BLASTN (for nucleotide sequence comparison) and BLASTP (for polypeptide sequence comparison), which programs are based on the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90(12):5873-5877, 1993). When utilizing "BLAST 2 Sequences," parameters that were default parameters as of September 1, 2004, can be used for word size (3), open gap penalty (11), extension gap penalty (1), gap dropoff (50), expect value (10) and any other required parameter including but not limited to matrix option. Two nucleotide or amino acid sequences are considered to have "substantially similar sequence identity" or "substantial sequence identity" if the two sequences have at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity relative to each other.

[0070] As used herein, unless otherwise provided, a position of an amino acid residue in a variable region of an immunoglobulin molecule is numbered according to the IMGT numbering convention (Brochet, X, *et al*, Nucl. Acids Res. (2008) 36, W503-508), and a position of an amino acid residue in a constant region of an immunoglobulin molecule is numbered according to EU nomenclature (Ward *et al.*, 1995 *Therap. Immunol.* 2:77-94). Other numbering conventions are known in the art (e.g., the Kabat numbering convention (Kabat, Sequences of Proteins of Immunological Interest, 5th ed. Bethesda, MD: Public Health Service, National Institutes of Health (1991)).

[0071] As used herein, the term "dimer" refers to a biological entity that consists of two subunits associated with each other via one or more forms of intramolecular forces, including covalent bonds (*e.g.*, disulfide bonds) and other interactions (*e.g.*, electrostatic interactions, salt bridges, hydrogen bonding, and hydrophobic interactions), and is stable under appropriate conditions (*e.g.*, under physiological conditions, in an aqueous solution suitable for expressing, purifying, and/or storing recombinant proteins, or under conditions for non-denaturing and/or non-reducing electrophoresis). A "heterodimer" or "heterodimeric protein," as used herein, refers to a dimer formed from two different polypeptides. A heterodimer does not include an antibody formed from four polypeptides (*i.e.*, two light chains and two heavy chains). A "homodimer" or "homodimeric protein," as used herein, refers to a dimer formed from two identical polypeptides. All disclosure of the polypeptide, including characteristics and activities (such as binding and RTCC) should be understood to include the polypeptide in its dimer form as well as other multimeric forms.

PGR2026-00039
Biocon Ex.1002
Page 4621 of 4945

[0072] When a polypeptide of the disclosure is in dimeric form (i.e., a dimeric protein), it contains two binding sites at the amino-terminus and two binding sites at the carboxyl terminus. The binding domains are thus considered bivalent (i.e., two binding portions at each terminus) when the single chain polypeptides are dimerized.

[0073] An "immunoglobulin constant region" or "constant region" is a term defined herein to refer to a peptide or polypeptide sequence that corresponds to or is derived from part or all of one or more constant domains of an immunoglobulin. In certain embodiments, the constant region comprises IgG CH2 and CH3 domains, *e.g.*, IgG1 CH2 and CH3 domains. In certain embodiments, the constant region does not comprise a CH1 domain. In certain embodiments, the constant domains making up the constant region are human. In some embodiments, the constant region of a fusion protein of this disclosure lacks or has minimal effector functions while retaining the ability to bind some Fc receptors such as the neonatal Fc receptor (FcRn) and retaining a relatively long half-life *in vivo*. For example, the constant region of a fusion protein of this disclosure do not result in, or substantially reduce the induction of antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), complement activation, and/or complement-dependent cytotoxicity (CDC). In other variations, a fusion protein of this disclosure comprises constant domains that retain one or more effector functions, such as of one or both of ADCC and CDC. In certain embodiments, a binding domain of this disclosure is fused to a human IgG1 constant region, wherein the IgG1 constant region has one or more of the following amino acids mutated: leucine at position 234 (L234), leucine at position 235 (L235), glycine at position 237 (G237), glutamate at position 318 (E318), lysine at position 320 (K320), lysine at position 322 (K322), or any combination thereof (numbering according to EU). For example, any one or more of these amino acids can be changed to alanine. In a further embodiment, an IgG1 Fc domain has each of L234, L235, G237, E318, K320, and K322 (according to EU numbering) mutated to an alanine (*i.e.*, L234A, L235A, G237A, E318A, K320A, and K322A, respectively), and optionally an N297A mutation as well (*i.e.*, essentially eliminating glycosylation of the CH2 domain).

[0074] The terms "light chain variable region" (also referred to as "light chain variable domain" or "V_L") and "heavy chain variable region" (also referred to as "heavy chain variable domain" or "V_H") refer to the variable binding region from an antibody light and heavy chain, respectively. The variable binding regions are made up of

PGR2026-00039
Biocon Ex.1002
Page 4622 of 4945

discrete, well-defined sub-regions known as "complementarity determining regions" (CDRs) and "framework regions" (FRs). In one embodiment, the FRs are humanized. The term "CL" refers to an "immunoglobulin light chain constant region" or a "light chain constant region," i.e., a constant region from an antibody light chain. The term "CH" refers to an "immunoglobulin heavy chain constant region" or a "heavy chain constant region," which is further divisible, depending on the antibody isotype into CH1, CH2, and CH3 (IgA, IgD, IgG), or CH1, CH2, CH3, and CH4 domains (IgE, IgM). A "Fab" (fragment antigen binding) is the part of an antibody that binds to antigens and includes the variable region and CH1 domain of the heavy chain linked to the light chain via an inter-chain disulfide bond.

[0075] As used herein, the term "linker" generally refers to a short polypeptide sequence connecting two sub-domains of a polypeptide. Non-limiting examples of linkers include flexible linkers comprising glycine-serine repeats, and linkers derived from (a) an interdomain region of a transmembrane protein (*e.g.*, a type I transmembrane protein); or (b) an immunoglobulin hinge. In some embodiments, a linker provides a spacer function compatible with interaction of the two sub-binding domains so that the resulting polypeptide retains a specific binding affinity to the same target molecule as an antibody that comprises the same light and heavy chain variable regions. In certain embodiments, a linker is comprised of five to about 35 amino acids, for instance, about 15 to about 25 amino acids. As used herein, the phrase a "linker between CH3 and CH1 or CL" refers to one or more amino acid residues (*e.g.*, about 2-12, about 2-10, about 4-10, about 5-10, about 6-10, about 7-10, about 8-10, about 9-10, about 8-12, about 9-12, or about 10-12) between the C-terminus of a CH3 domain (*e.g.*, a wild type CH3 or a mutated CH3) and the N-terminus of a CH1 domain or CL domain (*e.g.*, Cκ).

[0076] In some embodiments, depending on context, a linker may refer to (1) a polypeptide region between V_H and V_L regions in a single-chain Fv (scFv) or (2) a polypeptide region between a first binding domain and a second binding domain in a multispecific polypeptide comprising two binding domains. In the later example, wherein a linker connects two or more binding domains, such a linker is referred to herein as a "Fc-binding domain linker." In some embodiments, a Fc-binding domain linker may directly link or connect two or more binding domains, resulting in a construct comprising the following structure: binding domain – Fc-binding domain linker – binding domain. In some embodiments, the multispecific polypeptides described

PGR2026-00039
Biocon Ex.1002
Page 4623 of 4945

herein comprise, in order from amino-terminus to carboxyl-terminus (i) a first binding domain, (ii) a Fc-binding domain linker, and (iii) a second binding domain. In some embodiments, a multispecific polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a second binding domain, (ii) a Fc-binding domain linker, and (iii) a first binding domain. In some embodiments, a Fc-binding domain linker may link or connect two or more binding domains by linking at least one binding domain to a non-binding domain polypeptide, such as an immunoglobulin Fc domain (*i.e.*, a polypeptide comprising the structure: Ig hinge – Ig constant region). In such embodiments, the resulting constructs may comprise the following structure: binding domain – Fc domain – Fc-binding domain linker – binding domain. In some embodiments, the multispecific polypeptides described herein comprise, in order from amino-terminus to carboxyl-terminus: (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, (iv) a Fc-binding domain linker, and (v) a second binding domain. In some embodiments, a multispecific polypeptide comprises, in order from amino-terminus to carboxyl-terminus (i) a second binding domain, (ii) a Fc-binding domain linker, (iii) an immunoglobulin constant region, (iv) a hinge region, and (v) a first binding domain. A polypeptide region between an immunoglobulin constant region and a second binding domain in a multispecific polypeptide comprising two binding domains (*e.g.*, a Fc-binding domain linker) may also be referred to as a "carboxyl-terminus linker" or an "amino-terminus linker" depending on the orientation of the domains within the multispecific polypeptide. Non-limiting examples of linkers are provided in Table 1.

[0077] In some embodiments, a "hinge" or a "hinge region" refers to a polypeptide derived from an immunoglobulin hinge region and located between a binding domain and an immunoglobulin constant region in a polypeptide described herein. A "wild-type immunoglobulin hinge region" refers to a naturally occurring upper and middle hinge amino acid sequences interposed between and connecting the CH1 and CH2 domains (for IgG, IgA, and IgD) or interposed between and connecting the CH1 and CH3 domains (for IgE and IgM) found in the heavy chain of an antibody. In certain embodiments, a wild type immunoglobulin hinge region sequence is human, and can comprise a human IgG hinge region (*e.g.*, and IgG1, IgG2, IgG3, or IgG4 hinge region).

[0078] An "altered immunoglobulin hinge region" or "variant immunoglobulin hinge region" refers to a hinge region polypeptide with one or more mutations, substitutions, insertions, or deletions compared to a corresponding parental wild-type immunoglobulin hinge region. In certain embodiments, an altered immunoglobulin

PGR2026-00039
Biocon Ex.1002
Page 4624 of 4945

hinge region is at least about 70% identical to a wild-type immunoglobulin hinge region (*e.g.*, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or at least about 99% identical). In certain embodiments, an altered immunoglobulin hinge region is a fragment of a wild type immunoglobulin hinge region that has a length of about 5 amino acids (*e.g.*, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, or more amino acids) up to about 120 amino acids (for instance, having a length of about 10 to about 40 amino acids or about 15 to about 30 amino acids or about 15 to about 20 amino acids or about 20 to about 25 amino acids). Typically, an altered immunoglobulin hinge region that is a fragment of a wild type immunoglobulin hinge region comprises an IgG core hinge region (*e.g.*, a polypeptide comprising the sequence C-X-X-C, wherein X is any amino acid (SEQ ID NO: 390)) as disclosed in U.S. Patent Application Publication Nos. 2013/0129723 and 2013/0095097. Non-limiting examples of hinges are provided in Table 2.

[0079] As used herein, the term "humanized" refers to a process of making an antibody or immunoglobulin binding proteins and polypeptides derived from a non-human species (*e.g.*, mouse or rat) less immunogenic to humans, while still retaining antigen-binding properties of the original antibody, using genetic engineering techniques. In some embodiments, the binding domain(s) of an antibody or immunoglobulin binding proteins and polypeptides (*e.g.*, light and heavy chain variable regions, Fab, scFv) are humanized. Non-human binding domains can be humanized using techniques known as CDR grafting (Jones *et al.*, Nature 321:522 (1986)) and variants thereof, including "reshaping" (Verhoeyen, *et al.*, 1988 Science 239:1534-1536; Riechmann, *et al.*, 1988 Nature 332:323-337; Tempest, *et al.*, Bio/Technol 1991 9:266-271), "hyperchimerization" (Queen, *et al.*, 1989 Proc Natl Acad Sci USA 86:10029-10033; Co, *et al.*, 1991 Proc Natl Acad Sci USA 88:2869-2873; Co, *et al.*, 1992 J Immunol 148:1149-1154), and "veneering" (Mark, *et al.*, "Derivation of therapeutically active humanized and veneered anti-CD18 antibodies." In: Metcalf BW, Dalton BJ, eds. Cellular adhesion: molecular definition to therapeutic potential. New York: Plenum Press, 1994; 291-312). If derived from a non-human source, other regions of the antibody or immunoglobulin binding proteins and polypeptides, such as the hinge region and constant region domains, can also be humanized.

PGR2026-00039
Biocon Ex.1002
Page 4625 of 4945

[0080] An "immunoglobulin dimerization domain" or "immunoglobulin heterodimerization domain", as used herein, refers to an immunoglobulin domain of a polypeptide chain that preferentially interacts or associates with a different immunoglobulin domain of a second polypeptide chain, wherein the interaction of the different immunoglobulin heterodimerization domains substantially contributes to or efficiently promotes heterodimerization of the first and second polypeptide chains (*i.e.*, the formation of a dimer between two different polypeptide chains, which is also referred to as a "heterodimer"). The interactions between immunoglobulin heterodimerization domains "substantially contributes to or efficiently promotes" the heterodimerization of first and second polypeptide chains if there is a statistically significant reduction in the dimerization between the first and second polypeptide chains in the absence of the immunoglobulin heterodimerization domain of the first polypeptide chain and/or the immunoglobulin heterodimerization domain of the second polypeptide chain. In certain embodiments, when the first and second polypeptide chains are co-expressed, at least 60%, at least about 60% to about 70%, at least about 70% to about 80%, at least 80% to about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% of the first and second polypeptide chains form heterodimers with each other. Representative immunoglobulin heterodimerization domains include an immunoglobulin CH1 domain, an immunoglobulin CL domain (*e.g.*, Cκ or Cλ isotypes), or derivatives thereof, including wild type immunoglobulin CH1 and CL domains and altered (or mutated) immunoglobulin CH1 and CL domains, as provided therein.

[0081] The terms patient and subject are used interchangeably herein. As used herein, the term "patient in need" or "subject in need" refers to a patient or subject at risk of, or suffering from, a disease, disorder or condition that is amenable to treatment or amelioration with a binding protein or multispecific polypeptide or a composition thereof provided herein. "Patient" and "subject" are used interchangeably herein.

[0082] As used herein, the term "pharmaceutically acceptable" refers to molecular entities and compositions that do not generally produce allergic or other serious adverse reactions when administered using routes well known in the art. Molecular entities and compositions approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans are considered to be "pharmaceutically acceptable."

PGR2026-00039
Biocon Ex.1002
Page 4626 of 4945

[0083] As used herein, the terms "nucleic acid," "nucleic acid molecule," or "polynucleotide" refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the terms encompass nucleic acids containing analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.* (1991) *Nucleic Acid Res.* 19:5081; Ohtsuka *et al.* (1985) *J. Biol. Chem.* 260:2605-2608; Cassol *et al.* (1992); Rossolini *et al.* (1994) *Mol. Cell. Probes* 8:91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene. As used herein, the terms "nucleic acid," "nucleic acid molecule," or "polynucleotide" are intended to include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments and homologs thereof.

[0084] The term "expression" refers to the biosynthesis of a product encoded by a nucleic acid. For example, in the case of nucleic acid segment encoding a polypeptide of interest, expression involves transcription of the nucleic acid segment into mRNA and the translation of mRNA into one or more polypeptides.

[0085] The terms "expression unit" and "expression cassette" are used interchangeably herein and denote a nucleic acid segment encoding a polypeptide of interest and capable of providing expression of the nucleic acid segment in a host cell. An expression unit typically comprises a transcription promoter, an open reading frame encoding the polypeptide of interest, and a transcription terminator, all in operable configuration. In addition to a transcriptional promoter and terminator, an expression unit can further include other nucleic acid segments such as, *e.g.*, an enhancer or a polyadenylation signal.

[0086] The term "expression vector," as used herein, refers to a nucleic acid molecule, linear or circular, comprising one or more expression units. In addition to one or more expression units, an expression vector can also include additional nucleic acid segments such as, for example, one or more origins of replication or one or more

PGR2026-00039
Biocon Ex.1002
Page 4627 of 4945

selectable markers. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both.

[0087] As used herein, a "polypeptide," "polypeptide chain," or "protein" refers to a contiguous arrangement of covalently linked amino acids. Polypeptides can form one or more intrachain disulfide bonds. With regard to polypeptides as described herein, reference to modifications or alterations of amino acid residues corresponding to those specified by SEQ ID NO includes post-translational modifications of such residues. The terms polypeptide and protein also encompass embodiments where two polypeptide chains link together in a non-linear fashion, such as via an interchain disulfide bond. For example, a native immunoglobulin molecule is comprised of two heavy chain polypeptides and two light chain polypeptides.

[0088] As used herein, a "multispecific polypeptide" refers to a polypeptide comprising two or more binding domains each capable of specifically binding to a target antigen. For example, the polypeptides described herein may comprise 2, 3, 4, or more binding domains and may be able to bind 2, 3, 4, or more target antigens. In some embodiments, a multispecific polypeptide is a bispecific polypeptide. Herein, a "bispecific polypeptide" comprises two binding domains and capable of binding to two distinct target antigens. In some embodiments, the bispecific polypeptides described herein comprise a first binding domain that specifically binds to a cell surface antigen expressed on a target cell. In some embodiments, the bispecific polypeptides described herein comprise a binding domain that specifically binds to a cell surface antigen expressed on an effector cell. A binding domain may be derived from an antibody (e.g., a variable heavy chain and/or variable light change, scFv), a ligand, or a receptor.

[0089] Multispecific polypeptides are disclosed, for instance, in PCT Publication Nos. WO 2007/146968; WO 2010/040105; WO 2010/003108; WO 2016/094873; WO 2017/053469; U.S. Patent Application Publication No. 2006/0051844; and U.S. Patent Nos. 7,166,707; and 8,409,577, which are each incorporated herein by reference in their entirety. In certain embodiments, the multispecific polypeptides described herein are bispecific polypeptides and may comprise an scFv-Fc-scFv structure, also referred to herein as an ADAPTIR™ polypeptide. The structure of a polypeptide comprising such a structure comprises, from N-terminus to C-terminus: a first scFv binding domain – an immunoglobulin (Ig) hinge region – an Ig constant region – a second scFv binding domain.

PGR2026-00039
Biocon Ex.1002
Page 4628 of 4945

[0001] A protein or polypeptide may be an antibody or an antigen-binding fragment of an antibody. In some embodiments, a protein may be a recombinant multispecific protein. In other embodiments, a multispecific protein may be produced by chemically linking two different monoclonal antibodies or by fusing two hybridoma cell lines to produce a hybrid-hybridoma. Other multivalent formats that can be used include, for example, quadromas, KA-bodies, dAbs, diabodies, TandAbs, nanobodies, Small Modular ImmunoPharmaceutials (SMIPs™), DOCK-AND-LOCKs® (DNLs®), CrossMab Fabs, CrossMab VH-VLs, strand-exchange engineered domain bodies (SEEDbodies), Affibodies, Fynomers, Kunitz Domains, Albu-dabs, two engineered Fv fragments with exchanged VHs (e.g., a dual-affinity re-targeting molecules (D.A.R.T.s)), scFv x scFv (e.g., BiTE), DVD-IG, Covx-bodies, peptibodies, scFv-Igs, SVD-Igs, dAb-Igs, Knobs-in-Holes, IgG1 antibodies comprising matched mutations in the CH3 domain (e.g., DuoBody antibodies) and triomAbs. Exemplary bispecific formats are discussed in Garber et al., Nature Reviews Drug Discovery 13:799-801 (2014), which is herein incorporated by reference in its entirety. Additional exemplary bispecific formats are discussed in Liu et al. Front. Immunol. 8:38 doi: 10.2289/fimmu.2017.00038, and Brinkmann and Kontermann, MABS 9: 2, 182–212 (2017), each of which is herein incorporated by reference in its entirety. In certain embodiments, a bispecific antibody can be a F(ab')2 fragment. A F(ab')2 fragment contains the two antigen-binding arms of a tetrameric antibody molecule linked by disulfide bonds in the hinge region.

[0090] As will be appreciated by one of skill in the art, proteins and polypeptides are defined herein in terms of the amino acid sequences of the individual polypeptide chains, which are indicated by the SEQ ID NOs referenced throughout this disclosure. For example, in some embodiments an scFv-Fc-scFv protein or polypeptide described herein is comprised of two scFv-Fc-scFv polypeptide chains associated by interchain bonds (*e.g.*, interchain disulfide bonds) to form a dimeric scFv-Fc-scFv protein (*e.g.*, a homodimeric or heterodimeric scFv-Fc-scFv protein). In such embodiments, the scFv-Fc-scFv protein is defined by the amino acid sequences of the individual scFv-Fc-scFv polypeptide chains. Polypeptides and proteins can also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents can be added to a protein or polypeptide by the cell in which the protein is produced, and will vary with the type of cell. Proteins and polypeptides are

PGR2026-00039
Biocon Ex.1002
Page 4629 of 4945

defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

[0091] The terms "light chain variable region" (also referred to as "light chain variable domain" or "VL" or V_L) and "heavy chain variable region" (also referred to as "heavy chain variable domain" or "VH" or V_H) refer to the variable binding region from an antibody light and heavy chain, respectively. The variable binding regions are made up of discrete, well-defined sub-regions known as "complementarity determining regions" (CDRs) and "framework regions" (FRs), generally comprising in order FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 from amino-terminus to carboxyl-terminus. In one embodiment, the FRs are humanized. The term "CL" refers to an "immunoglobulin light chain constant region" or a "light chain constant region," i.e., a constant region from an antibody light chain. The term "CH" refers to an "immunoglobulin heavy chain constant region" or a "heavy chain constant region," which is further divisible, depending on the antibody isotype into CH1, CH2, and CH3 (IgA, IgD, IgG), or CH1, CH2, CH3, and CH4 domains (IgE, IgM). A "Fab" (fragment antigen binding) is the part of an antibody that binds to antigens and includes the variable region and CH1 domain of the heavy chain linked to the light chain via an inter-chain disulfide bond.

[0092] The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl-terminus of the reference sequence, but is not necessarily at the carboxyl-terminus of the complete polypeptide.

[0093] As used herein, the term "transformation," "transfection," and "transduction" refer to the transfer of nucleic acid (i.e., a nucleotide polymer) into a cell. As used herein, the term "genetic transformation" refers to the transfer and incorporation of DNA, especially recombinant DNA, into a cell. The transferred nucleic acid can be introduced into a cell via an expression vector.

[0094] "Antibody-dependent cell-mediated cytotoxicity" and "ADCC," as used herein, refer to a cell-mediated process in which nonspecific cytotoxic cells that express FcγRs (*e.g.*, monocytic cells such as natural killer (NK) cells and macrophages) recognize bound antibody (or other protein capable of binding FcγRs)

PGR2026-00039
Biocon Ex.1002
Page 4630 of 4945

on a target cell and subsequently cause lysis of the target cell. In principle, any effector cell with an activating FcγR can be triggered to mediate ADCC. The primary cells for mediating ADCC are NK cells, which express only FcγRIII, whereas monocytes, depending on their state of activation, localization, or differentiation, can express FcγRI, FcγRII, and FcγRIII. For a review of FcγR expression on hematopoietic cells, see, *e.g.*, Ravetch et al., 1991, Annu. Rev. Immunol., 9:457-92.

[0095] The term "having ADCC activity," as used herein in reference to a polypeptide or protein, means that the polypeptide or protein, for example, one comprising an Fc domain (*e.g.*, an immunoglobulin hinge region and an immunoglobulin constant region having CH2 and CH3 domains) such as derived from IgG (*e.g.*, IgG1), is capable of mediating antibody-dependent cell-mediated cytotoxicity (ADCC) through binding of a cytolytic Fc receptor (*e.g.*, FcγRIII) on a cytolytic immune effector cell expressing the Fc receptor (*e.g.*, an NK cell). In some embodiments, a multispecific polypeptide or protein comprising an Fc domain may lack effector function (*e.g.*, null ADCC activity) as the result of mutations in the CH2 and/or CH3 domain.

[0096] "Complement-dependent cytotoxicity" and "CDC," as used herein, refer to a process in which components in normal serum ("complement"), together with an antibody or other C1q-complement-binding protein bound to a target antigen, exhibit lysis of a target cell expressing the target antigen. Complement consists of a group of serum proteins that act in concert and in an orderly sequence to exert their effect.

[0097] The terms "classical complement pathway" and "classical complement system," as used herein, are synonymous and refer to a particular pathway for the activation of complement. The classical pathway requires antigen-antibody complexes for initiation and involves the activation, in an orderly fashion, of nine major protein components designated C1 through C9. For several steps in the activation process, the product is an enzyme that catalyzes the subsequent step. This cascade provides amplification and activation of large amounts of complement by a relatively small initial signal.

[0098] The term "having CDC activity," as used herein in reference to a polypeptide or protein, means that the polypeptide or protein, for example, one comprising an Fc domain (*e.g.*, an immunoglobulin hinge region and an immunoglobulin constant region having CH2 and CH3 domains) such as derived from IgG (*e.g.*, IgG1) is capable of

PGR2026-00039
Biocon Ex.1002
Page 4631 of 4945

mediating complement-dependent cytotoxicity (CDC) through binding of C1q complement protein and activation of the classical complement system. In some embodiments, a multispecific polypeptide or protein may lack effector function (*e.g.*, null CDC activity) as the result of one or more mutations in the CH2 and/or CH3 domains.

[0099] "Enhanced effector cell activation" as used herein, refers to the increase, prolonging, and/or potentiation of an effector cell response by the polypeptides or proteins described herein. In some embodiments, enhanced effector cell activation refers to an increase in the cytotoxic activity of an effector cell. In some embodiments, enhanced effector cell activation refers to an increase in cytokine production, cell proliferation, or a change in cell-surface molecule expression such that the ability of the effector cell to lyse a target cell is enhanced.

[0100] As used herein, the term "effector cell" refers to a cell of the immune system that is capable of lysing or killing a target cell, such as a tumor cell. Herein, an effector cell may refer to a lymphocyte, such as a T cell, a natural killer (NK) cell, or an NKT cell, a monocyte, a macrophage, a dendritic cell, or a granulocyte. In particular embodiments, the term effector cell refers to a T cell, an NK cell, or an NKT cell.

[0101] As used herein, the terms "treatment," "treating," or "ameliorating" refers to either a therapeutic treatment or prophylactic/preventative treatment. A treatment is therapeutic if at least one symptom of disease in an individual receiving treatment improves or a treatment can delay worsening of a progressive disease in an individual, or prevent onset of additional associated diseases.

[0102] As used herein, the term "therapeutically effective amount (or dose)" or "effective amount (or dose)" of a polypeptide or protein described herein or a composition thereof refers to that amount of the compound sufficient to result in amelioration of one or more symptoms of the disease being treated in a statistically significant manner or a statistically significant improvement in organ function. When referring to an individual active ingredient, administered alone, a therapeutically effective dose refers to that ingredient alone. When referring to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered serially or simultaneously (in the same formulation or concurrently in separate formulations).

Pharmaceutical Compositions

PGR2026-00039
Biocon Ex.1002
Page 4632 of 4945

[0103] Described herein are stable pharmaceutical formulations of protein therapeutics, such as multispecific polypeptides, that prevent denaturation and/or prevent or substantially reduce the formation of aggregates, especially upon freezing. In addition to a therapeutic protein, the pharmaceutical compositions described herein may further comprise one or more of a buffer, an excipient, and a surfactant. In some embodiments, the compositions comprise, consist of, or consist essentially of a buffer, an excipient and a surfactant, wherein the multispecific protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, and (iv) a second binding domain; and the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

[0104] In some embodiments, the composition comprises from about 0.1 mg/ml to about 10 mg/ml of the multispecific protein. In some embodiments, the composition comprises from about 1 mg/ml to about 5 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 2 mg/ml of the multispecific protein. In some embodiments, the composition comprises about 2 mg/ml of the multispecific protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80.

[0105] In some embodiments, wherein the composition substantially prevents degradation of the multispecific protein. In some embodiments, the composition slows or reduces the degradation of the multispecific polypeptide as compared to an identical multispecific polypeptide stored in histidine buffer under identical storage conditions. In some embodiments, the composition is substantially stable for at least 1 year at 4°C. In some embodiments, the composition is substantially resistant to formation of aggregates of multispecific protein.

[0106] In some embodiments, the composition is capable of withstanding freeze to thaw conditions. In some embodiments, the composition slows or reduces degradation of the multispecific polypeptide in freeze to thaw conditions as compared to a multispecific polypeptide stored in a histidine buffer under identical freeze to thaw conditions.

[0107] In other embodiments, a CD123 x CD3 targeting multispecific polypeptide undergoes little to no degradation after lyophilization when formulated as disclosed herein. For instance, a CD123 x CD3 targeting multispecific polypeptide may be

PGR2026-00039
Biocon Ex.1002
Page 4633 of 4945

formulated in a succinate and sucrose formulation that exhibits reduced degradation after lyophilization as compared to an identical polypeptide formulated with a histidine buffer. Also provided herein is a lyophilized anti-CD123 x anti-CD3 multispecific polypeptide, including but not limited to TRI130 and TRI129, formulated in about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80. In some embodiments, the composition is lyophilized.

Buffer

[0108] As used herein, the term "buffer" or "buffering agent" refers to one or more components that when added to an aqueous solution is able to protect the solution against variations in pH when adding acid or alkali, or upon dilution with a solvent.

[0109] In some embodiments, the buffer comprises, consists of, or consists essentially of any pharmaceutically acceptable buffer. For example, the buffer may be potassium phosphate, acetic acid/sodium acetate, citric acid/sodium citrate, succinic acid/sodium succinate, tartaric acid/sodium tartrate, histidine/histidine HCl, glycine, Tris, glutamate, acetate, mixtures thereof, or pharmaceutically acceptable salts or acids thereof. In particular embodiments, the buffer comprises, consists of, or consists essentially of succinate or a pharmaceutically acceptable salt or acid thereof.

[0110] In some embodiments, the concentration of the buffer in the composition is from about 1 mM to about 500 mM, from about 1mM to about 100 mM, from about 1mM to about 50 mM, from about 1 to about 10 mM, from about 5 mM to about 50 mM, or from about 5mM to about 20 mM from about 5 mM to about 10mM. In some embodiments, the composition comprises from about 1 mM to about 10 mM succinate or a pharmaceutically acceptable salt or acid thereof. In some embodiments, the composition comprises about 5 mM succinate or a pharmaceutically acceptable salt or acid thereof.

[0111] In some embodiments, the pH of the composition is 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 4.75, 5.0, 5.25, 5.5, 5.75, 6.0, 6.25, 6.5, 6.75, 7.0, 7.25, 7.5, 7.75, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, 9.5, 9.75, 10.0, 10.25, 10.5, 10.75, 11.0, 11.25 or 11.5. In some embodiments, the pH of the composition is about 3.0 to about 6.0. In some embodiments, the composition has a pH from about 4.0 to about 5.5. In some embodiments, the pH of the composition is about 4.8.

Excipient

PGR2026-00039
Biocon Ex.1002
Page 4634 of 4945

[0112] As referred to herein, an excipient is a pharmacologically inactive substance formulated alongside the active pharmaceutical ingredient of a composition. Excipients might aid in lubricity, flowability, disintegration, or taste and may confer some form of antimicrobial function.

[0113] Exemplary excipients which may be used in the compositions disclosed herein include pharmaceutical binders, diluents, release retarding excipients, lubricant, glidants, gas generating agents, coating systems, solvents, and coloring agents. Suitable excipients include the substances mentioned as excipients in the Handbook of Pharmaceutical Excipients, Third Edition, Edited by A. H. Kibbe, American Pharmaceutical Association and Pharmaceutical Press (2000), and Tables 3-5 in E. T. Cole et al., Advanced Drug Delivery Reviews 60 (2008), 747-756. For example, an excipient may be selected from the group consisting of polypropylene glycol, polyethylene glycol, polyoxyethylene castor oil derivatives, polyoxyethylene glycerol oxystearate, saturated polyglycolized glycerides, polyethylene polypropylene glycol, Vitamin E, and Vitamin E TPGS (d-alpha - tocopheryl polyethylene glycol 1000 succinate).

[0114] In some embodiments, the composition comprises from about 1% weight/volume (w/v) to about 20% w/v, about 1% w/v to about 10% w/v, about 5% w/v to about 15% w/v, or about 10% w/v of the excipient. In some embodiments, the composition comprises from about 1% w/v to about 12% w/v of the excipient, such as about 6.5% w/v of the excipient.

[0115] In some embodiments, the excipient the excipient comprises, consists of, or consists essentially of a sugar. In some embodiments, the composition comprises from about 1% w/v to about 12% w/v of the sugar. In some embodiments, the composition comprises about 4% to about 8% w/v of the sugar. In some embodiments, the composition comprises about 6.5% w/v of the sugar. In some embodiments, the sugar is sucrose.

Surfactant

[0116] As described herein, a "surfactant" is a surface active molecule containing both a hydrophobic portion (e.g., alkyl chain) and a hydrophilic portion (e.g., carboxyl and carboxylate groups).

[0117] Surfactants suitable for use in the compositions described herein include, but are not limited to, polysorbates (e.g. polysorbates 20 or 80); poloxamers (e.g.

PGR2026-00039
Biocon Ex.1002
Page 4635 of 4945

poloxamer 188); sorbitan esters and derivatives; Triton; sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetadine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauramidopropyl-cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropylbetaine (e.g., lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate); and the MONAQUAT™ series (Mona Industries, Inc., Paterson, N.J.), polyethylene glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g., Pluronics, PF68 etc.). In particular embodiments, the surfactant comprises or consists of polysorbate 80.

[0118] In some embodiments, the composition comprises from about 0.001% w/v to about 1% w/v, about 0.01% w/v to about 0.5% w/v, or about 0.01% w/v to about 0.1% w/v of the surfactant. In some embodiments, the composition comprises about 0.02% w/v of the surfactant.

[0119] In some embodiments, the composition comprises from about 0.001% w/v to about 1% w/v, about 0.01% w/v to about 0.5% w/v, or about 0.01% w/v to about 0.1% w/v of polysorbate 80. In some embodiments, the composition comprises about 0.02% w/v of polysorbate 80.

Therapeutic Proteins

[0120] The compositions described herein may be used in connection with many different protein therapeutics as described herein.

Binding domains

[0121] In some embodiments, the therapeutic proteins comprise a binding domain. The binding domain may provide for specific binding to at least one cell-surface molecule (e.g., a cell-surface receptor). The binding domain can be in the form of an antibody, or fragment thereof, or a fusion protein of any of a variety of different formats (*e.g.*, the fusion protein can be in the form of a bispecific or multispecific molecule). In other embodiments, the binding domain can comprise, for example, a particular cytokine or a molecule that targets the binding domain polypeptide to, for example, a particular cell type, a toxin, an additional cell receptor, or an antibody.

[0122] In some embodiments, a binding domain described herein is derived from an antibody and comprises a variable heavy chain (V_H) and a variable light chain (V_L).

PGR2026-00039
Biocon Ex.1002
Page 4636 of 4945

For example, an scFv comprising a V_H and V_L chain. These binding domains and variable chains may be arranged in any order that still retains some binding to the target(s). In some embodiments, a binding domain comprises (i) an immunoglobulin heavy chain variable region (V_H) comprising HCDR1, HCDR2, and HCDR3; and (ii) an immunoglobulin light chain variable region (V_L) comprising LCDR1, LCDR2, and LCDR3.

[0123] In some embodiments, the polypeptides and proteins described herein comprise binding domains that are scFvs. In such embodiments, the binding domains may be referred to as scFv domains. In some embodiments, a binding domain is a single-chain Fv fragment (scFv) that comprises V_H and V_L regions specific for a target of interest. In certain embodiments, the V_H and V_L regions are human or humanized. In some variations, a binding domain is a single-chain Fv (scFv) comprising V_L and V_H regions joined by a peptide linker.

[0124] In certain embodiments, the binding domains of the polypeptides described herein comprise (i) an immunoglobulin light chain variable region (V_L) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (V_H) comprising CDRs HCDR1, HCDR2, and HCDR3. In some embodiments, amino acid sequences provided for polypeptide constructs do not include the human immunoglobulin leader sequences. CDR sequences and amino acid substitution positions shown are those defined using the IMGT criteria (Brochet et al, Nucl. Acids Res. (2008) 36, W503-508).

[0125] In certain embodiments, a binding domain V_L and/or V_H region of the present disclosure is derived from a V_L and/or V_H of a parent V_L and/or V_H region (*e.g.*, 1618/1619 as described in PCT Application Publication No. WO 2016/185016) and optionally contains about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) insertions, about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) deletions, about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) amino acid substitutions (*e.g.*, conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared to the V_L and/or V_H sequence of a known monoclonal antibody. The insertion(s), deletion(s) or substitution(s) can be anywhere in the V_L and/or V_H region, including at the amino- or carboxyl-terminus or both ends of this region, provided that each CDR comprises zero changes or at most one, two, or three changes. In some embodiments, the binding domain containing the modified

PGR2026-00039
Biocon Ex.1002
Page 4637 of 4945

V_L and/or V_H region can still specifically bind its target with an affinity similar to or greater than the parent binding domain.

[0126] The use of peptide linkers for joining V_L and V_H regions is well-known in the art, and a large number of publications exist within this particular field. In some embodiments, a peptide linker is a 15mer consisting of three repeats of a Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 128) amino acid sequence ($(Gly_4Ser)_3$) (SEQ ID NO: 59). Other linkers have been used, and phage display technology, as well as selective infective phage technology, has been used to diversify and select appropriate linker sequences (Tang *et al.*, *J. Biol. Chem.* 271, 15682-15686, 1996; Hennecke *et al.*, *Protein Eng.* 11, 405-410, 1998). In certain embodiments, the V_L and V_H regions are joined by a peptide linker having an amino acid sequence comprising the formula $(Gly_4Ser)_n$, wherein n = 1-5 (SEQ ID NO: 129). For instance, in some embodiments, the linker comprises $(Gly4Ser)_4$ (SEQ ID NO: 61). Other suitable linkers can be obtained by optimizing a simple linker through random mutagenesis. In some embodiments, the V_H region of the scFv described herein may be positioned N-terminally to a linker sequence. In some embodiments, the V_L region of the scFvs described herein may be positioned C-terminally to the linker sequence.

[0127] In some embodiments, the binding domain may bind to a tumor antigen, such as CD123, PSMA, CD19, CD33, 5T4, or HER2. In some embodiments, the binding domain may be a CD3 binding domain. In some embodiments, the binding domain may bind to 4-1-BB. In some embodiments, the binding domain may bind to OX40. In some embodiments, a formulated multispecific protein binds to both 4-1BB and OX40.

Hinge

[0128] In addition to a binding domain, the therapeutic polypeptides may further comprise a hinge region. In some embodiments, the hinge is an altered immunoglobulin hinge in which one or more cysteine residues in a wild type immunoglobulin hinge region are substituted with one or more other amino acid residues (*e.g.*, serine or alanine). Exemplary altered immunoglobulin hinges, carboxyl-terminus linkers, and amino-terminus linkers include an immunoglobulin human IgG1 hinge region having one, two or three cysteine residues found in a wild type human IgG1 hinge substituted by one, two or three different amino acid residues (*e.g.*, serine or alanine). An altered immunoglobulin hinge can additionally have a proline

PGR2026-00039
Biocon Ex.1002
Page 4638 of 4945

substituted with another amino acid (*e.g.*, serine or alanine). For example, the above-described altered human IgG1 hinge can additionally have a proline located carboxyl-terminal to the three cysteines of wild type human IgG1 hinge region substituted by another amino acid residue (*e.g.*, serine, alanine). In one embodiment, the prolines of the core hinge region are not substituted. In certain embodiments, a hinge, a carboxyl-terminus linker, or an amino-terminus linker polypeptide comprises or is a sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a wild type immunoglobulin hinge region, such as a wild type human IgG1 hinge, a wild type human IgG2 hinge, or a wild type human IgG4 hinge.

Immunoglobulin Constant Domain

[0129] The therapeutic proteins may also comprise an immunoglobulin constant (Fc) domain (also referred to herein as a constant region, Fc domain, Fc region, and the like). In come embodiments, the constant region comprises IgG CH2 and CH3 domains, e.g., IgG1 CH2 and CH3 domains. In some embodiments, the constant region does not comprise a CH1 domain. In some embodiments, the immunoglobulin constant region is a human Fc domain. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to reduce or prevent binding to FcγR1, FcγRIIa, FcγRIIb, FcγRIIa, and FcγRIIIb. In some embodiments, the constant domains making up the constant region are human or derived from human sequences. In some embodiments, the Fc domain comprises one or more mutations the Fc region to reduce or prevent complement fixation and interaction with Fcγ receptors. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce Fc-mediated T-cell activation. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce CDC activity. In some embodiments, the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce ADCC activity.

PGR2026-00039
Biocon Ex.1002
Page 4639 of 4945

[0130] In some embodiments, the Fc region comprises one or more mutations at positions 234, 235, 237 and 322 of the CH2 domain, according to the EU numbering system. In some embodiments, the Fc domain comprises mutations at positions 234, 235, 237, 318, 320 and 322 of the CH2 domain, according to the EU numbering system. In some embodiments, the Fc domain comprises mutations L234A, L235A, G237A and K322A of the CH2 domain, according to the EU numbering system. In some embodiments, the Fc domain comprises mutations L234A, L235A, G237A, E318A, K320A, and K322A of the CH2 domain, according to the EU numbering system. In some embodiments, the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions E233P, L234A, L235A, G237A, and K322A and a deletion of G236, according to the EU numbering system. In some embodiments, the Fc domain is derived from human IgG1. In some embodiments, the two or more mutations in the IgG1 Fc domain prevent or substantially reduce signaling through Fc-mediated cross-linking.

[0131] In some embodiments, the immunoglobulin constant region comprises an amino acid sequence of any one of SEQ ID NO:32-35, or a variant thereof. The inclusion of an immunoglobulin constant region slows clearance of the polypeptides and proteins of the present disclosure from circulation after administration to a subject. By mutations or other alterations, an immunoglobulin constant region further enables relatively easy modulation of polypeptide effector functions (*e.g.*, ADCC, ADCP, CDC, complement fixation, and binding to Fc receptors), which can either be increased or decreased depending on the disease being treated, as known in the art and described herein. In certain embodiments, the polypeptides and proteins described herein comprise an immunoglobulin constant region capable of mediating one or more of these effector functions. In other embodiments, one or more of these effector functions are reduced or absent in an immunoglobulin constant region of a polypeptide or protein described in the present disclosure, as compared to a corresponding wild-type immunoglobulin constant region.

[0132] An immunoglobulin constant region present in the polypeptides and proteins of the present disclosure can comprise or can be derived from part or all of: a CH2 domain, a CH3 domain, a CH4 domain, or any combination thereof. For example, an immunoglobulin constant region can comprise a CH2 domain, a CH3 domain, both CH2 and CH3 domains, both CH3 and CH4 domains, two CH3 domains, a CH4 domain, two CH4 domains, and a CH2 domain and part of a CH3 domain. In certain

PGR2026-00039
Biocon Ex.1002
Page 4640 of 4945

embodiments, the polypeptides or proteins described herein do not comprise a CH1 domain.

[0133] A polypeptide or protein described herein may comprise a wild type immunoglobulin CH2 domain or an altered immunoglobulin CH2 domain from certain immunoglobulin classes or subclasses (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, or IgD) and from various species (including human, mouse, rat, and other mammals). In certain embodiments, a CH2 domain of a polypeptide or a protein described herein is a wild type human immunoglobulin CH2 domain, such as wild type CH2 domains of human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, or IgD, as set forth in SEQ ID NOs: 115, 199-201 and 195-197, respectively, of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, the CH2 domain is a wild type human IgG1 CH2 domain as set forth in SEQ ID NO: 115 of U.S. Patent Application Publication No. US 2013/0129723 (said sequence incorporated by reference herein).

[0134] In certain embodiments, an altered CH2 region in a polypeptide or a protein of the present disclosure comprises or is a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to a wild type immunoglobulin CH2 region, such as the CH2 region of wild type human IgG1, IgG2, or IgG4, or mouse IgG2a (*e.g.*, IGHG2c).

[0135] An altered immunoglobulin CH2 region in a polypeptide or protein of the present disclosure can be derived from a CH2 region of various immunoglobulin isotypes, such as IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, and IgD, from various species (including human, mouse, rat, and other mammals). In certain embodiments, an altered immunoglobulin CH2 region in a fusion protein of the present disclosure can be derived from a CH2 region of human IgG1, IgG2 or IgG4, or mouse IgG2a (*e.g.*, IGHG2c), whose sequences are set forth in SEQ ID NOs: 115, 199, 201, and 320 of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, an altered CH2 domain of a polypeptide or a protein described herein is an altered human IgG1 CH2 domain with mutations known in the art that enhance or reduce immunological activities (*i.e.*, effector functions) such as ADCC, ADCP, CDC, complement fixation, Fc receptor binding, or any combination thereof.

PGR2026-00039
Biocon Ex.1002
Page 4641 of 4945

[0136] In certain embodiments, a CH2 domain of a polypeptide or a protein described herein is an altered immunoglobulin CH2 region (*e.g.*, an altered human IgG1 CH2 domain) that comprises one or more amino acid deletions or substitutions. In some embodiments, the CH2 domain comprises an amino acid substitution at the asparagine of position 297 (*e.g.*, asparagine to alanine). Such an amino acid substitution reduces or eliminates glycosylation at this site and abrogates efficient Fc binding to FcγR and C1q. The sequence of an altered human IgG1 CH2 domain with an Asn to Ala substitution at position 297 is set forth in SEQ ID NO: 324 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein). In some embodiments, the altered CH2 domain comprises at least one substitution or deletion at positions 234 to 238. For example, an immunoglobulin CH2 region can comprise a substitution at position 234, 235, 236, 237 or 238; positions 234 and 235; positions 234 and 236; positions 234 and 237; positions 234 and 238; positions 234-236; positions 234, 235 and 237; positions 234, 236 and 238; positions 234, 235, 237, and 238; positions 236-238; or any other combination of two, three, four, or five amino acids at positions 234-238. In some embodiments, an altered CH2 region comprises one or more (*e.g.*, two, three, four or five) amino acid deletions at positions 234-238, for instance, at one of position 236 or position 237 while the other position is substituted. In certain embodiments, the amino acid residues at one or more of positions 234-238 has been replaced with one or more alanine residues. In further embodiments, only one of the amino acid residues at positions 234-238 have been deleted while one or more of the remaining amino acids at positions 234-238 can be substituted with another amino acid (*e.g.*, alanine or serine).

[0137] In some embodiments, the above-noted mutation(s) decrease or eliminate the ADCC activity or Fc receptor-binding capability of a polypeptide that comprises the altered CH2 domain.

[0138] In certain other embodiments, a CH2 domain of a polypeptide or a protein described herein is an altered immunoglobulin CH2 region (*e.g.*, an altered human IgG1 CH2 domain) that comprises one or more amino acid substitutions at positions 253, 310, 318, 320, 322, and 331. For example, an immunoglobulin CH2 region can comprise a substitution at position 253, 310, 318, 320, 322, or 331, positions 318 and 320, positions 318 and 322, positions 318, 320 and 322, or any other combination of two, three, four, five or six amino acids at positions 253, 310, 318, 320, 322, and 331.

PGR2026-00039
Biocon Ex.1002
Page 4642 of 4945

In such embodiments, the above-noted mutation(s) decrease or eliminate the CDC activity of a polypeptide comprising the altered CH2 domain.

[0139] In certain other embodiments, in addition to the amino acid substitution at position 297, an altered CH2 region of a polypeptide or a protein described herein (*e.g.*, an altered human IgG1 CH2 domain) can further comprise one or more (*e.g.*, two, three, four, or five) additional substitutions at positions 234-238. For example, an immunoglobulin CH2 region can comprise a substitution at positions 234 and 297, positions 234, 235, and 297, positions 234, 236 and 297, positions 234-236 and 297, positions 234, 235, 237 and 297, positions 234, 236, 238 and 297, positions 234, 235, 237, 238 and 297, positions 236-238 and 297, or any combination of two, three, four, or five amino acids at positions 234-238 in addition to position 297. In addition or alternatively, an altered CH2 region can comprise one or more (*e.g.*, two, three, four or five) amino acid deletions at positions 234-238, such as at position 236 or position 237. The additional mutation(s) decreases or eliminates the ADCC activity or Fc receptor-binding capability of a polypeptide comprising the altered CH2 domain. In certain embodiments, the amino acid residues at one or more of positions 234-238 have been replaced with one or more alanine residues. In further embodiments, only one of the amino acid residues at positions 234-238 has been deleted while one or more of the remaining amino acids at positions 234-238 can be substituted with another amino acid (*e.g.*, alanine or serine).

[0140] In certain embodiments, in addition to one or more (*e.g.*, 2, 3, 4, or 5) amino acid substitutions at positions 234-238, a mutated CH2 region of a polypeptide or a protein described herein (*e.g.*, an altered human IgG1 CH2 domain) in a fusion protein of the present disclosure can contain one or more (*e.g.*, 2, 3, 4, 5, or 6) additional amino acid substitutions (*e.g.*, substituted with alanine) at one or more positions involved in complement fixation (*e.g.*, at positions I253, H310, E318, K320, K322, or P331). Examples of mutated immunoglobulin CH2 regions include human IgG1, IgG2, IgG4 and mouse IgG2a CH2 regions with alanine substitutions at positions 234, 235, 237 (if present), 318, 320 and 322. An exemplary mutated immunoglobulin CH2 region is mouse IGHG2c CH2 region with alanine substitutions at L234, L235, G237, E318, K320, and K322.

[0141] In still further embodiments, in addition to the amino acid substitution at position 297 and the additional deletion(s) or substitution(s) at positions 234-238, an altered CH2 region of a polypeptide or a protein described herein (*e.g.*, an altered

PGR2026-00039
Biocon Ex.1002
Page 4643 of 4945

human IgG1 CH2 domain) can further comprise one or more (*e.g.*, two, three, four, five, or six) additional substitutions at positions 253, 310, 318, 320, 322, and 331. For example, an immunoglobulin CH2 region can comprise a (1) substitution at position 297, (2) one or more substitutions or deletions or a combination thereof at positions 234-238, and one or more (*e.g.*, 2, 3, 4, 5, or 6) amino acid substitutions at positions I253, H310, E318, K320, K322, and P331, such as one, two, three substitutions at positions E318, K320 and K322. The amino acids at the above-noted positions can be substituted by alanine or serine.

[0142] In certain embodiments, an immunoglobulin CH2 region of a polypeptide or a protein described herein comprises: (i) an amino acid substitution at the asparagines of position 297 and one amino acid substitution at position 234, 235, 236 or 237; (ii) an amino acid substitution at the asparagine of position 297 and amino acid substitutions at two of positions 234-237; (iii) an amino acid substitution at the asparagine of position 297 and amino acid substitutions at three of positions 234-237; (iv) an amino acid substitution at the asparagine of position 297, amino acid substitutions at positions 234, 235 and 237, and an amino acid deletion at position 236; (v) amino acid substitutions at three of positions 234-237 and amino acid substitutions at positions 318, 320 and 322; or (vi) amino acid substitutions at three of positions 234-237, an amino acid deletion at position 236, and amino acid substitutions at positions 318, 320 and 322.

[0143] Exemplary altered immunoglobulin CH2 regions with amino acid substitutions at the asparagine of position 297 include: human IgG1 CH2 region with alanine substitutions at L234, L235, G237 and N297 and a deletion at G236 (SEQ ID NO: 325 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), human IgG2 CH2 region with alanine substitutions at V234, G236, and N297 (SEQ ID NO: 326 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at F234, L235, G237 and N297 and a deletion of G236 (SEQ ID NO: 322 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at F234 and N297 (SEQ ID NO: 343 of U.S. Patent Application Publication No. US 2013/0129723, said sequence incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at L235 and N297 (SEQ ID NO: 344 of U.S. Patent Application Publication No. 2013/0129723, said sequence

PGR2026-00039
Biocon Ex.1002
Page 4644 of 4945

incorporated by reference herein), human IgG4 CH2 region with alanine substitutions at G236 and N297 (SEQ ID NO: 345 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), and human IgG4 CH2 region with alanine substitutions at G237 and N297 (SEQ ID NO: 346 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein). These CH2 regions can be used in a polypeptide of the present disclosure.

[0144] In certain embodiments, in addition to the amino acid substitutions described above, an altered CH2 region of a polypeptide or a protein described herein (*e.g.*, an altered human IgG1 CH2 domain) can contain one or more additional amino acid substitutions at one or more positions other than the above-noted positions. Such amino acid substitutions can be conservative or non-conservative amino acid substitutions. For example, in certain embodiments, P233 can be changed to E233 in an altered IgG2 CH2 region (*see, e.g.*, SEQ ID NO: 326 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein). In addition or alternatively, in certain embodiments, the altered CH2 region can contain one or more amino acid insertions, deletions, or both. The insertion(s), deletion(s) or substitution(s) can be anywhere in an immunoglobulin CH2 region, such as at the N- or C-terminus of a wild type immunoglobulin CH2 region resulting from linking the CH2 region with another region (*e.g.*, a binding domain or an immunoglobulin heterodimerization domain) via a hinge.

[0145] In certain embodiments, an altered CH2 domain of a polypeptide or protein described herein is a human IgG1 CH2 domain with alanine substitutions at positions 235, 318, 320, and 322 (*i.e.*, a human IgG1 CH2 domain with L235A, E318A, K320A and K322A substitutions) (SEQ ID NO: 595 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), and optionally an N297 mutation (*e.g.*, to alanine). In certain other embodiments, an altered CH2 domain is a human IgG1 CH2 domain with alanine substitutions at positions 234, 235, 237, 318, 320 and 322 (*i.e.*, a human IgG1 CH2 domain with L234A, L235A, G237A, E318A, K320A and K322A substitutions) (SEQ ID NO: 596 of U.S. Patent Application Publication No. 2013/0129723, said sequence incorporated by reference herein), and optionally an N297 mutation (*e.g.*, to alanine).

[0146] In some embodiments, an immunoglobulin constant region of a polypeptide or a protein described herein comprises a human IgG1 CH2 domain comprising the

PGR2026-00039
Biocon Ex.1002
Page 4645 of 4945

substitutions L234A, L235A, G237A, and K322A, according to the EU numbering system.

[0147] The CH3 domain that can form an immunoglobulin constant region of a polypeptide or a protein described herein can be a wild type immunoglobulin CH3 domain or an altered immunoglobulin CH3 domain thereof from certain immunoglobulin classes or subclasses (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, IgM) of various species (including human, mouse, rat, and other mammals). In certain embodiments, a CH3 domain of a polypeptide described herein is a wild type human immunoglobulin CH3 domain, such as wild type CH3 domains of human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM as set forth in SEQ ID NOs: 116, 208-210, 204-207, and 212, respectively of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, the CH3 domain is a wild type human IgG1 CH3 domain as set forth in SEQ ID NO: 116 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein).

[0148] In certain embodiments, a CH3 domain of a polypeptide described herein is an altered human immunoglobulin CH3 domain, such as an altered CH3 domain based on or derived from a wild-type CH3 domain of human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM antibodies. For example, an altered CH3 domain can be a human IgG1 CH3 domain with one or two mutations at positions H433 and N434 (positions are numbered according to EU numbering). The mutations in such positions can be involved in complement fixation. In certain other embodiments, an altered CH3 domain of a polypeptide described herein can be a human IgG1 CH3 domain but with one or two amino acid substitutions at position F405 or Y407. The amino acids at such positions are involved in interacting with another CH3 domain. In certain embodiments, an altered CH3 domain of polypeptide described herein can be an altered human IgG1 CH3 domain with its last lysine deleted. The sequence of this altered CH3 domain is set forth in SEQ ID NO: 761 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein).

[0149] In certain embodiments, a polypeptide or a protein described herein comprises a CH3 domain that comprises so called "knobs-into-holes" mutations (*see*, Marvin and Zhu, Acta Pharmacologica Sinica 26:649-58, 2005; Ridgway *et al.*, Protein Engineering 9:617-21, 1966). More specifically, mutations can be introduced into each of the CH3 domains of each polypeptide chain so that the steric complementarity

PGR2026-00039
Biocon Ex.1002
Page 4646 of 4945

required for CH3/CH3 association obligates these two CH3 domains to pair with each other. For example, a CH3 domain in one single chain polypeptide of a polypeptide heterodimer can contain a T366W mutation (a "knob" mutation, which substitutes a small amino acid with a larger one), and a CH3 domain in the other single chain polypeptide of the polypeptide heterodimer can contain a Y407A mutation (a "hole" mutation, which substitutes a large amino acid with a smaller one). Other exemplary knobs-into-holes mutations include (1) a T366Y mutation in one CH3 domain and a Y407T in the other CH3 domain, and (2) a T366W mutation in one CH3 domain and T366S, L368A and Y407V mutations in the other CH3 domain.

[0150] The CH4 domain that can form an immunoglobulin constant region a polypeptide or a protein described herein can be a wild type immunoglobulin CH4 domain or an altered immunoglobulin CH4 domain thereof from IgE or IgM molecules. In certain embodiments, the CH4 domain of a polypeptide described herein is a wild type human immunoglobulin CH4 domain, such as wild type CH4 domains of human IgE and IgM molecules as set forth in SEQ ID NOs: 213 and 214, respectively, of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). In certain embodiments, a CH4 domain of a polypeptide described herein is an altered human immunoglobulin CH4 domain, such as an altered CH4 domain based on or derived from a CH4 domain of human IgE or IgM molecules, which have mutations that increase or decrease an immunological activity known to be associated with an IgE or IgM Fc region.

[0151] In certain embodiments, an immunoglobulin constant region of a polypeptide or a protein described herein comprises a combination of CH2, CH3 or CH4 domains (*i.e.*, more than one constant region domain selected from CH2, CH3 and CH4). For example, the immunoglobulin constant region can comprise CH2 and CH3 domains or CH3 and CH4 domains. In certain other embodiments, the immunoglobulin constant region can comprise two CH3 domains and no CH2 or CH4 domains (*i.e.*, only two or more CH3). The multiple constant region domains that form an immunoglobulin constant region of the polypeptides described herein can be based on or derived from the same immunoglobulin molecule, or the same class or subclass immunoglobulin molecules. In certain embodiments, the immunoglobulin constant region is an IgG CH2-CH3 (*e.g.*, IgG1 CH2-CH3, IgG2 CH2-CH3, and IgG4 CH2-CH3) and can be a human (*e.g.*, human IgG1, IgG2, and IgG4) CH2CH3. For example, in certain embodiments, the immunoglobulin constant region of a polypeptide described

PGR2026-00039
Biocon Ex.1002
Page 4647 of 4945

herein comprises (1) wild type human IgG1 CH2 and CH3 domains, (2) human IgG1 CH2 with N297A substitution (*i.e.*, CH2(N297A)) and wild type human IgG1 CH3, or (3) human IgG1 CH2(N297A) and an altered human IgG1 CH3 with the last lysine deleted. Alternatively, the multiple constant region domains of a polypeptide or a protein described herein can be based on or derived from different immunoglobulin molecules, or different classes or subclasses immunoglobulin molecules. For example, in certain embodiments, an immunoglobulin constant region comprises both human IgM CH3 domain and human IgG1 CH3 domain. The multiple constant region domains that form an immunoglobulin constant region of a polypeptide described herein can be directly linked together or can be linked to each other via one or more (*e.g.*, about 2-10) amino acids.

[0152] Exemplary immunoglobulin constant regions that can be used in a polypeptide or a protein described herein are set forth in SEQ ID NOs: 305-309, 321, 323, 341, 342, and 762 of U.S. Patent Application Publication No. 2013/0129723 (said sequences incorporated by reference herein). Further exemplary immunoglobulin constant regions that can be used in a polypeptide or a protein described herein are provided in the table below.

Table 1: Exemplary immunoglobulin constant regions

Name	DNA Sequence	DNA SEQ ID NO:	AA Sequence	AA SEQ ID NO:
SS-Fc domain	TCGAGTGAGCCCAAATCTTCTGACAAAAC TCACACATGCCCACCGTGCCCAGCACCTG AAGCCGCGGGTGCACCGTCAGTCTTCCTC TTCCCCCCAAAACCCAAGGACACCCTCAT GATCTCCCGGACCCCTGAGGTCACATGCG TGGTGGTGGACGTGAGCCACGAAGACCCT GAGGTCAAGTTCAACTGGTACGTGGACGG CGTGGAGGTGCATAATGCCAAGACAAAGC CGCGGGAGGAGCAGTACAACAGCACGTAC CGTGTGGTCAGCGTCCTCACCGTCCTGCA CCAGGACTGGCTGAATGGCAAGGAATACA AGTGCGCGGTCTCCAACAAAGCCCTCCCA GCCCCCATCGAGAAAACCATCTCCAAAGC CAAAGGGCAGCCCCGAGAACCACAGGTGT ACACCCTGCCCCCATCCCGGGATGAGCTG ACCAAGAACCAGGTCAGCCTGACCTGCCT GGTCAAAGGCTTCTATCCAAGCGACATCG CCGTGGAGTGGGAGAGCAATGGGCAGCCG GAGAACAACTACAAGACCACGCCTCCCGT GCTGGACTCCGACGGCTCCTTCTTCCTCT ACAGCAAGCTCACCGTGGACAAGAGCAGG TGGCAGCAGGGGAACGTCTTCTCATGCTC CGTGATGCATGAGGCTCTGCACAACCACT	32	SSEFR33DKTHTCPPC PAPEAAGAPSVFLFPP KPKDTLMLSRTPEVTC VVVDVSHEDPEVKFNW YVDGVEVHNAKTKPRE EQYNSTYRVVSVLTVL HQDWLNGKEYKCAVSN KALPAPIEKTISKAKG QPPEPQVYTLPPSRDE LTKNQVSLTCLVKGFY PSDIAVEWESNGQPEN NYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGN VFSCSVMHEALHNHYT QKSLSLSPG	33

PGR2026-00039
Biocon Ex.1002
Page 4648 of 4945

	ACACGCAGAAGAGCCTCTCCCTGTCTCCG GGT			
Delta SS-Fc domain	GAGCCCAAATCTTCTGACAAAACTCACAC ATGCCCACCGTGCCCAGCACCTGAAGCCG CGGGTGCACCGTCAGTCTTCCTCTTCCCC CCAAAACCCAAGGACACCCTCATGATCTC CCGGACCCCTGAGGTCACATGCGTGGTGG TGGACGTGAGCCACGAAGACCCTGAGGTC AAGTTCAACTGGTACGTGGACGGCGTGGA GGTGCATAATGCCAAGACAAAGCCGCGGG AGGAGCAGTACAACAGCACGTACCGTGTG GTCAGCGTCCTCACCGTCCTGCACCAGGA CTGGCTGAATGGCAAGGAATACAAGTGCG CGGTCTCCAACAAAGCCCTCCCAGCCCCC ATCGAGAAAACCATCTCCAAAGCCAAAGG GCAGCCCCGAGAACCACAGGTGTACACCC TGCCCCCATCCCGGGATGAGCTGACCAAG AACCAGGTCAGCCTGACCTGCCTGGTCAA AGGCTTCTATCCCAGCGACATCGCCGTGG AGTGGGAGAGCAATGGGCAGCCGGAGAAC AACTACAAGACCACGCCTCCCGTGCTGGA CTCCGACGGCTCCTTCTTCCTCTACAGCA AGCTCACCGTGGACAAGAGCAGGTGGCAG CAGGGGAACGTCTTCTCATGCTCCGTGAT GCATGAGGCTCTGCACAACCACTACACGC AGAAGAGCCTCTCCCTGTCTCCGGGT	34	EPKSSDKTHTCPPCPA PEAAGAPSVFLFPPKP KDTLMISRTPEVTCVV VDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQ DWLNGKEYKCAVSNKA LPAPIEKTISKAKGQP REPQVYTLPPSRDELT KNQVSLTCLVKGFYPS DIAVEWESNGQPENNY KTTPPVLDSDGSFFLY SKLTVDKSRWQQGNVF SCSVMHEALHNHYTQK SLSLSPG	35

[0153] In certain embodiments, the immunoglobulin constant regions of each polypeptide chain of a homodimeric or heterodimeric protein described herein are identical to each other. In certain other embodiments, the immunoglobulin constant region of one polypeptide chain of a heterodimeric protein is different from the immunoglobulin constant region of the other polypeptide chain of the heterodimer. For example, one immunoglobulin constant region of a heterodimeric protein can contain a CH3 domain with a "knob" mutation, whereas the other immunoglobulin constant region of the heterodimeric protein can contain a CH3 domain with a "hole" mutation.

Fc-Binding Domain Linker

[0154] In some embodiments, the polypeptide may further comprise a Fc-binding domain linker. In some embodiments, the Fc-binding domain linker can be used to link the immunoglobulin constant region to a C-terminal binding domain (e.g., a CD3 binding domain). In some embodiments, the Fc-binding domain linker can be used as a hinge domain and/or incorporated into an scFv. In some embodiments, the Fc-binding domain linker is a Gly_4Ser linker (SEQ ID NO: 128). In some embodiments, the Fc-binding domain linker is a 20mer consisting of four repeats of a Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 128) amino acid sequence ($(Gly_4Ser)_4$) (SEQ ID NO:61). In some embodiments, the Fc-binding domain linker comprises an amino acid sequence

PGR2026-00039
Biocon Ex.1002
Page 4649 of 4945

selected from any one of SEQ ID NOs 50-70. Other linkers have been used, and phage display technology, as well as selective infective phage technology, has been used to diversify and select appropriate linker sequences (Tang *et al.*, *J. Biol. Chem.* 271, 15682-15686, 1996; Hennecke *et al.*, *Protein Eng.* 11, 405-410, 1998). In certain embodiments, the V_L and V_H regions are joined by a peptide linker having an amino acid sequence comprising the formula $(Gly_4Ser)_n$, wherein n = 1-5 (SEQ ID NO: 129). Other suitable linkers can be obtained by optimizing a simple linker through random mutagenesis. In some embodiments, bispecific molecules do not comprise a hinge region or a constant region.

[0155] In certain embodiments, a Fc-binding domain linker is a flexible linker sequence comprising glycine-serine (*e.g.*, Gly_4Ser, SEQ ID NO: 128) repeats. In certain embodiments, the linker comprises three Gly_4Ser repeats (SEQ ID NO: 59) followed by a proline residue. In certain embodiments the proline residue is followed by an amino acid selected from the group consisting of glycine, arginine and serine. In some embodiments, a Fc-binding domain linker comprises or consists of a sequence selected from SEQ ID NO: 50-70.

[0156] Some exemplary hinge, Fc-binding domain linker sequences suitable for use in accordance with the present disclosure are shown in Table 2. Additional exemplary hinge and linker regions are set forth in SEQ ID NOs: 241-244, 601, 78, 763-791, 228, 379-434, 618-749 of U.S. 2013/0129723 (said sequences incorporated by reference herein).

Table 2: Exemplary hinges and linkers

Name	Amino Acid Sequence	SEQ ID NO
sss(s)-hIgG1 hinge	EPKSSDKTHTSPPSS	SEQ ID NO:36
csc(s)-hIgG1 hinge	EPKSCDKTHTSPPCS	SEQ ID NO:37
ssc(s)-hIgG1 hinge	EPKSSDKTHTSPPCS	SEQ ID NO:38
scc(s)-hIgG1 hinge	EPKSSDKTHTCPPCS	SEQ ID NO:39
css(s)-hIgG1 hinge	EPKSCDKTHTSPPSS	SEQ ID NO:40

PGR2026-00039
Biocon Ex.1002
Page 4650 of 4945

Name	Amino Acid Sequence	SEQ ID NO
scs(s)-hIgG1 hinge	EPKSSDKTHTCPPSS	SEQ ID NO:41
ccc(s)-hIgG1 hinge	EPKSCDKTHTSPPCS	SEQ ID NO:42
ccc(p)-hIgG1 hinge	EPKSCDKTHTSPPCP	SEQ ID NO:43
sss(p)-hIgG1 hinge	EPKSSDKTHTSPPSP	SEQ ID NO:44
csc(p)-hIgG1 hinge	EPKSCDKTHTSPPCP	SEQ ID NO:45
ssc(p)-hIgG1 hinge	EPKSSDKTHTSPPCP	SEQ ID NO:46
scc(p)-hIgG1 hinge	EPKSSDKTHTCPPCP	SEQ ID NO:47
css(p)-hIgG1 hinge	EPKSCDKTHTSPPSP	SEQ ID NO:48
scs(p)-hIgG1 hinge	EPKSSDKTHTCPPSP	SEQ ID NO:49
Scppcp	SCPPCP	SEQ ID NO:50
STD1	NYGGGGSGGGGSGGGGSGNS	SEQ ID NO:51
STD2	NYGGGGSGGGGSGGGGSGNYGGGGSGGGGSGGGGSGNS	SEQ ID NO:52
H1	NS	--
H2	GGGGSGNS	SEQ ID NO:53
H3	NYGGGGSGNS	SEQ ID NO:54
H4	GGGGSGGGGSGNS	SEQ ID NO:55
H5	NYGGGGSGGGGSGNS	SEQ ID NO:56
H6	GGGGSGGGGSGGGGSGNS	SEQ ID NO:57
H7	GCPPCPNS	SEQ ID NO:58
$(G_4S)_3$	GGGGSGGGGSGGGGS	SEQ ID NO:59
H10S	SGGGGSGGGGSGGGGS	SEQ ID NO:60
$(G_4S)_4$	GGGGSGGGGSGGGGSGGGGS	SEQ ID NO:61

PGR2026-00039
Biocon Ex.1002
Page 4651 of 4945

Name	Amino Acid Sequence	SEQ ID NO
$(G_4S)_5$	GGGGSGGGGSGGGGS GGGGSGGGGS	SEQ ID NO: 62
H94	SGGGGSGGGGSGGGG SPNS	SEQ ID NO:68
H111	SGGGGSGGGGSGGGG SPGS	SEQ ID NO:69
H114	GGGGSGGGGSGGGGS PS	SEQ ID NO:70

[0157] In addition to the aforementioned domains, the therapeutic polypeptides can further comprise immunoglobulin dimerization/heterodimerization domains, junctional amino acids, tags, additional binding domains, etc. In some embodiments, the polypeptides and proteins described herein are conjugated to a drug or a toxic moiety.

Bispecific/Multispecific Proteins

[0158] In some embodiments, a therapeutic protein may be a bispecific or multispecific protein. Non-limiting examples of bispecific molecules include an scFv-Fc-scFv molecule, an scFv-Ig molecule and an scFv-scFv molecule. In some embodiments, the bispecific molecules described herein comprise or consist of a first binding domain scFv linked to a second binding domain scFv and do not include other sequences such as an immunoglobulin constant region. In some embodiments, a therapeutic protein may be a bispecific or multispecific protein that comprises, from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus, (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, (iv) (optionally) a Fc-binding domain linker, and (v) a second binding domain.

[0159] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, a CD3 binding domain, a hinge region, an immunoglobulin constant region, and a tumor antigen binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0160] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, a tumor antigen binding domain, a hinge region, an immunoglobulin constant region, and a CD3 binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

PGR2026-00039
Biocon Ex.1002
Page 4652 of 4945

[0161] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, the 4-1-BB binding domain, a hinge region, an immunoglobulin constant region, and a tumor antigen binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

[0162] In some embodiments, a multispecific protein may comprise, from N-terminus to C-terminus, a tumor antigen binding domain, a hinge region, an immunoglobulin constant region, and a 4-1-BB binding domain. The tumor antigen binding domain may bind to, for example, CD123, PSMA, CD19, CD33, 5T4, or HER2.

Homodimers/Heterodimers

[0163] In some embodiments, a therapeutic protein may be a homodimer or a heterodimer. In some embodiments, a therapeutic protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus (i) a first binding domain, (ii) a hinge region, and (iii) an immunoglobulin constant region, (iv) (optionally) a Fc-binding domain linker, and (v) a second binding domain. In some embodiments, the bispecific or multispecific protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus: (i) a first binding domain, (ii) a hinge region, (iii) an immunoglobulin constant region, (iv) (optionally) a Fc-binding domain linker, and (v) a second binding domain. In other embodiments, the bispecific proteins described herein are diabodies.

