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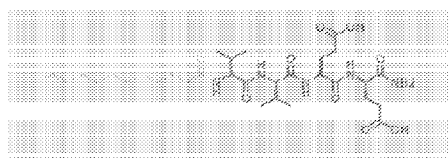
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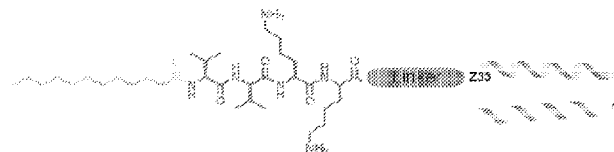
(54) Title: HIGH-AFFINITY SUPRAMOLECULAR POLYMERS FOR BINDING-TRIGGERED ANTIBODY PRECIPITATION AND PURIFICATION

A Filler molecule: C12-VVEE



FIGS. 1A

Ligand molecule: C12-VVKK[Linker]Z33
Z33=FNMQQQRRFYCALHDPNLNCCQRNAKIKSIRDD



(57) Abstract: The disclosure is directed to systems and methods for purifying proteins containing an Fc region, such as antibodies or Fc fusion proteins. The system includes one or more filler molecules comprising a linear hydrocarbon chain conjugated to a peptide amino acid sequence, and one or more ligand molecules comprising a linear hydrocarbon chain, a peptide amino acid sequence, a linker, and a Z33 peptide of *Staphylococcus aureus* Protein A. The filler molecules and ligand molecules self-assemble into immunofibers (IFs) under physiological conditions.



HIGH-AFFINITY SUPRAMOLECULAR POLYMERS FOR BINDING-TRIGGERED ANTIBODY PRECIPITATION AND PURIFICATION

[0001] This application claims priority to United States provisional patent application serial number 63/223,792, filed July 20, 2021, which is incorporated herein by reference in its entirety.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] The text of the computer readable sequence listing filed herewith, titled “JHU-39610-601_SQL”, created July 18, 2022, having a file size of 5,022 bytes, is hereby incorporated by reference in its entirety.

BACKGROUND

[0003] Supramolecular assembly is a bottom-up approach to construct hierarchical and functional nanostructures. Among the recently developed supramolecular polymers, peptide-based materials are notably appealing because of their biocompatibility, biodegradability, and low toxicity. The presentation of bioactive epitopes on a supramolecular substrate enables the active-targeting of the nanostructures for efficient molecular/cellular recognition and signaling, as well as the precise delivery and accumulation of therapeutics at desired sites. In light of the steric hindrance introduced by anchoring these epitopes on supramolecular polymer surfaces, the epitope spatial arrangement plays an important role in regulating their functionality. Co-assembly of bioactive building units with an inert molecule has been shown to effectively modulate the epitope density and achieve enhanced bioactivities. Meanwhile, the use of flexible linkers for spacing out the epitope in the radial direction of the resultant nanostructures is equally essential to improve the epitope accessibility and their interactions with target biomolecules.

[0004] Affinity precipitation has been increasingly explored as a promising alternative to protein A chromatography for the purification of monoclonal antibodies (mAbs) and other therapeutic proteins, as the conventional protein A-based affinity chromatography method suffers from limited production capacities and high media cost. The use of salt to trigger or aid in the precipitation of antibodies is prevalent during the purification process. However,

high salt concentration is known to increase the risks of protein instability and the non-specific precipitation of impurities present in the solution.

[0005] There remains a need for more efficient systems and methods for capturing and purifying antibodies and other proteins.

BRIEF SUMMARY OF THE DISCLOSURE

[0006] The disclosure provides a system comprising: (a) one or more filler molecules comprising a linear hydrocarbon chain conjugated to an amino acid sequence of 2-5 amino acids; (b) one or more ligand molecules comprising a linear hydrocarbon chain, an amino acid sequence of 2-5 amino acids, a linker, and a Z33 peptide of *Staphylococcus aureus* Protein A, or an antibody-binding fragment thereof, conjugated to the linker, wherein the one or more filler molecules and one or more ligand molecules have the ability to self-assemble into immunofibers (IFs) under physiological conditions. Also provided are methods of purifying an antibody and/or fusion Fc protein using the aforementioned system.

BRIEF DESCRIPTION OF THE DRAWING(S)

[0007] Figures 1A-1C include schematic diagrams illustrating the molecular design of the immunofiber (IF) building units with OEG (or PEG) linkers and the formation of IFs for monoclonal antibody (mAb) capture. Figure 1A shows the chemical structures of the filler molecule, C12-VVEE, and the ligand molecules, C12-VVKK[Linker]Z33. Figure 1B is a schematic illustrating the ligand molecule design with various linkers and the effect of linker length on the presentation of Z33 peptide on co-assembled IFs. Figure 1C illustrates co-assembly of filler and ligand molecules into supramolecular IFs and four possible states of mAbs in an IF solution: (1) two Fc-portions of a mAb bound to two ligands from two different IFs; (2) two Fc-portions of a mAb bound to two adjacent ligands from the same IF; (3) one Fc-portion of a mAb bound to one ligand on an IF; (4) free mAbs in the solution bound to zero, one, or two monomer ligands that are not assembled into IFs.

[0008] Figures 2A-2H show the characterization of filler and ligand molecules. Figures 2A-2G are representative TEM images of self-assembled filler and ligand molecules (2A:C12-VVEE, 2B:O4; 2C:O8; 2D:O12; 2E:O16; 2F:O36; and 2G:P2000) in PBS with a diameter of 7.4 ± 0.6 nm, 11.1 ± 0.9 nm, 12.6 ± 1.2 nm, 13.6 ± 1.2 nm, 14.9 ± 1.3 nm, 18.5 ± 1.8 nm, and 19.3 ± 2.0 nm, respectively. Diameters are given as mean $\pm$ SD ($n = 30$). Scale bars: 100 nm. Figure 2H is a graph illustrating normalized CD spectra of self-assembled

ligand molecules with different OEG (or PEG) linkers, suggesting the formation and retention of α -helix secondary structures.

[0009] Figures 3A-3D are graphs illustrating ITC profiles and binding curves for the stepwise injection of 100 μ M mAb1 into 40 μ M O4 (3A), O8 (3B), O12 (3C), O16 (3D) at 25 °C in PBS, pH 7.4.

[0010] Figures 4A-4E show comparisons of mAb precipitation performance of IFs with different ligand molecules. Figures 4A and 4B are graphs illustrating mAb precipitation yields for 20 μ M and 40 μ M mAb1, respectively, incubated with various co-assembled IFs (2.5 mM filler, 100 μ M ligand) under 1 M salt or no salt conditions. The data point for G2 with 1 M salt in Figure 4A was replotted from reference. Figures 4C and 4D are graphs illustrating precipitated mAb mass from 100 μ L samples of 20 μ M (0.3 mg) (4C) and 40 μ M (0.6 mg) (4D) mAb1 incubated with various 100 IFs (2.5 mM filler, 100 μ M ligand) under 1 M salt or no salt conditions. Figure 4E includes photographs of co-assembled IFs (2.5 mM filler, 100 μ M ligand) after mixing with 40 μ M mAb for 5 minutes under 1 M salt or no salt conditions. All experiments were performed in triplicate and the data is shown as mean $\pm$ standard deviation.

[0011] Figures 5A-5F show optimization of mAb precipitation yield. Figures 5A and 5B are graphs illustrating the yield and mass, respectively, of mAb precipitation for 100 μ L mAb1 at 40 μ M (0.6 mg), 80 μ M (1.2 mg), and 133 μ M (2 mg) incubated with IFs at various O16 concentrations. Figure 5C is a graph illustrating a comparison of mAb precipitation yield for 40 μ M mAb1 incubated with optimized IFs (2.5 mM filler, 250 μ M O16) under 1 M salt and no salt conditions. Figure 5D is a schematic diagram showing a proposed mechanism of mAb-IF agglomeration as the ligand concentration increases for a fixed mAb concentration. mAbs at States 3, State 1, and State 2 are dominant at low, medium, and high ligand concentrations, respectively. Figure 5E is a graph showing turbidity of 40 μ M mAb with optimized IFs (2.5 mM filler, 250 μ M O16) in 2 hours. Figure 5F is a graph showing the effect of binding time on the mAb precipitation yield for 40 μ M mAb1 incubated with optimized IFs (2.5 mM filler, 250 μ M O16). All experiments were performed in triplicate and the data is shown as mean $\pm$ standard deviation.

[0012] Figures 6A-6F show purification of mAb from clarified bulk cell culture harvest (abbreviated as “mAb CB”) and separation of mAbs at high titers. Figure 6A is a schematic illustration of the mAb purification process using IFs in PBS solution. Figure 6B is a graph showing mAb precipitation yield, yield loss at wash step, and elution yield for 40 μ M and 80

μ M pure mAb1 after two sequential precipitations under no salt conditions. Figure 6C is a graph showing mAb precipitation yields for 40 μ M and 80 μ M mAb1 CB after two sequential precipitations with IF solutions under no salt conditions. Figure 6D is a schematic illustration of the high titer mAb purification process using solid IFs in lyophilized powder form. Figures 6E and 6F are graphs showing precipitation yields for 21 mg/mL (6E) and 31 mg/mL (6F) pure mAb1 using sequential precipitation with lyophilized IFs under no salt conditions. All experiments were performed in triplicate and the data is shown as mean $\pm$ standard deviation.

[0013] Figure 7 is a graph illustrating mAb precipitation yields for 100 μ L mAb1 at 10 μ M and 20 μ M after an incubation with 100 μ L IFs at various O16 concentrations. IFs with 200 μ M O16 gave the best mAb precipitation yield for both mAb concentrations.

DEFINITIONS

[0014] To facilitate an understanding of the present technology, a number of terms and phrases are defined below. Additional definitions are set forth throughout the detailed description.

[0015] The terms “nucleic acid,” “polynucleotide,” “nucleotide sequence,” and “oligonucleotide” are used interchangeably herein and refer to a polymer or oligomer of pyrimidine and/or purine bases, preferably cytosine, thymine, and uracil, and adenine and guanine, respectively (See Albert L. Lehninger, *Principles of Biochemistry*, at 793-800 (Worth Pub. 1982)). The terms encompass any deoxyribonucleotide, ribonucleotide, or peptide nucleic acid component, and any chemical variants thereof, such as methylated, hydroxymethylated, or glycosylated forms of these bases. The polymers or oligomers may be heterogenous or homogenous in composition, may be isolated from naturally occurring sources, or may be artificially or synthetically produced. In addition, nucleic acids may be DNA or RNA, or a mixture thereof, and may exist permanently or transitionally in single-stranded or double-stranded form, including homoduplex, heteroduplex, and hybrid states. In some embodiments, a nucleic acid or nucleic acid sequence comprises other kinds of nucleic acid structures such as, for instance, a DNA/RNA helix, peptide nucleic acid (PNA), morpholino nucleic acid (see, e.g., Braasch and Corey, *Biochemistry*, 41(14): 4503-4510 (2002) and U.S. Patent 5,034,506), locked nucleic acid (LNA; see Wahlestedt et al., *Proc. Natl. Acad. Sci. U.S.A.*, 97: 5633-5638 (2000)), cyclohexenyl nucleic acids (see Wang, *J. Am. Chem. Soc.*, 122: 8595-8602 (2000)), and/or a ribozyme. The terms “nucleic acid” and “nucleic acid sequence” may also encompass a chain comprising non-natural nucleotides,

modified nucleotides, and/or non-nucleotide building blocks that can exhibit the same function as natural nucleotides (e.g., “nucleotide analogs”).

