

TETRAHEDRAL ANTIBODIES

[0001] This application claims the benefit of U.S. Provisional Application No. 63/050,738, filed July 10, 2021, the contents of which is hereby incorporated by reference.

[0002] Throughout this application, various publications are referenced, including referenced in parenthesis. The disclosures of all publications mentioned in this application in their entireties are hereby incorporated by reference into this application in order to provide additional description of the art to which this invention pertains and of the features in the art which can be employed with this invention.

REFERENCE TO SEQUENCE LISTING

[0003] This application incorporates-by-reference nucleotide sequences which are present in the file named “210712_91300-B-PCT_SequenceListing_DH.txt”, which is 14.4 megabytes in size, and which was created on July 12, 2021 in the IBM-PC machine format, having an operating system compatibility with MS-Windows, which is contained in the text file filed July 12, 2021 as part of this application.

BACKGROUND OF THE INVENTION

[0004] Antibodies are a diverse family of vertebrate proteins comprising a Y-shaped structure consisting of two antigen binding (Fab) domains and one effector cell binding (Fc) domain. The arrangement of these three domains around a central hinge region bears a striking resemblance to trigonal molecular geometries in which a central atom is bonded to three peripheral atoms arranged at the corners of a triangle. Such a planar configuration of binding domains is sufficient for antibodies to carry out their normal functions. As such, efforts to-date to engineer antibodies and antibody-like molecules have also generally adopted the natural planar configuration of antibody binding domains. However, when the binding domains of an engineered antibody and antibody-like molecule are intended to engage multiple targets, a planar configuration of binding domains is not ideally suited to permit the simultaneous engaging of multiple targets by the binding domains.

SUMMARY OF THE INVENTION

[0005] This invention provides a tetrahedral antibody comprising a first, second, third, and fourth domain, wherein:

- a. each of the first and second domains are selected from the group consisting of a Fab domain and an Fc domain,
- b. each of the first and second domains comprise:
 - i. a first polypeptide chain comprising a first N-terminus of the domain, and
 - ii. a second polypeptide chain comprising a second N-terminus of the domain,
- c. the first N-terminus of the first domain and the first N-terminus of the second domain are joined to each other by a non-peptidyl linkage wherein the non-peptidyl linkage is:
 - i. a covalent linkage, or
 - ii. a non-covalent linkage between
 - 1. a first dimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the first domain, and
 - 2. a second dimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the second domain,wherein the first and second dimerizing polypeptides are not immunoglobulin polypeptides,
- d. the third domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the first domain, or
 - ii. the N-terminus of the first dimerizing polypeptide, and
- e. the fourth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the second domain, or
 - ii. the N-terminus of the second dimerizing polypeptide.

[0006] This invention also provides an octahedral antibody comprising a first, second, third, fourth, fifth and sixth domain, wherein:

- a. each of the first, second and third domains are selected from the group consisting of a Fab domain and an Fc domain,

- b. each of the first, second and third domains comprise:
 - i. a first polypeptide chain comprising a first N-terminus of the domain, and
 - ii. a second polypeptide chain comprising a second N-terminus of the domain,
- c. the first N-terminus of the first domain, the first N-terminus of the second domain and the first N-terminus of the third domain are joined to each other by a non-peptidyl linkage wherein the non-peptidyl linkage is:
 - i. a branched covalent linkage, or
 - ii. a non-covalent linkage between
 - 1. a first trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the first domain,
 - 2. a second trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the second domain, and
 - 3. a third trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the third domain,wherein the first, second, and third trimerizing polypeptides are not immunoglobulin polypeptides,
- d. the fourth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the first domain, or
 - ii. the N-terminus terminus of the first trimerizing polypeptide,
- e. the fifth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the second domain, or
 - ii. the N-terminus of the second trimerizing polypeptide, and
- f. the sixth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the third domain, or
 - ii. the N-terminus of the third trimerizing polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] **Figure 1:** General schematic structure of tetrahedral antibodies showing the positions of domains D1, D2, D3, D4 (A-D), and D5 and D6 (D), and the positions of the covalent linkage (A) and non-covalent linkage (B-D).

[0008] **Figure 2:** Schematic structure of tetrahedral antibodies with covalent linkages shown in Figure 1, Panel A, wherein (A) D1, D2, D3 and D4 are all different; (B) D1 and D2 are different; D3 and D4 are the same; (C) D1 and D2 are the same; D3 and D4 are different; (D) D1 and D2 are the same; D3 and D4 are the same.

[0009] **Figure 3:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel B, wherein (A) D1, D2, D3 and D4 are all different; (B) D1 and D2 are different; D3 and D4 are the same; (C) D1 and D2 are the same; D3 and D4 are different; (D) D1 and D2 are the same; D3 and D4 are the same. A single pair of dimerizing polypeptides form a heterodimeric pair in (A-C), and a homodimeric pair in (D).

[0010] **Figure 4:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel C, wherein (A) D1, D2, D3 and D4 are all different; (B) D1 and D2 are different; D3 and D4 are the same; (C) D1 and D2 are the same; D3 and D4 are different; (D) D1 and D2 are the same; D3 and D4 are the same. A single pair of dimerizing polypeptides form a heterodimeric pair in (A-C), and a homodimeric pair in (D).

[0011] **Figure 5:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel D, wherein D1 and D2 are different, and wherein among the D3-D6 domains (A) D3-D6 are all different; (B) D4 and D5 are the same and D3 and D6 are different; (C) D4 and D6 are the same and D3 and D5 are different; (D) D3 and D4 are the same and D5 and D6 are different. A single pair of dimerizing polypeptides form a heterodimeric pair in (A-D).

[0012] **Figure 6:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel D, wherein D1 and D2 are different, and wherein among the D3-D6 domains (A) D3 and D6 are the same, and D4 and D5 are the same; (B) D3 and D5 are the same, and D4 and D6 are the same; (C) D3 and D4 are the same, and D5 and D6 are the same; (D) D3-D6 are the same. A single pair of dimerizing polypeptides form a heterodimeric pair in (A-D).

[0013] **Figure 7:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel D, wherein D1 and D2 are different, there is no D6 domain, and wherein among the D3-D5 domains (A) D3-D5 are all different; (B) D4 and D5 are the same, and D3 is different; (C) D3 and D5 are the same, and D4 is different; (D) D3-D5 are all the same. A single pair of dimerizing polypeptides form a heterodimeric pair in (A-D).

[0014] **Figure 8A:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel D, wherein D1 and D2 are the same, and wherein among the D3-D6 domains (A) D3-D6 are all the same of a first type; (B) D3-D6 are all the same of a second type; (C) D3 and D4 are of the first type and D5 and D6 are of the second type, (D) D3 and D5 are of the first type and D4 and D6 are of the second type. A single pair of dimerizing polypeptides form a homodimeric pair in (A-D).

[0015] **Figure 8B:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel D, wherein D1 and D2 are the same, and wherein among the D3-D6 domains (A) D3-D6 are all the same of a first type; (B) D3-D6 are all the same of a second type; (C) D3 and D4 are of the first type and D5 and D6 are of the second type, (D) D3 and D4 are of the second type and D5 and D6 are of the first type. A single pair of dimerizing polypeptides form a homodimeric pair in (A-D).

[0016] **Figure 9:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel D, wherein D1 and D2 are the same, and wherein among the D3-D5 domains (A) D3-D6 are all the same of a first type, and (B) D3-D6 are all the same of a second type; (C) D4 and D5 are of the first type and D3 and D6 are of the second type, (D) D3 and D6 are of the first type and D4 and D5 are of the second type. Each of two pairs of dimerizing polypeptides form a heterodimeric pair in (A-D).

[0017] **Figure 10:** Schematic structure of tetrahedral antibodies with non-covalent linkages shown in Figure 1, Panel D, (A-B) wherein D1 and D2 are the same, and wherein among the D3-D5 domains (A) D3-D6 are all the same of a first type, and (B) D3-D6 are all the same of a second type; (C-D) wherein D1 and D2 are different, and wherein among the D3-D5 domains (C) D3 and D4 are of the first type and D5 and D6 are of the second type, (D) D3 and D5 are of the first type and D4 and D6 are of the second type. Each of two pairs of dimerizing polypeptides form a

homodimeric pair in (A-B). Each of two distinct pairs of dimerizing polypeptides form separate homodimeric pairs in (C-D).