[0164] In certain embodiments, a hinge present in a polypeptide that forms a heterodimer with another polypeptide chain can be an immunoglobulin hinge, such as a wild-type immunoglobulin hinge region or an altered immunoglobulin hinge region thereof. In certain embodiments, a hinge of one polypeptide chain of a heterodimeric protein is identical to a corresponding hinge of the other polypeptide chain of the heterodimer. In certain other embodiments, a hinge of one chain is different from that of the other chain (in their length or sequence). The different hinges in the different chains allow different manipulation of the binding affinities of the binding domains to which the hinges are connected, so that the heterodimer is able to preferentially bind to the target of one binding domain over the target of the other binding domain.

[0165] In other embodiments, the polypeptides and proteins described herein include a heterodimerization domain that is capable of heterodimerization with a

PGR2026-00039
Biocon Ex.1002
Page 4653 of 4945

different heterodimerization domain in a second, non-identical polypeptide chain. In certain variations, the second polypeptide chain for heterodimerization includes a second binding domain. Accordingly, in certain embodiments of the present disclosure, two non-identical polypeptide chains, one comprising the polypeptide comprising a first binding domain and the second optionally comprising a second binding domain, dimerize to form a heterodimeric binding protein. Dimerization/heterodimerization domains can be used where it is desired to form heterodimers from two non-identical polypeptide chains, where one or both polypeptide chains comprise a binding domain. In certain embodiments, one polypeptide chain member of certain heterodimers described herein does not contain a binding domain. Examples of types of heterodimers include those described in U.S. Patent Application Publication Nos. 2013/0095097 and 2013/0129723, and International PCT Publication No. WO 2016/094873.

[0166] In certain embodiments, the first and second polypeptide chains dimerize via the inclusion of an "immunoglobulin dimerization domain" or "immunoglobulin heterodimerization domain." An "immunoglobulin dimerization domain" or "immunoglobulin heterodimerization domain" refers herein to an immunoglobulin domain of a first polypeptide chain that preferentially interacts or associates with a different immunoglobulin domain of a second polypeptide chain, wherein the interaction of the different immunoglobulin domains substantially contributes to or efficiently promotes heterodimerization of the first and second polypeptide chains (*i.e.*, the formation of a dimer between two different polypeptide chains, which is also referred to as a "heterodimer"). The immunoglobulin heterodimerization domains in the polypeptide chains of a heterodimer are different from each other and thus can be differentially modified to facilitate heterodimerization of both chains and to minimize homodimerization of either chain. Immunoglobulin heterodimerization domains provided herein allow for efficient heterodimerization between different polypeptides and facilitate purification of the resulting heterodimeric protein.

[0167] As provided herein, immunoglobulin heterodimerization domains useful for promoting heterodimerization of two different polypeptide chains according to the present disclosure include wild-type and altered immunoglobulin CH1 and CL domains, for instance, human CH1 and CL domains. In certain embodiments, an immunoglobulin heterodimerization domain is a wild-type CH1 domain, such as a wild type IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM CH1 domain, for example,

PGR2026-00039
Biocon Ex.1002
Page 4654 of 4945

as set forth in SEQ ID NOs: 114, 186-192 and 194, respectively, of U.S. Patent Application Publication No. 2013/0129723 or SEQ ID NO: 114 of U.S. Patent Application Publication No. 2013/0129723 (said sequence incorporated by reference herein). In further embodiments, a cysteine residue of a wild-type CH1 domain (*e.g.*, a human CH1) involved in forming a disulfide bond with a wild type immunoglobulin CL domain (*e.g.*, a human CL) is deleted or substituted in the altered immunoglobulin CH1 domain such that a disulfide bond is not formed between the altered CH1 domain and the wild-type CL domain.

[0168] Polypeptides and proteins described herein may be made using scaffolding as generally disclosed in U.S. Patent Application Publication Nos. 2013/0129723 and 2013/0095097, which are each incorporated herein by reference in their entirety. The polypeptides described herein may comprise two non-identical polypeptide chains, each polypeptide chain comprising an immunoglobulin heterodimerization domain. The interfacing immunoglobulin heterodimerization domains are different. In one embodiment, the immunoglobulin heterodimerization domain comprises a CH1 domain or a derivative thereof. In another embodiment, the immunoglobulin heterodimerization domain comprises a CL domain or a derivative thereof. In one embodiment, the CL domain is a Cκ or Cλ isotype or a derivative thereof.

Exemplary protein therapeutics: Anti-CD123 x Anti-CD3 Polypeptides and Dimers thereof

[0169] An exemplary protein therapeutic may bind both CD123-expressing cells and the T-cell receptor complex on T-cells to induce target-dependent T-cell cytotoxicity, activation and proliferation.

[0170] Thus, in certain embodiments, the therapeutic protein used in connection with the methods and compositions described herein is a bispecific single chain molecule comprising a CD123 binding domain and a CD3 binding domain. In some embodiments, a CD123 and/or a CD3 binding domain is derived from an antibody and comprises a variable heavy chain (VH) and a variable light chain (VL). For example, the CD123 and/or CD3 binding domains may be an scFv that comprises a VH and VL. These binding domains and variable chains may be arranged in any order that still retains some binding to the target(s). For example, the variable domains may be arranged in the order such as (VH CD123)-(VL CD123)-(VH CD3)-(VL CD3); (VL

PGR2026-00039
Biocon Ex.1002
Page 4655 of 4945

CD123)-(VH CD123)-(VH CD3)-(VL CD3); (VH CD123)-(VL CD123)-(VL CD3)-(VH CD3); (VL CD123)-(VH CD123)-(VL CD3)-(VH CD3); (VH CD3)-(VL CD3)-(VH CD123)-(VL CD123); (VL CD3)-(VH CD3)-(VL CD123)-(VH CD123); (VH CD3)-(VL CD3)-(VL CD123)-(VH CD123); or (VL CD3)-(VH CD3)-(VH CD123)-(VL CD123). The pairs of VH regions and VL regions in the binding domain binding to CD3 may be in the format of a single chain antibody (scFv). The VH and VL regions may be arranged in the order VH-VL or VL-VH. In some embodiments, the scFv may bind to CD123 more effectively than the antibody comprising the same VH and VL region sequences in the same orientation. In certain embodiments, the scFv may bind more effectively to CD123 in the VL-VH orientation than in the VH-VL orientation, or vice versa. The VH-region may be positioned N-terminally to a linker sequence. The VL region may be positioned C-terminally to the linker sequence. The domain arrangement in the CD3 binding domain of the bispecific single chain molecule may be VH-VL, with the CD3 binding domain located C-terminally to the CD123-binding domain. A bispecific molecule may comprise an scFv binding to CD123 linked to an scFv binding to CD3. These scFvs may be linked with a short peptide. In some embodiments, bispecific single chain molecules do not comprise a hinge region or a constant region (see, for example, US 2013/0295121, WO 2010/037836, WO 2004/106381 and WO 2011/121110; each incorporated herein by reference in its entirety).

[0171] The CD123-bispecific binding construct may comprise one or more sequences shown in Table 3, Table 4, and/or Table 5.

Table 3: Binding Polypeptide Sequences and Components

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
OMT1 variable light chain domain	gacatcgtgatgacccagtctccagactccctggctgtgtctctgggcgag agggccaccatcaactgcaagtccagccacagtgttttatacagctccaa caataagaactacttagcttggtaccagcagaaaccaggacagcctccta agctgctcatttactgggcatctacccgggaatccggggtccctgaccgat tcagtggcagcgggtctgggacagatttcactctcaccatcagcagcctgc aggctgaagatgtggcagttattactgtcagcaatattatagtactcctcc gaccactttcggcggagggaccaaggtggagatcaaa	divmtqspdslavslger atinckssqsvlyssnnk nylawyqqkpgqppkll iywastresgvpdrfsgs gsgtdftltissiqaedva vyycqqyystpptffggg tkveik	SEQ ID NO:133 (SEQ ID NO:134)

PGR2026-00039
Biocon Ex.1002
Page 4656 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
OMT1 variable heavy chain domain	gaggtgcagctgttggagtctgggggaggcttggtacagcctgggggtc cctgagactctcctgtgcagcctctggatttcaccttttagcagctatggcatg agctgggtccgccaggctccagggaaggggctggaggggtctcagcta ttagtggtagtggtggtagcacatactacgcagactccgtgaagggccgg ttcaccatctccagagacaattccaagaacacgctgtatctgcaaatgaa cagcctgagagccgaggacacggccgtatattactgtgcgaaagaaaag ttacgatattttgactggttatccgatgcttttgatatctggggccaaggga caatggtcaccgtctcttca	evqllesgggivqpggslr lscaasgftfssygmswv rqapgkglegvsaisgsg gstyyadsvkgrftisrdn sknt1ylqmnslraedta vyycakekiryfdwlsda fdiwgqgtmvtvss	SEQ ID NO:135 (SEQ ID NO:136)
OMT1 CDR L1	cacagtgtttatacagctccaacaataagaactac	HSVLYSSNNKNY	SEQ ID NO:137 (SEQ ID NO:138)
OMT1 CDR L2	tgggcatct	WAS	SEQ ID NO:139 (SEQ ID NO:140)
OMT1 CDR L3	cagcaatattatagtactcctccgaccact	QQYYSTPPTT	SEQ ID NO:141 (SEQ ID NO:142)
OMT1 CDR H1	ggatttcacctttagcagctatggc	GFTFSSYG	SEQ ID NO:143 (SEQ ID NO:144)
OMT1 CDR H2	attagtggtagtggtggtagcaca	ISGSGGST	SEQ ID NO:145 (SEQ ID NO:146)
OMT1 CDR H3	gcgaaagaaaagttacgatattttgactggttatccgatgcttttgatatc	AKEKLRYFDWLSDA FDI	SEQ ID NO:147 (SEQ ID NO:148)

PGR2026-00039
Biocon Ex.1002
Page 4657 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
MT1 VHVL x T5C456 scFv-Fc-scFv TRI129	atggaagcaccagcgcagcttctcttcctcctgctactctggctcccagat accaccggtgaggtgcagctgttggagtctgggggaggcttggtacagcc tggggggtccctgagactctcctgtgcagcctctggattcaccttiagcagc tatggcatgagctgggtccgccaggctccagggaaggggctggaggggg tctcagctattagtggtagtggtggtagcacatactacgcagactccgtga agggccggttcaccatctccagagacaattccaagaacacgctgtatctg caaatgaacagcctgagagccgaggacacggccgtatattactgtgcga aagaaaagttacgatattttgactggttatccgatgcttttgatatctgggg ccaagggacaatggtcaccgtctcttcaggtggaggcggttcaggcggag gtggatccggcggtggcggctccggtggcggcggatctgacatcgtgatg acccagtctccagactccctggctgtgtctctgggcgagagggccaccatc aactgcaagtccagccagtgttttatacagctccaacaataagaaacta cttagcttggtaccagcagaaaccaggcagcctcctaagctgctcattta ctgggcatctacccgggaatccggggtccctgaccgattcagtggcagcg ggtctgggacagatttcactctcaccatcagcagcctgcaggctgaagat gtggcagtttattactgtcagcaatattatagtactcctccgaccactttcg gcggagggaccaaggtggagatcaaatcctcgagtgagcccaaatcttct gacaaaactcacacatgcccaccgtgcccagcacctgaagccgcgggtg caccgtcagtcttcttcttcccccaaaacccaaggacaccctcatgatct cccggacccctgaggtcacatgcgtggtggtggacgtgagccacgaaga ccctgaggtcaagttcaactggtacgtggacggcgtggaggtgcataatg ccaagacaaagccgcgcgggaggagcagtacaacagcacgtaccgtgtgg tcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggaatac aagtgcgcggtctccaacaaagccctcccagcccccatcgagaaaacca tctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcc cccatcccgggatgagctgaccaagaaccaggtcagcctgacctgcctgg tcaaaggcttctatccagcgacatcgccgtggagtgggagagcaatgg gcagccggagaacaactacaagaccacgcctcccgtgctggactccgac ggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggca gcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaacc actacacgcagaagagcctctccctgtctccgggttccggaggaggggat tcaggtgggggaggttctggcggcgggggaagccctttcacaggtgcaact ggtgcagcgtggacccgaggttaaaaaaccagggtcctccgttaaggtta gctgcaaagcctctggctacacatttccaggagtacaatgcactgggtga ggcaggctcctggacagggactcgagtggatcgggtatatcaaccccatct agcgcctataccaattacaaccaaaagtttaaggaccgagttaccattac cgctgacaaatccaccagtacagcttatatggagctgtcatctcttaggtc cgaggacactgctgtttattactgcgctcgtcctcaggttcactatgactat aatggttttccctactggggtcagggaaccctggtgactgtctcttctggcg gtggaggcagcggtgggggtggctctggaggcggtggcagtggcggcgg aggctctgatattcagatgactcagtctcctagcactctcagcgccagcgt gggggatcgtgtgacaatgacttgctccgctagcagtagtgtgtcttacat gaattggtatcagcagaagcccgggaaagcacctaagcgctggatctat gactcttccaagctggcaagtggtgtcccctcacggttctctggctcaggtt ctggtactgactatactttgactatctcctccctccagcccgatgatttcgct acctattattgtcagcagtggagccgtaacccacccactttcggaggcggt accaaagtggagatcaagaggtcataa	evqliesgggivqpggslr lscaasgftfssygmswv rqapgkglegvsaisgsg gstyyadsvkgrftisrdn sknilylqmnsiraedta vyycakekiryfdwlsde fdiwgqgtmvtvssggg gsggggsggggsgggs divmtqspdslavslger atinckssqsvlyssnnk nylawyqqkpgqppkll iywastresgvpdrfsgs gsgtdftlticslqaedva vyycqqyystpptffggg tkveikssepkssdkth tcppcpapeaagapsvf lfppkpkdtlmisrtpev tcvvvdvshedpevkfn wyvdgvevhnaktkpr eeqynstyrvvsvltvlh qdwlngkeykcavsnk alpapiektiskakgqpr epqvytlppsrdeltknq vsltclvkgfypsdiave wesngqpennyklttpp vldsdgsfflyskltvdksr wqqgnvfscsvmheal hnhytqkslslspgsggg gsggggsggggspsqvq lvqsgpevkkpgssvkv sckasgytfsrstmhwv rqapgqglewigyinps saytnynqkfkdrvtita dkststaymelssirsed tavyycarpqvhydyng fpywgqgtlvtvssggg gsggggsggggsgggs diqmtqspstlsasvgdr vtmtcsasssvsymnw yqqkpgkapkrwiyds sklasgvpsrfsgsgsgtd ytlticslqpddfatyycq qwsrnpptfgggtkvei krs	SEQ ID NO:309 (SEQ ID NO:310)

PGR2026-00039
Biocon Ex.1002
Page 4658 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
OMT1 VLVH x TSC456 scFv-Fc-scFv TRI130	atggaagcaccagcgcagcttctcttcctcctgctactctggctcccagat accaccggtgacatcgtgatgacccagtctccagactccctggctgtgtct ctgggcgagagggccaccatcaactgcaagtccagccacagtgttttata cagctccaacaataagaactacttagcttggtaccagcagaaaccagga cagcctcctaagctgctcatttactgggcatctaccccgggaatccggggtc cctgaccgattcagtggcagcgggtctgggacagatttcactctcaccatc agcagcctgcaggctgaagatgtggcagtttattactgtcagcaatattat agtactcctccgaccacttttcggcggagggaccaaggtggagatcaaag gtggaggcggttcaggcggaggtggatccggcggtggcggctccggtgga cggcggatctgaggtgcagctgttggagtctgggggaggcttggtacagc ctgggggtccctgagactctcctgtgcagcctctggattcacctttagcag ctatggcatgagctgggtccgccaggctccagggaaggggctggagggg gtctcagctattagtggtagtggtggtagcacatactacgcagactccgtg aagggccggttcaccatctccagagacaattccaagaacacgctgtatct gcaaatgaacagcctgagagccgaggacacggccgtatattactgtgcg aaagaaaagttacgatattttgactggttatccgatgctttgatatctggg gccaagggacaatggtcaccgtctcctcgagtgagcccaaatcttctgac aaaactcacacatgcccaccgtgcccagcacctgaagccgcgggtgcac cgtcagtcttcctcttccccccaaaacccaaggacaccctcatgatctccc ggacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccct gaggtcaagttcaactggtacgtggacggcgtggaggtgcataatgccaa gacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcag cgtcctcaccgtcctgcaccaggactggctgaatggcaaggaatacaagt gcgcggtctccaacaaagccctcccagcccccatcgagaaaaccatctcc aaagccaaagggcagccccgagaaccacaggtgtacaccctgcccccat cccgggatgagctgaccaagaaccaggtcagcctgacctgcctggtcaa aggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcag ccggagaacaactacaagaccacgcctcccgtgctggactccgacggctc cttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcagg ggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactaca cgcagaagagcctctccctgtctccgggttccggaggaggggggttcaggt gggggaggttcggcggcgggggaagcccttcacaggtgcaactggtgc agagtggacccgaggttaaaaaaaccagggtcctccgttaaggttagctgc aaagcctctggctacacatttccaggagtacaatgcactgggtgaggca ggctcctggacagggactcgagtggatcgggtatatcaacccatctagcg cctataccaattacaaccaaaagtttaaggaccgagttaccattaccgctg acaaatccaccagtacagcttatatggagctgtcatctcttaggtccgagg acactgctgttttattactgcgctcgtcctcaggttcactatgactataatggt tttccctactggggtcagggaaccctggtgactgtctcttctggcggtggag gcagcggtgggggtgggtctggaggcggtggcagtggcggcggaggctc tgatattcagatgactcagtctcctagcactctcagcgccagcgtgggga tcgtgtgacaaatgacttgctccgctagcagtagtgtgtcttacatgaattgg tatcagcagaagcccgggaaagcacctaagcgctggatctatgactcttc caagctggcaagtggtgtcccctcacggttctctggctcaggttctggtact gactatactttgactatctcctcccctccagcccgatgatttcgctacctatta ttgtcagcagtggagccgtaaccccacccactttcggaggcggtaccaaag tggagatcaagaggtcataa	divmtqspdslavslger atinckssqsvlysanrk nylawyqqkpgqppkll iywastresgvpdrfsgs gsgtdftlticsslqaedva vyycqqyystpptfggg tkveikggggsggggsgg ggsggggsevqllesggg lvqpggslrlscaasgftfs sygmswvrqapgkgle gvsaisgsggstyyadsv kgrftisrdnskntlylqm nslraedtavyycakekl ryfdwlsdafdiwgqgt mvtvsssepkssdkthtt cppcpapeaagapsvfl fppkpkdtlmisrtpevt cvvvdvshedpevkfn wyvdgvevhnaktkpr eeqynstyrvvsvltvlh qdwlngkeykcavsnk alpapiektiskakgqpr epqvytlppsrdeltknq vsltclvkgfypsdiave wesngqpennyklttpp vldsdgsfflyskltvdksr wqqgnvfscsvmheal hnhytqkslslspgsggg gsggggsggggspsqvq lvqsgpevkkpgssvkv sckasgytfsrstmhwv rqapgqglewigyinps saytnynqkfkdrvtita dkststaymelssirsed tavyycarpqvhydyng fpywgqgtlvtvssggg gsggggsggggsggggs diqmtqspstlsasvgdr vtmtcsasssvsymnw yqqkpgkapkrwiyds sklasgvpsrfsgsgsgtd ytlticsslqpddfatyycq qwsrnpptfgggtkvei krs	SEQ ID NO:311 (SEQ ID NO:312)
Cris7 and DRA222 VH CDR1 (Kabat)		RSTMH	(SEQ ID NO:345)

PGR2026-00039
Biocon Ex.1002
Page 4659 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
Cris7 and DRA222 VH CDR2 (Kabat)		YINPSSAYTNYNQKFK	(SEQ ID NO:346)
Cris7 and DRA222 VH CDR3 (Kabat)		QVHYDYNGFPY	(SEQ ID NO:347)
Cris7 and DRA222 VL CDR1 (Kabat)		SASSSVSYMN	(SEQ ID NO:348)
Cris7 and DRA222 VL CDR2 (Kabat)		DSSKLAS	(SEQ ID NO:349)
Cris7 and DRA222 VL CDR3 (Kabat)		QQWSRNPPT	(SEQ ID NO:350)
Cris7 and DRA222 VH CDR1 (IMGT)		GYTFTRST	(SEQ ID NO:351)
Cris7 and DRA222 VH CDR2 (IMGT)		INPSSAYT	(SEQ ID NO:352)
Cris7 and DRA222 VH CDR3 (IMGT)		QQWSRNPPT	(SEQ ID NO:353)
Cris7 and DRA222 VL CDR1 (IMGT)		ASSSVSY	(SEQ ID NO:354)
Cris7 and DRA222 VL CDR2 (IMGT)		DSS	(SEQ ID NO:355)
Cris7 and DRA222 VL CDR3 (IMGT)		QQWSRNPPT	(SEQ ID NO:356)

PGR2026-00039
Biocon Ex.1002
Page 4660 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
Cris-7 variable light chain sequence		QVVLTQSPAIMSA FPGEKVTMTCSAS SSVSYMNWYQQK SGTSPKRWIYDSS KLASGVPARFSGS GSGTSYSLTISSME TEDAATYYCQQW SRNPPTFGGGTKL QITR	(SEQ ID NO: 341)
Cris-7 variable heavy chain sequence		QVQLQQSGAELA RPGASVKMSCKA SGYTFTRSTMHW VKQRPGQGLEWI GYINPSSAYTNYN QKFKDKATLTADK SSSTAYMQLSSLTS EDSAVYYCASPQV HYDYNGFPYWGQ GTLVTVSA	(SEQ ID NO: 342)
HuM291 variable light chain sequence		DIQMTQSPSSLSA SVGDRVTITCSASS SVSYMNWYQQK PGKAPKRLIYDTSK LASGVPSRFSGSG SGTDFTLTISSLQP EDFATYYCQQWS SNPPTFGGGTKVE IK	(SEQ ID NO: 343)
HuM291 variable heavy chain sequence		QVQLVQSGAEVK KPGASVKVSCKAS GYTFISYTMHWV RQAPGQGLEWM GYINPRSGYTHYN QKLKDKATLTADK SASTAYMELSSLRS EDTAVYYCARSAY YDYDGFAYWGQG TLVTVSS	(SEQ ID NO: 344)
I2C VH CDR1 (Kabat)		KYAMN	(SEQ ID NO:357)
I2C VH CDR2 (Kabat)		RIRSKYNNYATYYAD SVKD	(SEQ ID NO:358)
I2C VH CDR3 (Kabat)		HGNFGNSYISYWAY	(SEQ ID NO:359)

PGR2026-00039
Biocon Ex.1002
Page 4661 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
I2C VL CDR1 (Kabat)		GSSTGAVTSGNYPN	(SEQ ID NO:360)
I2C VL CDR2 (Kabat)		GTKFLAP	(SEQ ID NO:361)
I2C VL CDR3 (Kabat)		VLWYSNRWV	(SEQ ID NO:362)
I2C VH CDR1 (IMGT)		GFTFNKYA	(SEQ ID NO:363)
I2C VH CDR2 (IMGT)		IRSKYNNYAT	(SEQ ID NO:364)
I2C VH CDR3 (IMGT)		VRHGNFGNSYISYWAY	(SEQ ID NO:365)
I2C VL CDR1 (IMGT)		TGAVTSGNY	(SEQ ID NO:366)
I2C VL CDR2 (IMGT)		GTK	(SEQ ID NO:367)
I2C VL CDR3 (IMGT)		VLWYSNRWV	(SEQ ID NO:368)
HuM291 VH CDR1 (Kabat)		SYTMH	(SEQ ID NO:369)
HuM291 VH CDR2 (Kabat)		YINPRSGYTHYNQKLKD	(SEQ ID NO:370)
HuM291 VH CDR3 (Kabat)		SAYYDYDGFAY	(SEQ ID NO:371)
HuM291 VL CDR1 (Kabat)		SASSSVSYMN	(SEQ ID NO:372)
HuM291 VL CDR2 (Kabat)		DTSKLAS	(SEQ ID NO:373)
HuM291 VL CDR3 (Kabat)		QQWSSNPPT	(SEQ ID NO:374)
HuM291 VH CDR1 (IMGT)		GYTFISYT	(SEQ ID NO:375)
HuM291 VH CDR2 (IMGT)		INPRSGYT	(SEQ ID NO:376)

PGR2026-00039
Biocon Ex.1002
Page 4662 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
HuM291 VH CDR3 (IMGT)		ARSAYYDYDGFAY	(SEQ ID NO:377)
HuM291 VL CDR1 (IMGT)		ASSSVSY	(SEQ ID NO:378)
HuM291 VL CDR2 (IMGT)		DTS	(SEQ ID NO:379)
HuM291 VL CDR3 (IMGT)		QQWSSNPPT	(SEQ ID NO:380)
TSC455 (anti-CD3) TSC394 F87Y scFv		QVQLVQSGPEVKKP GSSVKVSCKASGYTF SRSTMHWVRQAPG QGLEWIGYINPSSAY TNYNQKFKDRVTIT ADKSTSTAYMELSSL RSEDTAVYYCARPQ VHYDYNGFPYWGQ GTLVTVSSGGGGSG GGGSGGGGSGGGG SDIQMTQSPSTLSAS VGDRVTMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFSGSGSGTEY TLTISSLQPDDFATYY CQQWSRNPPTFGG GTKVEIKRSSS	(SEQ ID NO:381)
TSC456 (anti-CD3) TSC394 E86D F87Y scFv		QVQLVQSGPEVKKP GSSVKVSCKASGYTF SRSTMHWVRQAPG QGLEWIGYINPSSAY TNYNQKFKDRVTIT ADKSTSTAYMELSSL RSEDTAVYYCARPQ VHYDYNGFPYWGQ GTLVTVSSGGGGSG GGGSGGGGSGGGG SDIQMTQSPSTLSAS VGDRVTMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFSGSGSGTD YTLTISSLQPDDFATY YCQQWSRNPPTFG GGTKVEIKRSSS	(SEQ ID NO:382)

PGR2026-00039
Biocon Ex.1002
Page 4663 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
TSC455 and TSC456 variable heavy domain		QVQLVQSGPEVKKP GSSVKVSCKASGYTF SRSTMHWVRQAPG QGLEWIGYINPSSAY TNYNQKFKDRVTIT ADKSTSTAYMELSSL RSEDTAVYYCARPQ VHYDYNGFPYWGQ GTLVTVSS	(SEQ ID NO:383)
TSC455 variable light domain		DIQMTQSPSTLSAS VGDRVTMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFSGSGSGTEY TLTISSLQPDDFATYY CQQWSRNPPTFGG GTKVEIKRS	(SEQ ID NO:384)
TSC456 variable light domain		DIQMTQSPSTLSAS VGDRVTMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPSRFSGSGSGTD YTLTISSLQPDDFATY YCQQWSRNPPTFG GGTKVEIKRS	(SEQ ID NO:385)
DRA222 (anti-CD3) scFv		QVQLVESGGGVVQ PGRSLRLSCKASGYT FTRSTMHWVRQAP GQGLEWIGYINPSS AYTNYNQKFKDRFTI SADKSKSTAFLQMD SLRPEDTGVYFCARP QVHYDYNGFPYWG QGTPVTVSSGGGGS GGGGSGGGGSAQD IQMTQSPSSLSASV GDRVTMTCSASSSV SYMNWYQQKPGK APKRWIYDSSKLAS GVPARFSGSGSGTD YTLTISSLQPEDFATY YCQQWSRNPPTFG GGTKLQITSS	(SEQ ID NO:386)

PGR2026-00039
Biocon Ex.1002
Page 4664 of 4945

Name	Nucleotide Sequence	Amino Acid Sequence	SEQ ID NOs: nucleotide (amino acid)
DRA222 variable heavy domain		QVQLVESGGGVVQ PGRSLRLSCKASGYT FTRSTMHWVRQAP GQGLEWIGYINPSS AYTNYNQKFKDRFTI SADKSKSTAFLQMD SLRPEDTGVYFCARP QVHYDYNGFPYWG QGTPVTVSS	(SEQ ID NO:387)
DRA222 variable light domain		DIQMTQSPSSLSAS VGDRVTMTCSASSS VSYMNWYQQKPG KAPKRWIYDSSKLAS GVPARFSGSGSGTD YTLTISSLQPEDFATY YCQQWSRNPPTFG GGTKLQITS	(SEQ ID NO:388)

Table 4: Composition of Humanized Constructs

Construct ID	scFv Orientation	Nucleotide SEQ ID NO	Amino acid SEQ ID NO
TRI129	VHVL	309	310
TRI130	VLVH	311	312

Table 5: Amino acid sequences of exemplary binding protein construct

Construct name	Sequence	SEQ ID NO
TRI130 (CD123 binding domain in bold, CD3 binding domain in italics) (CDR sequences	MEAPAQLLFLLLLWLPDTTG**DIVMTQSPDSLAVSLGERATINCKS SHSVLYSSNNKNYLAWYQQKPGQPPKLLIYWASTRESGVPDR FSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTPPTTFGGGTKV EIKGGGGSGGGGSGGGGSGGGGSEVQLLESGGGLVQPGGSL RLSCAASGFTFSSYGMSWVRQAPGKGLEGVSAISGSGGSTYY ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKEKLRY FDWLSDAFDIWGQGTMVTVSS**SEPKSSDKTHTCPPCPAPEAAG APSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAV	337

PGR2026-00039
Biocon Ex.1002
Page 4665 of 4945

are single-underlined)	SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGSGGGGSGG GGSGGGGSPSQVQLVQSGPEVKKPGSSVKVSCKASGYTFSRS TMHWVRQAPGQGLEWIGYINPSSAYTNYNQKFKDRVTITADKST STAYMELSSLRSEDTAVYYCARPQVHYDYNGFPYWGQGTLVTV SSGGGGSGGGGSGGGGSGGGGSDIQMTQSPSTLSASVGDRV TMTCSASSSVSYMNWYQQKPGKAPKRWIYDSSKLASGVPSRFS GSGSGTDYTLTISSLQPDDFATYYCQQWSRNPPTFGGGTKVEIK RS	

[0172] In certain embodiments, the CD123-binding domain comprises (i) an immunoglobulin light chain variable region (VL) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (VH) comprising CDRs HCDR1, HCDR2, and HCDR3 with HCDR1 comprising an amino acid sequence as set forth in SEQ ID NO:144, with HCDR2 comprising an amino acid sequence as set forth in SEQ ID NO:146 and with HCDR3 comprising an amino acid sequence as set forth in SEQ ID NO:148. In certain embodiments, the CD123-binding domain comprises (i) an immunoglobulin light chain variable region (VL) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (VH) comprising CDRs HCDR1, HCDR2, and HCDR3. In some such embodiments, (i) the LCDR1 has an amino acid sequence set forth in SEQ ID NO:138 or a sequence that differs from SEQ ID NO:138 by at least one amino acid substitution; (ii) the LCDR2 has an amino acid sequence set forth in SEQ ID NO:140 or a sequence that differs from SEQ ID NO:140 by at least one amino acid substitution; (iii) the LCDR3 has an amino acid sequence set forth in SEQ ID NO:142 or a sequence that differs from SEQ ID NO:142 by at least one amino acid substitution; (iv) the HCDR1 has an amino acid sequence set forth in SEQ ID NO:144 or a sequence that differs from SEQ ID NO:144 by at least one amino acid substitution; (v) the HCDR2 has an amino acid sequence set forth in SEQ ID NO:146 or a sequence that differs from SEQ ID NO:146 by at least one amino acid substitution; and (vi) the HCDR3 has an amino acid sequence set forth in SEQ ID NO:148 or a sequence that differs from SEQ ID NO:148 by at least one

PGR2026-00039
Biocon Ex.1002
Page 4666 of 4945

amino acid substitution. The amino acid substitution described above may be a conservative or a non-conservative amino acid substitution. In some embodiments, an LCDR1, LCDR2, LCDR3, HCDR1, HCDR2, and/or HCDR3 differs from a recited sequence by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids. In certain embodiments, a CDR of the present disclosure contains about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) insertions, about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) deletions, about one or more (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10) amino acid substitutions (e.g., conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared to the CDR sequence of a known monoclonal antibody. For instance, the disclosure includes a recombinant polypeptide comprising (i) the LCDR1 has an amino acid sequence set forth in SEQ ID NO:138 or a sequence that differs from SEQ ID NO:138 by one or two amino acid substitutions; (ii) the LCDR2 has an amino acid sequence set forth in SEQ ID NO:140 or a sequence that differs from SEQ ID NO:140 by one or two amino acid substitutions; (iii) the LCDR3 has an amino acid sequence set forth in SEQ ID NO:142 or a sequence that differs from SEQ ID NO:142 by one or two amino acid substitutions; (iv) the HCDR1 has an amino acid sequence set forth in SEQ ID NO:144 or a sequence that differs from SEQ ID NO:144 by one or two amino acid substitutions; (v) the HCDR2 has an amino acid sequence set forth in SEQ ID NO:146 or a sequence that differs from SEQ ID NO:146 by one or two amino acid substitutions; and (vi) the HCDR3 has an amino acid sequence set forth in SEQ ID NO:148 or a sequence that differs from SEQ ID NO:148 by one or two amino acid substitutions. The amino acid substitution described above may be a conservative or a non-conservative amino acid substitution.

[0173] In related embodiments, a recombinant polypeptide of the disclosure comprises or is a sequence that is at least about 80%, at least about 85%, at least about 88%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% identical to an amino acid sequence of a light chain variable region (V_L) (*e.g.*, SEQ ID NO:134) or to a heavy chain variable region (V_H) (*e.g.*, SEQ ID NO:136), or both. In one embodiment, the CD123-binding domain of the recombinant polypeptide is an scFv comprising a variable heavy chain comprising SEQ ID NO:136 and a variable light chain comprising SEQ ID NO:134 in the VHVL orientation. In another embodiment, the CD123-binding domain of the recombinant polypeptide is an scFv comprising a variable light chain

PGR2026-00039
Biocon Ex.1002
Page 4667 of 4945

comprising SEQ ID NO:134 and a variable heavy chain comprising SEQ ID NO:136 in the VLVH orientation. For instance, in certain embodiments, the polypeptide of the disclosure comprises an amino acid sequence of SEQ ID NO:337. The instant disclosure includes a recombinant polypeptide that is at least about 80%, at least about 85%, at least about 88%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% identical to an amino acid sequence of SEQ ID NO:337.

[0174] In certain embodiments, the CD123-binding domain comprises (i) an immunoglobulin light chain variable region (V_L) comprising CDRs LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region (V_H) comprising CDRs HCDR1, HCDR2, and HCDR3. In some such embodiments, (i) the LCDR1 has an amino acid sequence set forth in SEQ ID NO:154 or a sequence that differs from SEQ ID NO:154 by at least one amino acid substitution; (ii) the LCDR2 has an amino acid sequence set forth in SEQ ID NO:156 or a sequence that differs from SEQ ID NO:156 by at least one amino acid substitution; (iii) the LCDR3 has an amino acid sequence set forth in SEQ ID NO:158 or a sequence that differs from SEQ ID NO:158 by at least one amino acid substitution; (iv) the HCDR1 has an amino acid sequence set forth in SEQ ID NO:160 or a sequence that differs from SEQ ID NO:160 by at least one amino acid substitution; (v) the HCDR2 has an amino acid sequence set forth in SEQ ID NO:162 or a sequence that differs from SEQ ID NO:162 by at least one amino acid substitution; and (vi) the HCDR3 has an amino acid sequence set forth in SEQ ID NO:164 or a sequence that differs from SEQ ID NO:164 by at least one amino acid substitution. The amino acid substitution described above may be a conservative or a non-conservative amino acid substitution. In some embodiments, an LCDR1, LCDR2, LCDR3, HCDR1, HCDR2, and/or HCDR3 differs from a recited sequence by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids. In certain embodiments, a CDR of the present disclosure contains about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) insertions, about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) deletions, about one or more (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10) amino acid substitutions (*e.g.*, conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared to the CDR sequence of a known monoclonal antibody.

PGR2026-00039
Biocon Ex.1002
Page 4668 of 4945

[0175] In related embodiments, a CD123-binding domain comprises or is a sequence that is at least about 80%, at least about 85%, at least about 88%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% identical to an amino acid sequence of a light chain variable region (V_L) (*e.g.*, SEQ ID NO:17) or to a heavy chain variable region (V_H) (*e.g.*, SEQ ID NO: 16), or both.

[0176] In certain embodiments, a CD123-binding domain comprises humanized immunoglobulin V_L and/or V_H regions. Techniques for humanizing immunoglobulin V_L and V_H regions are known in the art and are discussed, for example, in U.S. Patent Application Publication No. 2006/0153837. In certain embodiments, a CD123-binding domain comprises human immunoglobulin V_L and/or V_H regions.

[0177] Essentially, humanization by CDR grafting involves recombining only the CDRs of a non-human antibody onto a human variable region framework and a human constant region. Theoretically, this should substantially reduce or eliminate immunogenicity (except if allotypic or idiotypic differences exist). However, it has been reported that some framework residues of the original antibody also may need to be preserved (Reichmann *et al.*, *Nature*, 332:323 (1988); Queen *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:10,029 (1989)).

[0178] The framework residues that need to be preserved are amenable to identification through computer modeling. Alternatively, critical framework residues can potentially be identified by comparing known antigen-binding site structures (Padlan, *Molec. Immunol.*, 31(3):169-217 (1994), incorporated herein by reference).

[0179] The residues that potentially affect antigen binding fall into several groups. The first group comprises residues that are contiguous with the antigen site surface, which could therefore make direct contact with antigens. These residues include the amino-terminal residues and those adjacent to the CDRs. The second group includes residues that could alter the structure or relative alignment of the CDRs, either by contacting the CDRs or another peptide chain in the antibody. The third group comprises amino acids with buried side chains that could influence the structural integrity of the variable domains. The residues in these groups are usually found in the same positions (Padlan, 1994, *supra*) although their positions as identified may differ depending on the numbering system (*see* Kabat *et al.*, "Sequences of proteins of

PGR2026-00039
Biocon Ex.1002
Page 4669 of 4945

immunological interest, 5th ed., Pub. No. 91-3242, U.S. Dept. Health & Human Services, NIH, Bethesda, Md., 1991).

[0180] Knowledge about humanized antibodies in the art is applicable to the polypeptides according to the disclosure, even if these polypeptides are not antibodies.

[0181] In some embodiments, an anti-CD123 scFv comprises a HCDR1 that comprises SEQ ID NO: 10, a HCDR2 that comprises SEQ ID NO: 11, and a HDCR3 that comprises SEQ ID NO: 12; and a LCDR1 that comprises SEQ ID NO: 13, a LCDR2 that comprises SEQ ID NO: 14, and a LCDR3 that comprises SEQ ID NO: 15. In some embodiments, the anti-CD123 scFv comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 136, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 134. In some embodiments, the anti-CD123 scFv comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 16. In some embodiments, the anti-CD123 scFv comprises a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 17. In some embodiments, the tumor antigen binding domain is an anti-CD123 scFv, and wherein the scFv comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 18.

[0182] In some embodiments, the disclosure relates to CD123-binding domains wherein (i) the immunoglobulin light chain variable region comprises an amino acid sequence that is at least 88%, at least 90%, at least 92%, at least 95%, at least 97%, at least 98% or at least 99% identical to the amino acid sequence set forth in SEQ ID NO:134 and the immunoglobulin heavy chain variable region comprises an amino acid sequence that is at least 85%, at least 90%, at least 92%, at least 95%, at least 97%, at least 98% or at least 99% identical to the amino acid sequence set forth in SEQ ID NO:136.

[0183] In further embodiments, each CDR comprises no more than one, two, or three substitutions, insertions or deletions, as compared to that from a monoclonal antibody or fragment or derivative thereof that specifically binds to a target of interest (*e.g.*, CD123).

[0184] In certain embodiments, a CD123-binding domain does not inhibit IL-3 binding to CD123.

[0185] In certain embodiments, a CD123-binding molecule or protein can comprise a T-cell binding domain for recruitment of T-cells to target cells expressing CD123. In

PGR2026-00039
Biocon Ex.1002
Page 4670 of 4945

certain embodiments, a CD123-binding protein as described herein can comprise (i) a binding domain that specifically binds a TCR complex or a component thereof (*e.g.*, TCRα, TCRβ, CD3γ, CD3δ, and CD3ε) and (ii) another binding domain that specifically binds to CD123. A CD123-binding protein can utilize essentially any binding domain that binds a T-cell, e.g., an antibody derived binding domain. Exemplary anti-CD3 antibodies from which the CD3 binding domain can be derived include the CRIS-7 monoclonal antibody (Reinherz, E. L. *et al.* (eds.), *Leukocyte typing II.*, Springer Verlag, New York, (1986); V_L and V_H amino acid sequences respectively shown in SEQ ID NO: 341 (QVVLTQSPAIMSAFPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDSSKLA SGVPARFSGSGSGTSYSLTISSMETEDAATYYCQQWSRNPPTFGGGTKLQITR) and SEQ ID NO: 342 (QVQLQQSGAELARPGASVKMSCKASGYTFTRSTMHWVKQRPGQGLEWIGYINP SSAYTNYNQKFKDKATLTADKSSSTAYMQLSSLTSEDSAVYYCASPQVHYDYNGF PYWGQGTLVTVSA)); HuM291 (Chau *et al.* (2001) *Transplantation* 71:941-950; V_L and V_H amino acid sequences respectively shown in SEQ ID NO:343 (DIQMTQSPSSLSASVGDRVTITCSASSSVSYMNWYQQKPGKAPKRLIYDTSKLAS GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSSNPPTFGGGTKVEIK) and SEQ ID NO: 344 (QVQLVQSGAEVKKPGASVKVSCKASGYTFISYTMHWVRQAPGQGLEWMGYINPR SGYTHYNQKLKDKATLTADKSASTAYMELSSLRSEDTAVYYCARSAYYDYDGFAY WGQGTLVTVSS)); BC3 monoclonal antibody (Anasetti *et al.* (1990) *J. Exp. Med.* 172:1691); OKT3 monoclonal antibody (Ortho multicenter Transplant Study Group (1985) *N. Engl. J. Med.* 313:337) and derivatives thereof such as OKT3 ala-ala (also referred to as OKT3 AA-FL or OKT3 FL), a humanized, Fc variant with alanine substitutions at positions 234 and 235 (Herold *et al.* (2003) *J. Clin. Invest.* 11:409); visilizumab (Carpenter *et al.* (2002) *Blood* 99:2712), G19-4 monoclonal antibody (Ledbetter *et al.*, 1986, *J. Immunol.* 136:3945), 145-2C11 monoclonal antibody (Hirsch *et al.* (1988) *J. Immunol.* 140: 3766) and I2C monoclonal antibody (see, e.g., US 2011/0293619 and US20120244162). For example, a CD3 binding domain may comprise a CD3 binding domain disclosed in U.S. Patent Application Publication No. 2012/0244162, including a CD3 binding domain comprising a VL region selected from SEQ ID NO: 17, 21, 35, 39, 53, 57, 71, 75, 89, 83, 107, 111, 125, 129, 143, 147, 161, 165, 179 and 183 of US 2012/0244162 and/or a VH region selected from SEQ ID

PGR2026-00039
Biocon Ex.1002
Page 4671 of 4945

NO:15, 19, 33, 37, 51, 55, 69, 73, 87, 91, 105, 109, 123, 127, 141, 145, 159, 163, 177 and 181 of US 2012/0244162. In some embodiments, a CD3 binding domain comprises an amino acid sequence selected from SEQ ID NO: 23, 25, 41, 43, 59, 61, 77, 79, 95, 97, 113, 115, 131, 133, 149, 151, 167, 169, 185, and 187 of US 2012/0244162. In some embodiments, a CD3 binding domain is one described in WO2004/106380, WO2005/040220A1, US 2014/0099318 or derived from a CD3 binding domain thereof. An exemplary anti-TCR antibody is the BMA031 monoclonal antibody (Borst *et al.* (1990) *Human Immunology* 29:175-188). The CD3 binding domain may be derived from any of the antibodies or sequences described in WO 2013/158856 (incorporated herein by reference in its entirety).

[0186] In some embodiments, the second binding domain of a CD123-binding polypeptide described herein comprises: (i) an immunoglobulin light chain variable region comprising LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region comprising HCDR1, HCDR2, and HCDR3, wherein (a) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs:348, 349 and 350, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 345, 346 and 347, respectively; or (b) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NO:354, SEQ ID NO:355, and SEQ ID NO:356, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NO: 351, SEQ ID NO:352, and SEQ ID NO:353, respectively. In some embodiments, the second binding domain of a CD123-binding polypeptide described herein comprises: (i) an immunoglobulin light chain variable region comprising LCDR1, LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region comprising HCDR1, HCDR2, and HCDR3, wherein (a) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 182, 183 and 184, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 351, 352 and 353, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 357, 359 and 359, respectively; or (b) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 359, 367 and 368, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 363, 364 and 365, respectively. In some embodiments, the second binding domain of a CD123-binding polypeptide described herein comprises: (i) an immunoglobulin light chain variable region comprising LCDR1,

PGR2026-00039
Biocon Ex.1002
Page 4672 of 4945

LCDR2, and LCDR3, and (ii) an immunoglobulin heavy chain variable region comprising HCDR1, HCDR2, and HCDR3, wherein (a) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 372, 373 and 374, respectively; or (b) the LCDR1, LCDR2 and LCDR3 has the amino acid sequences set forth in SEQ ID NOs: 378, 379 and 380, respectively, and the HCDR1, HCDR2, and HCDR3 has the amino acid sequences set forth in SEQ ID NOs: 375, 376 and 377, respectively. In some embodiments, the second binding domains comprising the CDR sequences recited in this paragraph are humanized.

[0187] In some embodiments of a CD123-binding protein comprising a second binding domain that specifically binds CD3ε, the second binding domain competes for binding to CD3ε with the CRIS-7, HuM291 or I2C monoclonal antibody. In some embodiments, the CD3-binding domain comprises an immunoglobulin light chain variable region (V_L) and an immunoglobulin heavy chain variable region (V_H) derived from the CRIS-7, HuM291 or I2C monoclonal antibody (*e.g.*, the V_L and V_H of the second binding domain can be humanized variable regions comprising, respectively, the light chain CDRs and the heavy chain CDRs of the monoclonal antibody). A second binding domain may comprise the light chain variable region, the heavy chain variable region, or both, of the DRA222, TSC455, or TSC456 CD3-binding domains. The amino acid sequences of DRA222, TSC455, and TSC456 are provided in Table 4. The DRA222 binding domains are also described in WO 2013/158856. TSC455 may also be referred to as TSC394 F87Y. TSC455 may also be referred to as TSC394 E86D F87Y or TSC394 DY.

[0188] In some embodiments, the second binding domain specifically binds CD3 and comprises an immunoglobulin light chain variable region and an immunoglobulin heavy chain variable region; wherein the immunoglobulin light chain variable region comprises an amino acid sequence that is at least about 93% identical, at least about 95% identical, at least about 97% identical, at least about 98% identical or at least about 99% identical to the amino acid sequence in SEQ ID NO:384; or at least about 94% identical, at least about 95% identical, at least about 97% identical, at least about 98% identical or at least about 99% identical to the amino acid sequence in SEQ ID NO:385; and wherein the immunoglobulin heavy chain variable region comprises an amino acid sequence that is at least about 82% identical, at least about 85% identical, at least about 87% identical, at least about 90% identical, at least about 92% identical,

PGR2026-00039
Biocon Ex.1002
Page 4673 of 4945

at least about 95% identical, at least about 97% identical, at least about 98% identical or at least about 99% identical to the amino acid sequence in SEQ ID NO:383.

[0189] In some embodiments, the second binding domain is a CD3 binding domain that comprises a HCDR1 that comprises SEQ ID NO: 19, a HCDR2 that comprises SEQ ID NO: 20, and a HDCR3 that comprises SEQ ID NO: 21; and a LCDR1 that comprises SEQ ID NO: 22, a LCDR2 that comprises SEQ ID NO: 23, and a LCDR3 that comprises SEQ ID NO: 24. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 383 or 387, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 384. In some embodiments, the CD3 binding domain comprises a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 25. In some embodiments, the CD3 binding domain comprises a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 26. In some embodiments, the CD3 binding domain is an anti-CD3 scFv that comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 27.

[0190] In some embodiments, a CD123-binding polypeptide or protein further comprising a CD3-binding domain may have a low level of high molecular weight aggregates produced during recombinant expression of the polypeptide or protein. A CD123-binding polypeptide or protein further comprising a CD3-binding domain may exhibit a relatively long stability in human serum, depending on the CD3-binding domain present in the polypeptide or protein.

[0191] In certain variations, the CD3-binding domain and comprises one or more of the CD3-binding sequences (e.g., CDRs or variable regions) disclosed in US 2013/0129730, US 2011/0293619, US 7,635,472, WO 2010/037836, WO 2004/106381, or WO 2011/121110; each incorporated herein by reference in its entirety. In some embodiments, a CD3-binding domain comprises one or more of the sequences shown in Table 6.

Table 6: Exemplary CD3-binding domain light chain CDRs

LCDR1	LCDR2	LCDR3
GSSTGAVTSGYYPN (SEQ ID NO:110)	GTKFLAP (SEQ ID NO:113)	ALWYSNRWV (SEQ ID NO:116)
RSSTGAVTSGYYPN (SEQ ID NO:111)	ATDMRPS (SEQ ID NO:114)	ALWYSNRVV (SEQ ID NO:117)

PGR2026-00039
Biocon Ex.1002
Page 4674 of 4945

GSSTGAVTSGNYPN (SEQ ID NO:112)	GTKFLAP (SEQ ID NO:115)	VLWYSNRWV (SEQ ID NO:118)

[0192] In various embodiments, a CD3-binding domain comprises one or more of the sequences shown in Table 7.

Table 7: Exemplary CD3-binding domain heavy chain CDRs

HCDR1	HCDR2	HCDR3
IYAMN (SEQ ID NO:119)	RIRSKYNNYATYYADSVKS (SEQ ID NO:122)	HGNFGNSYVSFFAY (SEQ ID NO:125)
KYAMN (SEQ ID NO:120)	RIRSKYNNYATYYADSVKD (SEQ ID NO:123)	HGNFGNSYISYWAY (SEQ ID NO:126)
SYAMN (SEQ ID NO:121)	RIRSKYNNYATYYADSVKG (SEQ ID NO:124)	HGNFGNSYLSFWAY (SEQ ID NO:127)

[0193] In some embodiments, a therapeutic protein comprises, in order from amino terminus to carboxyl terminus a first binding domain, a hinge region, an immunoglobulin constant region, and a second binding domain. In some embodiments, the immunoglobulin constant region comprises immunoglobulin CH2 and CH3 domains of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgD. In some embodiments, the first binding domain comprises: an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3. In some embodiments, the HCDR1 comprises SEQ ID NO: 10, the HCDR2 comprises SEQ ID NO: 11, and the HDCR3 comprises SEQ ID NO: 12. In some embodiments, the LCDR1 comprises SEQ ID NO: 13, the LCDR2 comprises SEQ ID NO: 14, and the LCDR3 comprises SEQ ID NO: 15. In some embodiments, the HCDR1 comprises SEQ ID NO: 10, the HCDR2 comprises SEQ ID NO: 11, and the HDCR3 comprises SEQ ID NO: 12; and the LCDR1 comprises SEQ ID NO: 13, the LCDR2 comprises SEQ ID NO: 14, and the LCDR3 comprises SEQ ID NO: 15. In some embodiments, the first binding domain comprises a sequence at least 95% identical to SEQ ID NO: 18. In some embodiments, the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and (ii) an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3. In some embodiments, the HCDR1 comprises SEQ ID NO: 19, the HCDR2 comprises SEQ ID NO: 20, and the HDCR3

PGR2026-00039
Biocon Ex.1002
Page 4675 of 4945

comprises SEQ ID NO: 21. In some embodiments, the LCDR1 comprises SEQ ID NO: 22, the LCDR2 comprises SEQ ID NO: 23, and the LCDR3 comprises SEQ ID NO: 24. In some embodiments, the HCDR1 comprises SEQ ID NO: 19, the HCDR2 comprises SEQ ID NO: 20, and the HDCR3 comprises SEQ ID NO: 21; and the LCDR1 comprises SEQ ID NO: 22, the LCDR2 comprises SEQ ID NO: 23, and the LCDR3 comprises SEQ ID NO: 24. In some embodiments, the second binding domain comprises a sequence at least 95% or 100% identical to SEQ ID NO: 27. In some embodiments, the therapeutic protein comprises the sequence of SEQ ID NO: 31.

[0194] The structural format of multispecific anti-CD123 and anti-CD3 molecules disclosed herein induces potent tumor cell lysis but reduced cytokine release compared to multispecific anti-CD123 and anti-CD3 molecules in alternative structural formats. Without being bound by any theory, the polypeptide structural format disclosed herein (e.g., in order from amino terminus to carboxyl terminus): (a) a first binding domain that is a CD123-binding domain; (b) a hinge region; (c) an immunoglobulin constant region; and (d) a second binding domain that is a human or humanized binding domain that specifically binds a T-cell, CD3, CD3ε or a T-cell receptor (TCR) complex) induces a moderate level of T-cell Receptor (TCR) stimulation, compared to other T-cell engagers. It has been extensively documented that the strength or magnitude of the TCR signal regulates the outcome of T-cell activation. TCR stimulation triggers a number of cellular events that include initiation of effector function (*e.g.*, cytolytic granzymes), and cytokine secretion and cell division (Corse, Gottschalk and Allison. J Immunol 2011, 186:5039-5045). These distinct cellular events can proceed with different kinetics and reach variable maximum levels, depending on the intensity of the TCR stimulus and additional factors. The multispecific structural format disclosed herein is sufficiently potent to cause lysis of tumor cells over multiple days and to induce multiple rounds of T-cell division but moderate enough to limit the amount of cytokine secretion.

[0195] In some embodiments, the multispecific polypeptide comprising a CD123-binding domain and a CD3-binding domain when bound to a CD3 protein on a T cell induces reduced cytokine release from said T cell as compared to an OKT3 antibody control. In some embodiments, the multispecific polypeptide comprising a CD123-binding domain and CD3-binding domain induces reduced cytokine release from said T cell as compared to a multispecific polypeptide comprising an CD3-binding domain derived from OKT3 or I2C. In some embodiments, the multispecific polypeptide

PGR2026-00039
Biocon Ex.1002
Page 4676 of 4945

comprising a CD123-binding domain (e.g., a CD123-binding domain comprising an amino acid sequence at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to SEQ ID NO: 312 and/or SEQ ID NO:337) and a CD3-binding domain and in the scFv-Fc-scFv format induces reduced cytokine release in a non-human primate or human as compared to a bispecific polypeptide comprising a CD123-binding domain and I2C derived CD3-binding domain in a bispecific T-cell engager (scFv-scFv) format or dual affinity re-targeting format.

[0196] Also provided herein are pharmaceutical compositions comprising the therapeutic proteins described herein. In some embodiments, the compositions comprise 1-20 mg/m, 2.5-12 mg/ml, or 5-10 mg/ml of a therapeutic protein. In some embodiments, the compositions comprise from about 2.5 mg/ml to about 12 mg/ml, or from about 5 mg/ml to about 10 mg/ml of a therapeutic protein. In some embodiments, the compositions comprise about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, or about 12 mg/ml of a therapeutic protein. In some embodiments, the compositions comprise about 5 mg/ml of a therapeutic protein.

Methods of Use

[0197] The present disclosure provides methods for treating a subject with a disease or disorder, the methods comprising administering a therapeutically effective amount of at least one composition of the disclosure to the subject.

[0198] In some embodiments, the disease or disorder may be cancer. The cancer may be selected from, for example, acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm, B-cell acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML).

[0199] In some embodiments, the disease or disorder may be an inflammatory disease or disorder. In embodiments, the inflammatory disease or disorder may be an autoimmune disease or disorder. In some embodiments, the autoimmune disease or disorder is selected from irritable bowel syndrome, inflammatory bowel disease (e.g. Crohn's disease or ulcerative colitis), psoriasis, rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, asthma, multiple sclerosis, dermatomyositis, polymyositis, pernicious anaemia, primary biliary cirrhosis, acute disseminated encephalomyelitis (ADEM), Addison's disease,

PGR2026-00039
Biocon Ex.1002
Page 4677 of 4945

ankylosing spondylitis, antiphospholipid antibody syndrome (aPL), autoimmune hepatitis, diabetes mellitus type 1, Goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome (GBS), Hashimoto's disease, idiopathic thrombocytopenic purpura, pemphigus vulgaris, Sjögren's syndrome, temporal arteritis, autoimmune hemolytic anemia, bullous pemphigoid, vasculitis, celiac disease, endometriosis, hidradenitis suppurativa, interstitial cystitis, morphea, scleroderma, narcolepsy, neuromyotonia, vitiligo, autoimmune inner ear disease and myasthenia gravis. In some embodiments, the inflammatory disease or disorder is psoriasis.

[0200] In some embodiments, the inflammatory disease or disorder may be a "neuroimmune disease" such as neuropathic pain, osteoarthritis, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer's disease.

[0201] In some embodiments, the inflammatory disease or disorder may be an adverse transplant associated event, i.e. transplant rejection, allograft disease or graft-versus-host disease.

[0202] In some embodiments, for treatment methods and uses described herein, a protein or polypeptide described herein is delivered in a manner consistent with conventional methodologies associated with management of the disease or disorder for which treatment is sought. In accordance with the disclosure herein, a therapeutically effective amount of the protein or polypeptide is administered to a subject in need of such treatment for a time and under conditions sufficient to prevent or treat the disease or disorder.

[0203] Subjects for administration of a protein of the present disclosure include patients at high risk for developing a particular disorder as well as patients presenting with an existing such disorder. Typically, the subject has been diagnosed as having the disorder for which treatment is sought. Further, subjects can be monitored during the course of treatment for any change in the disorder (*e.g.*, for an increase or decrease in clinical symptoms of the disorder). Also, in some variations, the subject does not suffer from another disorder requiring treatment.

[0204] In prophylactic applications, pharmaceutical compositions or medicants comprising a protein of the present disclosure are administered to a patient susceptible to, or otherwise at risk of, a particular disorder in an amount sufficient to eliminate or reduce the risk or delay the onset of the disorder. In therapeutic applications, compositions or medicants comprising a protein of the present disclosure are administered to a patient suspected of, or already suffering from such a disorder in an

PGR2026-00039
Biocon Ex.1002
Page 4678 of 4945

amount sufficient to cure, or at least partially arrest, the symptoms of the disorder and its complications. An amount adequate to accomplish this is referred to as a therapeutically effective dose or amount. In both prophylactic and therapeutic regimes, agents are usually administered in several dosages until a sufficient response (*e.g.*, inhibition of inappropriate angiogenesis activity) has been achieved. Typically, the response is monitored and repeated dosages are given if the desired response starts to fade.

[0205] To identify subject patients for treatment according to the methods of the disclosure, accepted screening methods can be employed to determine risk factors associated with specific disorders or to determine the status of an existing disorder identified in a subject. Such methods can include, for example, determining whether an individual has relatives who have been diagnosed with a particular disorder. Screening methods can also include, for example, conventional work-ups to determine familial status for a particular disorder known to have a heritable component. For example, various cancers are also known to have certain inheritable components. Inheritable components of cancers include, for example, mutations in multiple genes that are transforming (*e.g.*, Ras, Raf, EGFR, cMet, and others), the presence or absence of certain HLA and killer inhibitory receptor (KIR) molecules, or mechanisms by which cancer cells are able to modulate immune suppression of cells like NK cells and T-cells, either directly or indirectly (*see, e.g.*, Ljunggren and Malmberg, *Nature Rev. Immunol.* 7:329-339, 2007; Boyton and Altmann, *Clin. Exp. Immunol.* 149:1-8, 2007). Toward this end, nucleotide probes can be routinely employed to identify individuals carrying genetic markers associated with a particular disorder of interest. In addition, a wide variety of immunological methods are known in the art that are useful to identify markers for specific disorder. For example, various ELISA immunoassay methods are available and well-known in the art that employ monoclonal antibody probes to detect antigens associated with specific tumors. Screening can be implemented as indicated by known patient symptomology, age factors, related risk factors, etc. These methods allow the clinician to routinely select patients in need of the methods described herein for treatment.

[0206] For administration, the pharmaceutical compositions of the disclosure may comprise: (i) therapeutic protein/polypeptide; and (ii) a pharmaceutically acceptable carrier, diluent or excipient. In some embodiments, the pharmaceutical composition

PGR2026-00039
Biocon Ex.1002
Page 4679 of 4945

may comprise (i) a therapeutic protein/peptide, (ii) a buffer, (iii) an excipient, and (iv) a surfactant.

[0207] A pharmaceutical composition comprising a polypeptide or protein described herein may be formulated in a dosage form selected from the group consisting of: an oral unit dosage form, an intravenous unit dosage form, an intranasal unit dosage form, a suppository unit dosage form, an intradermal unit dosage form, an intramuscular unit dosage form, an intraperitoneal unit dosage form, a subcutaneous unit dosage form, an epidural unit dosage form, a sublingual unit dosage form, and an intracerebral unit dosage form. The oral unit dosage form may be selected from the group consisting of: tablets, pills, pellets, capsules, powders, lozenges, granules, solutions, suspensions, emulsions, syrups, elixirs, sustained-release formulations, aerosols, and sprays.

[0208] A pharmaceutical composition comprising polypeptide or protein described herein may be administered to a subject in a therapeutically effective amount. According to the methods of the present disclosure, polypeptide or protein described herein can be administered to subjects by a variety of administration modes, including, for example, by intramuscular, subcutaneous, intravenous, intra-atrial, intra-articular, parenteral, intranasal, intrapulmonary, transdermal, intrapleural, intrathecal, and oral routes of administration. For prevention and treatment purposes, an antagonist can be administered to a subject in a single bolus delivery, via continuous delivery (*e.g.*, continuous transdermal delivery) over an extended time period, or in a repeated administration protocol (*e.g.*, on an hourly, daily, weekly, or monthly basis).

[0209] Determination of effective dosages in this context is typically based on animal model studies followed up by human clinical trials and is guided by determining effective dosages and administration protocols that significantly reduce the occurrence or severity of the subject disorder in model subjects. Effective doses of the compositions of the present disclosure vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, whether treatment is prophylactic or therapeutic, as well as the specific activity of the composition itself and its ability to elicit the desired response in the individual. Usually, the patient is a human, but in some diseases, the patient can be a nonhuman mammal. Typically, dosage regimens are adjusted to provide an optimum therapeutic response, *i.e.*, to optimize safety and efficacy.

PGR2026-00039
Biocon Ex.1002
Page 4680 of 4945

[0210] Also provided herein are uses of the compositions of the disclosure in the manufacture of a medicament for treating cancer. For instance, compositions of the disclosure may be used for treatment of acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Also provided are methods comprising administering a composition comprising a multispecific polypeptide comprising a CD123 binding domain and a CD3 binding domain to a patient by IV infusion at a weekly dose of about 0.3, about 1, about 3, about 6, about 9, about 12, about 18, about 20, about 24, about 30, about 36, about 50, about 48, about 60, about 75, or about 100 µg. Typically a patient is treated once or twice a week for 4 to 6 weeks. The patient may receive the same dosage each week or the dosage may be increased, for instance, each week.

[0211] In some embodiments, the dosage is increased each week, with the first dosage being less that what a patient would be expected to tolerate. This type of step-up treatment regimen reduces the risk that the patient will develop an infusion related reaction or cytokine release syndrome. In some embodiments, a multispecific protein comprising a CD123 binding domain and a CD3 binding domain (e.g., TRI130 or TRI129) may be administered to a patient intravenously such that the dosage is increased each week for at least the first two or first three doses. For instance, a composition of the disclosure may be administered by IV infusion according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; and week 4 dosage and subsequent week dosages: 12 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; and week 4 dosage and subsequent week dosages: 18 µg. In some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage: 6 µg; and week 2 and subsequent week dosages: 9 µg, and in some embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule week 1 dosage: 9 µg; and week 2 and subsequent week dosages: 12 µg. In other embodiments, the composition is administered to a patient intravenously according to the weekly treatment schedule: week 1 dosage 12 µg, and week 2 and subsequent week dosages: 18 µg.

[0212] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week

PGR2026-00039
Biocon Ex.1002
Page 4681 of 4945

1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; week 4 dosage, and subsequent week doses: 12 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 9 µg; week 3 dosage: 12 µg; week 4 dosage, and subsequent week doses: 18 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 12 µg; week 4 dosage, and subsequent week doses: 12 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 24 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 36 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 48 µg. In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following weekly treatment schedule: week 1 dosage: 6 µg; week 2 dosage: 12 µg; week 3 dosage: 18 µg; week 4 dosage, and subsequent week doses: 60 µg.

[0213] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 9 µg; day 3: 12 µg; day 4: 18 µg; day 8: 18 µg; day 11: 18 µg; day 15: 36 µg; day 22: 36 µg; followed by weekly doses of 36 µg.

[0214] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 12 µg; day 3: 18 µg; day 4: 24 µg; day 8: 24 µg; day 11: 24 µg; day 15: 48 µg; day 22: 48 µg; followed by weekly doses of 48 µg.

[0215] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 12 µg; day 3: 24 µg; day 4: 36 µg; day 8: 36 µg; day 11: 36 µg; day 15: 60 µg; day 22: 60 µg, followed by weekly doses of 60 µg.

PGR2026-00039
Biocon Ex.1002
Page 4682 of 4945

[0216] In some embodiments, a patient may be administered a composition of the disclosure intravenously according to the following treatment schedule: day 1: 6 µg; day 2: 12 µg; day 3: 24 µg; day 4: 36 µg; day 8: 48 µg; day 11: 48 µg; day 15: 100 µg; day 22: 100 µg, followed by weekly doses of 100 µg.

[0217] In some embodiments, a method for treating a patient in need thereof comprises administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the patient on days 1, 8, 15, and 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 12 µg is administered on day 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 18 µg is administered on day 22. In some embodiments, 6 µg is administered on day 1, 9 µg is administered on day 8, 9 µg is administered on day 15, and 9 µg is administered on day 22. In some embodiments, 9 µg is administered on day 1, 12 µg is administered on day 8, 12 µg is administered on day 15, and 12 µg is administered on day 22. In some embodiments, 12 µg is administered on day 1, 18 µg is administered on day 8, 18 µg is administered on day 15, and 18 µg is administered on day 22.

[0218] In some embodiments, a patient treated according to the methods of the disclosure exhibits a decrease in bone marrow blast percentage, and in some embodiments, a patient exhibits a decrease in absolute blast counts in the blood. In some embodiments, the treatment results in reduction in patient blast levels by at least 0.5%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50% or more, compared to the patient's levels immediately before the treatment.

[0219] In some embodiments, a patient treated according to the methods of the disclosure exhibits a complete remission (CR). As used herein, complete remission refers to a reduction in bone marrow blasts to less than about 5%, absence of circulating blasts and blasts with Auer rods, absence of extramedullary disease, and absolute neutrophil count (ANC) ≥ 1.0×10^9/L (1,000/µL) and PLT ≥ 100×10^9/L (100,000/µL). In some embodiments, a patient treated according to the methods of the disclosure exhibits a CR without Minimal Residual Disease (CR_{MRD}). As used herein, CR_{MRD} refers to CR with negativity for a genetic marker by quantitative reverse transcription polymerase chain reaction (RT-qPCR) or CR with negativity by

PGR2026-00039
Biocon Ex.1002
Page 4683 of 4945

multiparameter flow cytometry. In some embodiments, a patient treated according to the methods of the disclosure exhibits a CR with Incomplete Hematologic Recovery (CR_i). CR_i includes all the criteria of CR, described above, except for residual neutropenia (ANC < 1.0×10^9/L [1,000/μL]) or thrombocytopenia (PLT < 100×10^9/L [100,000/μL]).

[0220] In some embodiments, a patient treated according to the methods of the disclosure exhibits a Morphologic Leukemia-Free State (MLFS). As used herein, MLFS refers to bone marrow blasts < 5% (i.e., marrow should not be merely "aplastic;" at least 200 cells should be enumerated or cellularity should be at least 10%); absence of blasts with Auer rods; and absence of extramedullary disease. No hematologic recovery is required.

[0221] In some embodiments, a patient treated according to the methods of the disclosure exhibits a Partial Remission (PR). As used herein, a PR includes all hematologic criteria of CR, described above, plus a decrease of bone marrow blast percentage to 5 to 25%, and at least 50% decrease of pretreatment bone marrow blast percentage.

[0222] In some embodiments, a patient treated according to the methods of the disclosure exhibits Stable Disease (SD), characterized by an absence of CR_{MRD}, CR, CR_i, PR, and MLFS, but without progressive disease (*i.e.*, increase in bone marrow blast percentage and/or increase of absolute blast counts in the blood).

[0223] A patient treated with the CD123 x CD3 targeting multispecific polypeptide (e.g., TRI130 or TRI129) at either the same dosage each week or with a step-up treatment regimen may also have infusion times (i.e., the length of the infusion) modified to further reduce the likelihood of an infusion reaction or cytokine release syndrome. To reduce the risk of an adverse event, the first dose is administered by IV to the patient over several hours, e.g., 20-24 hours. In some embodiments, the first dose of the composition is administered over a period of about 20-24 hours, the second dose is administered over a period of about 8 hours, the third dose is administered over a period of about 6 hours, and the fourth dose and subsequent doses are administered over a period of about 4 hours. In some embodiments, the first dose of the composition is administered over a period of about 20-24 hours, the second dose is administered over a period of about 8 hours, the third dose is administered over a period of about 6 hours, and the fourth dose and subsequent doses are administered over a period of about 4 hours, wherein each of the first,

PGR2026-00039
Biocon Ex.1002
Page 4684 of 4945

second, third, and fourth dose are the same. The composition can also be administered to a subject by continuous IV infusion, e.g., continuous IV infusion up to about 72 hours in duration.

[0224] A patient treated with the CD123 x CD3 targeting multispecific polypeptide (e.g., TRI130 or TRI129) may also be treated with one or more additional therapeutic agents. The one or more additional therapeutic agents may be administered at or around the same time as the CD123 x CD3 targeting multispecific polypeptide. In some embodiments, the one or more additional therapeutic agents are administered before (i.e., as a "premedication") administration of the multispecific polypeptide, such as about 1-3 hours before administration thereof. In some embodiments, the one or more additional therapeutic agents are administered after administration of one or more doses of the multispecific polypeptide.

[0225] In some embodiments, the one or more additional therapeutic agents are diphenhydramine, acetaminophen, and/or dexamethasone. In some embodiments, the one or more additional therapeutic agents may be administered intravenously or orally. In some embodiments, dexamethasone may be administered at a dose of about 10 to about 20 mg. In some embodiments, methylprednisolone may be administered at a dose of about 1 mg/kg. In some embodiments, acetaminophen may be administered at a dose of about 650 or about 1,000 mg. In some embodiments, the acetaminophen may be administered three times a day for 1 day, with the first dose administered 1 to 3 hours before administration of the CD123 x CD3 targeting multispecific polypeptide. In some embodiments, the one or more additional therapeutic agents may comprise an antihistamine such as diphenhydramine. Diphenhydramine may be administered at a dose of about 50 mg. In some embodiments, the one or more therapeutic agents may comprise allopurinol. In some embodiments, allopurinol is administered at least 2 days prior to administration of the CD123 x CD3 targeting multispecific polypeptide. In some embodiments, the one or more additional therapeutic agents may comprise tocilizumab.

