:* A "conservative" amino acid substitution is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine (K), arginine (R), histidine (H)); acidic side chains (e.g., aspartic acid (D), glutamic acid (E)); uncharged polar side chains (e.g., asparagine (N), glutamine (Q) , serine (S), threonine (T), tyrosine (Y), cysteine (C)); nonpolar side chains (e.g., glycine (G); alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), methionine (M), tryptophan (W), beta-branched side chains (e.g., threonine (T), valine (V), isoleucine (I)); and aromatic side chains (e.g., tyrosine (Y), phenylalanine (F), tryptophan (W), histidine (H)). For example,

substitution of a phenylalanine for a tyrosine is a conservative substitution. In some embodiments, conservative amino acid substitutions in the sequence of a ligand confer or improve specific binding of the ligand to a target of interest. In some embodiments, conservative amino acid substitutions in the sequence of a ligand do not reduce or abrogate the binding of the ligand to a target of interest. In some embodiments, conservative amino acid substitutions do not significantly affect specific binding of a ligand to a target of interest. Methods of identifying nucleotide and amino acid conservative substitutions and non-conservative substitutions which confer, alter or maintain selective binding affinity are known in the art (see, e.g., Brummell, Biochem. 32:1180-1187 (1993); Kobayashi, *Protein Eng.* 12(10):879-884 (1999); and Burks, *PNAS* 94:412-417 (1997)). In some embodiments, non-conservative amino acid substitutions in the sequence of a ligand confer or improve specific binding of the ligand to a target of interest. In some embodiments, non-conservative amino acid substitutions in the sequences of a ligand do not reduce or abrogate the binding of the ligand to a target of interest. In some embodiments, non-conservative amino acid substitutions do not significantly affect specific binding of a ligand to a target of interest.

[0023] *Linker:* As used herein a "linker" refers to a peptide or other chemical linkage that functions to link otherwise independent functional domains, entities, or moieties. In some embodiments, a linker is located between a ligand and another polypeptide component containing an otherwise independent functional domain. In some embodiments, a linker is a peptide or other chemical linkage located between a ligand and a surface, such as a solid surface or solid support. In particularly preferred embodiments, the ligand is chemically conjugated (*i.e.*, covalently bound) to the solid surface or solid support through a bond formed between the thiol group of a cysteine of the ligand (preferably, an N- or C-terminal cysteine, more preferably a C-terminal cysteine) and the solid support. For example, the ligand may be covalently bound to the solid support through a bond formed by nucleophilic addition of a thiol group of a cysteine (*e.g.*, a C-terminal cysteine) of the ligand to a maleimide group on the solid surface or solid support. Other methods for covalently or non-covalently attaching ligands (*e.g.*, ligands comprising polypeptide(s)) to a solid surface or solid support are well known and routinely employed in the art. For example, any of the methods and linkages described in Greg T. Hermanson, "Bioconjugate Techniques", 3rd edition, 2013 (which is incorporated herein by reference in its entirety) can be used for covalently binding the ligand to the solid support.

[0024] *Naturally occurring:* The term "naturally occurring", when used in connection with biological materials, such as nucleic acid molecules, polypeptides, and host cells, refers to those which are found in nature and not modified by a human being. Conversely, "non-natural" or "synthetic" when used in connection with biological materials refers to those which are not found in nature and/or have been modified by a human being.

[0025] *"Non-natural amino acids," "amino acid analogs" and "non-standard amino acid residues"* are used interchangeably herein. Non-natural amino acids that can be substituted in a ligand as provided herein are known in the art. In some embodiments, a non-natural amino acid is 4-hydroxyproline which can be substituted for proline; 5-hydroxylysine which can be substituted for lysine; 3-methylhistidine which can be substituted for histidine; homoserine which can be substituted for serine; and ornithine which can be substituted for lysine. Additional examples of non-natural amino acids that can be substituted in a polypeptide ligand include, but are not limited to molecules such as: D-isomers of the common amino acids, 2,4-diaminobutyric acid, alpha-amino isobutyric acid, A-aminobutyric acid, Abu, 2-amino butyric acid, gamma-Abu, epsilon-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, beta-alanine, lanthionine, dehydroalanine, γ-aminobutyric acid, selenocysteine and pyrrolysine fluoro-amino acids, designer amino acids such as beta-methyl amino acids, C alpha-methyl amino acids, and N alpha-methyl amino acids.

[0026] *"Polynucleotide" and "nucleic acid molecule":* As used interchangeably herein, polynucleotide and nucleic acid molecule refer to a polymeric form of nucleotides of any length, comprising or consisting of either ribonucleotides or deoxyribonucleotides, or both. These terms include, but are not limited to, DNA, RNA, cDNA (complementary DNA), mRNA (messenger RNA), rRNA (ribosomal RNA), shRNA (small hairpin RNA), snRNA (small nuclear RNA), snoRNA (short nucleolar RNA), miRNA (microRNA), genomic DNA, synthetic DNA, synthetic RNA, and/or tRNA.

[0027] *Operably linked:* The term "operably linked," as used herein, indicates that two molecules are attached so as to each retain functional activity. Two molecules are "operably linked" whether they are attached directly or indirectly.

[0028] *Peptide tag:* The term "peptide tag" as used herein refers to a peptide sequence that is part of or attached (for instance through genetic engineering) to another protein, to provide a function

to the resultant fusion. Peptide tags are usually relatively short in comparison to a protein to which they are fused. In some embodiments, a peptide tag is four or more amino acids in length, such as, 5, 6, 7, 8, 9, 10, 15, 20, or 25 or more amino acids. In some embodiments, a ligand is a protein that contains a peptide tag. Numerous peptide tags that have uses as provided herein are known in the art. Examples of peptide tags that may be a component of a ligand fusion protein or a target of interest bound by a ligand (e.g., a ligand fusion protein) include, but are not limited to, HA (hemagglutinin), c-myc, the Herpes Simplex virus glycoprotein D (gD), T7, GST, GFP, MBP, Strep-tags, His-tags, Myc-tags, TAP-tags and FLAG tag (Eastman Kodak, Rochester, N.Y.). Likewise, antibodies to the tag epitope allow detection and localization of the fusion protein in, for example, affinity purification, Western blots, ELISA assays, and immunostaining of cells.

[0029] *Polypeptide:* The term "polypeptide" as used herein refers to a sequential chain of amino acids linked together via peptide bonds. The term is used to refer to an amino acid chain of any length, but one of ordinary skill in the art will understand that the term is not limited to lengthy chains and can refer to a minimal chain comprising two amino acids linked together via a peptide bond. Thus, for the sake of the present disclosure, it is understood that the terms "peptide" and "polypeptide" may be used interchangeably. As is known to those skilled in the art, polypeptides may be processed and/or modified.

[0030] *Protein:* The term "protein" as used herein refers to one or more polypeptides that function as a discrete unit. If a single polypeptide is the discrete functioning unit and does not require permanent or temporary physical association with other polypeptides in order to form the discrete functioning unit, the terms "polypeptide" and "protein" may be used interchangeably. If the discrete functional unit is comprised of more than one polypeptide that physically associate with one another, the term "protein" refers to the multiple polypeptides that are physically coupled and function together as the discrete unit.

[0031] *Specifically binds:* As used herein in reference to ligands, the term "specifically binds" or "has selective affinity for" means a ligand reacts or associates more frequently, more rapidly, with greater duration, with greater affinity, or combinations of the above to a particular epitope, protein, or target molecule than with alternative substances, including unrelated proteins. Because of the sequence identity between homologous proteins in different species, specific binding can include a binding agent that recognizes a protein or target in more than one species. Likewise, because of

homology within certain regions of polypeptide sequences of different proteins, specific binding can include a binding agent that recognizes more than one protein or target. It is understood that, in certain embodiments, a binding agent that specifically binds a first target may or may not specifically bind a second target. As such, "specific binding" does not necessarily require (although it can include) exclusive binding, i.e., binding to a single target. Thus, a ligand or affinity agent may, in certain embodiments, specifically bind more than one target. In certain embodiments, multiple targets may be bound by the same antigen-binding site on an affinity agent.