[0018] **Figure 11:** Schematic structures of tetrahedral antibodies (A) Rc6-P4-Rc6, (B) Rc66SIDE-P4-Rc66SIDE, (C) Rc66SIDE-P16-Rc66SIDE, (D) Rc66SIDE-P28-Rc66SIDE, (E) B19c66-P4-Rc6m (F) Soc66-P28-6Ec66, (G) HA9c66AAC9-P4-HA9c66AAC9, and (H) HA9c66AAC9-P4-IL15c6AAC9.

[0019] **Figure 12:** Preparation of tetrahedral antibody Rc6-P4-Rc6.

[0020] **Figure 13:** Preparation of tetrahedral antibodies with non-covalent linkages as shown in Panel B of Figure 1.

[0021] **Figure 14:** Preparation of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG.

[0022] **Figure 15:** Analysis of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG by SE-HPLC.

[0023] **Figure 16:** Analysis of tetrahedral antibodies by SE-HPLC with different peptide linkers (L-1) ACE2RQ740c60PGRF-ACE2RQ740c60PGRF, (L-185) ACE2RQ740c60PGRF185-ACE2RQ740c60PGRF185, (L-198) ACE2RQ740c60PGRF198-ACE2RQ740c60PGRF198, (L-208) ACE2RQ740c60PGRF208-ACE2RQ740c60PGRF208, (L-212) ACE2RQ740c60PGRF235-ACE2RQ740c60PGRF235, (L-235) ACE2RQ740c60PGRF235-ACE2RQ740c60PGRF235, (L-240) ACE2RQ740c60PGRF240-ACE2RQ740c60PGRF240.

[0024] **Figure 17:** Analysis of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG by SE-HPLC/MALS.

[0025] **Figure 18:** Stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG and ACE monomer ACE2RQ615c60PG.

[0026] **Figure 19:** Preparation of tetrahedral antibody ACE2740FcG9-ACE2740FcG9.

[0027] **Figure 20:** Analysis of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 by SE-HPLC.

[0028] **Figure 21:** Analysis of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 by SE-HPLC/MALS.

[0029] **Figure 22:** Analysis of tetrahedral antibody ACE2RQ740FcPG-ACE2RQ740FcPG by SE-HPLC/MALS.

[0030] **Figure 23:** Stoichiometric binding analysis of an impure preparation of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 and ACE2 dimer ACE2740FcG9.

[0031] **Figure 24:** Stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 and ACE2 dimer ACE2-740Fc-G9.

[0032] **Figure 25:** Stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2RQ740FcPG-ACE2RQ740FcPG and ACE2 dimer ACE2-740Fc-G9.

[0033] **Figure 26:** Stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 and ACE2 dimer ACE2-615Fc-G9.

[0034] **Figure 27:** Inhibition of SARS-CoV-2-VSV pseudotype virus infection by ACE2 tetrahedral antibodies ACE2740FcG9-ACE2740FcG9 and ACE2RQ740FcPG-ACE2RQ740FcPG, and by ACE2 dimers ACE2-615Fc-G9 and ACE2RQ615FcPG.

[0035] **Figure 28:** Inhibition of SARS-CoV-2-VSV pseudotype virus infection by ACE2 tetrahedral antibodies ACE2740FcG9-ACE2740FcG9 and ACE2RQ740FcPG-ACE2RQ740FcPG, and by ACE2 dimers ACE2-740Fc-G9 and ACE2RQ740FcPG.

[0036] **Figure 29A:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and each of D3 and D4 is a Fab domain that specifically binds to a first target.

[0037] **Figure 29B:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, each of D3 and D4 is a Fab domain that specifically binds to a first target, and each of D5 and D6 is a variable region exchanged Fab domain that specifically binds to a second target.

[0038] **Figure 29C:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, each of D3 and D4 is a Fab domain that specifically binds to a first target, and each of D7 and D8 is a domain that specifically binds to a second target. D7 and D8 are each depicted as a single-chain TNFSF fusion polypeptide but may be any domain.

[0039] **Figure 29D:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, each of D3 and D4 is a Fab domain that specifically binds to a first target, each of D5 and

D6 is a variable region exchanged Fab domain that specifically binds to a second target, and each of D7 and D8 is a domain that specifically binds to a third target. D7 and D8 are depicted as a single-chain TNFSF fusion polypeptide but may be any domain.

[0040] **Figure 30A:** Octahedral antibody comprising a first, second and third trimerizing polypeptide that form a homotrimeric non-covalent linkage, wherein each of D1, D2 and D3 is a heterodimeric Fc domain, and each of D4, D5 and D5 is a Fab domain that specifically binds to a first target.

[0041] **Figure 30B:** Octahedral antibody comprising a first, second and third trimerizing polypeptide that form a homotrimeric non-covalent linkage, wherein each of D1, D2 and D3 is a heterodimeric Fc domain, each of D4, D5 and D6 is a Fab domain that specifically binds to a first target, and each of D7, D8 and D9 is a variable region exchanged Fab domain that specifically binds to a second target.

[0042] **Figure 30C:** Octahedral antibody comprising a first, second and third trimerizing polypeptide that form a homotrimeric non-covalent linkage, wherein each of D1, D2 and D3 is a heterodimeric Fc domain, each of D4, D5 and D6 is a Fab domain that specifically binds to a first target, and each of D10, D11 and D12 specifically binds to a second target. D10, D11 and D12 each are depicted as a single-chain TNFSF fusion polypeptide but may be any domain.

[0043] **Figure 30D:** Octahedral antibody comprising a first, second and third trimerizing polypeptide that form a homotrimeric non-covalent linkage, wherein each of D1, D2 and D3 is a heterodimeric Fc domain, each of D4, D5 and D6 is a Fab domain that specifically binds to a first target, each of D7, D8 and D9 is a variable region exchanged Fab domain that specifically binds to a second target and each of D10, D11 and D12 specifically binds to a third target. D10, D11 and D12 are each depicted as a single-chain TNFSF fusion polypeptide but may be any domain.

[0044] **Figure 31:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3, D4, D5 and D6 specifically bind to a first target (tetravalent, monospecific), (B) D3 and D4 specifically bind to a first target, and D5 and D6 are Fab domains that specifically bind to a second target (tetravalent, 2+2 bispecific), (C) D3 and D4 are Fab domains that specifically bind to a first target, and D5 and D6 specifically bind to a second target (tetravalent, 2+2 bispecific), and (D) D3, D4, D5 and D6 are Fab domains that specifically bind to

a first target (tetravalent, monospecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], and second H2 chain [D2/D6], (B) six chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L2 chain [D5], and a second L2 chain [D6], (C) six chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], and a second L1 chain [D4], and (D) eight chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], a second L1 chain [D4], a first L2 chain [D5], and a second L2 chain [D6].

[0045] **Figure 32:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3 and D4 specifically bind to a first target, and D5 and D6 specifically bind to a second target (tetravalent, 2+2 bispecific), (B) D5 and D6 specifically bind to a first target, and D3 and D4 specifically bind to a second target (tetravalent, 2+2 bispecific), (C) D3 and D4 are Fab domains that specifically bind to a first target, and D5 and D6 are variable region exchanged Fab domains that specifically bind to a second target (tetravalent, 2+2 bispecific), and (D) D5 and D6 are Fab domains that specifically bind to a first target, and D3 and D4 are variable region exchanged Fab domains that specifically bind to a second target (tetravalent, 2+2 bispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], and a second H2 chain [D2/D6], (B) four chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], and a second H2 chain [D2/D6], (C) eight chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], a second L1 chain [D4], a first L2 chain [D5], and a second L2 chain [D6], and (D) eight chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], a second L1 chain [D4], a first L2 chain [D5], and a second L2 chain [D6].