[0226] In some embodiments, a method for treating a disorder characterized by overexpression of CD123 in a patient in need thereof comprises administering to the patient an effective amount of a pharmaceutical composition comprising a recombinant polypeptide comprising a CD123 binding domain and a CD3 binding domain (e.g., TRI130 or TRI129) at any of the doses or regimens described herein. In some embodiments, a method for treating a disorder characterized by overexpression

PGR2026-00039
Biocon Ex.1002
Page 4685 of 4945

of CD123 in a patient in need thereof comprises administering to the patient an effective amount of a pharmaceutical composition comprising a recombinant polypeptide comprising a CD123 binding domain and a CD3 binding domain (e.g., TRI130 or TRI129); wherein the administration of the pharmaceutical composition induces reduced cytokine levels in the subject as compared to administration of (a) a dual affinity re-targeting antibody comprising the CD123 binding domain and the CD3 binding domain of the recombinant polypeptide; or (b) a bispecific T-cell engager molecule comprising the CD123 binding domain and the CD3 binding domain of the recombinant polypeptide. In some embodiments, the disorder is cancer, such as AML or MDS. In some embodiments, the subject was previously treated with a different CD123-binding molecule, and wherein the subject experienced an adverse event after the previous treatment. In some embodiments, the adverse event was excessive cytokine release. In some embodiments, the cytokine levels were levels of IFN-γ, TNF-α, IL-6, IL-2, IL-8, IL-10, IL-17, GM-CSF, IL-4, IL-12, IL-13 or IL-1β, or any combination thereof. In some embodiments, the cytokine levels were levels of IFN-γ, IL-2, TNF-α and IL-10. In some embodiments, cytokine levels are measured in an *in vitro* activated T cell assay.

[0227] Pharmaceutical compositions comprising the proteins and polypeptides described herein can be supplied as a kit comprising a container that comprises the pharmaceutical composition as described herein. A pharmaceutical composition can be provided, for example, in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Such a kit can further comprise written information on indications and usage of the pharmaceutical composition. In some embodiments, the kit comprises the pharmaceutical composition and an IV stabilizing solution (0.1 M succinate buffer, and 0.08 % weight/volume polysorbate 80, at pH 6.0 or similar solution that is designed to prevent or reduce the likelihood that the multispecific polypeptides will adhere to plastic tubing and bags).

[0228] The disclosure will be further clarified by the following examples, which are intended to be purely exemplary of the disclosure and in no way limiting.

PGR2026-00039
Biocon Ex.1002
Page 4686 of 4945

EXAMPLES

[0229] The invention is further described in detail by reference to the following examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

[0230] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLE 1: Formulation of Anti-CD123 x Anti-CD3 Bispecific Proteins

[0231] It is critical for therapeutic protein drugs to maintain their product quality during long term storage at distribution centers, shipping, and storage and use as the site of dose administration. Proteins may be exposed to a variety of temperatures following production and formulation, including frozen (-80 °C, -20 °C), refrigerated (4 °C) and room temperature (25 °C). Conditions impacting protein drug stability include pH, protein concentration and the concentration of salts and excipients that may be included in the formulation.

[0232] To evaluate the 5 mM succinate, 6.5% sucrose, 0.02% weight/volume polysorbate-80 (abbreviated SSuT), pH 4.8 formulation for anti-CD123 x anti-CD3 bispecifics (anti-CD123 – hinge region – CH2-CH3 – anti-CD3), Chinese Hamster Ovary (CHO) cells were stably transfected with DNA plasmids encoding either TRI-129 or TRI-130 along with a selection marker. Following transfection, these cells were grown under selective pressure to assure stable integration of the bispecific protein-encoding gene into the CHO cell genome and to kill cells that did not contain the selection marker.

[0233] The TRI-129 and TRI-130 CHO pools were initially grown in shake flasks, then transferred to 10L bioreactors and cultured in defined, animal component-free media. After approximately two weeks of culture, the cell culture supernatant was

PGR2026-00039
Biocon Ex.1002
Page 4687 of 4945

clarified using a combination of depth and sterile filtration, then purified using a combination of affinity and mixed mode column chromatography. The two-step purified protein was diafiltered into succinate buffer using a Tangential Flow Filtration (TFF) apparatus, then spiked with the appropriate volumes of concentrated stock solutions of sucrose and polysorbate-80 to achieve the defined composition. The TRI-129 and TRI-130 ADAPTIR proteins were evaluated in the preferred formulation buffer at both 2 mg/mL and 10 mg/mL. The stability in SSuT was compared to protein in Dulbecco's phosphate-buffered saline (dPBS).

[0234] In one experiment, TRI-129 and TRI-130 were formulated either in SSut or PBS and samples were stored at 4 ºC and 25 ºC. The purity of the sample was determined at the beginning of the study and after 9 days of storage using an analytical Size Exclusion Chromatography (SEC) assay. The change in the %purity of the drug product after 9 days is reported in Table 8 below. The negative values for the samples in dPBS indicate that the purity of the sample is declining and is indicative of product peak area redistributing from the main peak to aggregate/higher molecular weight species (HMW). After 9 days, both TRI-129 and TRI-130 in SSuT show much smaller changes in purity as compared to the samples in dPBS, indicating that the formulation has significant stabilizing properties.

Table 8. Comparison of PBS versus SSut, stability of TRI-129 and TRI-130 at 2 mg/mL after 9 days

Construct ID	dPBS Change in % MP 9 days @ 4 ˚C	dPBS Change in % MP 9 days @ 25 ˚C	SSuT Change in % MP 9 days @ 4 ˚C	SSuT Change in % MP 9 days @ 25 ˚C
TRI129	-1.2	-4.1	-0.4	-0.6
TRI130	-1.7	-3.4	0.0	-0.3

[0235] It is common for protein therapeutics to be submitted to one or more freeze-thaw cycles from either -80 or -20 ˚C to 4 ˚C or room temperature during production and/or storage. It is important that the selected bulk drug substance and drug product formulations stabilize the protein and prevent drastic changes in drug quality. TRI-129 and TRI-130 formulated in SSuT were examined for their ability to resist cryoaggregation, which is the formation of aggregation caused by freezing and thawing protein solutions. The stability of TRI-129 and TRI-130 were investigated at

PGR2026-00039
Biocon Ex.1002
Page 4688 of 4945

2 and 10 mg/mL. The purity of the samples was assessed by analytical SEC, then 200 µL were placed at -80 or -20 ˚C for several hours to allow for sufficient time for the samples to completely freeze. The samples were removed from the freezers and allowed to completely thaw at room temperature. The samples were submitted to a total of three freeze-thaw cycles at -80 or -20 ˚C, then retested by SEC to determine the change in %MP. As show in Table 9 below, both TRI-129 and TRI-130 showed minimal change in %MP at either -80 or -20 °C. The higher protein concentration samples at 10 mg/mL showed slightly greater changes in %MP than the samples at 2 mg/mL.

Table 9. Analysis of Freeze-Thaw stability of TRI-129 and TRI-130 in SSuT formulation buffer, at 2 and 10 mg/mL.

Construct ID	2 mg/mL Change in % MP -80 °C FT x 3	2 mg/mL Change in % MP -20 °C FT x 3,	10 mg/mL Change in % MP -80 °C FT x 3,	10 mg/mL Change in % MP -20 °C FT x 3
TRI129	-0.1	-0.1	-0.4	-0.4
TRI130	0.0	0.0	-0.1	-0.1

EXAMPLE 2: Determination of the No Observed Adverse Effect Level (NOAEL) and the Minimum Anticipated Biological Effect Level (MABEL) for TRI130

The No Observed Adverse Effect Level (NOAEL)

[0236] A 28-day repeat-dose toxicology study with a 5-week recovery period was conducted in non-human primates (NHP). Animals in four study groups received weekly doses of vehicle, or 0.5, 2.5, or 10 mg/kg of TRI130. Parameters measured included safety pharmacology, laboratory evaluations, and necropsy with full histopathology. There were no clinical adverse findings, no changes in animal weights or organ weights, and no abnormal macroscopic or microscopic findings in histopathology related to TRI130. Minimal cytokines were detected after dosing and were attenuated after the second dose compared to the first dose. The expected pharmacodynamic effect of the anti-CD3 binding domain of the molecule was observed with transient redistribution of T cells. The elimination half-life was approximately 73 hours at the high dose. The no observed adverse effect level

PGR2026-00039
Biocon Ex.1002
Page 4689 of 4945

(NOAEL) was 10 mg/kg in NHP, which translates into a human equivalent dose (HED) of approximately 3.2 mg/kg.

Minimum Anticipated Biological Effect Level (MABEL)

[0237] TRI130 mobilizes T cells to lyse tumor cells expressing the target antigen CD123, and the maximum activity occurs at very low levels of TCR occupancy (data not shown). To calculate the minimum anticipated biologic effect level (MABEL), in vitro activity assays were used in place of in vitro receptor occupancy.

[0238] The MABEL was determined using the effective concentration necessary to elicit 10% activity (EC10) (Muller et al., 2009) in a human T-cell activation in vitro assay. While the potency of TRI130 in these T-cell activation assays may vary depending on the degree of activation of T cells and the effector-to-target (E:T) cell ratio, the activation assay represents a more sensitive assay for calculation of MABEL, compared to the redirected T cell cytotoxicity (RTCC) assay. Both RTCC and T-cell activation were evaluated as possible assays to predict the MABEL for the starting clinical dose. The RTCC assay, using purified T cells at a 10:1 E:T ratio with CD123+ KG1a tumor cell line had an average EC10 of 5.8 pM, assessed from 5 donors (a range of 4.4 to 6.7 pM across the donors evaluated). In contrast, the T-cell activation assay was a more sensitive assay to estimate the MABEL. To measure T-cell activation induced in vitro, T cells were isolated from peripheral blood mononuclear cells and incubated with TRI130 in the presence of CD123+ tumor cells (MOLM-13). Upregulation of CD69 and CD25 on T cells was monitored at 20 hours using multi-color flow cytometry, after gating on live CD4+ and CD8+ T cells. These assays assessed three donors, for activation of both CD4 and CD8 T cells. The average EC10 calculation for these assays was 1.2 pM for CD4 T cells (range 0.7 to 1.6 pM) and 1.3 pM for CD8 T cells (range 0.9 to 2.0 pM). This assay represents a more conservative estimate of MABEL and was used to estimate the starting dose for patients. For TRI130, 0.7 pM (0.113 ng/ml) was the most conservative approach to MABEL, based on the CD4+ T-cell response from the donors.

[0239] Clearance and volume estimates for Group 4 in the single dose NHP study were determined using a WinNonlin (v6.4) precompiled 2 compartment model for intravenous (IV) dosing. Allometric scaling was used to predict human clearance and volume parameter estimates that could be used to simulate dosing strategies that would result in a Cmax below the EC10 (MABEL) value of 0.7 pM, the lowest EC10

PGR2026-00039
Biocon Ex.1002
Page 4690 of 4945

determined from an individual donor used in the activation assay. With this modeling, a dose of about 0.005 µg/kg would have a Cmax below the MABEL concentration of 0.113 ng/mL (0.7 pM).

[0240] A flat or fixed dose, instead of a weight-based dose is being utilized in this study. Several studies have shown that flat dosing compared to weight-based dosing performed similarly across a number of monoclonal antibodies and that the PK variability introduced by either dosing regimen is moderate relative to the variability generally observed in pharmacodynamics, efficacy, and safety (Wang et al., 2009). Assuming a 60-kg patient and a MABEL dose of 0.005 µg/kg, the starting dose for the study is 0.3 µg.

EXAMPLE 3: Administration of TRI130, a formulated anti-CD123 x anti-CD3 therapeutic candidate, to patients

[0241] Formulated TRI130 (5 mM succinate, 6.5% sucrose, 0.02% weight/volume polysorbate-80, pH 4.8) is being administered to patients in an ongoing Phase 1/1b open-label, dose-escalation study of patients with relapsed or refractory acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). The study is being conducted in two parts. The first part is a Phase 1 open-label, dose escalation study to determine the recommended dose for Phase 1b. Phase 1b is an open-label expansion study to assess the clinical activity and safety of the drug at the recommended dose. The study design is outlined in **FIG. 2**. Endpoints include safety, immunogenicity, pharmacokinetics, pharmacodynamics, and clinical activity.

[0242] Table 10 is a dosing schedule that sets forth the amount that cohorts 1-10 are being dosed weekly (by IV administration) (dose escalation cohorts). In both parts of the study, patients receive drug intravenously weekly for six 28-day cycles, unless disease progression, intolerable toxicity, or withdrawal of consent occurs earlier. There is an option for longer treatment if the patient is responding.

Table 10: Dose Escalation Cohorts 1 to 10

Cohort	1st IV Dose	2nd IV Dose	3rd IV Dose	≥ 4th IV Dose	Number of Patients
1	0.3 µg	0.3 µg	0.3 µg	0.3 µg	1
2	1 µg	1 µg	1 µg	1 µg	1

PGR2026-00039
Biocon Ex.1002
Page 4691 of 4945

3	3 µg	3 µg	3 µg	3 µg	1
4	9 µg	9 µg	9 µg	9 µg	3 + 3
5	6 µg	9 µg	12 µg	12 µg	3 + 3
6	Cohort 6 will consist of 2 dose Cohorts conducted concurrently, 6a and 6b				
6a	6 µg	9 µg	12 µg	18 µg	3 + 3
6b	6 µg	12 µg	12 µg	12 µg	3 + 3
7	6 µg	12 µg	18 µg	24 µg	3 + 3
8	6 µg	12 µg	18 µg	36 µg	3 + 3
9	6 µg	12 µg	18 µg	48 µg	3 + 3
10	6 µg	12 µg	18 µg	60 µg	3 + 3

[0243] Drug is supplied in single-use vials containing 2 mg drug product in 1 mL of liquid at a concentration of 2 mg/mL. The drug product is mixed with an IV stabilizing solution to prevent drug product from adhering to IV bags and IV tubing sets. The stabilizing solution is supplied sterile and refrigerated (2 to 8°C) in 10 mL vials comprised of 0.1 M succinate buffer, and 0.08 % weight/volume polysorbate 80, at pH 6.0.

[0244] For Cohorts 1 through 4, administration is by IV infusion over approximately 20 to 24 hours for the first dose (Cycle 1, Day 1), over 8 hours (± 1 hour) for the second dose (Cycle 1, Day 8), over 6 hours (± 1 hour) for the third dose (Cycle 1, Day 15) and over 4 hours (± 1 hour) for all subsequent doses (Cycle 1, Day 22 and onwards). For Cohorts 5 and above, administration is by IV infusion over 20 to 24 hours for the first dose and every time the dose is increased. The second time the same dose is administered it is infused over 8 hours (± 30 minutes), for the third time over 6 hours (± 30 minutes), and for the fourth time and all further times over 4 hours (± 30 minutes).

[0245] If necessary, to manage or prevent any adverse event, and in particular, infusion related reaction (IRR) or cytokine release syndrome (CRS), any dose infusion may be slowed and/or interrupted, with the administration time extended up to 72 hours. If an infusion is extended past 60 hours, then the patient must be observed for 12 hours after the infusion is completed. Table 10 discloses a stepped dosing regimen with the potential to reduce the likelihood of IRR and/or CRS (starting at cohort 5).

[0246] The frequency of patient dosing is weekly for up to 6 months. Dosing on a weekly schedule was selected based on the cynomolgus monkey toxicology study of escalating TRI130 doses (data not shown). The half-life of TRI130 following a single dose ranged from approximately 25 to 113 hours for individual animals dosed with

PGR2026-00039
Biocon Ex.1002
Page 4692 of 4945

0.25 to 1 mg/kg; the longer half-life estimates were associated with animals in the high dose group (1 mg/kg).

[0247] To mitigate infusion related reactions (IRRs) and cytokine release syndrome (CRS), patients are administered the following pre-medications: diphenhydramine, acetaminophen, and dexamethasone. All premedications are administered 1 to 3 hours before the infusion is initiated. The dose of any of the premedications may be reduced, if, in the opinion of the Investigator, comorbidities require it. Dexamethasone is optional after Cycle 2, Day 15, so long as the patient has not experienced any IRRs or CRS with earlier doses. The doses of the 3 premedications are:

1. Dexamethasone 10 to 20 mg IV, or methylprednisolone 1 mg/kg IV, or equivalent; the dose is at the Investigator's discretion based on patient's comorbidities;
2. Acetaminophen 650 or 1,000 mg, or equivalent, orally (PO) three times a day for 1 day (650- or 1,000-mg dose at the Investigators discretion), with the first dose administered 1 to 3 hours before the study drug infusion; and
3. Antihistamine: diphenhydramine 50 mg PO or IV; or equivalent.

If an investigator elects to administer allopurinol for tumor lysis prophylaxis, then it must be started at least 2 days prior to the start of study drug.

Phase 1 - Dose Escalation Study

[0248] Dosing was started at the minimum anticipated biologic effect level (MABEL) in patient cohorts. Patients enrolled had either: 1) relapsed or refractory AML and refused or were not eligible for intensive chemotherapy or an allogeneic stem cell transplant, or 2) relapsed or refractory MDS and had > 5% blasts in the marrow or any circulating blasts in the peripheral blood and had failed a prior hypomethylating agent (HMA); failure is defined as intolerance to HMA, lack of response (no CR by at least 6 cycles), or IWG defined progressive disease during or after treatment with an HMA. Demographics for 32 patients enrolled in the ongoing Phase 1 dose escalation study (through Cohort 7) are shown below in Table 11. For patients enrolled through Cohort 7, the median age was 67 years, and 79% of the patients had AML. The average number of doses administered per patient was 8.5 with an average treatment duration of 54 days.

PGR2026-00039
Biocon Ex.1002
Page 4693 of 4945

Table 11: Demographics and exposure, patients enrolled through cohort 7

Characteristic	Statistic	All Patients (N=32)
Age	Median (Range)	67 (18, 81)
Female	N (%)	15 (47%)
Prior Lines of Therapy	Median (Range)	3 (1, 13)
Disease		
MDS	N (%)	7 (22%)
AML Primary	N (%)	21 (66%)
AML Therapy Related	N (%)	4 (13%)
Cytogenetic Risk		
Favorable	N (%)	3 (9%)
Intermediate	N (%)	7 (22%)
Poor	N (%)	6 (19%)
Unknown	N (%)	16 (50%)

Summary of Treatment Exposure	32 Patients Mean (SD)
Doses Administered	8.5 (8.3)
Treatment Duration (days)	54 (59)

[0249] Treatment-Related Adverse Events for the 32 patients enrolled in the ongoing Phase 1 dose escalation study are shown below in Table 12. 34% of patients experienced one or more IRR/CRS events (Grade ≥ 3 reported in 16%). The most common symptoms were dyspnea, fever, hypotension, hypoxia, tachycardia, and rigors/chills. Notably, IRR/CRS was the only treatment-related serious adverse event to occur in two or more patients. 3 out of the 11 patients that experienced an IRR/CRS event received tocilizumab to treat the same.

Table 12: Treatment-Related Adverse Events

Treatment-Related Adverse Events*	All (N=32)	Grade ≥ 3 (N=32)
IRR/CRS**	11 (34%)	5 (16%)
Fatigue	5 (16%)	1 (3%)
Diarrhea	3 (9%)	

PGR2026-00039
Biocon Ex.1002
Page 4694 of 4945

Fever	3 (9%)	
Anemia	2 (6%)	2 (6%)
Flushing	2 (6%)	
Hypotension	2 (6%)	
Nausea	2 (6%)	1 (3%)
Peripheral Edema	2 (6%)	
Rigors/Chills	2 (6%)	
Treatment-Related Serious Adverse Events		
IRR/CRS	8 (25%)	4 (13%)
*Treatment-related AE and SAE events occurring in 2 or more patients **Infusion Related Reaction/Cytokine Release Syndrome Toxicity grading is based on CTCAE criteria version 5.0		

[0250] The percentage of blasts in bone marrow aspirates was monitored over time for patients. As shown in **FIG. 3A-3D**, a reduction of bone marrow blasts was observed in several patients that received a highest dose ≥ 12 µg. Two patients each had reductions in bone marrow blasts from 29% to 0% (Cohort 6b, **FIG. 3C**), and from 33% to 4% (Cohort 6a, **FIG. 3B**). Absolute neutrophil and platelet counts met Complete Remission criteria. Both patients remain on study, as well as one patient with decreasing blasts in Cohort 7.

[0251] Serum cytokines were assessed at scheduled timepoints before and after administration of the highest dose in each patient, and were also evaluated at intervals during infusion related reactions or cytokine release syndrome events. As shown in **FIG. 4A-4D**, cytokines were not elevated during scheduled collections. Elevated cytokines, particularly IL-6, were observed during adverse events of IRR/CRS. Notably, in this small data set, no correlation was observed between the highest cytokine concentrations and dose level or grade of event.

[0252] Thus, in this preliminary study, administration of TRI130, through doses of 24 µg, was tolerated with a manageable safety profile. As noted above, two patients had complete remission (CR). Cytokines were not significantly elevated unless there was a concurrent adverse event of IRR/CRS. Preliminary data suggested no evidence of treatment-induced anti-drug antibodies (ADA).

PGR2026-00039
Biocon Ex.1002
Page 4695 of 4945

Phase 1 - Additional Dose-Escalation Cohorts

[0253] After completion of a dose-limiting toxicity (DLT) observation period for Cohort 7, four additional sequential cohorts (Cohorts A, B, C, and D) will enroll patients while Cohorts ≥ 8 are treated. These cohorts will run sequentially and are independent of Cohorts 8 to 10, and will achieve a more rapid dose escalation.

[0254] Patients in Cohorts A, B, C, and D will have continuous IV dosing (20-24 hours/day) for the first 4 days of Cycle 1, then twice a week dosing during Week 2, followed by once a week dosing thereafter in Cycle 1 and all subsequent cycles. Table 14 shows the dosing schedule for Cohorts A, B, C, and D. The first week of dosing in Cohort A utilizes doses tested in Cohort 6a (Day 1 of 6 µg, Day 2 of 9 µg, Day 3 of 12 µg, and Day 4 of 18 µg). During the second week, the dose of 18 µg is administered on Day 8 and Day 11. In Week 3 the dose is escalated to 36 µg and is maintained at that level. The advantage of escalating the daily dose during the first week is that the C_{max} is gradually increased, which may lower the propensity for the development of IRR/CRS. Aggressive treatment with tocilizumab will be administered for Grade ≥ 2 IRR or CRS that does not respond within 2 hours of symptomatic treatment and dose interruption.

Table 14: Dosing for Cohorts A, B, C and D

	Week 1				Week 2		Weeks 3 & 4	≥ Cycle 2	
Cohort	Day 1	Day 2	Day 3	Day 4	Day 8	Day 11	Days 15, & 22	Days 1, 8, 15, and 22	Number of Patients
A	6 µg	9 µg	12 µg	18 µg	18 µg	18 µg	36 µg	36 µg	3 + 3
B	6 µg	12 µg	18 µg	24 µg	24 µg	24 µg	48 µg	48 µg	3 + 3
C	6 µg	12 µg	24 µg	36 µg	36 µg	36 µg	60 µg	60 µg	3 + 3
D	6 µg	12 µg	24 µg	36 µg	48 µg	48 µg	100 µg	100 µg	3 + 3

[0255] For Cohorts A-D, administration is by IV infusion over 20 to 24 hours for the first dose and every time the dose is increased. The second time the same dose is

PGR2026-00039
Biocon Ex.1002
Page 4696 of 4945

administered it is infused over 8 hours (± 30 minutes), for the third time over 6 hours (± 30 minutes), and for the fourth time and all further times over 4 hours (± 30 minutes).

[0256] Premedications are administered before Cycle 1, Day 1; Cycle 1, Day 8; Cycle 1, Day 11; Cycle 1, Day 15, and Cycle 1, Day 22. For all subsequent doses, dexamethasone is optional, but acetaminophen and diphenhydramine are required.

Phase 1b - Expansion

[0257] The recommended-dose regimen will be further examined in two expansion cohorts consisting of the same type of patients: 1) Cohort 1 will consist of 24 patients with relapsed or refractory AML that are not eligible for intensive chemotherapy or an allogeneic transplant, and 2) Cohort 2 will consist of 24 patients with relapsed or refractory MDS that have > 5% blasts in the marrow or any circulating blasts in the peripheral blood and have failed a prior HMA; failure is defined as intolerance to HMA, lack of response (no CR by at least 6 cycles), or IWG-defined progressive disease during or after treatment with an HMA.

[0258] Serum samples will be collected for serial PK assessment for drug levels.

PGR2026-00039
Biocon Ex.1002
Page 4697 of 4945

CLAIMS

1. A composition comprising a multispecific protein, a buffer, an excipient and a surfactant, wherein

(a) the multispecific protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus:

(i) a first binding domain,

(ii) a hinge region,

(iii) an immunoglobulin constant region, and

(iv) a second binding domain; and

(b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

2. The composition of claim 1, wherein the composition comprises from about 1 mM to about 10 mM succinate or a pharmaceutically acceptable salt or acid thereof.

3. The composition of claim 2, wherein the composition comprises about 5 mM succinate or a pharmaceutically acceptable salt or acid thereof.

4. The composition of any one of claims 1-3, wherein the excipient comprises or consists of a sugar.

5. The composition of claim 4, wherein the sugar is sucrose.

6. The composition of claim 4 or 5, wherein the composition comprises from about 1% weight/volume (w/v) to about 12% w/v of the sugar.

7. The composition of claim 6, wherein the composition comprises about 6.5% (w/v) of the sugar.

8. The composition of any one of claims 1-7, wherein the surfactant comprises or consists of polysorbate 80.

PGR2026-00039
Biocon Ex.1002
Page 4698 of 4945

9. The composition of claim 8, wherein the composition comprises about 0.02% w/v polysorbate 80.

10. The composition of any one of claims 1-9, wherein the composition comprises from about 0.1 mg/ml to about 10 mg/ml of the multispecific protein.

11. The composition of claim 10, wherein the composition comprises from about 1 mg/ml to about 5 mg/ml of the multispecific protein.

12. The composition of claim 11, wherein the composition comprises about 2 mg/ml of the multispecific protein.

13. The composition of any one of claims 1-12, wherein the composition comprises about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80.

14. The composition of any one of claims 1-13, wherein the composition has a pH from about 4.0 to about 5.5.

15. The composition of claim 14, wherein the composition has a pH of about 4.8.

16. The composition of any one of claims 1-15, wherein the immunoglobulin constant region is a human Fc domain.

17. The composition of any one of claims 1-16, wherein the immunoglobulin constant region comprises immunoglobulin CH2 and CH3 domains of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgD.

18. The composition of any one of claims 1-17 wherein the first binding domain is a CD3 binding domain and the second binding domain is a tumor antigen binding domain.

19. The composition of claim 18, wherein the polypeptide comprises, from N-terminus to C-terminus, the CD3 binding domain, the hinge region, the immunoglobulin constant region, and the tumor antigen binding domain.

PGR2026-00039
Biocon Ex.1002
Page 4699 of 4945

20. The composition of any one of claims 1-17 wherein the first domain is a tumor antigen binding domain, and the second binding domain is a CD3 binding domain.

21. The composition of claim 20, wherein the polypeptide comprises, from N-terminus to C-terminus, the tumor antigen binding domain, the hinge region, the immunoglobulin constant region, and the CD3 binding domain.

22. The composition of any one of claims 1-17 wherein the first binding domain is a 4-1-BB binding domain and the second binding domain is a tumor antigen binding domain.

23. The composition of claim 22, wherein the polypeptide comprises, from N-terminus to C-terminus, the 4-1-BB binding domain, the hinge region, the immunoglobulin constant region, and the tumor antigen binding domain.

24. The composition of any one of claims 1-17 wherein the first binding domain is a tumor antigen binding domain, and the second binding domain is a 4-1-BB binding domain.

25. The composition of claim 24, wherein the polypeptide comprises, from N-terminus to C-terminus, tumor antigen binding domain, the hinge region, the immunoglobulin constant region, and the 4-1-BB binding domain.

26. The composition of any one of claims 18-25, wherein the tumor antigen binding domain binds to CD123, PSMA, CD19, CD33, or HER2.

27. The composition of any one of claims 1-17, wherein the first binding domain or the second binding domain is a 4-1BB binding domain.

28. The composition of any one of claims 1-17, wherein the first binding domain or the second binding domain is an OX40 binding domain.

29. The composition of any one of claims 1-17, wherein the first binding domain is a 4-1BB binding domain and wherein the second binding domain is an OX40 binding domain.

PGR2026-00039
Biocon Ex.1002
Page 4700 of 4945

30. The composition of any one of claims 1-17, wherein the first binding domain is an OX40 binding domain and wherein the second binding domain is a 4-1BB binding domain.

31. The composition of claim 29 or 30, wherein the 4-1BB binding domain is an scFv and the OX40 binding domain is an scFv.

32. The composition of any one of claims 1-17, wherein the first binding domain is an OX40 binding domain and the second binding domain is a tumor antigen binding domain.

33. The composition of any one of claims 1-17, wherein the first binding domain is a tumor antigen binding domain and the second binding domain is an OX40 binding domain.

34. The composition of any one of claims 1-33, wherein at least one of the first binding domain and the second binding domain comprises:

(i) an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and

(ii) an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3.

35. The composition of any one of claims 1-34, wherein at least one of the first binding domain and the second binding domain is a single chain variable fragment (scFv).

36. The composition of claim 35, wherein the light chain variable region of the scFv is carboxy-terminal to the heavy chain variable region of the scFv.

37. The composition of claim 35, wherein the light chain variable region of the scFv is amino-terminal to the heavy chain variable region of the scFv.

38. The composition of any one of claims 35-37, wherein the scFv comprises a linker polypeptide.

PGR2026-00039
Biocon Ex.1002
Page 4701 of 4945

39. The composition of claim 38, wherein the linker polypeptide is between the light chain variable region and the heavy chain variable region of the scFv.

40. The composition of any one of claims 38-39, wherein the linker polypeptide comprises a Gly_4Ser (SEQ ID NO: 128) linker.

41. The composition of claim 40, wherein the linker polypeptide comprises the formula $(Gly_4Ser)_n$, wherein n = 1-5 (SEQ ID NO: 129).

42. The composition of any one of claims 18-26, wherein the tumor antigen binding domain is an anti-CD123 scFv comprising:

a HCDR1 that comprises SEQ ID NO: 10, a HCDR2 that comprises SEQ ID NO: 11, and a HDCR3 that comprises SEQ ID NO: 12; and

a LCDR1 that comprises SEQ ID NO: 13, a LCDR2 that comprises SEQ ID NO: 14, and a LCDR3 that comprises SEQ ID NO: 15.

43. The composition of any one of claims 18-26, wherein the tumor antigen binding domain is an anti-CD123 scFv comprising a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 136, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 134.

44. The composition of any one of claims 18-26, wherein the tumor antigen binding domain is an anti-CD123 scFv, and wherein the scFv comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 27.

45. The composition of any one of claims 18 to 21, wherein the CD3 binding domain is an anti-CD3 scFv comprising:

a HCDR1 that comprises SEQ ID NO: 19, a HCDR2 that comprises SEQ ID NO: 20, and a HDCR3 that comprises SEQ ID NO: 21; and

a LCDR1 that comprises SEQ ID NO: 22, a LCDR2 that comprises SEQ ID NO: 23, and a LCDR3 that comprises SEQ ID NO: 24.

46. The composition of any one of claims 18-21, wherein the CD3 binding domain is an anti-CD3 scFv that comprises a VH comprising a sequence at least 90%, at least

PGR2026-00039
Biocon Ex.1002
Page 4702 of 4945

95%, or 100% identical to SEQ ID NO: 383 or 387, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 384.

47. The composition of any one of claims 18-21, wherein the CD3 binding domain is an anti-CD3 scFv that comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 27.

48. The composition of any one of claims 18-21 or 45-47, wherein each polypeptide comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 31.

49. The composition of any one of claims 1-48, wherein the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to reduce or prevent binding to FcγR1, FcγRIIa, FcγRIIb, FcγRIIa, and FcγRIIIb.

50. The composition of any one of claims 1-48, wherein the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce Fc-mediated T-cell activation.

51. The composition of any of claims 1-48, wherein the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce CDC activity.

52. The composition of any one of claims 1-48, wherein the immunoglobulin constant region comprises one, two, three or more amino acid substitutions compared to a wild-type immunoglobulin constant region to prevent or reduce ADCC activity.

53. The composition of any one of claims 1-52, wherein the immunoglobulin constant region comprises a human IgG1 CH2 domain with one, two, three or more amino acid substitutions compared to a wild-type human IgG1 CH2 domain.

PGR2026-00039
Biocon Ex.1002
Page 4703 of 4945

54. The composition of any one of claims 1-53, wherein the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, and K322A, according to the EU numbering system.

55. The composition of any one of claim 54, wherein the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, E318A, K320A and K322A, according to the EU numbering system.

56. The composition of any one of claims 1-53, wherein the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions E233P, L234A, L235A, G237A, and K322A and a deletion of G236, according to the EU numbering system.

57. The composition of any one of claims 1-56, wherein the hinge region is derived from an immunoglobulin hinge region.

58. The composition of any one of claims 1-57, wherein each polypeptide comprises and Fc-binding domain linker between the immunoglobulin constant region, and the second binding domain.

59. The composition of claim 58, wherein the Fc-binding domain linker comprises a Gly_4Ser (SEQ ID NO: 128) sequence.

60. The composition of claim 59, wherein the Fc-binding domain linker comprises the formula $(Gly_4Ser)_n$, wherein n = 1-5 (SEQ ID NO: 129).

61. The composition of any one of claims 1-60, wherein the composition is for intravenous or subcutaneous administration.

62. The composition of any one of claims 1 to 61, wherein the composition substantially prevents degradation of the multispecific protein.

63. The composition of any one of claims 1-62, wherein the composition slows or reduces the degradation of the multispecific protein as compared to an identical multispecific protein stored in histidine buffer under identical storage conditions.

PGR2026-00039
Biocon Ex.1002
Page 4704 of 4945

64. The composition of any one of claims 1 to 63, wherein the composition is substantially stable for at least 1 year at 4°C.

65. The composition of any one of claims 1 to 64, wherein the composition is substantially resistant to formation of aggregates of multispecific protein.

66. The composition of any one of claims 1-65, wherein the composition forms fewer aggregates as compared to an identical multispecific protein stored in histidine buffer under identical storage conditions.

67. A composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein:

(a) the fusion protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus:

(i) a first binding domain that specifically binds to CD123,

(ii) a hinge region,

(iii) an immunoglobulin constant region, and

(iv) a second binding domain that specifically binds to CD3; and

(b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

68. The composition of claim 67, wherein the composition comprises about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80.

69. The composition of claim 67 or 68, wherein the composition comprises from about 0.1 mg/ml to about 10 mg/ml of the multispecific protein.

70. The composition of claim 69, wherein the composition comprises from about 1 mg/ml to about 5 mg/ml of the multispecific protein.

71. The composition of claim 70, wherein the composition comprises about 2 mg/ml of the multispecific protein.

PGR2026-00039
Biocon Ex.1002
Page 4705 of 4945

72. The composition of any one of claims 67-71, wherein the composition has a pH from about 4.0 to about 5.5.

73. The composition of claim 72, wherein the composition has a pH of about 4.8.

74. The composition of any one of claims 67-73, wherein the immunoglobulin constant region is a human Fc domain.

75. The composition of any one of claims 67-74, wherein the immunoglobulin constant region comprises immunoglobulin CH2 and CH3 domains of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgD.

76. The composition of any one of claims 1-75, wherein the CH2 domain is a human IgG1 CH2 domain with one, two, three or more amino acid substitutions compared to a wild-type human IgG1 CH2 domain.

77. The composition of any one of claims 67-76, wherein the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, and K322A, according to the EU numbering system.

78. The composition of any one of claims 67-77, wherein the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions L234A, L235A, G237A, E318A, K320A and K322A, according to the EU numbering system.

79. The composition of any one of claims 67-76, wherein the immunoglobulin constant region comprises a human IgG1 CH2 domain comprising the substitutions E233P, L234A, L235A, G237A, and K322A and a deletion of G236, according to the EU numbering system.

80. The composition of any one of claims 67-79, wherein the hinge region is derived from an immunoglobulin hinge region.

PGR2026-00039
Biocon Ex.1002
Page 4706 of 4945

81. The composition of any one of claims 67-80, wherein each polypeptide comprises an Fc-binding domain linker between the immunoglobulin constant region and the second binding domain.

82. The composition of claim 81, wherein the Fc-binding domain linker comprises a Gly_4Ser (SEQ ID NO: 128) sequence.

83. The composition of claim 82, wherein the Fc-binding domain linker comprises the formula $(Gly_4Ser)_n$, wherein n = 1-5 (SEQ ID NO: 129).

84. The composition of any one of claims 67-83, wherein the composition is for intravenous or subcutaneous administration.

85. The composition of any one of claims 67-84, wherein at least one of the first binding domain and the second binding domain comprises:

(i) an immunoglobulin heavy chain variable region (VH) comprising HCDR1, HCDR2, and HCDR3; and

(ii) an immunoglobulin light chain variable region (VL) comprising LCDR1, LCDR2, and LCDR3.

86. The composition of any one of claims 67-85, wherein at least one of the first binding domain and the second binding domain is a single chain variable fragment (scFv).

87. The composition of claim 86, wherein the light chain variable region of the scFv is carboxy-terminal to the heavy chain variable region of the scFv.

88. The composition of claim 86, wherein the light chain variable region of the scFv is amino-terminal to the heavy chain variable region of the scFv.

89. The composition of any one of claims 86-88, wherein the scFv comprises a linker polypeptide.

90. The composition of claim 89, wherein the linker polypeptide is between the light chain variable region and the heavy chain variable region of the scFv.

PGR2026-00039
Biocon Ex.1002
Page 4707 of 4945

91. The composition of claim 89 or 90, wherein the linker polypeptide comprises a Gly_4Ser (SEQ ID NO: 128) linker.

92. The composition of claim 91, wherein the linker polypeptide comprises the formula $(Gly_4Ser)_n$, wherein n = 1-5 (SEQ ID NO: 129).

93. The composition of any one of claims 67-92, wherein the first binding domain is an anti-CD123 scFv comprising:

a HCDR1 comprising the sequence of SEQ ID NO: 10, a HCDR2 comprising the sequence of SEQ ID NO: 11, and a HDCR3 comprising the sequence of SEQ ID NO: 12; and

a LCDR1 comprising the sequence of SEQ ID NO: 13, a LCDR2 comprising the sequence of SEQ ID NO: 14, and a LCDR3 comprising the sequence of SEQ ID NO: 15.

94. The composition of any one of claims 67-92, wherein the first binding domain is an anti-CD123 scFv comprising a VH comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 136, and a VL comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 134.

95. The composition of any one of claims 67-92, wherein the first binding domain is an anti-CD123 scFv comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 18.

96. The composition of any one of claims 67-95, wherein the second binding domain is an anti-CD3 scFv comprising:

a HCDR1 comprising the sequence of SEQ ID NO: 19, a HCDR2 comprising the sequence of SEQ ID NO: 20, and a HDCR3 comprising the sequence of SEQ ID NO: 21; and

a LCDR1 comprising the sequence of SEQ ID NO: 22, a LCDR2 comprising the sequence of SEQ ID NO: 23, and a LCDR3 comprising the sequence of SEQ ID NO: 24.

PGR2026-00039
Biocon Ex.1002
Page 4708 of 4945

97. The composition of any one of claims 67-95, wherein the second binding domain is an anti-CD3 scFv comprising a VH that comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 383 or 387, and a VL that comprises a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 384.

98. The composition of any one of claims 67-95, wherein the second binding domain is an anti-CD3 scFv comprising a sequence at least 90%, at least 95%, or 100% identical to SEQ ID NO: 27.

99. The composition of any one of claims 67-95, wherein each polypeptide comprises the sequence of SEQ ID NO: 31, or comprises a sequence at least 90%, at least 95%, or at least 98% identical to SEQ ID NO: 31.

100. The composition of any one of claims 67-95, wherein:

the first binding domain is an anti-CD123 scFv comprising an immunoglobulin heavy chain variable region (VH) comprising a HCDR1 of SEQ ID NO: 10, a HCDR2 of SEQ ID NO: 11, and a HCDR3 of SEQ ID NO: 12, and an immunoglobulin light chain variable region (VL) comprising a LCDR1 of SEQ ID NO: 13, a LCDR2 of SEQ ID NO: 14, and a LCDR3 of SEQ ID NO: 15, and

the second binding domain is an anti-CD3 scFv comprising an immunoglobulin heavy chain variable region (VH) comprising a HCDR1 of SEQ ID NO: 19, a HCDR2 of SEQ ID NO: 20, and a HCDR3 of SEQ ID NO: 21, and an immunoglobulin light chain variable region (VL) comprising a LCDR1 of SEQ ID NO: 22, a LCDR2 of SEQ ID NO: 23, and a LCDR3 of SEQ ID NO: 24.

101. The composition of any one of claims 67 to 100, wherein the composition substantially prevents degradation of the multispecific protein.

102. The composition of any one of claims 67 to 101, wherein the composition slows or reduces the degradation of the multispecific protein as compared to an identical multispecific protein stored in histidine buffer under identical storage conditions.

103. The composition of any one of claims 67 to 102, wherein the composition is substantially stable for at least 1 year at 4°C.

PGR2026-00039
Biocon Ex.1002
Page 4709 of 4945

104. The composition of any one of claims 67 to 103, wherein the composition is substantially resistant to formation of aggregates of multispecific protein.

105. The composition of any one of claims 67 to 104, wherein the composition forms fewer aggregates as compared to an identical multispecific protein stored in histidine buffer under identical storage conditions.

106. A composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein

(a) the fusion protein comprises:

(i) a first binding domain that specifically binds to CD123,

wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15; and

(ii) a second binding domain that specifically binds to CD3, wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24; and

(b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

107. A composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein the fusion protein comprises a Fab, Fab', F(ab')2, scFv, disulfide linked Fv, scFv x scFv (BiTE), scFv-Fc (SMIP), scFv-Fc-scFv, quadroma, Kλ-body, dAbs, diabody, nanobody, DOCK-AND-LOCKs® (DNLs®), CrossMab Fabs, CrossMab VH-VL, strand-exchange engineered domain bodies (SEEDbodies), Affibodies, Fynomers, Kunitz Domains, Albu-dabs, two engineered Fv fragments with exchanged VHs (e.g., a dual-affinity re-targeting molecules (D.A.R.T.s)), DVD-IG, Covx-body, peptibody, SVD-Igs, dAb-Igs,

PGR2026-00039
Biocon Ex.1002
Page 4710 of 4945

Knobs-in-Holes, IgG1 antibodies comprising matched mutations in the CH3 domain (e.g., DuoBody antibody) and triomAb.

108. A composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein

(a) the fusion protein comprises:

(i) a first binding domain that specifically binds to CD123,

wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15; and

(ii) a second binding domain that specifically binds to CD3,

wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24.

109. A composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein

(a) the fusion protein comprises:

(i) a first binding domain that specifically binds to CD123,

wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising SEQ ID NO: 136; and an immunoglobulin light chain variable region (VL) comprising SEQ ID NO: 134; and

(ii) a second binding domain that specifically binds to CD3,

wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising SEQ ID NO: 383 or 387; and an immunoglobulin light chain variable region (VL) comprising SEQ ID NO: 384; and

and

(b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

PGR2026-00039
Biocon Ex.1002
Page 4711 of 4945

110. A composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein

(a) the fusion protein comprises:

(i) a first binding domain that specifically binds to CD123, wherein the first binding domain comprises SEQ ID NO: 18; and

(ii) a second binding domain that specifically binds to CD3, wherein the second binding domain comprises SEQ ID NO: 27; and

(b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

111. A composition comprising a fusion protein, a buffer, an excipient and a surfactant, wherein

(a) the fusion protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus, or in order from carboxyl-terminus to amino-terminus:

(i) a first binding domain that specifically binds to CD123,

wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15,

(ii) a hinge region of SEQ ID NO: 47,

(iii) an immunoglobulin constant region of SEQ ID NO: 131,

(iv) a Fc-binding domain linker of SEQ ID NO: 132, and

(v) a second binding domain that specifically binds to CD3,

wherein the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24; and

(b) the buffer comprises or consists of succinate or a pharmaceutically acceptable salt or acid thereof.

PGR2026-00039
Biocon Ex.1002
Page 4712 of 4945

112. A composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80, wherein:

the fusion protein is a dimer of two identical polypeptides, wherein each polypeptide comprises, in order from amino-terminus to carboxyl-terminus:

(i) a first binding domain that specifically binds to CD123,

wherein the binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 10, HCDR2 of SEQ ID NO: 11, and HCDR3 of SEQ ID NO: 12; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 13, LCDR2 of SEQ ID NO: 14, and LCDR3 of SEQ ID NO: 15,

(ii) a hinge region of SEQ ID NO: 47,

(iii) an immunoglobulin constant region of SEQ ID NO: 131,

(iv) a Fc-binding domain linker of SEQ ID NO: 132, and

(v) a second binding domain that specifically binds to CD3,

wherein the second binding domain comprises an immunoglobulin heavy chain variable region (VH) comprising HCDR1 of SEQ ID NO: 19, HCDR2 of SEQ ID NO: 20, and HCDR3 of SEQ ID NO: 21; and an immunoglobulin light chain variable region (VL) comprising LCDR1 of SEQ ID NO: 22, LCDR2 of SEQ ID NO: 23, and LCDR3 of SEQ ID NO: 24.

113. A composition comprising a fusion protein, about 5 mM succinate, about 6.5% weight/volume (w/v) sucrose and about 0.02% w/v polysorbate 80,

wherein the fusion protein comprises or consists of SEQ ID NO: 31;

wherein the composition comprises about 2 mg/ml of the fusion protein; and

wherein the composition has a pH of about 4.8.

114. A method for treating an autoimmune disorder in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the composition of any one of claims 1-113.

115. The method of claim 114, wherein the autoimmune disorder is irritable bowel syndrome, inflammatory bowel disease, psoriasis, rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, asthma, multiple sclerosis, dermatomyositis, polymyositis, pernicious anaemia, primary biliary

PGR2026-00039
Biocon Ex.1002
Page 4713 of 4945

cirrhosis, acute disseminated encephalomyelitis (ADEM), Addison's disease, ankylosing spondylitis, antiphospholipid antibody syndrome (aPL), autoimmune hepatitis, diabetes mellitus type 1, Goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome (GBS), Hashimoto's disease, idiopathic thrombocytopenic purpura, pemphigus vulgaris, Sjögren's syndrome, temporal arteritis, autoimmune hemolytic anemia, bullous pemphigoid, vasculitis, celiac disease, endometriosis, hidradenitis suppurativa, interstitial cystitis, morphea, scleroderma, narcolepsy, neuromyotonia, vitiligo, autoimmune inner ear disease or myasthenia gravis.

116. A method for treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the composition of any one of claims 1-113.

117. The method of claim 116, wherein the cancer is selected from a carcinoma or sarcoma.

118. The method of claim 116 wherein the cancer is selected from a melanoma, kidney cancer, pancreatic cancer, lung cancer, intestinal cancer, prostate cancer, breast cancer, liver cancer, brain cancer, colon cancer, ovarian cancer, or a hematological cancer.

119. The method of claim 118, wherein the hematological cancer is an acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), hairy cell leukemia (HCL), blastic plasmacytoid dendritic cell neoplasm, B-cell acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML).

120. Use of the composition of any one of claims 1-113 for treating autoimmune disorder in a subject.

121. Use of the composition of any one of claims 1-113 in the manufacture of a medicament for treating an autoimmune disorder.

122. Use of the composition of any one of claims 1-113 for treating cancer in a subject.

PGR2026-00039
Biocon Ex.1002
Page 4714 of 4945

123. Use of the composition of any one of claims 1-113 in the manufacture of a medicament for treating cancer.

124. A method of treating a cancer in a subject comprising administering a composition comprising a multispecific protein comprising a CD123 binding domain and a CD3 binding domain to the subject by IV infusion at a dose of 0.3, 1, 3, 6, 9, 12, 18, 20, 24, 30, 36, 48, 50, 60, 75, or 100 µg.

125. A method of treating a cancer in a subject comprising administering a composition of any one of claims 42-48, 93-100, and 108-111 to a patient by IV infusion at a dose of 0.3, 1, 3, 6, 9, 12, 18, 20, 24, 30, 36, 48, 60, 50, 75, or 100 µg.

126. The method of claim 124 or 125, wherein the first dose of the composition is administered to the patient by IV infusion over a 20-24 hour period of time.

127. The method of claim 126, wherein a second dose of the composition is administered to the patient by IV infusion over an 8 hour period of time, wherein the second dose is the same as the first dose.

128. The method of claim 127, wherein a third dose of the composition is administered to the patient by IV infusion over a 6 hour period of time, wherein the third dose is the same as the first and the second dose.

129. The method of any one of claims 124-128, wherein the fourth dose and subsequent doses of the composition are administered to the patient by IV infusion over about a 2 to about a 4 hour period of time.

130. The method of any one of claims 124-125, wherein the composition is administered to the patient by continuous IV infusion up to 72 hours.

131. The method of any one of claims 124-130, wherein the composition is administered on days 1, 8, 15, and 22.

PGR2026-00039
Biocon Ex.1002
Page 4715 of 4945

132. The method of claim 131, wherein 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 12 µg is administered on day 22.

133. The method of claim 131, wherein 6 µg is administered on day 1, 9 µg is administered on day 8, 12 µg is administered on day 15, and 18 µg is administered on day 22.

134. The method of claim 131, wherein 6 µg is administered on day 1, 9 µg is administered on day 8, 9 µg is administered on day 15, and 9 µg is administered on day 22.

135. The method of claim 131, wherein 9 µg is administered on day 1, 12 µg is administered on day 8, 12 µg is administered on day 15, and 12 µg is administered on day 22.

136. The method of claim 131, wherein 12 µg is administered on day 1, 18 µg is administered on day 8, 18 µg is administered on day 15, and 18 µg is administered on day 22.

137. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg;
Week 2 dosage: 9 µg;
Week 3 dosage: 12 µg; and
Week 4 dosage and subsequent week dosages: 12 µg.

138. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg;
Week 2 dosage: 9 µg;
Week 3 dosage: 12 µg; and
Week 4 dosage and subsequent week dosages: 18 µg.

PGR2026-00039
Biocon Ex.1002
Page 4716 of 4945

139. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg;
Week 2 dosage: 12 µg;
Week 3 dosage: 12 µg; and
Week 4 dosage and subsequent week dosages: 12 µg.

140. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg;
Week 2 dosage: 12 µg;
Week 3 dosage: 18 µg; and
Week 4 dosage and subsequent week dosages: 24 µg.

141. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg;
Week 2 dosage: 12 µg;
Week 3 dosage: 18 µg; and
Week 4 dosage and subsequent week dosages: 36 µg.

142. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg;
Week 2 dosage: 12 µg;
Week 3 dosage: 18 µg; and
Week 4 dosage and subsequent week dosages: 48 µg.

143. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg;
Week 2 dosage: 12 µg;
Week 3 dosage: 18 µg; and
Week 4 dosage and subsequent week dosages: 60 µg.

PGR2026-00039
Biocon Ex.1002
Page 4717 of 4945

144. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 6 µg; and
Week 2 and subsequent week dosages: 9 µg.

145. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 9 µg; and
Week 2 and subsequent week dosages: 12 µg.

146. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV according to the following weekly treatment schedule:
Week 1 dosage: 12 µg; and
Week 2 and subsequent week dosages: 18 µg.

147. The method of any one of claims 124-130, wherein the composition is administered to a patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 9 µg of the multispecific protein is administered on day 2, 12 µg of the multispecific protein is administered on day 3, 18 µg of the multispecific protein is administered on day 4, 18 µg of the multispecific protein is administered on day 8, 18 µg of the multispecific protein is administered on day 11, 36 µg of the multispecific protein is administered on day 15, and 36 µg of the multispecific protein is administered on day 22 of the first 28-day cycle.

148. The method of claim 147, wherein the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 36 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

149. The method of any one of claims 124-130, wherein the composition is administered to a patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 12 µg of the multispecific protein is administered on day 2, 18 µg of the multispecific protein is administered on day 3, 24 µg of the multispecific protein is administered on day 4, 24 µg of the multispecific protein is

PGR2026-00039
Biocon Ex.1002
Page 4718 of 4945

administered on day 8, 24 µg of the multispecific protein is administered on day 11, 48 µg of the multispecific protein is administered on day 15, and 48 µg of the multispecific protein is administered on day 22 of the first 28-day cycle.

150. The method of claim 149, wherein the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 48 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

151. The method of any one of claims 124-130, wherein the composition is administered to a patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 12 µg of the multispecific protein is administered on day 2, 24 µg of the multispecific protein is administered on day 3, 36 µg of the multispecific protein is administered on day 4, 36 µg of the multispecific protein is administered on day 8, 36 µg of the multispecific protein is administered on day 11, 60 µg of the multispecific protein is administered on day 15, and 60 µg of the multispecific protein is administered on day 22 of the first 28-day cycle.

152. The method of claim 151, wherein the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle, wherein 60 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

153. The method of any one of claims 124-130, wherein the composition is administered to a patient during a first 28-day cycle, wherein 6 µg of the multispecific protein is administered on day 1, 12 µg of the multispecific protein is administered on day 2, 24 µg of the multispecific protein is administered on day 3, 36 µg of the multispecific protein is administered on day 4, 48 µg of the multispecific protein is administered on day 8, 48 µg of the multispecific protein is administered on day 11, 100 µg of the multispecific protein is administered on day 15, and 100 µg of the multispecific protein is administered on day 22 of the first 28-day cycle.

154. The method of claim 153, wherein the method further comprises administering the multispecific protein to the patient during at least one additional 28-day cycle,

PGR2026-00039
Biocon Ex.1002
Page 4719 of 4945

wherein 100 µg of the multispecific protein is administered on each of days 1, 8, 15, and 22 of the at least one additional 28-day cycle.

155. The method of any one of claims 124-130, wherein the composition is administered to a patient by IV and the dosage is increased each week for the first weeks.

156. The method of any one of claims 124-130, wherein the patient is administered the composition once, twice, three, or four times each week.

157. The method of any one of claims 124-156, wherein the composition is administered to the patient with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS).

PGR2026-00039
Biocon Ex.1002
Page 4720 of 4945

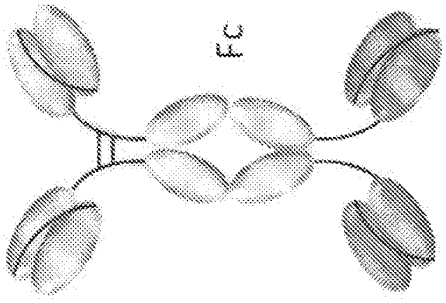


FIG. 1B

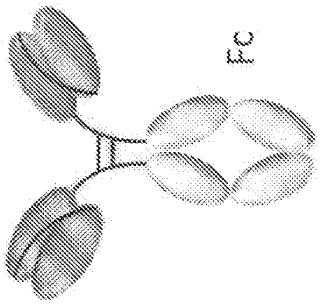


FIG. 1A

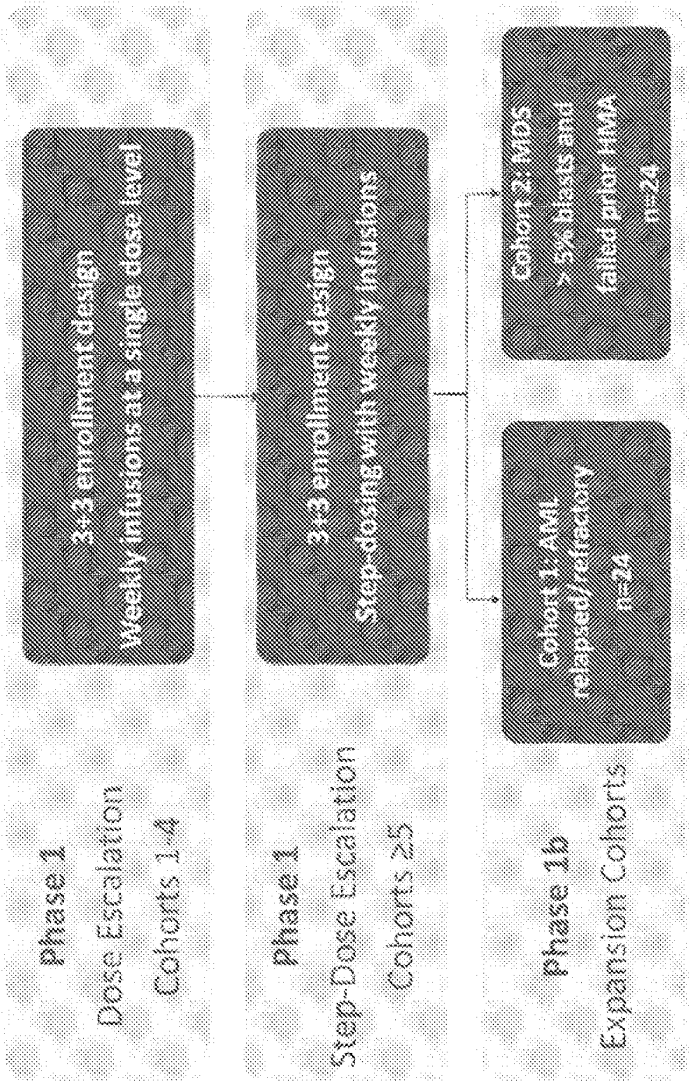

Phase 1
Dose Escalation
Cohorts 1-4
3+3 enrollment design
Weekly infusions at a single dose level
Phase 1
Step-Dose Escalation
Cohorts 25
3+3 enrollment design
Step-dosing with weekly infusions
Phase 1b
Expansion Cohorts
Cohort 11 AML
relapsed/refractory
n=22
Cohort 24 MDS
< 5% blasts and
failed prior HMA
n=24

FIG. 2

FIG. 3B

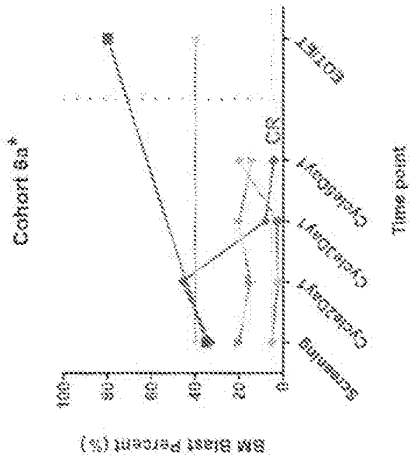


FIG. 3D

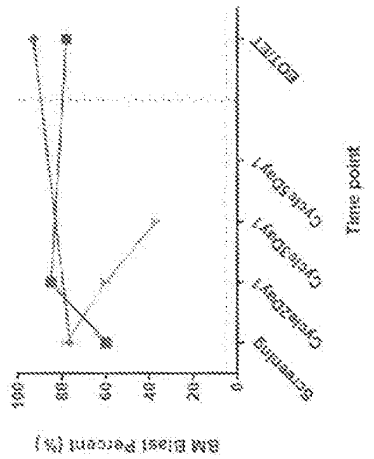


FIG. 3A

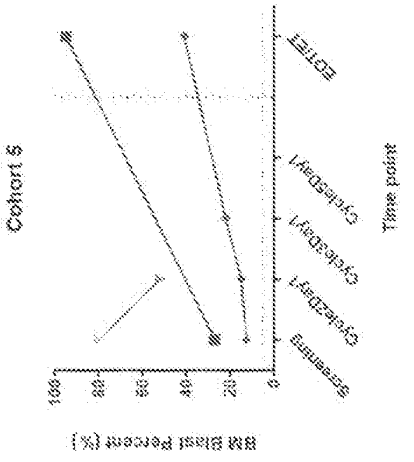
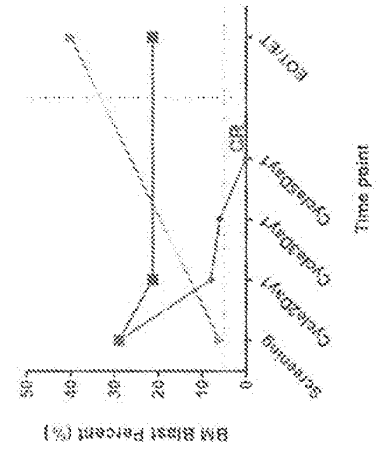


FIG. 3C



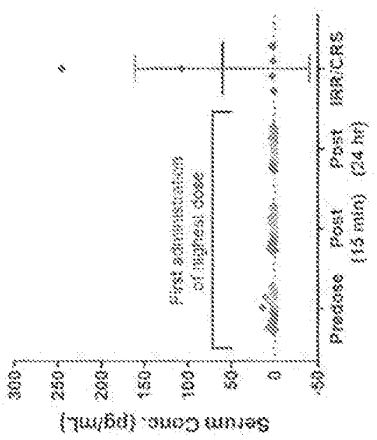


FIG. 4A

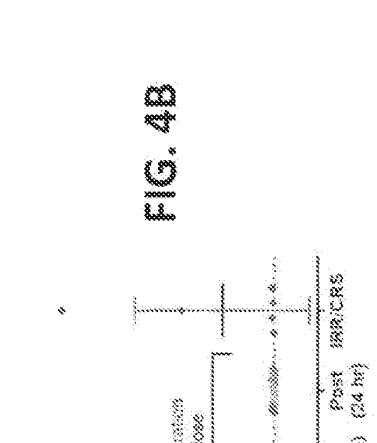


FIG. 4B

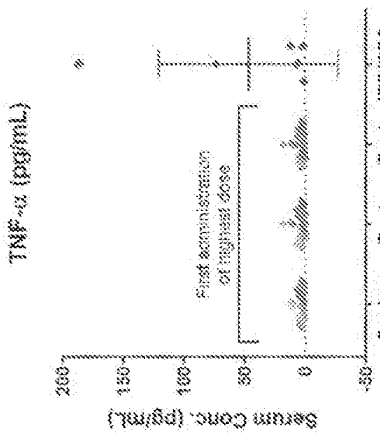


FIG. 4C



FIG. 4D

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/013294

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 A61P35/02 C07K16/28 C07K16/46
ADD.

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)
A61K A61P C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MANIKWAR PRAKASH ET AL: "Characterization of a Novel Bispecific Antibody With Improved Conformational and Chemical Stability", JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 109, no. 1, 7 July 2019 (2019-07-07), pages 220-232, XP055794177, US ISSN: 0022-3549, DOI: 10.1016/j.xphs.2019.06.025 Retrieved from the Internet: URL:https://www.jpharmsci.org/article/S0022-3549(19)30428-9/pdf> page 221, left-hand column, paragraph 2; figures 1a,2 page 222, left-hand column, paragraph 2 ----- -/--	1-41, 49-66, 76,107

[X] Further documents are listed in the continuation of Box C. [X] 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

"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)

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"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	Date of mailing of the international search report
14 April 2021	22/04/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax. (+31-70) 340-3016	Cilensek, Zoran

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X,P	Watts Justin M: "Paper: Preliminary Results from a Phase 1 Study of APVO436, a Novel Anti-CD123 x Anti-CD3 Bispecific Molecule, in Relapsed/Refractory Acute Myeloid Leukemia and Myelodysplastic Syndrome", , 7 December 2020 (2020-12-07), XP055794863, Retrieved from the Internet: URL:https://ash.confex.com/ash/2020/webprogram/Paper141619.html [retrieved on 2021-04-13] table 1	114-157
A	Comeau Michael R ET AL: "Group Number Test Material APVO436, a Bispecific anti-CD123 x anti-CD3 ADAPTIR(TM) Molecule for Redirected T-cell Cytotoxicity with Limited Cytokine Release, is Well Tolerated in Repeat Dose Toxicology Studies in Cynomolgus Macaques", , 1 April 2019 (2019-04-01), page 1, XP055794845, Retrieved from the Internet: URL:https://aptevotherapeutics.gcs-web.com/static-files/95dfda00-d096-4244-92e0-b1dad8dfc582 [retrieved on 2021-04-13] the whole document	1-157
A	Anonymous: "Preliminary Results Show Flotetuzumab Well-Tolerated and Effective in Relapsed/Refractory AML and MDS - ASH Clinical News", , 2 February 2018 (2018-02-02), XP055794869, Retrieved from the Internet: URL:https://www.ashclinicalnews.org/on-location/preliminary-results-show-flotetuzumab-well-tolerated-effective-relapsedrefractory-aml-mds/ [retrieved on 2021-04-13] the whole document	1-157
A	WO 2019/050521 A1 (MACROGENICS INC [US]) 14 March 2019 (2019-03-14) example 2	1-157

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/013294

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YOANN LE BASLE ET AL: "Physicochemical Stability of Monoclonal Antibodies: A Review", JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 109, no. 1, 1 January 2020 (2020-01-01), pages 169-190, XP055697789, US ISSN: 0022-3549, DOI: 10.1016/j.xphs.2019.08.009 the whole document	1-157

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2021/013294

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International Bureau

(43) International Publication Date
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WIPO | PCT

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A61K 39/00 (2006.01) *A61K 47/26* (2006.01)
A61K 39/395 (2006.01) *A61K 9/08* (2006.01)
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(54) Title: PREVENTION OF VISIBLE PARTICLE FORMATION IN AQUEOUS PROTEIN SOLUTIONS

(57) Abstract: The present invention provides methods to prevent the formation of visible particles in aqueous protein formulations, as well as compositions and pharmaceutical products obtained with said method.

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PREVENTION OF VISIBLE PARTICLE FORMATION IN AQUEOUS PROTEIN SOLUTIONS

The present invention relates to the field of aqueous protein compositions, in particular pharmaceutical antibody formulations, which are stabilized against the formation of visible particles comprising free fatty acids.

BACKGROUND OF THE INVENTION

Surfactants are crucial excipients in protein formulations as they protect the labile protein from interfacial stress that may lead to protein aggregation. Proteins, such as monoclonal antibodies (mAb), are administered parenterally, which limits the choice of the surfactant, including one of the most commonly used surfactants polysorbate 20 (PS20), but also polysorbate 80, poloxamer 188, and Kolliphor/Solutol® HS 15 (poly-oxyethylene ester of 12-hydroxystearic acid).[1] PS20 can degrade over the shelf-life of a product either by oxidative degradation or by enzymatic, hydrolytic degradation. In particular, the latter yields free fatty acids (FFA) as degradation products, which can precipitate in solution and subsequently form sub-visible and visible particles.[2] Under conditions typically found in biopharmaceutical formulations, FFA can precipitate even below their solubility limit dependent on temperature but the time point of particle precipitation is poorly understood even for well-characterized degradation profiles. This suggests the involvement of nucleation factors.

There is thus a need to provide efficient solutions to prevent the formation of visible particles in aqueous protein solution, especially for long term storage. The present invention provides mitigation options for FFA particle formation below their solubility limit by selection and treatment of primary packaging material thus reducing the amount of glass leachables acting as nucleation factor.

Previous work demonstrated heterogeneity of glass surfaces for single vial lots, which could translate in differences in glass leaching upon storage.[3] For this

invention, glass leachables were studied as nucleation factors for FFA particle formation.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Identification of visible particles by FTIR as free fatty acid (FFA) after spiking of myristic acid to glass leachables solution generated from Expansion 51 vials filled with 20 mM glycine solution (pH 10) after three times terminal sterilization. Only a small selection of FFA particles are highlighted.

Figure 2: Representative FFA particle with Aluminium (few highlighted in dark circles) and Magnesium (dashed circle) on gold filter by SEM-EDX. The chemical composition of the particle is summarized in the table below. The particle was identified after spiking of glass leachables (generated from Exp33 vials with glycine solution) to aged protein solution (22M 5°C) containing degraded PS20/ mixtures of free fatty acids.

Figure 3: Proposed mechanism of FFA particle formation dependent on nucleation factors exemplarily shown for myristic acid and aluminium.

Figure 4: Historical real-time glass leachables data from Exp51 vials generated from three different placebo solutions dependent on storage time and vial format justifying leachables concentrations at end of shelf life.

Figure 5: PS 20 concentration of mAb1 and mAb2 dependent on storage time and temperature.

Figure 6: Myristic acid (A) and lauric acid (B) concentration of mAb1 and mAb2 dependent on storage time and temperature. The presence of visible particles is indicated by a dashed grey box in the first graph. The samples used for the spiking experiments are indicated by a dashed black box.

DETAILED DESCRIPTION OF THE INVENTION

Formation of visible particles composing of FFA as a result of surfactant degradation, especially PS20 and/ or PS80 degradation, represents a major challenge in the biopharmaceutical industry as there is limited choice for parenteral surfactants. Reducing PS20 degradation and degradation products such as FFA by various means is key as FFA can precipitate above their solubility limit without specific nucleation factors. Below their solubility limit however, nucleation factors can induce precipitation of FFA and limit the shelf life of the product.

Surfactants are essential components in protein formulations protecting against interfacial stress and subsequent protein aggregation. One of the current industry-wide challenges is enzymatic degradation of parenteral surfactants such as polysorbate 20 (PS20), which potentially leads to the formation of free fatty acids (FFA) forming visible particles over the course of the shelf life of a commercial protein containing preparation such as, for example, a commercial aqueous antibody formulation. While concentration of FFAs can reliably be quantified, the time point of particle formation in solution stored in glass vials remains unpredictable. The present inventors therefore studied the influence of inorganic ions, such as glass leachables, for example, as nucleation factors for FFA particle formation.

Table A, below, summarizes the concentrations of the most relevant glass leachables for different primary packaging material depending on the stored solution, preparation of glass material (e.g., terminal sterilization), solution storage time, and temperature clearly highlighting the reduction of leachables for surface-modified glass vials in comparison to uncoated glass vials (Exp33/Duran® and Exp51/Fiolax® vials) Among the surface modified vials are Siliconized vials, TopLyo® vials (Si-O-C-H layer, https://www.schott.com/d/pharmaceutical_packaging/7f629b7e-e978-4417-a15e-8621f969d225/1.4/schott-datasheet-schott-toplyo-english-14062017.pdf), and Type I plus® vials (covalently bound SiO_2 layer,

https://www.schott.com/d/pharmaceutical_packaging/ff592e9e-4a7f-495f-9952-965c4d7b1ed8/1.4/schott-datasheet-schott-type-i-plus-english-14062017.pdf)

Table A: Concentration of glass leachables Aluminium, Boron, Silicon, and Sodium in different glass types with different solutions dependent on storage temperature, time, and glass preparation. Concentrations are compared to the initial concentration of glass leachables of the solutions and their limit of quantification (LOQ).