[0016] The term “amino acid” refers to natural amino acids, unnatural amino acids, and amino acid analogs, all in their D and L stereoisomers, unless otherwise indicated, if their structures allow such stereoisomeric forms.

[0017] Natural amino acids include alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), Lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V).

[0018] Unnatural amino acids include, but are not limited to, azetidinecarboxylic acid, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, naphthylalanine (“naph”), aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, tertiary-butylglycine (“tBuG”), 2,4-diaminoisobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, homoproline (“hPro” or “homoP”), hydroxylysine, allo-hydroxylysine, 3-hydroxyproline (“3Hyp”), 4-hydroxyproline (“4Hyp”), isodesmosine, allo-isoleucine, N-methylalanine (“MeAla” or “Nime”), N-alkylglycine (“NAG”) including N-methylglycine, N-methylisoleucine, N-alkylpentylglycine (“NAPG”) including N-methylpentylglycine. N-methylvaline, naphthylalanine, norvaline (“Norval”), norleucine (“Norleu”), octylglycine (“OctG”), ornithine (“Orn”), pentylglycine (“pG” or “PGly”), pipecolic acid, thioproline (“ThioP” or “tPro”), homoLysine (“hLys”), homoArginine (“hArg”), (S)-N-Fmoc-2-(4'-pentenyl) alanine, Fmoc-2,2-bis(4'-pentenyl) glycine. Other unnatural amino acids that may be employed are disclosed in, e.g., International Patent Application Publication WO 2018/106937.

[0019] The terms “polypeptide” and “protein” are used interchangeably herein and refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones.

[0020] The term, “peptide,” as used herein, includes a sequence of from four to 100 amino acid residues in length, preferably about 10 to 80 residues in length, more preferably, 15 to 65 residues in length, and in which the α -carboxyl group of one amino acid is joined by

an amide bond to the main chain (α - or β -) amino group of the adjacent amino acid. A peptide may comprise natural amino acids, non-natural amino acids, amino acid analogs, and/or modified amino acids. A peptide may be a subsequence of naturally occurring protein or a non-natural (synthetic) sequence.

[0021] As used herein, the term “immuno-amphiphiles” means a molecule that can spontaneously associate into discrete, stable supramolecular nanostructures termed “immunofibers.” Generally, the IFs can assemble in a pH range between about 2.8 to about 7.5. However, the binding properties are also pH dependent. Those IFs which are more positively charged are easier to associate with higher pH solutions, and conversely, negatively charged IFs will associate easier in lower pH solutions.

[0022] As used herein, the term “antibody” refers to an immunoglobulin molecule that is typically composed of two identical pairs of polypeptide chains, each pair having one “light” (L) chain and one “heavy” (H) chain. Human light chains are classified as kappa and lambda light chains. Heavy chains are classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a “J” region of about 12 or more amino acids, with the heavy chain also including a “D” region of about 3 or more amino acids. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH_1 , CH_2 and CH_3 . Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L . The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of each heavy/light chain pair (V_H and V_L), respectively, form the antibody binding site. The term “antibody” encompasses an antibody that is part of an antibody multimer (a multimeric form of antibodies), such as dimers, trimers, or higher-order multimers of monomeric antibodies. It also encompasses an antibody that is linked or attached to, or

otherwise physically or functionally associated with, a non-antibody moiety. Further, the term “antibody” is not limited by any particular method of producing the antibody. For example, it includes, *inter alia*, recombinant antibodies, synthetic antibodies, monoclonal antibodies, polyclonal antibodies, bi-specific antibodies, and multi-specific antibodies.

[0023] The term “monoclonal antibody,” as used herein, refers to an antibody produced by a single clone of B lymphocytes that is directed against a single epitope on an antigen. Monoclonal antibodies typically are produced using hybridoma technology, as first described in Köhler and Milstein, *Eur. J. Immunol.*, 5: 511-519 (1976). Monoclonal antibodies may also be produced using recombinant DNA methods (see, e.g., U.S. Patent 4,816,567), isolated from phage display antibody libraries (see, e.g., Clackson et al. *Nature*, 352: 624-628 (1991)); and Marks et al., *J. Mol. Biol.*, 222: 581-597 (1991)), or produced from transgenic mice carrying a fully human immunoglobulin system (see, e.g., Lonberg, *Nat. Biotechnol.*, 23(9): 1117-25 (2005), and Lonberg, *Handb. Exp. Pharmacol.*, 181: 69-97 (2008)). In contrast, “polyclonal” antibodies are antibodies that are secreted by different B cell lineages within an animal. Polyclonal antibodies are a collection of immunoglobulin molecules that recognize multiple epitopes on the same antigen.

[0024] The terms “Fc region,” “Fc fragment,” or “fragment crystallizable region,” can be used interchangeably herein to refer to the region of a monoclonal antibody comprising the hinge and constant heavy-chain domains (CH2 and CH3). The Fc region mediates downstream effector functions via its interaction with Fc-receptors on (innate) immune cells or with C1q, the recognition molecule of the complement system. The interaction with Fc-receptors can exert a broad range of immunomodulatory functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), in response to infectious agents.

[0025] The terms “fragment of an antibody,” “antibody fragment,” and “functional fragment of an antibody” are used interchangeably herein to mean one or more fragments of an antibody that retain the ability to specifically bind to an antigen (see, generally, Holliger et al., *Nat. Biotech.*, 23(9): 1126-1129 (2005)). An antibody fragment desirably comprises, for example, one or more CDRs, the variable region (or portions thereof), the constant region (or portions thereof), or combinations thereof. Examples of antibody fragments include, but are not limited to, (i) a Fab fragment, which is a monovalent fragment consisting of the V_L, V_H, C_L, and C_{H1} domains, (ii) a F(a')₂ fragment, which is a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region, (iii) a F_v fragment consisting of

the V_L and V_H domains of a single arm of an antibody, (iv) a Fab' fragment, which results from breaking the disulfide bridge of an $F(ab')_2$ fragment using mild reducing conditions, (v) a disulfide-stabilized Fv fragment (dsFv), and (vi) a domain antibody (dAb), which is an antibody single variable region domain (V_H or V_L) polypeptide that specifically binds antigen.

[0026] As used herein, the term “antibody binding peptide” means a peptide that has the ability to bind an antibody, or a specific portion of an antibody molecule, for example, the Fc portion, with high specificity, such as having a K_d of between about 10^{-6} M to about 10^{-10} M.

[0027] As used herein, the term “sample” means any sample, solution, fluid, or mixture containing an antibody of interest or an Fc region-containing protein of interest (e.g., an Fc fusion protein) which can be bound using the immunofibers of the present invention. In some embodiments, the sample can be a biological sample. For example, the sample includes, for example, cell cultures, cell lysates, and/or clarified bulk (e.g., clarified cell culture supernatant). Optionally, the sample is produced from a host cell or organism that expresses the antibody or Fc region-containing protein of interest (either naturally or recombinantly). For example, the cells in a cell culture include host cells transfected with an expression construct containing a nucleic acid that encodes an antibody or Fc fusion protein of interest. These host cells can be bacterial cells, fungal cells, insect cells or, preferably, animal cells grown in culture. The terms “biological sample” and “biological fluid,” as used herein, refers to any quantity of a substance from a living or formerly living patient or mammal or from cultured cells. Such substances include, but are not limited to, blood, serum, plasma, urine, cells, organs, tissues, bone, bone marrow, lymph, lymph nodes, synovial tissue, chondrocytes, synovial macrophages, endothelial cells, skin, cell cultures, cell lysates, and clarified bulk (e.g., clarified cell culture supernatant).

[0028] The terms “monomer,” “monomeric subunit” and “monomeric unit” are used interchangeably herein and refer to one of the basic structural units of a polymer or oligomer. An “oligomer” is a molecule possessing from about 1 to about 30 monomers. The architecture of an oligomer can be, e.g., linear, branched, or forked. An oligomer is a type of polymer. The term “polymer,” as used herein, refers to a substance or material containing large molecules, or macromolecules, composed of many monomers (e.g., hundreds or thousands).

DETAILED DESCRIPTION

[0029] The present disclosure is predicated, at least in part, on the construction of a supramolecular immunofiber (IF) system by co-assembling rationally-designed filler and ligand molecules for the affinity precipitation and purification of monoclonal antibodies (mAbs). In some embodiments, the ligand molecule comprises a protein A mimicking peptide that binds to the Fc-portion of immunoglobulin G (IgG) from most mammalian species in a pH-specific manner. In addition, a series of linkers may be incorporated into the ligand design to increase flexibility and accessibility of the protein A mimicking peptide. Given the dual Fc-binding sites on monoclonal antibodies, specifically IgG, and the multivalence of the immunofibers provided herein, the resulting enhanced multivalent mAb-IF binding could potentially result in IF crosslinking into large complexes and facilitate monoclonal antibody precipitation under low salt or no salt conditions.

[0030] In this regard, the disclosure provides a system comprising one or more filler molecules and one or more ligand molecules that have the ability to self-assemble into immunofibers (IFs) under physiological conditions. The terms “filler” or “filler molecule,” as used herein, refer to a molecule, particle, or compound that is added to a composition that can improve specific properties of the composition. In the context of the present disclosure, the one or more filler molecules are designed to modulate the distribution of the ligand molecule in the co-assembled immunofibers. The terms “ligand” or “ligand molecule,” as used herein, refer to a substance that forms a complex with a biomolecule to produce a biological effect. In some embodiments, a ligand produces a signal by binding to a site on a target protein (e.g., a receptor). Alternatively, a ligand may be a small molecule, ion, or protein which binds to a nucleic acid molecule, such as a DNA double helix. Other types of ligands include, but are not limited to, steroid hormones, growth factors, neurotransmitters, and other peptides.