[0032] *Substantially:* As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0033] It is also to be understood that when reference is made herein to a "noun" in singular (e.g., a ligand, a bead etc.), the plural of such terms (e.g., ligands, beads etc.) is preferably meant to also be included and envisaged herein, unless specified otherwise. Thus, in preferred embodiments, wherever applicable, the reference to a noun in singular is intended to alternatively also refer to "at least one", "one or more" or "a plurality" of the referred term.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] **Figure 1** shows an exemplary affinity agent comprising a 3 helical bundle protein, as well as examples of how the structure of a 3 helical bundle protein can be envisaged as a triangular prism, with each triangle vertex representing a helix.

[0035] **Figure 2** shows an example sensorgram for an affinity agent of the present invention. A ligand corresponding to biotinylated ligand SEQ ID NO: 2 was immobilized on the sensor to determine the binding to a target analyte, a trimeric vaccine protein.

[0036] **Figure 3** shows the stability of one affinity agent of the present invention. The ligand corresponded to biotinylated SEQ ID NO: 7.

[0037] Figure 4 shows the stability of one affinity agent of the present invention in 0.1 M NaOH. The affinity resin contained ligand SEQ ID NO: 2.

[0038] Figure 5 shows a chromatogram of absorbance at wavelength of 280 nm during the purification of a trimeric vaccine protein using an affinity agent of the present invention. The affinity resin contained ligand SEQ ID NO: 7.

[0039] Figure 6 shows an SDS-PAGE gel of a trimeric vaccine protein purified using an affinity agent of the present invention. The affinity resin contained ligand SEQ ID NO: 7. The load (L), wash (W) elution (E) and strip (S) fractions were loaded on the gel. A molecular weight reference standard (Std) is included. The molecular weight of the trimeric vaccine protein being purified is 180 kDa.

DETAILED DESCRIPTION

[0040] The present disclosure encompasses, *inter alia*, the recognition that affinity agents prepared from identified and characterized peptide ligands are shown to generate highly purified preparations of one or more targets of interest, for example, in some embodiments, a trimeric protein, preferably a trimeric vaccine protein. In some embodiments, affinity agents (e.g., affinity resins or affinity beads) described herein are useful for, *inter alia*, removal of protein product related impurities as well as host cell-derived contaminants.

Ligand binding to targets of interest for use in an affinity agent

[0041] The characteristics of a ligand binding to a target can be determined using known or modified assays, bioassays, and/or animal models known in the art for evaluating such activity. In accordance with the affinity agent of the invention, the recited feature that said affinity agent “binds” a target of interest (e.g., a trimeric protein, preferably a trimeric vaccine protein) may, alternatively, also be more specifically defined as that the ligand(s) comprised in said affinity agent “binds” said target of interest (e.g., a trimeric protein, preferably a trimeric vaccine protein). In other words, it will be understood that the capacity of the affinity agent to bind a target of interest is provided by the ligand comprised in said affinity agent. Moreover, the term “binds”, as used in the context of the binding to a target of interest, is intended to mean, and may thus be interchangeably defined as, “capable of binding” to said target of interest. In preferred embodiments, the affinity agent (or the ligand comprised in said affinity

agent) binds "specifically" to a trimeric protein, preferably a trimeric vaccine protein, or a trimerization domain comprised therein.

[0042] As used herein, terms such as "binding affinity for a target", "binding to a target" and the like refer to a property of a ligand which may be directly measured, for example, through the determination of affinity constants (e.g., the amount of ligand that associates and dissociates at a given antigen concentration). Several methods are available to characterize such molecular interactions, for example, competition analysis, equilibrium analysis and microcalorimetric analysis, and real-time interaction analysis based on surface plasmon resonance interaction (for example using a BIACORE instrument). These methods are well-known to those of skill in the art and are discussed in publications such as Neri D et al. (1996) *Tibtech* 14:465-470 and Jansson M et al. (1997) *J Biol Chem* 272:8189-8197.

[0043] Affinity requirements for a given ligand binding event are contingent on a variety of factors including, but not limited to, the composition and complexity of the binding matrix, the valency and density of both the ligand and target molecules, and the functional application of the ligand. In some embodiments, a ligand binds a target of interest (e.g., a trimeric protein, preferably a trimeric vaccine protein, or a trimerization domain as referred to herein) with a dissociation constant (K_D) of less than or equal to 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, or 10^{-5} M. In some embodiments, a ligand binds a target of interest with a K_D of less than or equal to 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7}M, 5×10^{-8} M, or 10^{-8} M. In some embodiments, a ligand binds a target of interest with a K_D less than or equal to 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M. In some embodiments, a ligand generated by methods disclosed herein has a dissociation constant with respect to the binding to a target of interest of from about 10^{-4} M to about 10^{-5} M, from about 10^{-5} M to about 10^{-6} M, from about 10^{-6} M to about 10^{-7} M, from about 10^{-7} M to about 10^{-8} M, from about 10^{-8} M to about 10^{-9} M, from about 10^{-9} M to about 10^{-10} M, from about 10^{-10} M to about 10^{-11} M, or from about 10^{-11} M to about 10^{-12} M.

[0044] Binding experiments to determine K_D and off-rates can be performed in a number of conditions. The buffers in which to make these solutions can readily be determined by one of skill in the art and depend largely on the desired pH of the final solution. Low pH solutions (<pH 5.5) can be made, for example, in citrate buffer, glycine-HCl buffer, or in succinic acid buffer. High pH solutions can be made, for example, in Tris-HCl, phosphate buffers, or sodium bicarbonate buffers. A number of

conditions may be used to determine K_D and off-rates for the purpose of determining, for example, optimal pH and/or salt concentrations.

[0045] In some embodiments, a ligand specifically binds a target of interest with a k_{off} ranging from 0.1 to 10^{-7} sec^{-1}, 10^{-2} to 10^{-7} sec^{-1}, or 0.5 x 10^{-2} to 10^{-7} sec^{-1}. In some embodiments, a ligand binds a target of interest with an off rate (k_{off}) of less than 5 x10^{-2} sec^{-1}, 10^{-2} sec^{-1}, 5 x10^{-3} sec^{-1}, or 10^{-3} sec^{-1}. In some embodiments a ligand binds a target of interest with an off rate (k_{off}) of less than 5 x10^{-4} sec^{-1}, 10^{-4} sec^{-1}, 5 x10^{-5} sec^{-1}, or 10^{-5} sec^{-1}, 5 x10^{-6} sec^{-1}, 10^{-6} sec^{-1}, 5 x10^{-7} sec^{-1}, or 10^{-7} sec^{-1}.

In some embodiments, a ligand specifically binds a target of interest with a k_{on} ranging from about 10^3 to 10^7 $M^{-1}sec^{-1}$, 10^3 to 10^6 $M^{-1}sec^{-1}$, or 10^3 to 10^5 $M^{-1}sec^{-1}$. In some embodiments, a ligand (e.g., a ligand fusion protein) binds the target of interest with an on rate (k_{on}) of greater than 10^3 $M^{-1}sec^{-1}$, 5 x10^3 $M^{-1}sec^{-1}$, 10^4 $M^{-1}sec^{-1}$, or 5 x10^4 $M^{-1}sec^{-1}$. In an additional embodiment, a ligand, binds a target of interest with a k_{on} of greater than 10^5 $M^{-1}sec^{-1}$, 5 x10^5 $M^{-1}sec^{-1}$, 10^6 M^{-1} sec^{-1}, 5 x10^6 M^{-1} sec^{-1}, or 10^7 M^{-1} sec^{-1}.