TS = terminal sterilized, n.m. = not measured

Time point	Vial type	Matrix	Elemental concentration (ug/mL)			
			Al	B	Si	Na
Initial	-	WFI	0.001	0.012	<LOQ	0.095
		Glycine pH 10	0.057	0.18	1	n.m.
		Placebo	<LOQ	<LOQ	<LOQ	<LOQ
	Exp33	WFI	<LOQ	0.16	0.2	0.23
		Glycine pH 10	0.053	0.34	2.7	n.m.
		Placebo	<LOQ	0.26	<LOQ	<LOQ
		1xTS Glycine pH 10	0.24	1.5	12	n.m.
		1xTS Placebo	<LOQ	0.82	<LOQ	1
	Exp51	WFI	<LOQ	0.028	0.05	0.2
		Glycine pH 10	0.065	0.19	1.7	n.m.
		Placebo	<LOQ	0.09	<LOQ	<LOQ
		1xTS Glycine pH 10	0.37	0.43	4.3	n.m.
		1xTS Placebo	0.026	0.22	<LOQ	1.1
	TopLyo®	WFI	<LOQ	<LOQ	0.13	0.018
		Glycine pH 10	0.046	0.17	2.3	n.m.
		Placebo	<LOQ	<LOQ	<LOQ	<LOQ
		1xTS Glycine pH 10	0.045	0.17	2	n.m.
		1xTS Placebo	<LOQ	0.05	<LOQ	<LOQ
	Siliconized	WFI	0.002	0.066	0.07	0.2
		Glycine pH 10	0.063	0.23	1.8	n.m.
		Placebo	<LOQ	0.12	<LOQ	<LOQ
		1xTS Glycine pH 10	0.25	0.42	3.6	n.m.
		1xTS Placebo	<LOQ	0.2	<LOQ	0.93
		WFI	<LOQ	<LOQ	0.06	0.016

	Type I plus®	Glycine pH 10	0.033	0.17	1.8	n.m.
		Placebo	<LOQ	0.05	<LOQ	<LOQ
		1xTS Glycine pH 10	0.028	0.22	9.2	n.m.
		1xTS Placebo	<LOQ	0.056	<LOQ	<LOQ
	Exp33	WFI	0.002	1	2.2	0.78
		Glycine pH 10	0.019	12	67	n.m.
		Placebo	0.055	1.1	1.7	1.1
		1xTS Glycine pH 10	0.017	12	69	n.m.
		1xTS Placebo	0.09	1.3	2.8	1.2
	ExpS1	WFI	0.026	0.1	0.73	0.55
		Glycine pH 10	0.97	0.97	8.8	n.m.
		Placebo	0.12	0.2	1	0.66
		1xTS Glycine pH 10	0.99	1	8.9	n.m.
		1xTS Placebo	0.21	0.28	1.6	1.1
	TopLyo®	WFI	<LOQ	<LOQ	1.2	0.012
		Glycine pH 10	0.054	0.17	3.2	n.m.
6W 40°C		Placebo	<LOQ	<LOQ	0.59	<LOQ
		1xTS Glycine pH 10	0.05	0.17	3.5	n.m.
		1xTS Placebo	0.068	<LOQ	0.76	<LOQ
	Siliconized	WFI	0.018	0.2	1.1	0.67
		Glycine pH 10	0.79	0.99	9.1	n.m.
		Placebo	0.069	0.18	0.79	0.58
		1xTS Glycine pH 10	0.81	0.98	9	n.m.
		1xTS Placebo	0.16	0.28	1.4	0.96
	Type I plus®	WFI	<LOQ	<LOQ	0.5	0.014
		Glycine pH 10	0.15	0.3	18	n.m.
		Placebo	<LOQ	<LOQ	5.6	<LOQ
		1xTS Glycine pH 10	0.19	0.41	19	n.m.
		1xTS Placebo	<LOQ	<LOQ	2.3	<LOQ
	-	WFI	0.001	0.005	0.05	0.005
LOQ		Glycine pH 10	0.01	0.1	0.5	n.m.
		Placebo	0.02	0.05	0.5	0.5

In accordance with the present invention, it has now been found that FFA particles, identified by FTIR, were a result of precipitation with inorganic ions/components, e g. glass leachables such as aluminium, as elucidated by further characterization of

the chemical composition by SEM-EDX. This suggests the involvement of inorganic elements in the formation of these particles. Silicon dioxide, boron trioxide, and aluminium trioxide are typical glass network formers of type I borosilicate glass used for parenteral products. Different glass network modifiers like alkali oxides (e.g , sodium, potassium), and oxides of alkaline earth metals (e g., calcium and magnesium) are added during the glass manufacturing process to decrease the melting temperature of the glass. Without being bound to theory, it may be concluded that inorganic elements leaching from the glass vials depending on glass type, formulation, and storage condition may act as nucleation seeds for FFA particle formation. In the present study, lauric acid and myristic acid were used as main degradation products from enzymatic PS20 degradation[4] and the study targeted different glass leachables as well as mixtures of the same to verify the hypothesis that free fatty acids below their solubility limit precipitate in their presence.

In accordance with the present invention, it has surprisingly been found that inorganic salts especially $NaAlO_2$ and $CaCl_2$ initiate the formation of visible particles in presence of myristic or lauric acid *below* their solubility limit. These salts mimic leachables from type I borosilicate glass typically used for parenteral products. In particular surprisingly, relevant glass leachables in mixtures obtained by autoclavation cycles in different glass types with different formulations and at representative leachable's concentrations for a proteinaceous drug product over its shelf life of 2-3 years at 5°C confirmed particle formation with lauric/myristic acid the major degradation products of polysorbates, such as PS20, in commercial parenteral antibody formulations. Particles in different formulations in Exp33 vials and Exp51 vials were identified as FFA salts with different glass leachables, such as aluminium or silicon. In addition, the present invention in particular demonstrated FFA particle formation depending on relevant aluminum concentrations. In one embodiment in accordance with the present invention, said aluminium concentrations are in the ppb range. The present findings were verified in two case studies with monoclonal antibody (mAb) formulations aged at recommended storage temperature (22M, 5°C) showing enzymatic PS20

degradation profiles resulting in mixtures of different FFA. In these, spiking of mixtures of glass leachables led to immediate visible particle formation, identified as a complex of glass leachables such as aluminium, silicon, magnesium, potassium, sodium, calcium and free fatty acid. Based on the present results, particle formation will be verified in protein formulations on long-term storage under real-time conditions.

Therefore, in one embodiment the present invention provides a stable aqueous composition comprising a protein together with pharmaceutically acceptable excipients such as, for example, buffers, stabilizers including antioxidants, and surfactants, wherein said composition further contains a mixture of one or several types of inorganic ions diffused out of the packaging material, such as a glass vial, and substances resulting from the degradation of said surfactants without forming visible particles. In one aspect said inorganic ions are selected from Aluminium, Boron, Silicon, Calcium, Magnesium, Potassium, and Sodium. In another aspect the concentration of said inorganic ions is any concentration up to the concentration disclosed in Table A (6M 40°C) for each ion and each vial type for non-surface-modified vials (referred to as Exp33 and exp51 vials), respectively. In yet another aspect, specifically for Exp51 vials of different vial formats, the concentration of said inorganic ions is any concentration up to the respective concentrations for each ion as disclosed in Fig.4.

In another embodiment, there is provided the composition as defined above, wherein the pH of said composition is in the range of 5 to 7. In one aspect the pH is about 6.

In another embodiment, the present invention provides a composition as defined herein before, wherein the protein is an antibody. In one aspect, the antibody is a monoclonal antibody. In another aspect the antibody is a human or humanized monoclonal, mono- or bispecific antibody.

In one aspect, the present invention provides a composition as defined herein before, further comprising one or several types of substances resulting from the degradation of surfactants present in said composition (degradation products). In

one aspect said surfactant is selected from polysorbates (PS). In another aspect, said surfactant is selected from PS20 or PS80. In another aspect, said degradation products are a mixture of different fatty acids of different chain length and saturation and remaining PS20 residues consisting of polymeric esters of different polar head groups, different fatty acid tails, and different degree of esterification. In one aspect said degradation products are free fatty acids as defined herein. In one aspect, said substances resulting from polysorbate degradation are free fatty acids in a concentration up to, but not above, their respective solubility level. In another aspect, said free fatty acid is selected as defined in USP in PS20. In another aspect, said free fatty acid is selected from lauric acid, myristic acid, palmitic/oleic acid, capric acid, and stearic acid. In another aspect, said free fatty acid is selected from lauric acid and/or myristic acid and the solubility level for lauric acid is 15 µg / ml, and the solubility level for myristic acid is 7 µg / ml in water at room temperature.

In one embodiment, the present invention provides a composition as defined herein before, wherein the concentration is up to 0.03 µg / ml aluminium, and/or up to 0.05 µg / ml boron, and/or up to 0.5 µg / ml silicon.

In another embodiment, the present invention provides a composition as defined herein before, wherein the stabilizer is selected from the group consisting of sugars, sugar alcohols, sugar derivatives, or amino acids. In one aspect the stabilizer is (1) sucrose, trehalose, cyclodextrines, sorbitol, mannitol, glycine, or/and (2) methionine, and/or (3) arginine, or lysine. In still another aspect, the concentration of said stabilizer is (1) up to 500 mM or (2) 5-25 mM, or/and (3) up to 350 mM, respectively.

In another embodiment, the present invention provides a composition as defined herein before, wherein the buffer is selected from the group consisting of acetate, succinate, citrate, arginine, histidine, phosphate, Tris, glycine, aspartate, and glutamate buffer systems. In one aspect the buffer composes of free histidine base and histidine-HCl or acetate or succinate and/or aspartate. Furthermore, within this embodiment, the histidine concentration of said buffer is from 5 to 50 mM.

In another embodiment, the present invention provides a composition as defined herein before, wherein the surfactant is selected from the group consisting of non-

ionic surfactants. In one aspect, the surfactant is a polysorbate (PS). In another aspect, the surfactant is PS20 or PS80 or Polyoxyl 15 Hydroxystearate. In yet another aspect, the concentration of said surfactant is from 0.01%-1%(w/v).

In another embodiment, the present invention provides a composition as defined herein before, wherein the pharmaceutically acceptable excipients are: 1000 U/mL hyaluronidase in 20mM HisHCl buffer pH 5.5, 105 mM Trehalose, 100 mM Sucrose, 10 mM Methionine, and 0.04%(w/v) Polysorbate 20.

In another embodiment, the present invention provides a composition as defined herein before, characterized in that it remains free of visible particles. In one aspect said visible particles consist of degradation products and inorganic ions, as defined herein. In one aspect said visible particles consist of free fatty acids and inorganic ions, as defined herein.

In another embodiment, the present invention provides a composition as defined herein before, wherein said composition remains free of said visible particles until the end of its authorized shelf life. In another aspect said composition remains free of said visible particles for up to 5 years, or for up to 3 years, or for up to 24 months, or for up to 18 months, or for up to 12 months.

In another embodiment, the present invention provides a method for obtaining a composition as defined herein, wherein said method comprises selecting a primary packaging material which prevents leaching of one or several inorganic ions as defined herein into said composition. In one aspect, said method prevents leaching of said one or several inorganic ions above the respective concentration given in Table A (6M 40°C, non-surface-modified vials) and/or Figure 4. In another embodiment the present method prevents leaching of up to 0.03 µg / ml aluminium, and/or up to 0.05 µg / ml boron, and/or up to 0.5 µg / ml silicon.

In one embodiment, the present invention provides the method for obtaining a composition as defined herein, wherein said primary packaging material is selected from

- Glass vials with inner surface coating
- Glass vials with covalently modified surface

- Glass vials from pure SiO_2 (>99%)
- Glass vials that are washed and sterilized as described below
- Polymer vials
- Polymer vials with inner surface coating or surface modification

In another embodiment, the present invention provides the method for obtaining a composition as defined herein, wherein said primary packaging material is selected from

- Siliconized vials,
- TopLyo® vials,
- Type I plus® vials,
- Pur Q® vials,
- Crystal Zenith® vials,
- SiO2 material science$_{SI}$ vials,
- Duran® vials washed and sterilized as described below, and/or
- Fiolax® vials washed and sterilized as described below.

In another embodiment, the present invention provides the method for obtaining a composition as defined herein, further comprising the steps of a) washing/drying and/or b) depyrogenation of the primary packaging material prior to its use, for example, prior to filling in the aqueous protein composition. In one aspect the washing is carried out at water temperatures above 50°C followed by a drying step allowing for residual water of <50 µl. In one aspect the depyrogenation is carried out at temperatures below or equal to 400°C. In another aspect the depyrogenation is carried out at temperatures between 180-340°C and residence time in the sterilization tunnel is limited to 8h.

In another embodiment, the present invention provides the method for obtaining a composition as defined herein, wherein said method provides stability of said composition against the formation of visible particles. In one aspect said visible particles comprise one or several degradation products and one or several types of inorganic ions as defined herein. In another aspect said visible particles consist of one or several free fatty acids and one or several inorganic ions, as defined herein. In yet another aspect, the method in accordance with the present invention provides

a composition, for example a commercial pharmaceutical antibody composition, which remains free of visible particles until the expiry of its authorized shelf life. In another aspect the present method provides a composition which remains free of said visible particles for up to 5 years, or for up to 3 years, or for up to 24 months, or for up to 18 months, or for up to 12 months.

In another embodiment, the present invention provides a pharmaceutical dosage form comprising a composition as defined herein, for example an aqueous antibody composition, in a container, wherein the concentration of one or several inorganic ions in that composition remains substantially constant during the authorized shelf life of said pharmaceutical dosage form. In one aspect said concentration of one or several inorganic ions remains substantially constant for up to 5 years, or 3years, or 24 months, or 18 months, or 12 months of storage, when compared to the concentration(s) of the same ion(s) measured in a pharmaceutical dosage form comprising the same composition in the same container at the beginning of storage, for example after 2 weeks, or immediately after filling said composition into said container or packaging material. In one aspect the container is a glass vial or a primary packaging material as defined herein. In one aspect the inorganic ions are selected from Aluminium, Boron, Silicon, Calcium, Magnesium, Potassium, and Sodium.

In another embodiment, the present invention provides the pharmaceutical dosage form as defined herein before, for example an aqueous antibody formulation in a container, wherein the increase in concentration of one or several inorganic ions in said dosage form remains below the respective concentration given for each ion and each vial type in Table A (non-surface modified vials, 6M 40°C) and/or Figure 4. In another aspect, and independent of the vial type, the concentration of aluminium remains below 0.03 µg/ml, and/or the concentration of boron remains below 0.05 µg/ml, and/or the concentration of silicon remains below 0.5 µg/ml after up to 5 years, or 3 years, or 24 months, or 18 months, or 12 months of storage when compared to the concentration(s) of the same ion(s) measured in a pharmaceutical dosage form comprising the same composition in the same container at the beginning of storage, for example after 2 weeks, or immediately after filling said composition into said container or packaging material. In one

aspect the inorganic ions are selected from Aluminium, Boron, Silicon, Calcium, Magnesium, Potassium, and Sodium. In one aspect the container is a glass vial, or a primary packaging material as defined herein.

In another embodiment, the present invention provides the use of a "primary packaging material", as defined herein before, for the storage of aqueous antibody preparations. In another embodiment, the present invention provides the use of said "primary packaging material", as defined herein, to reduce or avoid formation of visible particles, for example particles comprising FFA's, during storage of aqueous antibody preparations. In one embodiment, said primary packaging material is a polymer vial, as defined herein. In another embodiment, said primary packaging material is a surface modified glass vial as defined herein. In still another embodiment, said antibody is a monoclonal antibody. In another embodiment, said storage is characterized in that said antibody preparation remains free of visible particles for at least the authorized shelf life of the corresponding antibody product. In another embodiment said storage is characterized in that said antibody preparation remains free of visible particles for up to 5 years, or 3 years, or 24 months, or 18 months, or 12 months of storage.

The term "excipient" refers to an ingredient in a pharmaceutical composition or formulation, other than an active ingredient, which is nontoxic to a subject. An excipient includes, but is not limited to, a buffer, stabilizer including antioxidant, surfactant, or preservative.

The term "inorganic ions" is well known to a person of skill in the art of inorganic chemistry. Inorganic ions as used herein means aluminium, boron, silicon, sodium, magnesium, potassium, and calcium. Preferred inorganic ions are Aluminium, calcium, and magnesium. In accordance with the present invention, said inorganic ions can be present in a concentration of up to 0.03 µg / ml aluminium, and/or up to 0.05 µg / ml boron, and/or up to 0.5 µg / ml silicon..

The term "buffer" is well known to a person of skill in the art of organic chemistry or pharmaceutical sciences such as, for example, pharmaceutical preparation development. Buffer as used herein means acetate, succinate, citrate, arginine, histidine, phosphate, Tris, glycine, aspartate, and glutamate buffer systems.

Furthermore, within this embodiment, the histidine concentration of said buffer is from 5 to 50 mM. Preferred buffers are free histidine base and histidine-HCl or acetate or succinate and/or aspartate. Furthermore, within this embodiment, the histidine concentration of said buffer is from 5 to 50 mM.

The term "surfactant" is well known to a person of skill in the art of organic chemistry. Surfactants as used herein means non-ionic surfactants. Preferred surfactants are polysorbates, especially PS20 or PS80. In accordance with the present invention, said surfactant can be present in a concentration from 0.01%-1% (w/v).

The term "stabilizer" is well known to a person of skill in the art of organic chemistry or pharmaceutical sciences such as, for example, pharmaceutical preparation development. A stabilizer in accordance with the present invention is selected from the group consisting of sugars, sugar alcohols, sugar derivatives, or amino acids. In one aspect the stabilizer is (1) sucrose, trehalose, cyclodextrines, sorbitol, mannitol, glycine, or/and (2) methionine, and/or (3) arginine, or lysine. In still another aspect, the concentration of said stabilizer is (1) up to500 mM or (2) 5-25 mM, or/and (3) up to 350 mM, respectively

The term "substances resulting from the degradation of polysorbates" or "degradation products" as used herein means any substance resulting from the degradation of polysorbates know to the skilled person. In one aspect, said substances are free Fatty Acids. The term "Fatty Acid" (or "FA") is well known to a person of ordinary skill in organic chemistry. In one aspect, fatty acids means any carboxylic acid with an aliphatic chain, which is saturated or unsaturated, linear or branched and contains from 4 to 28; or from 8 to 24; or from 10 to 22; or from 12 to 20 carbon atoms. In one aspect, said free fatty acid is selected as defined in USP in PS20. In one aspect, said free fatty acid is selected from lauric acid, myristic acid, palmitic/oleic acid, capric acid, and stearic acid. In another aspect, said free fatty acid is selected from lauric acid and/or myristic acid.. In accordance with the present invention, said substances resulting from the degradation of polysorbates can be present in a concentration up to their respective solubility level in water at room temperature. In another aspect such substances are present at any concentration up to but not including their solubility level in water at room

temperature. The term "room temperature" as used herein has its ordinary meaning. In one aspect room temperature means from 20 to 28, preferably from 22 to 26 degrees Celsius.

The term "packaging material" or "primary packaging material" as used herein means material in contact with the product. In one embodiment the term primary packaging material means

- Glass vials with inner surface coating,
- Glass vials with covalently modified surface,
- Glass vials from pure SiO_2 (>99%),
- Glass vials that are washed and sterilized as described below,
- Polymer vials,
- Polymer vials with inner surface coating or surface modification,

In one embodiment the term primary packaging material means

- Siliconized vials,
- TopLyo® vials,
- Type I plus® vials,
- Pur Q® vials,
- Crystal Zenith® vials,
- SiO_2 material science$_{SM}$ vials,
- Duran® vials washed and sterilized as described below, and/or
- Fiolax® vials washed and sterilized as described below

In certain embodiments, the packaging material is washed and/or depyrogenated prior to receiving said stable aqueous protein composition. Washing of said packaging material can be carried out by any means known to the skilled person. Preferably said washing is carried out using at water temperatures above 50°C followed by a drying step allowing for residual water of <50uL. Depyrogenation of said packagaing material can be carried out by any means known to the skilled person. Preferably said depyrogenation is carried out using at temperatures below or equal to 400°C. More preferably said depyrogenation is carried out at

temperatures between 180-340°C and residence time in the sterilization tunnel is limited to 8h

The term "protein" as used herein means any therapeutically relevant polypeptide. In one embodiment, the term protein means an antibody. In another embodiment, the term protein means an immunocunjugate.

The term "antibody" herein is used in the broadest sense and encompasses various antibody classes or structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity. In one embodiment, any of these antibodies is human or humanized. In one aspect, the antibody is selected from alemtuzumab (LEMTRADA®), atezolizumab (TECENTRIQ®), bevacizumab (AVASTIN®), cetuximab (ERBITUX®), panitumumab (VECTIBIX®), pertuzumab (OMNITARG®, 2C4), trastuzumab (HERCEPTIN®), tositumomab (Bexxar®), abciximab (REOPRO®), adalimumab (HUMIRA®), apolizumab, aselizumab, atlizumab, bapineuzumab, basiliximab (SIMULECT®), bavituximab, belimumab (BENLYSTA®) briankinumab, canakinumab (ILARIS®), cedelizumab, certolizumab pegol (CIMZIA®), cidfusituzumab, cidtuzumab, cixutumumab, clazakizumab, crenezumab, daclizumab (ZENAPAX®), dalotuzumab, denosumab (PROLIA®, XGEVA®), eculizumab (SOLIRIS®), efalizumab, epratuzumab, erlizumab, emicizumab (HEMLIBRA®), felvizumab, fontolizumab, golimumab (SIMPONI®), ipilimumab, imgatuzumab, infliximab (REMICADE®), labetuzumab, lebrikizumab, lexatumumab, lintuzumab, lucatumumab, lulizumab pegol, lumretuzumab, mapatumumab, matuzumab, mepolizumab, moganulizumab, motavizumab, motovizumab, muronomab, natalizumab (TYSABRI®), necitumumab (PORTRAZZA®), nimotuzumab (THERACIM®), nolovizumab, numavizumab, olokizumab, omalizumab (XOLAIR®), onartuzumab (also known as MetMAb), palivizumab (SYNAGIS®), pascolizumab, pecfusituzumab, pectuzumab, pembrolizumab (KEYTRUDA®), pexelizumab, priliximab, ralivizumab, ranibizumab (LUCENTIS®), reslivizumab, reslizumab, resyvizumab, robatumumab, rontalizumab, rovelizumab, ruplizumab, sarilumab, secukinumab, seribantumab, sifalimumab, sibrotuzumab, siltuximab (SYLVANT®) siplizumab,

sontuzumab, tadocizumab, talizumab, tefibazumab, tocilizumab (ACTEMRA®), toralizumab, tucusituzumab, umavizumab, urtoxazumab, ustekinumab (STELARA®), vedolizumab (ENTYVIO®), visilizumab, zanolimumab, zalutumumab.

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')2; diabodies; linear antibodies; single-chain antibody molecules (e.g., scFv, and scFab); single domain antibodies (dAbs); and multispecific antibodies formed from antibody fragments. For a review of certain antibody fragments, see Holliger and Hudson, Nature Biotechnology 23.1126-1136 (2005).

The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g , IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. In certain aspects, the antibody is of the IgG1 isotype. In certain aspects, the antibody is of the IgG1 isotype with the P329G, L234A and L235A mutation to reduce Fc-region effector function. In other aspects, the antibody is of the IgG2 isotype. In certain aspects, the antibody is of the IgG4 isotype with the S228P mutation in the hinge region to improve stability of IgG4 antibody. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called a, d, e, g, and m, respectively. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human CDRs and amino acid residues from human FRs. In certain aspects, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

The term "hypervariable region" or "HVR" as used herein refers to each of the regions of an antibody variable domain which are hypervariable in sequence and which determine antigen binding specificity, for example "complementarity determining regions" ("CDRs") Generally, antibodies comprise six CDRs: three in the VH (CDR-H1, CDR-H2, CDR-H3), and three in the VL (CDR-L1, CDR-L2, CDR-L3). Exemplary CDRs herein include:

(a) hypervariable loops occurring at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987));

(b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)); and

(c) antigen contacts occurring at amino acid residues 27c-36 (L1), 46-55 (L2), 89-96 (L3), 30-35b (H1), 47-58 (H2), and 93-101 (H3) (MacCallum et al. J. Mol. Biol. 262: 732-745 (1996)).

Unless otherwise indicated, the CDRs are determined according to Kabat et al., supra. One of skill in the art will understand that the CDR designations can also be determined according to Chothia, supra, McCallum, supra, or any other scientifically accepted nomenclature system.

An "immunoconjugate" is an antibody conjugated to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain aspects, the individual or subject is a human.

An "isolated" antibody is one which has been separated from a component of its natural environment. In some aspects, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g , SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC) methods. For a review of methods for assessment of antibody purity, see, e.g., Flatman et al., J. Chromatogr. B 848:79-87 (2007).

The term "pharmaceutical composition" or "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the pharmaceutical composition would be administered.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical composition or formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to an excipient as defined herein.

A. Chimeric and Humanized Antibodies

In certain aspects, an antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed

from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

In certain aspects, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which the CDRs (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some aspects, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the CDR residues are derived), e.g., to restore or improve antibody specificity or affinity.

Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, Front. Biosci. 13:1619-1633 (2008), and are further described, e.g., in Riechmann et al.,

Nature 332:323-329 (1988); Queen et al., Proc. Nat'l Acad. Sci. USA 86:10029-10033 (1989); US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri et al., Methods 36:25-34 (2005) (describing specificity determining region (SDR) grafting); Padlan, Mol. Immunol. 28:489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., Methods 36:43-60 (2005) (describing "FR shuffling"); and Osbourn et al., Methods 36:61-68 (2005) and Klimka et al., Br. J. Cancer, 83:252-260 (2000) (describing the "guided selection" approach to FR shuffling).

Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims et al. J. Immunol. 151:2296 (1993)); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter et al. Proc. Natl. Acad. Sci. USA, 89:4285 (1992); and Presta et al. J. Immunol., 151:2623 (1993)); human mature (somatically mutated) framework regions or human germline framework regions

(see, e.g., Almagro and Fransson, Front. Biosci. 13:1619-1633 (2008)); and framework regions derived from screening FR libraries (see, e.g., Baca et al., J. Biol. Chem. 272:10678-10684 (1997) and Rosok et al., J. Biol. Chem. 271:22611-22618 (1996)).

B. Human Antibodies

In certain aspects, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, Curr. Opin. Pharmacol. 5: 368-74 (2001) and Lonberg, Curr. Opin. Immunol. 20:450-459 (2008).

Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, Nat. Biotech. 23:1117-1125 (2005). See also, e.g., U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSETM technology, U.S. Patent No. 5,770,429 describing HUMAB® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (See, e.g., Kozbor J. Immunol., 133: 3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987), and Boerner et al.,

J. Immunol., 147: 86 (1991).) Human antibodies generated via human B-cell hybridoma technology are also described in Li et al., Proc. Natl. Acad. Sci. USA, 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, Xiandai Mianyixue, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein, Histology and Histopathology, 20(3):927-937 (2005) and Vollmers and Brandlein, Methods and Findings in Experimental and Clinical Pharmacology, 27(3):185-91 (2005).

Human antibodies may also be generated by isolating variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described below.

C. Antibody Derivatives

In certain aspects, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not

limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

D. Immunoconjugates

The invention also provides immunoconjugates comprising an antibody herein conjugated (chemically bound) to one or more therapeutic agents such as cytotoxic agents, chemotherapeutic agents, drugs, growth inhibitory agents, toxins (e.g., protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

In one aspect, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more of the therapeutic agents mentioned above. The antibody is typically connected to one or more of the therapeutic agents using linkers. An overview of ADC technology including examples of therapeutic agents and drugs and linkers is set forth in Pharmacol Review 68:3-19 (2016).

In another aspect, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

In another aspect, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At211, I131, I125, Y90, Re186, Re188, Sm153, Bi212, P32, Pb212 and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc99m or I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic

resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

Conjugates of an antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Res. 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The immunoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S A).

E. Multispecific Antibodies

In certain aspects, an antibody provided herein is a multispecific antibody, e.g., a bispecific antibody. "Multispecific antibodies" are monoclonal antibodies that have binding specificities for at least two different sites, i.e., different epitopes on different antigens or different epitopes on the same antigen. In certain aspects, the

multispecific antibody has three or more binding specificities. Multispecific antibodies may be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, Nature 305: 537 (1983)) and "knob-in-hole" engineering (see, e.g., U.S. Patent No. 5,731,168, and Atwell et al., J. Mol. Biol. 270:26 (1997)). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (see, e.g., WO 2009/089004); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al., Science, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny et al., J. Immunol., 148(5):1547-1553 (1992) and WO 2011/034605); using the common light chain technology for circumventing the light chain mis-pairing problem (see, e.g., WO 98/50431); using "diabody" technology for making bispecific antibody fragments (see, e.g., Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (see, e.g., Gruber et al., J. Immunol., 152:5368 (1994)); and preparing trispecific antibodies as described, e.g., in Tutt et al. J. Immunol. 147: 60 (1991).

Engineered antibodies with three or more antigen binding sites, including for example, "Octopus antibodies", or DVD-Ig are also included herein (see, e.g., WO 2001/77342 and WO 2008/024715). Other examples of multispecific antibodies with three or more antigen binding sites can be found in WO 2010/115589, WO 2010/112193, WO 2010/136172, WO 2010/145792, and WO 2013/026831. The bispecific antibody or antigen binding fragment thereof also includes a "Dual Acting FAb" or "DAF" comprising an antigen binding site that binds to two different antigens, or two different epitopes of the same antigen (see, e.g., US 2008/0069820 and WO 2015/095539).

Multi-specific antibodies may also be provided in an asymmetric form with a domain crossover in one or more binding arms of the same antigen specificity, i.e. by exchanging the VH/VL domains (see e.g., WO 2009/080252 and WO 2015/150447), the CH1/CL domains (see e.g., WO 2009/080253) or the complete Fab arms (see e.g., WO 2009/080251, WO 2016/016299, also see Schaefer et al.

PNAS, 108 (2011) 1187-1191, and Klein at al., MAbs 8 (2016) 1010-20). In one aspect, the multispecific antibody comprises a cross-Fab fragment. The term "cross-Fab fragment" or "xFab fragment" or "crossover Fab fragment" refers to a Fab fragment, wherein either the variable regions or the constant regions of the heavy and light chain are exchanged. A cross-Fab fragment comprises a polypeptide chain composed of the light chain variable region (VL) and the heavy chain constant region 1 (CH1), and a polypeptide chain composed of the heavy chain variable region (VH) and the light chain constant region (CL). Asymmetrical Fab arms can also be engineered by introducing charged or non-charged amino acid mutations into domain interfaces to direct correct Fab pairing. See e.g., WO 2016/172485.

Various further molecular formats for multispecific antibodies are known in the art and are included herein (see e.g., Spiess et al., Mol Immunol 67 (2015) 95-106).

F. Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, e.g., as described in US 4,816,567. For these methods one or more isolated nucleic acid(s) encoding an antibody are provided.

In case of a native antibody or native antibody fragment two nucleic acids are required, one for the light chain or a fragment thereof and one for the heavy chain or a fragment thereof. Such nucleic acid(s) encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chain(s) of the antibody). These nucleic acids can be on the same expression vector or on different expression vectors.

In case of a bispecific antibody with heterodimeric heavy chains four nucleic acids are required, one for the first light chain, one for the first heavy chain comprising the first heteromonomeric Fc-region polypeptide, one for the second light chain, and one for the second heavy chain comprising the second heteromonomeric Fc-region polypeptide. The four nucleic acids can be comprised in one or more nucleic acid molecules or expression vectors. Such nucleic acid(s) encode an amino acid sequence comprising the first VL and/or an amino acid sequence comprising the first VH including the first heteromonomeric Fc-region and/or an amino acid

sequence comprising the second VL and/or an amino acid sequence comprising the second VH including the second heteromonomeric Fc-region of the antibody (e.g., the first and/or second light and/or the first and/or second heavy chains of the antibody). These nucleic acids can be on the same expression vector or on different expression vectors, normally these nucleic acids are located on two or three expression vectors, i.e. one vector can comprise more than one of these nucleic acids. Examples of these bispecific antibodies are CrossMabs (see, e.g., Schaefer, W. et al, PNAS, 108 (2011) 11187-1191). For example, one of the heteromonomeric heavy chain comprises the so-called "knob mutations" (T366W and optionally one of S354C or Y349C) and the other comprises the so-called "hole mutations" (T366S, L368A and Y407V and optionally Y349C or S354C) (see, e.g., Carter, P. et al., Immunotechnol. 2 (1996) 73) according to EU index numbering.

For recombinant production of an antibody, nucleic acids encoding the antibody, e.g., as described above, are isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acids may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody) or produced by recombinant methods or obtained by chemical synthesis.

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., US 5,648,237, US 5,789,199, and US 5,840,523. (See also Charlton, K.A., In: Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2003), pp. 245-254, describing expression of antibody fragments in E. coli.) After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized",

resulting in the production of an antibody with a partially or fully human glycosylation pattern. See Gerngross, T.U., Nat. Biotech. 22 (2004) 1409-1414; and Li, H. et al., Nat. Biotech. 24 (2006) 210-215.

Suitable host cells for the expression of (glycosylated) antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of Spodoptera frugiperda cells.

Plant cell cultures can also be utilized as hosts. See, e.g., US 5,959,177, US 6,040,498, US 6,420,548, US 7,125,978, and US 6,417,429 (describing PLANTIBODIESTM technology for producing antibodies in transgenic plants).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293T cells as described, e.g., in Graham, F.L. et al., J. Gen Virol. 36 (1977) 59-74), baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, J.P., Biol. Reprod. 23 (1980) 243-252); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells (as described, e.g., in Mather, J.P. et al., Annals N.Y. Acad. Sci. 383 (1982) 44-68); MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR- CHO cells (Urlaub, G. et al., Proc. Natl. Acad. Sci. USA 77 (1980) 4216-4220); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki, P. and Wu, A.M., Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2004), pp. 255-268.

The invention will now be further illustrated by the following, non-limiting working examples.

EXAMPLES

Material and Methods

FFA solutions (stock solutions)

Aqueous stock solutions were prepared in 0.02% PS20 (Croda, Edison, NJ, USA) at defined concentrations of (1) 5 mg/mL and (2) 12.5 mg/mL (lauric acid, Sigma-Aldrich/ Merck, Darmstadt, DE) or (1) 1.5 mg/mL and (2) 5 mg/mL (myristic acid, Sigma-Aldrich/ Merck, Darmstadt, DE) as previously described by Doshi et al.[6] The procedure was adapted that the FFA/PS20 stock solution was sterile filtered using 0.22 µm PVDF Steriflip filters (Merck Millipore, Darmstadt, DE) before 1:10 dilution and subsequently homogenized without a magnetic stirrer using a Heidolph Rotamax 120 orbital shaker (Schwabach, DE) at 100 rpm at 25°C for 1 hour. After 1:500 dilution, the solution was homogenized for 1 hour at 25°C before homogenization at 2-8°C overnight. 12-40 µL of stock solution were used for spiking experiments yielding final FFA concentrations of (1) 10 µg/mL and (2) 25 µg/mL (lauric acid) or (1) 3 µg/mL and (2) 10 µg/mL (myristic acid). Dilutions with stock solutions 1 yield FFA concentration below their solubility limit, whereas stock solutions 2 act as positive control confirmed by the formation of visible particles. FFA concentrations were verified for selected by LC-MS samples acc. Honemann et al.[7]

Inorganic salt solutions

Aqueous stock solutions of different salts were prepared at 1 mg/mL and used for spiking experiments to final concentrations between 250 mg/mL and 1 g/mL. NaCl, $NaAlO_2$, $NaBO_2$, B_2O_3, and $CaCl_2$ (Sigma-Aldrich/ Merck, Darmstadt, DE) were selected as their dissolution products (ions) represent typical glass leachables from Type I borosilicate glass. The pH of the $NaAlO_2$ and $NaBO_2$ stock solutions was adjusted to pH 6 using HCl and subsequently filtered using 0.22 µm PVDF Sterivex filters (Merck Millipore, Darmstadt, DE) and true elemental concentrations were determined by inductively-coupled plasma mass spectrometry (ICP-MS). Elemental concentrations were 0.048 µg/mL Aluminium and 295 µg/mL sodium and 78 µg/mL boron and 168 µg/mL, respectively. Spiking experiments with FFA were performed in duplicates.

Glass leachable's solutions

Representative mixtures of glass leachables were obtained from three different types of glass vials, e.g. Exp 33 and Exp51 (Schott AG, Müllheim, DE, and Schott North America Inc., NY, USA) in the 6 mL format, by three autoclavation cycles (121°C, 20 min) representing accelerate aging conditions. Vials were filled with 6 mL of either water for injection (WFI), 20 mM glycine solution pH 10 or a typical placebo solution used for protein formulations consisting of 20 mM Histidine/Histdine-HCl buffer pH 6.0, 10 mM Methionine, 240 mM sucrose (Ferro Pfanstiehl, Waukegan, IL, USA), and 0.02% PS20. The pH of the glycine solutions was adapted after autoclavation with HCl to pH 6.0 and filtered through a 0.22 µm PVDF Sterivex filters (Merck Millipore, Darmstadt, DE) Leachable concentrations were verified ICP-MS (Table 1) as described by Ditter et al.[8] Spiking experiments with FFA were performed in triplicates.

Table 1: Concentration of selected glass leachables in spiking solutions.

Vial type	Matrix	Elemental concentration (µg/mL)					
		Al	B	Si	Na	Ca	K
Exp33	3xTS WFI	<LOQ	1	0.31	1.10	<LOQ	0.05
	3xTS Placebo	<LOQ	0.25	<LOQ	0.78	<LOQ	<LOQ
	3xTS Glycine pH 6	0.56	4.7	33	361	<LOQ	1.1
Exp51	3xTS WFI	0.08	0.12	1.4	0.80	0.06	<LOQ
	3xTS Placebo	0.03	<LOQ	0.5	2.70	0.09	<LOQ
	3xTS Glycine pH 6	0.02	1.9	19	397	0.28	0.71
LOQ	3xTS WFI	*0.05*	*0.05*	*0.05*	*0.1*	*0.05*	*0.05*
	3xTS Placebo	*0.01*	*0.2*	*0.5*	*0.3*	*0.05*	*0.2*
	3xTS Glycine pH 6	*0.01*	*0.1*	*0.5*	*0.5*	*0.1*	*0.1*

mAB formulations

mAb1 (IgG_1, Mw=145.5 kDa, pertuzumab) and mAb2 (IgG_1, Mw=148 kDa, trastuzumab) were obtained from F. Hoffmann-La Roche and formulated with 1000 U/mL hyaluronidase in 20mM HisHCl buffer pH 5.5, 105 mM Trehalose, 100 mM Sucrose, 10 mM Methionine, and 0.04% Polysorbate 20. The corresponding placebo was the same formulation without proteins. Formulations were stored at 5°C and 25°C for 24 months. Spiking experiments including respective controls with placebo were performed in triplicates using either inorganic salt solutions

($CaCl_2$ and $NaAlO_2$ in 10+1 dilution) or glass leachable's from stock solutions in 10+1 or 100+1 dilution.

Analytical characterization

Samples were analyzed by visual inspection on a Seidenader V 90-T instrument (Seidenader Maschinenbau GmbH, Markt Schwaben, DE) as previously described by Ditter et al.[9] and using a black/white panel according Ph. Eur. 2.9.20.[10] and classified as Many particles (>7), Few particles (4-7), or Practically free of particles (0 – 4) in E/P box, and Many particles (>10), Few particles (6-10), Essentially free of particles (1-5), or Free of particles (0) by Seidenader.

Sub-visible particles (SVP) were determined by light obscuration according Ph. Eur. 2.9.19.[11] using a HIAC/ROYCO 9703 Liquid Syringe Sampler 3000A with a HRLD-150 sensor (Skan AG, Allschwil, CH) as previously described by Ditter et al.[9]

Turbidity was determined as outlined in Ph. Eur. 2.2.1.[12] using a Hach 2100AN turbidimeter (Hach Company, Loveland, Co) in the ratio mode.

Particles >20 µm were further identified by Fourier transformed infrared spectroscopy (FTTIR) using a Nicolet™ iNTM10 Infrared Microscope (Thermo Fisher Scientific) by comparison to reference spectra. Samples were filtrated under laminar air flow through gold-coated polycarbonate filters (pore size 0.8 µm, diameter 13 mm, Sterlitech). Filter conditioning included few droplets of ethanol followed by 1 mL of particle-free water. After filtration of the samples, ~1 mL of cooled particle-free water was used as a final washing step before analysis.

Chemical compositions of selected particles was verified by Scanning electron microscopy associated with Energy Dispersive X-ray spectroscopy (SEM-EDX) using a Phenom XL instrument from LOT Quantum Design GmbH.

pH of all solutions was verified. Samples were visually inspected immediately after spiking and regularly for up to 7 days. Further characterization by HIAC, turbidity, FTIR, and SEM-EDX was performed on day1 only. All samples were analyzed when equilibrated to room temperature (4h).

Polysorbate content for the mAb samples was determined by mixed mode HPLC using evaporative light scattering detection.

Example 1 Artificial glass leachables (salts) lead to FFA particle formation

Different inorganic salt solutions, i.e. $CaCl_2$, $NaAlO_2$, $NaBO_2$, B_2O_3, and NaCl, were prepared simulating artificial glass leachables. Myristic and lauric acid, as main degradation product from hydrolytic PS20 degradation, were added at concentrations below their solubility limit and samples were analyzed for visible particles, SVP, and turbidity. Samples were compared against relevant controls and the pH verified at pH 6.

Immediate formation of visible particles after spiking was observed for both myristic and lauric acid with $NaAlO_2$ depending on the salt concentration. Immediate particle formation was in particular seen directly after spiking with myristic acid with $CaCl_2$ for all salt concentrations tested. Increasing particle formation correlated in general with increasing incubation time for both fatty acid solutions as well as for with increasing $NaAlO_2$ and $CaCl_2$ concentrations, respectively. Particles were even visible in E/P box in particular for myristic acid in presence of Calcium as summarized in Table 1 depending on the time point of inspection. Particles were subsequently identified by FTIR as FFA particles (data not shown). For $NaBO_2$ and B_2O_3 solutions, visible particles were obtained in Seidenader for both myristic and lauric acid dependent on the salt concentration over time, but to a much lesser extent compared to $CaCl_2$ and $NaAlO_2$. No particle formation was observed when adding sodium chloride up to a salt concentration of 1 mg/mL.

The spiking experiments demonstrate feasibility of FFA particle formation in the visible range in presence of salts simulating relevant glass leachables from borosilicate glass typically used as primary packaging for parenteral products. Particle formation was found to be highly dependent on type and concentration of the ion/ salt, like Ca^{2+} or Al^{3+}, as well as on incubation time. Besides adequate controls, the time point of inspection and equilibration of the samples/ temperature of the solution are crucial for these experiments: FFA solubility is highly

dependent on temperature and more particles are detected at lower temperatures, e.g., equilibration time to room temperature of 1 versus 4 h. The solubility limit of FFA is also heavily dependent on pH. Thus, experiments were performed at pH 6. However, $NaAlO_2$ and $NaBO_2$ initially form hydroxides in solution (~pH 10). Thus, the pH of the spiking solutions need to be adjusted and the remaining ion concentrations (after filtration) subsequently checked by ICP-MS. The exact ion concentrations present in the spiking solutions are outlined in the method section. In particular for Aluminium, concentrations were used in relevant leachable concentration comparable to real-time data obtained from Expansion 51 vials for a relevant placebo formulation as summarized in Fig 4.

Table 2: Visible particles after spiking of (A) myristic acid and (B) lauric acid below their solubility limit to different anorganic salt solutions ($CaCl_2$ and $NaAlO_2$) at different salt concentrations. Data from duplicates are presented, which were verified against the negative controls (without salt) and positive controls (FFA above solubility limit). Particles were classified as Many particles (>7, xxx), Few particles (4-7, xx), or Practically free of particles (0 – 4, /) in E/P box, and Many particles (>10, xxx), Few particles (6-10, xx), Essentially free of particles (1-5, x), or Free of particles (0, /) by Seidenader. d = day of inspection. d0 = directly after spiking. * Nominal salt concentration before pH adjustment and filtration.

(A) Myristic acid

$CaCl_2$ spiking solution												
Salt conc. (µg/mL)	Ca^{2+} conc. (µg/mL)	Sample	E/P	d0	d1	d2	d7	Seidenader	d0	d1	d2	d7
1000	360	1		/	xxx	xxx	xxx		xxx	xxx	xxx	xxx
		2		/	xxx	xxx	xxx		xxx	xxx	xxx	xxx
500	180	1		/	xx	xx	xxx		xxx	xxx	xxx	xxx
		2		/	xx	/	xxx		xxx	xxx	xxx	xxx
250	90	1		/	/	/	xxx		xxx	xxx	xxx	xxx
		2		/	/	/	xxx		xxx	xxx	xxx	xxx
$NaAlO_2$ spiking solution												
Salt conc.* (µg/mL)	Al^{3+} conc. (µg/mL)	Sample	E/P	d0	d1	d2	d7	Seidenader	d0	d1	d2	d7
1000	0.048	1		/	/	xx	xxx		x	xxx	xxx	xxx
		2		/	/	/	xxx		x	xx	x	xxx
500	0.024	1		/	/	/	xx		x	xx	x	xxx
		2		/	/	/	/		/	x	x	xxx
250	0.012	1		/	/	/	/		x	x	x	xxx
		2		/	/	/	/		/	x	x	xxx

(B) Lauric acid

$CaCl_2$ spiking solution												
Salt conc. (µg/mL)	Ca^{2+} conc. (µg/mL)	Sample		d0	d1	d2	d7		d0	d1	d2	d7
1000	360	1	F/P	/	xxx	xx	/	Seidenader	x	xxx	xxx	xxx
		2		/	xx	xx	/		/	xx	xxx	xx
500	180	1		/	xxx	/	/		/	xxx	xxx	xxx
		2		/	/	/	/		/	xx	xxx	xxx
250	90	1		/	xxx	/	/		/	xxx	xxx	xxx
		2		/	/	/	/		/	xx	xxx	xxx
$NaAlO_2$ spiking solution												
Salt conc.* (µg/mL)	Al^{3+} conc. (µg/mL)	Sample		d0	d1	d2	d7		d0	d1	d2	d7
1000	0.048	1	F/P	/	/	/	/	Seidenader	x	x	x	xxx
		2		/	/	/	/		/	x	/	xxx
500	0.024	1		/	/	/	/		x	x	x	x
		2		/	/	/	/		x	x	/	/
250	0.012	1		/	/	/	/		/	x	x	x
		2		/	/	/	/		/	x	x	/

Example 2: 'Real' glass leachables (mixtures) lead to FFA particle formation

Glass leachables were generated from different types of glass vials, e.g. Exp33 and Exp51 vials, with different matrix solutions including WFI, a glycine solution adjusted to pH 6, and a placebo solution representative for a mAb formulation. Concentrations of glass leachables are provided in Table 1. Defined amounts of myristic and lauric acid below their solubility limit were added to the solutions/ mixtures of glass leachables and analyzed. Visible particles in Seidenader are summarized in Table 3 and were detected for all samples in contrast to various controls. Particle formation was dependent on the glass leachable solution and dependent on incubation time. No clear trends were determined for SVP and turbidity at the time points of inspection (d1), however the data suggest an increase in SVP if no visible particles have formed yet. An example for the dependency of particle formation on incubation time is provided in Table 3 for myristic acid in glass leachable solution from Exp51 vials/glycine solution. The example highlights the kinetics of particle formation with no particles directly after spiking and more than 10 particles at incubation day 5 and 7. For selected samples, particles were further characterized and identified by FTIR confirming the presence of free fatty

acids. FFA were not confirmed by FTIR for glass leachable solutions generated with WFI, which is linked to the time point of FTIR analysis at d1 and the late onset of particle formation for these samples. Placebo samples were not analyzed further by FTIR as the positive control was found negative attributed to the presence of additional 0.02% PS20, potentially solubilizing the FFA seeds. Characterization of the particles by SEM-EDX confirmed the presence of glass leachables, like Aluminium or silicon on the surface of the FFA particles. Figure 1 shows a typical picture of a gold filter after FTIR analysis highlighting a few FFA particles of different size and a representative FFA spectrum. The spiking study highlights that mixtures of 'real' glass leachables lead to precipitation of FFA and particle formation in the visible range dependent on mixture and amount of glass leachables as well as dependent on incubation time.

Table 3: Visible particles (Seidenader) and particle identification of selected samples by FTIR and SEM-EDX (d1). Visible particles are reported after spiking of lauric and myristic acid below their solubility limit to different glass leachable containing solutions generated by 3 autoclavation cycles in different glass vials. Results from triplicates are reported in relative ranking to each other from less to most particle fromation (+, ++, +++) over incubation time of up to 7 days. The dependency of particle formation on incubation time is exemplarily shown for myristic acid in Exp51/glycine matrix. n.t. =not tested

Vial type	Matrix	FFA	Visible particles	FTIR	Glass leachables in SEM-EDX
Exp33 vials	3xTS WFI	Myristic acid	+	n.t.	n.t.
		Lauric acid	+	FFA	+
	3xTS Placebo	Myristic acid	+	n.t.	n.t.
		Lauric acid	+++	n.t.	n.t.
	3xTS Glycine pH 6	Myristic acid	++	FFA	+
		Lauric acid	+	FFA	+
Exp51 vials	3xTS WFI	Myristic acid	+	n.t.	n.t.
		Lauric acid	++	n.t.	n.t.
	3xTS Placebo	Myristic acid	+	n.t.	n.t.
		Lauric acid	+++	n.t.	n.t.
	3xTS Glycine pH 6	Myristic acid	+++	FFA	+

		Lauric acid	++	FFA	+
Example for dependency on incubation time:				Visible particles	
		Sample no.	d0	d1	d5/7
Exp51 vials	3xTS Glycine pH 6, Myristic acid	1	-	-	>10 particles
		2	1-5 particles	1-5 particles	>10 particles
		3	-	1-5 particles	>10 particles

Example 3: Verification of FFA particle formation in presence of glass leachables in aged matrix (case study)

Precipitation of FFA particle in a protein matrix was further studied in aged mAb1 and mAb2 solutions (22M, 5°C) by addition of different concentrations of 'real' glass leachables. In this experiment, the presence of FFA were a result of PS20 degradation forming over shelf-life of the drug product. Mab1 and mAb2 were formulated in the same matrix but differ in the type of mAb (CDRs). Originating from different drug substance processes and purification processes, the PS20 degradation rates were found different (Figure 5) as well as the type and concentrations of FFAs as a subsequent result (Figure 6). Interestingly, mAb2 showed visible particles characterized as FFA and Aluminium after 12M storage at 25°C, whereas mAb1 did not. After 12M storage at 25°C *plus* 10M storage at 5°C, both formulations showed visible particles identified as a complex of FFA and different glass leachables.

Products were characterized as free of visible particles before the experiment (stored for 22M at 5°C). For both formulations, visible particle formation in Seideander inspection machine was observed after incubation with already 50 or 500 µL of different mixtures of glass leachables (Table 4). Results were compared to various controls like the initial time point and in comparison to a spiked placebo solution, which remained free of visible particles. Selected particles were further identified as FFA particles (FTIR) in combination with a mixture of inorganic ions by high resolution SEM-EDX. Figure 2 shows a representative SEM picture of a FFA particle highlighting the presence of Aluminium and Magnesium. The chemical composition is summarized indicating the presence of a variety of glass

leachables. This suggests the precipitation of FFA in presence of the spiked glass leachables acting as nucleation factors. Based on these findings, but without being bound to theory, a potential mechanism of particle formation is illustrated in Figure 3. FFAs exist in equilibrium of their protonated and deprotonated forms at relevant pH values for biopharmaceuticals. Taking the example of aluminum, triple charged aluminum ions may react with a deprotonated FFA and form highly insoluble aluminum-fatty acid-tri-carboxylates, which would act as nucleating seed. The hydrophobic chain of FFAs may further interact by hydrophobic interaction, fostering seed growing. As displayed in Figure 3, the proposed mechanism is shown for myristic acid in the presence of aluminum. Finally, particles may precipitate due to increasing hydrophobicity.

Table 4: Visible particles (Seidenader). Aged mAb1 and mAb2 formulation (22M, 5°C) in comparison to placebo after spiking of different mixtures and amounts of glass leachables. *d= day of inspection*

Product	Glass leachable's solution			Visible particles			
	Type	Added volume		initial	after spiking/ shaking	d1 1h	d1 4h
		500 µL	50 µL				
Placebo	Glycin 3TS Exp33	x	-	-	-	-	-
	Glycin 3TS Exp33	-	x	-	-	-	-
	WFI 3TS Exp33	x	-	-	-	-	-
	WFI 3TS Exp33	-	x	-	-	-	-
	Glycin 3TS Exp51	x	-	-	-	-	-
	Glycin 3TS Exp51	-	x	-	-	-	-
	$CaCl_2$ 1 mg/mL	x	-	-	-	-	-
	$NaAlO_2$ pH 6	x	-	-	-	-	-
mAb1	Glycin 3TS Exp33	x	-	-	+	+	+
	Glycin 3TS Exp33	-	x	-	+	-	+
	WFI 3TS Exp33	x	-	-	+	+	+
	WFI 3TS Exp33	-	x	-	-	+	-
	Glycin 3TS Exp51	x	-	-	+	+	+
	Glycin 3TS Exp51	-	x	-	+	+	+
	$CaCl_2$ 1 mg/mL	x	-	-	+	+	+
	$NaAlO_2$ pH 6	x	-	-	+	+	+
mAb2	Glycin 3TS Exp33	x	-	-	-	+	-
	Glycin 3TS Exp33	-	x	-	+	+	+
	WFI 3TS Exp33	x	-	-	+	+	+
	WFI 3TS Exp33	-	x	-	+	+	+
	Glycin 3TS Exp51	x	-	-	+	+	+
	Glycin 3TS Exp51	-	x	-	+	+	+
	$CaCl_2$ 1 mg/mL	x	-	-	+	+	+
	$NaAlO_2$ pH 6	x	-	-	+	+	+

PGR2026-00039
Biocon Ex.1002
Page 4765 of 4945

Example 4: Influence of washing and sterilization procedures on nucleation of particles as a result of polysorbate degradation

Preparation of glass vials

Expansion 51 glass vials (Fiolax®) in the 20cc configuration were purchased from Schott North America Inc. (NY, USA). The vials comply with type I glass as per European Pharmacopoeia (Ph. Eur.). After washing and depyrogenation as described below, vials were filled with 12.2 mL of a placebo buffer (20 mM His/His-HCl, 240 mM sucrose, 10 mM methionine, pH 5.5) containing (1.) no further excipients (negative control, NC), (2.) 0.4 mg/mL polysorbate 20 (PS20) or (3.) 0.4 mg/mL PS20 with additionally spiked $AlCl_3$ to a final Al^{3+} concentration of ~250 ppb (positive control, PC). Beforehand, the 0.4 mg/mL PS20 was degraded by ~10% with C. Antarctica-coupled beads as described previously[13] . Vials were stored upright at 5°C, 25°C / 60% RH, and 40°C / 70% RH.

Washing and depyrogenation

Vials were washed with water for injection (WFI) at 70°C (water and air pressure 1 bar, final air pressure 2.5 bar) using a FAW1020 vial washing machine (Bausch & Stroebel, Germany). Subsequently, vials were placed in stainless steel boxes and dried for 96 h under laminar air flow.

Vials were further processed according to either best or worst case sterilization conditions as specified in Table 5. Depyrogenation of all vials was conducted in a DHT2550 sterilization tunnel (Bausch & Stroebel, Germany). The process conditions differ in presence of residual moisture, heating zone temperature, sterilization temperature, residence time in the tunnel and conveyer belt speed. Vials processed according to the worst case conditions were filled with 281 µL of WFI prior to depyrogenation to simulate the presence of residual moisture. In addition, the conveyor belt was stopped after the vials had entered the sterilization zone for prolonged residence time in the tunnel. The time in the heating, sterilization, and cooling zone was calculated to be 41 min, 43.5 min, and 50 min, respectively, for the best case conditions (134.5 min total), compared to 3 min, 16 h, and 3.5 min, respectively, for the worst case conditions (966.5 min total).

PGR2026-00039
Biocon Ex.1002
Page 4766 of 4945

Table 5. Depyrogenation process parameters

Conditions reffered to as:	Residual moisture (µL)	Temperature of heating zone (°C)	Conveyor speed (entry) (mm/min)	Sterilization Temperature (°C)	Residence time in tunnel (h)	Conveyor speed (out) (mm/min)
Best case	-	60	20 (min. 5)	180	-	20 (min. 5)
Worst case	281*	45	280 (max. 300)	350	16 ± 1	280 (max. 300)

* volume normalized to surface area of 2cc vials with 80 µL residual moisture as default. Normalized volume = surface area * F; with F = 80 µL / surface area of 2cc vial

Visual inspection

Particles were identified by visual inspection using both a black/white panel according to Ph. Eur. 2.9.20 and a Seidenader V 90-T instrument (Seidenader Maschinenbau GmbH, Markt Schwaben, Germany). The latter is referred to as enhanced visual inspection in this study. For enhanced visual inspection, samples were illuminated from behind as well as from the bottom and the top. Containers were rotated and inspected through 2-fold magnifying glass. For both instruments, samples were inspected after allowing the containers to equilibrate to room temperature for 3 h. The number of particles as mean of 5 vials is reported.

Results:

Particle formation was observed for worst case sterilized samples after storage at 25°C and 40°C starting from 2 months on when analyzed by enhanced visual inspection (Table 6A). For best case samples, particle formation starts at 40°C after 3 months, but to a smaller extent. Given that particle formation is a stochastic event, trends from analysis by E/P box (Table 6B) follow the results from enhanced visual inspection, which is more sensitive. Particle formation started for the 40°C worst case sterilized vials after 3 months. In general, no particle formation was observed at 5°C storage up to 3 months for none of the visual inspection methods and samples tested. Negative controls have been confirmed for absence of particles and positive controls have been confirmed for presence of particles using both visual inspection methods, for all temperatures, and time points of analysis.

It can thus be concluded that the washing and sterilization procedures has a major influence on nucleation of particles as a result of polysorbate degradation.

Table 6: Summary of visual inspection results.

(A) Enhanced visual inspection (Seidenader)						
Sterilization process	Storage temperature	Sample	Initial	1M	2M	3M
Best case	5°C	NC	0	0	0	0
		PC	>10	>10	>10	>10
		sample	0	0	0	0
	25°C	NC	0	0	0	0
		PC	>10	>10	>10	>10
		sample	0	2	0	0
	40°C	NC	0	0	0	0
		PC	>10	>10	>10	>10
		sample	0	1	0	6
Worst Case	5°C	NC	0	0	0	0
		PC	>10	>10	>10	>10
		sample	0	0	0	2
	25°C	NC	0	0	0	0
		PC	>10	>10	>10	>10
		sample	0	2	8	6
	40°C	NC	0	0	0	0
		PC	>10	>10	>10	>10
		sample	0	4	>10	>10

(B) Visual inspection (Ph. Eur.)						
Sterilization process	Storage temperature	Sample	Initial	1M	2M	3M
Best case	5°C	NC	0	0	0	0
		PC	2	>7	>7	>7
		sample	0	0	0	0
	25°C	NC	0	0	0	0
		PC	3	>7	>7	>7
		sample	0	0	0	1
	40°C	NC	0	0	0	0
		PC	>7	>7	>7	>7
		sample	0	0	0	>7
Worst Case	5°C	NC	0	0	0	0
		PC	0	>7	>7	>7
		sample	0	0	0	1
	25°C	NC	0	0	0	0
		PC	2	>7	>7	>7
		sample	0	0	1	>7
	40°C	NC	0	0	0	0
		PC	>7	>7	>7	>7
		sample	0	0	>7	>7

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CLAIMS

1. A stable aqueous composition comprising a protein together with pharmaceutically acceptable excipients such as, for example, buffers, stabilizers including antioxidants, and surfactants, wherein said composition further contains a mixture of one or several types of inorganic ions diffused out of the packaging material, such as a glass vial, and substances resulting from the degradation of said surfactants without forming visible particles.

2. A composition according to claim 2, wherein said inorganic ions are selected from Aluminium, Boron, Silicon, Calcium, Magnesium, Potassium, and Sodium.

3. A composition according to any one of claims 1 or 2, wherein the pH of said composition is in the range of 5 to 7, preferably around 6.

4. A composition according to any one of claims 1 to 3, wherein the protein is an antibody, preferably a monoclonal antibody.

5. A composition according to any one of claims 1 to 4, comprising a concentration of up to 0.03 µg/ml aluminium, and/or up to 0.05 µg/ml boron, and/or up to 0.5 µg/ml silicon.

6. A composition according to any one of claims 1 to 5, wherein the stabilizer is selected from the group consisting of sugars, sugar alcohols, sugar derivatives, or amino acids.

7. A composition according to any one of claims 1 to 6, wherein the buffer is selected from the group consisting of acetate, succinate, citrate, arginine, histidine, phosphate, Tris, glycine, aspartate, and glutamate buffer systems.

8. A composition according to any one of claims 1 to 7, wherein the surfactant is selected from the group consisting of non-ionic surfactants, preferably polysorbates.

9. A composition according to any one of claim 1 to 8, wherein the substances resulting from the degradation of said surfactants are free fatty acids, below their solubility level in water at room temperature.

10. A composition according to any one of claims 1 to 9, wherein the pharmaceutically acceptable excipients are: 1000 U/mL hyaluronidase in 20mM HisHCl buffer pH 5.5, 105 mM Trehalose, 100 mM Sucrose, 10 mM Methionine, and 0.04% Polysorbate 20.

11. A method for obtaining a composition according to any one of claims 1 to 10, wherein said method comprises selecting a primary packaging material which prevents leaching of one or several of the inorganic ions as defined in claim 1 into said composition.

12. The method according to claim 11, wherein said primary packaging material is a glass or a polymer vial.

13. The method according to claim 11 or 12, further comprising the steps of a) washing and/or b) depyrogenation of the primary packaging material prior to its use.

14. The method according to any one of claims 11 to 13, wherein said method provides stability of said composition against the formation of visible particles.

15. A pharmaceutical dosage form comprising a composition according to any one of claims 1 to 10 in a container, wherein the concentration of one or several inorganic ions selected from Aluminium, Boron, Silicon, Calcium, Magnesium, Potassium, and Sodium in that composition remains substantially constant during the time of its authorized shelf life.

***

Fig. 1

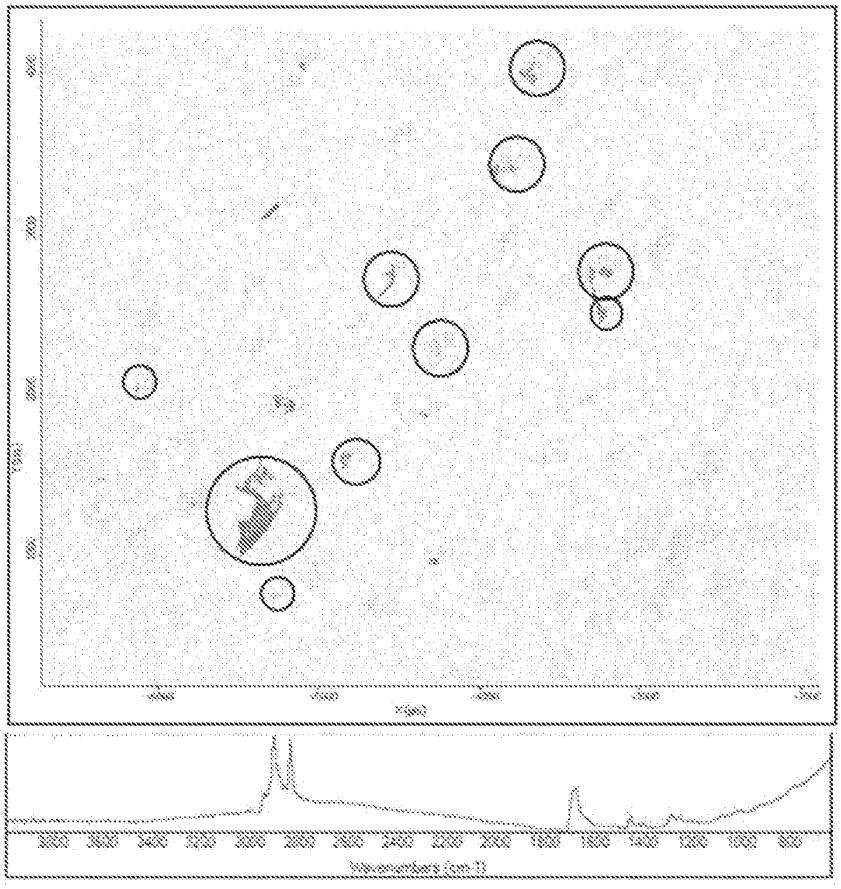
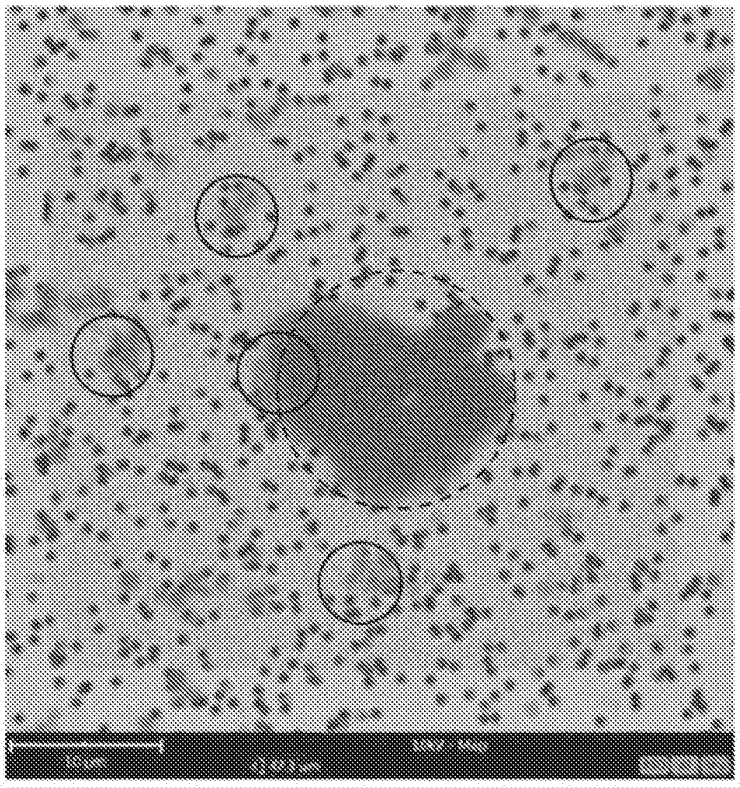


Fig. 2



Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
79	Au	Gold	33.33	89.37
6	C	Carbon	53.16	8.07
7	N	Nitrogen	8.36	1.15
8	O	Oxygen	4.05	0.83
14	Si	Silicon	0.91	0.33
13	Al	Aluminium	0.57	0.20
12	Mg	Magnesium	0.48	0.15
11	Na	Sodium	0.31	0.09
20	Ca	Calcium	0.02	0.01

Fig. 3

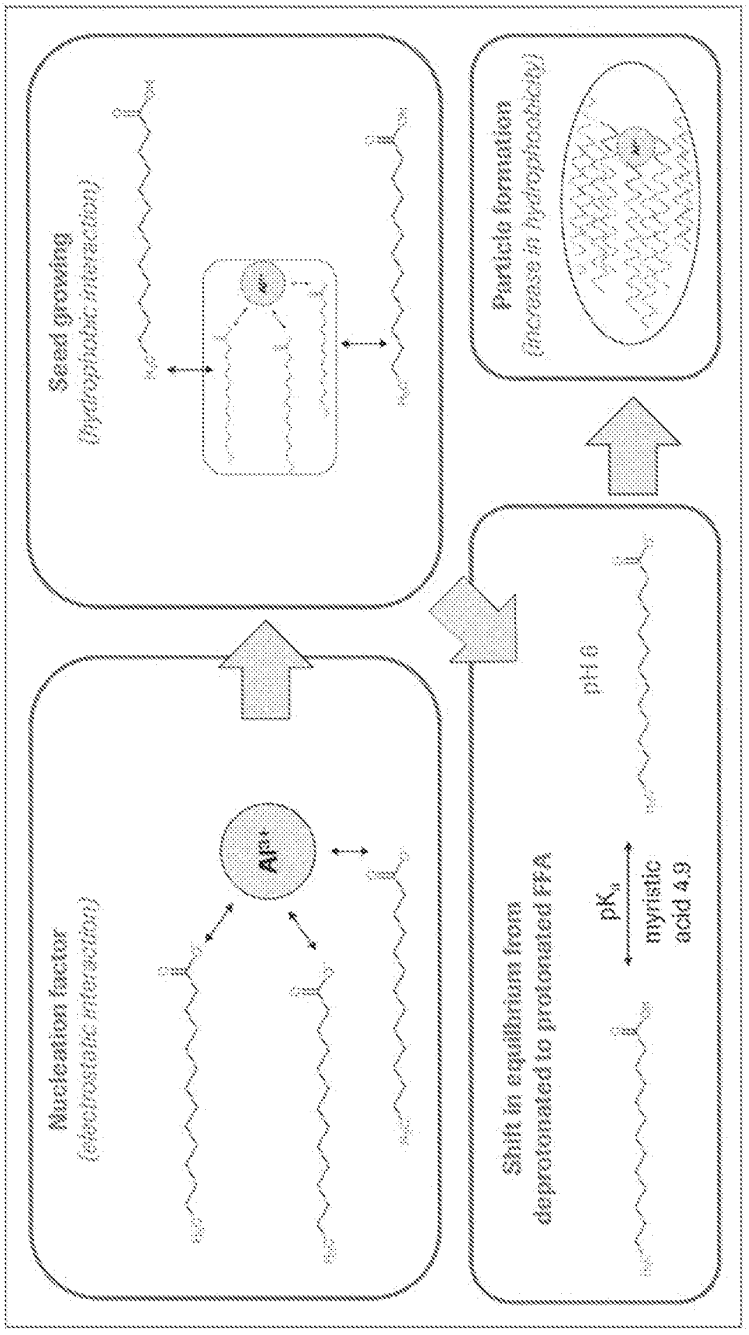

Nucleation factor
(electrostatic interaction)
Af+
Seed growing
(hydrophobic interaction)
Particle formation
(increase in hydrophobicity)
Shift in equilibrium from
deprotonated to protonated FFA
pH 6
pK$_a$
myristic
acid 4.9

Fig. 4

	Composition	Vial format	Storage time (months)	Average value from duplicates [µg/ml]							Standard deviation (* single measurements)						
				B	Mg	Al	Si	K	Ca	B	Mg	Al	Si	K	Ca	
Placebo 1	10 mM NaPO4 pH 6, 40 mM NaSO4, 10 mM Met, 30 mg/mL Mannitol, 0.01% (w/v) Poloxamer 188	2ml	30	0.380	0.020	0.073	1.950	0.155	0.290	0.042	0.001	0.001	0.212	0.037	0.028	
		6ml	30	0.415	0.012	0.047	2.100	0.150	0.200	0.021	0.001	0.001	0.000	0.014	0.028	
		20ml	30	0.355	0.012	0.049	1.450	0.150	0.175	0.007	0.001	0.001	0.071	0.000	0.035	
		2mL	21	0.115	0.005	0.074	1.050	0.075	0.175	0.007	0.006	0.003	0.071	0.007	0.007	
		blind value	0	0.070	0.000	0.019	0.450	0.000	0.000	-	-	-	-	-	-	
Placebo 2	51 mM NaPO4 pH 6.2, 6% trehalose, 0.04% PS20	20ml	62	0.355	0.009	0.235	1.900	0.090	0.105	0.021	0.001	0.007	0.000	0.014	0.021	
		2mL	21	0.110	0.013	0.140	0.910	0.180	0.170	*	*	*	*	*	*	
		blind value	0	0.070	0.000	0.010	0.000	0.000	0.000	-	-	-	-	-	-	
Placebo 3	20 mM HisHCl pH 6, 240 mM trehalose, 0.02% PS20	20ml	30	0.080	0.007	0.031	1.050	0.000	0.060	0.014	0.001	0.008	0.071	0.000	0.000	
		20ml	40	0.035	0.001	0.024	1.100	0.090	0.035	0.007	0.000	0.001	0.000	0.014	0.007	
		20ml	30	0.055	0.007	0.034	0.900	0.000	0.060	0.007	0.001	0.001	0.000	0.000	0.014	
		6ml	18	0.055	0.010	0.019	0.850	0.000	0.080	0.007	0.001	0.003	0.071	0.000	0.014	
		2mL	21	0.060	0.008	0.028	0.450	0.050	0.090	0.014	0.003	0.006	0.000	0.000	0.000	
		blind value	0	0.030	0.000	0.000	0.000	0.000	0.000	-	-	-	-	-	-	

Fig. 5

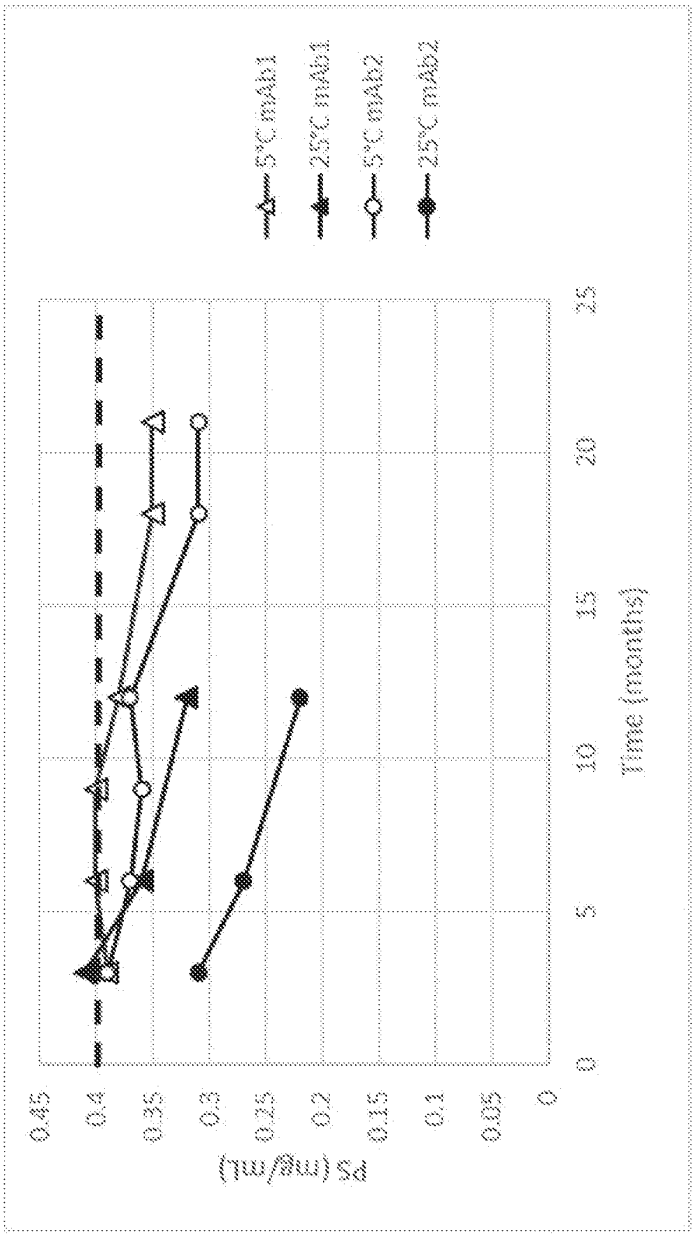

5°C mAb1
25°C mAb1
5°C mAb2
25°C mAb2
PS (mg/mL)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
Time (months)

Fig. 6(A)

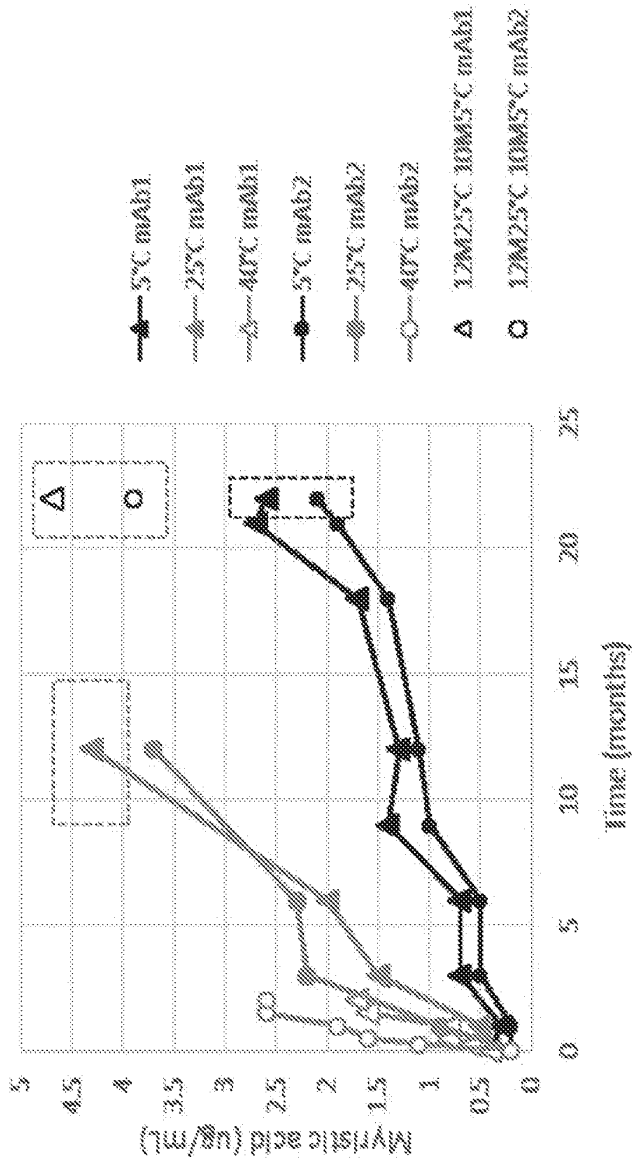
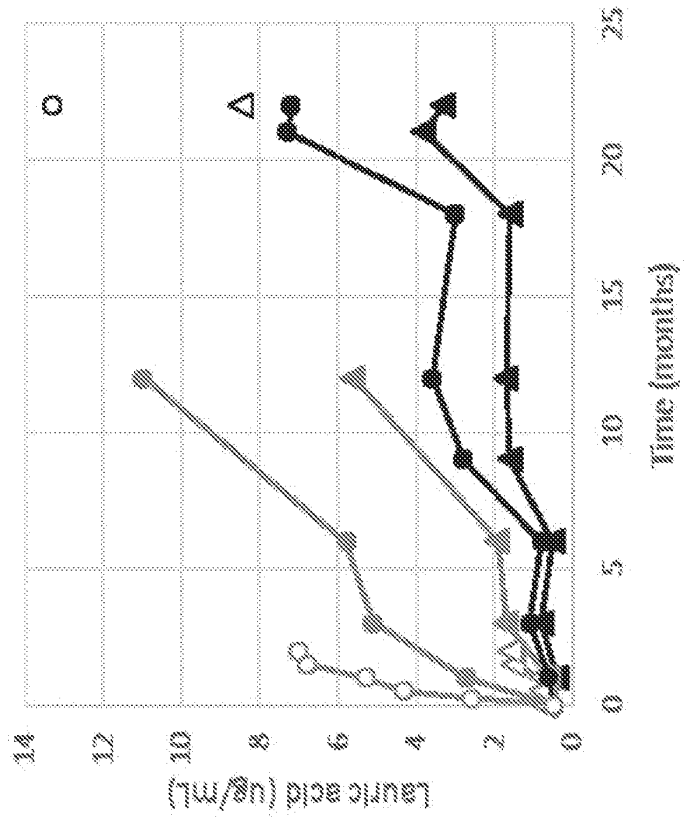

5°C mAb1
25°C mAb1
40°C mAb1
5°C mAb2
25°C mAb2
40°C mAb2
Δ 12M25°C 10M5°C mAb1
o 12M25°C 10M5°C mAb2
Myristic acid (ug/mL)
Time (months)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
5
10
15
20
25

Fig. 6(B)


5°C mAb1
25°C mAb1
40°C mAb1
5°C mAb2
25°C mAb2
40°C mAb2
12M25°C 10M5°C mAb1
12M25°C 10M5°C mAb2
Lauric acid (ug/mL)
Time (months)
0
2
4
6
8
10
12
14
0
5
10
15
20
25

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/081999

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61J1/20 A61K39/00 A61K39/395 A61K47/12 A61K47/18
A61K47/26 A61K9/08
ADD.
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)
A61K A61J C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2019/255173 A1 (FRANCIS DONNY [DE] ET AL) 22 August 2019 (2019-08-22) claims 1, 5, 12, 13, 15, 16 paragraphs [0166], [0185], [0187], [0188], [0195] tables 3, 5 -----	1-15

☐ 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

"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	Date of mailing of the international search report
4 February 2021	11/02/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vázquez Lantes, M

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/081999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2019255173 A1	22-08-2019	AU 2017293103 A1	21-02-2019
		BR 112019000135 A2	16-04-2019
		CA 3029642 A1	11-01-2018
		CN 109661240 A	19-04-2019
		EP 3481420 A1	15-05-2019
		JP 2019520390 A	18-07-2019
		KR 20190027375 A	14-03-2019
		RU 2019102943 A	05-08-2020
		SG 11201900043T A	27-02-2019
		US 2019255173 A1	22-08-2019

Electronic Acknowledgement Receipt

EFS ID:	44429811
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes/Brandi Henley
Filer Authorized By:	Lance A. Termes
Attorney Docket Number:	125984-266215
Receipt Date:	03-DEC-2021
Filing Date:	23-JUL-2021
Time Stamp:	13:48:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	10430US02_IDS-Transmittal.pdf	87215 e75f3b95445152 4e3622722e8ab28595980fdf56	no	3

Warnings:

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	10430US02_IDS-SB08.pdf	175384 17d0c394fe5e9987ead365b39cea234a3437ff23	no	18
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	397_WO2021125852_EngTrans.pdf	4773873 19d726df9231959e73795c64cfa20af6b07504db	no	20
Warnings:					
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4	Foreign Reference	399_WO2021168218A1.pdf	3198858 64fe7358c9dbf7898a6d82f878330abf5cf592c0	no	60
Warnings:					
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5	Foreign Reference	400_WO2021203769_EngTrans.pdf	1638442 b8a987299d88a9beeabcb47f5d6b682f328a8d11	no	34
Warnings:					
Information:					
6	Foreign Reference	401_WO2021216916A1.pdf	20967823 daca0475e17ff9d5a3166e457cb713d8eae6b2b6	no	380
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Information:					
7	Foreign Reference	398_WO2021146328A1.pdf	23818492 d6ab839aab5f121206f1f26ba0218897df0a14ce	no	130
Warnings:					
Information:					
8	Foreign Reference	396_WO2021094508A1.pdf	7576959 4fcefb88858f3a97726eca97b11795cc54f7c192	no	53
Warnings:					
Information:					

Total Files Size (in bytes):	62237046

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PATENT
Attorney Docket No.: 125984-266215 / 10430US02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth S. Graham, et al. Application No.: 17/384,070 Filed: Jul-23-2021 For: HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS Customer No.: 147803	Confirmation No.: 1061 Examiner: Not Yet Assigned Art Unit: Not Yet Assigned INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR §1.97 and §1.98 Date of Transmission: December 3, 2021

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The references cited on the attached IDS are being called to the attention of the Examiner. Copies of cite Nos. 396-401 are submitted herewith in compliance with the requirements of 37 CFR §1.98(a)(2). Copies of all other citations required to be submitted pursuant to 37 CFR §1.98(a)(2) were previously submitted to, or cited by, the Office in U.S. Application No. 16/409,631, filed May 10, 2019, which is relied on for an earlier effective filing date under 35 USC §120. Pursuant to 37 CFR §1.98(d)(1) and 37 CFR §1.98(d)(2), copies of these previously cited references are not being submitted.