[0046] **Figure 33:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3, D4 and D5 specifically bind to a first target, and D6 specifically binds to a second target (tetravalent, 3+1 bispecific), (B) D3, D4 and D5 specifically bind to a first target, and D6 is a Fab domain that specifically binds to a second target (tetravalent, 3+1

bispecific), (C) D3, D4 and D5 are Fab domains that specifically bind to a first target, and D6 specifically binds to a second target (tetravalent, 3+1 bispecific), and (D) D3, D4 and D5 are Fab domains that specifically bind to a first target, and D6 is a variable region exchanged Fab domain that specifically binds to a second target (tetravalent, 3+1 bispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], and a first H3 chain [D2/D6], (B) five chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a first H3 chain [D2/D6], and a first L3 chain [D6], (C) seven chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a first H3 chain [D2/D6], a first L1/L2 chain [D3], a second L1/L2 chain [D4], and a third L1/L2 chain [D5], and (D) eight chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a first H3 chain [D2/D6], a first L1/L2 chain [D3], a second L1/L2 chain [D4], a third L1/L2 chain [D5], and a first L3 chain [D6].

[0047] **Figure 34:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3 and D4 specifically bind to a first target, and D6 specifically binds to a second target (trivalent, 2+1 bispecific), (B) D3 and D4 specifically bind to a first target, and D6 is a Fab domain that specifically binds to a second target (trivalent, 2+1 bispecific), (C) D3 and D4 are Fab domains that specifically bind to a first target, and D6 specifically binds to a second target (trivalent, 2+1 bispecific), and (D) D3 and D4 are Fab domains that specifically bind to a first target, and D6 is a variable region exchanged Fab domain that specifically binds to a second target (trivalent, 2+1 bispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D2/D6], and a first Fc chain [D1], (B) five chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D2/D6], a first Fc chain [D1], and a first L2 chain [D6], (C) six chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D2/D6], a first Fc chain [D1], a first L1 chain [D3], and a second L1 chain [D4], and (D) seven chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D2/D6], a first Fc chain [D1], a first L1 chain [D3], a second L1 chain [D4], and a first L2 chain [D6].

[0048] **Figure 35:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3 and D4 specifically bind to a first target, D5 specifically binds to a

second target, and D6 specifically binds to a third target (tetravalent, 2+1+1 trispecific), (B) D3 and D4 specifically bind to a first target, D5 specifically binds to a second target, and D6 is a Fab domain that specifically binds to a third target (tetravalent, 2+1+1 trispecific), (C) D3 and D4 are Fab domains that specifically bind to a first target, D5 specifically binds to a second target, and D6 specifically binds to a third target (tetravalent, 2+1+1 trispecific), and (D) D3 and D4 are Fab domains that specifically bind to a first target, D5 specifically binds to a second target, and D6 is a variable region exchanged Fab domain that specifically binds to a third target (tetravalent, 2+1+1 trispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], and a first H3 chain [D2/D6], (B) five chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a first H3 chain [D2/D6], and a first L3 chain [D6], (C) six chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a first H3 chain [D2/D6], a first L1 chain [D3], and a second L1 chain [D4], and (D) seven chains: a first H1 chain [D1/D3], a second H1 chain [D2/D4], a first H2 chain [D1/D5], a first H3 chain [D2/D6], a first L1 chain [D3], a second L1 chain [D4], and a first L3 chain [D6].

[0049] **Figure 36:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein D1 is a heterodimeric Fc domain and D2 is a Fab domain, and wherein (A) D2 is a Fab that binds to a first target, and D3, D4 and D5 specifically bind to a second target (tetravalent, 3+1 bispecific), (B) D2 is a Fab that binds to a first target, D3 and D4 specifically bind to a second target, and D5 is a variable region exchanged Fab that specifically bind to a third target (tetravalent, 2+1+1 trispecific), (C) D2 is a Fab that binds to a first target, D3 and D4 are variable region exchanged Fab domains that specifically bind to a second target, and D5 specifically binds to a third target (tetravalent, 2+1+1 trispecific), and (D) D2 is a Fab that binds to a first target, D3, D4 and D5 are variable region exchanged Fab domains that specifically bind to a second target (tetravalent, 4+1 bispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], and a first Fab chain [D2], (B) five chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], a first Fab chain [D2], and a first L2 chain [D5], (C) six chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], a first Fab chain [D2], a first L1 chain [D3], and a second L1 chain [D4], (D) seven chains: a first H1

chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], a first Fab chain [D2], a first L1/L2 chain [D3], a second L1/L2 chain [D4], and a third L1/L2 chain [D5].

[0050] **Figure 37:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein D1 is a heterodimeric Fc domain and D2 is a Fab domain, and wherein (A) D2 is a variable region exchanged Fab that binds to a first target, and D3, D4 and D5 specifically bind to a second target (tetravalent, 3+1 bispecific), (B) D2 is a variable region exchanged Fab that binds to a first target, D3 and D4 specifically bind to a second target, and D5 is a Fab that specifically bind to a third target (tetravalent, 2+1+1 trispecific), (C) D2 is a variable region exchanged Fab that binds to a first target, D3 and D4 are Fab domains that specifically bind to a second target, and D5 specifically binds to a third target (tetravalent, 2+1+1 trispecific), and (D) D2 is a variable region exchanged Fab that binds to a first target, D3, D4, and D5 are Fab domains that specifically bind to a second target (tetravalent, 3+1 bispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], and a first Fab chain [D2], (B) five chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], a first Fab chain [D2], and a first L2 chain [D5], (C) six chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], a first Fab chain [D2], a first L1 chain [D3], and a second L1 chain [D4], and (D) seven chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first H2 chain [D1/D5], a first Fab chain [D2], a first L1/L2 chain [D3], a second L1/L2 chain [D4], and a third L1/L2 chain [D5].

[0051] **Figure 38:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein D1 is a heterodimeric Fc domain and D2 is a Fab domain, and wherein (A) D2 is a Fab that binds to a first target, and D3 and D4 specifically bind to a second target (trivalent, 2+1 bispecific), (B) D2 is a variable region exchanged Fab that binds to a first target, and D3 and D4 specifically bind to a second target, (C) D2 is a Fab that binds to a first target, and D3 and D4 are variable region exchanged Fab domains that specifically bind to a second target (trivalent, 2+1 bispecific), and (D) D2 is a variable region exchanged Fab that binds to a first target, D3 and D4 are Fab domains that specifically bind to a second target (trivalent, 2+1 bispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first Fc chain [D1], and a first Fab chain [D2], (B) four chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first Fc chain [D1], and a

first Fab chain [D2], (C) six chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first Fc chain [D1], a first Fab chain [D2], a first L1 chain [D3], and a second L1 chain [D4], and (D) six chains: a first H1 chain [D1/D3], a first H1Fab chain [D2/D4], a first Fc chain [D1], a first Fab chain [D2], a first L1 chain [D3], and a second L1 chain [D4].

[0052] **Figure 39:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3, D4, D5 and D6 specifically bind to a first target, and D7 and D8 specifically bind to a second target (hexavalent, 4+2 bispecific), (B) D3 and D4 specifically bind to a first target, D5 and D6 are Fab domains that specifically bind to a second target, and D7 and D8 specifically bind to a third target (hexavalent, 2+2+2 trispecific), (C) D3 and D4 are Fab domains that specifically bind to a first target, D5 and D6 specifically bind to a second target, and D7 and D8 specifically bind to a third target (hexavalent, 2+2+2 trispecific), and (D) D3, D4, D5 and D6 are Fab domains that specifically bind to a first target, and D7 and D8 specifically bind to a second target (hexavalent, 4+2 bispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], and second H2 chain [D2/D6], (B) six chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L2 chain [D5], and a second L2 chain [D6], (C) six chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], and a second L1 chain [D4], and (D) eight chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], a second L1 chain [D4], a first L2 chain [D5], and a second L2 chain [D6]. D7 and D8 may be attached at the C-termini of the first and second H1 chains (as shown), or the C-termini of the first and second H2 chains. D7 and D8 are depicted as a single-chain TNFSF ligand fusion polypeptides but may be any domain.