[0031] In some embodiments, each of the one or more filler molecules and each of the one or more ligand molecules comprises a linear hydrocarbon chain conjugated to an amino acid sequence. It will be appreciated that a “hydrocarbon” is an organic compound consisting entirely of hydrogen and carbon. The linear hydrocarbon chain may be of any suitable length. In some embodiments, a linear hydrocarbon chain comprises from 1 to 24 carbon atoms, for example, 1 to 16 carbon atoms, 1 to 14 carbon atoms, 1 to 12 carbon atoms, 1 to 10 carbon atoms, 1 to 8 carbon atoms, 1 to 6 carbon atoms, or 1 to 4 carbon atoms. In some embodiments, the linear hydrocarbon chain comprises 10-15 carbon atoms (e.g., 10, 11, 12,

13, 14, or 15 carbon atoms). Ideally, the linear hydrocarbon chain comprises 12 carbon atoms. One of ordinary skill in the art will appreciate that there is an upper limit to the number of carbons included in the linear hydrocarbon chain to maintain solubility in an aqueous solution.

[0032] Each of the one or more filler molecules and each of the one or more ligand molecules also comprises an amino acid sequence conjugated to the linear hydrocarbon chain. The amino acid sequence present in the one or more filler molecules may be the same as or different than the amino acid sequence present in the one or more ligand molecules. Ideally, the amino acid sequences present in the filler and ligand molecules are designed to promote interaction among the molecules during co-assembly. The amino acid sequences may be of any suitable length. In some embodiments, the amino acid sequence present in a filler molecule and/or a ligand molecules comprises 1-20 amino acids, such as 1-5 amino acids, 5-10 amino acids, 1-10 amino acids, 10-15 amino acids, or 15-20 amino acids. For example, each of the one or more filler molecules comprises an amino acid sequence of 2-5 amino acids (e.g., 2, 3, 4, or 5 amino acids), and, in some aspects, 4 amino acids. Similarly, each of the one or more ligand molecules comprises an amino acid sequence of 2-5 amino acids (e.g., 2, 3, 4, or 5 amino acids), and, in some aspects, 4 amino acids. An exemplary amino acid sequence for inclusion in the one or more filler molecules and/or the one or more ligand molecules comprises VVXX (SEQ ID NO: 2, with “X” indicating any amino acid). In some embodiments, each of the one or more filler molecules comprises the amino acid sequence VVEE (SEQ ID NO: 3), and each of the one or more ligand molecules comprises the amino acid sequence VVKK (SEQ ID NO: 4). The disclosure is not limited to these particular amino acid sequences, however.

[0033] In addition to the linear hydrocarbon chain and the amino acid sequence, each of the one or more ligand molecules further comprises a linker and a Z33 peptide of *Staphylococcus aureus* Protein A, or an antibody-binding fragment thereof, conjugated to the linker. A “linker” is any chemical moiety that is capable of linking one compound to another compound (e.g., a cell-binding agent such as a peptide ligand or antibody) in a stable, covalent manner. Linkers can be susceptible to, or be substantially resistant to, acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and/or disulfide bond cleavage. In some embodiments, the linker can be any amino acid with a side chain having a free amino, carboxyl or disulfide group. Exemplary amino acids useful as amino acid linkers in the one or more filler molecules and/or one or more ligand molecules

of the present invention include lysine (K), glutamic acid (E), arginine (R) and cysteine (C). Other suitable linkers are well known in the art and include, for example, disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, and esterase labile groups. Linkers also include charged linkers and hydrophilic forms thereof as described herein and known in the art. In some embodiments, the linker may be a cleavable linker, a non-cleavable linker, a hydrophilic linker, and a dicarboxylic acid-based linker.

[0034] In some embodiments, the linker is a hydrophilic linker comprising one or more poly(ethylene glycol) (PEG) molecules or one or more oligo(ethylene glycol) (OEG) molecules. Unless otherwise indicated, a “PEG oligomer” or an oligoethylene glycol (OEG) is one in which all of the monomer subunits are ethylene oxide subunits. Typically, substantially all, or all, monomeric subunits are ethylene oxide subunits, though the oligomer may contain distinct end capping moieties or functional groups, e.g., for conjugation. Typically, PEG oligomers for use in the present disclosure will comprise one of the two following structures: “—(CH₂CH₂O)_n—” or “—(CH₂CH₂O)_{n-1}CH₂CH₂—,” depending upon whether or not the terminal oxygen(s) has been displaced, e.g., during a synthetic transformation. In some embodiments, the variable (n) ranges from 1 to 30, and the terminal groups and architecture of the overall PEG or OEG can vary.

[0035] PEGylation is a process through which polyethylene glycol chains are conjugated to proteins (e.g., therapeutic proteins), peptides, aptamers, enzymes, small molecule drugs, antibodies, and other molecules. Through the PEGylation process, the molecular mass of the conjugated protein is increased, resulting in reduced degradation and increased stability *in vivo*. PEGylation also reduces the immunogenicity of the protein to which it is conjugated. PEG and OEG linkers and related conjugation methods are described in, e.g., U.S. Patent 6,716,821; U.S. Patent 9,388,104; U.S. Patent Application Publication 2009/0285780; and Harris, J.M. and Chess, R.B., *Nature Reviews Drug Discovery*, 2: 214-221 (2003).

[0036] The linker may comprise any suitable number of PEG or OEG molecules, units, or monomers. In some embodiments, the linker comprises 2-50 (e.g., 5, 10, 15, 20, 25, 30, 35, 40, or 45) PEG or OEG molecules. In other embodiments, the linker comprises 10-20 (e.g., 10, 11, 13, 14, 15, 16, 17, 18, 19, or 20) PEG or OEG molecules, or 30-40 (e.g., 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40) PEG or OEG molecules. For example, the linker may comprise 16 OEG or PEG molecules, or 36 OEG or PEG molecules.

[0037] The ligand molecule disclosed herein further comprises a Z33 peptide of *Staphylococcus aureus* Protein A, or an antibody-binding fragment thereof, conjugated to the

linker. Staphylococcal protein A (SPA) is a protein originally found in the cell wall of *Staphylococcus aureus*. It is composed of five homologous domains that fold into a three-helix bundle. Protein A plays an important role in immunology due to its specific binding to the Fc-portion of immunoglobulin G (IgG) from most mammalian species, including human. Extensive structural and biochemical studies of protein A have been conducted. The Z-58 domain of protein A was the first protein A domain widely used in affinity chromatography and affinity precipitation. The minimized binding domain Z-33 was later developed without significantly changing the function of the molecule. The Z33 peptide is a Protein A mimic having the amino acid sequence FNMQQRRFYALHDPNLNEEQRNAKIKSIRDD (SEQ ID NO: 1), and contains a motif of two α -helices. The disclosure also encompasses a ligand molecule comprising any antigen-binding fragment of a Z33 peptide, or a peptide having an amino acid sequence that is at least 95% (e.g., 95%, 96%, 97%, 98%, or 99%) identical to SEQ ID NO: 1. The degree of nucleic acid and/or amino acid identity can be determined using any method known in the art, such as the BLAST sequence database.

[0038] The disclosure further provides a method for purifying an antibody or an Fc fusion protein, which comprises (a) dissolving the above-described system in an aqueous solution at physiological pH and aging overnight, whereby the one or more filler molecules and one or more ligand molecules self-assemble into immunofibers (IFs); (b) mixing a sample containing a protein comprising an Fc region with the IFs under conditions whereby the IFs bind to the Fc region to form an immunofiber-protein complex in solution; (c) separating the immunofiber-protein complex from the solution by adding salt and/or centrifugation; and (d) dissociating the protein comprising an Fc region from the IFs. Any suitable protein that contains an Fc region may be purified using the methods disclosed herein. For example, the Fc region-containing protein may be an antibody, such as a monoclonal antibody. In other embodiments, the Fc region-containing protein may be an Fc fusion protein. An “Fc fusion protein” is a bioengineered polypeptide that joins the crystallizable fragment (Fc) domain of an antibody with another biologically active protein domain or peptide to generate a molecule with unique structure-function properties and, in some cases, therapeutic potential. The gamma immunoglobulin (IgG) isotype is often used as the basis for generating Fc-fusion proteins because of favorable characteristics such as recruitment of effector function and increased plasma half-life. In some embodiments, the system described herein may be used to capture and purify other proteins that do not comprise an Fc region by incorporating customized binding pairs into the design of the filler and ligand molecules.

[0039] “Physiologic” or “physiological” pH is the pH that typically occurs in human cells (e.g., human cells *in vivo*). In this regard, physiologic pH of the human body ranges between 7.35 to 7.45, with the average physiologic pH at 7.40. Once the filler molecules and ligand molecules are dissolved in the aqueous solution at the appropriate pH, the dissolved system is incubated or “aged” for a time sufficient for the one or more filler molecules and one or more ligand molecules to self-assemble into immunofibers (IFs). The incubation or “aging” of the filler and ligand molecules in solution may be for any suitable period of time. In some embodiments, the dissolved system is aged for at least two hours, but not more than 48 hours. For example, the dissolved system may be incubated or aged for 2-8 hours, 8-12 hours, 12-16 hours, 16-20 hours, 20-24 hours, 24-36 hours, or 36-48 hours. In some embodiments, the dissolved system is aged overnight (e.g., about 6, 7, 8, 9, 10, 11, or 12 hours).

[0040] Following a sufficient aging period and formation of IFs, the disclosed method comprises mixing a sample containing an antibody or an Fc fusion protein with the IFs under conditions whereby the IFs bind to the Fc region of the protein (e.g., an antibody or Fc fusion protein) to form an immunofiber-protein complex in solution. Suitable conditions for affinity-based antibody purification methods are known in the art and may be employed in the disclosed method. For example, reagents and conditions suitable for Protein A-based purification are known in the art and may be used in the context of the present disclosure (see, e.g., Proteus Protein A Antibody Purification Handbook, Bio-Rad (2016); and Fishman, J.B., and Berg, E.A., Cold Spring Harb Protoc; doi:10.1101/pdb.prot099143). The complexes formed can then be separated from the unbound immunofibers and Fc region-containing proteins (e.g., antibodies or Fc fusion proteins) and other components in the sample by many known separation means, including, for example, salt-induced precipitation and centrifugation. The separated complexes can then be introduced into another solution at an acidic pH, where the immunofibers lose their binding affinity for the Fc region-containing proteins (e.g., antibodies or Fc fusion proteins).

[0041] The antibody or Fc fusion protein can then be dissociated from the immunofibers by filtration, such as diafiltration, microfiltration, or other means. In some embodiments, the antibody or Fc fusion protein is dissociated from the IFs by lowering the pH to elution conditions (e.g., pH 2.5-4.0) and filtration or microfiltration. In other embodiments, the antibody or Fc fusion protein may be dissociated from the IFs using sequential precipitation. In this regard, a sample containing an Fc region-containing protein (e.g., an antibody or an Fc fusion protein) may be mixed with a first IF solution (prepared as described above), aged or

incubated for any suitable amount of time (e.g., 2, 3, 4, 5, or 6 hours), and centrifuged (e.g., at 10,000-20,000 rpm). The resulting supernatant, which contains immunofiber complexed with Fc region-containing protein, may then be mixed with a second IF solution (prepared as described above), aged or incubated for any suitable amount of time (e.g., 2, 3, 4, 5, or 6 hours), and centrifuged a second time (e.g., at 10,000-20,000 rpm). The aforementioned process may be repeated any number of times until a desired yield of Fc region-containing protein (e.g., antibody or Fc fusion protein) is reached, and the final precipitate is then washed and resuspended in elution buffer. In some embodiments, the eluted antibodies or Fc fusion proteins may be fully recovered using membrane separation methods.