It is respectfully requested that the cited references be expressly considered during the prosecution of this application, and the references be made of record therein and appear among the "references cited" on any patent to issue therefrom.

The cited references may include office actions from related cases. The Examiner is encouraged to review the full prosecution history from related cases. Applicant understands that the prosecution history can be viewed electronically on PALM. Nevertheless, if the Examiner wishes Applicant to provide copies of any documents from related applications to the extent not already provided, he or she is requested to contact the undersigned.

Applicant cites the following applications, which are directed to related subject matter and are commonly owned or under an assignment obligation to be commonly owned:

Attorney Docket No.	Application No.	Filing Date	Publication No.	Status
125984-248591	62/669,506	05-10-2018	N/A	Expired
125984-248592	62/752,127	10-29-2018	N/A	Expired
125984-248593	62/769,876	11-20-2018	N/A	Expired
125984-248594	62/813,882	03-05-2019	N/A	Expired
125984-248595	16/409,631	05-10-2019	US 11,103,552	Issued
125984-248596	PCT/US2019/031879	05-10-2019	WO 2019/217927	Published

As provided for by 37 CFR §1.97(g) and (h), no inference should be made that the information and references cited are prior art merely because they are in this statement and no representation is being made that a search has been conducted or that this statement encompasses all the possible relevant information.

Applicant believes that no fee is required for submission of this statement. However, if a fee is required in connection with this paper, the Commissioner is authorized to deduct such fee from the undersigned's Deposit Account No. 500393.

Respectfully submitted,

/Lance A. Termes/

Lance A. Termes
Reg. No. 43184

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1211 SW 5th Avenue, Suite 1900
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/384,070	07/23/2021	Kenneth S. Graham	125984-266215

CONFIRMATION NO. 1061

147803
Schwabe W&W, PC / Regeneron Pharmaceuticals, Inc.
PacWest Center
1211 SW Fifth Avenue, Suite 1900
Portland, OR 97204

PUBLICATION NOTICE

OC000000129891375

Title: High Concentration VEGF Receptor Fusion Protein Containing Formulations

Publication No.US-2021-0353714-A1
Publication Date:11/18/2021

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 17/384,070

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	700
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	18 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 480 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1820

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
17/384,070	07/23/2021		1820	125984-266215	18	1

CONFIRMATION NO. 1061

FILING RECEIPT

147803
Schwabe W&W, PC / Regeneron Pharmaceuticals, Inc.
PacWest Center
1211 SW Fifth Avenue, Suite 1900
Portland, OR 97204

OC000000127352990

Date Mailed: 08/10/2021

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

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Inventor(s)

Kenneth S. Graham, Pleasant Valley, NY;
Saurabh WADHWA, Nanuet, NY;

Applicant(s)

Regeneron Pharmaceuticals, Inc., Tarrytown, NY;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 16/409,631 05/10/2019
which claims benefit of 62/669,506 05/10/2018
and claims benefit of 62/752,127 10/29/2018
and claims benefit of 62/769,876 11/20/2018
and claims benefit of 62/813,882 03/05/2019

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

PGR2026-00039
Biocon Ex.1002
Page 4790 of 4945

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/384,070**
Projected Publication Date: 11/18/2021
Non-Publication Request: No
Early Publication Request: No
Title

High Concentration VEGF Receptor Fusion Protein Containing Formulations

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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PGR2026-00039
Biocon Ex.1002
Page 4791 of 4945

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I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on July 23, 2021.

SCHWABE, WILLIAMSON & WYATT PC

By: /Lance A. Termes/
Lance A. Termes

PATENT
Attorney Docket No.: 125984-266215

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. Unassigned
Kenneth S. Graham et al.	Examiner: Unassigned
Application No.: Unassigned	Art Unit: Unassigned
Filed: Herewith	
For: HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS	PRELIMINARY AMENDMENT
Customer No.: 147803	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Prior to examination of the above-identified application, please enter the following amendments and remarks:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 5 of this paper.

PGR2026-00039
Biocon Ex.1002
Page 4793 of 4945

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-29. (Canceled)

30. (Currently Amended) A method for treating an angiogenic eye disorder in a human patient~~subject~~ in need thereof comprising administering an intravitreal~~intraocular~~ injection of at least~~more than~~ about 8[[2]] mg of a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobulin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of a VEGFR2, and a multimerizing component, to the eye of the patient~~subject~~.

31. (Currently Amended) The method of claim 30 wherein, following said intravitreal injection, the patient maintains or achieves a reduction in central retinal thickness but does not experience a significant increase in blood pressure~~comprising administering about 4 mg, about 6 mg, about 8 mg or about 10 mg of the VEGF receptor fusion protein to the eye of the subject~~.

32. (Currently Amended) The[[A]] method of claim 30 wherein, following said intravitreal injection, the patient maintains or achieves an improvement in best corrected visual acuity but does not experience a significant increase in blood pressure~~for treating an angiogenic eye disorder in a subject in need thereof, the method comprising intraocularly injecting more than about 2 mg of VEGF receptor fusion protein in a formulation of any one of claims 1-23 and 28 into an eye of the subject~~.

33-34. (Canceled)

35. (Currently Amended) The method of claim 30~~any one of claims 30-34~~, wherein the angiogenic eye disorder is age-related macular degeneration (wet), macular edema, macular edema following retinal vein occlusion, retinal vein occlusion (RVO), central retinal vein occlusion (CRVO) branch retinal vein occlusion (BRVO), diabetic macular edema (DME),

PGR2026-00039
Biocon Ex.1002
Page 4794 of 4945

choroidal neovascularization (CNV), iris neovascularization, neovascular glaucoma, post-surgical fibrosis in glaucoma, proliferative vitreoretinopathy (PVR), optic disc neovascularization, corneal neovascularization, retinal neovascularization, vitreal neovascularization, pannus, pterygium, vascular retinopathy, diabetic retinopathy, non-proliferative diabetic retinopathy and/or proliferative diabetic retinopathy.

36-38. (Canceled)

39. (New) The method of claim 35 wherein the angiogenic eye disorder is wet age-related macular degeneration.

40. (New) The method of claim 35 wherein the angiogenic eye disorder is diabetic macular edema.

41. (New) The method of claim 35 wherein the angiogenic eye disorder is diabetic retinopathy.

42. (New) The method of claim 35 wherein the angiogenic eye disorder is non-proliferative diabetic retinopathy.

43. (New) The method of claim 35 wherein the angiogenic eye disorder is proliferative diabetic retinopathy.

44. (New) The method of claim 35 wherein the angiogenic eye disorder is macular edema following retinal vein occlusion.

45. (New) The method of claim 30 wherein the VEGF antagonist is aflibercept.

46. (New) The method of claim 35 wherein the VEGF antagonist is aflibercept.

47. (New) The method of claim 39 wherein the VEGF antagonist is aflibercept.

48. (New) The method of claim 40 wherein the VEGF antagonist is aflibercept.

PGR2026-00039
Biocon Ex.1002
Page 4795 of 4945

49. (New) The method of claim 41 wherein the VEGF antagonist is aflibercept.

50. (New) The method of claim 42 wherein the VEGF antagonist is aflibercept.

51. (New) The method of claim 43 wherein the VEGF antagonist is aflibercept.

52. (New) The method of claim 44 wherein the VEGF antagonist is aflibercept.

PGR2026-00039
Biocon Ex.1002
Page 4796 of 4945

REMARKS/ARGUMENTS

Upon entry of the present amendment, claims 30-32, 35 and 39-52 will be pending in the application. Original claims 1-29, 33, 34 and 36-38 are hereby canceled without prejudice. Claims 30-32 and 35 are hereby amended, and new claims 39-52 are added.

Support for the amendments and new claims can be found in the specification and original claims at, *e.g.*, the following locations:

Claim 30 – original claims 33 and 34, and paragraphs 0019, 0066 and 0208;

Claims 31 and 32 – paragraphs 0068 and 0207;

Claims 39 to 44 – original claim 35; and

Claims 45 to 52 – paragraph 0019.

Claim 35 is amended to remove multiple dependencies. No new matter is added.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 541-749-4085.

Respectfully submitted,

/Lance A. Termes/

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PGR2026-00039
Biocon Ex.1002
Page 4797 of 4945

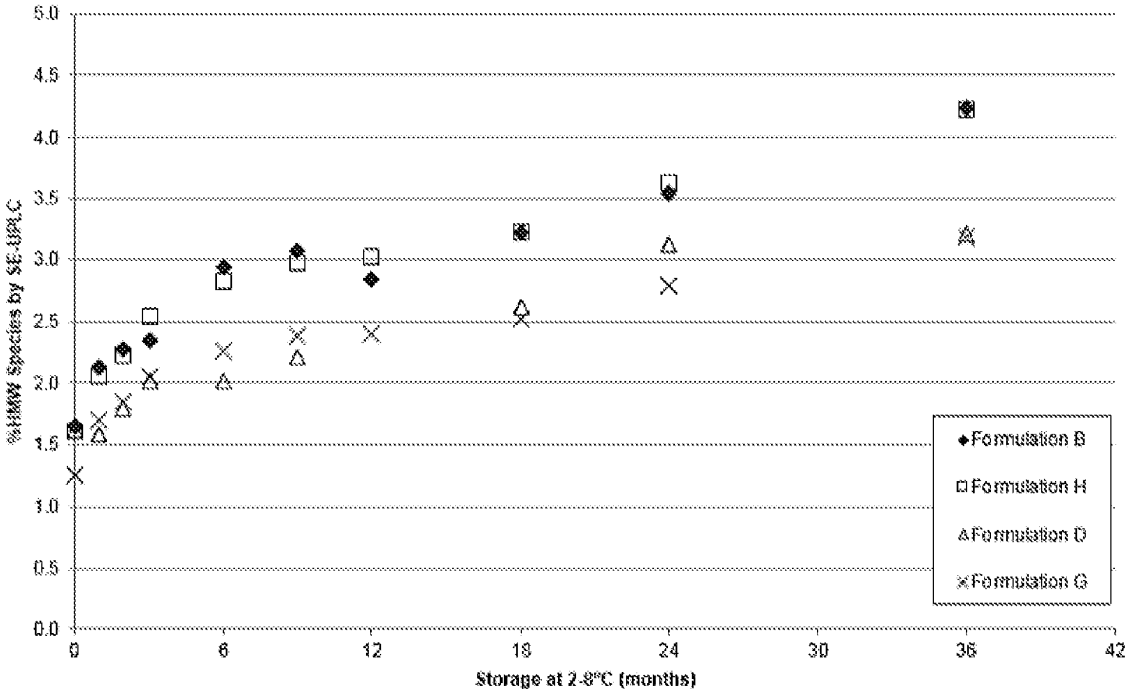

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
%HMW Species by SE-HPLC
0
6
12
18
24
30
36
42
Storage at 2-8°C (months)
Formulation B
Formulation H
Formulation D
Formulation G

FIG. 1A

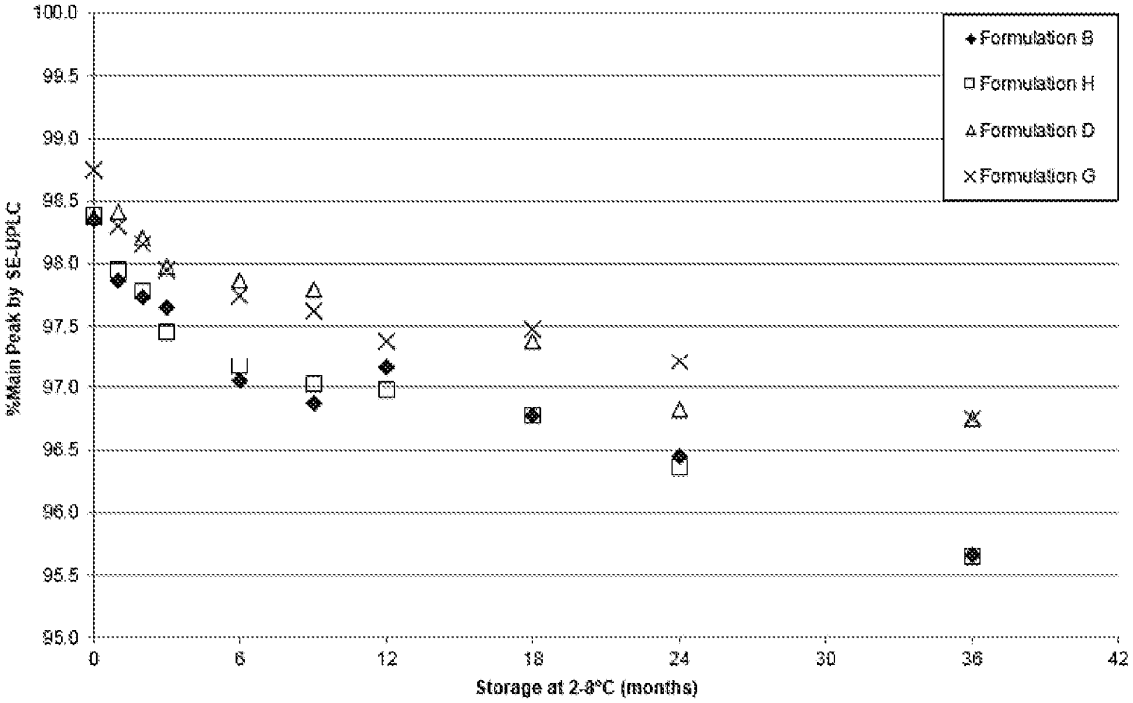

100.0
99.5
99.0
98.5
98.0
97.5
97.0
96.5
96.0
95.5
95.0
%Main Peak by SE-UPLC
0
6
12
18
24
30
36
42
Storage at 2-8°C (months)
Formulation B
Formulation H
Formulation D
Formulation G

FIG. 1B

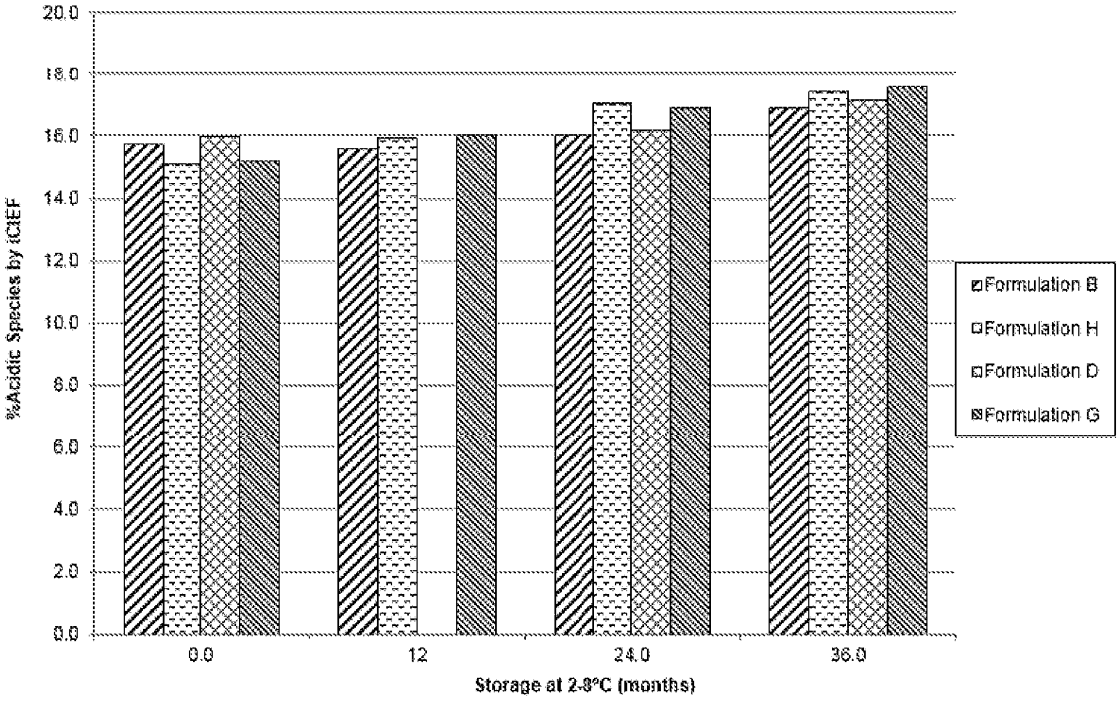

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
%Acidic Species by iCIEF
0.0
12
24.0
36.0
Storage at 2-8°C (months)
Formulation B
Formulation H
Formulation D
Formulation G

FIG. 1C

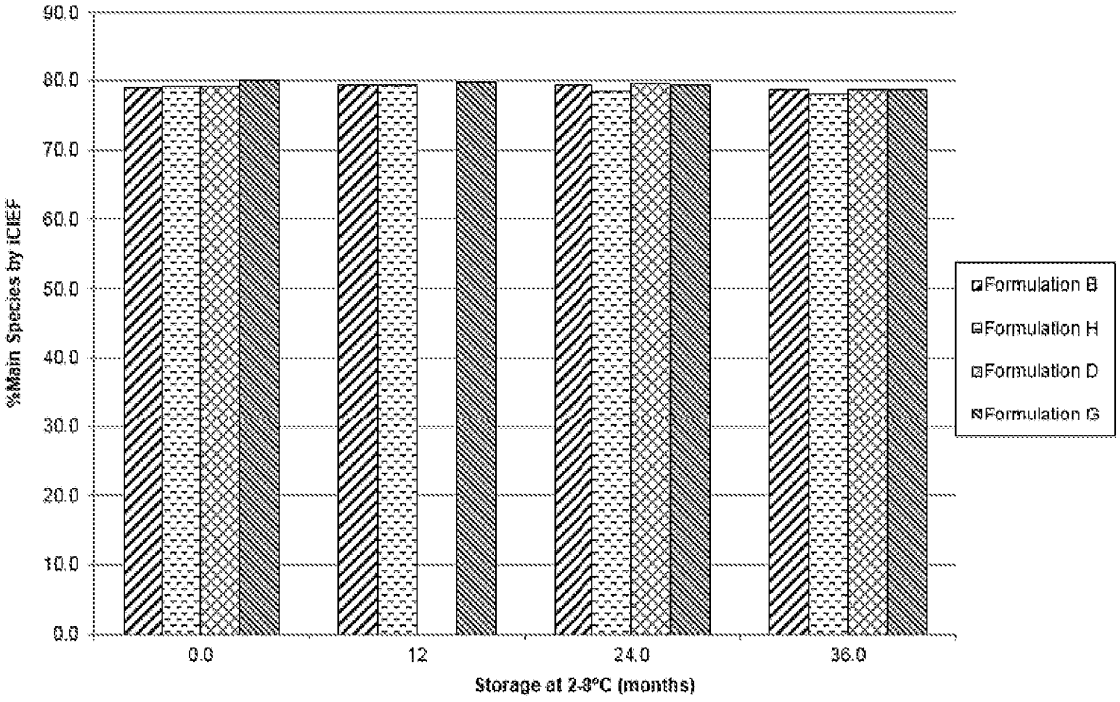

90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
%Main Species by iCIEF
0.0
12
24.0
36.0
Storage at 2-8°C (months)
Formulation B
Formulation H
Formulation D
Formulation G

FIG. 1D

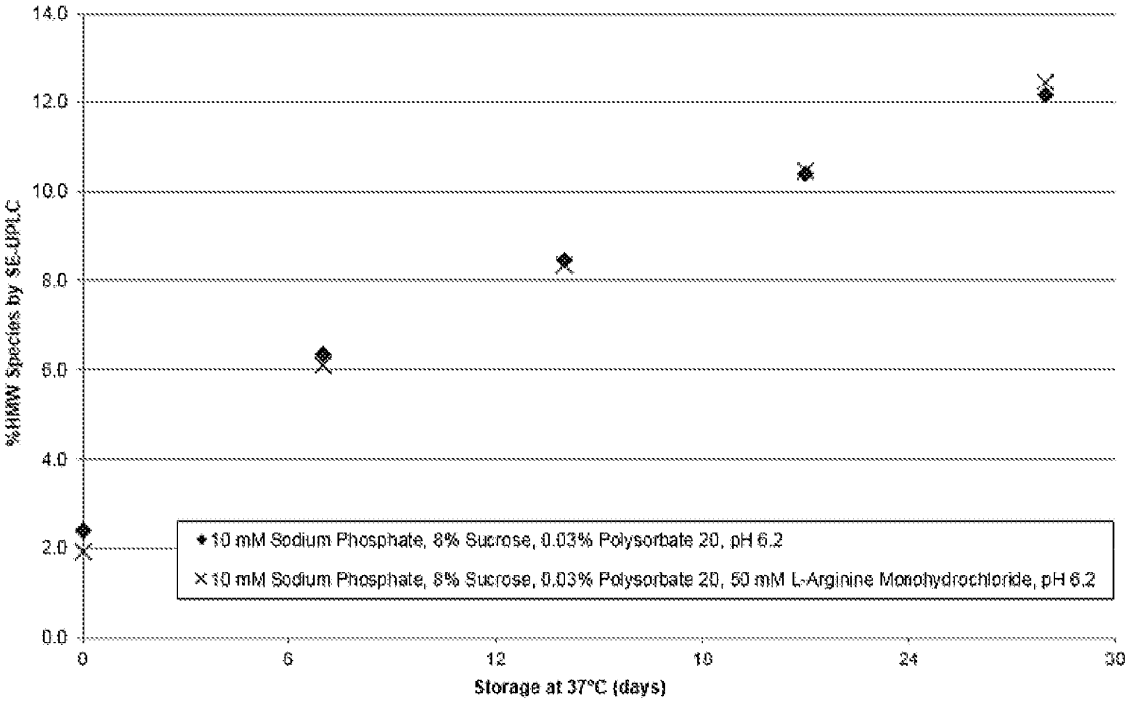

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
%HMW Species by SE-UPLC
0
6
12
18
24
30
Storage at 37°C (days)
10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2
10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2

FIG. 2A

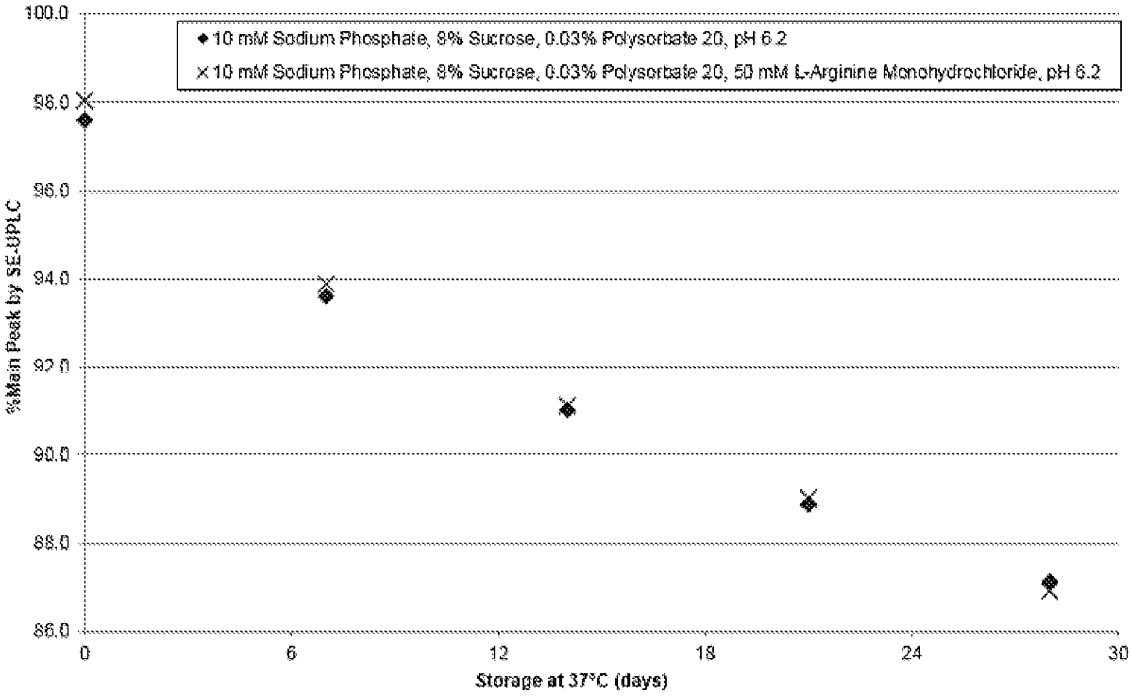

10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2
10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2
%Main Peak by SE-UPLC
100.0
98.0
96.0
94.0
92.0
90.0
88.0
86.0
0
6
12
18
24
30
Storage at 37°C (days)

FIG. 2B

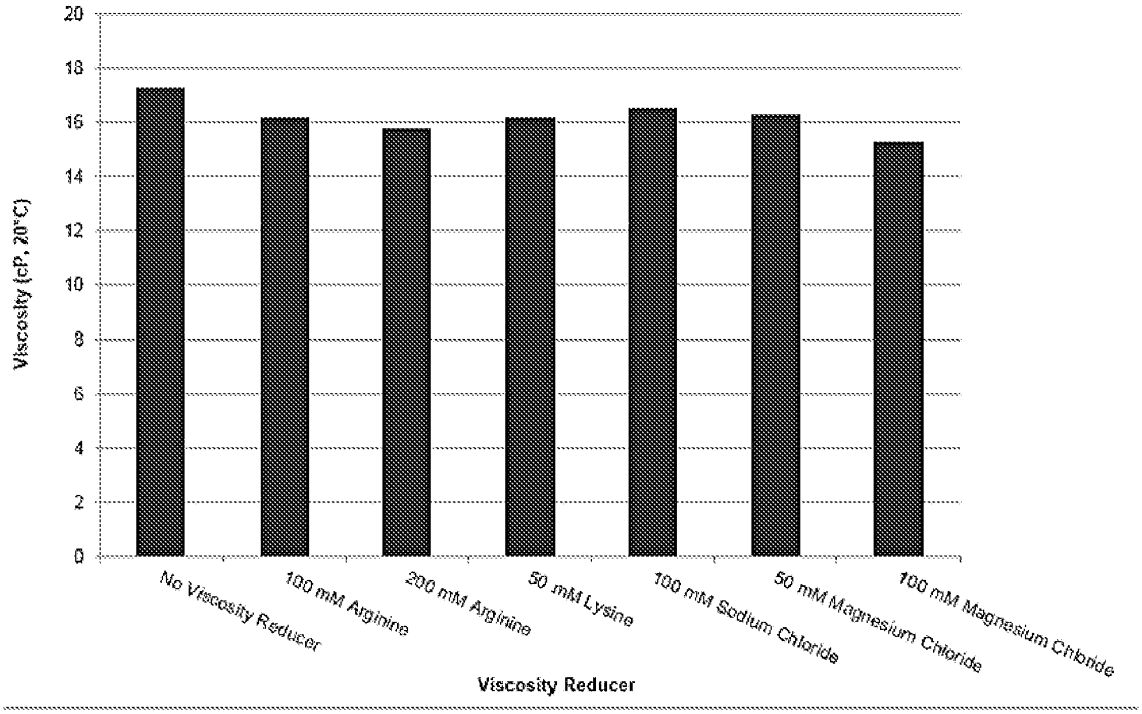

20
18
16
14
12
10
8
6
4
2
0
Viscosity (cP, 20°C)
No Viscosity Reducer
100 mM Arginine
200 mM Arginine
50 mM Lysine
100 mM Sodium Chloride
50 mM Magnesium Chloride
100 mM Magnesium Chloride
Viscosity Reducer

FIG. 3A

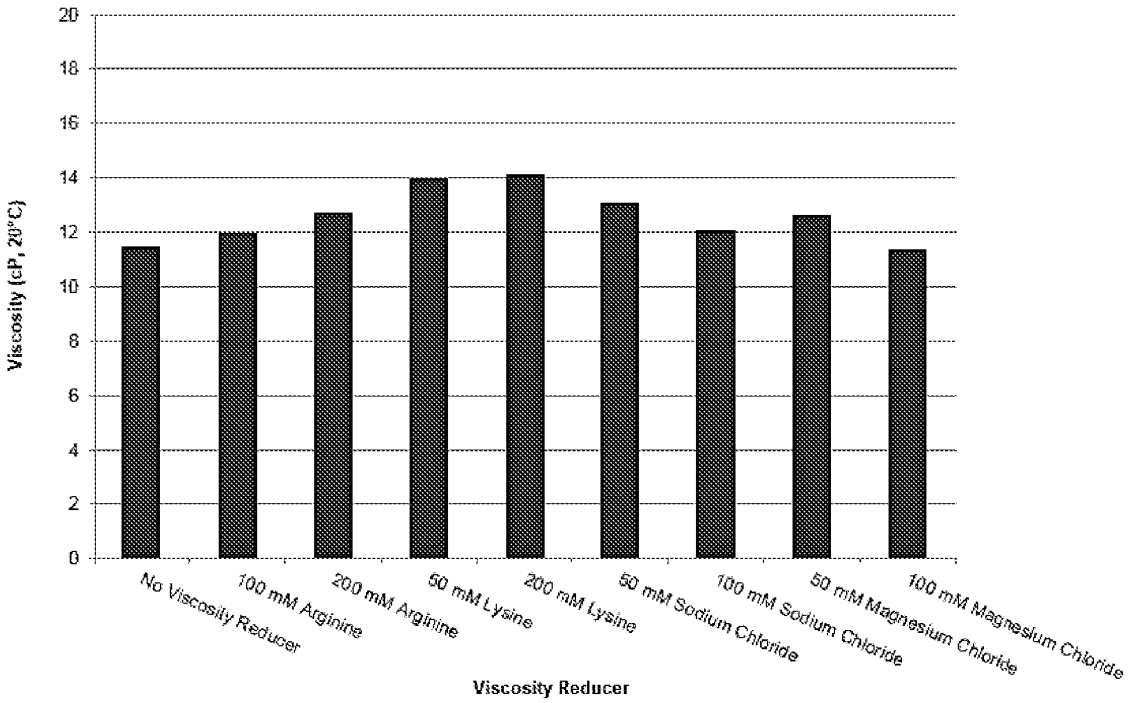

Viscosity (cP, 20°C)
20
18
16
14
12
10
8
6
4
2
0
No Viscosity Reducer
100 mM Arginine
200 mM Arginine
50 mM Lysine
200 mM Lysine
50 mM Sodium Chloride
100 mM Sodium Chloride
50 mM Magnesium Chloride
100 mM Magnesium Chloride
Viscosity Reducer

FIG. 3B

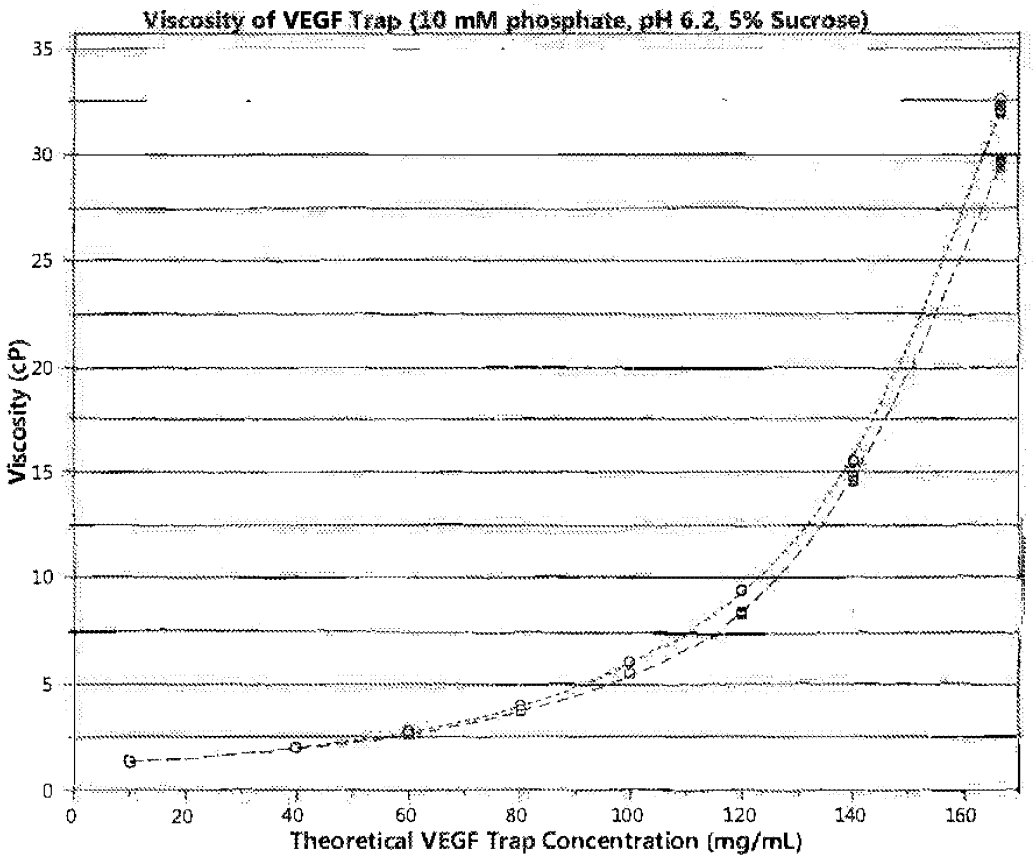

Viscosity of VEGF Trap (10 mM phosphate, pH 6.2, 5% Sucrose)
Viscosity (cP)
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
140
160
Theoretical VEGF Trap Concentration (mg/mL)
Arginine Concentration / Temperature
0 mM / 20°C
50 mM / 20°C
0 mM / 20°C Smoother
50 mM / 20°C Smoother

FIG. 3C

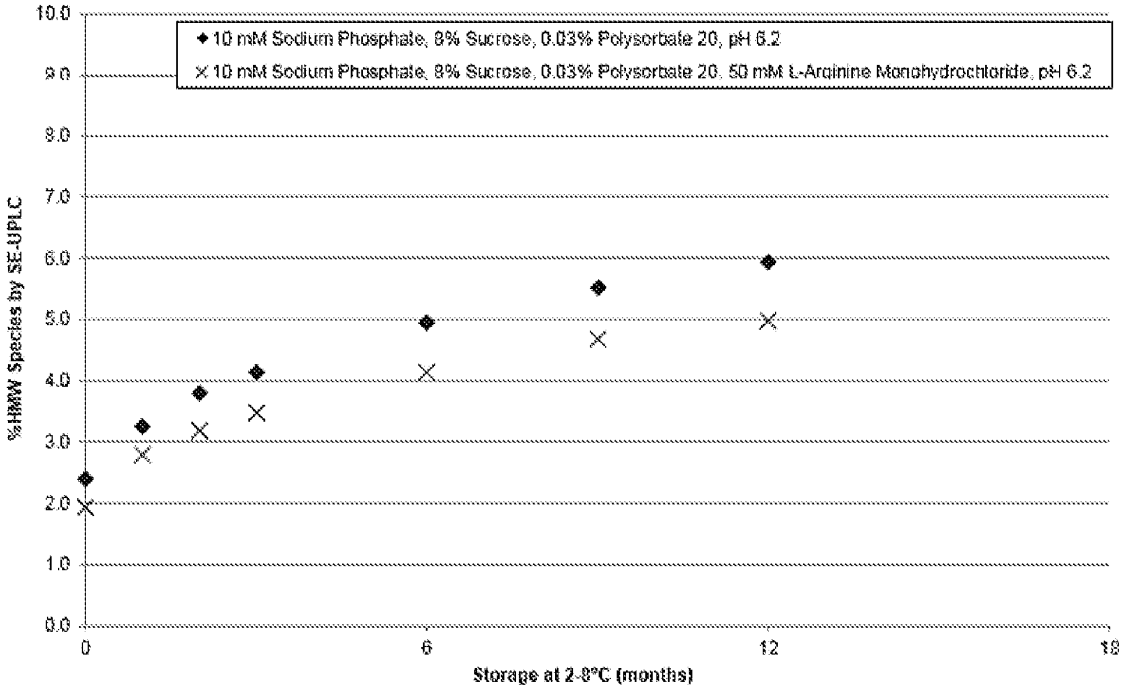

10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2
10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2
%HMW Species by SE-UPLC
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
6
12
18
Storage at 2-8°C (months)

FIG. 4A

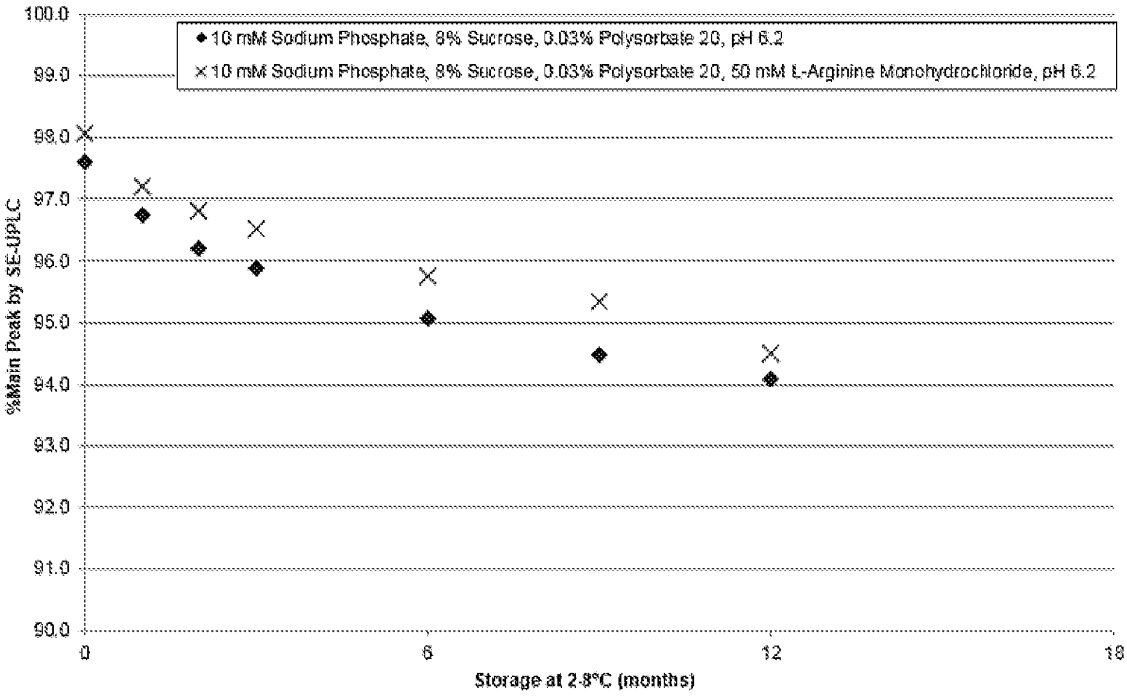

10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2
10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2
%Main Peak by SE-UPLC
100.0
99.0
98.0
97.0
96.0
95.0
94.0
93.0
92.0
91.0
90.0
0
6
12
18
Storage at 2-8°C (months)

FIG. 4B

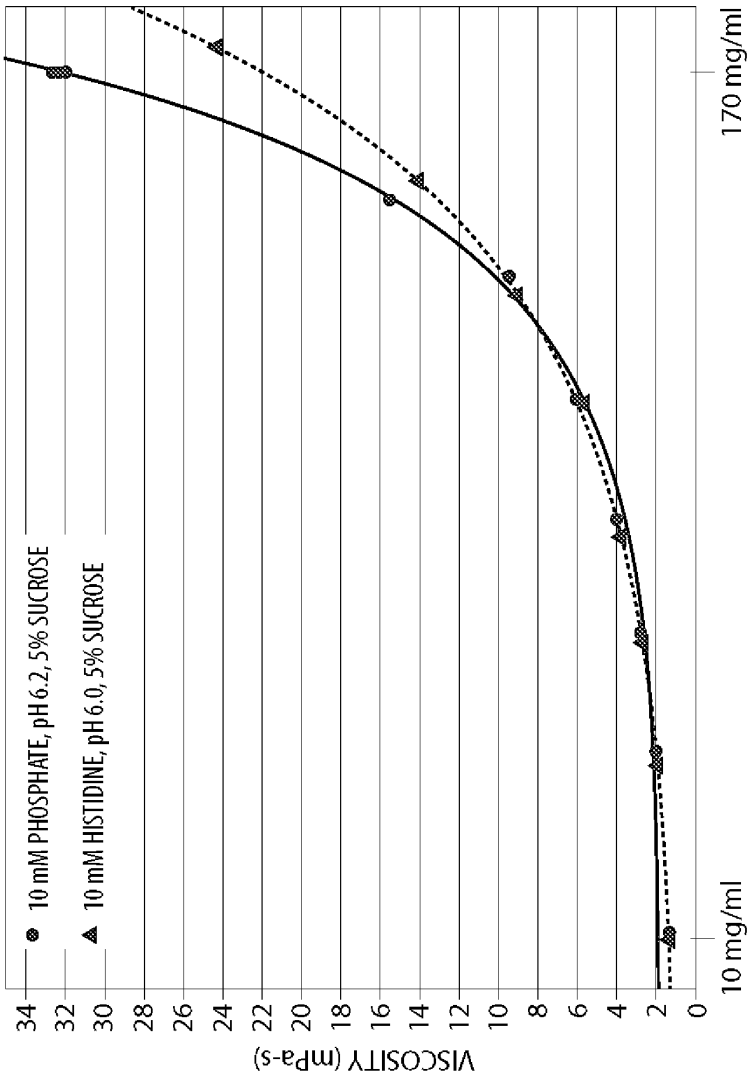

VISCOSITY (mPa-s)
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
10 mg/ml
170 mg/ml
10 mM PHOSPHATE, pH 6.2, 5% SUCROSE
10 mM HISTIDINE, pH 6.0, 5% SUCROSE

FIG. 5

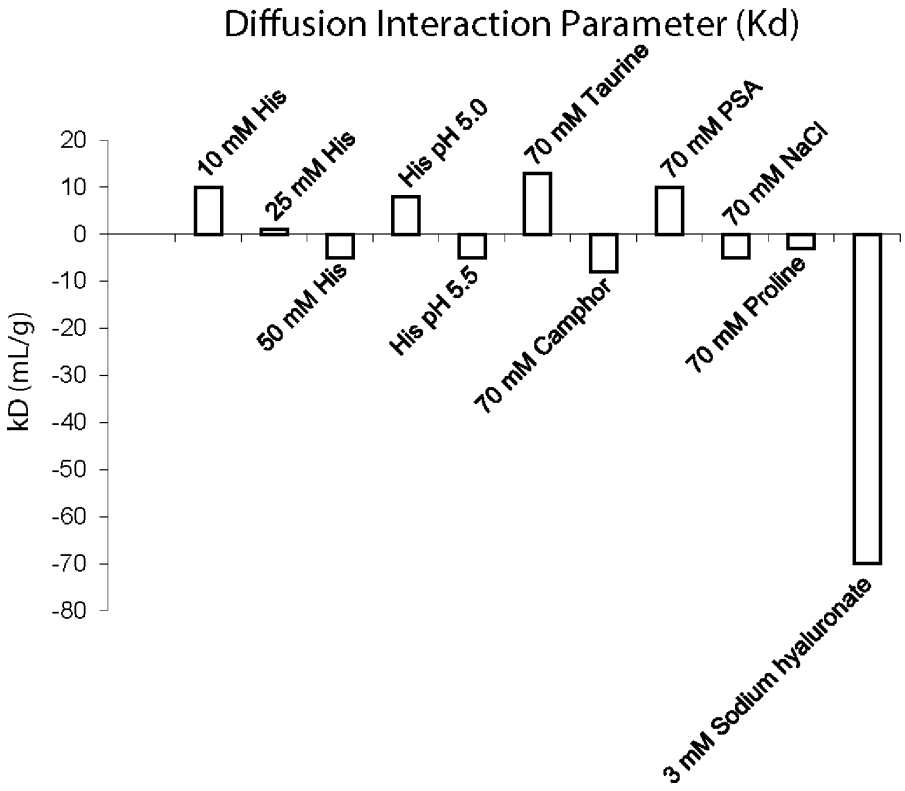

Diffusion Interaction Parameter (Kd)
kD (mL/g)
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
10 mM His
25 mM His
50 mM His
His pH 5.0
His pH 5.5
70 mM Taurine
70 mM Camphor
70 mM PSA
70 mM NaCl
70 mM Proline
3 mM Sodium hyaluronate

FIG. 6

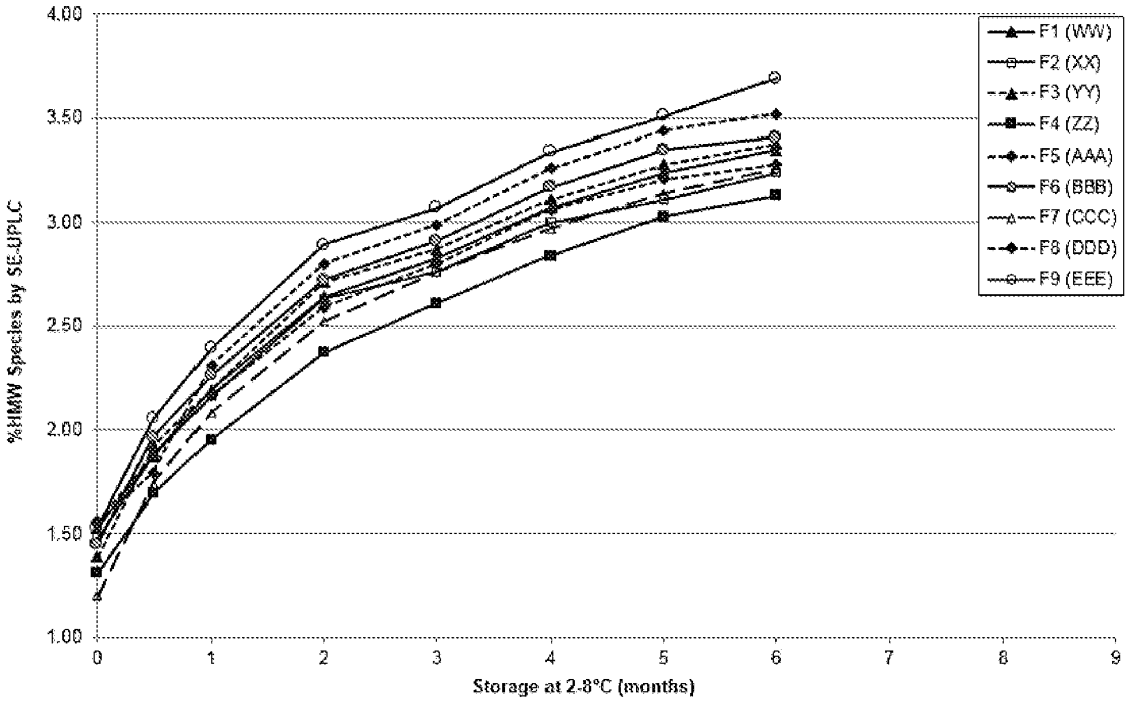

4.00
3.50
3.00
2.50
2.00
1.50
1.00
%HMW Species by SE-UPLC
0
1
2
3
4
5
6
7
8
9
Storage at 2-8°C (months)
F1 (WW)
F2 (XX)
F3 (YY)
F4 (ZZ)
F5 (AAA)
F6 (BBB)
F7 (CCC)
F8 (DDD)
F9 (EEE)

FIG. 7

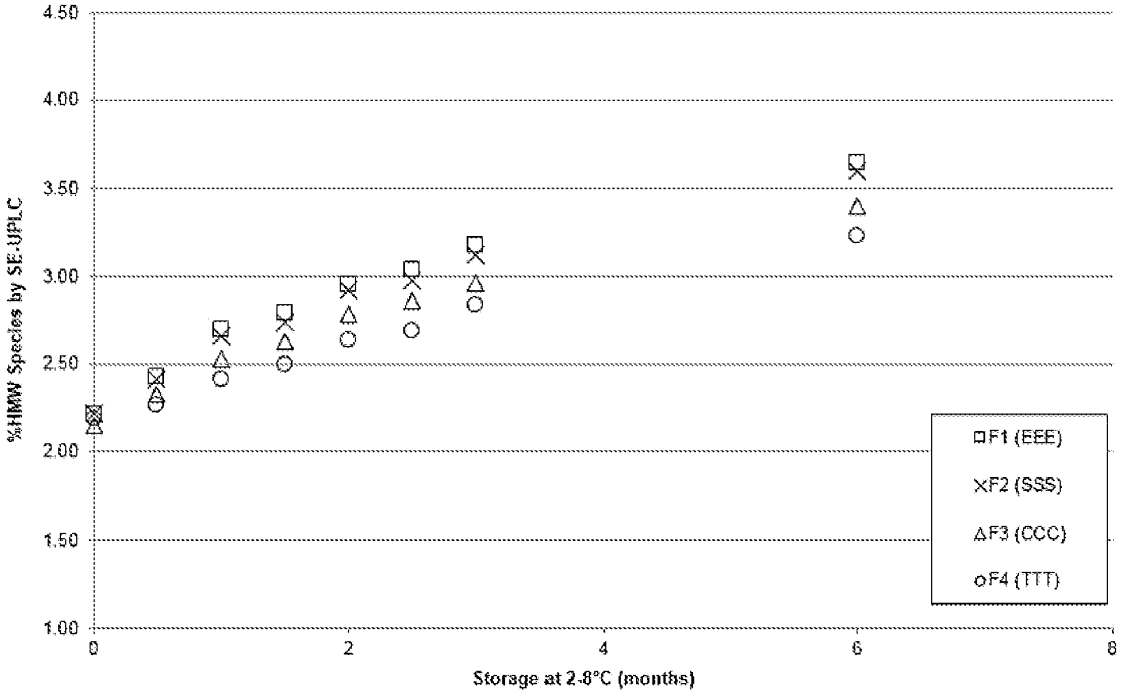

4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
%HMW Species by SE-UPLC
0
2
4
6
8
Storage at 2-8°C (months)
F1 (EEE)
F2 (SSS)
F3 (CCC)
F4 (TTT)

FIG. 8A

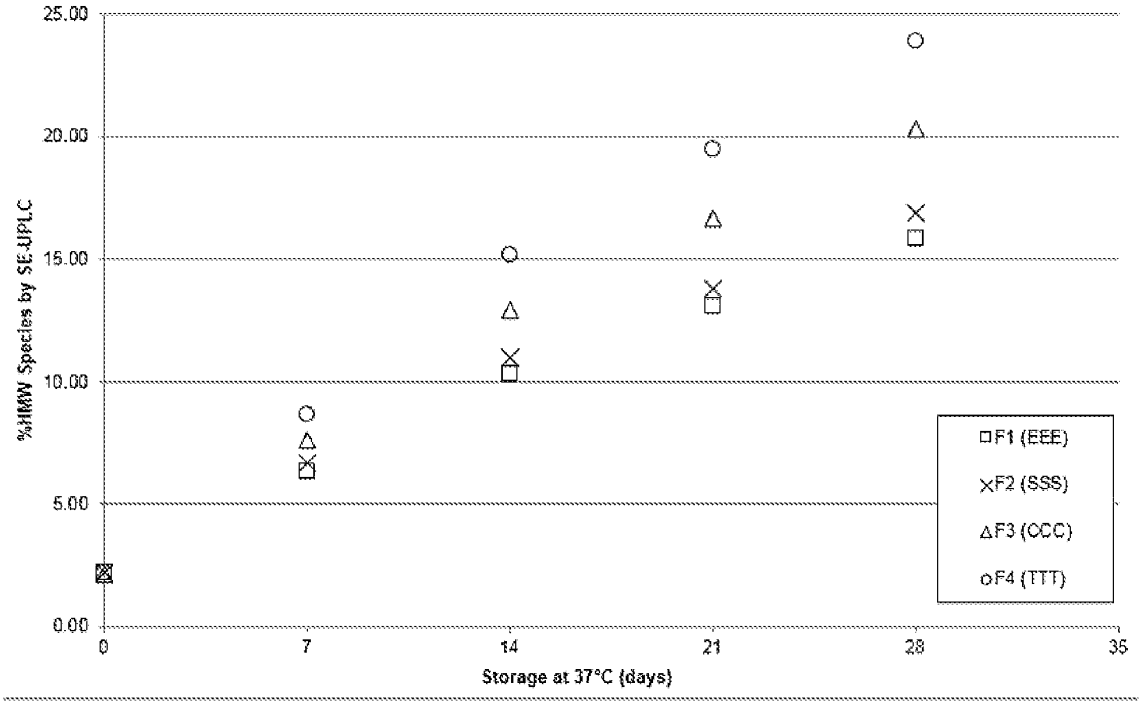

25.00
20.00
15.00
10.00
5.00
0.00
%HMW Species by SEJPLC
0
7
14
21
28
35
Storage at 37°C (days)
F1 (EEE)
F2 (SSS)
F3 (CCC)
F4 (TTT)

FIG. 8B

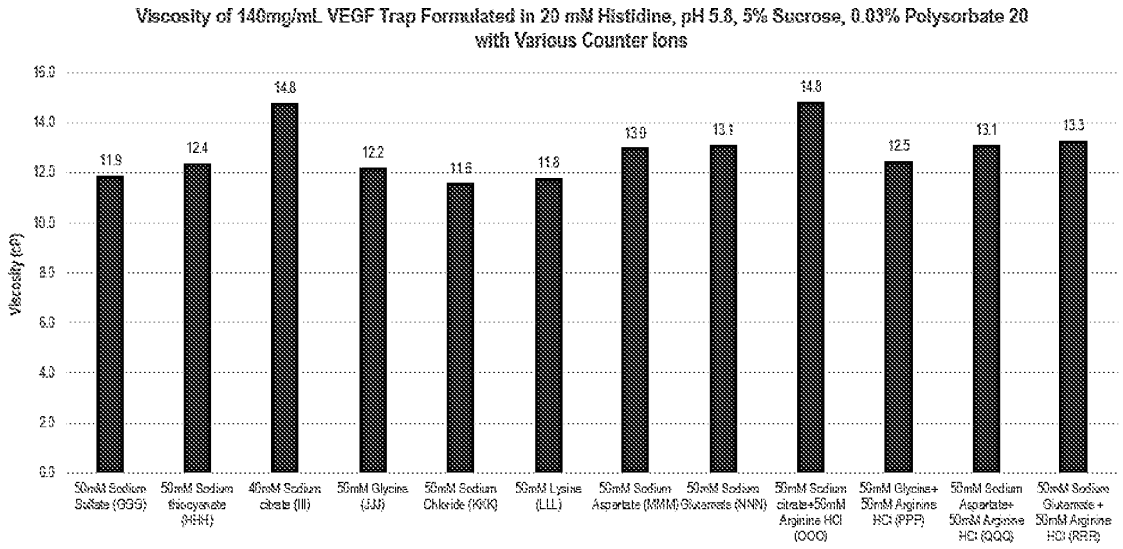

Viscosity of 140mg/mL VEGF Trap Formulated in 20 mM Histidine, pH 5.8, 5% Sucrose, 0.03% Polysorbate 20 with Various Counter Ions
Viscosity (cP)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
11.9
12.4
14.8
12.2
11.6
11.8
13.0
13.1
14.8
12.5
13.1
13.3
50mM Sodium Sulfate (SSS)
50mM Sodium thiocyanate (HHH)
40mM Sodium citrate (III)
50mM Glycine (JJJ)
50mM Sodium Chloride (KKK)
50mM Lysine (LLL)
50mM Sodium Aspartate (MMM)
50mM Sodium Glutamate (NNN)
50mM Sodium citrate+50mM Arginine HCl (OOO)
50mM Glycine+ 50mM Arginine HCl (PPP)
50mM Sodium Aspartate+ 50mM Arginine HCl (QQQ)
50mM Sodium Glutamate + 50mM Arginine HCl (RRR)

FIG. 9

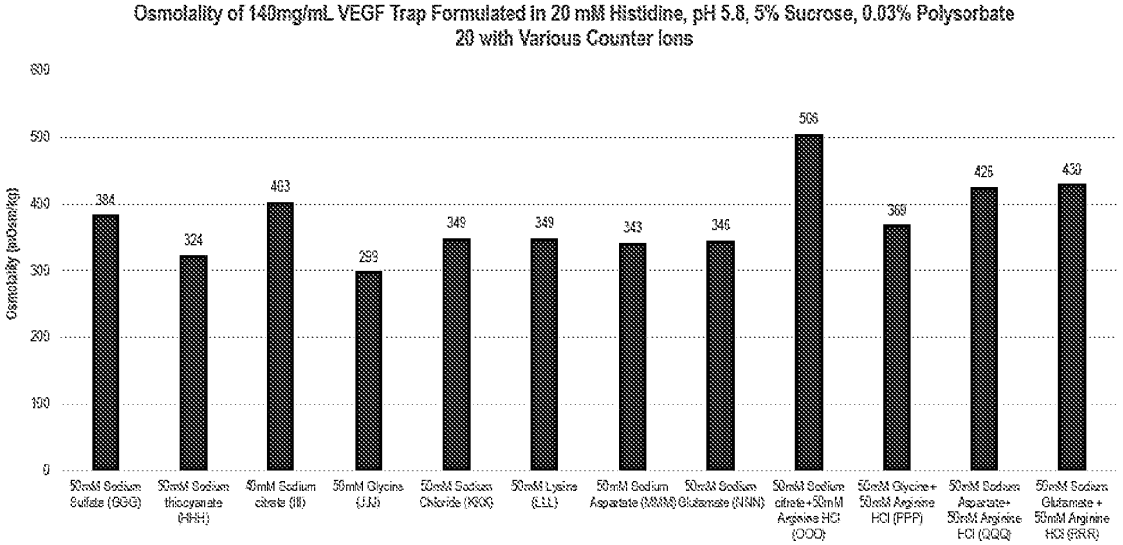

Osmolality of 140mg/mL VEGF Trap Formulated in 20 mM Histidine, pH 5.8, 5% Sucrose, 0.03% Polysorbate 20 with Various Counter Ions
Osmolality (mOsm/kg)
600
500
400
300
200
100
0
384
324
463
299
349
349
343
346
508
369
426
439
50mM Sodium Sulfate (SSS)
50mM Sodium thiocyanate (HHH)
40mM Sodium citrate (III)
50mM Glycine (JJJ)
50mM Sodium Chloride (KKK)
50mM Lysine (LLL)
50mM Sodium Aspartate (MMM)
50mM Sodium Glutamate (NNN)
50mM Sodium citrate+50mM Arginine HCl (OOO)
50mM Glycine+ 50mM Arginine HCl (PPP)
50mM Sodium Aspartate+ 50mM Arginine HCl (QQQ)
50mM Sodium Glutamate + 50mM Arginine HCl (RRR)

FIG. 10

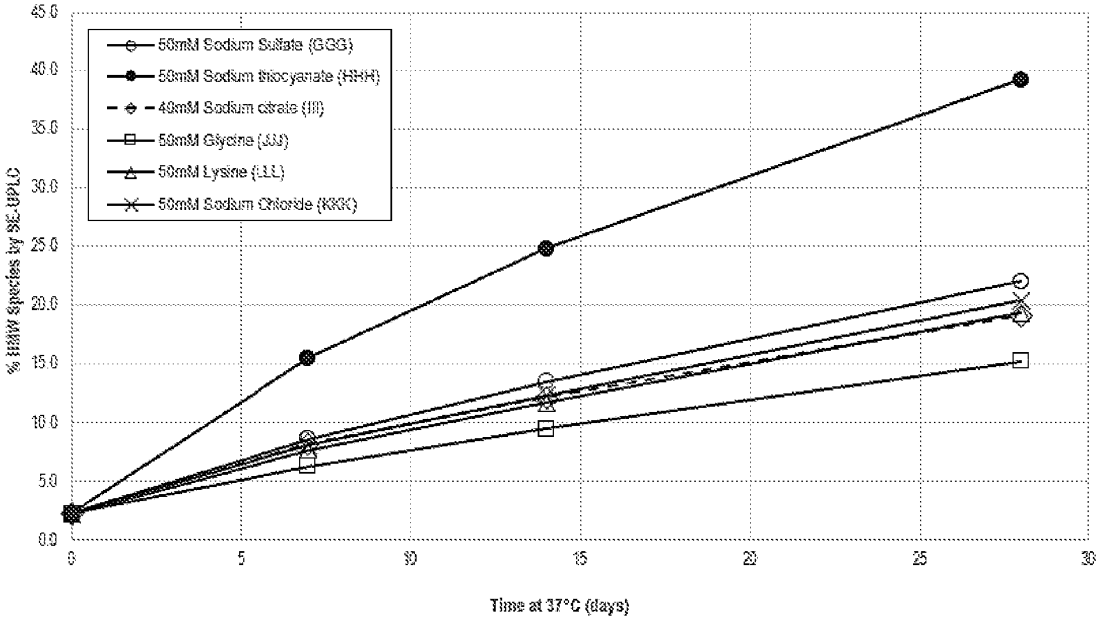


FIG. 11A

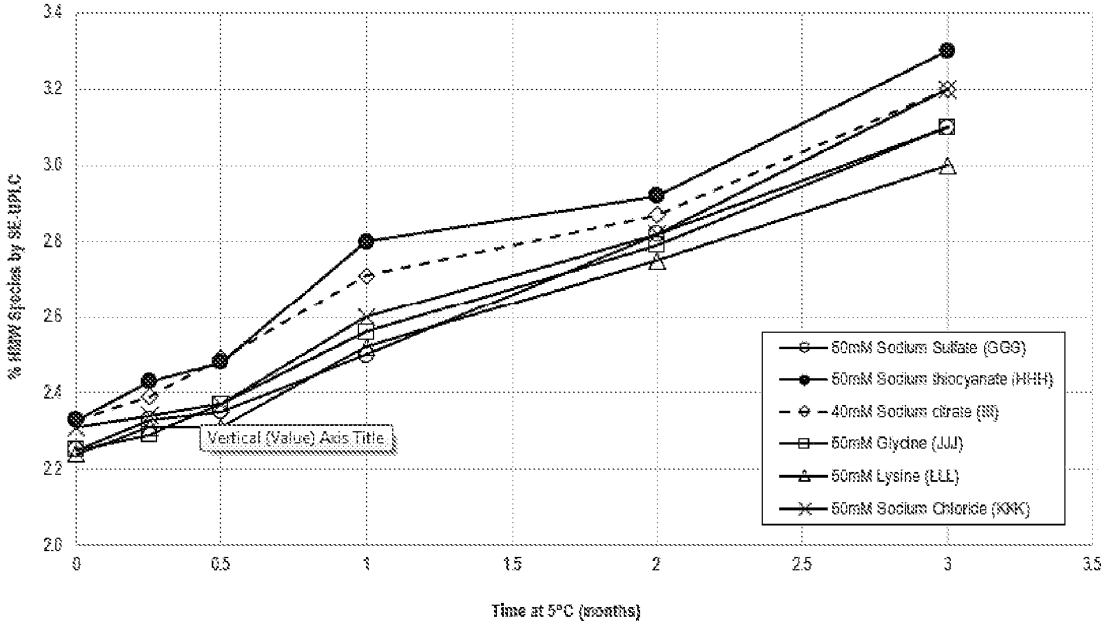


FIG. 11B

Formulations	Diffusion coefficient (cm2/sec)	Radius (nm)	% Pd
F1 (50mM Sodium Sulfate)	3.32E-07	7.4	15.3
F2(50mM Sodium thiocyanate)	2.98E-07	8.2	14.7
F3 (50mM Sodium citrate)	3.20E-07	7.7	16.3
F4 (50mM Glycine)	3.89E-07	6.3	18.9
F5 (50mM Sodium Chloride)	3.24E-07	7.6	13.8
F6 (50mM Lysine)	3.17E-07	7.7	13.5
F7 (50mM Sodium Aspartate)	3.32E-07	7.4	18.2
F8 (50mM Sodium Glutamate)	3.32E-07	7.4	15.0
F9 (50mM Sodium citrate+50mM Arginine HCl)	3.31E-07	7.4	14.4
F10 (50mM Glycine+ 50mM Arginine HCl)	3.31E-07	7.4	14.3
F11 (50mM Sodium Aspartate+ 50mM Arginine HCl)	3.24E-07	7.6	14.8
F12 (50mM Sodium Glutamate + 50mM Arginine HCl)	3.22E-07	7.6	13.3