[0053] **Figure 40:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3 and D4 specifically bind to a first target, D5 and D6 specifically bind to a second target, and D7 and D8 specifically bind to a third target (hexavalent, 2+2+2 trispecific), (B) D5 and D6 specifically bind to a first target, D3 and D4 specifically bind to a second target, and D7 and D8 specifically bind to a third target (hexavalent, 2+2+2 trispecific), (C) D3 and D4 are Fab domains that specifically bind to a first target, D5 and D6 are variable region exchanged

Fab domains that specifically bind to a second target, and D7 and D8 specifically bind to a third target (hexavalent, 2+2+2 trispecific), and (D) D5 and D6 are Fab domains that specifically bind to a first target, D3 and D4 are variable region exchanged Fab domains that specifically bind to a second target, and D7 and D8 specifically bind to a third target (hexavalent, 2+2+2 trispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], and a second H2 chain [D2/D6], (B) four chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], and a second H2 chain [D2/D6], (C) eight chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], a second L1 chain [D4], a first L2 chain [D5], and a second L2 chain [D6], and (D) eight chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D1/D5], a second H2 chain [D2/D6], a first L1 chain [D3], a second L1 chain [D4], a first L2 chain [D5], and a second L2 chain [D6]. D7 and D8 may be attached at the C-termini of the first and second H1 chains (as shown), or the C-termini of the first and second H2 chains. D7 and D8 are depicted as a single-chain TNFSF ligand fusion polypeptides but may be any domain.

[0054] **Figure 41:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein each of D1 and D2 is a heterodimeric Fc domain, and wherein (A) D3 and D4 specifically bind to a first target, D6 specifically binds to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific), (B) D3 and D4 specifically bind to a first target, D6 is a Fab domain that specifically binds to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific), (C) D3 and D4 are Fab domains that specifically bind to a first target, D6 specifically binds to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific), and (D) D3 and D4 are Fab domains that specifically bind to a first target, D6 is a variable region exchanged Fab domain that specifically binds to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D2/D6], and a first Fc chain [D1], (B) five chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D2/D6], an Fc chain [D1] and an L2 chain [D6], (C) six chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D2/D6], a first Fc chain [D1], a first L1 chain [D3], and a second L1 chain [D4], and (D) seven chains: a first H1

chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D2/D6], a first Fc chain [D1], a first L1 chain [D3], a second L1 chain [D4], and a first L2 chain [D6]. D7 and D8 may be attached at the C-termini of the first and second H1 chains (as shown), or the C-termini of the first and second H2 chains. D7 and D8 are depicted as a single-chain TNFSF ligand fusion polypeptides but may be any domain.

[0055] **Figure 42:** Tetrahedral antibody comprising a first and second dimerizing polypeptide that form a homodimeric non-covalent linkage, wherein D1 is a heterodimeric Fc domain and D2 is a Fab domain, and wherein (A) D2 is a Fab domain that specifically binds to a first target, D3 and D4 specifically bind to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific), (B) D2 is a variable region exchanged Fab domain that specifically binds to a first target, D3 and D4 specifically bind to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific), (C) D2 is a Fab domain that specifically binds to a first target, D3 and D4 are variable region exchanged Fab domains that specifically bind to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific), and (D) D2 is a variable region exchanged Fab domain that specifically binds to a first target, D3 and D4 are Fab domains that specifically bind to a second target, and D7 and D8 specifically bind to a third target (pentavalent, 2+2+1 trispecific). The tetrahedral antibodies of A-D comprise (A) four chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first Fc chain [D1], and a first Fab chain [D2]. (B) four chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first Fc chain [D1], and a first Fab chain [D2]. (C) six chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first Fc chain [D1], a first Fab chain [D2], a first L1 chain [D3], and a second L1 chain [D4], and (D) six chains: a first H1 chain [D1/D3/D7], a second H1 chain [D2/D4/D8], a first H2 chain [D2/D6], a first Fc chain [D1], a first Fab chain [D2], a first L1 chain [D3], and a second L1 chain [D4]. D7 and D8 may be attached at the C-termini of the first and second H1 chains (as shown), or the C-termini of the first and second H2 chains. D7 and D8 are depicted as a single-chain TNFSF ligand fusion polypeptides but may be any domain.

[0056] **Figure 43:** ACE2 tetrahedral antibodies. Four forms of ACE2 tetrahedral antibodies are shown in panels c, d, e and f. Each comprises domains 1-4 and a dimerizing polypeptide. The structures in panels a and b are standard Fc dimers.

[0057] **Figure 44:** Relative neutralization activity of ACE2 tetrahedral antibody on live virus. Upper panel: demonstrates neutralization of live SARS-CoV-2 virus. Lower panel: demonstrates neutralization of NL63 (alpha coronavirus). ACE2 superdimer (6-05 SD) neutralizes viruses approximately 3 orders of magnitude better than standard Fc fusion protein (ACE2Fc615). Also shown are purified dimer (6-05 D) and an impure mixture of dimer and superdimer (6-05 Impure).

[0058] **Figure 45:** Pharmacokinetic curves for constructs 13-21 and 13-22. 13-21 corresponds to wt ACE2-B13 superheterodimer tetrahedral antibody. 13-22 corresponds to a mutant ACE2-B13 superheterodimer tetrahedral antibody which has the H378A mutation.

[0059] **Figure 46:** Pharmacokinetic curves for constructs 10-05 and 13-21. 10-05 corresponds to the ACE2-ACE2 superheterodimer tetrahedral antibody. 13-21 corresponds to wt ACE2-B13 superheterodimer tetrahedral antibody.

[0060] **Figure 47:** Relative neutralization activity of ACE2 core dimer (10-59), B-13 (12-09), and ACE2-B-13 superdimer (15-16).

[0061] **Figure 48A-Figure 48P:** Relative neutralization activity of various antibodies against 12 different variant viruses is compared to the ACE2-B13 silent version and ACE2-B13 active version (denoted by diamonds and circles).

[0062] **Figure 49:** Body weight change of Hamsters infected with South African SARS-CoV-2 virus treated with PBS control (PBS), 25mg/kg of REGN10933, and REG-CoV-2 cocktail (25 mg/kg each of Regeneron antibody REGN10933 and REG108987), and 25 mg/kg Fc silent ACE2-B13 (HB1516).

[0063] **Figure 50A-Figure 50C:** Relative quantitation of correct pairing and mispairing in bispecific antibodies. Main peak represents correctly paired molecules. Arrows point to positions at which one would expect mispaired products. The main peak consists of two peaks owing to residual O-glycan remaining after de-glycosylation procedure.

[0064] **Figure 51A-Figure 51C:** Relative neutralization activity of various antibodies against N439K and South African variants. ACE2 superheterodimers (squares) are described in Tables 36, 37, and 38.

DETAILED DESCRIPTION OF THE INVENTION

Tetrahedral Antibodies

[0065] This invention provides a tetrahedral antibody comprising a first, second, third, and fourth domain, wherein:

- a. each of the first and second domains are selected from the group consisting of a Fab domain and an Fc domain,
- b. each of the first and second domains comprise:
 - i. a first polypeptide chain comprising a first N-terminus of the domain, and
 - ii. a second polypeptide chain comprising a second N-terminus of the domain,
- c. the first N-terminus of the first domain and the first N-terminus of the second domain are joined to each other by a non-peptidyl linkage wherein the non-peptidyl linkage is:
 - i. a covalent linkage, or
 - ii. a non-covalent linkage between
 - 1. a first dimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the first domain, and
 - 2. a second dimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the second domain,wherein the first and second dimerizing polypeptides are not immunoglobulin polypeptides,
- d. the third domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the first domain, or
 - ii. the N-terminus of the first dimerizing polypeptide, and
- e. the fourth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the second domain, or
 - ii. the N-terminus of the second dimerizing polypeptide.

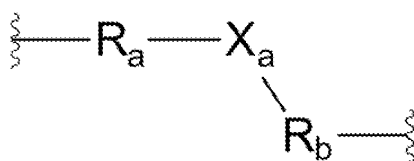
[0066] In embodiments of the invention:

- a. the first domain is a Fc domain and the second domain is a Fab domain,

- b. the first and second domains are Fc domains,
- c. the first and second domains are Fab domains,
- d. the third and fourth domains are Fab domains,
- e. the third and/or fourth domain are selected from the group consisting of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein,
- f. the third domain is selected from the group of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein, and the fourth domain is a Fab,
- g. the third domain is IL-15,
- h. the third domain is IL-15 and the fourth domain is an IL-15R α sushi domain,
- i. the third domain is IL-15 and the fourth domain is a Fab,
- j. the third and fourth domains are each the ACE2 peptidase domain (PD),
- k. the first and second domains are Fc domains, and the third and fourth domains are selected from the group consisting of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein,
- l. the first and second domains are Fc domains, the third domain is selected from the group consisting of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein, and the fourth domain is Fab,
- m. the first and second domains are Fc domains and the third and fourth domains are Fab domains,
- n. the first and second domains are Fc domains and the third and fourth domains are each the ACE2 peptidase domain (PD),
- o. the first domain is an Fc domain and the second, third and fourth domains are Fab domains,
- p. the first domain is an Fc domain, the second domain is a Fab domain, the third domain is IL-15, and the fourth domain is an IL-15R α sushi domain, or
- q. the first domain is an Fc domain, the second domain is a Fab domain, the third domain is IL-15, and the fourth domain is a Fab.