[0042] The system and methods described herein have several advantages as compared to other protein purification methods known in the art, particularly protein A chromatography methods in which the capture step is one of the major downstream bottlenecks due to resin capacity limitation and high production cost. Indeed, the IF system and methods described herein provide for high throughput purification of monoclonal antibodies, ease of handling, and reduced cost. In addition, the disclosed system and methods allow for rapid purification of proteins, in that antibody-IF binding and agglomeration can be completed in 30 minutes or less.

[0043] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLES

[0044] The following materials and methods were used in the experiments described in the Examples.

[0045] Materials. All Fmoc amino acids and resins were obtained from Advanced Automated Peptide Protein Technologies (AAPPTec, Louisville, KY, USA). The pure mAb1, and mAb1 and mAb2 in clarified Chinese hamster ovary (CHO) cell culture harvest were obtained from Bristol-Myers Squibb (Devens, MA, USA). The cell culture was clarified using depth filtration. The Fmoc-N-amido-O(P)EGn-acids were purchased from PurePEG (San Diego, CA, USA) and BroadPharm (San Diego, CA, USA). Lauric acid (C12) was obtained from MilliporeSigma (St. Louis, MO, USA). Unless otherwise specified, all other reagents were obtained from VWR (Radnor, PA, USA) and used as received without further purification.

[0046] Molecular synthesis. Filler and ligand molecules were synthesized using the method described previously. In brief, C12-VVEE and C12-VVKKO(P)EGnGGZ33 (n=4, 8, 12, 16, 36, 45) were synthesized on the Liberty Blue automatic microwave peptide synthesizer (CEM Corporation, Matthews, NC, USA) using standard 9-fluorenyl-methoxycarbonyl (Fmoc) solid phase synthesis protocols. Crude products were cleaved from the solid support using a mixture of trifluoroacetic acid (TFA)/ triisopropylsilane (TIS)/H₂O in a ratio of 92.5:5:2.5 for 2.5 hours. Excess TFA was removed by evaporation and ice-cold diethyl ether was added to precipitate the crude products, followed by centrifugation. The crude products were purified by preparative RP-HPLC using a Varian Polymeric Column (PLRP-S, 100 Å, 10 µm, 150×25 mm) at 25 °C on a Varian ProStar Model 325 preparative HPLC (Agilent Technologies, Santa Clara, CA, USA) monitored at 220 nm for the absorbance of peptide segments. Collected fractions were analyzed by MALDI-TOF (BrukerAutoflex III MALDI-TOF instrument, Billerica, MA, USA) and product-containing fractions were then lyophilized (LABCONCO™ FreeZone -105 °C, 4.5 L freeze dryer, Kansas City, MO, USA) and stored at -30 °C.

[0047] CMC measurement. The CMCs of the ligand molecules in PBS were determined using Nile Red, a hydrophobic dye that undergoes changes in both fluorescence intensity and emission wavelength (a blue-shift) upon partition into the hydrophobic domains of supramolecular assemblies. Nile Red was initially dissolved in acetone at 20 µM and 10 µl aliquots were loaded into several centrifuge tubes. After the acetone evaporated under room temperature, 500 µl fresh ligand solutions in PBS at various concentrations were added into the centrifuge tubes containing dry Nile Red and aged overnight for assembly. Fluorescent spectra of Nile Red were then monitored by a Fluorolog fluorometer (Jobin Yvon, Edison, NJ, USA) with fixed excitation wavelength at 560 nm; emission spectra were monitored at 580-720 nm. The ratio of the emission intensity at 635 nm (emission maximum of Nile red in a hydrophobic environment) to that at 660 nm (emission maximum of Nile red in an aqueous environment) was then plotted against the tested concentrations to obtain a transition curve, and the CMC value was determined by the intersection of the two fitting lines.

[0048] Self-Assembly, Co-Assembly, and TEM Imaging. For self-assembly, filler or ligand molecules were dissolved in PBS (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) to reach a final concentration of 2.5 mM or 400 µM, respectively, and aged for 24 hours at room temperature. To construct co-assembled IFs, filler and ligand molecules were pretreated with hexafluoroisopropanol (HFIP) to eliminate any pre-existing

nanostructures that could be possibly formed during the synthesis and purification process. After HFIP evaporation, filler and ligand molecules were dissolved in PBS (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) to reach desired concentrations and aged for 24 hours at room temperature. 10 μ l stock of each sample solution was then spotted on carbon film coated copper grids with 400 square mesh (Electron Microscopy Sciences, Hatfield, PA, USA) and the excess was removed with filter paper to leave a thin film of sample on the grid. After letting the sample dry for 5 minutes, 10 μ l 2% uranyl acetate was added to the sample grid, and the excess was removed after 30 seconds. All samples were dried for at least three hours before imaging. Bright-field TEM imaging was performed on a FEI Tecnai 12 TWIN Transmission Electron Microscope and all TEM images were required by a SIS Megaview III wide-angle CCD camera.

[0049] CD Spectroscopy. The CD spectra of self-assembled ligand molecules were collected on a Jasco J-710 spectropolarimeter (JASCO, Easton, MD, USA) using a 1 mm path length quartz UV-vis absorption cell (ThermoFisher Scientific, Pittsburgh, PA, USA) at 25 °C. A background spectrum of the solvent was acquired and subtracted from the sample spectrum. The collected data was averaged from three scans and normalized with respect to the ligand concentration.

[0050] ITC Experiment. ITC experiments were performed using a high precision VP-ITC titration calorimetric system (Microcal Inc.). 40 μ M ligand solution was titrated with 100 μ M mAb1 in PBS (pH 7.4) at 25 °C. The heat evolved after each injection was obtained from the integral of the calorimetric signal. The heat associated with the binding between ligand molecule and mAb1 was obtained by subtracting the heat of dilution. Analysis of the data was performed using MicroCal Origin package.

[0051] mAb Precipitation Experiment. IF stock solutions at desired filler and ligand concentrations were prepared one day before the precipitation experiment. In experimental groups, pure mAb1 from a concentrated solution (448 μ M, 64.5 g/L) was then added into 100 μ L IF solutions to reach a desired final concentration and incubated for 30 minutes at room temperature. The solution was then centrifuged at 15000 rpm for 15 minutes. For the 1M salt group, ammonium sulfate was added to the mAb-IF mixtures to reach a final concentration of 1M and incubated for another 30 minutes before centrifugation. The supernatant was taken out and analyzed by ProA-HPLC (POROS™ A 20 μ m Column, Stainless Steel, 2.1 x 30 mm, 0.1 mL) to determine the protein concentrations. The precipitated amount of each species was calculated by subtracting the amount in the

supernatant from the amount added. The precipitation yield was calculated by dividing the added amount by the precipitated amount.

[0052] Sequential Precipitation and mAb Elution Using IF Solutions. Three IF stock solutions, IF1(2.5 mM filler, 250 μ M O16), IF2 (2.5 mM filler, 750 μ M O16), and IF3 (2.5 mM filler, 200 μ M O16), were prepared one day before the precipitation experiment. Pure mAb1 from a concentrated solution (448 μ M, 64.5 g/L) was added into 100 μ L IF1 and 100 μ L IF2 to reach a desired final concentration of 40 μ M and 80 μ M, respectively. After a 4-hour incubation, the samples were then centrifuged at 15000 rpm for 15 minutes and the supernatant was transferred to 100 μ L IF3 for another 4-hour incubation and subsequent centrifugation. 200 μ L PBS and 400 μ L elution buffer (40 mM sodium acetate, pH 3.7) were then used to wash and resuspend the precipitates from the two precipitation steps. The supernatant from each precipitation step, wash step, and elution step were analyzed by ProA-HPLC (POROS™ A 20 μ m Column, Stainless Steel, 2.1 x 30 mm, 0.1 mL) to determine mAb concentration.

[0053] Purification of mAb1 from Clarified Cell Culture Harvest. mAb1 at clarified bulk state were incubated with optimized IFs (100 μ L) at a concentration of 40 μ M or 80 μ M for 30 minutes. Same procedures were performed to precipitate mAb1 and obtain the centrifuged pellets. The pellets were then resuspended in PBS (400 μ L, 40 mM sodium acetate, pH 3.7) and transferred to a dialysis tube (Pur-A-Lyzer Maxi, 50 kDa cut-off molecular weight, Sigma-Aldrich, St. Louis, MO, USA). The resuspended solution was then dialyzed against 40 mM sodium acetate (1 L) at pH 3.7 for 24 hours with dialysis buffer replaced three times. The protein, filler, and ligand concentrations were determined by ProA-HPLC and RP-UPLC.

[0054] Sequential Precipitation of High mAb Titers Using Lyophilized IFs. Six lyophilized IF stock powders (IF4-IF9) were prepared such that upon dissolution in 100 μ L, the concentrations of the filler and O16 would be 2.5 mM and 400 μ M, respectively. Lyophilized IF powders were prepared one day before the precipitation experiment. The IFs were allowed to co-assemble in water for 24 hours prior to lyophilization. Pure mAb1 from concentrated stock solutions of either (146 μ M, 21 g/L) or (215 μ M, 31 g/L) was added to the lyophilized IF4. After a 1-hour incubation, the samples were centrifuged at 15000 rpm for 15 minutes. Following centrifugation, the volume of supernatant was measured and fresh PBS was added until a final supernatant volume of 100 μ L was reached. The 100 μ L supernatant following the first precipitation step was then added to the next lyophilized IF stock powder

(IF5) and allowed to incubate for another hour. This process of incubation, centrifugation, and supernatant transfer was repeated until all six lyophilized IFs (IF4-IF9) had been used for mAb precipitation. The supernatants after the first, third, and sixth precipitation steps were analyzed using ProA-HPLC to measure the cumulative amount of mAbs remaining throughout the precipitation process.

EXAMPLE 1

[0055] This example describes the molecular design and characterization of the system disclosed herein.

[0056] An immunofiber system was formed by the co-assembly of filler and ligand molecules. The filler molecule, C12-VVEE, was designed to modulate the distribution of the ligand molecule in the co-assembled IFs (Figure 1A). The Z33 peptide, which possesses a two-helix motif, is conjugated on the C-terminus of the ligand molecule, C12-VVKK[Linker]Z33, for specific monoclonal antibody capture (Figure 1A). In a previous ligand design (G2), a double glycine (GG) segment was inserted as a linker between the IF surface and Z33. To further increase Z33 flexibility and accessibility, a series of ligand molecules, O4, O8, O12, O16, O36, and P2000, with various OEG (or PEG) linkers (n=4, 8, 12, 16, 36, 45) were designed as summarized in Table 1.