FIG. 12

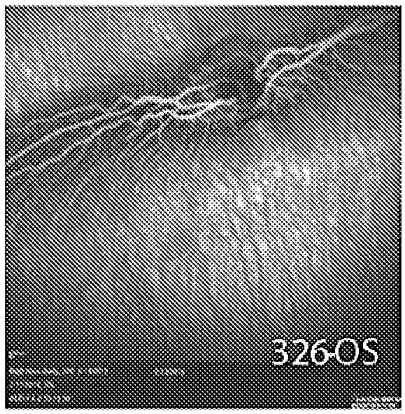


FIG. 13A

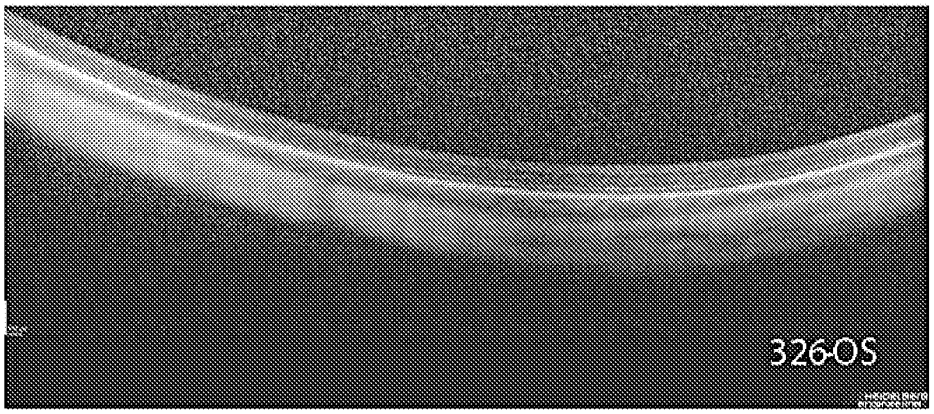


FIG. 13B

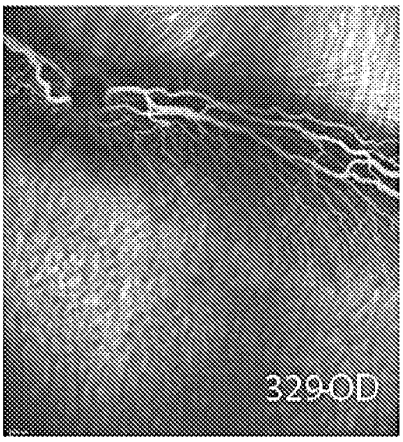


FIG. 13C

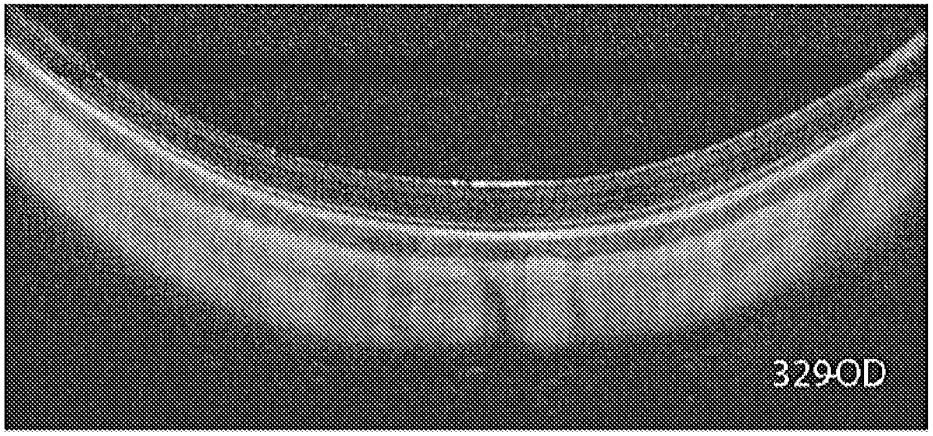


FIG. 13D

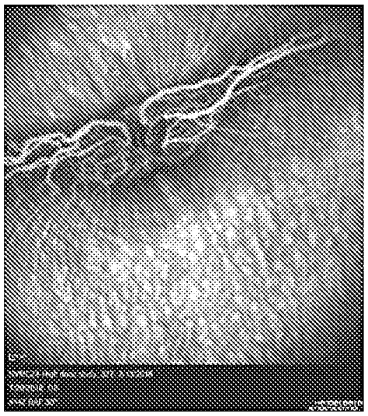


FIG. 14A

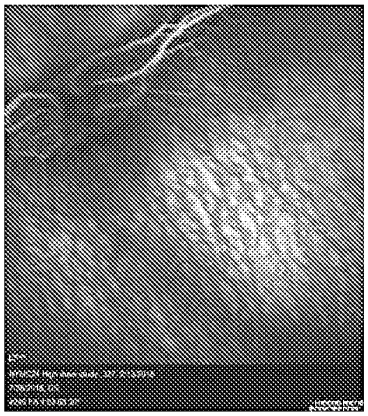


FIG. 14B

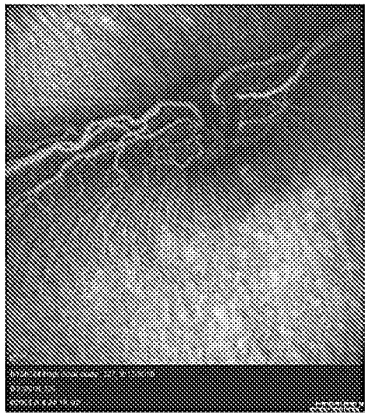


FIG. 14C

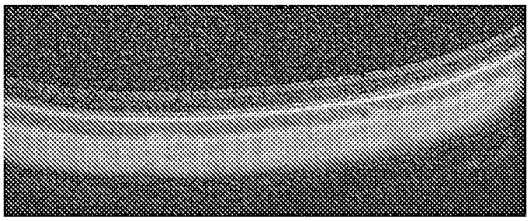


FIG. 14D

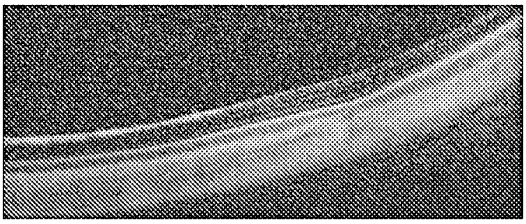


FIG. 14E

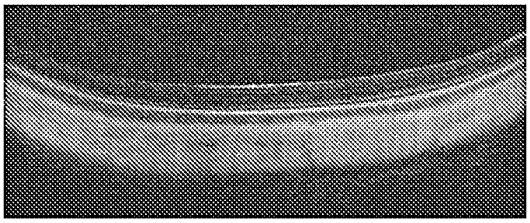


FIG. 14F

FA

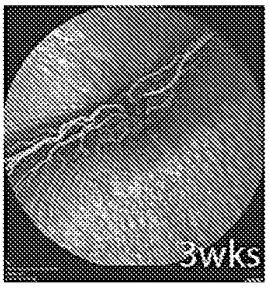


FIG. 15A

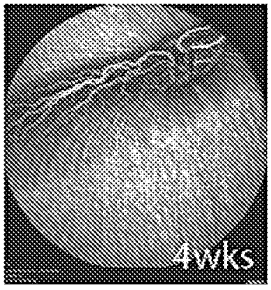


FIG. 15B

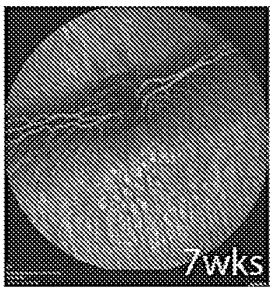


FIG. 15C

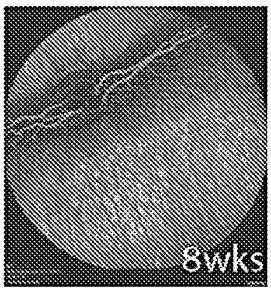


FIG. 15D

OCT

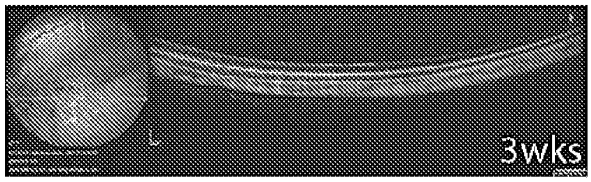


FIG. 15E

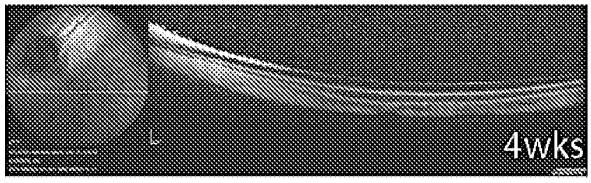


FIG. 15F

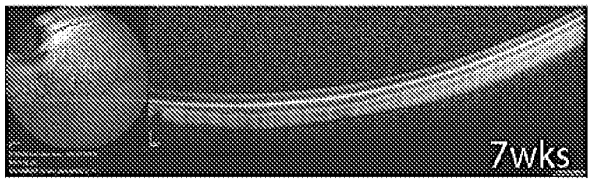


FIG. 15G

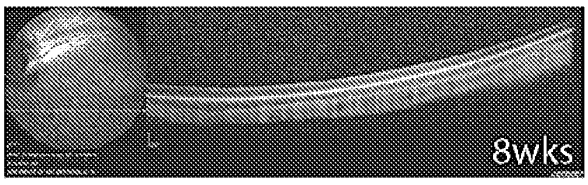


FIG. 15H

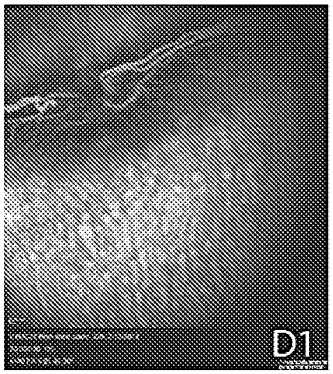


FIG. 16A



FIG. 16B

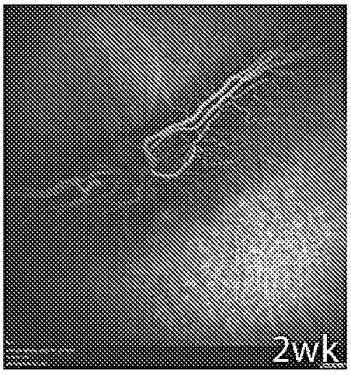


FIG. 16C

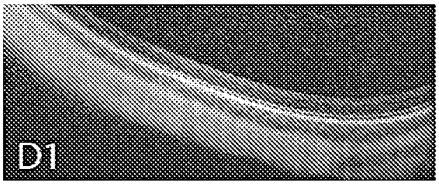


FIG. 16D

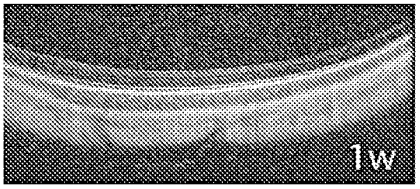


FIG. 16E

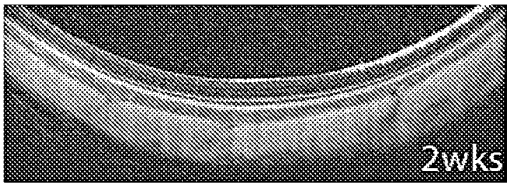


FIG. 16F

FA

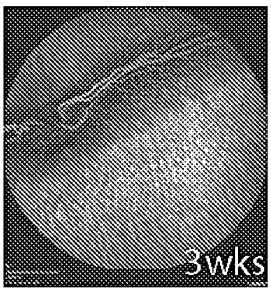


FIG. 17A

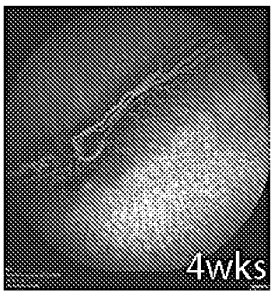


FIG. 17B

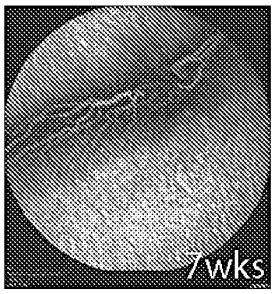


FIG. 17C

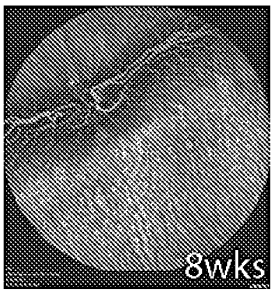


FIG. 17D

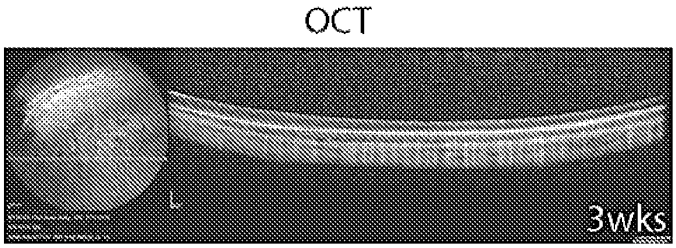


FIG. 17E

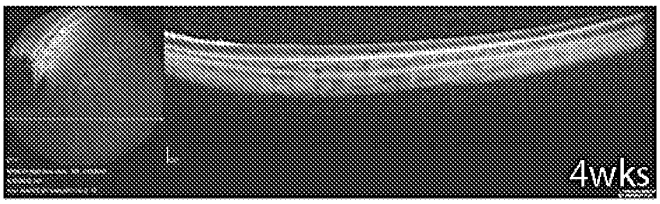


FIG. 17F

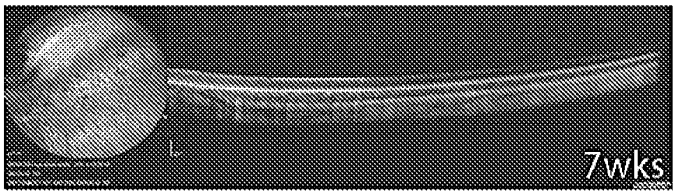


FIG. 17G

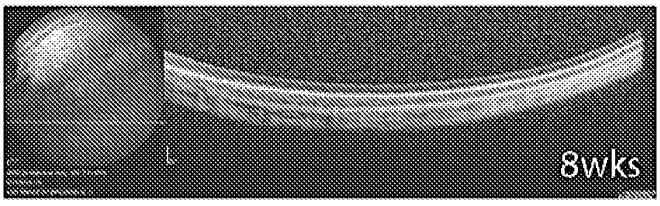


FIG. 17H

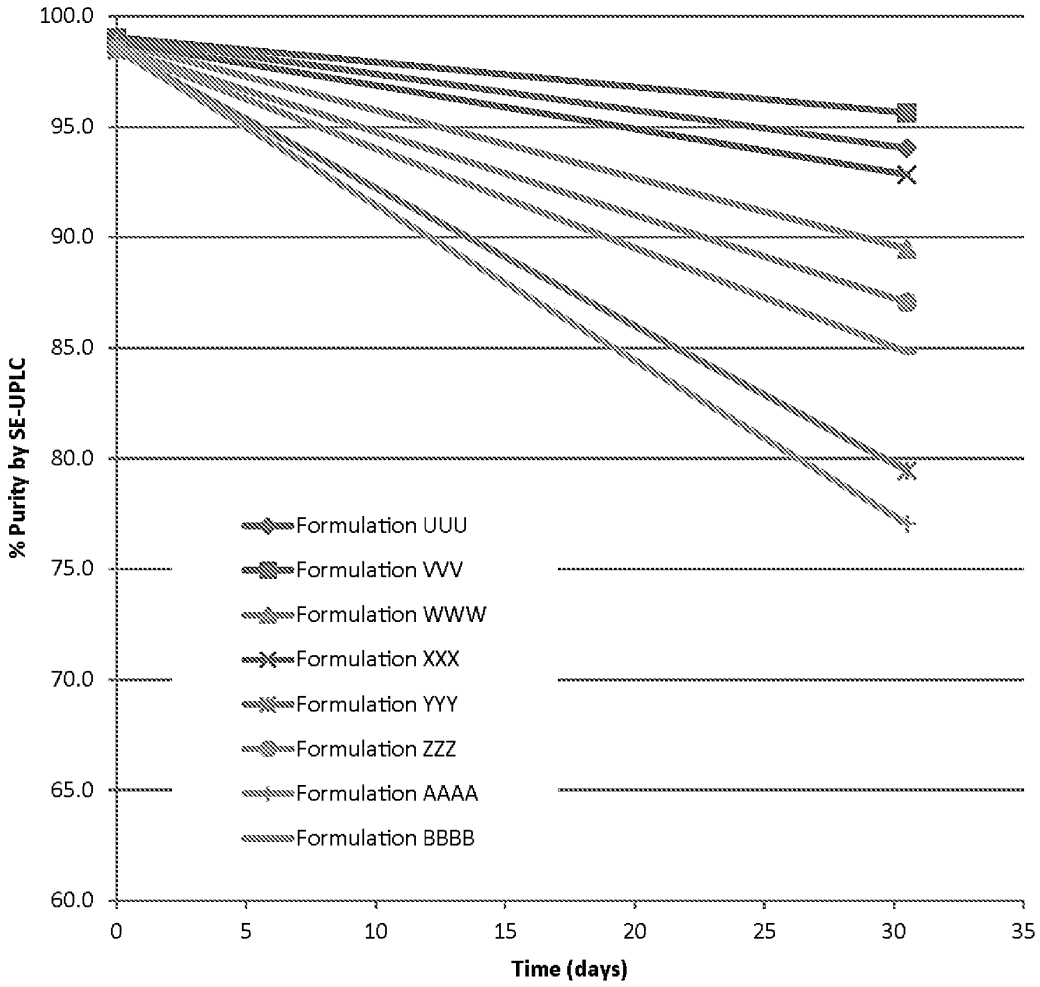

100.0
95.0
90.0
85.0
80.0
75.0
70.0
65.0
60.0
% Purity by SE-UPLC
0
5
10
15
20
25
30
35
Time (days)
Formulation UUU
Formulation VVV
Formulation WWW
Formulation XXX
Formulation YYY
Formulation ZZZ
Formulation AAAA
Formulation BBBB

FIG. 18A

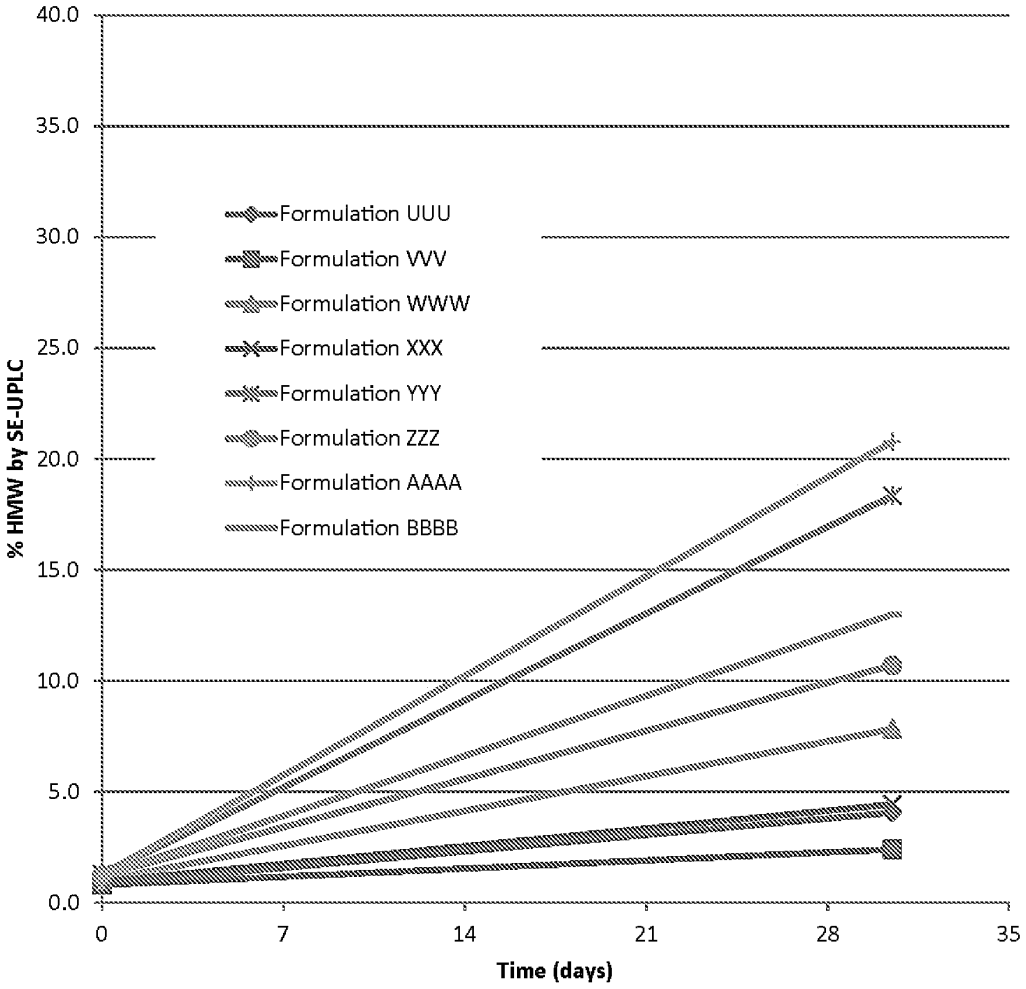

40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
% HMW by SE-UPLC
0
7
14
21
28
35
Time (days)
Formulation UUU
Formulation VVV
Formulation WWW
Formulation XXX
Formulation YYY
Formulation ZZZ
Formulation AAAA
Formulation BBBB

FIG. 18B

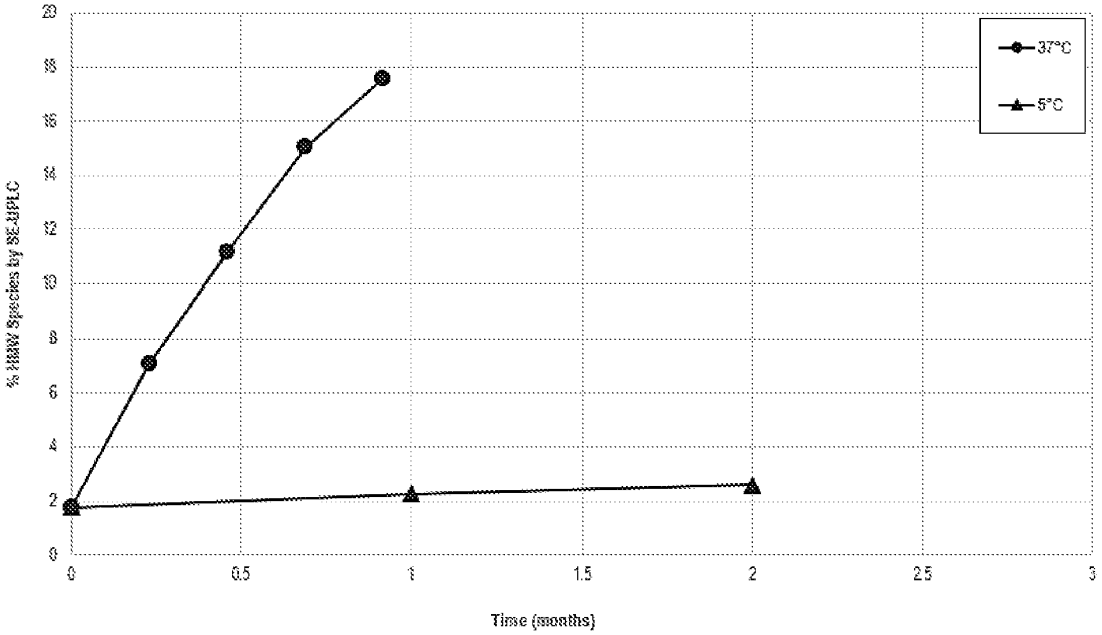


FIG. 19A

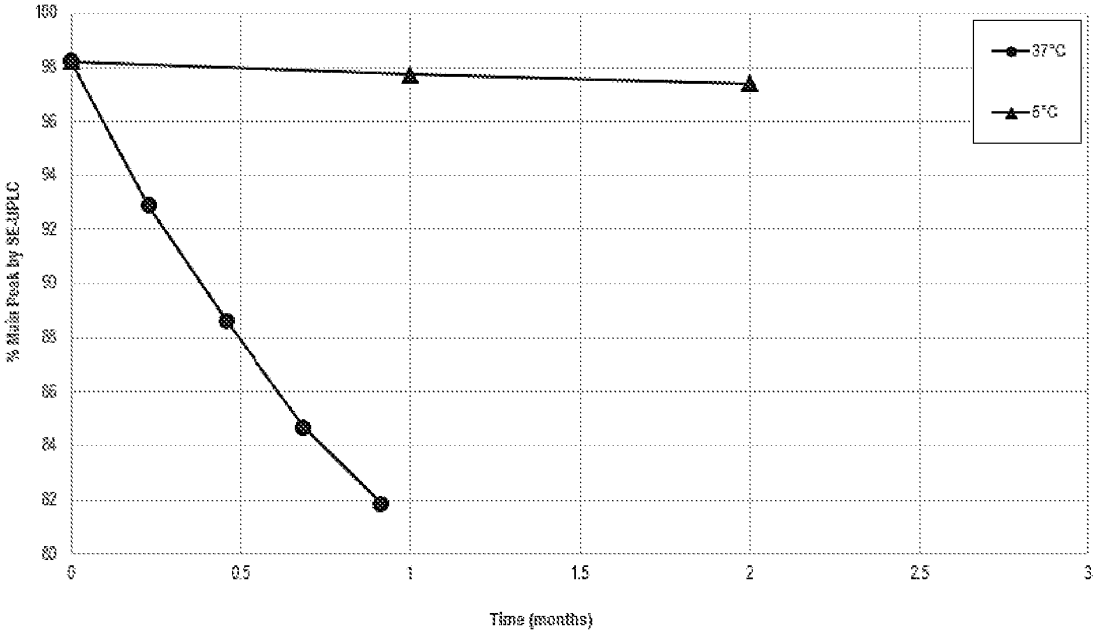


FIG. 19B

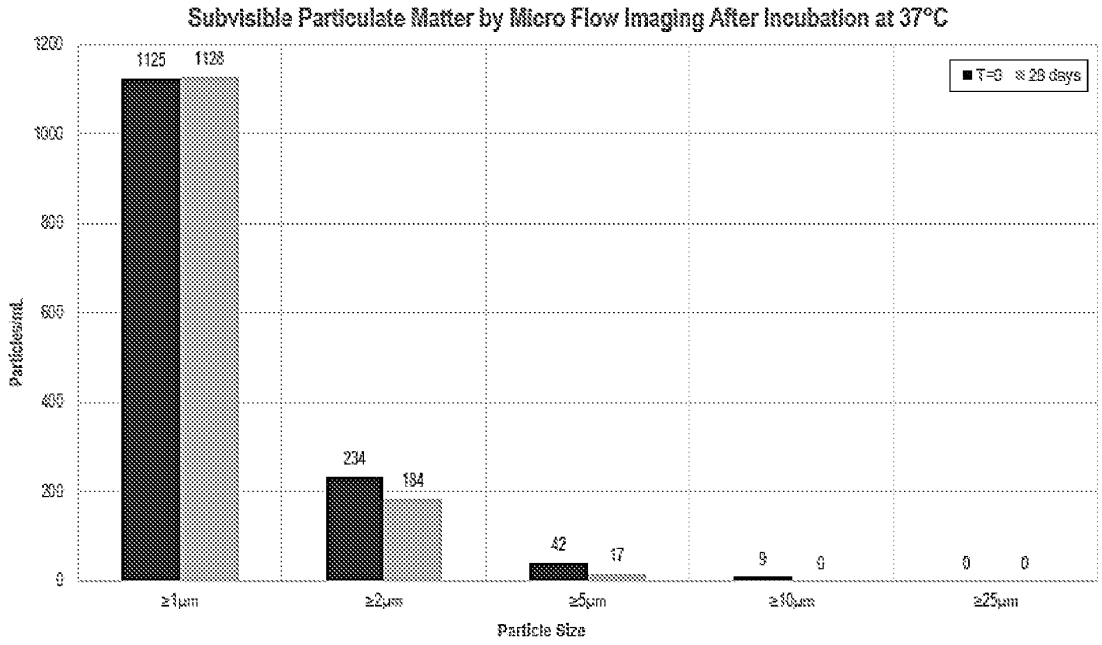


FIG. 19C

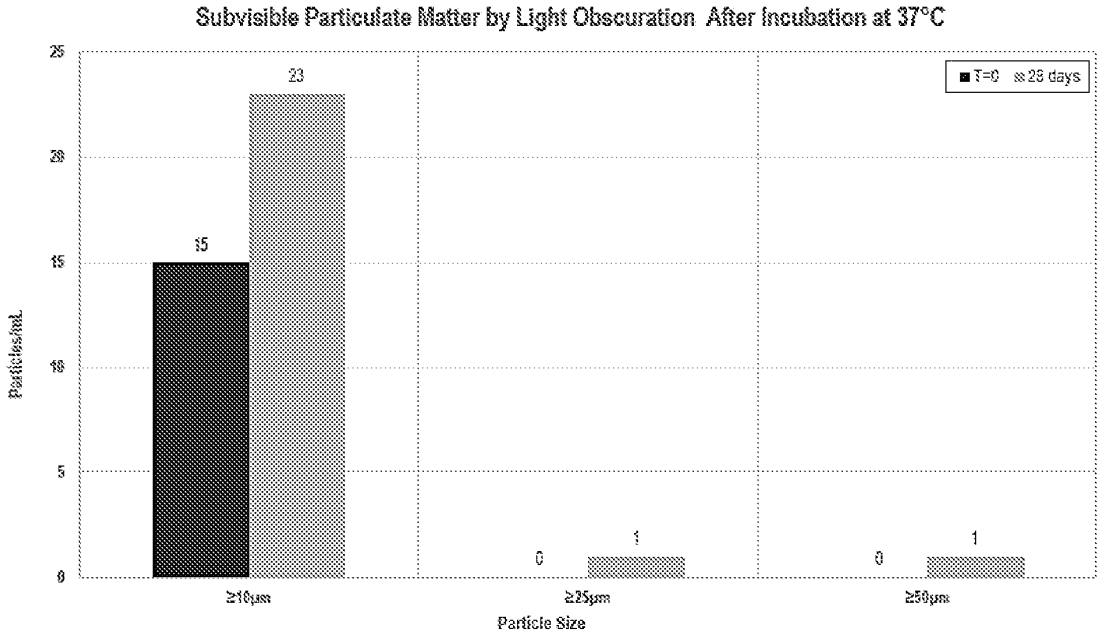


FIG. 19D

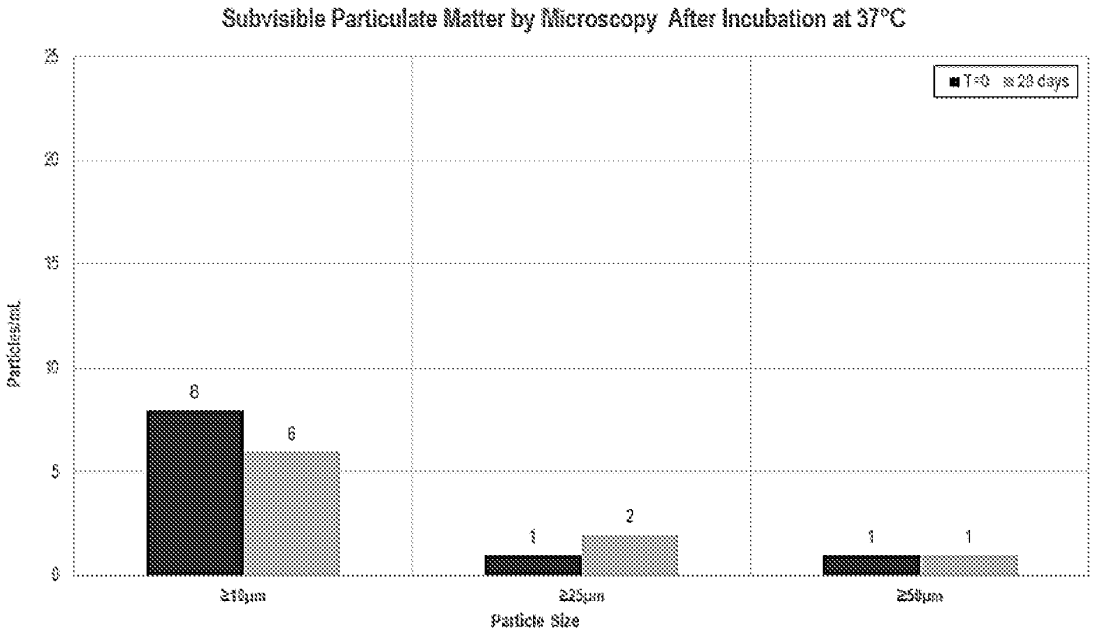

Subvisible Particulate Matter by Microscopy After Incubation at 37°C
T=0
28 days
Particles/mL
25
20
15
10
5
0
8
6
1
2
1
1
≥10µm
≥25µm
≥50µm
Particle Size

FIG. 19E

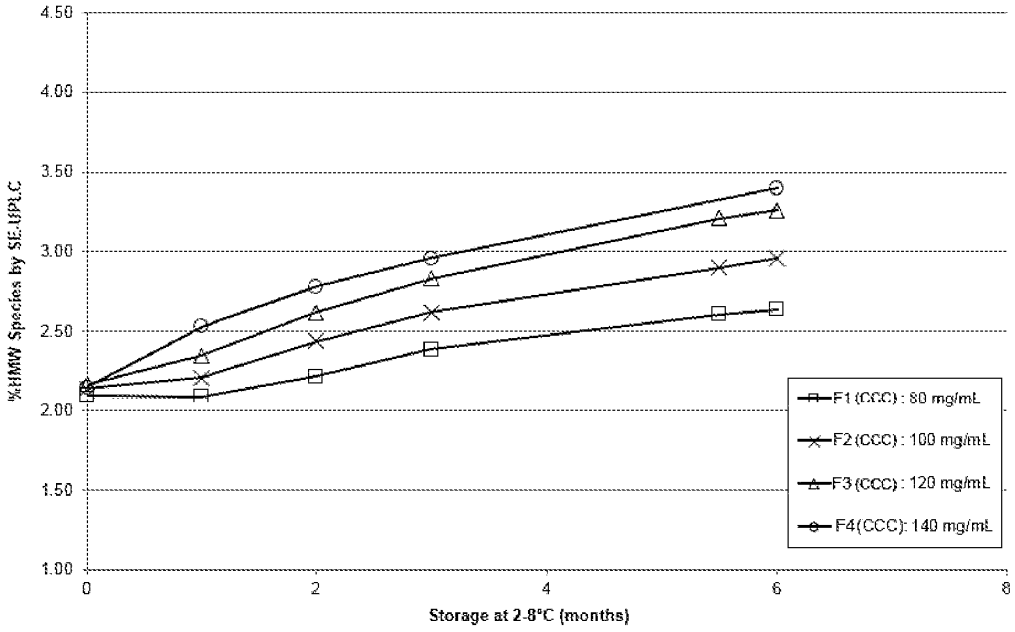

4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
%HMW Species by SE-UPLC
0
2
4
6
8
Storage at 2-8°C (months)
F1 (CCC) : 80 mg/mL
F2 (CCC) : 100 mg/mL
F3 (CCC) : 120 mg/mL
F4 (CCC): 140 mg/mL

FIG. 20A

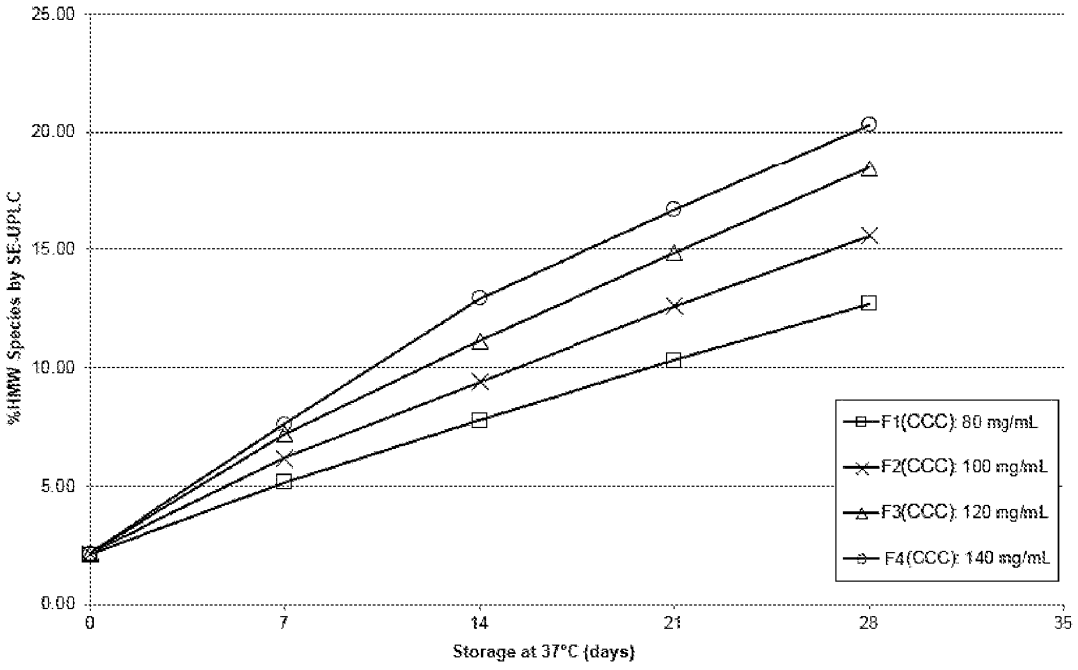

25.00
20.00
15.00
10.00
5.00
0.00
%HMW Species by SE-UPLC
0
7
14
21
28
35
Storage at 37°C (days)
F1(CCC): 80 mg/mL
F2(CCC): 100 mg/mL
F3(CCC): 120 mg/mL
F4(CCC): 140 mg/mL

FIG. 20B

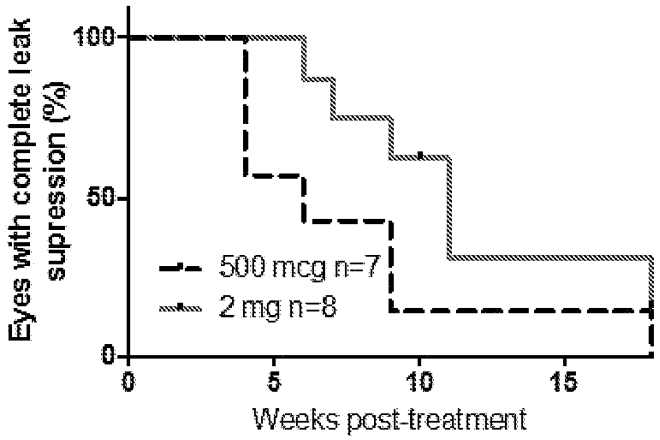

Eyes with complete leak supression (%)
100
50
0
0
5
10
15
Weeks post-treatment
500 mcg n=7
2 mg n=8

FIG. 21

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Filer:	Lance A. Termes
Attorney Docket Number:	125984-266215

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	320	320
UTILITY SEARCH FEE	1111	1	700	700
UTILITY EXAMINATION FEE	1311	1	800	800
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1820**

Electronic Acknowledgement Receipt	
EFS ID:	43331918
Application Number:	17384070
International Application Number:	
Confirmation Number:	1061
Title of Invention:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
First Named Inventor/Applicant Name:	Kenneth S. Graham
Customer Number:	147803
Filer:	Lance A. Termes
Filer Authorized By:	
Attorney Docket Number:	125984-266215
Receipt Date:	23-JUL-2021
Filing Date:	
Time Stamp:	15:14:52
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1820
RAM confirmation Number	E20217MF15330215
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	10430US02-ADS.pdf	132849 ef37349a9660ad0960aff636b599641bcb4bf297	no	6
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
2		10430US02-Application.pdf	566297 06f130b41da5e1c4bdd24f1a7bd30a44751519ae	yes	89

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Specification	1	76
	Claims	77	88
	Abstract	89	89

Warnings:					
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3	Oath or Declaration filed	10430US02-Declarations_Copy.pdf	222653 6a1f35681c36dd4c2f31c9dedd8aadbe2fa89f79	no	2
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4		10430US02-Preliminary_Amendment.pdf	106052 94c315756a1aee30fd6b188a9623d5b51edcc04f	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1

	Claims		2	4	
	Applicant Arguments/Remarks Made in an Amendment		5	5	
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5	Drawings-only black and white line drawings	10430US02-Figures.pdf	4552789 12a8b0a5b65aad7164ae9ccce7587f4dcb2f4511	no	37
Warnings:					
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6	Sequence Listing (Text File)	10430US02-Sequence.txt	6385	no	-
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7	Fee Worksheet (SB06)	fee-info.pdf	35202 b24699be058056e373943ed68f2e7a1d5e20b243	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	5622227		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Application Data Sheet

Application Information

Application number:	Unassigned
Filing Date (YYYY-MM-DD):	Herewith
Application Type:	Nonprovisional
Subject Matter:	Utility
Suggested classification:	
Suggested Group Art Unit:	
CD-ROM or CD-R??:	
Number of CD disks:	
Number of copies of CDs:	
Sequence Submission:	Yes
Computer Readable Form (CRF)?:	Yes
Number of copies of CRF:	1
Title:	High Concentration VEGF Receptor Fusion Protein Containing Formulations
Attorney Docket Number:	125984-266215
Request for Early Publication:	No
Request for Non-Publication:	No
Suggested Drawing Figure:	
Total Drawing Sheets:	37
Small Entity?:	No
Latin name:	
Variety denomination name:	
Petition included?:	No
Petition Type:	
Licensed US Govt. Agency:	
Contract or Grant Numbers One:	
Secrecy Order in Parent Appl.:	No

PGR2026-00039
Biocon Ex.1002
Page 4840 of 4945

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PGR2026-00039
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Page 4841 of 4945

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Email Address:

Publication Information

☐ Request Early Publication:
(Fee required at time of Request 37 CFR 1.219)

☐ Request Not to Publish:
I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information

Representative Customer Number: 147803

Domestic Benefit/National Stage Information

Application:	Continuity Type:	Prior Application No.:	Filing Date (YYYY-MM-DD):
This Application	Continuation of	16/409,631	2019-05-10
16/409,631	An application claiming the benefit under 35 USC 119(e)	62/669,506	2018-05-10
16/409,631	An application claiming the benefit under 35 USC 119(e)	62/752,127	2018-10-29
16/409,631	An application claiming the benefit under 35 USC 119(e)	62/769,876	2018-11-20
16/409,631	An application claiming the benefit under 35 USC 119(e)	62/813,882	2019-03-05

PGR2026-00039
Biocon Ex.1002
Page 4842 of 4945

Foreign Priority Information

Application Number:	Country:	Filing Date (YYYY-MM-DD):	Access Code (if applicable)

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

PGR2026-00039
Biocon Ex.1002
Page 4843 of 4945

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Applicant Information

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Person who shows sufficient proprietary interest:	
If applicant is the legal representative, indicate the Authority to file the patent application, the inventor is:	
Name of the Deceased or Legally Incapacitated Inventor:	
If the Applicant is an Organization check here:	☒
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PGR2026-00039
Biocon Ex.1002
Page 4844 of 4945

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If the Applicant is an Organization check here: ☐

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Submitted by:

Signature	**/Lance A. Termes/**	Date	July 23, 2021
Printed Name	Lance A. Termes	Registration Number	43,184

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HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application is a continuation of U.S. Application No. 16/409,631, filed May 10, 2019, which claims the benefit of U.S. Provisional Patent Application No. 62/813,882, filed March 5, 2019; U.S. Provisional Patent Application No. 62/769,876, filed November 20, 2018; U.S. Provisional Patent Application No. 62/752,127, filed October 29, 2018; and U.S. Provisional Patent Application No. 62/669,506, filed May 10, 2018; each of which is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[002] Embodiments herein relate generally to high concentration VEGF receptor fusion protein containing formulations suitable for ocular administration. More particularly, embodiments herein provide liquid pharmaceutical formulations for intravitreal administration, where the formulations include more than 40 mg/ml VEGF receptor fusion protein, and show pharmaceutically acceptable potency, stability, viscosity and pH.

SEQUENCE LISTING

[003] An official copy of the sequence listing is submitted concurrently with the specification electronically via EFS-Web as an ASCII formatted sequence listing with a file name of "10430US02-Sequence.txt", a creation date of July 23, 2021, and a size of 6,385 bytes. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[004] The development of a therapeutically useful liquid formulation requires a combination of ingredients, at differing amounts, to provide a functional and stable delivery vehicle for a drug of interest. This is particularly true where the drug is a protein, and furthermore, an antibody. Antibody formulations have long presented challenges to drug developers and manufacturers, as antibody activity and administration typically require a pharmaceutically acceptable potency, osmolality, protein stability, viscosity, and proper pH.

PGR2026-00039
Biocon Ex.1002
Page 4846 of 4945

These challenges are exacerbated when the antibody is at higher concentrations in the formulation, for example, beyond 20 to 40 mg/ml, the concentration of most pharmaceutically sold antibodies.

[005] Higher concentration antibody formulations allow for shorter injection times, smaller injection volumes, lower frequency of antibody administration, and more efficient manufacturing and storage utility. However, as noted above, the higher the antibody or protein concentration, the more difficult it is to maintain the proper activity and delivery parameters for the formulation. In particular, high concentration antibody and protein formulations often contend with increased protein aggregation and viscosity, which results in lower overall antibody or protein potency, and lower manufacturing and poorer storage stability. Because of the issues related to high concentration protein or antibody formulations, few such pharmaceutically acceptable formulations have been developed. There is a need in the art for preparing high concentration, high stability, antibody and protein formulations that include the proper potency, stability, viscosity, osmolality and pH.

[006] One such protein in need of high concentration formulations is the vascular endothelial growth factor (VEGF) receptor fusion protein. VEGF receptor fusion proteins are used to block VEGF function in a number of ophthalmic formulations, for example, EYLEA® (Regeneron Pharmaceuticals, Inc.). High concentration VEGF receptor fusion protein containing formulations could allow for shorter ocular injection times, smaller injection volumes, fewer possible injections per administration cycle, and more efficient manufacturing and storage.

[007] The present invention is directed toward overcoming one or more of the problems discussed above.

SUMMARY OF THE INVENTION

[008] Various embodiments described herein encompass high concentration protein containing formulations (*e.g.*, which are suitable for intravitreal administration), and in particular, high concentration vascular endothelial growth factor (VEGF) receptor fusion protein containing formulations. High concentration VEGF receptor fusion protein containing formulations provide a number of therapeutic and economic benefits, including, pharmaceutically acceptable potency, long term manufacturing and storage stability, and a viscosity and pH compatible with ocular injection. High concentration VEGF receptor fusion

PGR2026-00039
Biocon Ex.1002
Page 4847 of 4945

protein containing formulations also allow for reduced ocular administration volumes, a benefit for avoiding undesired effects on the limited volume of the eye.

[009] Embodiments herein provide formulations having a VEGF receptor fusion protein, a buffer, a thermal stabilizer, a viscosity reducing agent, and a surfactant. In other embodiments, the formulations do not include a viscosity reducing agent. Formulations of the present invention have a pH and viscosity suitable for injection, and in particular, for therapeutic ocular injection.

[0010] In an embodiment of the invention, a pharmaceutical formulation of the present invention is provided having a VEGF receptor fusion protein at a concentration of at least 41 mg/ml or a concentration that contains a single dose of VEGF receptor fusion protein (which is discussed herein) in less than about 100 µl, less than about 50 µl, about 50 µl, about 57 µl, about 60 µl, about 70 µl, or about 75 µl, a buffer, optionally a thermal stabilizer and/or a viscosity reducing agent, and a surfactant, where the formulation has a pH of from about 5.0 to about 6.8 (*e.g.*, 5.8).

[0011] In an embodiment of the invention, the VEGF receptor fusion protein concentration is about 30 mg/ml, 60 mg/ml, 114 mg/ml, 120 mg/ml or 140 mg/ml.

[0012] In an embodiment of the invention, the surfactant can be a non-ionic surfactant (*e.g.*, about 0.02% to about 0.1%; or about 0.03% (w/v)). In an embodiment of the invention, the surfactant is a non-ionic surfactant having a polyoxyethylene moiety, for example, polysorbate 20 (PS20), polysorbate 80 (PS80), poloxamer 188, polyethylene glycol 3350 or mixtures thereof.

[0013] In an embodiment of the invention, the buffer is a histidine-based buffer (*e.g.*, 10 mM or 20 mM), such as histidine, histidine HCl or histidine acetate; a phosphate-based buffer, such as sodium phosphate (*e.g.*, 10 mM); an acetate-based buffer, such as sodium acetate and acetic acid, or a citrate-based buffer, such as sodium citrate or citric acid. In an embodiment of the invention, if the buffer is phosphate buffer, the pH is from about 5.7 to about 8.0, about 5.8 to about 8.0, about 5.7 to about 7.0, about 5.8 to about 7.0, about 5.9 to about 7.0 or about 6.0 to about 7.0; if the buffer is histidine buffer, the pH is from about 5.5 to about 6.5; if the buffer is citrate buffer, the pH is from about 3.0 to about 6.2 or about 5.0 to about 6.0; and if the buffer is acetate buffer, the pH is from about 3.7 to about 5.6 or about 5.0 to about 6.0.

[0014] In an embodiment of the invention, the thermal stabilizer is a sugar, such as sucrose (*e.g.*, about 2.5%, 5% or 8%, 10% or 20% (w/v), *e.g.*, about 2-20%), mannitol, sorbitol,

PGR2026-00039
Biocon Ex.1002
Page 4848 of 4945

or trehalose; L-proline (*e.g.*, about 2%, 3% or 4% (w/v)); glycine (*e.g.*, about 50 mM), glycerol, taurine (*e.g.*, about 50 mM) or propane sulfonic acid (*e.g.*, about 50 mM) or any combination of the above.

[0015] In some embodiments of the invention, the pharmaceutical formulations of the present invention include a viscosity reducing agent, for example, arginine hydrochloride (*e.g.*, L-arginine monohydrochloride) (*e.g.*, 50 mM), lysine, sodium chloride (*e.g.*, 40 mM or 50 mM), or magnesium chloride. Alternatively, in other embodiments, the formulations of the present invention specifically exclude substantially all, if not all, viscosity reducing agents.

[0016] In an embodiment of the invention, the pharmaceutical formulations of the present invention comprise, consists of or consists essentially of the constituents in any one of formulations A-KKKK as set forth herein.

[0017] In aspects herein, the pharmaceutical formulations of the present invention may include a VEGF receptor fusion protein (*e.g.*, aflibercept or conbercept) at a concentration of from about 41 mg/ml to about 275 mg/ml, from about 80 mg/ml to about 275 mg/ml, about 140 mg/ml to about 150 mg/ml, about 140 mg/ml to about 159 or 160 mg/ml; including about 60 mg/ml, about 80 mg/ml, about 100 mg/ml, about 113.3 mg/ml, about 114.3 mg/ml, about 120 mg/ml, about 133.3 mg/ml, about 140 mg/ml, about 150 mg/ml, about 200 mg/ml or about 250 mg/ml. In an embodiment of the invention, the formulation comprises about 40 mg/ml of the VEGF receptor fusion protein.

[0018] Some aspects of the pharmaceutical formulations of the present invention include a thermal stabilizer at a concentration of from about 2% (w/v) to about 9% (w/v) or from about 4% (w/v) to about 9% (w/v), with the proviso, that when the thermal stabilizer is taurine or propane sulfonic acid, the stabilizer is at a concentration of from about 25 mM to about 100 mM, surfactant is at a concentration of from about 0.02% (w/v) to about 0.1% (w/v) (*e.g.*, about 0.03%), and buffer is of from about 5 mM to about 15 mM.

[0019] The VEGF receptor fusion protein can, for example:

- be encoded by the nucleic acid sequence of SEQ ID NO:1 or nucleotides 79-1374 or 79-1371 of SEQ ID NO: 1;
- comprise amino acids of SEQ ID NO: 2 or amino acids 27-457 or 27-458 of SEQ ID NO: 2;
- comprise:

PGR2026-00039
Biocon Ex.1002
Page 4849 of 4945

(1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2;

(2) a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2;

(3) a multimerization component ("FcΔC1(a)") comprising amino acids 232 to 457 or 458 of SEQ ID NO:2 (the C-terminal amino acids of SEQ ID NO:2, *i.e.*, K458, may or may not be included in the VEGF receptor fusion proteins). Note that amino acids 1 to 26 of SEQ ID NO:2 are the signal sequence;

- comprise an immunoglobin-like (Ig) domain 2 of a first VEGF receptor (*e.g.*, VEGFR1) and Ig domain 3 of a second VEGF receptor (*e.g.*, VEGFR2), optionally further including an Ig domain 4 of the second VEGF receptor (*e.g.*, VEGFR2) and a multimerizing component (*e.g.*, Fc domain of IgG);
- be conbercept; or
- be aflibercept.

VEGF Trap

atggtcagctactgggacaccggggtcctgctgtgcgcgctgctcagctgtctgcttctc
acaggatctagttccggaagtgataccggtagacctttcgtagagatgtacagtgaaatc
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cctaacatcactgttactttaaaaaagtttccacttgacactttgatccctgatggaaaa
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gaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaatga
(SEQ ID NO: 1)

MVSYWDTGVLLCALLSCLLLTGSSSGSDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIP
DGKRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLSPSHGIELSVGEKLVLNCTART
ELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEK
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP
SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO: 2)

AAs 1-26 = Signal Sequence

AAs 27-129 = Flt1-D2 (VEGFR1-D2)

AAs 130-231 = Flk1-D3 (VEGFR2-D3)

AAs 232-458 = FcΔC1

[0020] In another embodiment of the present invention, a container containing an aqueous solution of from about 5 mM to about 25 mM pharmaceutically acceptable buffer, from about 4% (w/v) to about 9% (w/v) of a pharmaceutically acceptable thermal stabilizer, from about 0.02% (w/v) to about 0.1% (w/v) of a pharmaceutically acceptable surfactant, and from about 41 mg/ml to about 275 mg/ml VEGF receptor fusion protein, is provided. When the thermal stabilizer includes either taurine or propane sulfonic acid, the taurine or propane sulfonic acid is at a concentration of from about 25 mM to about 100 mM. The aqueous solution has a pH of from about 5.0 to about 6.8, and can be from about 5.5 to about 6.2 (*e.g.*, 5.8). In some aspects, the container is a vial or syringe. In other aspects, the aqueous solution does not include an inorganic salt. When the thermal stabilizer is a sugar it can be sucrose, mannitol, sorbitol or trehalose, and can be present at between about 4% (w/v) and 9% (w/v); the surfactant can be a non-ionic surfactant, and can include a polyoxyethylene moiety, such as polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350, at a concentration of from about 0.02% to about 0.1% weight per volume (w/v), and more typically, 0.02% to about 0.04% (w/v). In an embodiment of the invention, the VEGF receptor fusion protein is encoded by the nucleic acid sequence of SEQ ID NO:1 or nucleotides 79-1374 or 79-1371 of SEQ ID NO: 1; comprises

amino acids of SEQ ID NO: 2 or amino acids 27-457 or 27-458 of SEQ ID NO: 2; comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2; and (3) a multimerization component ("FcΔC1(a)") comprising amino acids 232 to 457 of SEQ ID NO:2 (the C-terminal amino acids of SEQ ID NO:2, *i.e.*, K458, may or may not be included in the VEGF receptor fusion proteins); comprises an immunoglobin-like (Ig) domain 2 of a first VEGF receptor (*e.g.*, VEGFR1) and Ig domain 3 of a second VEGF receptor (*e.g.*, VEGFR2), optionally further including an Ig domain 4 of the second VEGF receptor (*e.g.*, VEGFR2) and a multimerizing component (*e.g.*, Fc domain of IgG); is conbercept or is aflibercept.

[0021] In still other embodiments, pharmaceutical formulations of the present invention are provided having a VEGF receptor fusion protein in an aqueous vehicle, where the aqueous vehicle has a viscosity of from about 10 cP to about 15 cP at 20°C, more typically about 10 cP to about 13 cP at 20°C, and most typically from about 11 cP to about 12 cP at 20°C (*e.g.*, about 6.0, 7.3, 11.5 or 12.0 cP at 20°C). In an embodiment of the invention, the viscosity is about 12 cP to about 15 cP at 20°C. The pH of the formulation can be from about 5.8 to about 6.5 (*e.g.*, about 5.8). In some cases, the formulations do not include a viscosity reducing agent, for example, do not include arginine hydrochloride, lysine, sodium chloride, or magnesium chloride. In other cases, the formulations include 10 mM histidine hydrochloride or 10 mM histidine-acetate. The sugar can be sucrose, mannitol, sorbitol or trehalose, and can be present at between about 4% (w/v) and 9% (w/v) (*e.g.*, about 5%); the surfactant can be a non-ionic surfactant, and can include a polyoxyethylene moiety, such as polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350, at a concentration of from about 0.02% to about 0.1% weight per volume (w/v), and more typically, 0.02% to about 0.04% (w/v) (*e.g.*, 0.03%). In addition, the formulation can additionally include a thermal stabilizer selected from either taurine or propane sulfonic acid at a concentration of between about 25 mM to about 100 mM, and more typically about, 50 mM to about 70 mM. In an embodiment of the invention, a formulation comprises VEGF receptor fusion protein, such as aflibercept, (*e.g.*, at a concentration of about 41-275 mg/ml, about 50 mg/ml, about 100 mg/ml, about 115 mg/ml, about 125 mg/ml, about 150 mg/ml, about 200 mg/ml or any of the "high" concentrations discussed herein), about 10 mM histidine-based buffer, about 5% (w/v) sucrose, about 0.03% (w/v) non-ionic surfactant such as polysorbate *e.g.*, polysorbate 20, and about 50 mM arginine, L-arginine or L-arginine

PGR2026-00039
Biocon Ex.1002
Page 4852 of 4945

monohydrochloride, with a pH of about 5.8. In an embodiment of the invention, the VEGF receptor fusion protein is encoded by the nucleic acid sequence of SEQ ID NO:1 or nucleotides 79-1374 or 79-1371 of SEQ ID NO: 1; comprises amino acids of SEQ ID NO: 2 or amino acids 27-457 or 27-458 of SEQ ID NO: 2; comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2; and (3) a multimerization component ("FcΔC1(a)") comprising amino acids 232 to 457 of SEQ ID NO:2 (the C-terminal amino acids of SEQ ID NO:2, *i.e.*, K458, may or may not be included in the VEGF receptor fusion proteins); comprises an immunoglobin-like (Ig) domain 2 of a first VEGF receptor (*e.g.*, VEGFR1) and Ig domain 3 of a second VEGF receptor (*e.g.*, VEGFR2), optionally further including an Ig domain 4 of the second VEGF receptor (*e.g.*, VEGFR2) and a multimerizing component (*e.g.*, Fc domain of IgG); is conbercept or is aflibercept.

[0022] In yet another embodiment, a pharmaceutical formulation of the present invention is provided that includes a VEGF receptor fusion protein at a concentration of from about 80 mg/ml to about 275 mg/ml in a pharmaceutically acceptable buffer. In some cases, the stable pharmaceutical formulations can also include taurine or propane sulfonic acid. The formulations can be stable at a temperature of about 2°C to about 8°C for about 24-36 months. In some cases, the VEGF receptor fusion protein shows less than about a 5% increase in high molecular weight species, and more typically, less than about 4.5% increase in high molecular weight species, less than about 4.0% increase in high molecular weight species, less than about 3.5% increase in high molecular weight species, less than about 3.0% increase in high molecular weight species, less than about 2.5% increase in high molecular weight species, less than about 2.0 increase in high molecular weight species and/or less than about 1.5% increase in high molecular weight species, when stored at these temperatures and for this amount of time. In other cases, the VEGF receptor fusion protein shows less than a 2.0% to 3.0% increase in high molecular weight species when the formulations are stored at these temperatures and for this amount of time.

[0023] Embodiments herein can also include pharmaceutical formulations of the present invention that include aflibercept, a pH buffered solution, a sugar and a surfactant, where the aflibercept is at a concentration of 41 mg/ml to about 275 mg/ml. Alternatively, embodiments can be stable liquid pharmaceutical formulations that include conbercept, a pH buffered solution, a sugar and a surfactant, where the conbercept is at a concentration of 41 mg/ml to about 275

PGR2026-00039
Biocon Ex.1002
Page 4853 of 4945

mg/ml. Stable liquid pharmaceutical formulations can also include taurine or propane sulfonic acid. In some aspects, the aflibercept or conbercept is at a concentration of either 80 mg/ml or 150 mg/ml. In other aspects, the formulations do not include an inorganic salt, sodium chloride for example.

[0024] Some embodiments are directed to sterile syringes such that the syringes are pre-filled with an aqueous solution having 80 mg/ml aflibercept, conbercept, 10 mM histidine hydrochloride, histidine acetate or sodium phosphate, 5% (w/v) sucrose, mannitol, sorbitol or trehalose , 0.03% (w/v) polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350, and 40 mM sodium chloride. Aspects herein include the further addition of about 25 mM to about 100 mM, and more typically about 50 mM to about 70 mM, of either taurine or propane sulfonic acid. In some aspects, the syringes are pre-filled with an aqueous solution having 80 mg/ml aflibercept, conbercept, 10 mM histidine HCl, histidine acetate or sodium phosphate, 8% (w/v) sucrose, mannitol, sorbitol or trehalose, and 0.03% (w/v) polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350. Aspects herein include the further addition of about 25 mM to about 100 mM, and more typically about 50 mM to about 70 mM, of either taurine or propane sulfonic acid. In still other aspects, the syringes are pre-filled with an aqueous solution having 150 mg/ml aflibercept, conbercept, 10 mM histidine hydrochloride, histidine acetate, or sodium phosphate, 5% (w/v) sucrose, mannitol, sorbitol or trehalose, 0.03% (w/v) polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350 and 40 mM sodium chloride, with a pH of about 6.2. Aspects herein can include the further addition of either taurine or propane sulfonic acid. In yet still other aspects, the syringes are pre-filled with an aqueous solution having 150 mg/ml aflibercept, conbercept, 10 mM histidine hydrochloride, histidine acetate, or sodium phosphate, 8% (w/v) sucrose, mannitol, sorbitol or trehalose, and 0.03% (w/v) polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350, with a pH of about 6.2 and the aqueous solution does not include an inorganic salt. Aspects can further include about 25 mM to about 100 mM, and more typically, about 50 mM to about 70 mM, of either taurine or propane sulfonic acid. In yet still other aspects, the syringes are pre-filled with an aqueous solution having 150 mg/ml aflibercept or conbercept, 10 mM sodium acetate or acetic acid, 5% (w/v) glycerol and 0.03% (w/v) polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350 and 40 mM sodium chloride, with a pH of about 6.2. In yet still other aspects, the syringes are pre-filled with an aqueous solution having 150 mg/ml aflibercept or

PGR2026-00039
Biocon Ex.1002
Page 4854 of 4945

conbercept, 10 mM sodium acetate or acetic acid, 8% (w/v) glycerol , and 0.03% (w/v) polysorbate 20, polysorbate 80, poloxamer 188, or polyethylene glycol 3350 and 40 mM sodium chloride, with a pH of about 6.2.

[0025] The present invention also provides a method for making any formulation set forth herein comprising the step of combining each component of the formulation into a single composition. Such a method may include the step of adding the resulting formulation into a vial or injection device. Any composition that is the product of such a method also forms part of the present invention. For example, embodiments herein also include methods for preparing a formulation by combining a histidine-based, citrate-based, acetate-based or phosphate-based buffer, with sucrose, polysorbate 20 and a VEGF receptor fusion protein; and, optionally, one or more additional components, *e.g.*, as discussed herein. In some cases, the formulation is prepared to also include taurine or propane sulfonic acid, but where it does not include taurine or propane sulfonic acid, it may also not include an inorganic salt. In aspects herein, the sucrose is at a weight per volume of from about 4% to about 10%, the polysorbate 20 has a weight per volume of about 0.02% to about 0.1%, and the receptor fusion protein is at a concentration of from about 41 mg/ml to about 275 mg/ml. Where the formulation includes taurine or propane sulfonic acid, it is present at, for example, about 25 mM to about 100 mM, but may also be included at 50 mM to 70 mM. The method can include loading a predetermined volume of prepared formulation into a sterile syringe, such that the volume has a 0.1 mg to 10 mg dose of the VEGF receptor fusion protein.

[0026] The present invention also provides a method for administering a formulation of the present invention to a subject (*e.g.*, a human) comprising intraocularly injecting (*e.g.*, intravitreal injection) the formulation into the eye of the subject. The present invention also provides a method for administering a formulation of the present invention to a subject (*e.g.*, a human) comprising implanting an intravitreal implant that contains a formulation of the present invention into the vitreous of the subject.

[0027] Embodiments herein also include methods for treating an angiogenic eye disorder, *e.g.*, age-related macular degeneration (wet), macular edema following retinal vein occlusion, retinal vein occlusion (RVO), central retinal vein occlusion (CRVO) branch retinal vein occlusion (BRVO), diabetic macular edema (DME), choroidal neovascularization (CNV), iris neovascularization, neovascular glaucoma, post-surgical fibrosis in glaucoma, proliferative

vitreoretinopathy (PVR), optic disc neovascularization, corneal neovascularization, retinal neovascularization, vitreal neovascularization, pannus, pterygium, vascular retinopathy or diabetic retinopathy (*e.g.*, non-proliferative diabetic retinopathy and/or proliferative diabetic retinopathy) in a subject in need thereof by intraocularly injecting at least about 2 mg (*e.g.*, 4 mg, 6 mg or 8 mg) of VEGF receptor fusion protein (*e.g.*, aflibercept or conbercept) into the eye of a subject in need thereof, for example, any formulation set forth herein. In an embodiment of the invention, the injection volume is 100 microliters or less (*e.g.*, 100 microliters, 50 microliters or 57 microliters). The method comprises an intravitreal injection of a pre-mixed aqueous solution having a VEGF receptor fusion protein at a concentration of from 41 mg/ml to about 275 mg/ml, a pharmaceutically acceptable sugar, a pharmaceutically acceptable buffer, and a pharmaceutically acceptable surfactant. Treatment methods using the pre-mixed aqueous solution do not require dilution. In aspects herein, the pre-mixed aqueous solution has a pH from about 6.0 and about 6.5, and has a viscosity of between about 10 cP and 13 cP.

[0028] In aspects of the treatment method, the VEGF receptor fusion protein comprises VEGFR1R2-FcΔC1(a) encoded by the nucleic acid sequence of SEQ ID NO:1, amino acids 27 to 129 of SEQ ID NO:2, a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2, or a multimerization component comprising amino acids 232 to 457 of SEQ ID NO:2, or any combination thereof. In an embodiment of the invention, the VEGF receptor fusion protein is aflibercept or conbercept.

[0029] Other embodiments in accordance with the disclosure herein will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] The above and other aspects, features and advantages of embodiments disclosed herein will become more apparent to those of ordinary skill in the art with reference to the accompanying drawings.

[0031] **Figure 1A** is a graph determining VEGF receptor fusion protein (aflibercept) stability in four different formulations (B, H, D and G) over the course of 36 months at 2°C to 8°C. VEGF receptor fusion protein stability was tested using size-exclusion ultra-performance liquid chromatography (SE-UPLC) to identify formation of high molecular weight (HMW) species (a sign of protein degradation).

PGR2026-00039
Biocon Ex.1002
Page 4856 of 4945

[0032] **Figure 1B** is also a graph determining VEGF receptor fusion protein (aflibercept) stability in four formulations (B, H, D and G) over the course of 36 months at 2°C to 8°C. VEGF receptor fusion protein stability was tested using SE-UPLC to identify the percent of the main species in each formulation.

[0033] **Figure 1C** is a bar graph showing formation of VEGF receptor fusion protein charge variants in four formulations (B, H, D and G) over the course of 36 months at 2°C to 8°C. The level of acidic species was tested at manufacture (0.0), 12 months, 24 months and 36 months using imaged capillary isoelectric focusing (iCIEF).

[0034] **Figure 1D** is a bar graph showing formation of VEGF receptor fusion protein charge variants in four formulations (B, H, D and G) over the course of 36 months at 2°C to 8°C. Identification of the main species was tested at manufacture (0.0), 12 months, 24 months and 36 months using iCIEF.

[0035] **Figure 2A** is a graph showing 150 mg/ml VEGF receptor fusion protein (aflibercept) stability in a 10 mM sodium phosphate, 8% (w/v) sucrose and 0.03% polysorbate 20 (w/v) formulation pH6.2 with and without L-arginine monohydrochloride over the course of 28 days at 37°C. VEGF receptor fusion protein stability was tested using SE-UPLC to identify formation of HMW species.

[0036] **Figure 2B** is a graph showing 150 mg/ml VEGF receptor fusion protein stability in a 10 mM sodium phosphate, 8% sucrose (w/v) and 0.03% polysorbate 20 (w/v) formulation pH6.2 with and without L-arginine monohydrochloride over the course of 28 days at 37°C. VEGF receptor fusion protein stability was tested using SE-UPLC to identify the percent of the main species in each formulation.

[0037] **Figure 3A** is a bar graph showing viscosity of 10 mM sodium phosphate buffered formulations having 155 mg/ml VEGF receptor fusion protein and no salt, 100 mM arginine, 200 mM arginine, 50 mM lysine, 200 mM lysine, 50 mM sodium chloride or 100 mM sodium chloride. Viscosity was measured in cP at 20°C.

[0038] **Figure 3B** is a bar graph showing viscosity of 10 mM histidine buffered formulations having 155 mg/ml VEGF receptor fusion protein and no salt, 100 mM arginine, 200 mM arginine, 50 mM lysine, 200 mM lysine, 50 mM sodium chloride or 100 mM sodium chloride. Viscosity was measured in cP at 20°C.