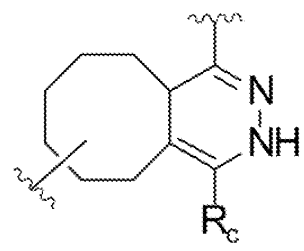
[0067] In embodiments of the invention, the non-peptidyl linkage between the first N-terminus of the first domain and the first N-terminus of the second domain is a covalent linkage.

[0068] In embodiments of the invention, the covalent linkage comprises the structure:



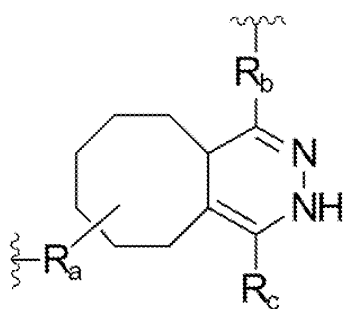
wherein:

- a. X_a is a chemical structure selected from the group consisting of:
 - i. a chemical structure which comprises a cyclooctane fused to a dihydropyridazine,
 - ii. a chemical structure which comprises a cyclooctene fused to a pyridazine,
- b. R_a is a bond or a chemical structure which connects X_a to the first N-terminus of the first domain, and
- c. R_b is a bond or a chemical structure which connects X_a to the first N-terminus of the second domain.



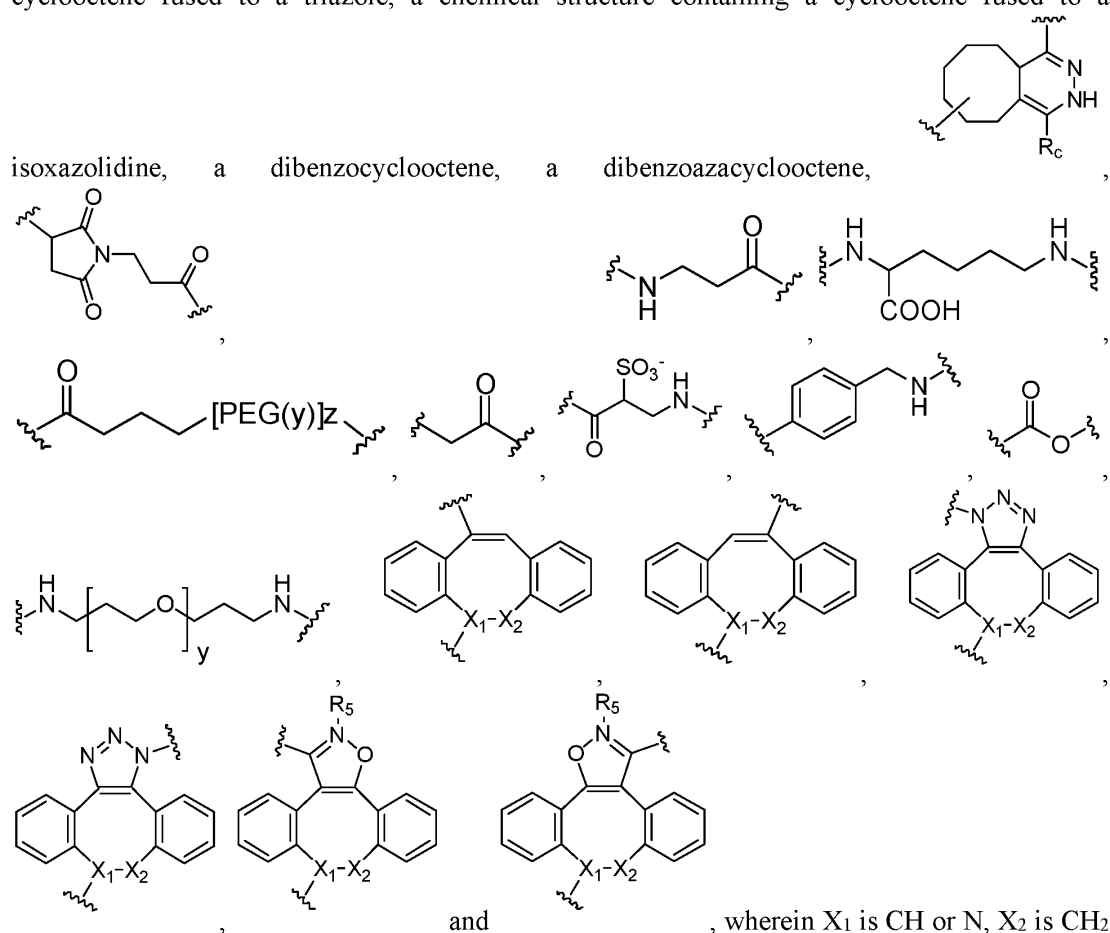
[0069] In embodiments of the invention, X_a comprises the structure wherein R_c is H, alkyl, or aryl, or a tautomer thereof.

[0070] In embodiments of the invention, the covalent linkage comprises the structure

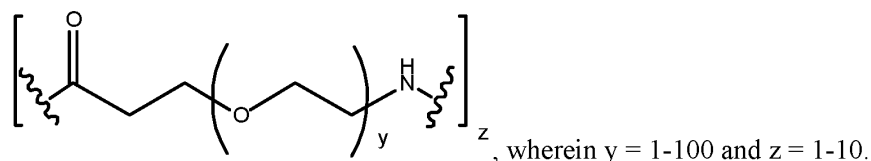


, wherein R_c is H, alkyl, or aryl, or a tautomer thereof.

[0071] In embodiments of the invention, R_a and R_b are, independently, a bond, or a chemical structure comprising or consisting of a chain of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more moieties, wherein each moiety is independently selected from the group consisting of [PEG(y)]_z, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C₁-C₁₀ alkyl, C₃-C₁₀ cycloalkane, C₂-C₁₀ alkene, C₅-C₁₀ cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C₂-C₅ acyl, C₂-C₅ acylamino, C₂-C₅ acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a