Table 1. Summary of ligand molecule design with various linkers.

Ligand Molecule C12- VVKK[Linker]Z33	Linker	Calculated Fully Extended Linker Length (nm)	Molecular Weight (kDa)	CMC (μ M)
G2	GG	0.7	4.9	2.0
O4	OEG4	1.9	5.0	5.1
O8	OEG8	3.5	5.2	6.0
O12	OEG12	5.1	5.3	8.5
O16	OEG16	6.7	5.5	10.5
O36	OEG36	14.6	6.4	9.1
P2000	PEG2000	18.2	6.8	8.1

[0057] As shown in Figure 1B, with increasing OEG (or PEG) length, the Z33 peptide was expected to be extended further away from the IF surface. It was hypothesized that the incorporation of OEG (or PEG) linkers would further reduce the steric hindrance in the radial direction of the IFs and improve mAb-IF interactions for both Fc binding sites. The filler and

ligand molecules were synthesized using automated solid-phase peptide synthesis (SPPS) methods.

[0058] Figure 1C illustrates the spontaneous co-assembly process of filler and ligand molecules in aqueous solution, forming filamentous IFs with Z33 displayed on the IF surface. Upon the addition of mAbs, there were four possible states of mAbs in the IF solution. States 1 and 2 represent two Fc-portions of a mAb bound to two ligands from two different IFs or the same IF, respectively. In State 3, only one Fc-portion of a mAb is bound to one ligand on an IF. Given the equilibrium between ligand monomers and co-assembled structures, it was also possible that in State 4 mAbs are bound to zero, one, or two monomer ligands in the solution instead of those assembled in IFs. mAb binding and precipitation represent two separate processes. Previous results showed that 1 M salt was not able to precipitate mAb1 directly, but it served as a strong charge screening agent to precipitate almost all IFs and thus precipitate mAbs that are bound to IFs (State 1-3). Under no salt conditions, however, binding to IFs may not be sufficient for mAbs to be precipitated. The key to the precipitation of mAbs under no salt conditions may lie in the formation of the cross-linked mAb-IF complexes, which is mainly triggered by mAbs in State 1.

EXAMPLE 2

[0059] This example describes the molecular assembly and characterization of immunofibers produced using the system and methods disclosed herein.

[0060] To estimate the behavior of the filler and ligand molecules in the co-assembled IFs, their self-assembly properties were first investigated in phosphate-buffered saline (PBS), pH 7.4. As shown in Figure 2A, the filler molecule was able to self-assemble into filamentous structures with a diameter of 7.4 ± 0.6 nm. With OEG (or PEG) linkers, the critical micelle concentrations (CMCs) of ligand molecules were relatively higher than the previous G2 ligand, ranging from 5.1 to 10.5 μ M (Table 1). Representative transmission electron microscopy (TEM) images revealed that each of the ligand molecules can self-assemble into filamentous structures with diameters of 11.1 ± 0.9 nm (O4), 12.6 ± 1.2 nm (O8), 13.6 ± 1.2 nm (O12), 14.9 ± 1.3 nm (O16), 18.5 ± 1.8 nm (O36), and 19.3 ± 2.0 nm (P2000), respectively (Figures 2B-2G). As expected, the IF diameter increased with the increase of the linker length. To gain insight into the molecular packing within the self-assembled ligand molecules, the circular dichroism (CD) spectra were collected for each of the newly designed ligand molecules. The negative peaks around 208 nm and 222 nm

suggest the preservation of α -helix secondary structures in all the self-assembled ligand molecules (Figure 2H), as shown in the original Z33 peptide. Isothermal titration calorimetry (ITC) were used to determine the binding affinity between a monoclonal human IgG1 (144 kDa, abbreviated as “mAb1”) and ligand molecules O4-O16. The dissociation constant K_d ranged between 50 nM and 70 nM, similar to the reported value (26 nM) for free Z33 peptide, showing that the conjugation of OEG linkers within ligand molecules would not significantly impair their mAb binding affinity (Figures 3A-3D).

EXAMPLE 3

[0061] This example demonstrates the impact of linker length and ligand selection on antibody yield.

[0062] To compare the ligand performance, 100 μ M ligand molecules were individually co-assembled with 2.5 mM filler molecules in 100 μ L PBS (pH 7.4) using similar methods described previously. The filler molecule at 2.5 mM (solubility limit) was found to give the best mAb precipitation results and was consistently used in experiments described herein. Pure mAb1 from a stock solution was then added into different IF solutions to reach a final concentration of 20 μ M and incubated for 30 minutes at room temperature. To investigate salt effects on the mAb precipitation yield, two parallel experiments were performed for each co-assembled IF system: one with 1 M salt and one without salt (Figure 4A). After centrifugation, the supernatant was analyzed by ProA-HPLC to determine the remaining mAb concentration and calculate the mAb precipitation yield. With 1M salt, the mAb precipitation yield showed significant improvement from 65% to higher than 88% when replacing the GG linker with O(P)EG linkers in the ligand design. In particular, greater than 95% mAb precipitation yields were obtained for O4-O36. Since there is no bias against the IF precipitation efficiency under 1M salt conditions, this mAb precipitation yield increase from G2 to the new ligand molecules with OEG (or PEG) linkers specifically reflects the significant improvement in the mAb binding efficiency as a result of the increased linker length and flexibility. A slight drop in the mAb precipitation yield was seen for P2000, likely due to the entanglement of long PEG chains that undermines the accessibility of the Z33 peptide for mAb capture.

[0063] Under no salt conditions, the mAb precipitation yields were generally lower than salt groups, while an upward trend was observed with an increase of the OEG linker length. Unlike the salt group, the mAb precipitation yield under no salt conditions was determined by

a combination of mAb binding efficiency and the precipitation efficiency of the mAb-IF complexes. From O4 to O36, there were no obvious differences in the mAb binding efficiency as indicated by the salt group data set. The increase in the mAb precipitation yield under no salt conditions represents an enhancement in the mAb precipitation efficiency, possibly caused by the improved crosslinking between IFs. More importantly, the yield difference between 1M salt and no salt groups was gradually diminished with increasing the linker length, leading to a less than 10% yield discrepancy for O36. This was considered very promising for mAb precipitation, in that the improved mAb-IF interactions achieved by using OEG or (PEG) linkers could potentially substitute for the salt contribution to mAb precipitation.

[0064] To confirm the above observation and have a preliminary understanding of the mAb binding capacity for this IF system, the experiments were repeated with a higher mAb1 concentration (40 μ M). Similar yield trends were observed, as shown in Figure 4B, except for a slightly upward trend in the 1M salt group for O4-O36, indicating an improvement in mAb binding efficiency with increasing the OEG linker length. Despite the consistent drop in the mAb precipitation yield from 20 to 40 μ M mAbs, the precipitated mAb mass for a 100 μ L sample (20 μ M:0.3 mg; 40 μ M:0.6 mg) were actually similar or higher for 40 μ M mAbs (Figures 4C-4D), showing that the Z33 peptide displayed on the IF surfaces were not fully saturated by 20 μ M mAb1 and could potentially be further utilized if more mAbs were added.

[0065] The difference in mAb precipitation yield can also be revealed by the cloudiness of the IF solution. IF solutions of O12, O16, O36, and P2000 became cloudy within 5 minutes after the mAb addition, which was not seen in the first three IF systems. Figure 4E shows the sample vials of O4-O16 IFs after being mixed with 40 μ M mAb1 for 5 minutes under 1 M salt or no salt conditions. Generally, the cloudiness of the salt groups was relatively higher than the no salt groups, suggesting a higher mAb precipitation yield for the salt groups. Meanwhile, the cloudiness increased from O4 to O16 in accordance with the yield trend shown in Figure 4B.

[0066] These results demonstrate that an increase of the linker length can simultaneously improve the mAb binding efficiency of the ligand molecules as well as the precipitation efficiency of the mAb-IF complex. Although O36 showed the best mAb precipitation yields in all conditions, O16 is more desirable in terms of the synthesis yield and material cost and was selected for the subsequent experiments.

EXAMPLE 4

[0067] This example describes the optimization of monoclonal antibody precipitation under no salt conditions using the methods disclosed herein.

[0068] In previous studies, the ligand:mAb molar ratio was shown to play the most significant role in the mAb precipitation yield. To optimize the mAb precipitation yield under no salt conditions and investigate whether the O16 IF system could be efficiently applied to high mAb concentrations, mAb1 at three concentrations, 40 μ M (6 mg/ml), 80 μ M (12 mg/ml), 133 μ M (20 mg/ml), was incubated with IFs formed by 2.5 mM filler molecule and O16 ligand molecule at various concentrations in 100 μ L PBS. Figures 5A and 5B show the plots of mAb precipitation yield and mass versus O16 concentration, respectively, and an optimal point was observed at a ligand:mAb molar ratio between 6:1 to 9:1 for all three mAb concentrations. At the optimal O16 concentration, the mAb precipitation yields for 40 and 80 μ M mAb were around 85%, while only about 65% mAb was precipitated for 133 μ M mAb, showing a decrease in the IF performance when scaling up for high mAb concentrations. To better understand why a 100% mAb precipitation yield was not reached at the optimal O16 concentration, parallel experiments were carried out for 40 μ M mAb with the optimal IFs (2.5 mM filler, 250 μ M O16) under both 1 M salt and no salt conditions (Figure 5C). More than 99% mAb1 was precipitated when 1 M salt was present, indicating a complete capture of mAb1 onto IFs. However, only 88% mAb1 was precipitated for the no salt group, which was attributed to the insufficient IF precipitation that may be caused by the limited IF crosslinking.

[0069] To understand the trend for mAb precipitation yield observed in Figures 5A and 5B, States 3, State 1, and State 2 likely are dominant at low, medium, and high ligand concentrations, respectively (Figure 5D). At low ligand concentration, all ligands are saturated by the excessive mAbs with little left for the binding of the second Fc site (State 3). The initial yield increase could be attributed to the increase in dual site mAb binding for IF crosslinking (State 1) as the ligand concentration increases. After the optimal operating point, further increasing ligand concentration resulted in a yield decline which could be caused by a decrease in the crosslinking efficiency, since chances for mAbs to bind to two Z33 from the same IF (State 2) would be much higher with decreased spacing of O16 ligands on IFs surfaces.

[0070] To gain insight into the kinetics of the agglomeration, the turbidity of a mixture of 40 μ M mAb1 and the optimized IFs (2.5 mM filler, 250 μ M O16) was monitored by the

absorbance at 350 nm for 24 hours. As shown in Figure 5E, the turbidity increased with time and plateaued within 30 minutes, suggesting rapid initial agglomeration. To correlate the solution turbidity to the mAb precipitation yield, the supernatant were analyzed for 30 minutes, 2 hours, and 24 hours (Figure 5F). Consistent with the turbidity study, the mAb-IF binding and agglomeration could nearly complete within 30 minutes to reach a high precipitation yield of 83%. Although a slight yield increase was seen for 2 hours (88%) and 24 hours (92%), which was not revealed by the lower-sensitivity turbidity study, it sacrificed the time efficiency of the mAb precipitation process.