Targets of interest

[0046] In accordance with various embodiments, a target of interest specifically bound by a ligand can be any molecule for which it is desirable for a ligand of an affinity agent to bind. For example, a target specifically bound by ligand can be any target of purification, manufacturing, formulation, therapeutic, diagnostic, or prognostic relevance or value. Non-limiting uses include therapeutic and diagnostic uses. A number of exemplary targets are provided herein, by way of example, and are intended to be illustrative and not limiting. It is well known in the art that viruses acquire mutations and the exemplary mutations described for the targets herein are provided herein, by way of example, and are intended to be illustrative and not limiting. A target of interest can be naturally occurring or synthetic. In some embodiments, a target comprises the receptor binding domain (RBD) of the CoV-2 virus spike protein (SARS-CoV-2S). In some embodiments, a target comprises the S1 protein of the CoV-2 virus. In some embodiments, a target comprises the spike protein of the CoV-2 virus. In some embodiments, a target comprises a trimeric RBD construct, S1 protein or spike protein of the CoV-2 virus. In some embodiments, a target comprises a CoV-2 virus particle. In preferred embodiments, a target of interest is a trimeric protein. Particularly preferred trimeric proteins include any of the chimeric polypeptides referred to in WO 2018/176103, the contents of which are herewith incorporated by reference in their

entirety. In particularly preferred embodiments, the trimeric protein is a trimeric vaccine protein. As used herein, the term "trimeric vaccine protein" generally refers to a trimeric protein which, based on the nature/origin of a comprised polypeptide/protein and consequently its antigenic capacity, may provide utility as a vaccine. Exemplary trimeric vaccine proteins include, in particular, trimeric proteins derived from trimeric virus surface proteins, such as, e.g., fusion proteins of class I or class III enveloped viruses or portions thereof (e.g., the ectodomain or antigenically active portions thereof). Particularly preferred trimeric vaccine proteins are described in WO 2018/176103, the contents of which are herewith incorporated by reference in their entirety. In further preferred embodiments, the target of interest (e.g., a trimeric protein, preferably a trimeric vaccine protein) comprises a trimerization domain. The term "trimerization domain", as used herein, generally refers to a domain that mediates the formation of a trimer out of three monomeric proteins/polypeptides or parts thereof. In a more specific embodiment, the term "trimerization domain" refers to an amino acid sequence within a polypeptide that promotes self-assembly by associating with two other trimerization domains to form a trimer. Various trimerization domains are known in the art and some particularly preferred representatives thereof are also referred to herein above. In particularly preferred embodiments, the trimerization domain is a polypeptide according to the "structure-stabilizing moiety" (also known as molecular clamp) as described in WO 2018/176103, the contents of which are herewith incorporated by reference in their entirety, wherein preferably said trimerization domain is fused C-terminally of a heterologous polypeptide (e.g., the ectodomain (or an antigenically portion thereof) of a fusion protein from an enveloped virus, e.g., an enveloped class I or III virus) and thereby mediates trimerization of the latter.

Linkers

[0047] The terms "linker" and "spacer" are used interchangeably herein to refer to a peptide or other chemical linkage that functions to link otherwise independent functional domains. In some embodiments, a linker is located between a ligand and another polypeptide component containing an otherwise independent functional domain. Suitable linkers for coupling two or more ligands may generally be any linker used in the art to link peptides, polypeptides, proteins or other organic molecules. In some embodiments, such a linker is suitable for constructing proteins or polypeptides that are intended for pharmaceutical use.

[0048] Suitable linkers for operably linking a ligand and an additional component of a ligand fusion protein in a single-chain amino acid sequence include but are not limited to, (poly)peptide linkers such as glycine linkers, serine linkers, mixed glycine/serine linkers, glycine- and serine-rich linkers or linkers composed of largely polar polypeptide fragments.

[0049] In some embodiments, a linker comprises a majority of amino acids selected from glycine, alanine, proline, asparagine, glutamine, and lysine. In some embodiments, a linker comprises a majority of amino acids selected from glycine, alanine, proline, asparagine, aspartic acid, threonine, glutamine, and lysine. In some embodiments, a ligand linker is made up of a majority of amino acids that are sterically unhindered. In some embodiments, a linker comprises a majority of amino acids selected from glycine, serine, and/or alanine. In some embodiments, a peptide linker is selected from polyglycines (such as, e.g., $(Gly)_5$ or $(Gly)_8$), poly(Gly-Ala), and polyalanines.

[0050] Linkers can be of any size or composition so long as they are able to operably link a ligand (with other ligands or with the solid support or molecules bound to the solid support) in a manner that permits the ligand to bind a target of interest. In some embodiments, linkers are from about 1 to 50 amino acids, from about 1 to 20 amino acids, from about 1 to 15 amino acids, from about 1 to 10 amino acids, from about 1 to 5 amino acids, from about 2 to 20 amino acids, from about 2 to 15 amino acids, from about 2 to 10 amino acids, or from about 2 to 5 amino acids. It should be clear that the length, the degree of flexibility and/or other properties of the linker(s) may influence certain properties of a ligand for use in an affinity agent, such as affinity, specificity or avidity for a target of interest, or for one or more other target proteins of interest, or for proteins not of interest (i.e., non-target proteins). In some embodiments, two or more linkers are utilized. In some embodiments, two or more linkers are the same. In some embodiments, two or more linkers are different.

[0051] In some embodiments, a linker is a non-peptide linker such as an alkyl linker, or a PEG linker. For example, alkyl linkers such as $-NH-(CH_2)_s-C(O)-$, wherein s=2-20 can be used. Any such linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6 alkyl), lower acyl (e.g., -CO-(C_1-C_5 alkyl)), halogen (e.g., Cl, Br, F, or I), CN, NH_2, phenyl, etc. An exemplary non-peptide linker is a PEG linker. In some embodiments, a PEG linker has a molecular weight of from about 100 to 5000 Da, or from about 100 to 500 Da. In some embodiments, a PEG linker has a molecular weight of from about 100 to 500 Da.

[0052] Linkers can be evaluated using techniques described herein and/or otherwise known in the art. In some embodiments, linkers do not alter (e.g., do not disrupt) the ability of a ligand to bind a target molecule.

Affinity agents comprising conjugated ligands

[0053] Ligands that promote specific binding to targets of interest can be chemically conjugated with a variety of chromatography compositions (e.g., beads, resins, gels, membrane, monoliths, etc.) to prepare an affinity agent. Affinity agents comprising ligands are particularly useful for purification and manufacturing applications.

[0054] In some embodiments, a ligand (e.g., a ligand fusion protein) contains at least one reactive residue. Reactive residues are useful, for example, as sites for the attachment of conjugates such as chemotherapeutic drugs. An exemplary reactive amino acid residue is lysine. A reactive residue (e.g., lysine) can be added to a ligand at either end, or within the ligand sequence and/or can be substituted for another amino acid in the sequence of a ligand. A suitable reactive residue (e.g., lysine, serine, tyrosine, hydroxytryptophan, etc.) can also be located within the sequence of an identified ligand without need for addition or substitution. In some embodiments, a reactive amino acid residue is cysteine. In some embodiments, a reactive amino acid residue is lysine. In some embodiments, a reactive amino acid residue is serine. In some embodiments, a reactive amino acid residue is tyrosine. In some embodiments, a reactive amino acid residue is hydroxytryptophan.

Attachment to solid surface

[0055]

The terms “solid surface”, “support”, “solid support”, or “matrix” are used interchangeably herein and refer to, without limitation, any column (or column material), resin, bead (e.g., agarose bead or Sepharose™ bead), test tube, microtiter dish, solid particle (for example, agarose or Sepharose™), microchip (for example, silicon, silicon-glass, or gold chip), or membrane of synthetic (e.g., a filter) or biological (e.g., liposome or vesicle) origin to which a ligand, affinity agent, antibody, or other protein may be attached (i.e., coupled, conjugated, linked, or adhered), either directly or indirectly (for example, through other binding partner intermediates, such as antibodies or Protein A or G), or in which a ligand or antibody may be embedded (for example, through a receptor or channel).

Reagents and techniques for attaching polypeptides to solid supports (e.g., matrices, resins, plastic, etc.) are well-known in the art. Suitable solid supports include, but are not limited to, a chromatographic resin or matrix (e.g., agarose or Sepharose™ (such as Sepharose 4 Fast Flow) beads), the wall or floor of a well in a plastic microtiter dish, a silica-based biochip, polyacrylamide, agarose, silica, nitrocellulose, paper, plastic, nylon, metal, and combinations thereof. Ligands and other compositions may be attached on a support material by a non-covalent association or by covalent bonding, using reagents and techniques known in the art. In exemplary preferred embodiments of a non-covalent association of a ligand to a solid support, the ligand comprises one member (i.e., a first member) of a binding pair (e.g., an affinity tag) and the solid support comprises the corresponding other member (i.e., a second member) of the binding pair (e.g., an affinity matrix specific for binding of the affinity tag). For example, in preferred aspects of the latter embodiments, the ligand comprises a poly-histidine-tag (e.g., a hexa-histidine-tag (6xHis-tag or His_6-tag)) and the solid support comprises a chelating agent, preferably a nitrilotriacetic acid (NTA) agarose resin or derivative thereof. In further preferred embodiments, the ligand comprises, or additionally comprises, a biotinylation-tag (e.g., an Avi-tag™) and the solid support comprises avidin (and/or streptavidin and/or neutravidin). In other embodiments, the ligand is attached to the solid support by means of an antibody which specifically binds the ligand. In the latter embodiments, the antibody may be attached to the solid support by means of Protein A and/or Protein G comprised in (or itself attached or conjugated to) the solid support. A multitude of further suitable binding pairs are well known in the art, each of which may be employed for the herein disclosed purposes. In some embodiments, a ligand is coupled to a solid surface or solid support (e.g., a chromatography material, such as a bead or resin made of, e.g., agarose or Sepharose™) via a linker.