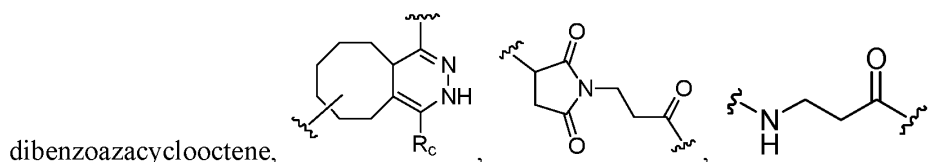


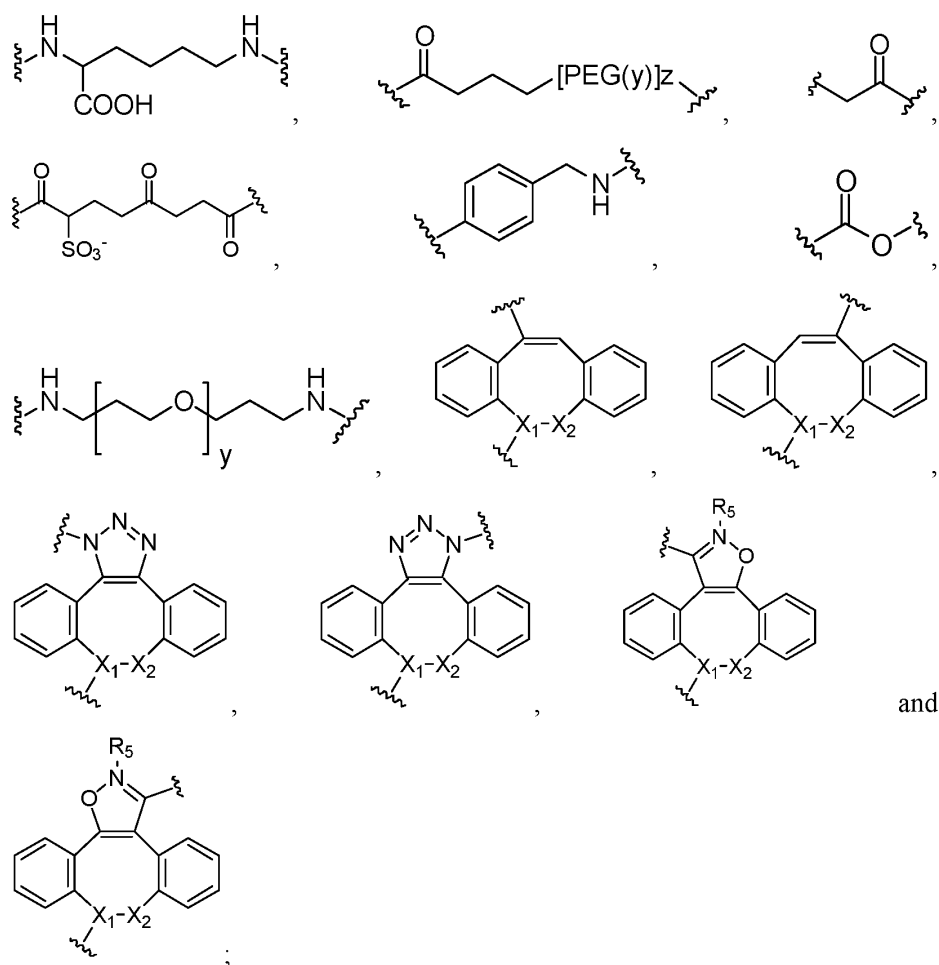
or a carbonyl group, and R_5 is an aryl or alkyl group, wherein $[PEG(y)]_z$ is:



[0072] In embodiments of the invention, R_a and/or R_b each independently:

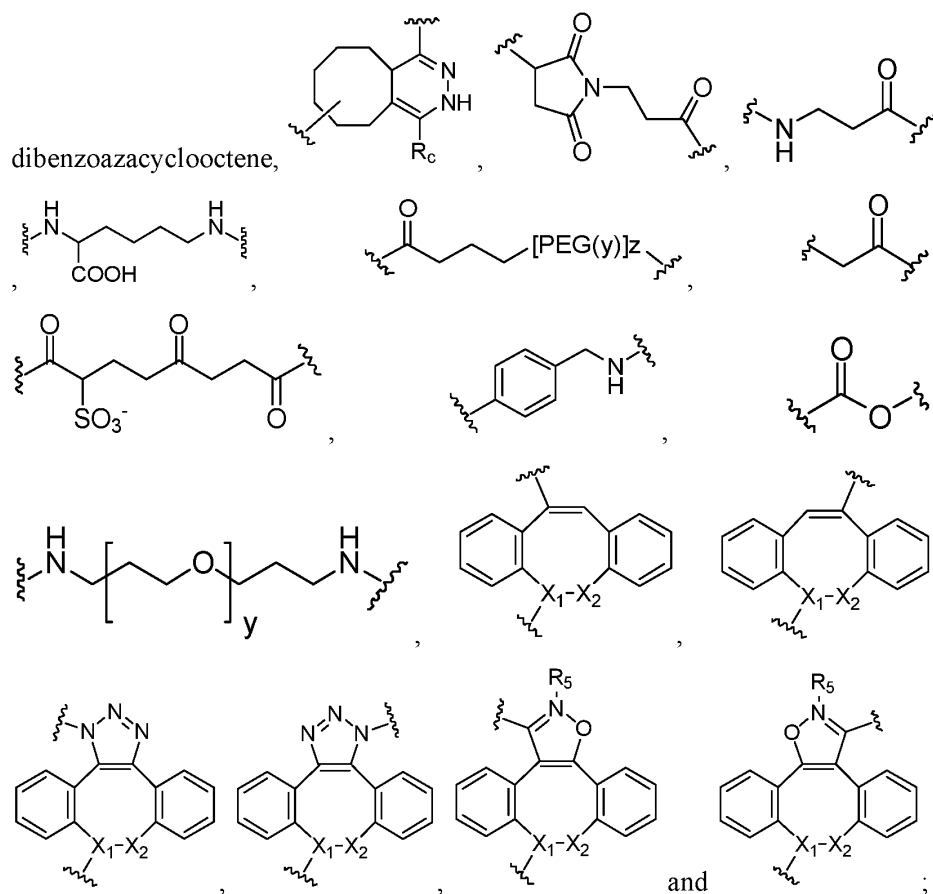
- a. comprise a $[PEG(y)]_z$ group;
- b. comprise a polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), or polysaccharide group;
- c. comprise a C_1 - C_4 alkyl group;
- d. comprise a succinimide;
- e. comprise an amine;
- f. comprise a succinyl, malonyl, glutaryl, phthalyl or adipoyl;
- g. comprise a malonyl;
- h. comprise an amino acid;
- i. comprise a cysteine;
- j. comprise a lysine;
- k. consist of a chain of 3 moieties selected from the group consisting of $[PEG(y)]_z$, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C_1 - C_{10} alkyl, C_3 - C_{10} cycloalkane, C_2 - C_{10} alkene, C_5 - C_{10} cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C_2 - C_5 acyl, C_2 - C_5 acylamino, C_2 - C_5 acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a isoxazolidine, a dibenzocyclooctene, a



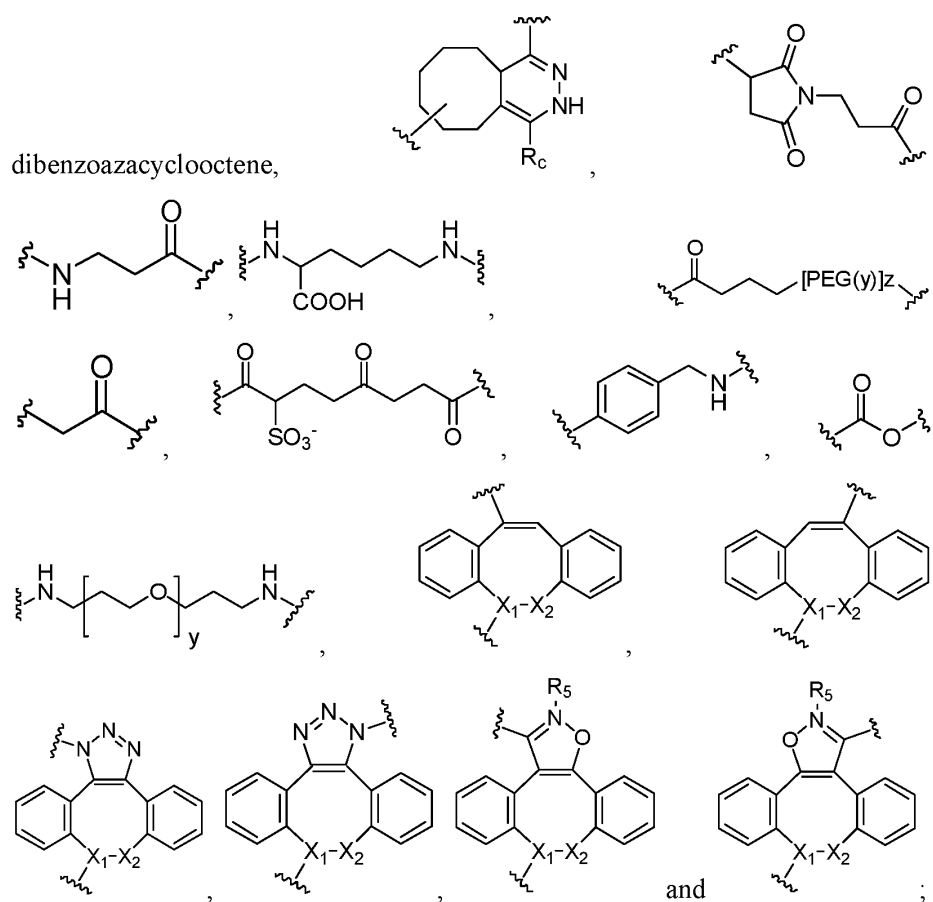


and

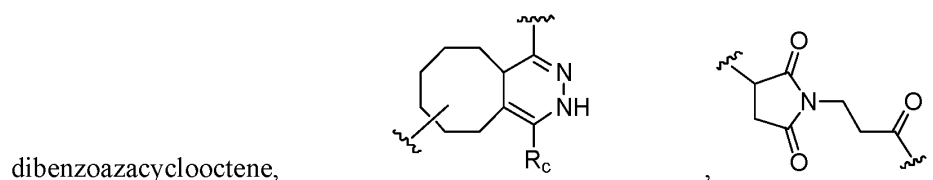
1. consist of a chain of 4 moieties selected from the group consisting of [PEG(y)]z, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C₁-C₁₀ alkyl, C₃-C₁₀ cycloalkane, C₂-C₁₀ alkene, C₅-C₁₀ cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C₂-C₅ acyl, C₂-C₅ acylamino, C₂-C₅ acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a isoxazolidine, a dibenzocyclooctene, a