EXAMPLE 5

[0071] This example describes sequential precipitation and elution of monoclonal antibodies.

[0072] To further improve the mAb precipitation yield, a two-step sequential precipitation was conducted to precipitate the remaining mAbs. As depicted in Figure 6A, the supernatant from the first precipitation step was added into fresh IF solution. A wash step was then carried out using PBS to remove the non-specifically bound impurities in the precipitates from the two precipitation steps. To resuspend the precipitated mAbs, elution buffer (40 mM sodium acetate, pH 3.7) was added to dissociate the mAb-IF complexes.

[0073] As a proof of concept, the sequential precipitation was carried out with pure mAb1 at 40 μ M and 80 μ M. For the first precipitation, 40 μ M (6 mg/mL) and 80 μ M (12 mg/mL) mAb1 were incubated with IFs containing 250 μ M and 750 μ M O16, respectively, the optimized condition indicated by Figure 5A. More than 82% mAb1 was precipitated for both groups while 8~20 μ M mAb1 remained in the supernatant (Figure 6B). Based on another set of O16 concentration optimizations shown Figure 7, IFs containing 200 μ M O16 was used for the second precipitation step. Eventually, a final precipitation yield of greater than 97% and ~88% elution yield were achieved for both mAb concentrations with little yield loss during the wash step (Figure 6B). To fully recover the eluted mAbs, a membrane separation step using a 50 kDa cut-off membrane will be conducted to separate the mAb1 (~144 kDa) and dissociated IFs (monomeric size <6 kDa) (Figure 6A). This sequential precipitation using dissolved IFs was then applied to the separation of mAb1 from clarified cell culture harvest at 40 μ M and 80 μ M, where more than 86% and 90% of mAb1 precipitation yield was achieved for the respective mAb titers (Figure 6C).

[0074] To achieve high mAb precipitation yields at mAb titers of over 20 mg/mL, sequential precipitation was carried out using fresh lyophilized IFs containing 2.5 mM filler and 400 μ M O16 mixed with 100 μ L of either 21 mg/mL or 31 mg/mL pure mAb. The mAbs were incubated with lyophilized IFs for 1 hour prior to centrifugation. Following the centrifugation step, the supernatant, still containing mAbs, was measured and fresh PBS was added until a final volume of 100 μ L was reached. The precipitation step was repeated with freshly prepared lyophilized IFs at the same concentration until a total of six precipitation steps had been completed (Figure 6D). The supernatants after the first, third, and sixth precipitation steps were analyzed using ProA-HPLC to measure the cumulative amount of mAbs remaining throughout the precipitation process. As shown in Figure 6E and 6F, nearly all the mAbs were precipitated after six incubation and centrifugation steps, with yields reaching 96% and 98% for 21 mg/mL and 31 mg/mL mAb titers, respectively. Furthermore, after only three precipitation steps, mAb precipitation yields increased drastically from around 20% to 80%, indicating that optimal ligand:protein ratios are reached early in the process as more proteins are captured and separated with each successive precipitation step.

[0075] The above Examples illustrate the design and construction of a series of supramolecular IF systems containing OEG (or PEG) linkers and demonstrate the impact of epitope topography in the radial direction of IFs on the IF bioactivity. The described results reveal that increasing the linker length to OEG16 can simultaneously improve monoclonal binding and precipitation efficiency under no salt conditions. Linkers that are too long, however, show an adverse impact on the function of the resultant supramolecular polymers. Importantly, the mAb precipitation yield under no salt conditions may be efficiently optimized by tuning the ligand concentration to reach desired monoclonal antibody binding states. The strategy of engineering linkers for better epitope presentation sheds important light on the design of supramolecular polymers for specific molecular recognition and targeted drug delivery. The supramolecular IF system described herein can serve as an efficient alternative for the purification of monoclonal antibodies, and may be applied to the capture and purification of other molecules of interest by incorporating customized binding pairs into the system design.

References

[0076] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were

individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

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[0077] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following

claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0078] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIM(S):

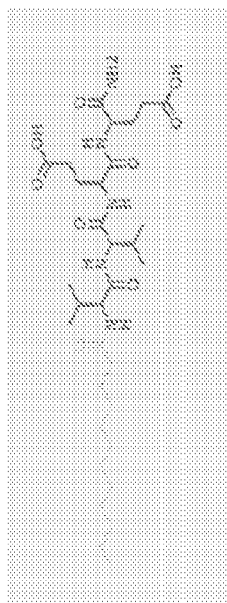
1. A system comprising:
 - (a) one or more filler molecules comprising a linear hydrocarbon chain conjugated to an amino acid sequence of 2-5 amino acids;
 - (b) one or more ligand molecules comprising a linear hydrocarbon chain, an amino acid sequence of 2-5 amino acids, a linker, and a Z33 peptide of *Staphylococcus aureus* Protein A, or an antibody-binding fragment thereof, conjugated to the linker, wherein the one or more filler molecules and one or more ligand molecules have the ability to self-assemble into immunofibers (IFs) under physiological conditions.
2. The system of claim 1, wherein the Z33 peptide comprises the amino acid sequence FNMQQRRFYALHDPNLNEEQRNAKIKSIRDD (SEQ ID NO: 1).
3. The system of claim 1 or claim 2, wherein each of the one or more filler molecules and one or more ligand molecules comprises a linear hydrocarbon chain of 12 carbons in length.
4. The system of any one of claims 1-3, wherein each of the one or more filler molecules and the one or more ligand molecules comprises an amino acid sequence of VVXX (SEQ ID NO: 2).
5. The system of claim 4, wherein the filler molecule comprises the amino acid sequence VVEE (SEQ ID NO: 3).
6. The system of claim 4, wherein the ligand molecule comprises the amino acid sequence VVKK (SEQ ID NO: 4).
7. The system of any one of claims 1-6, wherein the linker comprises one or more oligo(ethylene glycol) (OEG) molecules.
8. The system of any one of claims 1-6, wherein the linker comprises one or more polyethylene glycol (PEG) molecules.
9. The system of claim 7 or claim 8, wherein the linker comprises 2-50 OEG or PEG molecules.

10. The system of claim 9, wherein the linker comprises 36 OEG or PEG molecules.
11. The system of claim 9, wherein the linker comprises 16 OEG or PEG molecules.
12. A method for purifying a protein comprising an Fc region of an antibody, which comprises
 - (a) dissolving the system of any one of claims 1-11 in an aqueous solution at physiological pH and aging overnight, whereby the one or more filler molecules and one or more ligand molecules self-assemble into immunofibers (IFs);
 - (b) mixing a sample containing a protein comprising an Fc region with the IFs under conditions whereby the IFs bind to the Fc region to form an immunofiber-protein complex in solution;
 - (c) separating the immunofiber-protein complex from the solution by adding salt and/or centrifugation; and
 - (d) dissociating the protein comprising an Fc region from the IFs.
13. The method of claim 12, wherein the protein comprising an Fc region is an antibody.
14. The method of claim 12, wherein the protein comprising an Fc region is an Fc fusion protein
15. The method of any one of claims 12-14, wherein the protein comprising an Fc region is dissociated from the IFs by lowering the pH to elution condition and filtration or microfiltration.
16. The method of any one of claims 12-15, which is completed in 30 minutes or less.

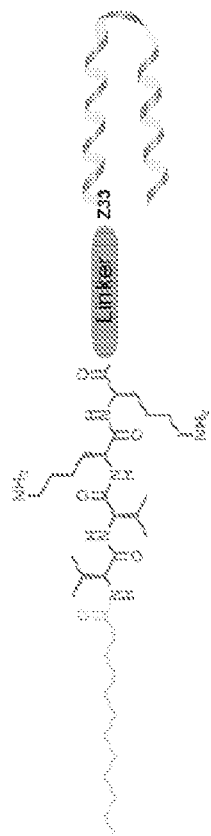
Attiny Docket No. JHU-39610.601
Client Ref. No. C16521_P16521-02

FIGS. 1A-1C

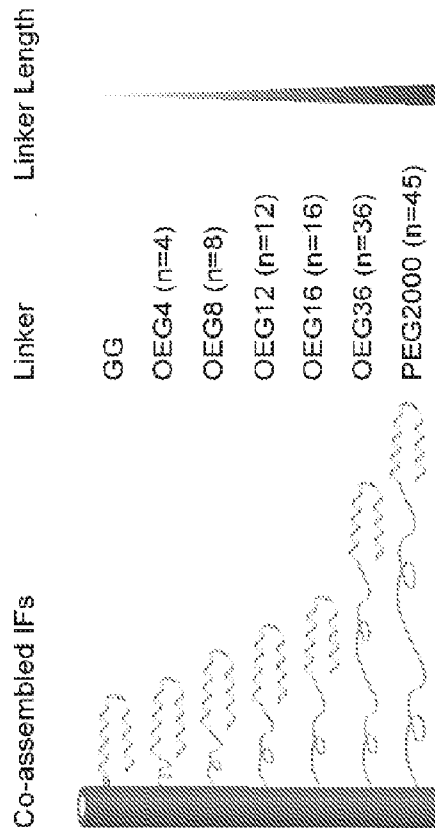
A Filler molecule: C12-WEE



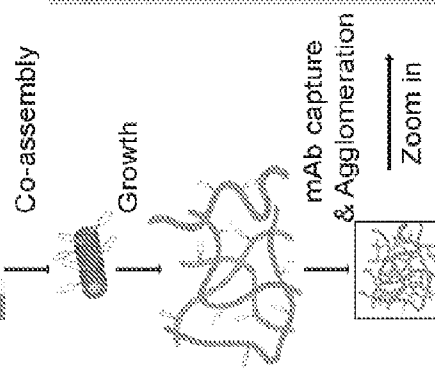
Ligand molecule: C12-VVKK[Linker]Z33
Z33=FNMQQRRFYALHDPNLNEEQRNAKIKSIRDD