Production of ligands

[0056] The production of a ligand, useful in practicing several embodiments of provided methods and uses, may be carried out using a variety of standard techniques for chemical synthesis, semi-synthetic methods, and recombinant DNA and protein expression and purification methodologies known in the art. Also provided are methods for producing a ligand, individually or as part of multi-domain fusion protein, as soluble agents and cell associated proteins. In some embodiments, the overall production scheme for a ligand comprises obtaining a reference protein scaffold and identifying a plurality of residues within the scaffold for modification. Depending on the embodiment, the reference scaffold

may comprise a protein structure with one or more alpha-helical regions, or other tertiary structure. Once identified, any of a plurality of residues can be modified, for example by substitution of one or more amino acids. In some embodiments, one or more conservative substitutions are made. In some embodiments, one or more non-conservative substitutions are made. In some embodiments a natural amino acid (e.g., one of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine) is substituted into a reference scaffold at targeted positions for modification. In some embodiments, modifications do not include substituting in either a cysteine or a proline. After modifications have been made at identified positions desired in a particular embodiment, the resulting modified polypeptides (e.g., candidate ligands) can be recombinantly expressed, for example in a plasmid, bacteria, phage, or other vector (e.g., to increase the number of each of the modified polypeptides). The modified polypeptides can then be purified and screened to identify those modified polypeptides that have specific binding to a particular target of interest. Modified polypeptides may show enhanced binding specificity for a target of interest as compared to a reference scaffold, or may exhibit little or no binding to a given target of interest (or to a non-target protein). In some embodiments, depending on the target of interest, the reference scaffold may show some interaction (e.g., nonspecific interaction) with a target of interest, while certain modified polypeptides will exhibit at least about two-fold, at least about five-fold, at least about 10-fold, at least about 20-fold, at least about 50-fold, or at least about 100-fold (or more) increased binding specificity for the target of interest. Additional details regarding production, selection, and isolation of ligand are provided in more detail below.

Recombinant expression of ligands

[0057] In some embodiments, a ligand such as a ligand fusion protein is "recombinantly produced," (i.e., produced using recombinant DNA technology). Exemplary recombinant methods available for synthesizing ligand fusion proteins include, but are not limited to, polymerase chain reaction (PCR) based synthesis, concatemerization, seamless cloning, and recursive directional ligation (RDL) (see, e.g., Meyer et al., *Biomacromolecules* 3:357-367 (2002), Kurihara et al., *Biotechnol. Lett.* 27:665-670 (2005), Haider et al., *Mol. Pharm.* 2:139-150 (2005); and McMillan et al., *Macromolecules* 32(11):3643-3646 (1999)).

[0058] Nucleic acids comprising a polynucleotide sequence encoding a ligand are also provided. Such polynucleotides optionally comprise one or more expression control elements. For example, a polynucleotide can comprise one or more promoters or transcriptional enhancers, ribosomal binding sites, transcription termination signals, and polyadenylation signals, as expression control elements. A polynucleotide can be inserted within any suitable vector, which can be contained within any suitable host cell for expression.

[0059] The expression of nucleic acids encoding ligands is typically achieved by operably linking a nucleic acid encoding the ligand to a promoter in an expression vector. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence. Exemplary promoters useful for expression in *E. coli* include, for example, the T7 promoter.

[0060] Methods known in the art can be used to construct expression vectors containing the nucleic acid sequence encoding a ligand along with appropriate transcriptional/ translational control signals. These methods include, but are not limited to, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic recombination. The expression of the polynucleotide can be performed in any suitable expression host known in the art including, but not limited to, bacterial cells, yeast cells, insect cells, plant cells or mammalian cells. In some embodiments, a nucleic acid sequence encoding a ligand is operably linked to a suitable promoter sequence such that the nucleic acid sequence is transcribed and/or translated into ligand in a host.

[0061] A variety of host-expression vector systems can be utilized to express a nucleic acid encoding a ligand. A vector containing the nucleic acid encoding a ligand (e.g., individual ligand subunits or ligand fusions) or portions or fragments thereof, may include a plasmid vector, a single-stranded phage vector, a double-stranded phage vector, a single-stranded RNA or DNA viral vector, or a double-stranded RNA or DNA viral vector. Phage and viral vectors may also be introduced into host cells in the form of packaged or encapsulated virus using known techniques for infection and transduction. Moreover, viral vectors may be replication competent or alternatively, replication defective. Alternatively, cell-free translation systems may also be used to produce the ligand using RNAs derived from the DNA expression constructs (see, e.g., WO86/05807 and WO89/01036; and U.S. Pat. No. 5,122,464).

[0062] Generally, any type of cell or cultured cell line can be used to express a ligand provided herein. In some embodiments, a background cell line used to generate an engineered host cell is a bacterial cell, a yeast cell or a mammalian cell. A variety of host-expression vector systems may be used to express the coding sequence of a ligand fusion protein. A mammalian cell can be used as a host cell system transfected with recombinant plasmid DNA or a cosmid DNA expression vector containing the coding sequence of the target of interest and the coding sequence of the fusion polypeptide. A cell can be a primary isolate from an organism, culture, or cell line of transformed or transgenic nature.

[0063] Suitable host cells include, but are not limited to, microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing ligand coding sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing ligand coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., Baculovirus) containing ligand coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing ligand coding sequences.

[0064] Prokaryotes useful as host cells in producing a ligand may include gram-negative or gram-positive organisms, such as *E. coli* and *B. subtilis*. Expression vectors for use in prokaryotic host cells generally contain one or more phenotypic selectable marker genes (e.g., genes encoding proteins that confer antibiotic resistance or that supply an autotrophic requirement). Examples of useful prokaryotic host expression vectors include the pKK223-3 (Pharmacia, Uppsala, Sweden), pGEMI (Promega, Wis., USA), pET (Novagen, Wis., USA) and pRSET (Invitrogen, Calif., USA) series of vectors (see, e.g., Studier, J. Mol. Biol. 219:37 (1991) and Schoepfer, *Gene* 124:83 (1993)). Exemplary promoter sequences frequently used in prokaryotic host cell expression vectors include T7 (Rosenberg et al., *Gene* 56:125-135 (1987)), beta-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature* 275:615 (1978)); and Goeddel et al., *Nature* 281 :544 (1979)), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, (1980)), and tac promoter (Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

[0065] In some embodiments, a eukaryotic host cell system is used. In some embodiments, the eukaryotic host cell system is a yeast cell transformed with a recombinant yeast expression vector

containing the coding sequence of a ligand. Exemplary yeast that can be used to produce compositions of the invention, include yeast from the genera *Saccharomyces*, *Pichia*, *Actinomycetes* and *Kluyveromyces*. Yeast vectors typically contain an origin of replication sequence from a 2mu yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Examples of promoter sequences in yeast expression constructs include promoters from metallothionein, 3-phosphoglycerate kinase (Hitzeman, *J. Biol. Chem.* 255:2073 (1980)) and other glycolytic enzymes, such as, enolase, glyceraldehyde-3 -phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phospho glycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Additional suitable vectors and promoters for use in yeast expression as well as yeast transformation protocols are known in the art. See, e.g., Fleer, *Gene* 107:285-195 (1991) and Hinnen, *PNAS* 75:1929 (1978).

[0066] Insect and plant host cell culture systems are also useful for producing the ligands of the invention. Such host cell systems include for example, insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the coding sequence of a ligand; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the coding sequence of a ligand, including, but not limited to, the expression systems taught in U.S. Pat. No. 6,815,184; U.S. Publ. Nos. 60/365,769, and 60/368,047; and WO2004/057002, WO2004/024927, and WO2003/078614.