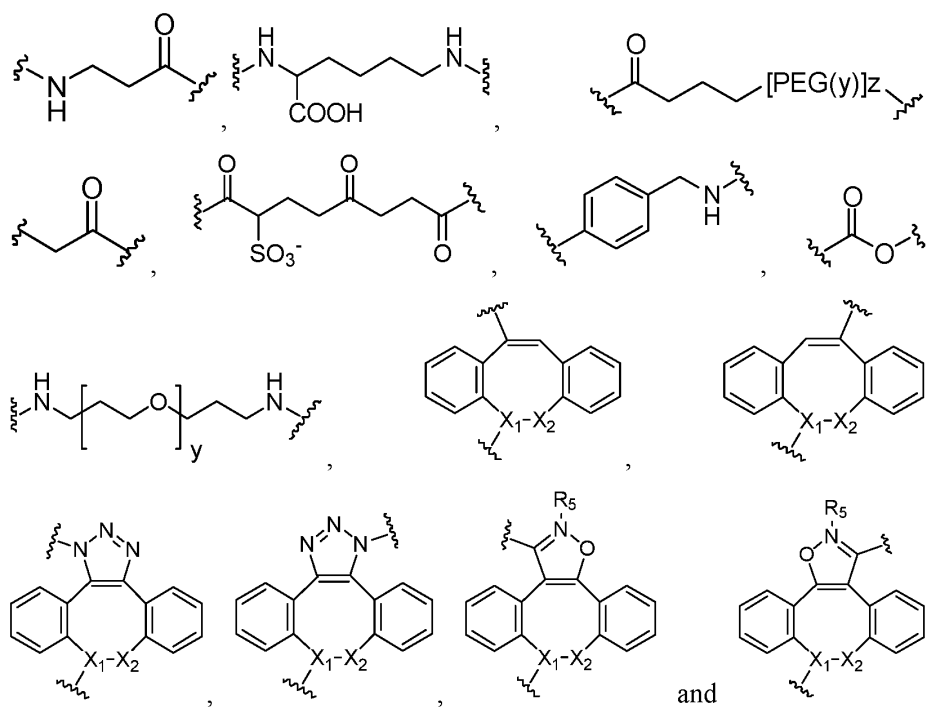


- m. consist of a chain of 5 moieties selected from the group consisting of [PEG(y)]z, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C₁-C₁₀ alkyl, C₃-C₁₀ cycloalkane, C₂-C₁₀ alkene, C₅-C₁₀ cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C₂-C₅ acyl, C₂-C₅ acylamino, C₂-C₅ acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a isoxazolidine, a dibenzocyclooctene, a



- n. comprise a [PEG(y)]z group bonded to a lysine;
- o. comprise a C₁-C₄ acyl group bonded to a succinimide group;
- p. comprise a lysine bonded to a C₁-C₄ acyl
- q. comprise a [PEG(y)]z group, which is bonded to a glutaryl;
- r. consist of a chain of three, four or five moieties selected from the group consisting of [PEG(y)]z, C₂-C₅ acyl, succinyl, malonyl, glutaryl, an amino acid, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to an isoxazolidine, a dibenzocyclooctene, a





wherein X_1 is CH or N, X_2 is CH_2 or a carbonyl group, and R_5 is an aryl or alkyl

group, wherein $[PEG(y)]_z$ is:

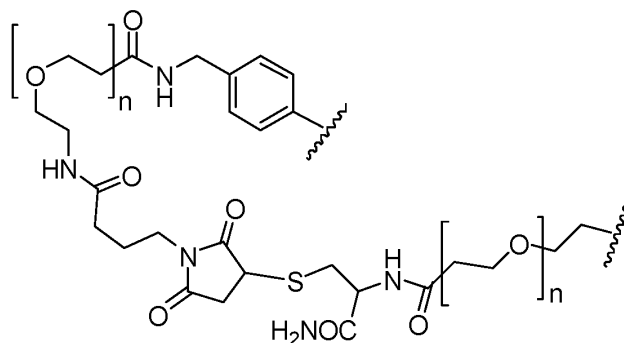
$$\left[\text{C}(=\text{O})\text{CH}_2\text{CH}_2\left(\text{OCH}_2\text{CH}_2\right)_y\text{NH} \right]_z$$

wherein $y = 1-100$ and $z = 1-10$;

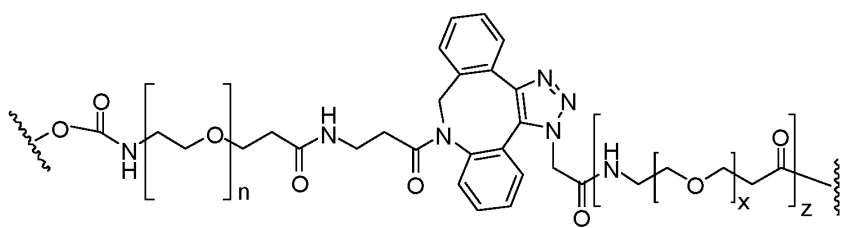
- s. is a bond;
- t. is a cysteine;
- u. has a linear structure; or
- v. has a branched structure;

w. has the structure:

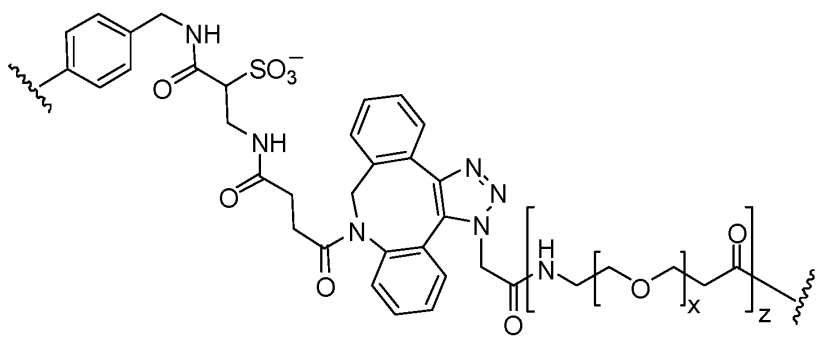
;



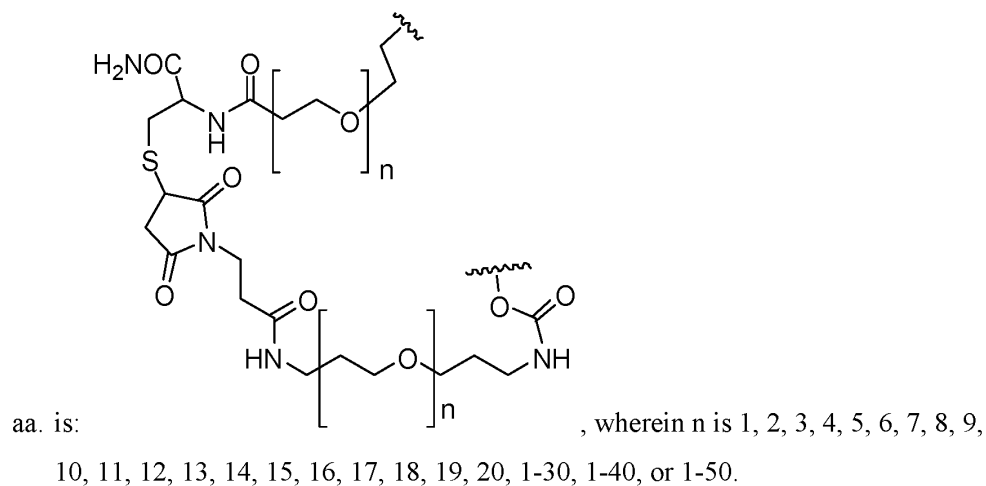
x. is: , wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50;



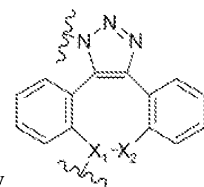
y. is: , wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50, x is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50 and z is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50;



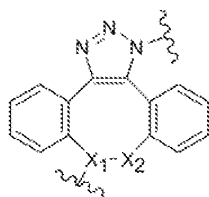
z. is: , wherein x is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50 and z is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50; or



[0073] In embodiments of the invention, R_a and/or R_b comprise the moiety



or



wherein X_1 is CH or N and X_2 is CH_2 or a carbonyl group.