PGR2026-00039
Biocon Ex.1002
Page 4857 of 4945

[0039] **Figure 3C** is a graph showing viscosity of various concentrations of VEGF receptor fusion protein under different formulation conditions (with or without 50 mM arginine hydrochloride). Viscosity was measured in cP at 20°C.

[0040] **Figure 4A** is a graph showing 150 mg/ml VEGF receptor fusion protein stability in 10 mM sodium phosphate, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, pH6.2 with and without 50 mM L-arginine monohydrochloride over 12 months at 5°C. The VEGF receptor fusion protein was at 150 mg/ml. VEGF receptor fusion protein stability was tested using SE-UPLC to identify formation of HMW species.

[0041] **Figure 4B** is a graph showing 150 mg/ml VEGF receptor fusion protein stability in 10 mM sodium phosphate, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, pH6.2 with and without 50 mM L-arginine monohydrochloride over 12 months at 5°C. The VEGF receptor fusion protein was at 150 mg/ml. VEGF receptor fusion protein stability was tested using SE-UPLC to identify the percent of the main species in each formulation.

[0042] **Figure 5** is a graph that shows viscosity of formulations under two buffer conditions at 20°C, 10 mM phosphate buffer or 10 mM histidine buffer. The VEGF receptor fusion protein concentration is varied from 10 mg/ml to 170 mg/ml. Viscosity was measured in mPa-S.

[0043] **Figure 6** shows a dynamic light scattering screen for a number of different formulation conditions, each having from 2 to 10 mg/ml protein. The diffusion interaction parameter indicated that taurine and PSA (propane sulfonic acid) improved Kd.

[0044] **Figure 7** is a graph monitoring the percentage of high molecular weight (HMW) species in formulations F1-F9 (formulations WW-EEE) over time as measured by size-exclusion ultra-performance liquid chromatography (SE-UPLC) after storage at 5°C for 6 months. Formulations F1-F9 are set forth in Table 7-1 herein.

[0045] **Figures 8A and 8B** measure the percentage of high molecular weight species, by SE-UPLC, after storage at 5°C for 3 months (Fig. 8A) or incubation at 37°C for 28 days (Fig. 8B). Formulations F1-F4 (EEE, SSS, CCC (140 mg/ml) and TTT) are set forth in Table 8-1 below.

[0046] **Figure 9** shows the viscosity (cp) for Formulations F1-F12, at 20°C, at the initial time point. Formulations F1-F12 are set forth in Table A below.

PGR2026-00039
Biocon Ex.1002
Page 4858 of 4945

Table A. Formulations F1-F12.

Formulation #	VEGF Trap (mg/mL)	Buffer	Other Excipients	Screening Excipients (mM)
F1 (GGG)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium sulfate
F2 (HHH)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium thiocyanate
F3 (III)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	40mM Sodium citrate
F4 (JJJ)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Glycine
F5 (KKK)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium chloride
F6 (LLL)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Lysine
F7 (MMM)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium aspartate
F8 (NNN)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium glutamate
F9 (OOO)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium Citrate + 50mM Arginine hydrochloride
F10 (PPP)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Glycine + 50mM Arginine hydrochloride
F11 (QQQ)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium aspartate + 50mM Arginine hydrochloride
F12 (RRR)	140	20 mM Histidine, pH 5.8	5% Sucrose, 0.03% Polysorbate 20	50mM Sodium glutamate + 50mM Arginine hydrochloride

PGR2026-00039
Biocon Ex.1002
Page 4859 of 4945

[0047] **Figure 10** shows the osmolality (mmol/Kg) of Formulations F1-F12 (set forth in Table A herein).

[0048] **Figures 11A and 11B** measure the percentage of high molecular weight species in formulations F1-F6 (Formulations GGG, HHH, III, JJJ, LLL and KKK) over time, by SE-UPLC, after storage at 37°C for up to 28 days (Fig. 11A) or 5°C for up to 3 months (Fig. 11B) over time.

[0049] **Figure 12** shows the dynamic light scattering (diffusion coefficient (cm^2/second), radius (nm) and %Pd) at the initial time point of the formulations shown in Table 9-3 (GGG-RRR).

[0050] **Figures 13A, 13B, 13C and 13D** show the baseline FA images and OCT (30 degree lens) for two different rabbits (326-OS and 329-OD) before administration of aflibercept (OD=*oculus dexter* (right eye); OS=*oculus sinister* (left eye)). (Fig. 13A) FA image for rabbit 326, left eye; (Fig. 13B) OCT image for rabbit 326, left eye; (Fig. 13C) FA image for rabbit 329, right eye; (Fig. 13D) OCT image for rabbit 329, right eye.

[0051] **Figures 14A, 14B, 14C, 14D, 14E and 14F** show a time course of rabbit FA images at days 1 (Fig. 14A), 7 (Fig. 14B) and 14 (Fig. 14C) and the OCT at days 1 (Fig. 14D), 7 (Fig. 14E) and 14 (Fig. 14F) for one rabbit (326-OS) administered the histidine buffer formulation (30 degree lens) (7 mg/eye).

[0052] **Figures 15A, 15B, 15C, 15D, 15E, 15F, 15G and 15H** show a time course of rabbit FA images at 3 weeks (Fig. 15A), 4 weeks (Fig. 15B), 7 weeks (Fig. 15C) and 8 weeks (Fig. 15D); and the OCT at 3 weeks (Fig. 15E), 4 weeks (Fig. 15F), 7 weeks (Fig. 15G) and 8 weeks (Fig. 15H) for one rabbit (326-OS) administered the histidine buffer formulation (55 degree lens) (7 mg/eye).

[0053] **Figures 16A, 16B, 16C, 16D, 16E and 16F** show a time course of rabbit FA images at day 1 (Fig. 16A), week 1 (Fig. 16B) and week 2 (Fig. 16C) and the OCT at day 1 (Fig. 16D), week 1 (Fig. 16E) and week 2 (Fig. 16F) for one rabbit (329-OS) administered the phosphate buffer formulation (30 degree lens) (7 mg/eye).

[0054] **Figures 17A, 17B, 17C, 17D, 17E, 17F, 17G and 17H** show a time course of rabbit FA images at week 3 (Fig. 17A), week 4 (Fig. 17B), week 7 (Fig. 17C) and week 8 (Fig. 17D) and the OCT at week 3 (Fig. 17E), week 4 (Fig. 17F), week 7 (Fig. 17G) and week 8 (Fig.

PGR2026-00039
Biocon Ex.1002
Page 4860 of 4945

17H) for one rabbit (329-OS) administered the phosphate buffer formulation (55 degree lens) (7 mg/eye).

[0055] **Figures 18A and 18B** show the purity (percentage of native species) (Fig. 18A) and percentage of high molecular weight (HMW) species (Fig. 18B) in formulations UUU-BBBB over time at 37ºC (up to one month) (see Table 11-1) as analyzed by SE-UPLC.

[0056] **Figures 19A, 19B, 19C, 19D and 19E** show stability and purity analysis of a formulation having 114.3 mg/mL VEGF Trap (aflibercept) formulated in 10 mM Histidine, pH 5.8, 5% Sucrose, 0.03% Polysorbate 20, 50 mM Arginine Monohydrochloride by size exclusion chromatographic determination of the percentage of (i) high molecular weight species (HMW) after incubation at 5ºC or 37ºC for up to 2 months (Fig. 19A) and (ii) main species (Main peak) after incubation at 5ºC or 37ºC for up to 2 months (Fig. 19B); as well as by microflow imaging to determine the presence of subvisible particulate matter after incubation at 37°C for up to 28 days (Fig. 19C), by particle light obscuration analysis to determine the presence of subvisible particulate matter after incubation at 37°C for up to 28 days (Fig. 19D), and by microscopic determination of subvisible particulate matter after incubation at 37°C for up to 28 days (Fig. 19E).

[0057] **Figures 20A and 20B** show stability and purity analysis of formulation CCC, having 80, 100, 120 or 140 mg/ml aflibercept, by SE-UPLC determination of the percentage of high molecular weight species (HMW) after incubation at 2-8ºC for up to 6 months (Fig. 20A) or 37ºC for up to 28 days (Fig. 20B).

[0058] **Figure 21** shows the percentage of eyes in rabbits tested with complete leak suppression over time, when administered 500 micrograms or 2 mg aflibercept, over time (Gehan-Breslow-Wilcoxon test (P 0.0453)).

DETAILED DESCRIPTION

[0059] The present invention provides formulations having high concentrations of VEGF receptor fusion proteins (*e.g.*, aflibercept), showing excellent functional and storage properties which were developed in spite of significant technical hurdles. For example, a common method for identifying suitable excipients for a formulation that includes a polypeptide drug (such as a VEGF receptor fusion protein) is by assessment of polypeptide stability under accelerated stress conditions, such as high temperature (*e.g.*, 37ºC). Excipients, which are not suitable under non-

PGR2026-00039
Biocon Ex.1002
Page 4861 of 4945

stress conditions (*e.g.*, a low temperature such as 5ºC), will typically cause an undesired effect, within a short period of time, while under stress, *e.g.*, protein aggregation. This approach is common in the biotechnology and pharmaceutical industry insofar as it expedites the elimination of excipients unlikely to stabilize the drug product. See *e.g.*, Magari, *Assessing Shelf Life Using Real-Time and Accelerated Stability Tests*, Biopharm Intl. 16(11): 36-48 (2003). In some cases, a product may be released based on accelerated stability data, but this must be done in parallel with real-time shelf-life analysis (non-accelerated). Magari (2003) and FDA, *Guidelines for Submitting Documentation for the Stability of Human Drugs and Biologics*, Rockville, MD (1987). Here, however, at 5ºC, the presence of arginine in histidine formulations was stabilizing, though arginine, it appeared, tended to decrease stability when under temperature stress (37ºC). See Figure 8 (A-B). This property of the formulations set forth herein would have presented a technical difficulty guiding a practitioner away from choosing arginine; thus, making it unlikely that a practitioner would have chosen arginine as an excipient. Nevertheless, the formulations set forth herein resulted from the overcoming of technical hurdles such as this to achieve a formulation with high stability. Formulation of VEGF trap in histidine buffer also led to a beneficial decrease in viscosity relative to that observed with a phosphate buffer formulation. Since small needle bores are preferred for performing intravitreal injections (due to decreased patient discomfort and eye trauma), a relative low viscosity is desirable. A lower viscosity formulation requires less force to push the formulation through the needle, thus making injection of the formulation through the needle easier for the treating physician. In addition, histidine and arginine containing formulation were well tolerated in rabbit eyes.

[0060] Reference will now be made in detail to representative embodiments. It should be understood that the following descriptions are not intended to limit the embodiments to one preferred embodiment. To the contrary, they are intended to cover alternatives, modifications, and equivalents as can be included within the spirit and scope of the described embodiments as defined in the appended claims.

[0061] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure pertains. As used herein, the term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. So

PGR2026-00039
Biocon Ex.1002
Page 4862 of 4945

for example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (*e.g.*, 99.1%, 99.2%, 99.3%, 99.4%, *etc.*).

[0062] In an embodiment of the invention, a pharmaceutical formulation of the present invention is compliant with USP<789> for small volume injection (SVP) of ophthalmic solutions, *e.g.*, contains less than about 50 particles ≥10 μm in diameter per ml; contains less than about 5 particles ≥25 μm in diameter per ml; or contains less than about 2 particles ≥50 μm in diameter per ml.

[0063] Note that for purposes herein, "intravitreal injection" refers to injection into the vitreous of the eye (near the retina at the back of the eye). The expressions “suitable for intravitreal administration,” “suitable for intravitreal injection,” and the like mean that the formulation in question can be safely injected into the vitreous of a subject’s eye without causing any adverse reactions beyond those known to be associated with intravitreal injection of EYLEA.

[0064] The term "pharmaceutical formulation" herein refers to formulations including pharmaceutically acceptable carriers, *e.g.*, used to administer VEGF receptor fusion proteins (*e.g.*, aflibercept or conbercept) to a subject for a therapeutic/medicinal use.

[0065] The term "pharmaceutically acceptable" refers to a formulation that within the scope of sound medical judgement is suitable for administration with the eye.

[0066] The term "subject" herein refers to any mammalian (*e.g.*, rabbit, mouse, rat or monkey) subject, particularly a human, *e.g.*, for whom diagnosis, prognosis or therapy is desired with the formulations as described herein.

[0067] The term “aqueous” formulation refers to a formulation which includes water.

[0068] The term "treat" or "treatment" refers to a therapeutic measure that reverses, stabilizes or eliminates an undesired disease or disorder (*e.g.*, an angiogenic eye disorder or cancer), for example, by causing the regression, stabilization or elimination of one or more symptoms or indicia of such disease or disorder by any clinically measurable degree, *e.g.*, with regard to an angiogenic eye disorder, by causing a reduction in or maintenance of diabetic retinopathy severity score (DRSS), by improving or maintaining vision (*e.g.*, in best corrected visual acuity *e.g.*, as measured by an increase in ETDRS letters), increasing or maintaining visual field and/or reducing or maintaining central retinal thickness and, with respect to cancer, stopping or reversing the growth, survival and/or metastasis of cancer cells in the subject.

PGR2026-00039
Biocon Ex.1002
Page 4863 of 4945

Typically, the therapeutic measure is administration of one or more doses of a therapeutically effective amount of VEGF receptor fusion protein to the subject with the disease or disorder.

[0069] "Prevent" or "prevention" refer to a prophylactic measure to cease the development of an undesired disease or disorder (*e.g.*, an angiogenic eye disorder).

[0070] SE-UPLC can be used in the present invention to quantitate the presence of high molecular weight species in a formulation. SE refers to size exclusion chromatography. UPLC refers to ultra-performance liquid chromatography. Suitable SE columns, which may be used in a UPLC system, to characterize such HMW species of VEGF receptor fusion proteins (*e.g.*, aflibercept or conbercept) in a formulation, are capable of resolving molecules in the molecular weight range of about 10,000–450,000 Daltons. See for example, the ACQUITY UPLC Protein BEH SEC 200Å Column. In an embodiment of the invention, two of such columns are connected in tandem when quantitating HMW species in a formulation. UPLC brings improvements over HPLC (high performance liquid chromatography) in sensitivity and resolution. UPLC uses instrumentation that operates at high pressures and uses finer particles (typically less than about 2.5µm) than that used in HPLC. Moreover, the UPLC mobile phases operate at high linear velocities than HPLC.

[0071] Embodiments herein include formulations that comprise a high concentration (*e.g.*, about 60 mg/ml, about 80 mg/ml, about 90 mg/ml, about 100 mg/ml, about 113.3 mg/ml, about 114.3 mg/ml, about 120 mg/ml, about 133.3 mg/ml, about 140 mg/ml, about 150 mg/ml, about 200 mg/ml or about 250 mg/ml) of VEGF receptor fusion protein (*e.g.*, aflibercept or conbercept). Suitable formulations included herein comprise a high concentration of VEGF receptor fusion protein, a buffer, a thermal stabilizer and a surfactant. In some aspects, a suitable formulation further includes a viscosity reducing agent. In other aspects, the suitable formulation substantially excludes all viscosity reducing agents. Typical formulations have a pH of from about 5.0 to about 6.8 (*e.g.*, 5.8), but can include any pH useful for administration of the VEGF receptor fusion protein to the eye of a subject.

[0072] The present invention includes formulations that include a VEGF receptor fusion protein (*e.g.*, aflibercept or conbercept) in association with one or more further therapeutic agents (*e.g.*, an Ang-2 inhibitor (*e.g.*, an anti-ANG2 antibody or antigen-binding fragment thereof or nesvacumab), a Tie-2 receptor activator, an anti-PDGF, PDGF receptor or PDGF receptor beta antibody or antigen-binding fragment thereof and/or an additional VEGF antagonist such as

PGR2026-00039
Biocon Ex.1002
Page 4864 of 4945

bevacizumab, ranibizumab, pegaptanib or a soluble form of human vascular endothelial growth factor receptor-3 (VEGFR-3) comprising extracellular domains 1-3, expressed as an Fc-fusion protein) as well as methods of prevention or treatment comprising administering such formulations as discussed herein. In an embodiment of the invention, a formulation of the present invention includes a VEGF receptor fusion protein, such as aflibercept, but excludes any further therapeutic agent (*e.g.*, which is an antibody or antigen-binding fragment thereof).

[0073] The term "in association with" indicates that a formulation and a further therapeutic agent can be formulated into a single composition, *e.g.*, for simultaneous delivery, or formulated separately into two or more compositions (*e.g.*, a kit). The further therapeutic agent may, itself, be formulated in its own pharmaceutical formulation. Each can be administered to a subject at the same time as the other or at a different time than when the other is administered; for example, each administration may be given non-simultaneously (*e.g.*, separately or sequentially) at intervals over a given period of time. Moreover, the formulation and the further therapeutic agent may be administered to a subject by the same or by a different route.

[0074] In an embodiment of the invention, a formulation of the present invention includes any one or more of sodium sulfate (*e.g.*, 50 mM); sodium thiocyanate (*e.g.*, 50 mM); sodium citrate (*e.g.*, 40 mM); glycine (*e.g.*, 50 mM); sodium chloride (*e.g.*, 50 mM); lysine (*e.g.*, 50 mM); sodium aspartate (*e.g.*, 50 mM); and/or sodium glutamate (*e.g.*, 50 mM). For example, in an embodiment the formulation comprises a combination of sodium citrate (*e.g.*, 50 mM) and arginine hydrochloride (*e.g.*, 50 mM); glycine (*e.g.*, 50 mM) and arginine hydrochloride (*e.g.*, 50 mM); sodium aspartate (*e.g.*, 50 mM) and arginine hydrochloride (*e.g.*, 50 mM); or sodium glutamate (*e.g.*, 50 mM) and arginine hydrochloride (*e.g.*, 50 mM).

VEGF Receptor Fusion Proteins and Other VEGF inhibitors

[0075] For purposes herein, a “VEGF receptor fusion protein” refers to a molecule that comprises one or more VEGF receptors or domains thereof, fused to another polypeptide, which interferes with the interaction between VEGF and a natural VEGF receptor, *e.g.*, wherein two of such fusion polypeptides are associated thereby forming a homodimer or other multimer. Such VEGF receptor fusion proteins may be referred to as a "VEGF-Trap" or “VEGF Trap”. VEGF receptor fusion proteins within the context of the present disclosure that fall within this definition include, chimeric polypeptides which comprise two or more immunoglobulin (Ig)-like domains

PGR2026-00039
Biocon Ex.1002
Page 4865 of 4945

of a VEGF receptor such as VEGFR1 (also known as Flt1) and/or VEGFR2 (also known as Flk1 or KDR), and may also contain a multimerizing domain (for example, an Fc domain).

[0076] An exemplary VEGF receptor fusion protein is a molecule referred to as VEGF1R2-FcΔC1(a) which is encoded by the nucleic acid sequence of SEQ ID NO:1 or nucleotides 79-1374 or 79-1371 thereof.

[0077] VEGF1R2-FcΔC1(a) comprises three components:

(1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2;

(2) a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2; and

(3) a multimerization component ("FcΔC1(a)") comprising amino acids 232 to 457 of SEQ ID NO:2 (the C-terminal amino acids of SEQ ID NO:2, *i.e.*, K458, may or may not be included in the VEGF receptor fusion proteins, see U.S. Patent No. 7,396,664 or 7,354,579, incorporated herein for all purposes). Note that amino acids 1 to 26 of SEQ ID NO:2 are the signal sequence. In an embodiment of the invention, the VEGF receptor fusion protein comprises amino acids 27-458 or 27-457 of SEQ ID NO: 2.

[0078] In an embodiment of the invention, the VEGF receptor fusion protein comprises

(1) an immunoglobin-like (Ig) domain 2 of a first VEGF receptor (*e.g.*, VEGFR1), and

(2) an Ig domain 3 of a second VEGF receptor (*e.g.*, VEGFR2),

(3) and, optionally, further including an Ig domain 4 of the second VEGF receptor (*e.g.*, VEGFR2) and

(4) a multimerizing component (*e.g.*, Fc domain of IgG).

For example, in an embodiment of the invention, the VEGF receptor fusion protein has the following arrangement of said domains:

- [VEGFR1 Ig domain 2]-[VEGFR2 Ig domain 3]-[MC] (*e.g.*, a homodimer thereof) or
- [VEGFR1 Ig domain 2]-[VEGFR2 Ig domain 3]-[VEGFR2 Ig domain 4]-[MC] (*e.g.*, a homodimer thereof).

[0079] In an embodiment of the invention, the VEGF receptor fusion protein is a VEGF mini-trap which is a VEGF trap molecule with a truncated multimerizing component (*e.g.*, Fc), *e.g.*, wherein the mini-trap still includes an Fc hinge region. See *e.g.*, WO2005/00895 or U.S. Patent No. 7,396,664.

PGR2026-00039
Biocon Ex.1002
Page 4866 of 4945

[0080] Note that the present disclosure also includes, within its scope, high concentration formulations including, instead of a VEGF receptor fusion proteins, VEGF binding molecules and anti-VEGF antibodies and antigen-binding fragments thereof,

- bevacizumab (*e.g.*, at a concentration of about 80-90 or 88 mg/ml),
- ranibizumab (*e.g.*, at a concentration of about 20-40 mg/ml, *e.g.*, 21-35, 21 or 35 mg/ml),
- an anti-VEGF aptamer such as pegaptanib (*e.g.*, pegaptanib sodium),
- a single chain (*e.g.*, V_L-V_H) anti-VEGF antibody such as brolucizumab (*e.g.*, at a concentration of about 200-400 or 200, 210, 400 or 420 mg/ml),
- an anti-VEGF DARPin such as the Abicipar Pegol DARPin (*e.g.*, at a concentration of about 70-140, 70 or 140 mg/ml), or
- a bispecific anti-VEGF antibody, *e.g.*, which also binds to ANG2, such as RG7716 (*e.g.*, at a concentration of about 100-400, 100, 105, 400 or 420 mg/ml).

In order to minimize the repetitiveness of the embodiments discussed herein, it is contemplated that the scope of the present invention includes embodiments wherein any of the formulations discussed herein include, in place of a VEGF receptor fusion protein, an anti-VEGF antibody or antibody fragment or other VEGF binding molecule as discussed herein (*e.g.*, substituted with an anti-VEGF DARPin) at any of the concentrations discussed herein. For example, the present invention includes a formulation having 35 or 80 mg/ml ranibizumab, a buffer, a thermal stabilizer, a viscosity reducing agent and a surfactant.

[0081] DARPins are Designed Ankyrin Repeat Proteins. DARPins generally contain three to four tightly packed repeats of approximately 33 amino acid residues, with each repeat containing a β-turn and two anti-parallel α-helices. This rigid framework provides protein stability whilst enabling the presentation of variable regions, normally comprising six amino acid residues per repeat, for target recognition.

[0082] An "anti-VEGF" antibody or antigen-binding fragment of an antibody refers to an antibody or fragment that specifically binds to VEGF.

[0083] Illustrative VEGF receptor fusion proteins include aflibercept (EYLEA®, Regeneron Pharmaceuticals, Inc.) or conbercept (sold commercially by Chengdu Kanghong Biotechnology Co., Ltd.). See International patent application publication no. WO2005/121176 or WO2007/112675. The terms "aflibercept" and "conbercept" include biosimilar versions thereof. A biosimilar version of a reference product (*e.g.*, aflibercept) generally refers to a

PGR2026-00039
Biocon Ex.1002
Page 4867 of 4945

product comprising the identical amino acid sequence, but includes products which are biosimilar under the U.S. Biologics Price Competition and Innovation Act.

[0084] Pharmaceutical formulations of the present invention are “high concentration”. High concentration pharmaceutical formulations of the present invention include VEGF receptor fusion protein at a concentration of at least 41 mg/ml, of at least 80 mg/ml, of at least 100 mg/ml, of at least 125 mg/ml, of at least 140 mg/ml, of at least 150 mg/ml, of at least 175 mg/ml, of at least 200 mg/ml, of at least 225 mg/ml, of at least 250 mg/ml, or of at least 275 mg/ml. Alternatively, "high concentration” can refer to formulations that include a concentration of VEGF receptor fusion protein of from about 140 mg/ml to about 160 mg/ml, at least about 140 mg/ml but less than 160 mg/ml, from about 41 mg/ml to about 275 mg/ml, from about 70 mg/ml to about 75 mg/ml or from about 80 mg/ml to about 250 mg/ml. In some aspects, the VEGF receptor fusion protein concentration in the formulation is about any of the following concentrations: 41 mg/ml; 42 mg/ml; 43 mg/ml; 44 mg/ml; 45 mg/ml; 46 mg/ml; 47 mg/ml; 48 mg/ml; 49 mg/ml; 50mg/ml; 51 mg/ml; 52 mg/ml; 53 mg/ml; 54 mg/ml; 55 mg/ml; 56 mg/ml; 57 mg/ml; 58 mg/ml; 59 mg/ml; 60 mg/ml; 61 mg/ml; 62 mg/ml; 63 mg/ml; 64 mg/ml; 65 mg/ml; 66 mg/ml; 67 mg/ml; 68 mg/ml; 69 mg/ml; 70 mg/ml; 71 mg/ml; 72 mg/ml; 73 mg/ml; 74 mg/ml; 75 mg/ml; 76 mg/ml; 77 mg/ml; 78 mg/ml; 79 mg/ml; 80 mg/ml; 81 mg/ml; 82mg/ml; 83 mg/ml; 84 mg/ml; 85 mg/ml; 86 mg/ml; 87mg/ml; 88 mg/ml; 89 mg/ml; 90 mg/ml; 91 mg/ml; 92 mg/ml; 93 mg/ml; 94 mg/ml; 95mg/ml; 96 mg/ml; 97 mg/ml; 98 mg/ml; 99 mg/ml; 100 mg/ml; 101 mg/ml; 102 mg/ml; 103 mg/ml; 104 mg/ml; 105 mg/ml; 106mg/ml; 107 mg/ml; 108 mg/ml; 109 mg/ml; 110 mg/ml; 111 mg/ml; 112 mg/ml; 113 mg/ml; 113.3 mg/ml; 114 mg/ml; 114.1 mg/ml; 114.2 mg/ml; 114.3 mg/ml; 114.4 mg/ml; 114.5 mg/ml; 114.6 mg/ml, 114.7 mg/ml, 114.8 mg/ml; 114.9 mg/ml; 115 mg/ml; 116 mg/ml; 117 mg/ml; 118 mg/ml; 119 mg/ml; 120 mg/ml; 121 mg/ml; 122 mg/ml; 123 mg/ml; 124 mg/ml; 125 mg/ml; 126 mg/ml; 127mg/ml; 128 mg/ml; 129 mg/ml; 130 mg/ml; 131 mg/ml; 132 mg/ml; 133 mg/ml; 133.3 mg/ml; 133.4 mg/ml, 134 mg/ml; 135 mg/ml; 136 mg/ml; 137 mg/ml; 138 mg/ml; 139 mg/ml; 140 mg/ml; 141 mg/ml; 142 mg/ml; 143 mg/ml; 144 mg/ml; 145 mg/ml; 146 mg/ml; 147 mg/ml; 148 mg/ml; 149 mg/ml; 150 mg/ml; 151 mg/ml; 152 mg/ml; 153 mg/ml; 154mg/ml; 155 mg/ml; 156 mg/ml; 157mg/ml; 158 mg/ml; 159 mg/ml; 160 mg/ml; 161 mg/ml; 162 mg/ml; 163 mg/ml; 164 mg/ml; 165 mg/ml; 166 mg/ml; 167 mg/ml; 168 mg/ml; 169 mg/ml; 170 mg/ml; 171 mg/ml; 172 mg/ml;

PGR2026-00039
Biocon Ex.1002
Page 4868 of 4945

173 mg/ml; 174 mg/ml; 175 mg/ml; 176 mg/ml; 177 mg/ml; 178 mg/ml; 179 mg/ml; 180 mg/ml; 181 mg/ml; 182 mg/ml; 183 mg/ml; 184 mg/ml; 185 mg/ml; 186 mg/ml; 187 mg/ml; 188 mg/ml; 189 mg/ml; 190 mg/ml; 191 mg/ml; 192 mg/ml; 193 mg/ml; 194 mg/ml; 195 mg/ml; 196 mg/ml; 197 mg/ml; 198 mg/ml; 199 mg/ml; 200 mg/ml; 201 mg/ml; 202 mg/ml; 203 mg/ml; 204 mg/ml; 205 mg/ml; 206 mg/ml; 207 mg/ml; 208 mg/ml; 209 mg/ml; 210 mg/ml; 211 mg/ml; 212 mg/ml; 213 mg/ml; 214 mg/ml; 215 mg/ml; 216 mg/ml; 217 mg/ml; 218 mg/ml; 219 mg/ml; 220 mg/ml; 221 mg/ml; 222 mg/ml; 223 mg/ml; 224 mg/ml; 225 mg/ml; 226 mg/ml; 227 mg/ml; 228 mg/ml; 229 mg/ml; 230 mg/ml; 231 mg/ml; 232 mg/ml; 233 mg/ml; 234 mg/ml; 235 mg/ml; 236 mg/ml; 237mg/ml; 238 mg/ml; 239 mg/ml; 240 mg/ml; 241 mg/ml; 242 mg/ml; 243 mg/ml; 244 mg/ml; 245 mg/ml; 246 mg/ml; 247 mg/ml; 248 mg/ml; 249 mg/ml; 250 mg/ml; 251 mg/ml; 252 mg/ml; 253 mg/ml; 254 mg/ml; 255 mg/ml; 256 mg/ml; 257 mg/ml; 258 mg/ml; 259 mg/ml; 260 mg/ml; 261 mg/ml; 262 mg/ml; 263 mg/ml; 264 mg/ml; 265 mg/ml; 266 mg/ml; 267 mg/ml; 268 mg/ml; 269 mg/ml; 270 mg/ml; 271 mg/ml; 272 mg/ml; 273 mg/ml; 274 mg/ml; or 275 mg/ml. Other VEGF receptor fusion protein concentrations are contemplated herein, as long as the concentration functions in accordance with embodiments herein.

[0085] In an embodiment of the invention, a pharmaceutical formulation of the present invention is of such a concentration as to contain about 4, 6, 8, 10, 12, 14, 16, 18 or 20 mg VEGF receptor fusion protein (*e.g.*, aflibercept), or the amount of such protein in any of the acceptable doses thereof which are discussed herein, in about 100 µl or less, about 75 µl or less or about 70 µl or less, *e.g.*, about 50 µl; 51 µl; 52 µl; 53 µl; 54 µl; 55 µl; 56 µl; 57 µl; 58 µl; 59 µl; 60 µl; 61 µl; 62 µl; 63 µl; 64 µl; 65 µl; 66 µl; 67 µl; 68 µl; 69 µl; 70 µl; 71 µl; 72 µl; 73 µl; 74 µl; 75 µl; 76 µl; 77 µl; 78 µl; 79 µl; 80 µl; 81 µl; 82 µl; 83 µl; 84 µl; 85 µl; 86 µl; 87 µl; 88 µl; 89 µl; 90 µl; 91 µl; 92 µl; 93 µl; 94 µl; 95 µl; 96 µl; 97 µl; 98 µl; 99 µl; or 100 µl.

[0086] The present invention includes any of the formulations set forth under "*Illustrative Formulations*" herein, but wherein the concentration of the VEGF receptor fusion protein (*e.g.*, aflibercept) is substituted with a concentration which is set forth in this section ("VEGF Receptor Fusion Proteins and Other VEGF inhibitors").

PGR2026-00039
Biocon Ex.1002
Page 4869 of 4945

Buffers

[0087] Buffers for use herein refer to solutions that resist pH change by use of acid-base conjugates. Buffers are capable of maintaining pH in the range of from about 5.0 to about 6.8, and more typically, from about 5.8 to about 6.5, and most typically, from about 6.0 to about 6.5. In some cases, the pH of the formulation of the present invention is about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, or about 6.8. Example buffers for inclusion in formulations herein include histidine-based buffers, for example, histidine, histidine hydrochloride, and histidine acetate. Buffers for inclusion in formulations herein can alternatively be phosphate-based buffers, for example, sodium phosphate, acetate-based buffers, for example, sodium acetate or acetic acid, or can be citrate-based, for example, sodium citrate or citric acid. It is also recognized that buffers can be a mix of the above, as long as the buffer functions to buffer the formulations in the above described pH ranges. In some cases, the buffer is from about 5 mM to about 25 mM, or more typically, about 5 mM to about 15 mM. Buffers can be about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 11 mM, about 12 mM, about 13 mM, about 14 mM, about 15 mM, about 16 mM, about 17 mM, about 18 mM, about 19 mM, about 20 mM, about 21 mM, about 22 mM, about 23 mM, about 24 mM, or about 25 mM.

[0088] In an embodiment of the invention, a histidine-based buffer is prepared using histidine and histidine monohydrochloride.

Surfactants

[0089] Surfactant for use herein refers to ingredients that protect the higher concentration of VEGF receptor fusion protein from various surface and interfacial induced stresses. As such, surfactants can be used to limit or minimize VEGF receptor fusion protein aggregation, and promote protein solubility. Suitable surfactants herein have been shown to be non-ionic, and can include surfactants that have a polyoxyethylene moiety. Illustrative surfactants in this category include: polysorbate 20, polysorbate 80, poloxamer 188, polyethylene glycol 3350, and mixtures thereof. Surfactants in the formulations can be present at from about 0.02% to about 0.1% weight per volume (w/v), and more typically, about 0.02% to about 0.04% (w/v). In some cases, the surfactant is about 0.02% (w/v), about 0.03% (w/v), about 0.04% (w/v), about 0.05%

PGR2026-00039
Biocon Ex.1002
Page 4870 of 4945

(w/v), about 0.06% (w/v), about 0.07% (w/v), about 0.08% (w/v), about 0.09% (w/v), or about 0.1% (w/v).

Thermal Stabilizers

[0090] Thermal stabilizers for use herein refers to ingredients that provide thermal stability against thermal denaturation of the VEGF receptor fusion protein, as well as protect against loss of VEGF receptor fusion protein potency or activity. Suitable thermal stabilizers include sugars, and can be sucrose, trehalose, sorbitol or mannitol, or can be amino acids, for example L-proline, L-arginine (*e.g.*, L-arginine monohydrochloride), or taurine. Additionally, thermal stabilizers may also include substituted acrylamides or propane sulfonic acid, or may be compounds like glycerol.

[0091] In some cases, the formulations herein include both a sugar and taurine, a sugar and an amino acid, a sugar and propane sulfonic acid, a sugar and taurine, glycerol and taurine, glycerol and propane sulfonic acid, an amino acid and taurine, or an amino acid and propane sulfonic acid. In addition, formulations can include a sugar, taurine and propane sulfonic acid, glycerol, taurine and propane sulfonic acid, as well as L-proline, taurine and propane sulfonic acid.

[0092] Embodiments herein typically have thermal stabilizers present alone, each independently present at a concentration of, or present in combination at a total concentration of, from about 2% (w/v) to about 10% (w/v) or 4% (w/v) to about 10% (w/v), or about 4% (w/v) to about 9% (w/v), or about 5% (w/v) to about 8% (w/v). Thermal stabilizers in the formulation can be at a concentration of about 2% (w/v), about 2.5% (w/v), about 3% (w/v), about 4% (w/v), about 5% (w/v), about 6% (w/v), about 7% (w/v), about 8% (w/v), about 9% (w/v), about 10% (w/v) or about 20% (w/v).

[0093] With respect to taurine and propane sulfonic acid, in an embodiment of the invention, these thermal stabilizers can be present in the formulations at about from 25 mM to about 100 mM, and more typically from about 50 mM to about 75 mM (as compared to the other thermal stabilizers).

PGR2026-00039
Biocon Ex.1002
Page 4871 of 4945

Viscosity Reducing Agents

[0094] Viscosity reducing agents typically are used to reduce or prevent protein aggregation. Viscosity reducing agents for inclusion herein include: sodium chloride, magnesium chloride, D- or L-arginine (*e.g.*, L-arginine monohydrochloride), lysine, or mixtures thereof. When present herein, viscosity reducing agents can be present at from about 10 mM to about 100 mM, and more typically from about 30 mM to about 75 mM, and even more typically from about 40 mM to about 70 mM. In some cases the viscosity reducing agent is present at about 10 mM, about 15 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, about 55 mM, about 60 mM, about 65 mM, about 70 mM, about 75 mM, about 80 mM, about 85 mM, about 90 mM, about 95 mM or about 100 mM.

Formulation Viscosity

[0095] Formulations in accordance with embodiments herein can also have a pharmaceutically acceptable viscosity for ocular administration, for example, intravitreal injection. Viscosity generally refers to the measure of resistance of a fluid which is being deformed by either shear stress or tensile stress (typically measured by techniques known in the art, viscometer or rheometer, for example). Typical viscosities of formulations in accordance with embodiments herein are from about 5.0 cP (centipoise) to about 15 cP, from about 11 cP to about 14 cP, from about 12 cP to about 15 cP or from about 11 cP to about 12 cP. As such, formulation viscosity herein can be about 5.0 cP, about 6.0, about 7.1 cP, about 7.2 cP, about 7.3 cP, about 7.4 cP, about 7.5 cP, about 7.6 cP, about 10 cP, about 10.5 cP, about 11.0 cP, about 11.5 cP, about 12.0 cP, about 12.5 cP, about 13.0 cP, about 13.5 cP, about 14.0 cP, about 14.5 cP, or about 15.0 cP (*e.g.*, when measured at 20°C).

[0096] Various embodiments herein do not require inclusion of an inorganic salt, or other viscosity reducing agent, to maintain these highly useful viscosities. Typically, high concentration protein solutions require viscosity reducing agents to avoid protein aggregation and higher viscosity, making the formulations difficult for intravitreal injection and reducing the potency of the VEGF receptor fusion protein. As such, embodiments herein include formulations that have had substantially no, or no added, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), D- or L-arginine hydrochloride, lysine or other viscosity reducing agent.

PGR2026-00039
Biocon Ex.1002
Page 4872 of 4945

Formulation osmolality

[0097] Osmolality is a critical attribute for injectable formulations. It is desirable to have products match physiological osmotic conditions. Furthermore, osmolality provides confirmation of soluble content in solution. In an embodiment of the invention, the osmolality of a formulation of the present invention is less than or equal to about 506 mmol/Kg or from about 250 to about 506 mmol/Kg., *e.g.*, about 250, 260, 270, 280, 290, 299, 300, 310, 314, 315, 316, 324, 343, 346, 349, 369, 384, 403, 426, 430 or 506 mmol/Kg. In an embodiment of the invention, the osmolality is lower than about 250 mmol/Kg.

Purity and Stability of Formulations

[0098] High concentration VEGF receptor fusion protein containing formulations described herein are stable during manufacture and storage. The term "stable" herein refers to formulations that include VEGF receptor proteins that retain both chemical and physical stability over the period of formulation manufacturing and storage, *e.g.*, that maintains integrity and has minimal degradation, denaturation, or unfolding. VEGF receptor protein stability can be determined using analytical techniques available in the art at different temperatures and over different periods of time. In particular, VEGF receptor chemical stability (potency) can be determined using various bioassays (for example, a BAF/3 VEGFR1/EPOR cell line used to determine $VEGF_{165}$ binding by the VEGF receptor fusion proteins herein), and physical stability, by size exclusion (SE) chromatographic analysis, UPLC (ultra-performance liquid chromatography) size exclusion (SE) chromatography, visual appearance, OD, pH, charge variant formation, and rate of high molecular weight (HMW) species formation. A stable VEGF receptor fusion protein is one that shows limited change in its OD, pH, formation of charge variants, and formation of HMW species.

[0099] A “high molecular weight” (HMW) species as used herein, with reference to a formulation containing a given VEGF Trap (*e.g.*, aflibercept), refers to any species of polypeptide or polypeptide complex in the formulation which elutes from a size exclusion column (*e.g.*, SE-UPLC) ahead of (*e.g.*, with a higher molecular weight than) VEGF Trap polypeptide and/or a homodimer thereof. The percentage of HMW species refers to the percentage of such species relative to the overall quantity of polypeptides in the formulation, *e.g.*, by SE-UPLC analysis.

PGR2026-00039
Biocon Ex.1002
Page 4873 of 4945

[00100] In an embodiment of the invention, as discussed more fully in the Examples below, stable formulations show significant VEGF receptor fusion protein potency and physical stability for a period of up to 12 months, up to 24 months, and/or up to 36 months when stored at about 2°C to about 8°C.

[00101] In an embodiment of the invention, formulation of the present invention is one that:

- After about 28 days at about 37°C has about a 3, 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 30, 35 or 37% (or 10-15% or 15-20% or 10-20%) increase in high molecular weight species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 28 days at about 37°C has about a 5, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22% (or 5-20% or 5-10% or 10-15% or 15-20%) decrease in main species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 28 days at about 37°C, has about 80, 81, 82, 83, 84, 85, 86 or 87% (or about 80-85%) or more protein as main species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 28 days at about 37°C has about a 1 or 1.5 or 2% (or 1-2%) increase in low molecular weight species (*e.g.*, as measured by SE-UPLC or SEC)
- After about one month at about 37°C has about a 16% increase in high molecular weight species; and/or about a 17% decrease in main species and/or about a 0.5% or <1% increase in low molecular weight species; and/or after about two months at about 5°C, has about a 1% decrease in main species, and/or about a 1% increase in high molecular weight species and/or no significant or detectable amounts low molecular weight (LMW) species; and/or after about 2 months at about 5°C, has about 97% main species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 12 months at about 2-8°C has about a 3-3.5% increase in high molecular weight species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 12 months at about 2-8°C has less than about a 1% or a 1, 2, 3 or 4% (*e.g.*, 1-4% or 3-4%) decrease in main species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 12 months at about 2-8°C, has about 94 or 95% or more protein as main species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 3 months at about 2-8°C has about a 1 or 2% increase in high molecular weight species (*e.g.*, as measured by SE-UPLC or SEC)

PGR2026-00039
Biocon Ex.1002
Page 4874 of 4945

- After about 6 months at about 2-8°C has about a <1, 1 or 2% increase in high molecular weight species (*e.g.*, as measured by SE-UPLC or SEC)
- After about $5^1/_2$ or 6 months at about 5°C has about 2.5, 3.0 or 3.5 (or about 2.5-3.5%) total high molecular weight species (*e.g.*, as measured by SE-UPLC or SEC).
- After about 6 months at about 2-8°C has less than about a 1% or 1 or 2% (or about 0.5-2% or 1-2%) decrease in main species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 6 months at about 2-8°C, has about 96 or 97 or 98% (or 96-98%) or more protein as main species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 24 months or 36 months at about 2-8°C, had less than about 5 or 6 or 7% (*e.g.*, about 1.5, 2, 3, 4 or 5%) (or 1.5-5% or 1.5-2.5%) increase in high molecular weight species; and/or has about 3.0, 3.25, 3.5, 4.0, 4.5 or 5% total high molecular weight species; and/or a decrease of about 2 or 3% (or 2-3%) main species; and/or a total amount of main species of about 95 or 96% or more (or 95-96%) (*e.g.*, as measured by SE-UPLC or SEC)
- After about 1 month at about 37°C, has about 97, 98, 99 or 100% (or 97-100%) of aflibercept in the formulation which is recoverable by RP-HPLC
- Immediately after manufacture and purification, has less than about 1.5, 2, 2.5, 3.0 or 3.5% high molecular weight species (*e.g.*, as measured by SE-UPLC or SEC)
- After about 6 months at about 37°C, has at least about 70% or 75% (*e.g.*, 70-75%) of aflibercept as main species/main peak (*e.g.*, as measured by capillary isoelectric focusing (cIEF) or imaged capillary isoelectric focusing) (non-acidic and non-basic species)
- After about 36 months at about 2-8°C, has about a 1 or 2% (or 1-2%) increase in acidic species (*e.g.*, as measured by capillary isoelectric focusing (cIEF) or imaged capillary isoelectric focusing)
- After about 36 months at about 2-8°C, has about a 1% or less decrease in main species/main peak (*e.g.*, as measured by capillary isoelectric focusing (cIEF) or imaged capillary isoelectric focusing)
- After about 36 months at about 2-8°C, has about 78-79% main species/main peak (*e.g.*, as measured by capillary isoelectric focusing (cIEF) or imaged capillary isoelectric focusing)

and/or

PGR2026-00039
Biocon Ex.1002
Page 4875 of 4945

- When administered intravitreally to a mammal, such as a human or rabbit or mouse (*e.g.*, with an angiogenic eye disorder such as wet AMD), does not cause any adverse events that are clinically different from those observed for EYLEA (*e.g.*, when EYLEA is dosed intravitreally at 0.5 or 2.0 mg); or does not cause clinically significant inflammation in the eye, prolonged intraocular pressure (IOP) increases, prolonged increases in blood pressure or decreases and/or retinal detachment.

[00102] Further, in an embodiment of the invention, high concentration VEGF receptor fusion proteins are stable as they show little or no formation of acidic charge variants over the course of manufacture and storage, for example, little or no formation of charge variants as tested by Imaged Capillary Isoelectric Focusing, for example.

[00103] In an embodiment of the invention, a formulation of the present invention exhibits less than or equal to about 8% low molecular weight (LMW) species.

[00104] In an embodiment of the invention, a formulation of the present invention has less than about 0.2, 0.4 or 0.5 EU (endotoxin units)/ml of endotoxin;

[00105] In an embodiment of the invention, a formulation of the present invention is essentially free of particulate matter or particulate matter of about 1, 2, 5, 10, 25 or 50 micrometers (or more) in size.

[00106] In an embodiment of the invention, when a formulation of the present invention is analyzed by non-reduced CE-SDS (SDS capillary gel electrophoresis), at least 97% of the total peak area is the main peak.

[00107] In an embodiment of the invention, when a formulation of the present invention is analyzed by size-exclusion UPLC (SE-UPLC), at least 93, 94 or 95% of the total peak area is the main peak and less than or equal to 3.5, 4, 5 or 6% is aggregate.

[00108] In an embodiment of the invention, a formulation of the present invention is at about 2°C, 3°C, 4°C, 5°C, 6°C, 7°C or 8°C, 2-8°C (*e.g.*, an average temperature of 5°C), 23°C, 25°C, 30°C or 37°C.

Illustrative Formulations

[00109] Illustrative high concentration VEGF receptor fusion protein containing formulations include the following:

PGR2026-00039
Biocon Ex.1002
Page 4876 of 4945

[00110] Formulation A: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00111] Formulation B: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00112] Formulation C: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00113] Formulation D: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.

[00114] Formulation E: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00115] Formulation F: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00116] Formulation G: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00117] Formulation H: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00118] Formulation I: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.0 3% (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00119] Formulation J: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00120] Formulation K: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00121] Formulation L: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

PGR2026-00039
Biocon Ex.1002
Page 4877 of 4945

[00122] Formulation M: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00123] Formulation N: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00124] Formulation O: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00125] Formulation P: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.

[00126] Formulation Q: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00127] Formulation R: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00128] Formulation S: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00129] Formulation T: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2 (*e.g.*, 6.2), and, optionally, specifically excluding a viscosity reducing agent.

[00130] Formulation U: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00131] Formulation V: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00132] Formulation W: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00133] Formulation X: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

PGR2026-00039
Biocon Ex.1002
Page 4878 of 4945

[00134] Formulation Y: 80 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00135] Formulation Z: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00136] Formulation AA: 80 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00137] Formulation BB: 80 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.

[00138] Formulation CC: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00139] Formulation DD: 80 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00140] Formulation EE: 80 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00141] Formulation FF: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00142] Formulation GG: 80 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00143] Formulation HH: 80 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00144] Formulation II: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00145] Formulation JJ: 80 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

PGR2026-00039
Biocon Ex.1002
Page 4879 of 4945

[00146] Formulation KK: 150 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00147] Formulation LL: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00148] Formulation MM: 150 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00149] Formulation NN: 150 mg/ml conbercept, 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2.

[00150] Formulation OO: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00151] Formulation PP: 150 mg/ml conbercept, 10 mM citrate-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

[00152] Formulation QQ: 150 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00153] Formulation RR: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00154] Formulation SS: 150 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00155] Formulation TT: 150 mg/ml conbercept, 10 mM histidine-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00156] Formulation UU: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

[00157] Formulation VV: 150 mg/ml conbercept, 10 mM citrate-based buffer, 8 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 80, with a pH of 5.8 to 6.2, and, optionally, specifically excluding a viscosity reducing agent.

PGR2026-00039
Biocon Ex.1002
Page 4880 of 4945

[00158] Formulation WW: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 50 mM taurine, with a pH of 5.8.

[00159] Formulation XX: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 4 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8.

[00160] Formulation YY: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 2.5 % (w/v) sucrose, 2.0 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM taurine, with a pH of 5.8.

[00161] Formulation ZZ: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM histidine-based buffer, 2.5 % (w/v) sucrose, 2.0 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8.

[00162] Formulation AAA: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8.

[00163] Formulation BBB: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 2.5 % (w/v) sucrose, 2.0 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8.

[00164] Formulation CCC: 80, 100, 120 or 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8.

[00165] Formulation DDD: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM histidine-based buffer, 4 % (w/v) proline, 0.03 % (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8.

[00166] Formulation EEE: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, and 0.03 % (w/v) polysorbate 20 and, optionally, no thermal stabilizer, with a pH of 5.8.

[00167] Formulation FFF: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10mM sodium phosphate, 5 % (w/v) sucrose and 0.03 % polysorbate 20 with a pH of 6.2.

[00168] Formulation GGG: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5% sucrose; 0.03 % polysorbate 20; 50 mM sodium sulfate

PGR2026-00039
Biocon Ex.1002
Page 4881 of 4945

[00169] Formulation HHH: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5% sucrose; 0.03 % polysorbate 20; 50 mM sodium thiocyanate

[00170] Formulation III: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose, 0.03 % polysorbate 20; 40 mM sodium citrate

[00171] Formulation JJJ: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5% Sucrose, 0.03 % polysorbate 20; 50 mM glycine

[00172] Formulation KKK: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose, 0.03 % polysorbate 20; 50 mM sodium chloride

[00173] Formulation LLL: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 50 mM lysine

[00174] Formulation MMM: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium aspartate

[00175] Formulation NNN: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 50 mM sodium glutamate

[00176] Formulation OOO: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium citrate; 50 mM arginine hydrochloride

[00177] Formulation PPP: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM glycine; 50 mM arginine hydrochloride

[00178] Formulation QQQ: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium aspartate; 50 mM arginine hydrochloride

[00179] Formulation RRR: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM histidine, pH 5.8; 5 % sucrose; 0.03% polysorbate 20; 50 mM sodium glutamate; 50 mM arginine hydrochloride

[00180] Formulation SSS: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM His, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 10 mM L-arginine hydrochloride

[00181] Formulation TTT: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept); 20 mM His, pH 5.8; 5 % sucrose; 0.03 % polysorbate 20; 100 mM L-arginine hydrochloride

PGR2026-00039
Biocon Ex.1002
Page 4882 of 4945

[00182] Formulation UUU: 30 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2

[00183] Formulation VVV: 30 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2

[00184] Formulation WWW: 60 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2

[00185] Formulation XXX: 60 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2

[00186] Formulation YYY: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2

[00187] Formulation ZZZ: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, pH 6.2

[00188] Formulation AAAA: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, 50 mM NaCl, pH 6.2

[00189] Formulation BBBB: 120 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 % sucrose, 10 mM phosphate, 0.03 % polysorbate 20, 50 mM NaCl, pH 6.2

[00190] Formulation CCCC: 140 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 10 mM sodium phosphate, 5 % sucrose, 40 mM sodium chloride, 0.03 % PS20, pH 6.2

[00191] Formulation DDDD: 80 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept), 20 mM histidine-based buffer, 5 % (w/v) sucrose, 0.03 % (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8.

[00192] Formulation EEEE: 120.0 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, $\pm$ 12 mg/ml), 20 mM histidine-based buffer (*e.g.*, $\pm$ 2 mM), 5 % (w/v) sucrose (*e.g.*, $\pm$ 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, $\pm$ 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

[00193] Formulation FFFF: 113.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, 102-125 mg/ml), 20 mM histidine-based buffer (*e.g.*, $\pm$ 2 mM), 5 % (w/v) sucrose (*e.g.*, $\pm$ 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, $\pm$ 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

[00194] Formulation GGGG: 114.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, 103-126 mg/ml), 10 mM histidine-based buffer (*e.g.*, $\pm$ 1 mM), 5 % (w/v)

PGR2026-00039
Biocon Ex.1002
Page 4883 of 4945

sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

[00195] Formulation HHHH: 100.0 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, ± 10 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5 % (w/v) sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

[00196] Formulation IIII: 133.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, ± 13 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5 % (w/v) sucrose (*e.g.*, ± 0.5%), 0.03 % (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1).

[00197] Formulation JJJJ: 150 mg/ml aflibercept (*e.g.*, aflibercept) (*e.g.*, ± 15 mg/ml), 10 mM sodium phosphate, 8% (w/v) sucrose (*e.g.*, ± 0.8%), 0.03% (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%) and 50 mM L-arginine hydrochloride, pH 6.2 (*e.g.*, 6.0-6.4 or 5.9-6.5).

[00198] Formulation KKKK: 114.3 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, ± 14 mg/ml), 20 mM histidine-based buffer (*e.g.*, ± 2 mM), 5% (w/v) sucrose (*e.g.*, ± 0.5%), 0.03% (w/v) polysorbate 20 (*e.g.*, 0.02-0.04%), and 50 mM L-arginine monohydrochloride (*e.g.*, ± 5 mM), with a pH of 5.8 (*e.g.*, 5.6-6.0 or 5.5-6.1);

[00199] or any formulation which is set forth herein.

[00200] In an embodiment of the invention, the concentration of any formulation constituent (*e.g.*, all constituents) listed above (*e.g.*, any one of formulations A-KKKK) or which is discussed herein is ± about 3%, 5% or about 10% of the concentration specifically mentioned.

Methods of Manufacture

[00201] Embodiments herein include methods of manufacturing a pharmaceutical formulation of the present invention including a VEGF receptor fusion protein (*e.g.*, any of formulations A-KKKK as set forth herein) comprising combining the components of the formulation into a single composition, and, optionally, introducing the formulation into a vessel or device *e.g.*, a vial, or delivery device, *e.g.*, a pre-filled syringe. The formulations, vials or devices that are the product of such methods are part of the present invention.

[00202] In an embodiment of the invention, the method of manufacturing a VEGF receptor fusion protein containing pharmaceutical formulation of the present invention (*e.g.*, any

PGR2026-00039
Biocon Ex.1002
Page 4884 of 4945

of formulations A-KKKK as set forth herein) comprises the steps of culturing a host cell (*e.g.*, Chinese hamster ovary cell) comprising one or more polynucleotides encoding a VEGF receptor fusion protein (*e.g.*, aflibercept) in a culture medium and under conditions whereby the protein is expressed; and, purifying the protein from the host cell and/or culture medium and combining a portion of the protein with excipients of a pharmaceutical formulation as set forth herein. Again, the formulations, vials or devices that are the product of such methods are part of the present invention.

[00203] In an embodiment of the invention, a determination is made as to the amount and type of VEGF receptor fusion protein needed in the formulation to make it a high concentration and for its end-use. The same determination is made regarding the amount and type of buffer, the amount and type of surfactant, the amount and type of thermal stabilizer, and the inclusion, or specific exclusion, of a viscosity reducing agent. These components are combined and mixed, making sure that the formulation pH is at the desired value, *e.g.*, between about 5.0 and about 6.8 (*e.g.*, 5.8), and/or that the viscosity is at a desired value, *e.g.*, about 6.0, 7.3, 11.5 or 12.0 cP at 20°C. In an embodiment of the invention, high concentration VEGF receptor fusion protein containing formulations can be sterilized and stored in a stable condition, *e.g.*, for up to 24 or 36 months at 2°C to 8°C (*e.g.*, 5°C).

[00204] See, *e.g.*, Hardman, *et al.* (2001) Goodman and Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, N.Y.; Gennaro (2000) Remington: The Science and Practice of Pharmacy, Lippincott, Williams, and Wilkins, New York, N.Y.; Avis, *et al.* (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Disperse Systems, Marcel Dekker, NY; Weiner and Kotkoskie (2000) Excipient Toxicity and Safety, Marcel Dekker, Inc., New York, N.Y.

Angiogenic Eye Disorders and Cancer

[00205] The pharmaceutical formulations of the present invention comprising VEGF receptor fusion proteins (*e.g.*, any of pharmaceutical formulations A-KKKK) can be used in the treatment or prevention of any angiogenic eye disorder by administration of a therapeutically effective amount of VEGF receptor fusion protein in a formulation of the present invention to a subject in need thereof, *e.g.*, by intravitreal injection. Angiogenic eye disorders herein refer to

PGR2026-00039
Biocon Ex.1002
Page 4885 of 4945

any disease of the eye which is caused by or associated with the growth or proliferation of blood vessels and/or by blood vessel leakage. Non-limiting examples of angiogenic eye disorders that are treatable or preventable using the formulations and methods herein, include:

- age-related macular degeneration (wet),
- macular edema,
- macular edema following retinal vein occlusion,
- retinal vein occlusion (RVO),
- central retinal vein occlusion (CRVO),
- branch retinal vein occlusion (BRVO),
- diabetic macular edema (DME),
- choroidal neovascularization (CNV),
- iris neovascularization,
- neovascular glaucoma,
- post-surgical fibrosis in glaucoma,
- proliferative vitreoretinopathy (PVR),
- optic disc neovascularization,
- corneal neovascularization,
- retinal neovascularization,
- vitreal neovascularization,
- pannus,
- pterygium,
- vascular retinopathy,
- diabetic retinopathies (*e.g.*, non-proliferative diabetic retinopathy (*e.g.*, characterized by a Diabetic Retinopathy Severity Scale (DRSS) level of about 47 or 53) or proliferative diabetic retinopathy; *e.g.*, in an subject that does not suffer from DME) and
- Diabetic retinopathy in a patient who has diabetic macular edema (DME).

[00206] The pharmaceutical formulations of the present invention comprising VEGF receptor fusion proteins (*e.g.*, any of pharmaceutical formulations A-KKKK) can be used in the treatment or prevention of any cancer by administration of a therapeutically effective amount of

PGR2026-00039
Biocon Ex.1002
Page 4886 of 4945

VEGF receptor fusion protein in a formulation of the present invention to a subject in need thereof, *e.g.*, by intramuscular, intratumoral, subcutaneous or intravenous injection. Cancers include those whose growth, proliferation, survival and/or metastasis is dependent, to a degree, on angiogenesis. In an embodiment of the invention, the cancer is colorectal cancer, lung cancer, skin cancer, breast cancer, brain cancer, stomach cancer, renal cancer, prostate cancer, liver cancer or pancreatic cancer.

[00207] Thus, the present invention provides methods for treating or preventing an angiogenic eye disorder in a subject in need thereof comprising administering a therapeutically effective amount of VEGF receptor fusion protein (*e.g.*, aflibercept) (*e.g.*, about 4, 6, or 8.0, 8.1, 8.4 or 8.5 mg), *e.g.*, in a pharmaceutical formulation according to the present invention, intraocularly, *e.g.*, into the vitreous, of an eye of the subject. In an embodiment of the invention, both eyes are administered the VEGF receptor fusion protein. In an embodiment of the invention, therapeutically effective doses of the VEGF receptor fusion protein are administered about every 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 17 weeks 18 weeks, 19 weeks, 20 weeks, 21, weeks, 22 weeks, 23 weeks or 24 weeks. In an embodiment of the invention, such methods of treatment or prevention are done in the absence of significant increases in blood pressure (systolic and/or diastolic) and/or the development of hypertension (*e.g.*, Grade 1, Grade 2 or Grade 3) and/or abnormally high intraocular pressure in the subject. In an embodiment of the invention, the method includes the step of, following said administration, monitoring the subject for significant increases in blood pressure (systolic and/or diastolic) and/or the development of hypertension (*e.g.*, Grade 1, Grade 2 or Grade 3) and/or abnormally high intraocular pressure.

Modes of Administration

[00208] The pharmaceutical formulations of the present invention which include a VEGF receptor fusion protein may be administered in accordance with known medically approved delivery systems. In embodiments herein, these delivery systems may include administering the formulations to the patient by ocular, intraocular, intrachoroidal, intravitreal or subconjunctival injection. Alternatively, pharmaceutical formulations of the present invention can also be administered to the patient by topical routes, *e.g.*, eye drops, eye gels, eye ointments, and the

PGR2026-00039
Biocon Ex.1002
Page 4887 of 4945

like. Other possible routes of delivery for the formulations herein include intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural and oral.

[00209] In an embodiment of the invention, intravitreal injection of a pharmaceutical formulation of the present invention includes the step of piercing the eye with a syringe and needle (*e.g.*, 30-gauge injection needle) containing the formulation and injecting the formulation (*e.g.*, less than or equal to about 100 microliters; about 40, 50, 55, 56, 57, 57.1, 58, 60, 70 or 75 microliters) into the vitreous of the eye (*e.g.*, with a sufficient volume as to deliver a therapeutically effective amount of VEGF receptor fusion protein, *e.g.*, of about 4, 5, 6, 7, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8 or 8.9, 9, 10, 12, 14, 16, 18 or 20 mg VEGF receptor fusion protein). Optionally, the method includes the steps of administering a local anesthetic (*e.g.*, proparacaine, lidocaine or tetracaine), an antibiotic (*e.g.*, a fluoroquinolone), antiseptic (*e.g.*, povidone-iodine) and/or a pupil dilating agent to the eye being injected. In an embodiment of the invention, a sterile field around the eye to be injected is established before the injection. In an embodiment of the invention, following intravitreal injection, the subject is monitored for elevations in intraocular pressure and/or blood pressure. In an embodiment of the invention, the other eye is also injected by the same procedure.

Amounts of VEGF Receptor Fusion Protein to be Administered

[00210] Each dose of high concentration VEGF receptor fusion protein to a subject over the course of a treatment may contain the same or substantially the same amount of fusion protein. Alternatively, the quantity of any one dose may be different or variable over a treatment course.

[00211] An effective or therapeutically effective amount of VEGF receptor fusion protein for treating or preventing cancer (*e.g.*, which is mediated, at least in part, by angiogenesis) or an angiogenic eye disorder refers to the amount of the VEGF receptor fusion protein sufficient to cause the regression, stabilization or elimination of the cancer or angiogenic eye disorder, *e.g.*, by regressing, stabilizing or eliminating one or more symptoms or indicia of the cancer or angiogenic eye disorder by any clinically measurable degree, *e.g.*, with regard to an angiogenic eye disorder, by causing a reduction in or maintenance of diabetic retinopathy severity score (DRSS), by improving or maintaining vision (*e.g.*, in best corrected visual acuity *e.g.*, as measured by an increase in ETDRS letters), increasing or maintaining visual field and/or

PGR2026-00039
Biocon Ex.1002
Page 4888 of 4945

reducing or maintaining central retinal thickness and, with respect to cancer, stopping or reversing the growth, survival and/or metastasis of cancer cells in the subject. In an embodiment of the invention, an effective or therapeutically effective amount of VEGF receptor fusion protein for treating or preventing an angiogenic eye disorder is about 0.5 mg to about 10 mg or 0.5 mg to about 20 mg per dose, including: about 0.5 mg or more; or about 2 mg or more, *e.g.*, about 2.1 mg; 2.2 mg; 2.3 mg; 2.4 mg; 2.5 mg; 2.6 mg; 2.7 mg; 2.8 mg; 2.9 mg; 3.0 mg; 3.1 mg; 3.2 mg; 3.3 mg; 3.4 mg; 3.5 mg; 3.6 mg; 3.7 mg; 3.8 mg; 3.9 mg; 4.0 mg; 4.1 mg; 4.2 mg; 4.3 mg; 4.4 mg; 4.5 mg; 4.6 mg; 4.7 mg; 4.8 mg; 4.9 mg; 5.0 mg; 5.1 mg; 5.2 mg; 5.3 mg; 5.4 mg; 5.5 mg; 5.6 mg; 5.7 mg; 5.8 mg; 5.9 mg; 6.0 mg; 6.1 mg; 6.2 mg; 6.3 mg; 6.4 mg; 6.5 mg; 6.6 mg; 6.7 mg; 6.8 mg; 6.9 mg; 7.0 mg; 7.1 mg; 7.2 mg; 7.3 mg; 7.4 mg; 7.5 mg; 7.6 mg; 7.7 mg; 7.8 mg; 7.9 mg; 8.0 mg; 8.1 mg; 8.2 mg; 8.3 mg; 8.4 mg; 8.5 mg; 8.6 mg; 8.7 mg; 8.8 mg; 8.9 mg; 9 mg; 9.1 mg; 9.2 mg; 9.3 mg; 9.4 mg; 9.5 mg; 9.6 mg; 9.7 mg; 9.8 mg; 9.9 mg, 10.0 mg, 10.1 mg; 10.2 mg; 10.3 mg; 10.4 mg; 10.5 mg; 10.6 mg; 10.7 mg; 10.8 mg; 10.9 mg; 11 mg; 11.1 mg; 11.2 mg; 11.3 mg; 11.4 mg; 11.5 mg; 11.6 mg; 11.7 mg; 11.8 mg; 11.9 mg; 12 mg; 12.1 mg; 12.2 mg; 12.3 mg; 12.4 mg; 12.5 mg; 12.6 mg; 12.7 mg; 12.8 mg; 12.9 mg; 13 mg; 13.1 mg; 13.2 mg; 13.3 mg; 13.4 mg; 13.5 mg; 13.6 mg; 13.7 mg; 13.8 mg; 13.9 mg; 14 mg; 14.1 mg; 14.2 mg; 14.3 mg; 14.4 mg; 14.5 mg; 14.6 mg; 14.7 mg; 14.8 mg; 14.9 mg; 15 mg; 15.1 mg; 15.2 mg; 15.3 mg; 15.4 mg; 15.5 mg; 15.6 mg; 15.7 mg; 15.8 mg; 15.9 mg; 16 mg; 16.1 mg; 16.2 mg; 16.3 mg; 16.4 mg; 16.5 mg; 16.6 mg; 16.7 mg; 16.8 mg; 16.9 mg; 17 mg; 17.1 mg; 17.2 mg; 17.3 mg; 17.4 mg; 17.5 mg; 17.6 mg; 17.7 mg; 17.8 mg; 17.9 mg; 18 mg; 18.1 mg; 18.2 mg; 18.3 mg; 18.4 mg; 18.5 mg; 18.6 mg; 18.7 mg; 18.8 mg; 18.9 mg; 19 mg; 19.1 mg; 19.2 mg; 19.3 mg; 19.4 mg; 19.5 mg; 19.6 mg; 19.7 mg; 19.8 mg; 19.9 mg; or 20 mg. In an embodiment of the invention, an effective or therapeutically effective amount of VEGF receptor fusion protein for treating or preventing cancer is about 4 mg/kg (*e.g.*, intravenously). This dose may be administered, for example, every 2 weeks.

[00212] In an embodiment of the invention, a VEGF receptor fusion protein is administered in a volume sufficient to deliver the desired dose of fusion protein, *e.g.*, as discussed above. In an embodiment of the invention, the volume delivered (*e.g.*, for treating or preventing an angiogenic eye disorder, *e.g.*, by intravitreal injection) is less than or equal to about 100 microliters (*e.g.*, about any of the following volumes: 25 microliters; 26 microliters; 27 microliters; 28 microliters; 29 microliters; 30 microliters; 31 microliters; 32 microliters; 33

PGR2026-00039
Biocon Ex.1002
Page 4889 of 4945

microliters; 34 microliters; 35 microliters; 36 microliters; 37 microliters; 38 microliters; 39 microliters; 40 microliters; 41 microliters; 42 microliters; 43 microliters; 44 microliters; 45 microliters; 46 microliters; 47 microliters; 48 microliters; 49 microliters; 50 microliters; 51 microliters; 52 microliters; 53 microliters; 54 microliters; 55 microliters; 56 microliters; 57 microliters; 58 microliters; 59 microliters; 60 microliters; 61 microliters; 62 microliters; 63 microliters; 64 microliters; 65 microliters; 66 microliters; 67 microliters; 68 microliters; 69 microliters; 70 microliters; 71 microliters; 72 microliters; 73 microliters; 74 microliters; 75 microliters; 76 microliters; 77 microliters; 78 microliters; 79 microliters; 80 microliters; 81 microliters; 82 microliters; 83 microliters; 84 microliters; 85 microliters; 86 microliters; 87 microliters; 88 microliters; 89 microliters; 90 microliters; 91 microliters; 92 microliters; 93 microliters; 94 microliters; 95 microliters; 96 microliters; 97 microliters; 98 microliters or 99 microliters).

[00213] In an embodiment of the invention, the formulation is administered (*e.g.*, for treating or preventing an angiogenic eye disorder, *e.g.*, by intravitreal injection) in a volume which is about 60 microliters or less, about 70 microliters or less or about 75 microliters or less or about 100 microliters or less. For example, in an embodiment of the invention, about 2, 4, 6, 8.0, 8.1, 8.0-8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8 or 8.9, 9.0 or 10 mg of VEGF receptor fusion protein is administered in about 50, 60, 70 or 75 microliters.

[00214] The scope of the present invention also encompasses methods for delivering 0.5 or 2 mg of VEGF receptor fusion protein (*e.g.*, for treating or preventing an angiogenic eye disorder, *e.g.*, by intravitreal injection) in pharmaceutical formulation of the present invention in a low volume such as less than 50 μl (*e.g.*, about 1, 5, 10, 17, 17.5, 18, 18.5, 20, 30, 40 or 45 μl).

[00215] The present invention also includes a composition including or consisting or consisting essentially of a "single dosage volume" of pharmaceutical formulation of the present invention, *i.e.*, a volume containing a single dose of VEGF receptor fusion protein (*e.g.*, aflibercept) in a pharmaceutical formulation of the present invention (*e.g.*, about 50 μl or 60 μl, 70 μl or 75 μl or whatever volume contains about 4, 6, 8 or 10 mg of VEGF receptor fusion protein). As discussed below, a container (*e.g.*, vial or injection device) comprising a single dosage volume, optionally including a small overfill volume of formulation, is also part of the present invention.

PGR2026-00039
Biocon Ex.1002
Page 4890 of 4945

Containers and Injection Devices

[00216] High concentration VEGF receptor fusion protein formulations in accordance with embodiments herein (*e.g.*, any of formulations A-KKKK) can be pre-packaged or pre-loaded in various useful containers and injection devices. Thus, the present invention includes containers and injection devices containing such formulations. In one embodiment herein, the container is a vial which may be sterile. In another embodiment herein, the container is a test tube which may be sterile. In an embodiment of the invention, an injection device (which may be sterile) is a syringe (*e.g.*, a pre-filled syringe or autoinjector). In an embodiment of the invention, the injection device is an intravitreal implant, *e.g.*, a refillable intravitreal implant.

[00217] A "pre-filled" syringe is a syringe which is filled with a formulation of the present invention prior to sale or use by the physician or patient.

[00218] "Sterile" herein refers to aseptic or free from substantially all, or all, living microorganisms and their spores.

[00219] Syringes as used herein include barrels made, for example, of glass or polymer, for example, cycloolefin as described in U.S. Patent Publication No. 2017/0232199, incorporated herein for all purposes, a plunger and a needle.

[00220] Containers and injection devices may be coated with silicone (*e.g.*, silicone oil or baked-silicone (*e.g.*, $\leq$40 μg or $\leq$100 μg)).

[00221] In an embodiment of the invention, a container or injection device is substantially metal-free or substantially tungsten-free or low-tungsten.

[00222] In an embodiment of the invention, the syringe contains one or more dose line graduations and/or is a dose metering system.

[00223] Containers in accordance with embodiments herein can hold the high concentration VEGF receptor fusion protein formulations. In some aspects, the container or injection device can include a label stating indications of use. In some instances, the container or injection device herein can include package inserts with instructions for use as described throughout this specification.