[0067] In some embodiments, a host cell system may be used. In some embodiments, the host cell system is an animal cell system infected with recombinant virus expression vectors (e.g., adenoviruses, retroviruses, adeno-associated viruses, herpes viruses, lentiviruses). In some embodiments, the host cell system is a cell line engineered to contain multiple copies of the DNA encoding a ligand either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines). In some embodiments, a vector comprising a polynucleotide(s) encoding a ligand is polycistronic. Exemplary mammalian cells useful for producing these compositions include HEK293 cells (e.g., 293T and 293F), CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 (Crucell, Netherlands) cells VERY, HeLa cells, COS cells, MDCK cells, 3T3 cells, W138 cells, BT483 cells, Hs578T cells, HTB2 cells, BT20 cells, T47D cells,

CRL7O30 cells, HsS78Bst cells, hybridoma cells, and other mammalian cells. Additional exemplary mammalian host cells that are useful in practicing the invention include, but are not limited, to T cells. Exemplary expression systems and selection methods are known in the art and may include those described in the following references and references cited therein: Borth et al., *Biotechnol. Bioen.* 71(4):266-73 (2000), in Werner et al., *Arzneimittelforschung/Drug Res.* 48(8):870-80 (1998), Andersen et al., *Curr. Op. Biotechnol.* 13:117-123 (2002), Chadd et al., *Curr. Op. Biotechnol.* 12:188-194 (2001), and Giddings, Curr. Op. Biotechnol. 12:450-454 (2001). Additional examples of expression systems and selection methods are described in Logan et al., *PNAS* 81:355-359 (1984), Birtner et al. *Methods Enzymol.* 153:51-544 (1987)). Transcriptional and translational control sequences for mammalian host cell expression vectors are frequently derived from viral genomes. Commonly used promoter sequences and enhancer sequences in mammalian expression vectors include, sequences derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus (CMV). Exemplary commercially available expression vectors for use in mammalian host cells include pCEP4 (Invitrogen) and pcDNA3 (Invitrogen).

[0068] Physical methods for introducing a nucleic acid into a host cell (e.g., a mammalian host cell) include, but are not limited to, calcium phosphate precipitation, lipofection, particle bombardment, microinjection, and electroporation. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

[0069] Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian cells (e.g., human cells). Other viral vectors may be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses, and adeno-associated viruses. See, for example, U.S. Pat, Nos. 5,350,674 and 5,585,362.

[0070] Methods for introducing DNA and RNA polynucleotides of interest into a host cell include, but are not limited to, the electroporation of a cell, in which an electrical field is applied to a cell in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or polynucleotides to be introduced into the cell. Ligand containing DNA or RNA constructs may be introduced into a mammalian or a prokaryotic cell using electroporation.

[0071] In some embodiments, electroporation of cells results in the expression of a ligand-CAR on the surface of T cells, NK cells, NKT cells. Such expression may be transient or stable over the life of the cell. Electroporation may be accomplished with methods known in the art including MaxCyte GT® and STX® Transfection Systems (MaxCyte, Gaithersburg, MD, USA).

[0072] Chemical means for introducing a polynucleotide into a host cell may include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (e.g., an artificial membrane vesicle). In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (*in vitro, ex vivo* or *in vivo*). In some embodiments, the nucleic acid is associated with a lipid. A nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which can be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

[0073] Lipids suitable for use can be obtained from commercial sources. For example, dimyristoylphosphatidylcholine ("DMPC") can be obtained from Sigma (St. Louis, MO); dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristoylphosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform may be used as the only

solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., *Glycobiology* 5:505-510 (1991)). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids can assume a micellar structure or merely exist as non-uniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

[0074] Regardless of the method used to introduce exogenous nucleic acids into a host cell, the presence of the recombinant nucleic acid sequence in the host cell can routinely be confirmed through a variety of assays known in the art. Such assays include, for example, "molecular biological" assays known in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

[0075] Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism, tissue, or cell and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes include, but are not limited to, genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., *FEBS Lett.* 479:79-82 (2000)). Suitable expression systems are known in the art and can be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions can routinely be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

[0076] A number of selection systems can be used in mammalian host-vector expression systems, including, but not limited to, the herpes simplex virus thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase (Lowy et al., *Cell* 22:817 (1980)) genes. Additionally, antimetabolite resistance can be used as the basis of selection for, e.g., dhfr, gpt, neo, hygro, trpB, hisD, ODC (ornithine decarboxylase), and the glutamine synthase system.

Ligand purification

[0077] Once a ligand or a ligand fusion protein has been produced by recombinant expression, it can be purified by methods known in the art for purification of a recombinant protein, for example, by chromatography (e.g., ion exchange, affinity, and size exclusion chromatography (SEC)), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In some embodiments, a ligand is optionally fused to a heterologous peptide or polypeptide sequence specifically disclosed herein (such as, for example, a His-tag (e.g., a 6xHis-tag) or a biotinylation-tag (e.g., an Avi-tag)) or otherwise known in the art to facilitate purification. In some embodiments, ligands (e.g., antibodies and other affinity matrices) for ligand affinity columns for affinity purification are removed from the composition prior to final preparation of the ligand using techniques known in the art. In some embodiments, the ligand or other components of the ligand fusion composition that are bound by these ligands are removed from the composition prior to final preparation of the ligand using techniques known in the art.

Chemical synthesis of ligand

[0078] In addition to recombinant methods, ligand production may also be carried out using organic chemical synthesis of the desired polypeptide using a variety of liquid and solid phase chemical processes known in the art. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Tam et al., *J. Am. Chem. Soc.*, 105:6442 (1983); Merrifield, *Science*, 232:341-347 (1986); Barany and Merrifield, The Peptides, Gross and Meienhofer, eds, Academic Press, New York, 1- 284; Barany et al., *Int. J. Pep. Protein Res.*, 30:705 739 (1987); Kelley et al. in Genetic Engineering Principles and Methods, Setlow, J. K., ed. Plenum Press, NY. 1990, vol. 12, pp. 1-19; Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co.,

San Francisco, 1989. One advantage of these methodologies is that they allow for the incorporation of non-natural amino acid residues into the sequence of the ligand.

[0079] The ligands that are used in the methods of the present invention may be modified during or after synthesis or translation, e.g., by glycosylation, acetylation, benzylation, phosphorylation, amidation, pegylation, formylation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule, hydroxylation, iodination, methylation, myristoylation, oxidation, prenylation, racemization, selenoylation, sulfation, ubiquitination, etc. (See, e.g., Creighton, Proteins: Structures and Molecular Properties, 2d Ed. (W.H. Freeman and Co., N.Y., 1992); Posttranslational Covalent Modification of Proteins, Johnson, ed. (Academic Press, New York, 1983), pp. 1-12; Seifter, *Meth. Enzymol.*, 182:626-646 (1990); Rattan, *Ann. NY Acad. Sci.*, 663:48-62 (1992)). In some embodiments, the peptides are acetylated at the N-terminus and/or amidated at the C-terminus.

[0080] Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, acetylation, formylation, etc. Additionally, derivatives may contain one or more non-classical amino acids.

[0081] In some embodiments, cyclization, or macrocyclization of the peptide backbone is achieved by sidechain-to-sidechain linkage formation. Methods for achieving this are well known in the art and may involve natural as well as unnatural amino acids. Approaches include disulfide formation, lanthionine formation or thiol alkylations (e.g., Michael addition), amidation between amino and carboxylate sidechains, click chemistry (e.g., azide – alkyne condensation), peptide stapling, ring closing metathesis and the use of enzymes.

Affinity agents for purification

[0082] In purification based on affinity chromatography, a target of interest (preferably a trimeric protein or trimeric vaccine molecule) is selectively isolated according to its ability to specifically and reversibly bind to a ligand that may be covalently coupled to a chromatographic matrix. In some embodiments, ligands may be used as reagents for affinity purification of targets of interest from either recombinant sources or natural sources such as biological samples (e.g., serum, a cell).

[0083] In some embodiments, a ligand that specifically binds a target of interest is immobilized on beads and then used to affinity purify the target.