B



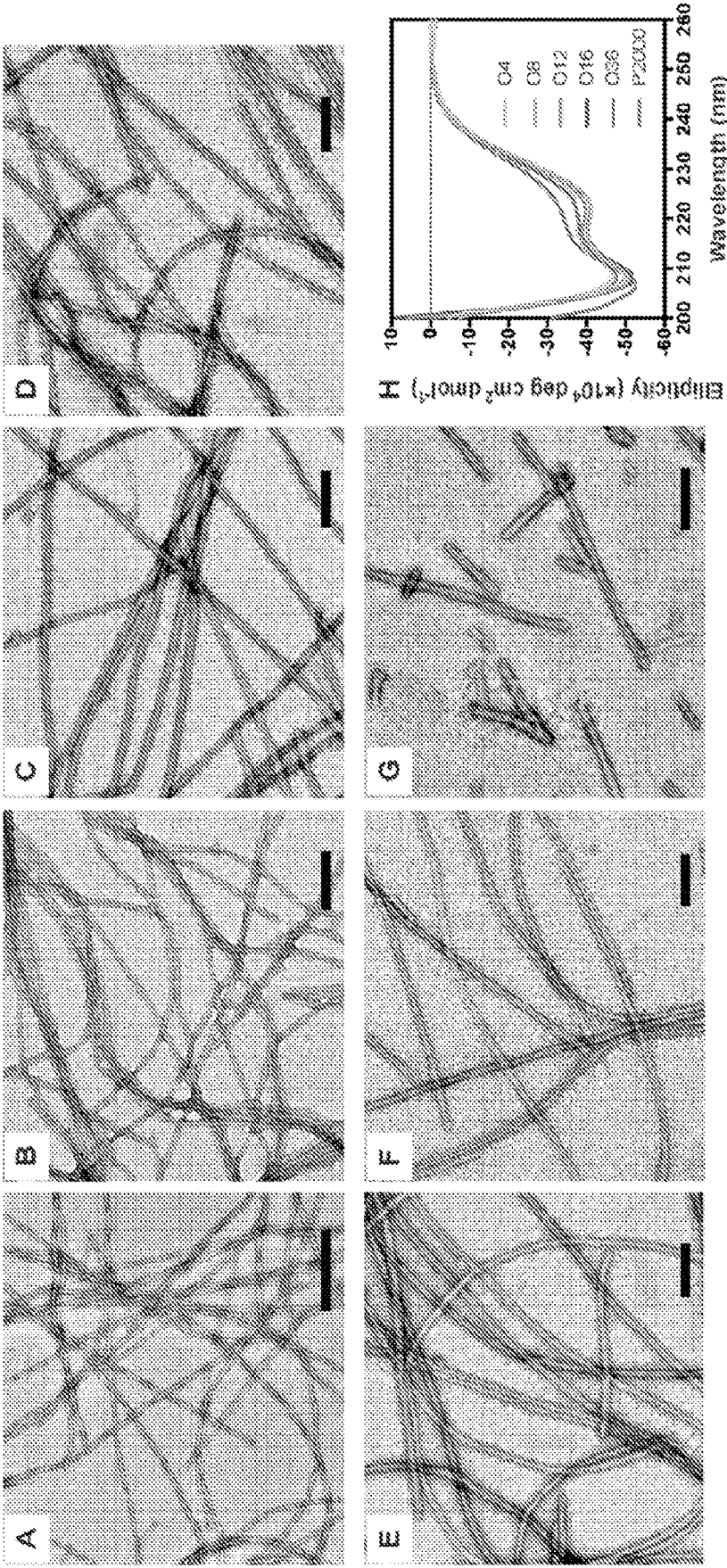
C



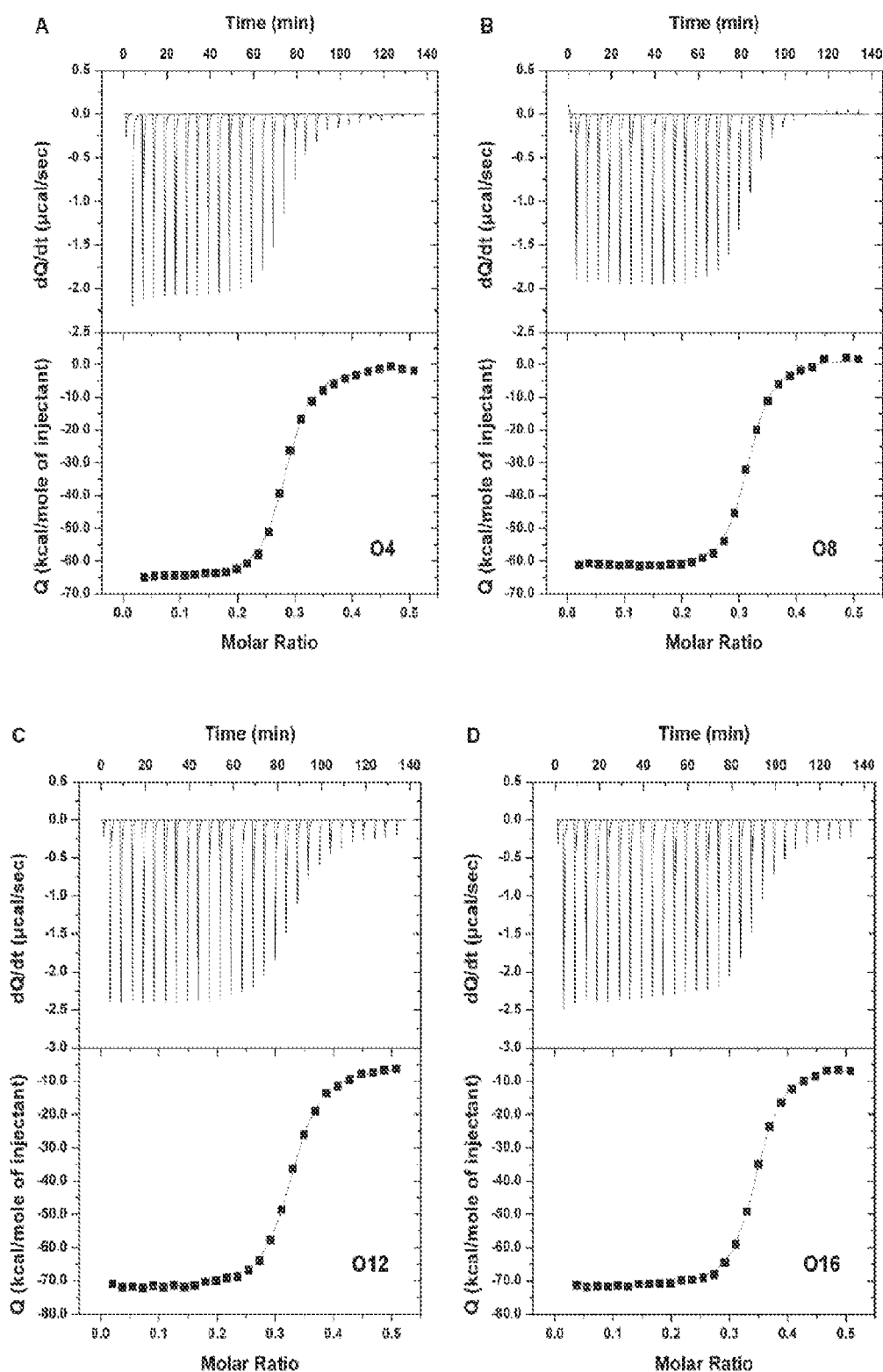
IF backbone GG O(P)EG linker Z33 Filler Ligand Immunofiber mAb