[00224] In other embodiments, a volume containing a single dose or multiple doses (*e.g.*, 2 or more than 2) of a high concentration VEGF receptor fusion protein (*e.g.*, wherein the dose is 2 mg, 4 mg, 6 mg, 8 mg or 10 mg of VEGF receptor fusion protein), as described above, can be pre-packaged into a container or injection device, *e.g.*, a sterile syringe, for example, for storage

PGR2026-00039
Biocon Ex.1002
Page 4891 of 4945

until use. In one example, the volume in the container includes a single dose of VEGF receptor fusion protein optionally further including a small amount of overfill volume. Sterile, pre-loaded syringes can be maintained under storage conditions (*e.g.*, 2°C-8°C), for example, for up to 12 months, 24 months or 36 months. Overfill is an excess volume that is meant to be sufficient to permit withdrawal and/or administration of the proper volume. In an embodiment of the invention, the container has a single dosage volume or multiple dosage volumes and about 5%-10% overfill volume.

[00225] Syringe sizes can be, for example, 0.3 cc, 0.5 cc or 1 cc, for example. Sterile syringes typically include a needle useful for ocular injection, typically about ½ inch, or 12.5 mm to 16 mm in length, and can be 29 gauge, 30 gauge, 31 gauge, 32 gauge or 33 gauge based on the patient and health care specialist preference. Other needle lengths and gauges can be used, as long as the needle is effective at accomplishing intravitreal injection.

[00226] While the invention has been particularly shown and described with reference to a number of embodiments, it would be understood by those skilled in the art that changes in the form and details may be made to the various embodiments disclosed herein without departing from the spirit and scope of the invention and that the various embodiments disclosed herein are not intended to act as limitations on the scope of the claims.

Additional formulations

[00227] The present invention includes the following formulations comprising greater than 40 mg/ml VEGF receptor fusion protein (*e.g.*, aflibercept or conbercept) and:

[00228] (a) a buffer including a histidine salt (*e.g.*, histidine-HCl or histidine-acetate, for example, 10 mM to 50 mM) and having pH ranging from 5.7 to 6.2; a sugar (*e.g.*, more than 6%, but not more than 10%) such as sucrose, trehalose, mannitol or glucose; a surfactant selected from the group consisting of polysorbate 20 and polysorbate 80 (*e.g.*, 0% to 0.1%);

[00229] (b) a histidine containing buffer such as L-histidine/histidine hydrochloride (*e.g.*, 10 mM); a non-ionic surfactant such as polysorbate 20 (*e.g.*, 0.03%), an inorganic salt such as NaCl (*e.g.*, 40 mM), and a carbohydrate such as sucrose (*e.g.*, 5%), *e.g.* pH 6.0-6.5 (*e.g.*, 6.2 or 6.5);

[00230] (c) citric acid (*e.g.*, 5 mM, 10 mM, 15 mM, 20 mM, 25 mM or 30 mM), sucrose (*e.g.*, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% or 10%), arginine (*e.g.*, 5 mM, 10 mM, 15 mM, 20

PGR2026-00039
Biocon Ex.1002
Page 4892 of 4945

mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM or 50 mM or 100 mM), and polysorbate 20 (*e.g.*, 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09% or 0.10%);

[00231] (d) a buffer such as phosphate, histidine, acetate, succinate, citrate, glutamate, and/or lactate (*e.g.*, at 5-20 or 5-50 mM); a non-ionic surfactant such as a polysorbate (*e.g.*, PS20 or PS80), a polyethylene glycol dodecyl ether, a poloxamer, 4-(l,l,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, an alkylsaccharide or an alkylglycoside, a tonicifying agent such as a polyol or an amino acid, for example, sucrose, trehalose, sorbitol, mannitol, glycerol, proline, arginine, methionine, glycine, or lysine, wherein the formulation has a final osmolality of about 300 mOsm/kg, and wherein the concentration of chloride anion is less than about 10 mM; pH 5.0-6.5; or

[00232] (e) 10 mM sodium phosphate, 40 mM sodium chloride, 0.03% polysorbate 20, and 5% sucrose, pH 6.2.

EXAMPLES

[00233] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention. Efforts have been made to ensure accuracy with respect to numbers used, but some experimental errors and deviations should be accounted for. Any formulations set forth in these examples are part of the present invention.

[00234] In these examples, when an experiment is conducted at 2-8°C, the temperature is targeted to 5°C with a tolerance of ±3°C variation.

Example 1: 80 mg/ml VEGF Receptor Fusion Protein Formulations Maintain Potency, Physical Stability and Charge Over A 36 Month Period.

[00235] A series of four different formulations having 80 mg/ml VEGF receptor fusion protein (aflibercept) were tested for long term potency, physical stability and development of charge variants. The ingredient listing of each of the four formulations is shown in Table 1-1. Each formulation was evaluated for VEGF receptor fusion protein HMW species formation over the course of storage at 2°C to 8°C for 36 months, as well as for the percent main species by SE-UPLC. Each VEGF receptor fusion protein containing formulation was also evaluated using Imaged Capillary Isoelectric Focusing over the same course of time and temperature. Finally,

PGR2026-00039
Biocon Ex.1002
Page 4893 of 4945

each formulation's potency was tested by bioassay over the same course of time and temperature.

Table 1-1. VEGF Receptor Fusion Protein Containing Formulations

Formulation	VEGFT (mg/mL)	Sodium phosphate (mM)	Histidine (mM)	Sucrose (w/v)	Polysorbate 20 (w/v)	Sodium Chloride (mM)	pH
F1 (B)	80	10	NA	5	0.03	40	6.2
F2 (H)	80	10	NA	8	0.03	0	6.2
F3 (D)	80	NA	10	5	0.03	40	6.2
F4 (G)	80	NA	10	8	0.03	0	6.2

Table 1-2. %HMW Species

	Formulation B	Formulation H	Formulation D	Formulation G
	Formulation 1	Formulation 2	Formulation 3	Formulation 4
Months at 2-8C				
0.0	1.7	1.6	1.6	1.3
1	2.1	2.1	1.6	1.7
2.0	2.3	2.2	1.8	1.8
3.0	2.4	2.5	2.0	2.1
6.0	2.9	2.8	2.0	2.3
9.0	3.1	3.0	2.2	2.4
12.0	2.8	3.0		2.4
18.0	3.2	3.2	2.6	2.5
24.0	3.6	3.6	3.1	2.8
36.0	4.2	4.2	3.2	3.2

PGR2026-00039
Biocon Ex.1002
Page 4894 of 4945

Table 1-3. %Main Peak

	Formulation B	Formulation H	Formulation D	Formulation G
	Formulation 1	Formulation 2	Formulation 3	Formulation 4
Months at 2-8C				
0.0	98.4	98.4	98.4	98.8
1	97.9	98.0	98.4	98.3
2.0	97.7	97.8	98.2	98.2
3.0	97.7	97.5	98.0	98.0
6.0	97.1	97.2	97.9	97.7
9.0	96.9	97.0	97.8	97.6
12.0	97.2	97.0		97.4
18.0	96.8	96.8	97.4	97.5
24.0	96.5	96.4	96.8	97.2
36.0	95.7	95.7	96.8	96.8

Table 1-4. %Acidic Species

	Formulation B	Formulation H	Formulation D	Formulation G
	Formulation 1	Formulation 2	Formulation 3	Formulation 4
Months at 2-8C				
0.0	15.8	15.1	16.0	15.2
12	15.6	15.9	NP	16.0
24.0	16.0	17.1	16.2	16.9
36.0	16.9	17.4	17.2	17.6

Table 1-5. %Main Species

	Formulation B	Formulation H	Formulation D	Formulation G
	Formulation 1	Formulation 2	Formulation 3	Formulation 4
Months at 2-8C				
0.0	78.9	79.1	79.2	79.9
12	79.3	79.3	NP	79.7
24.0	79.3	78.5	79.5	79.3
36.0	78.6	78.1	78.7	78.6

PGR2026-00039
Biocon Ex.1002
Page 4895 of 4945

[00236] Data is shown in Figures 1A, 1B, 1C and 1D. Figure 1A shows the percent of VEGF receptor fusion protein HMW species formed for each of the four formulations. VEGF TRAP shows a slightly slower rate of HMW species formation when the formulation is histidine-based, as compared to sodium phosphate. As shown in Figure 1A, the %HMW species after storage at 2^0 to 8^0C for 36 months increased by 2.6% in sodium phosphate formulations (Formulations 1 and 2), as compared to the 1.6 to 1.9% increase in histidine buffers (Formulations 3 and 4). The data was confirmed, as shown in Figure 1B, where SE-UPLC was used to identify the percent of the VEGF receptor fusion protein main species present over the same time frame and temperature range. As a comparison point, assays were run on the current EYLEA® formulation (40 mg/ml) having 10 mM sodium phosphate, which showed a 1.2% increase in the %HMW species after storage at 2^0 to 8^0C for 36 months (not shown). In addition, each of the four 80 mg/ml VEGF TRAP formulations was tested for potency and compared to EYLEA® activity (not shown). Potency was maintained by bioassay for all four formulations as well as EYLEA®. These data show that formulations herein are able to maintain VEGF receptor fusion protein stability comparable to EYLEA®.

[00237] Referring to Figures 1C and 1D, the percent of VEGF TRAP charge variants formed after storage at 2° to 8^oC for 36 months was also tested. Figure 1C shows that the VEGF TRAP in Formulations 1 - 4 showed no meaningful change in percent acidic species over the course of 36 months, and Figure 1D shows the same finding for percent main species over the course of 36 months. The finding that the VEGF TRAP maintained charge variant species during storage shows that the quality of the VEGF TRAP was maintained, and processes like deamination, N-terminal pyroglutamate formation, isomerization, aggregation, *etc.* did not occur over the course of the storage parameters.

[00238] The data in Example 1 shows that formulations having double the VEGF TRAP concentration found in EYLEA®, can maintain physical stability, quality and potency over the course of 36 months at 2° to 8^oC. Further, although all four formulations showed similar potency over the period, histidine containing formulations resulted in a slightly smaller increase in HMW species formation (sign of protein degradation), showing that under some circumstances, histidine may be a buffer of choice. However, the findings for both histidine and phosphate based buffers showed excellent stability over the course of the study.

PGR2026-00039
Biocon Ex.1002
Page 4896 of 4945

Example 2: 150 mg/ml VEGF TRAP Stability in Sodium Phosphate Buffer/Sucrose Formulations.

[00239] Two sodium phosphate formulations were tested for physical stability of 150 mg/ml VEGF Trap (aflibercept) at 37°C for 28 days. The ingredient listing of each of the two formulations is shown in Table 2-1. Each formulation was evaluated for HMW species formation over the course of storage at 37°C for the 28 days as well as for the percent main species by SE-UPLC.

Table 2-1. VEGF Receptor Fusion Protein (aflibercept) Containing Formulations for Example 2

Formulation	VEGFT (mg/mL)	Sodium phosphate (mM)	Sucrose (w/v)	Polysorbate 20 (w/v)	Arginine-HCl (mM)	pH
F1 (T)	150	10	8%	0.03	N/A	6.2
F2 (JJJJ)	150	10	8%	0.03	50	6.2

Table 2-2. %HMW Species

	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2
	Phosphate	Phosphate-Arginine
Days at 37C		
0.0	2.4	1.9
7	6.4	6.1
14.0	8.4	8.3
21.0	10.4	10.5
28.0	12.2	12.5

PGR2026-00039
Biocon Ex.1002
Page 4897 of 4945

Table 2-3. %Main Peak

	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2
	Phosphate	Phosphate-Arginine
Days at 37C		
0.0	97.6	98.1
7	93.6	93.9
14.0	91.0	91.1
21.0	88.9	89.0
28.0	87.1	86.9

[00240] Figure 2A shows that there was no meaningful difference of %HMW species formed between the two formulations during storage at 37°C for 28 days, which as confirmed by the percent of main species by SE-UPLC (Figure 2B). There was no change in the appearance, turbidity or pH of the two formulations over the course of the 28 days (not shown).

[00241] The results were then compared to formulations that included 40 mg/ml EYLEA®, and Formulation 1 from Example 1 (80 mg/ml VEGF TRAP in sodium phosphate buffer). As expected, the 150 mg/ml VEGF TRAP formulations showed a higher percent of HMW species formation as compared to the EYLEA® formulation, and a somewhat higher percent of HMW species formation as compared to Formulation 1 of Example 1.

[00242] Further testing was performed on high concentration VEGF TRAP containing formulations, and histidine containing formulations showed better protection of these molecules than did formulations based in sodium phosphate. Further, inclusion of sodium chloride into each of the tested formulations actually caused destabilization of the VEGF TRAP at 37°C. The data in Example 2 shows that even at high concentrations of 150 mg/ml VEGF receptor fusion protein, and fairly extreme temperature storage, the formulations herein provide excellent protection for physical stability.

PGR2026-00039
Biocon Ex.1002
Page 4898 of 4945

Example 3: Pharmaceutically Acceptable Formulation Viscosity Can be Attained for 150 mg/ml VEGF TRAP.

[00243] A number of VEGF Trap (aflibercept) containing formulations were tested for their viscosity behavior. Formulations having from between 10 mg/ml to 160 mg/ml VEGF Trap were tested in the presence and absence of various viscosity reducing agents.

[00244] Figure 3A shows formulations having 155 mg/ml VEGF Trap in 10 mM sodium phosphate buffer, 5% sucrose and a pH of 6.2 in combination with no inorganic salt, arginine, lysine, sodium chloride and magnesium chloride showed little difference in viscosity. In each case viscosity was measured in cP at 20°C. A range of viscosity between about 17 cP (no inorganic salt) and 15 cP (100 mM magnesium chloride) was observed, with little variation between which viscosity reducing agent was used, or with the presence of a viscosity reducing agent at all. Figure 3B shows a similar series of formulations, except that the base buffer is 10 mM histidine and pH 5.8. Here, the lack of any inorganic salt provided a formulation with a viscosity of 11.5 cP , and 50 mM lysine a viscosity of 14 cP. The histidine containing buffer provided excellent viscosity, one in line with other low-concentration biologic injectables.

[00245] Figure 3C shows viscosity of VEGF Trap between the concentrations of 10 mg/ml and 160 mg/ml, with 10 mM sodium phosphate, 5% sucrose at a pH of 6.2 in the absence of arginine, and in 50 mM of arginine hydrochloride. Inclusion of the viscosity reducing agent resulted in similar viscosities for all VEGF Trap concentrations tested. As shown in Figures 3A and 3B, the viscosity reducing agent had little positive effect on the formulation viscosity, even at higher protein concentrations. This is a surprising result, as arginine and other viscosity reducing agents have previously been shown to have beneficial properties on high concentration protein viscosity.

Table 3-1. Viscosity-Phosphate formulations (cP at 20°C)

Buffer Composition	10 mM Sodium Phosphate, 5% Sucrose, pH 6.2
No Viscosity Reducer	17.3
100 mM Arginine	16.2
200 mM Arginine	15.8
50 mM Lysine	16.2
100 mM Sodium Chloride	16.5
50 mM Magnesium Chloride	16.3
100 mM Magnesium Chloride	15.3

PGR2026-00039
Biocon Ex.1002
Page 4899 of 4945

Table 3-2. Viscosity-Histidine formulations (cP at 20°C)

Buffer Composition	10 mM Histidine, pH 5.8
No Viscosity Reducer	11.5
100 mM Arginine	12.0
200 mM Arginine	12.7
50 mM Lysine	14.0
200 mM Lysine	14.1
50 mM Sodium Chloride	13.1
100 mM Sodium Chloride	12.1
50 mM Magnesium Chloride	12.6
100 mM Magnesium Chloride	11.4

Example 4: Arginine Hydrochloride Improves Stability of 150 mg/ml VEGF TRAP In Sodium Phosphate Buffer/Sucrose Formulations.

[00246] Two sodium phosphate formulations were tested for physical stability of 150 mg/ml VEGF Trap (aflibercept) at 2-8°C for 12 months. The ingredient listing of each of the two formulations is shown in Table 4-1. Formulation 2 further includes 50 mM arginine hydrochloride. Each formulation was evaluated for HMW species formation over the course of storage at 37°C for the 12 months as well as for the percent main species by SE-UPLC.

Table 4-1. VEGF Receptor Fusion Protein (aflibercept) Containing Formulations for Example 3

Formulation	VEGFT (mg/mL)	Sodium phosphate (mM)	Sucrose (w/v)	Polysorbate 20 (w/v)	pH	Arginine Hydrochloride (mM)
F1 (T)	150	10	8%	0.03	6.2	0
F2 (JJJJ)	150	10	8%	0.03	6.2	50

[00247] Figure 4A shows that inclusion of 50 mM arginine hydrochloride to the formulation improved the VEGF TRAP physical stability. This stabilizing attribute on the VEGF TRAP was confirmed in Figure 4B, where the percent main species remains higher for the VEGF TRAP in the presence of 50 mM arginine hydrochloride. This data shows that there are situations under which addition of arginine hydrochloride provides a stabilizing effect to the

PGR2026-00039
Biocon Ex.1002
Page 4900 of 4945

stored VEGF TRAP. Tabulated size-exclusion chromatography data are set forth in Table 4-2 and 4-3.

Table 4-2. %HMW Species

	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2
Months at 2-8C		
0.0	2.4	1.9
1	3.3	2.8
2.0	3.8	3.2
3.0	4.1	3.5
6.0	4.9	4.1
9.0	5.5	4.7
12.0	5.9	5.0

Table 4-3. %Main Peak

	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, pH 6.2	10 mM Sodium Phosphate, 8% Sucrose, 0.03% Polysorbate 20, 50 mM L-Arginine Monohydrochloride, pH 6.2
Months at 2-8C		
0.0	97.6	98.1
1	96.8	97.2
2.0	96.2	96.8
3.0	95.9	96.5
6.0	95.1	95.8
9.0	94.5	95.3
12.0	94.1	94.5

PGR2026-00039
Biocon Ex.1002
Page 4901 of 4945

Example 5: Viscosity of 10 mg/ml to 170 mg/ml VEGF TRAP in Histidine and Phosphate Buffers

[00248] Figure 5 showed that 10 mM phosphate buffer and 10 mM histidine buffer both had highly useful viscosities for a range of VEGF Trap (aflibercept) concentrations. At higher VEGF Trap concentrations, the histidine buffer showed an improvement in viscosity (relative to phosphate). Viscosity was measured at 20°C.

Example 6: Taurine and Propane Sulfonic Acid Provide Improved Stability to High Concentration VEGF Trap (aflibercept) Formulations.

[00249] An experiment was conducted to determine the effect on protein stability with a number of formulation combinations. Figure 6 shows a dynamic light scattering plot for formulations having anywhere from 2 mg/ml protein to 10 mg/ml protein (aflibercept). Formulations that included 70 mM taurine or 70 mM PSA showed reduced self-interaction at higher concentrations. These data indicate that taurine and PSA can be useful inclusions in high concentration protein formulations, like the ones at issue in this case.

Example 7: Long Term Study of Stability of Various Histidine Containing Formulations.

[00250] A total of 9 different aflibercept formulations were tested with varying buffer concentration, thermal stabilizers and hydrophobic salts. The 9 formulations, F1-F9, are summarized below in Table 7-1.

PGR2026-00039
Biocon Ex.1002
Page 4902 of 4945

Table 7-1. Formulations F1-F9.

Formulation	VEGFT (mg/mL)	Buffer Type	Buffer Concentration (mM)	Sucrose (%w/v)	Proline (%w/v)	Polysorbate 20 (w/v)	Stabilizer (mM)	Stabilizer Concentration (mM)	pH
F1 (WW)	140	His	10	5	0	0.03	Taurine	50	5.8
F2 (XX)	140	His	20	0	4	0.03	Arginine HCl	50	5.8
F3 (YY)	140	His	20	2.5	2	0.03	Taurine	50	5.8
F4 (ZZ)	140	His	10	2.5	2	0.03	Arginine HCl	50	5.8
F5 (AAA)	140	His	20	5	0	0.03	PSA	50	5.8
F6 (BBB)	140	His	20	2.5	2	0.03	PSA	50	5.8
F7 (CCC)	140	His	20	5	0	0.03	Arginine HCl	50	5.8
F8 (DDD)	140	His	10	0	4	0.03	PSA	50	5.8
F9 (EEE)	140	His	20	5	0	0.03	NA	NA	5.8

VEGFT=VEGF Trap (aflibercept)

[00251] Fourteen (14) mL of each formulation was compounded in 15 mL Falcon tubes. The formulation was then sterile filtered using a 0.22 μM syringe filter and filled in sterile 2 mL Type 1 glass vials. Eight (8) vials were filled for each formulation with 0.4 mL volume in a laminar flow hood.

[00252] SE-UPLC (molecular weight species) was performed on all samples to determine the chemical stability of each formulation.

[00253] The percentage of HMW species over time for each of formulations F1-F9 are shown in Figure 7 (see also Table 7-2). Formulations F4 and F7 exhibited the lowest percentage of HMW species after 4 months at 5°C. The results showed that addition of excipients like arginine and proline significantly reduced the rate of %HMW species formation in VEGF Trap formulations.

PGR2026-00039
Biocon Ex.1002
Page 4903 of 4945

Table 7-2. SE-UPLC Analysis (2-8°C)

	F1 (WW)		F2 (XX)		F3 (YY)	
Months at 2-8C	%HMW	%Main Peak	%HMW	%Main Peak	%HMW	%Main Peak
0.0	1.53	98.46	1.48	98.52	1.39	98.61
0.5	1.87	98.13	1.87	98.13	1.92	98.08
1.0	2.19	97.81	2.16	97.84	2.19	96.15
2.0	2.64	97.36	2.63	97.37	2.71	97.29
3.0	2.83	97.17	2.76	97.24	2.87	97.13
4.0	3.07	96.93	3.00	97.00	3.11	96.89
5.0	3.24	96.76	3.11	96.89	3.28	96.72
6.0	3.35	96.65	3.24	96.76	3.38	96.62

F4 (ZZ)		F5 (AAA)		F6 (BBB)	
%HMW	%Main Peak	%HMW	%Main Peak	%HMW	%Main Peak
1.31	98.69	1.55	98.45	1.45	98.55
1.69	98.31	1.79	98.21	1.96	98.04
1.95	98.05	2.31	97.69	2.26	97.74
2.37	97.63	2.80	97.20	2.72	97.28
2.61	97.39	2.99	97.01	2.91	97.09
2.84	97.16	3.26	96.74	3.17	96.83
3.03	96.97	3.44	96.56	3.35	96.65
3.13	96.87	3.52	96.48	3.41	96.59

F7 (CCC)		F8 (DDD)		F9 (EEE)	
%HMW	%Main Peak	%HMW	%Main Peak	%HMW	%Main Peak
1.20	98.80	1.56	98.44	1.53	98.47
1.74	98.26	1.87	98.13	2.05	97.95
2.08	97.92	2.16	97.84	2.39	97.61
2.52	97.48	2.59	97.41	2.89	97.11
2.76	97.24	2.80	97.20	3.07	96.93
2.97	97.03	3.06	96.94	3.34	96.66
3.14	96.86	3.21	96.79	3.51	96.49
3.26	96.74	3.28	96.72	3.69	96.31

PGR2026-00039
Biocon Ex.1002
Page 4904 of 4945

Example 8: Stability Study of 140 mg/mL VEGF Trap with Varying Arginine Concentrations.

[00254] The impact of varying the concentration of arginine-HCl on the stability of four 140 mg/mL VEGF Trap (aflibercept) eye drug product formulations was studied. The evaluation of stability was performed at the storage condition of 2-8°C. The drug product (DP) was also incubated under stress (37°C) conditions. The four formulations being evaluated in this stability study (F1-F4) are described in Table 8-1 set forth below.

Table 8-1. Formulations F1-F4

Formulation Number	Composition					
	VEGFT (mg/mL)	Histidine (mM)	Sucrose (w/v)	Polysorbate 20	L-Arginine-	pH
F1	140	20	5	0.03	0	5.8
F2					10	
F3					50	
F4					100	

[00255] Approximately 145 mL of 187 mg/mL VEGF Trap histidine formulations were thawed. Thirty six (36) mL of each formulation, with the exception of F3, was compounded; 39 mL of bulk F3 was compounded. Each formulation was filter sterilized within a LFH (laminar flow hood) using a 0.22 μm Durapore PVDF sterilizing filter prior to filling. Clean depyrogenated 2 mL type I Schott glass vials stoppered with 13 mm serum stoppers (S2-F451 4432/50B2-40) were used to perform stability studies.

[00256] SE-UPLC analysis of the formulations was performed as set forth above. The %HMW measurements, using SE-UPLC, in each formulation over time are set forth in Figure 8 (A-B). See also Tables 8-2 and 8-3.

[00257] When stored at 5°C, these formulations exhibited a reduction in the rate of formation of %HMW species that was proportional to the concentration of arginine-HCl. Under stress, however, this effect was reversed. The formulations, at 37°C, exhibited an apparent increase in %HMW species proportional to the concentration of arginine-HCl. This property made the discovery of arginine's beneficial effects on aflibercept in the presence of histidine buffer highly unlikely. Typically, for formulation development in the biotechnology industry, the effect of various excipients on a drug is initially screened under stress (*e.g.*, at elevated

PGR2026-00039
Biocon Ex.1002
Page 4905 of 4945

temperature such as 37°C) for a short period of time. The purpose of this approach it to rapidly eliminate excipients which will not likely perform well in the absence of stress (*e.g.*, at lower temperature such as 5°C) over a longer period of time. Here, arginine was identified as a helpful excipient in spite of its effect on stability at 37°C. Since formulated drug product is typically stored for months at 4-5°C after manufacture, aflibercept in arginine and histidine buffer would be a highly valuable formulation for long term, stable drug product storage.

Table 8-2. SE-UPLC Analysis (2-8°C)

	F1 (EEE)		F2 (SSS)	
Months at 2-8C	%Main Peak	%HMW	%Main Peak	%HMW
0.0	97.79	2.21	97.78	2.22
0.5	97.56	2.43	97.59	2.41
1.0	97.30	2.70	97.34	2.66
1.5	97.21	2.79	97.26	2.74
2.0	97.05	2.95	97.08	2.92
2.5	96.96	3.04	97.02	2.98
3.0	96.82	3.18	96.88	3.12
6.0	96.35	3.65	96.40	3.60

F3 (CCC)		F4 (TTT)	
%Main Peak	%HMW	%Main Peak	%HMW
97.85	2.15	97.81	2.19
97.67	2.33	97.73	2.27
97.47	2.53	97.59	2.41
97.37	2.63	97.50	2.50
97.22	2.78	97.36	2.64
97.14	2.86	97.31	2.69
97.04	2.96	97.16	2.84
96.60	3.40	96.77	3.23

PGR2026-00039
Biocon Ex.1002
Page 4906 of 4945

Table 8-3. SE-UPLC Analysis (37°C)

	F1 (EEE)		F2 (SSS)	
Days at 37C	%Main Peak	%HMW	%Main Peak	%HMW
0.0	97.79	2.21	97.78	2.22
7	93.66	6.34	93.32	6.68
14.0	89.48	10.35	88.79	10.98
21.0	86.64	13.08	85.69	13.82
28.0	83.90	15.85	82.66	16.90

F3 (CCC)		F4 (TTT)	
%Main Peak	%HMW	%Main Peak	%HMW
97.85	2.15	97.81	2.19
92.38	7.61	91.32	8.68
86.90	12.91	84.61	15.21
83.11	16.65	80.11	19.47
79.44	20.29	75.80	23.91

Example 9: VEGF Trap Stability in the presence of counterions from the Hofmeister Series and other Excipients.

[00258] The effect of various counterions and other excipients on stability of several formulations was determined.

A. Counterion Screening

[00259] Counter ions (*e.g.*, sulfate, thiocyanate and citrate) were tested in the form of sodium salts with high concentration VEGF Trap (aflibercept) formulations (140 mg/ml). Additionally, other amino add excipients (*e.g.*, glycine and lysine) were also tested.

[00260] Approximately 42 ml of 187 mg/ml VEGF Trap eye drug substance were thawed. 50 ml of intermediate formulated drug substance (155.56 mg/ml), having a 110% concentration of the 140 mg/ml formulated drug substance and target excipient concentration (excluding the counterions, glycine and lysine), were prepared.

[00261] The intermediate formulated drug substance was further diluted with the 0.5 M stock excipient solutions (counterions, glycine or lysine) to produce the 140 mg/ml formulated drug substance formulations listed in Table 9-1 below. Each final formulation was filter sterilized with a 0.22 μm PVDF syringe filter within a laminar flow hood prior to filling into clean

PGR2026-00039
Biocon Ex.1002
Page 4907 of 4945

depyrogenated 2mL type I Schott glass vials stoppered with 13 mm serum stoppers S2-F451 4432/50 82-40 (ELN item# 19700004 Wash# 0 000078949). Sixty (60) vials were filled with 0.5 ml of each formulation. Six (6) vials were filled with 1.5 ml.

Table 9-1. Formulations for Counterion Screening

Excipient (mM)	Formulation	VEGF Trap conc.
Sodium sulfate 50mM	20mM Histidine/0.03%PS20/5% Sucrose/50mM Sodium Sulfate	140
Sodium thiocyanate 50mM	20mM Histidine/0.03%PS20/5% Sucrose/50mM Sodium thiocyanate	140
Sodium Citrate 50mM	20mM Histidine/0.03%PS20/5% Sucrose/50mM Sodium Citrate	140
Glycine 50mM	20mM Histidine/0.03%PS20/5% Sucrose/50mM Glycine	140
Sodium chloride 50mM	20mM Histidine/0.03%PS20/5% Sucrose/50mM sodium chloride	140
Lysine 50mM	20mM Histidine/0.03%PS20/5% Sucrose/50mM Lysine	140

B. Glutamate And Aspartate Screening

[00262] The stability of VEGF Trap (aflibercept) high concentration formulations (140 mg/ml) in the presence of organic counter ions in combination with arginine hydrochloride was tested. In addition, two new counter ions (glutamate and asparate) were tested for compatibility with high concentration VEGF Trap (in combination and without arginine hydrochloride). The formulations tested are summarized below in Table 9-2.

[00263] Approximately 50 ml of 187 mg/ml VEGF Trap drug substance were thawed. 60 ml of intermediate formulated drug substance (155.56 mg/ml) with a 110% of the 140 mg/ml formulated drug substance and target excipient concentration (excluding the counterions, citrate, glycine, glutamate and aspartate) was prepared. The intermediate formulated drug substance was further diluted with the 1M stock excipient solutions (arginine hydrochloride, sodium citrate, glycine, sodium glutamate and sodium aspartate) to produce the 140 mg/ml formulated drug substance formulations listed in the table below. Each final formulation was filter sterilized with a 0.22 μm PVDF syringe filter within a laminar flow hood prior to filling into clean

PGR2026-00039
Biocon Ex.1002
Page 4908 of 4945

depyrogenated 2 ml type I Schott glass vials stoppered with 13 mm serum stoppers S2-F451 4432/50 B2-40 (ELN item# 19700004 Wash# 0000078949). Sixty (60) vials were filled with 0.5 ml of each formulation. Six (6) vials were filled with 1.5 ml.

Table 9-2. Formulations for Glutamate And Aspartate Screening

Excipient (mM)	Formulation	VEGF Trap conc.
Sodium Citrate 50mM + Arginine Hydrochloride 50mM	20mM Histidine/0.03%PS20/5% Sucrose/ 50mM Sodium Citrate/50mM Arginine hydrochloride	140
Glycine 50mM + Arginine hydrochloride 50mM	20mM Histidine/0.03%PS20/5% Sucrose/ 50mM Glycine/50mM Arginine hydrochloride	140
Sodium aspartate 50mM + Arginine hydrochloride 50mM	20mM Histidine/0.03%PS20/5% Sucrose/ 50mM Sodium Aspartate/50mM Arginine hydrochloride	140
Sodium glutamate 50mM + Arginine hydrochloride 50mM	20mM Histidine/0.03%PS20/5% Sucrose/ 50mM Sodium Glutamate/50mM Arginine hydrochloride	140
Sodium aspartate 50mM	20mM Histidine/0.03%PS20/5% Sucrose/ 50mM sodium aspartate	140
Sodium glutamate 50mM	20mM Histidine/0.03%PS20/5% Sucrose/ 50mM Sodium glutamate	140

[00264] Viscosity testing was performed using a RHEOSENSE m-VROC® viscometer. Approximately, 0.5 mL of undiluted sample was loaded onto a glass syringe. The sample was equilibrated to the desired temperature and injected into a chip or measuring cell. Viscosity was calculated by measuring the pressure drop from inlet to the outlet which is correlated to the shear-stress at the wall of the chip. Results are expressed as mPas-1 or cp. See Figure 9 and Table 9-3.

[00265] Osmolality testing was performed using a VAPRO vapor pressure osmometer. Approximately, 10 µL of undiluted sample was inoculated into a paper disc. The dew point temperature depression, a function of the solution vapor pressure, was measured through a sensitive thermocouple and reported as osmolality of the solution. Results are expressed as mmol/kg or mOsm. See Figure 10 and Table 9-3.

PGR2026-00039
Biocon Ex.1002
Page 4909 of 4945

[00266] High molecular weight species in certain formulations over time, after storage at 37°C or 5°C, was also evaluated by SE-UPLC. See Figure 11 (A-B) and Tables 9-4 and 9-5.

[00267] Dynamic light scattering experiments were performed using a DynaPro® Plate Reader II. Approximately, 100 µL of undiluted sample was loaded onto a 96-well plate. Thirty five acquisitions were obtained for each well at 25°C. Analysis of autocorrelation function using regularization was performed on the DYNAMICS v7.1 software. Radius (nm) vs %intensity plots and % mass vs radius (nm) plots were generated to derive the average molecular radius and % polydispersity values (%Pd) for each sample. See Figure 12.

Table 9-3. Viscosity and Osmolality of Various Formulations

Parameter tested	50mM Sodium Sulfate (GGG)	50mM Sodium thiocyanate (HHH)	40mM Sodium citrate (III)	50mM Glycine (JJJ)	50mM Sodium Chloride (KKK)	50mM Lysine (LLL)
viscosity (cp)	11.9	12.4	14.8	12.2	11.6	11.8
Osmolality(mOsmol/Kg)	384	324	403	299	349	349

50mM Sodium Aspartate (MMM)	50mM Sodium Glutamate (NNN)	50mM Sodium citrate+50mM Arginine HCl (OOO)	50mM Glycine+ 50mM Arginine HCl (PPP)	50mM Sodium Aspartate+ 50mM Arginine HCl (QQQ)	50mM Sodium Glutamate + 50mM Arginine HCl (RRR)
13.0	13.1	14.8	12.5	13.1	13.3
343	346	506	369	426	430

Table 9-4. SE-UPLC Analysis; %HMW (37°C)

%HMW (Days at 37C)	50mM Sodium Sulfate (GGG)	50mM Sodium thiocyanate (HHH)	40mM Sodium citrate (III)	50mM Glycine (JJJ)	50mM Sodium Chloride (KKK)	50mM Lysine (LLL)
0	2.3	2.3	2.3	2.3	2.3	2.2
7	8.6	15.5	8.2	6.3	8.1	7.7
14	13.5	24.8	12.1	9.5	12.4	11.7
28	22.0	39.2	19.1	15.3	20.4	19.3

PGR2026-00039
Biocon Ex.1002
Page 4910 of 4945

Table 9-5. SE-UPLC Analysis; %HMW (5°C)

%HMW (Months at 5C)	50mM Sodium Sulfate (GGG)	50mM Sodium thiocyanate (HHH)	40mM Sodium citrate (III)	50mM Glycine (JJJ)	50mM Sodium Chloride (KKK)	50mM Lysine (LLL)
0	2.3	2.3	2.3	2.3	2.3	2.2
0.25	2.3	2.4	2.4	2.3	2.3	2.3
0.5	2.4	2.5	2.5	2.4	2.4	2.3
1	2.5	2.8	2.7	2.6	2.6	2.5
2	2.8	2.9	2.9	2.8	2.8	2.8
3	3.1	3.3	3.2	3.1	3.2	3.0

Example 10: Tolerability of IVT delivery of High Dose VEGF Trap (140mg/ml) in Normal Rabbits.

[00268] Anti-VEGF therapeutics, administered via intravitreal injection, are currently the standard of care for treatment of neovascular age-related macular degeneration, diabetic macular edema, and retinal vascular occlusive disease. However, the monthly or bi-monthly intravitreal injections represent a significant treatment burden on patients, caregivers, and physicians. There is a pressing need for more effective and durable therapies in clinical practice. This study is to investigate the tolerability of stably formulated high dose VEGF Trap (140 mg/ml, equivalently 14 folds of the clinical dose) in normal New Zealand white rabbit eyes.

[00269] Two formulations of high dose VEGF-Trap (aflibercept) in histidine buffer or in phosphate buffer were tested. Three rabbits per cohort received 7mg of VEGF Trap in 50 uL by single bilateral intravitreal injection of each formulation. Ocular inflammation signs were monitored by slit lamp, optical coherence tomography (OCT) and fundus angiograph at day 1, day 4, week 1 then weekly until 12 weeks post-intravitreal administration. Intraocular pressure was measured with Tonopen before and 10 minutes and 30 minutes post intravitreal injection, then at every follow up time point. Animals were euthanized at week 12.

[00270] The rabbit eyes were dosed with either:

(1) 140 mg/ml aflibercept, 20 mM Histidine, 5% Sucrose, 50 mM Arginine HCl, 0.03% PS20, pH 5.8;

or

(2) 140 mg/ml aflibercept, 10 mM Sodium phosphate, 5% Sucrose, 40 mM Sodium Chloride, 0.03% PS20, pH 6.2.

PGR2026-00039
Biocon Ex.1002
Page 4911 of 4945

[00271] Two of six eyes from the histidine buffer group showed dark shadows in the vitreous until 8 weeks post IVT, which were confirmed by slit-lamp examination as local cataract due to posterior lens injury, procedure related. One of 6 eyes the from phosphate buffer group showed dark shadows in the vitreous until 8 weeks post IVT, which were confirmed by slit-lamp examination as local cataract due to posterior lens injury (procedure related).

[00272] The high dose VEGF Trap (140 mg/ml) formulations tested were well tolerated in normal New Zealand White rabbit eyes within 12 weeks post-single intravitreal administration. After a single intravitreal injection of 7 mg of VEGF Trap, no signs of abnormalities or inflammation were seen in the fundus. The vitreous was clear and the retinal vascular pattern appeared normal. No retinal detachment, hemorrhage or optic nerve head changes were seen. There was no intraocular pressure change compared to baseline. The baseline fluorescein angiography (FA) and OCT images of rabbit eyes are represented in Figure 13(A-D). The time course FA and OCT images of rabbit eyes in each treatment group over 8 weeks is represented in figures 14, 15, 16 and 17. Data from the left eye (OS) or right eye (OD) of rabbits 326 and 329 are shown.

Example 11: Evaluation of Stability of Formulations UUU-BBBB

[00273] This study examined the stability of 60 mg/ml and 120 mg/ml VEGF Trap (aflibercept) in 10 mM phosphate, 10% or 20% sucrose (Suc.), 0 or 50 mM NaCl, 0.03% polysorbate 20, pH 6.2 when incubated at 37°C for up to 6 months. The formulations for this stability study are set forth below in Table 11-1.

Table 11-1. Formulations UUU-BBBB

Formulation Designation	[VEGF Trap] mg/mL	% Sucrose	mM NaCl
F1 (UUU)	30	10	-
F2 (VVV)	30	20	-
F3 (WWW)	60	10	-
F4 (XXX)	60	20	-
F5 (YYY)	120	10	-
F6 (ZZZ)	120	20	-

PGR2026-00039
Biocon Ex.1002
Page 4912 of 4945

F7 (AAAA)	120	10	50
F8 (BBBB)	120	20	50

Base formulation: 10 mM phosphate, 0.03% polysorbate 20, pH 6.2

VEGF Trap=aflibercept

[00274] The formulation was filter sterilized with a PVDF 0.2 µm filter in a laminar flow hood prior to dispensing.

[00275] Vials containing each formulation were stored at 37ºC for 1 month.

Table 11-2. Reverse-phase High Performance Liquid Chromatography (RP-HPLC) Results

Formulation	Storage Condition	Actual Incubation (months)	% VEGFT Recovered	[VGFT] mg/ml
30 mg/mL 10% sucrose (UUU)	37ºC	0.0	100.0	29.99
		1.0	97.9	29.37
30 mg/mL 20% sucrose (VVV)	37ºC	0.0	100.0	29.97
		1.0	98.2	29.44
60 mg/mL 10% sucrose (WWW)	37ºC	0.0	100.0	58.90
		1.0	98.3	57.89
60 mg/mL 20% sucrose (XXX)	37ºC	0.0	100.0	58.80
		1.0	98.9	58.18
120 mg/mL 10% sucrose (YYY)	37ºC	0.0	100.0	120.80
		1.0	97.4	117.63
120 mg/mL 20% sucrose (ZZZ)	37ºC	0.0	100.0	117.85
		1.0	98.9	116.51
120 mg/mL 10% sucrose 50 mM NaCl (AAAA)	37ºC	0.0	100.0	120.14
		1.0	97.9	117.64

PGR2026-00039
Biocon Ex.1002
Page 4913 of 4945

120 mg/mL 20% sucrose 50 mM NaCl (BBBB)	37°C	0.0	100.0	120.55
		1.0	97.0	116.94

VGFT=VEGF Trap, aflibercept

PGR2026-00039
Biocon Ex.1002
Page 4914 of 4945

Table 11-3. Size-exclusion Ultra Performance Liquid Chromatography (SE-UPLC) Results

Formulation	Storage Condition	Actual Incubation (months)	Total % HMW	%Native	Total % LMW
30 mg/mL VGFT 10% sucrose (UUU)	37°C	0	0.8	99.0	0.1
		1.0	4.1	94.0	1.9
30 mg/mL VGFT 20% sucrose (VVV)	37°C	0	0.8	99.0	0.2
		1.0	2.4	95.6	2.0
60 mg/mL VGFT 10% sucrose (WWW)	37°C	0	1.0	98.8	0.2
		1.0	7.8	89.5	2.7
60 mg/mL VGFT 20% sucrose (XXX)	37°C	0	1.0	98.8	0.2
		1.0	4.4	92.8	2.7
120 mg/mL VGFT 10% sucrose (YYY)	37°C	0	1.3	98.5	0.2
		1.0	18.3	79.5	2.2
120 mg/mL VGFT 20% sucrose (ZZZ)	37°C	0	1.2	98.5	0.2
		1.0	10.7	87.1	2.2
120 mg/mL VGFT 10% sucrose 50 mM NaCl (AAAA)	37°C	0	1.3	98.5	0.2
		1.0	20.8	77.1	2.2
120 mg/mL VGFT 20% sucrose 50 mM NaCl (BBBB)	37°C	0	1.2	98.5	0.3
		1.0	13.0	84.8	2.2

VGFT=VEGF Trap, aflibercept

PGR2026-00039
Biocon Ex.1002
Page 4915 of 4945

[00276] All of the formulations were essentially particle-free by visual inspection and optical density measurement. There was no significant protein loss in recovery by RP-HPLC after 1 month at 37°C (Table 11-2) and this trend continued out to 6 months.

[00277] The main degradation pathway for VEGF Trap was aggregation under these conditions (Figure 18 (A-B); Table 11-3). The stability of VEGF Trap was dependent on the protein and sucrose concentrations. Formulations with lower VEGF Trap concentrations and higher sucrose were more stable. Maintaining the same protein:sucrose ratio did not result in the same degradation rate. For example, F1 and F4 have the same protein:sucrose ratio of 3:1 but F4 was less stable than F1 due to its higher protein concentration. Similar trends were observed out to 6 months.

Example 12: Repeat-Dose Intravitreal Toxicology Study in Monkeys with Different Aflibercept Formulations.

[00278] The safety of various formulations in cynomolgus monkeys is assessed in this example. A total of 7 intravitreal doses are administered to both eyes approximately Q4W to Groups 1-3; a total of 3 intravitreal doses are administered to both eyes approximately Q4W to groups 4-9. A terminal necropsy is performed approximately one week after last dose (N=3/sex/group) with recovery necropsy (2/sex/group) approximately 12-week after last dose

[00279] The following assessments are performed:

- Safety assessment based on clinical signs, body weights, vital signs, electrocardiographic data (pre-dose end of treatment and end of recovery), blood pressure measurements (via tail cuff), clinical and anatomic pathology
- Comprehensive ocular exams at regular intervals during the dosing and recovery intervals including slit lamp microscopy, indirect ophthalmoscopy and intraocular pressure measurements
- Fundus photography, fluorescein angiography and electroretinography at pre-treatment, during study week 9 (all groups), week 26 (Groups 1-3 only) and end of recovery period
- Blood and vitreous samples (at termination) for bioanalytical, ADA analysis and toxicokinetic evaluation

PGR2026-00039
Biocon Ex.1002
Page 4916 of 4945

Table 13. Study group summary*

Group	Test Material	IVT Dose Volume (µL/dose/eye)	mg aflibercept/eye	N/sex	N/sex for terminal necropsy	N/sex for Recovery
1	20 mM Histidine, **50 mM Arginine**, 5% Sucrose, 0.03% PS20	50	0 (control)	5	3	2
2	**80 mg/mL Aflibercept** in 20 mM Histidine, **50mM Arginine**, 5% Sucrose, 0.03% PS20	50	4	5	3	2
3	**~140 mg/mL Aflibercept** in 20 mM Histidine, **50mM Arginine**, 5% Sucrose, 0.03% PS20	50	~7	5	3	2
4	20 mM Histidine, 5% Sucrose, 0.03% PS20	50	0 (control)	5	3	2
5	**80 mg/mL Aflibercept** in 20 mM Histidine, 5% Sucrose, 0.03% PS20	50	4	5	3	2
6	**~140 mg/mL Aflibercept** in 20 mM Histidine, 5% Sucrose, 0.03% PS20	50	~7	5	3	2
7	20 mM Histidine, **50 mM Glycine**, 5% Sucrose, 0.03% PS20	50	0 (control)	5	3	2
8	**80 mg/mL Aflibercept** in 20 mM Histidine, **50 mM Glycine**, 5% Sucrose, 0.03% PS20	50	4	5	3	2
9	**~140 mg/mL Aflibercept** in 20 mM Histidine, **50 mM Glycine**, 5% Sucrose, 0.03% PS20	50	~7	5	3	2

*each of said formulations are part of the present invention

[00280] It is expected that the formulations tested in any one or more of Groups 1, 2, 3, 4, 5, 6, 7, 8 and/or 9 will be determined to have a safety profile comparable to EYLEA in cynomolgous monkeys as measured in the assessments discussed above.

Example 13: Stability Study with VEGF Trap Product.

[00282] A formulation including 114.3 mg/mL VEGF Trap (aflibercept) and 10 mM Histidine, pH 5.8, 5% Sucrose, 0.03% Polysorbate 20, 50 mM Arginine Monohydrochloride was prepared within a LFH (laminar flow hood) using a 0.22 µm Durapore PVDF sterilizing filter prior to filling. Clean depyrogenated 3 mL type I Schott glass vials were filled with 0.3 mL of Drug Product and stoppered with 13 mm serum stoppers (S2-F451 4432/50B2-40). Samples were analyzed at various timepoints after storage at 37°C or 5°C for purity by size-exclusion chromatography (SEC) to determine the presence (%) of high molecular weight species (HMW), low molecular weight species (LMW) and the main peak (main); as well as microflow imaging, HIAC liquid particle counting (by light obscuration) and microscopically to determine the presence of particles of various sizes.

PGR2026-00039
Biocon Ex.1002
Page 4917 of 4945

[00283] Stability and purity data after storage at 37ºC, 25ºC and 2-8ºC are shown in Figure 19 (A-E). See also Tables 13-1 – 13-4.

Table 13-1. Size-Exclusion Chromatography (SEC) Purity Analysis

37°C			
Months	**%Main**	**%HMW**	**%LMW**
0	98.23	1.77	0
0.229	92.88	7.07	0.05
0.459	88.59	11.18	0.23
0.688	84.67	15.03	0.3
0.918	81.84	17.59	0.57
5°C			
Months	**%Main**	**%HMW**	**%LMW**
0	98.23	1.77	0
1	97.73	2.27	0
2	97.4	2.59	0

Table 13-2. Microflow Imaging/Subvisible Particles

37°C					
Months	**≥1µm**	**≥2µm**	**≥5µm**	**≥10µm**	**≥25µm**
T=0	1125	234	42	9	0
28 days	1128	184	17	0	0

Table 13-3. Subvisible Particulate Matter as Measured by Light Obscuration (HIAC)/Subvisible Particles

37°C			
Months	**≥10µm**	**≥25µm**	**≥50µm**
T=0	15	0	0
28 days	23	1	1

PGR2026-00039
Biocon Ex.1002
Page 4918 of 4945

Table 13-4. Microscopic Analysis of Subvisible Particles

37°C			
Months	**≥10µm**	**≥25µm**	**≥50µm**
T=0	8	1	1
28 days	6	2	1

Example 14: Stability Study with Varying VEGF Trap Concentrations.

[00284] The impact of varying the concentration of VEGF Trap on the stability of four VEGF Trap (aflibercept) eye drug product formulations was studied. The concentrations ranged between 80 and 140 mg/mL. The evaluation of stability was performed at the storage condition of 2-8ºC. The drug product (DP) was also incubated under stress (37ºC) conditions. The four formulations (each of which is named "CCC" herein) being evaluated in this stability study are described in Table 14-1 set forth below.

[00285] Approximately 27 mL of each formulation was prepared within a LFH (laminar flow hood) using a 0.22 µm Durapore PVDF sterilizing filter prior to filling. Clean depyrogenated 2 mL type I Schott glass vials were filled and stoppered with 13 mm serum stoppers (S2-F451 4432/50B2-40). Samples were analyzed at various timepoints after storage at 37ºC or 2-8ºC for purity by size-exclusion-ultraperformance liquid chromatography (SE-UPLC) to determine the presence (%) of high molecular weight species (HMW) and the main peak (main).

[00286] Data showing the percentage HMW after incubation at 2-8ºC or 37ºC for up to 6 months are set forth in Figure 20 (A-B). See also Tables 14-2 and 14-3. When stored at 37ºC and 5ºC, these formulations exhibited a positive correlation of the rate of HMW formation to the concentration of VEGF Trap. The 140 mg/mL formulation exhibited highest rate of %HMW species formation.

PGR2026-00039
Biocon Ex.1002
Page 4919 of 4945

Table 14-1. Formulations tested.

Formulation Number	composition					
	L-Arginine mM	Histidine (mM)	Sucrose (w/v)	Polysorbate 20 (w/v)	VEGFT (mg/mL)	pH
F1 (CCC)	50	20	5	0.03	80	5.8
F2 (CCC)					100	
F3 (CCC)					120	
F4 (CCC)					140	

Table 14-2. SE-UPLC Analysis (2-8°C)

	F1 (CCC): 80 mg/mL		F2 (CCC): 100 mg/mL		F4 (CCC): 120 mg/mL		F3 (CCC): 140 mg/mL	
Months at 2-8C	%Main Peak	%HMW	%Main Peak	%HMW	%Main Peak	%HMW	%Main Peak	%HMW
0.0	97.90	2.10	97.86	2.14	97.83	2.17	97.85	2.15
1	97.91	2.09	97.79	2.21	97.65	2.35	97.47	2.53
2.0	97.78	2.22	97.56	2.44	97.38	2.62	97.22	2.78
3.0	97.61	2.39	97.38	2.62	97.17	2.83	97.04	2.96
5.5	97.39	2.61	97.10	2.90	96.79	3.21		
6.0	97.36	2.64	97.04	2.96	96.74	3.26	96.60	3.40

Table 14-3. SE-UPLC Analysis (37°C)

	F1 (CCC): 80 mg/mL		F2 (CCC): 100 mg/mL		F4 (CCC): 120 mg/mL		F3 (CCC): 140 mg/mL	
Days at 37C	%Main Peak	%HMW	%Main Peak	%HMW	%Main Peak	%HMW	%Main Peak	%HMW
0.0	**97.90**	**2.10**	**97.86**	**2.14**	**97.83**	**2.17**	**97.85**	**2.15**
7	**94.83**	**5.15**	**93.82**	**6.17**	**92.81**	**7.18**	**92.38**	**7.61**
14.0	**92.00**	**7.77**	**90.31**	**9.39**	**88.67**	**11.13**	**86.90**	**12.91**
21.0	**89.47**	**10.31**	**87.00**	**12.58**	**85.04**	**14.85**	**83.11**	**16.65**
28.0	**86.76**	**12.71**	**83.98**	**15.55**	**81.19**	**18.46**	**79.44**	**20.29**

<u>Example 15</u>: High dose Eylea has Prolonged Duration of Action in the D,L-AAA Model of Sustained Neovascular Leak.

[00287] The effect of a four-fold increase in dose on duration of action of aflibercept to inhibit chronic retinal vascular leak in the D,L-AAA model was determined in this Example.

[00288] Animals used were New Zealand White rabbits 3 months post-DL-AAA (DL-α-aminoadipic acid) disease induction. The treatment groups were:

PGR2026-00039
Biocon Ex.1002
Page 4920 of 4945

- Placebo (formulation buffer) 50 mcl/eye; n= 6 eyes
- Aflibercept 500 mcg/eye in 50 mcl; n= 7 eyes
- Aflibercept 2 mg/eye in 50 mcl; n= 8 eyes

[00289] The formulation used in each treatment group was: 10 mM Histidine, 8% sucrose, 0.03% PS20, pH 5.8. Ophthalmic exams, which were performed at baseline and at weeks 1, 2, 4, 6, 7, 9, 10, 11, 13 and 18, were intra-ocular pressure (IOP), red-free (RF) imaging (to determine blood vessel morphology), fluorescein angiography (FA; to determine vascular leak), optical coherence tomography (OCT; to identify vitreal inflammation). Serum (ADA) and plasma (drug levels) were collected at baseline and at weeks 1, 2, 4, 6 and 9.

[00290] Data from FA exams of each group are set forth in Figure 21. These data demonstrated that vascular leak inhibition in the retinas of animals receiving higher doses of aflibercept lasted longer than that of animals receiving lower doses. The number of treated eyes in which vascular permeability was completely inhibited was significantly greater in the 2 mg dose group at all sampling times up to 18 weeks post dosing.

PGR2026-00039
Biocon Ex.1002
Page 4921 of 4945

What is claimed is:

1. An aqueous pharmaceutical formulation comprising:

a VEGF receptor fusion protein, anti-VEGF antibody or antigen-binding fragment or VEGF binding molecule at a concentration of at least about 100 mg/ml;

about 5% sucrose

a histidine buffer; and

about 0.03% surfactant;

optionally, in association with a further therapeutic agent;

wherein the formulation has a pH of about 5.0 to about 6.8.

2. The formulation of claim 1 which is suitable for intavitreal administration.

3. The formulation of any one of claims 1-2 further comprising sodium sulfate, sodium thiocyanate, glycine, NaCl, sodium aspartate, L-arginine and/or sodium glutamate.

4. The formulation of any one or claims 1-3, wherein the VEGF receptor fusion protein or anti-VEGF antibody or antibody fragment or VEGF binding molecule is:

(i) a VEGF receptor fusion protein comprising two polypeptides that comprise (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2;

(ii) a VEGF receptor fusion protein comprising two polypeptides that comprise immunoglobin-like (Ig) domain 2 of a first VEGF receptor and Ig domain 3 of a second VEGF receptor, and a multimerizing component;

(iii) a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobin-like (Ig) domain 2 of a first VEGF receptor, an Ig domain 3 of a second VEGF receptor, an Ig domain 4 of said second VEGF receptor and a multimerizing component;

(iv) a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of a VEGFR2, and a multimerizing component;

PGR2026-00039
Biocon Ex.1002
Page 4922 of 4945

(v) a VEGF receptor fusion protein comprising two polypeptides that comprise an immunoglobin-like (Ig) domain 2 of VEGFR1, an Ig domain 3 of VEGFR2, an Ig domain 4 of VEGFR2 and a multimerizing component;

(vi) a VEGF receptor fusion protein comprising two VEGFR1R2-FcΔC1(a) polypeptides encoded by the nucleic acid sequence of SEQ ID NO:1; or

(vii) selected from the group consisting of: bevacizumab, ranibizumab, pegaptanib, brolucizumab, an anti-VEGF DARPin and a bispecific anti-VEGF/ANG2 antibody.

5. The formulation of any one or claims 1-4, wherein the VEGF receptor fusion protein or anti-VEGF antibody or antibody fragment or VEGF binding molecule is a VEGF receptor fusion protein selected from the group consisting of: aflibercept and conbercept.

6. The formulation of any one of claims 1-5 wherein the VEGF receptor fusion protein or anti-VEGF antibody or antibody fragment or VEGF binding molecule is a VEGF receptor fusion protein is a VEGF receptor fusion protein at a concentration of from about 41 to about 275 mg/ml.

7. The formulation of any one of claims 1-6, wherein the VEGF receptor fusion protein or anti-VEGF antibody or antibody fragment or VEGF binding molecule is a VEGF receptor fusion protein is a VEGF receptor fusion protein at a concentration of:

- about 100 mg/ml;
- about 111.5 mg/ml;
- about 112.0 mg/ml;
- about 113.3 mg/ml;
- about 114.3 mg/ml;
- about 115.6 mg/ml;
- about 116.3 mg/ml;
- about 120 mg/ml;
- about 133 mg/ml;
- about 140 mg/ml;
- about 150 mg/ml;
- about 200 mg/ml;

PGR2026-00039
Biocon Ex.1002
Page 4923 of 4945

- about 250 mg/ml;
- that which contains about 4 mg VEGF receptor fusion protein in about 100 µl or less, about 70 µl or less, about 75 µl or less, about 50 µl or less or about 60 µl or less;
- that which contains about 6 mg VEGF receptor fusion protein in about 100 µl or less, about 70 µl or less, about 75 µl or less, about 50 µl or less or about 60 µl or less;
- that which contains about 8 mg VEGF receptor fusion protein in about 100 µl or less, about 70 µl or less, about 75 µl or less, about 50 µl or less or about 60 µl or less; or
- that which contains about 10 mg VEGF receptor fusion protein in about 100 µl or less, about 70 µl or less, about 75 µl or less, about 50 µl or less or about 60 µl or less.

8. The formulation of any one of claims 1-7 wherein:
(i) the osmolality is about 299 to about 506 mmol/Kg; and/or
(ii) the viscosity is from about 6-15 cP at 20°C.

9. The formulation of any one of claims 1-8 wherein the pH is 5.8 to 6.5.

10. The formulation of claim 9 wherein the pH is about 5.8.

11. The formulation of any one of claims 1-10, wherein the surfactant is a non-ionic surfactant.

12. The formulation of claim 11, wherein the non-ionic surfactant is selected from the group consisting of polysorbate 20, polysorbate 80, poloxamer 188, polyethylene glycol 3350 and mixtures thereof.

13. The formulation of any one of claims 1-12, wherein the histidine is histidine hydrochloride.

14. The formulation of claim 13 comprising about 10 mM to 20 mM histidine hydrochloride.

15. The formulation of any one of claims 1-14, comprising a thermal stabilizer which is a sugar.

PGR2026-00039
Biocon Ex.1002
Page 4924 of 4945

16. The formulation of claim 15, wherein the sugar is sucrose, mannitol, sorbitol or trehalose.

17. The formulation of any one of claims 15-16 comprising about 2.5% (w/v), about 5% (w/v), about 8% (w/v), about 10% (w/v) or about 20% (w/v) sucrose.

18. The formulation of any one of claims 1-17 comprising surfactant at a concentration of about 0.03% (w/v).

19. The formulation of any one of claims 1-18, comprising L-arginine monohydrochloride.

20. The formulation of any one of claims 1-19 wherein the VEGF receptor fusion protein or anti-VEGF antibody or antibody fragment or VEGF binding molecule is a VEGF receptor fusion protein which has less than about 3.5% high molecular weight species immediately after manufacture and purification and/or less than or equal to about 6% high molecular weight species after storage for about 24 months at about 2-8ºC.

21. A formulation comprising the components in a formulation selected from the group consisting of:

Formulation A: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation B: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation C: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation D: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2;

Formulation E: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation F: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2.

PGR2026-00039
Biocon Ex.1002
Page 4925 of 4945

Formulation G: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation H: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation I: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation J: 80 mg/ml aflibercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation K: 80 mg/ml aflibercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation L: 80 mg/ml aflibercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation M: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation N: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation O: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation P: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2;

Formulation Q: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation R: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation S: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation T: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation U: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

PGR2026-00039
Biocon Ex.1002
Page 4926 of 4945

Formulation V: 150 mg/ml aflibercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation W: 150 mg/ml aflibercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation X: 150 mg/ml aflibercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation Y: 80 mg/ml conbercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation Z: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation AA: 80 mg/ml conbercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation BB: 80 mg/ml conbercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2;

Formulation CC: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation DD: 80 mg/ml conbercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation EE: 80 mg/ml conbercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation FF: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation GG: 80 mg/ml conbercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation HH: 80 mg/ml conbercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation II: 80 mg/ml conbercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation JJ: 80 mg/ml conbercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

PGR2026-00039
Biocon Ex.1002
Page 4927 of 4945

Formulation KK: 150 mg/ml conbercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation LL: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation MM: 150 mg/ml conbercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation NN: 150 mg/ml conbercept, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 6.2;

Formulation OO: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation PP: 150 mg/ml conbercept, 10 mM citrate-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 80, and 40 mM sodium chloride, with a pH of 5.8 to 6.2;

Formulation QQ: 150 mg/ml conbercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation RR: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation SS: 150 mg/ml conbercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 20, with a pH of 5.8 to 6.2;

Formulation TT: 150 mg/ml conbercept, 10 mM histidine-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation UU: 150 mg/ml conbercept, 10 mM phosphate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation VV: 150 mg/ml conbercept, 10 mM citrate-based buffer, 8% (w/v) sucrose, and 0.03% (w/v) polysorbate 80, with a pH of 5.8 to 6.2;

Formulation WW: 140 mg/ml VEGF receptor fusion protein, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM taurine, with a pH of 5.8;

Formulation XX: 140 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 4% (w/v) proline, 0.03% (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8;

PGR2026-00039
Biocon Ex.1002
Page 4928 of 4945

Formulation YY: 140 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 2.5% (w/v) sucrose, 2.0 % (w/v) proline, 0.03% (w/v) polysorbate 20, and 50 mM taurine, with a pH of 5.8;

Formulation ZZ: 140 mg/ml VEGF receptor fusion protein, 10 mM histidine-based buffer, 2.5% (w/v) sucrose, 2.0% (w/v) proline, 0.03% (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8;

Formulation AAA: 140 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8;

Formulation BBB: 140 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 2.5% (w/v) sucrose, 2.0% (w/v) proline, 0.03% (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8;

Formulation CCC: 80, 100, 120 or 140 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM arginine hydrochloride, with a pH of 5.8;

Formulation DDD: 140 mg/ml VEGF receptor fusion protein, 10 mM histidine-based buffer, 4% (w/v) proline, 0.03% (w/v) polysorbate 20, and 50 mM PSA, with a pH of 5.8;

Formulation EEE: 140 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, and 0.03% (w/v) polysorbate 20 and no thermal stabilizer, with a pH of 5.8;

Formulation FFF: 140 mg/ml VEGF receptor fusion protein, 10 mM sodium phosphate, 5 % (w/v) sucrose and 0.03 % polysorbate 20 with a pH of 6.2;

Formulation GGG: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% Sucrose; 0.03% polysorbate 20; 50 mM sodium sulfate;

Formulation HHH: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50 mM sodium thiocyanate;

Formulation III: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose, 0.03% polysorbate 20; 40 mM sodium citrate;

Formulation JJJ: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% Sucrose, 0.03% polysorbate 20; 50 mM glycine;

Formulation KKK: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose, 0.03% polysorbate 20; 50 mM sodium chloride;

PGR2026-00039
Biocon Ex.1002
Page 4929 of 4945

Formulation LLL: 140 mg/ml VEGF receptor fusion protein; 20 mM Histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50 mM lysine;

Formulation MMM: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50 mM sodium aspartate;

Formulation NNN: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50 mM sodium glutamate;

Formulation OOO: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50 mM sodium Citrate; 50 mM arginine hydrochloride;

Formulation PPP: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50mM glycine; 50 mM arginine hydrochloride;

Formulation QQQ: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50 mM sodium aspartate; 50 mM arginine hydrochloride;

Formulation RRR: 140 mg/ml VEGF receptor fusion protein; 20 mM histidine, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 50 mM sodium glutamate; 50 mM arginine hydrochloride;

Formulation SSS: 140 mg/ml VEGF receptor fusion protein; 20 mM His, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 10 mM L-arginine hydrochloride;

Formulation TTT: 140 mg/ml VEGF receptor fusion protein; 20 mM His, pH 5.8; 5% sucrose; 0.03% polysorbate 20; 100 mM L-arginine hydrochloride;

Formulation UUU: 30 mg/ml VEGF receptor fusion protein, 10% sucrose, 10 mM phosphate, 0.03% polysorbate 20, pH 6.2;

Formulation VVV: 30 mg/ml VEGF receptor fusion protein, 20% sucrose, 10 mM phosphate, 0.03% polysorbate 20, pH 6.2;

Formulation WWW: 60 mg/ml VEGF receptor fusion protein, 10% sucrose, 10 mM phosphate, 0.03% polysorbate 20, pH 6.2;

Formulation XXX: 60 mg/ml VEGF receptor fusion protein, 20% sucrose, 10 mM phosphate, 0.03% polysorbate 20, pH 6.2;

Formulation YYY: 120 mg/ml VEGF receptor fusion protein, 10% sucrose, 10 mM phosphate, 0.03% polysorbate 20, pH 6.2;

PGR2026-00039
Biocon Ex.1002
Page 4930 of 4945

Formulation ZZZ: 120 mg/ml VEGF receptor fusion protein, 20% sucrose, 10 mM phosphate, 0.03% polysorbate 20, pH 6.2;

Formulation AAAA: 120 mg/ml VEGF receptor fusion protein, 10% sucrose, 10 mM phosphate, 0.03% polysorbate 20, 50 mM NaCl, pH 6.2;

Formulation BBBB: 120 mg/ml VEGF receptor fusion protein, 20% sucrose, 10 mM phosphate, 0.03% polysorbate 20, 50 mM NaCl, pH 6.2;

Formulation CCCC: 140 mg/ml VEGF receptor fusion protein, 10 mM sodium phosphate, 5% sucrose, 40 mM sodium chloride, 0.03% PS20, pH 6.2;

Formulation DDDD: 80.0 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8;

Formulation EEEE: 120.0 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8;

Formulation FFFF: 113.3 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8;

Formulation GGGG: 114.3 mg/ml VEGF receptor fusion protein, 10 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8;

Formulation HHHH: 100.0 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8; and

Formulation IIII: 133.3 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8;

Formulation JJJJ: 150 mg/ml aflibercept, 10 mM sodium phosphate, 8% (w/v) sucrose, 0.03% (w/v) polysorbate 20 and 50 mM arginine hydrochloride, pH 6.2;

Formulation KKKK: 114.3 mg/ml VEGF receptor fusion protein, 20 mM histidine-based buffer, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, and 50 mM L-arginine monohydrochloride, with a pH of 5.8;

PGR2026-00039
Biocon Ex.1002
Page 4931 of 4945

Formulation LLLL: 90 mg/ml VEGF receptor fusion protein, 10 mM histidine, 5% (w/v) sucrose, 0.03% (w/v) polysorbate 20, pH 5.75; and

Formulation MMMM: 90 mg/ml VEGF receptor fusion protein, 10 mM histidine, 7% (w/v) sucrose, 0.03% (w/v) polysorbate 20, pH 5.75.

22. The formulation of claim 21 wherein the VEGF receptor fusion protein is aflibercept or conbercept.

23. The formulation of any one of claims 21-22 wherein the VEGF receptor fusion protein has less than about 3.5% high molecular weight species immediately after manufacture and purification and/or less than or equal to about 6% high molecular weight species after storage for about 24 months at about 2-8°C.

24. A container or injection device comprising the formulation of any one of claims 1-23.

25. The container or injection device of claim 24, which is a vial, syringe, autoinjector or pre-filled syringe.

26. A method for preparing the formulation of any one of claims 1-23, comprising combining the components of said formulation into a single composition.

27. The method of claim 26, further comprising loading a volume of the formulation into a syringe.

28. A formulation which is the product of the method of any one of claims 26-27.

29. A method for administering a formulation of any one of claims 1-22 and 27 to a subject comprising intraocularly injecting the formulation into the eye of the subject.

30. A method for treating an angiogenic eye disorder in a subject in need thereof comprising administering an intraocular injection of more than about 2 mg of a VEGF receptor fusion protein to the eye of the subject.

PGR2026-00039
Biocon Ex.1002
Page 4932 of 4945

31. The method of claim 30 comprising administering about 4 mg, about 6 mg, about 8 mg or about 10 mg of the VEGF receptor fusion protein to the eye of the subject.

32. A method for treating an angiogenic eye disorder in a subject in need thereof, the method comprising intraocularly injecting more than about 2 mg of VEGF receptor fusion protein in a formulation of any one of claims 1-23 and 28 into an eye of the subject.

33. The method of claim 32, comprising injecting about 4 mg, about 6 mg, about 8 mg or about 10 mg of the VEGF receptor fusion protein into the eye of the subject.

34. The method of any one of claims 30-33, wherein the intraocular injection is an intravitreal injection.

35. The method of any one of claims 30-34, wherein the angiogenic eye disorder is age-related macular degeneration (wet), macular edema, macular edema following retinal vein occlusion, retinal vein occlusion (RVO), central retinal vein occlusion (CRVO) branch retinal vein occlusion (BRVO), diabetic macular edema (DME), choroidal neovascularization (CNV), iris neovascularization, neovascular glaucoma, post-surgical fibrosis in glaucoma, proliferative vitreoretinopathy (PVR), optic disc neovascularization, corneal neovascularization, retinal neovascularization, vitreal neovascularization, pannus, pterygium, vascular retinopathy, diabetic retinopathy, non-proliferative diabetic retinopathy and/or proliferative diabetic retinopathy.

36. The method of any one of claims 30-35 wherein the VEGF receptor fusion protein is administered in about 100 microliters or less, about 90 microliters, about 80 microliters, about 75 microliters, about 70 microliters, about 60 microliters or about 50 microliters.

37. The method of any one of claims 30-36, wherein the VEGF receptor fusion protein is contained within a formulation comprising: about 5% sucrose; a histidine buffer; and about 0.03% surfactant.

38. The method of any one or claims 30-37 wherein more than about 2.0 mg of the VEGF receptor fusion protein is injected once every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 weeks.

PGR2026-00039
Biocon Ex.1002
Page 4933 of 4945

ABSTRACT

The present invention provides ophthalmic formulations having high concentrations of vascular endothelial growth factor (VEGF) receptor fusion protein and high stability during storage. Methods for treating angiogenic eye disorders using the high concentration formulations are also provided.

PTO/AIA/01 (06-12)
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	HIGH CONCENTRATION VEGF RECEPTOR FUSION PROTEIN CONTAINING FORMULATIONS

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 16/409,631 filed on May 10, 2019.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

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LEGAL NAME OF INVENTOR

Inventor: Kenneth S. GRAHAM Date (Optional): 14 Jan 2021

Signature: Kenneth S Graham

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LEGAL NAME OF INVENTOR

Inventor: Saurabh WADHWA Date (Optional): 17 June 2021

Signature: /Swadhwa/

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
    370                 375                 380

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385                 390                 395                 400

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                405                 410                 415

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
            420                 425                 430

Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
        435                 440                 445

Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455
```

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 17384070 Document Date: 07/23/2021

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via DAV or via the SCORE web page.
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Form Revision Date: March 1, 2019

DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 17384070 Document Date: 07/23/2021

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

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Form Revision Date: March 1, 2019

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 17/384,070	Filing Date 07/23/2021	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $480 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	07/23/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 18	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	PROGRAM SUPPORT ASSISTANT
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/TINA J BARDEN/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.