[0084] Methods of covalently coupling proteins to a surface are known by those of skill in the art. Peptide tags that can be used to attach a ligand to a solid surface are known to those of skill in the art. Further, a ligand may be attached (i.e., coupled, conjugated, linked, or adhered) to a solid surface using any reagents or techniques known in the art. In some embodiments, a solid support comprises beads, glass, slides, chips and/or gelatin. Thus, a series of ligands can be used to make an array on a solid surface using techniques known in the art. For example, U.S. Publ. No. 2004/0009530 discloses methods for preparing arrays.

[0085] In some embodiments, a ligand is used to isolate a target of interest by affinity chromatography. In some embodiments, a ligand is immobilized on a solid support. The ligand can be immobilized on the solid support using techniques and reagents described herein or otherwise known in the art. Suitable solid supports are described herein or otherwise known in the art and, in specific embodiments, are suitable for packing a chromatography column. The immobilized ligand may be loaded or contacted with a solution (e.g., a sample comprising the target of interest) under conditions favorable to form a complex between the ligand and the target of interest. Non-binding materials may be washed away. Suitable wash conditions can readily be determined by one of skill in the art. Examples of suitable wash conditions are described in Shukla and Hinckley, *Biotechnol Prog.* 2008 Sep-Oct;24(5):1115-21. doi: 10.1002/btpr.50.

[0086] In some embodiments, chromatography is carried out by mixing a solution containing a target of interest and a ligand followed by isolation of complexes of a target of interest and a ligand. For example, a ligand is immobilized on a solid support such as beads, then separated from a solution along with a target of interest by filtration. In some embodiments, a ligand is a fusion protein (i.e., a ligand fusion protein) that contains a peptide tag, such as a poly-HIS tail or streptavidin binding region (e.g., a biotinylation tag, such as an Avi-tag), which can be used to isolate the ligand after complexes have formed using an immobilized metal affinity chromatographic resin or streptavidin-coated substrate. Once separated, a target of interest can be released from the ligand under elution conditions and recovered in a purified form.

[0087] In some embodiments, a ligand is isolated that includes the initiator N-terminal methionine since that is the protein sequence encoded by the DNA. In some embodiments, a ligand is isolated without the N-terminal methionine residue. In some embodiments, a mixture is obtained with only a proportion of the purified ligand containing the N-terminal methionine. It is understood by those

skilled in the art that the presence or absence of the N-terminal methionine does not affect the suitability of the ligands for the herein disclosed purposes.

EXAMPLES

Example 1

[0088] Recombinant protein ligands were expressed in *E. coli* and/or *Pichia pastoris* using standard techniques. Ligands were purified using multi-column chromatography. For his-tagged ligands, IMAC was used as the primary capture step. Biotinylated ligands were generated with the Avitag™ system (Avidity, Aurora, CO). Non-biotinylated ligands bearing the Avitag™ sequence were prepared by omitting exogenous biotin. The purity and identity of recombinant protein ligands was assessed by a combination of SDS-PAGE, RP UPLC, quadrupole time-of-flight mass spectrometry and SEC. In many instances, the ligand is isolated without the N-terminal methionine residue, which is presumed to be cleaved during expression. In many instances, a mixture is obtained with only a proportion of the purified ligand containing the N-terminal methionine. It is obvious to those skilled in the art that the presence or absence of the N-terminal methionine does not affect the conclusions herein. For clarity, we include the N-terminal methionine.

Example 2

[0089] This example demonstrates the binding of biotinylated ligands to target protein using biolayer interferometry (ForteBio, Menlo Park, CA). Biotinylated ligands were immobilized on sensors and incubated with solutions containing trimeric vaccine protein at various concentrations. An example sensorgram is shown in Figure 2.

Example 3

[0090] This example demonstrates the sodium hydroxide (NaOH) stability of the affinity ligands. Ligands were incubated in 0.1 M NaOH for a predetermined time and then neutralized. The binding of the NaOH treated ligands was measured as described in Example 2 and compared to untreated ligand. The binding retained was calculated according to the following formula:

$$\% \text{ binding retained} = (\text{measured response after NaOH treatment}) \div (\text{measured response of untreated}) \times 100$$

An example of the stability is shown in Figure 3.

Example 4

[0091] This example demonstrates the production and characterization of affinity agents comprising ligands identified and described herein. Affinity resins were prepared by conjugating ligands to activated agarose beads. After washing, ligands were conjugated to the beads at room temperature. Targeted ligand densities were varied from about 2 g/L to about 20 g/L. After washing, the beads were deactivated with excess thioglycerol. The actual ligand density for all resins was measured using a subtractive RP-HPLC method according to the following formula:

Actual Ligand Density = (Measured [ligand] in feed – Measured [ligand] in effluent).

Example 5

[0092] This example demonstrates the stability of the test resin after a cleaning-in-place (CIP) challenge with 0.1 M sodium hydroxide (NaOH). Resin was prepared from a ligand corresponding to SEQ ID NO: 7. The binding capacity of the resin pre- and post-incubation in 0.1 M NaOH were measured in a binding capacity assay and the results are shown in Figure 4.

Example 6

[0093] This example demonstrates use of affinity agents comprising binding ligands described herein for affinity purification of trimeric proteins. Clarified cell culture feed stream (CCCF) from the production of a trimeric vaccine protein was applied to a 30 mm internal diameter (ID) x 100 mm column packed with resin prepared from a ligand corresponding to SEQ ID NO: 7. The chromatographic method is shown in the following table and the resulting chromatogram is shown in Figure 5.

Step	Buffer	Residence Time (min)	Column volumes
Equilibration	Phosphate Buffered Saline (PBS)	1.4	2.8
Load	CCCF	10	32.1
Equilibration	PBS	4	7.1

Wash	100 mM Na-Octanoate, 25 mM HEPES pH 8	4	7.1
Equilibration	PBS	4	7.1
Elution	50 mM Glycine pH 3, 1 M Arginine	4	7.1
Equilibration	PBS	2	2.8
Strip	0.1 M NaOH, 1 M NaCl	2	7.5
Equilibration	PBS	1.4	14.1

The purity of the eluted material was demonstrated using an SDS-PAGE gel stained with Coomassie Blue and is shown in Figure 6.

[0094] The above examples demonstrate that the affinity resins can be fine-tuned to achieve different performance features that different applications and users may require.

[0095] It is contemplated that various combinations or sub-combinations of the specific features and aspects of the embodiments disclosed above may be made and still fall within the invention. Further, the disclosure herein of any particular feature, aspect, method, property, characteristic, quality, attribute, element, or the like in connection with an embodiment can be used in all other embodiments set forth herein. Accordingly, it should be understood that various features and aspects of the disclosed embodiments can be combined with, or substituted for, one another. Thus, it is intended that the scope of the invention described herein should not be limited by the particular disclosed embodiments described above. Moreover, while the invention is susceptible to various modifications, and alternative forms, specific examples thereof have been shown in the drawings and are herein described in detail. It should be understood, however, that the invention is not to be limited to the particular forms or methods disclosed, but to the contrary, the invention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the various embodiments described.

[0096] Any methods disclosed herein need not be performed in the order recited. The methods disclosed herein include certain actions taken by a practitioner; however, they can also include any third-party instruction of those actions, either expressly or by implication.

Table 6. SEQUENCES

SEQ ID NO:	Amino acid sequence
1	EQRRNFIENLRWDPSKSARLLARAKRFNDW
2	MVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQAPKAA ACGLNDIFEAQKIEWHEHHHHHH
3	MAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAAAHHHHHHC
4	MAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAAAAQHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKA QHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKHHHHHHC
5	MAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAAAAQHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKA QHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKHHHHHHC
6	MAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAAAHHHHHHC
7	MAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAAAAQHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKH HHHHHC
8	MAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAAAAQHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKC
9	MAQGTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQA PKAAAAQHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKA QHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKC
10	MAQHDKIQQAADKEILHLPNLTEEQRNKFRQSLRDDPSVSAEILAEAKKLNDAQAPKAMAQ GTVDAKFDKELEEARAEIERLPNLTEEQRRNFIENLRWDPSKSARLLARAKRFNDWQAPKAA AC

CLAIMS

1. An affinity agent comprising a ligand that binds a trimeric protein, wherein said ligand comprises at least one polypeptide, said polypeptide comprising or consisting of:
   (i) an amino acid sequence as defined by SEQ ID NO: 1; and/or
   (ii) an amino acid sequence that differs by no more than three, by no more than two, or by no more than one amino acid substitution(s), addition(s), or deletion(s) from the amino acid sequence as defined by SEQ ID NO: 1.