Attmy Docket No. JHU-39610.601
Client Ref. No. C16521_P16521-02

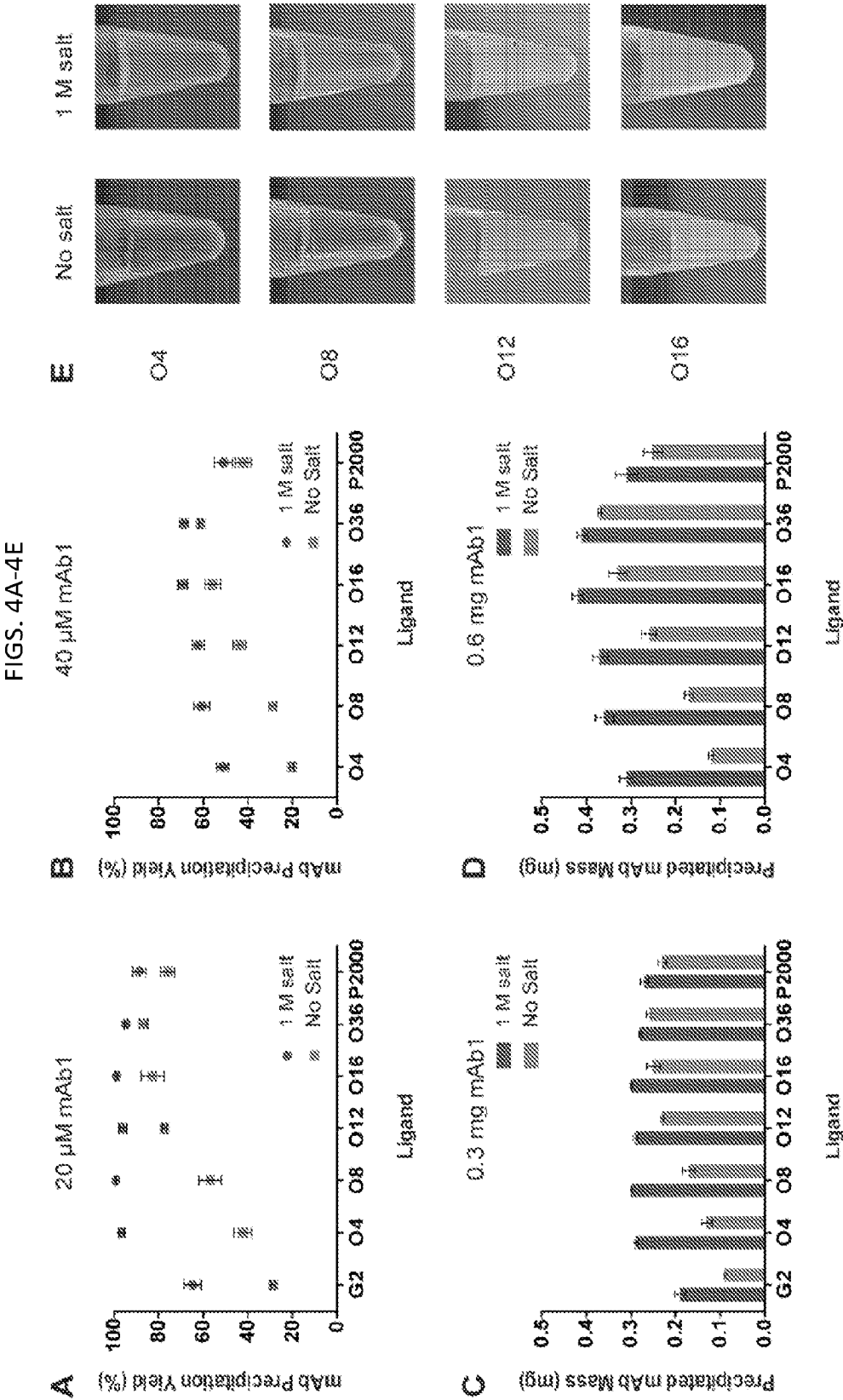
FIGS. 2A-2H



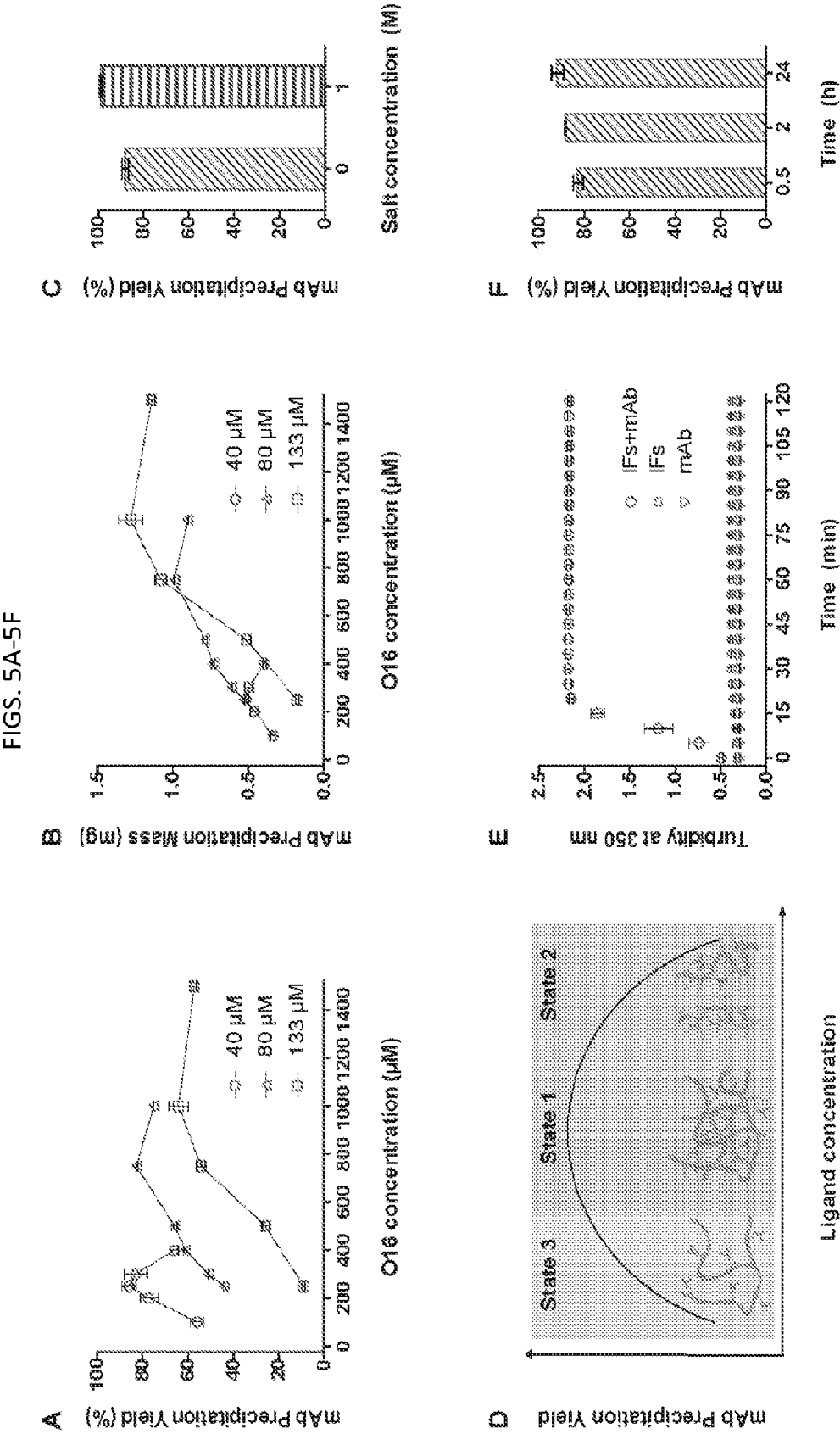
FIGS. 3A-3D



Attiny Docket No. JHU-39610.601
Client Ref. No. C16521_P16521-02



Attiny Docket No. JHU-39610.601
Client Ref. No. C16521_P16521-02



FIGS. 6A-6F

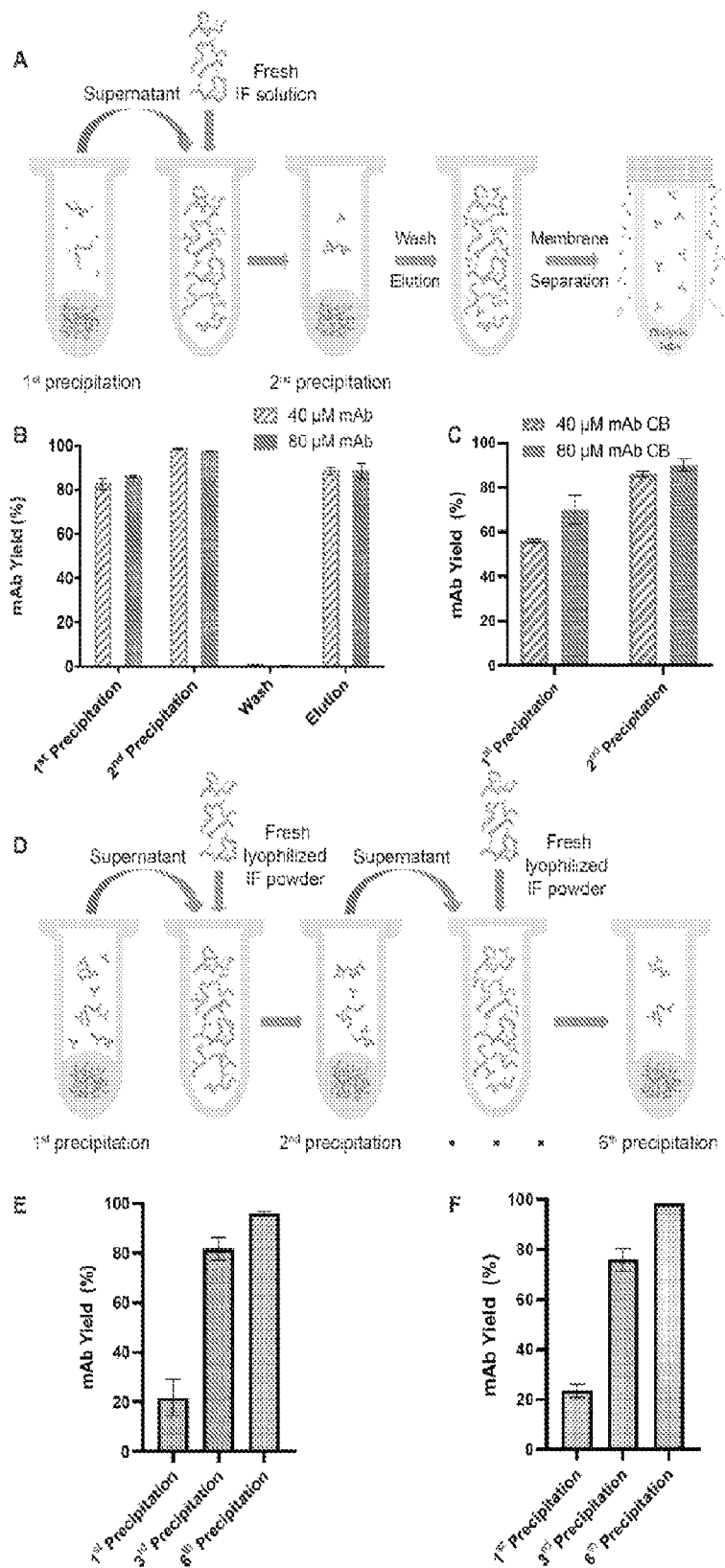
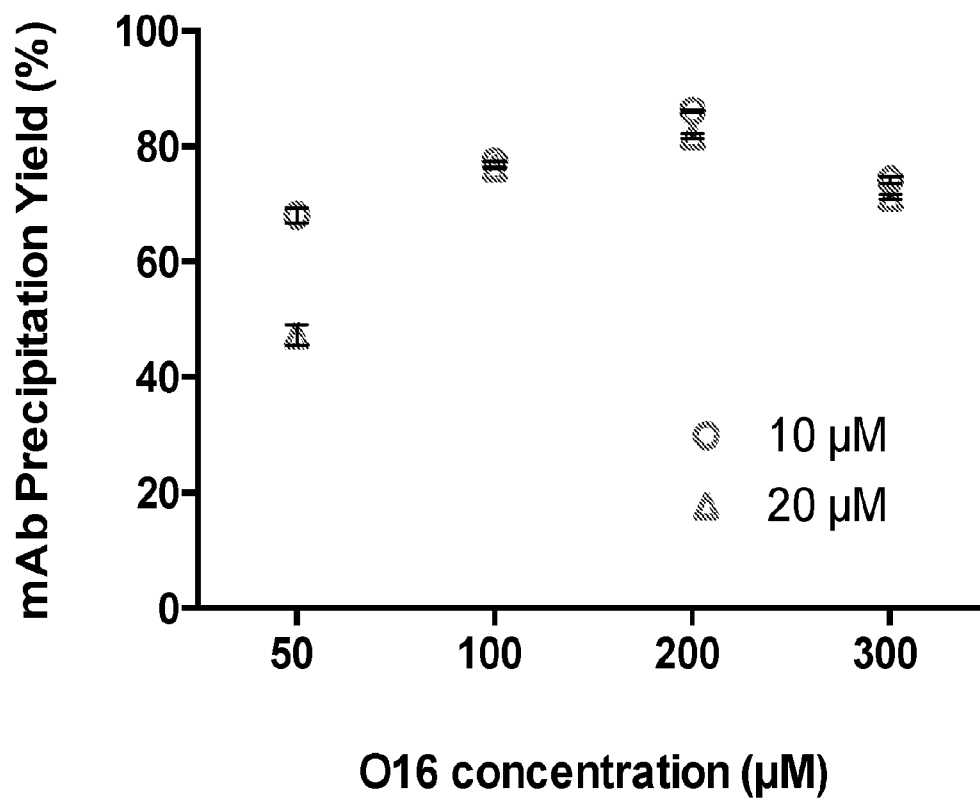


FIG. 7



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/73822

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. C07K 14/31, C07K 17/02, C07K 1/303, C07K 1/22, C07K 5/01 (2022.01)

ADD. C07K 16/06 (2022.01)

CPC - INV. C07K 14/31, C07K 17/02, C07K 1/303, C07K 1/22, C07K 5/01

ADD. C07K 16/065

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/036631 A1 (THE JOHNS HOPKINS UNIVERSITY et al.) 21 February 2019 (21.02.2019) para [0011], [0023], [0043]; claim 8	1-3
A	US 2010/0040646 A1 (NASSAL et al.) 18 February 2010 (18.02.2010); entire document	1-3



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 September 2022

Date of mailing of the international search report

OCT 19 2022

Name and mailing address of the ISA/US

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Authorized officer

Kari Rodriguez

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/73822

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/73822

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.