2. The affinity agent of claim 1, wherein said at least one polypeptide comprises or consists of:
   (i) an amino acid sequence as defined by any one of SEQ ID NOs: 2–10; and/or
   (ii) an amino acid sequence that differs by no more than three, by no more than two, or by no more than one amino acid substitution(s), addition(s), or deletions from the amino acid sequence as defined by any one of SEQ ID NOs: 2–10.

3. The affinity agent of claim 1 or 2, wherein said ligand comprises:
   a multimer polypeptide comprising at least two subunits, wherein each subunit comprises a polypeptide as defined in claim 1 or 2.

4. The affinity agent of claim 3, wherein the polypeptides comprised in the subunits are not identical in amino acid sequence.

5. The affinity agent of any one of claims 1 to 4, wherein the trimeric protein is a trimeric vaccine protein.

6. The affinity agent of any one of claims 1 to 5, wherein the trimeric protein or the trimeric vaccine protein comprises a trimerization domain.

7. The affinity agent of any one of claims 1 to 6, wherein the ligand is attached to a solid surface.

8. The affinity agent of claim 7, wherein the solid surface comprises or consists of a resin or bead.

9. The affinity agent of claim 7, wherein the solid surface comprises or consists of a membrane.

10. The affinity agent of claim 7, wherein the solid surface comprises or consists of a monolith.

11. The affinity agent of any one of claims 7 to 10, wherein the ligand is covalently or non-covalently conjugated to the solid surface.

12. The affinity agent of any one of claims 7 to 10, wherein the ligand is covalently conjugated to the solid surface via a linker.

13. Use of the affinity agent of any one of claims 1 to 12 for purifying one or more trimeric proteins from a sample comprising said one or more trimeric proteins.

14. The use of claim 13, wherein the trimeric protein is a trimeric vaccine protein.

15. A method of making an affinity agent, the method comprising conjugating a ligand as defined in any one of claims 1 to 6 to a solid surface.

16. The method of claim 15, wherein the ligand is conjugated to the solid surface via a linker.

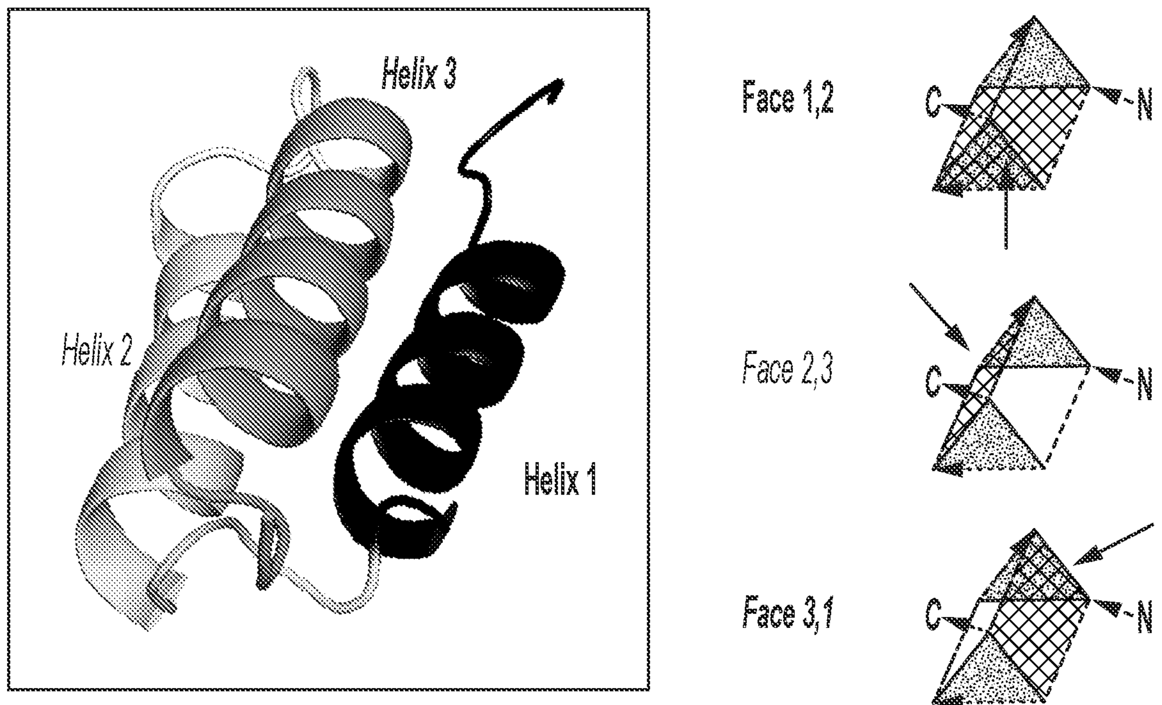

FIG. 1

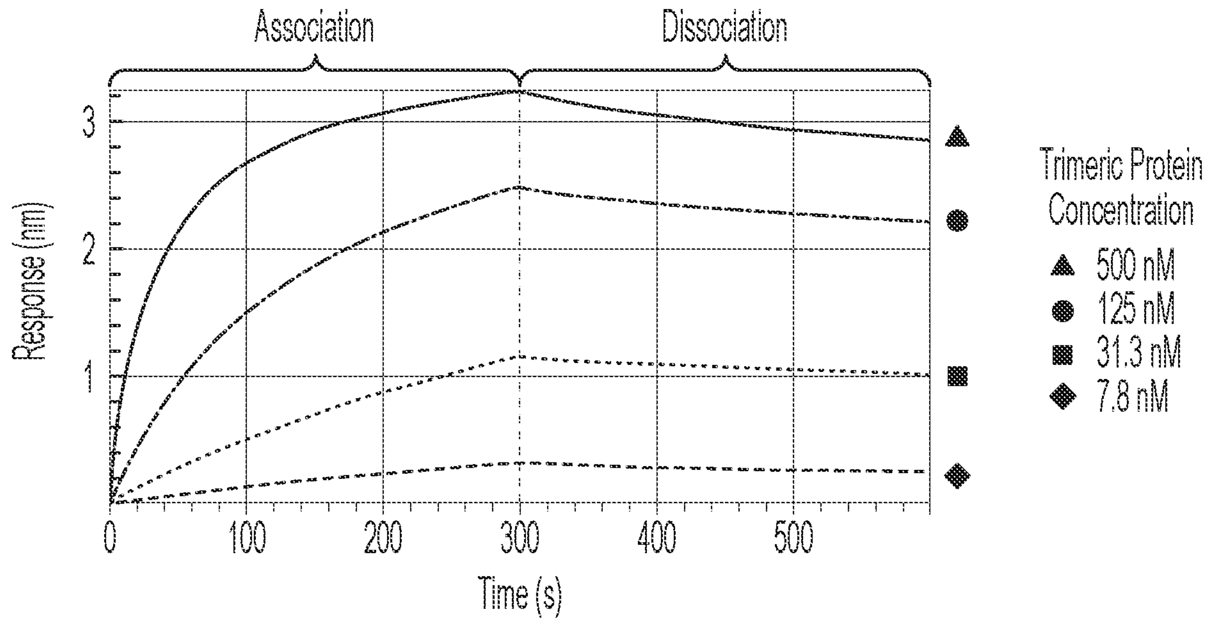

FIG. 2

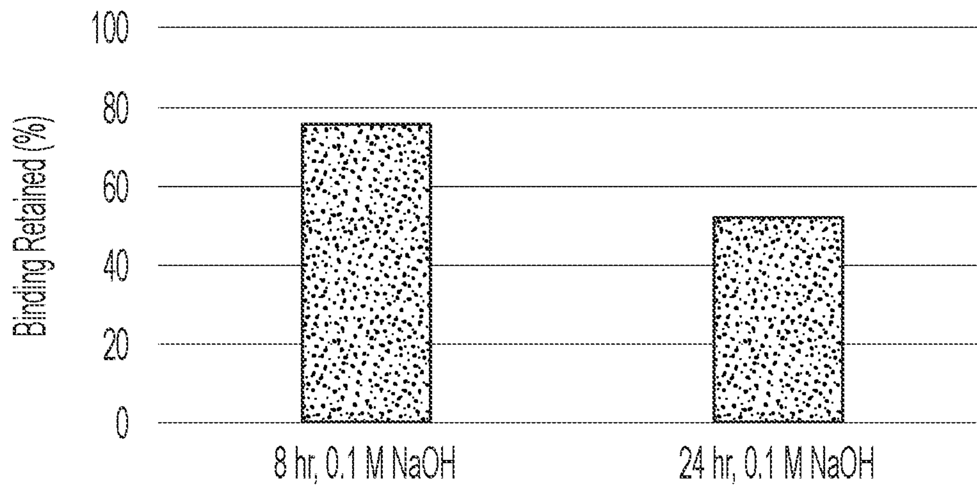

FIG. 3

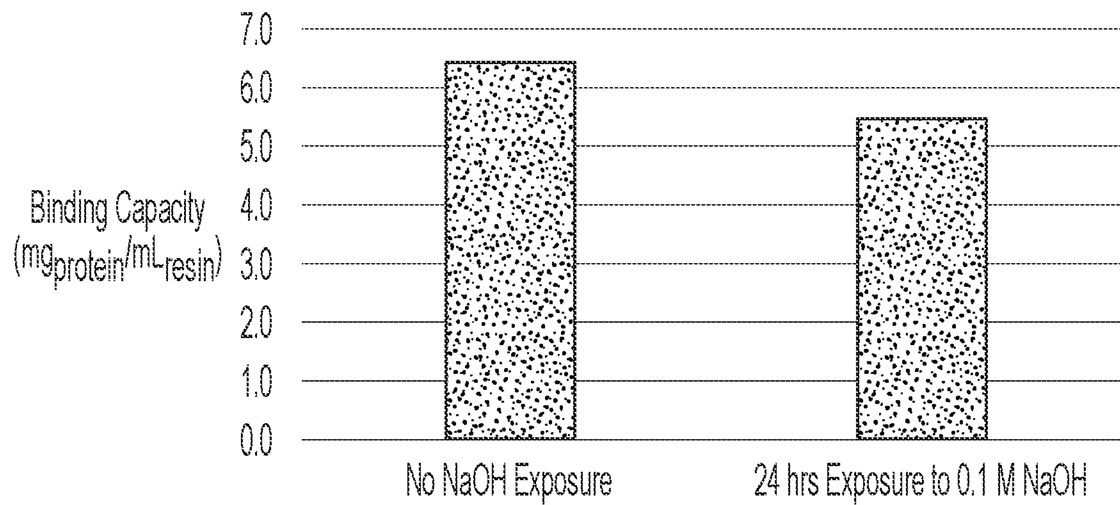

FIG. 4

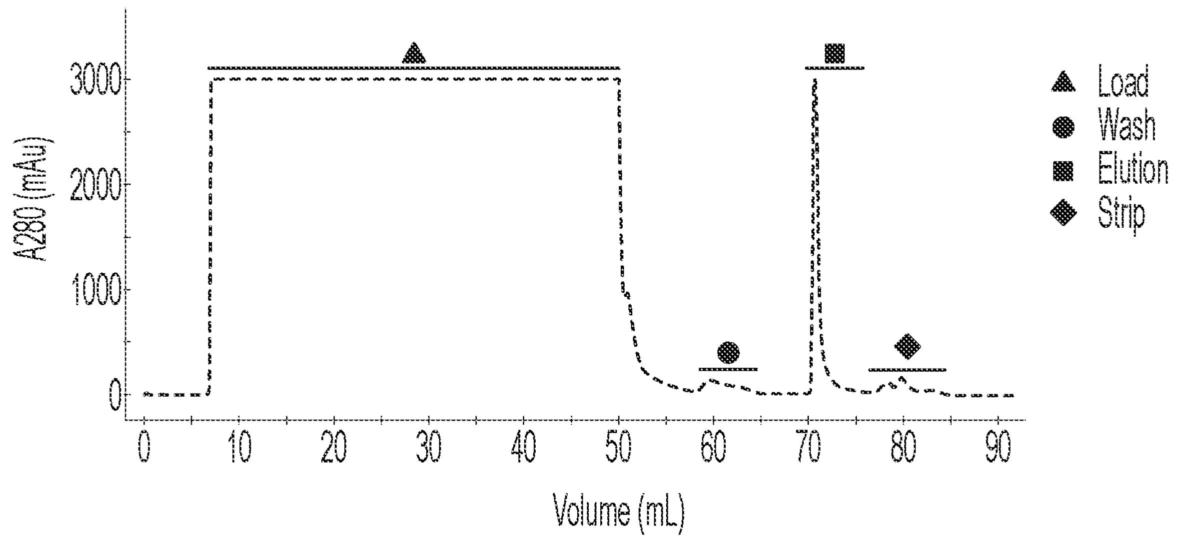

FIG. 5

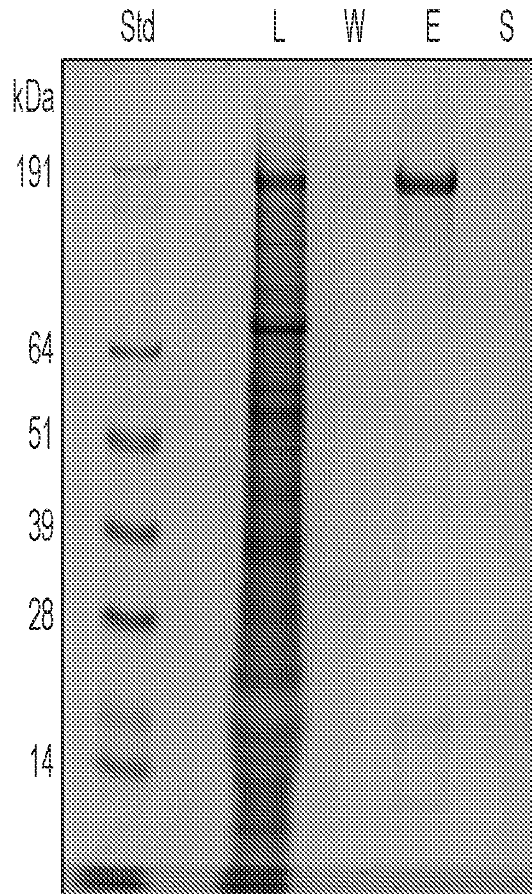

FIG. 6

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13*ter*.1(c) and (d) and 39)

Applicant's or agent's file reference **2011039-0112**	**IMPORTANT DECLARATION**
International application No. **PCT/US2023/032328**	Date of mailing *(day/month/year)* **25 January 2024 (25.01.2024)**
International filing date *(day/month/year)* **08 September 2023 (08.09.2023)**	(Earliest) Priority Date *(day/month/year)* **09 September 2022 (09.09.2022)**
International Patent Classification (IPC) or both national classification and IPC IPC: ***A61P 37/04*** (2023.01) CPC: ***A61P 37/04***	
Applicant **AVITIDE LLC**	

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☐ The subject matter of the international application relates to:
   - a. ☐ scientific theories
   - b. ☐ mathematical theories
   - c. ☐ plant varieties
   - d. ☐ animal varieties
   - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes
   - f. ☐ schemes, rules or methods of doing business
   - g. ☐ schemes, rules or methods of performing purely mental acts
   - h. ☐ schemes, rules or methods of playing games
   - i. ☐ methods for treatment of the human body by surgery or therapy
   - j. ☐ methods for treatment of the animal body by surgery or therapy
   - k. ☐ diagnostic methods practised on the human or animal body
   - l. ☐ mere presentations of information
   - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art
2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

   ☐ the description ☐ the claims ☐ the drawings
3. ☑ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:
   - ☑ furnish a sequence listing complying with WIPO Standard ST.26, and such listing was not available to the International Searching Authority in a form, language and manner acceptable to it.
   - ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a).
4. Further comments:

   An ST.26-compliant sequence listing was not timely received in response to the Invitation to Furnish Nucleotide and/or Amino Acid Sequence Listing (Form PCT/ISA/225), mailed by the ISA/US on 05 December 2023. Pursuant to PCT Rule 13*ter*.1(d),ISA/US cannot carry out a meaningful search of the

Name and mailing address of the ISA/US	Authorized officer
Commissioner for Patents **Mail Stop PCT, Attn: ISA/US** **P.O. Box 1450** **Alexandria, VA 22313-1450, United States of America**	**THOMAS SHANE**
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international application without the sequence listing as all of claims 1-16 contain limitations drawn to sequences.