[0074] In embodiments of the invention, the non-peptidyl linkage is a non-covalent linkage between a first dimerizing polypeptide attached to the first N-terminus of the first domain and a second dimerizing polypeptide attached to the first N-terminus of the second domain.

[0075] In embodiments of the invention, the first and second dimerizing polypeptides are selected from the group consisting of:

- a leucine zipper domain,
- a collectrin-like domain (CLD), and
- a collectrin domain (CD).

[0076] In embodiments of the invention:

- the first dimerizing polypeptide is the same as the second dimerizing polypeptide,
- and

- b. the dimerizing polypeptides form a homodimer.

[0077] In embodiments of the invention:

- a. the first dimerizing polypeptide is different than the second dimerizing polypeptide, and
- b. the first and second dimerizing polypeptides form a heterodimer.

[0078] In some of these embodiments of the invention, the first and second dimerizing polypeptides, when in the presence of each other, form less than 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% homodimers.

[0079] In embodiments of the invention:

- a. the third and fourth domains are each the ACE2 peptidase domain (PD), and
- b. the first and second dimerizing polypeptides are each the ACE2 collectrin-like domain (CLD).

[0080] In embodiments of the invention, the first and second domains are Fc domains.

[0081] In embodiments of the invention:

- a. the first and second domains are Fc domains and the third domain is a first type of Fab domain,
- b. the first and second domains are Fc domains and the third and fourth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
- c. the first domain is an Fc domain, and the second and third domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain, or
- d. the first domain is an Fc domain, and the second, third, and fourth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain.

[0082] In embodiments of the invention the tetrahedral antibody of additionally comprises a fifth domain, wherein the fifth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:

- a. the N-terminus of the first dimerizing polypeptide,

- b. the second N-terminus of the first domain, or
- c. the N-terminus of a third dimerizing polypeptide, wherein the third dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain.

[0083] In embodiments of the invention:

- a. the first and second domains are Fc domains and the fifth domain is a first type of Fab domain,
- b. the first and second domains are Fc domains, and the third and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
- c. the first and second domains are Fc domains, and the fourth and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
- d. the first and second domains are Fc domains, and the third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
- e. the first domain is an Fc domain, and the second and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
- f. the first domain is an Fc domain, and the second, third, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
- g. the first domain is an Fc domain, and the second, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain, or
- h. the first domain is an Fc domain, and the second, third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, a third type of Fab domain, and a fourth type of Fab domain.

[0084] In embodiments of the invention, the tetrahedral antibody additionally comprises a fifth and/or sixth domain, wherein:

- a. the fifth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the N-terminus of the first dimerizing polypeptide,
 - ii. the second N-terminus of the first domain, or
 - iii. the N-terminus of a third dimerizing polypeptide, wherein the third dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain,
- b. the sixth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the N-terminus of the second dimerizing polypeptide,
 - ii. the second N-terminus of the second domain, or
 - iii. the N-terminus of a fourth dimerizing polypeptide, wherein the fourth dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the second domain.

[0085] In embodiments of the invention:

- a. the first and second domains are Fc domains and the fifth domain is a first type of Fab domain,
- b. the first and second domains are Fc domains, and the third and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
- c. the first and second domains are Fc domains, and the fourth and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
- d. the first and second domains are Fc domains, and the third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
- e. the first domain is an Fc domain, and the second and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,

- f. the first domain is an Fc domain, and the second, third, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
- g. the first domain is an Fc domain, and the second, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain, or
- h. the first domain is an Fc domain, and the second, third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, a third type of Fab domain, and a fourth type of Fab domain.

[0086] In embodiments of the invention, the tetrahedral antibody further comprises a seventh and/or eighth domain, wherein:

- a. the seventh domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i. the first C-terminus of the first domain, or
 - ii. the second C-terminus of the first domain,
- b. the eighth domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i. the first C-terminus of the second domain, or
 - ii. the second C-terminus of the second domain.

[0087] In embodiments of the invention:

- a. the third, fourth, fifth and sixth domains are each the ACE2 PD and the dimerizing polypeptides are each the ACE2 CLD,
- b. the third and fourth domains are each the ACE2 PD, the fifth and sixth domains are each Fab domains, and the dimerizing polypeptides are each the ACE2 CLD,
- c. the third and fourth domains are each Fab domains, the fifth and sixth domains are each the ACE2 PD, and the dimerizing polypeptides are each the ACE2 CLD, or
- d. the first and second domains are Fc domains, the third and fourth domains are the ACE2 PD, the fifth and sixth domains are Fab domains, the dimerizing polypeptides are each the ACE2 CLD, and the first and second domains are each connected to their respective dimerizing polypeptides via a peptide linker,

preferably wherein such domains and linkers are characterized by one or more or all of the following features:

- i. the Fc domains are characterized by one or more or all of the following features:
 1. are heterodimers,
 2. are IgG1 Fc domains,
 3. comprise a silencing mutation such that the Fc domain lacks Fc gamma receptor binding activity, preferably wherein such mutation is a combination of the following mutations: P329G/L234A/L235A (PGLALA),
 4. comprise a mutation that enhances FcRn activity, preferably wherein such mutation extends the half-life of the tetrahedral antibody, preferably wherein the mutation is a combination the following mutations: L309D/Q311H/N434S (DHS),
 5. comprise a mutation that ablates their Protein A binding site, preferably wherein such mutation is H435R/Y436F (HY/RF),
- ii. the ACE2 peptidase domains comprise a mutation which blocks its angiotensin converting enzyme activity, preferably wherein such mutation is an H378A mutation,
- iii. the Fab domains are chimeric Fab domains comprising a murine variable region,
- iv. the peptide linkers each have a length of 23 amino acids and are derived from the stalk region of a TNF receptor, preferably wherein the TNF receptor is TNF receptor 1B, still more preferably wherein the peptide linker consists of the amino acid sequence set forth in SEQ ID NO: 4468,
- v. such domains and peptide linkers are formed by three different types of polypeptide chains, more preferably wherein the three different types of polypeptide chains are denoted H1, L2, and H2, the H1 chain comprises the amino acid sequence set forth in SEQ ID NO: 522, the L2 chain comprises the amino acid sequence set forth in SEQ ID NO: 465, and the H2 chain comprises the amino acid sequence set forth in SEQ ID NO: 534.

[0088] In embodiments of the invention:

- a. the third and fourth domains are each the ACE2 PD,
- b. the fifth domain, if present, is attached at its C-terminus by a peptide bond or via a peptide linker to the N-terminus of a third dimerizing polypeptide, wherein the third dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain,
- c. the sixth domain, if present, is attached at its C-terminus by a peptide bond or via a peptide linker to the N-terminus of a fourth dimerizing polypeptide, wherein the fourth dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the second domain, and
- d. the first, second, third, and fourth dimerizing polypeptides are each the ACE2 CLD.

[0089] In embodiments of the invention, the ACE2 PD comprises or consists of amino acids 18-615 of the ACE2 protein or a portion thereof.

[0090] In embodiments of the invention, the ACE2 CLD comprises or consists of amino acids 616-740 of the ACE2 protein or a portion thereof.

[0091] In embodiments of the invention, the ACE2 PD is catalytically active.

[0092] In embodiments of the invention, the ACE2 PD is catalytically inactive.

[0093] In embodiments of the invention, ACE2 PD comprises a R273Q, or a H378A mutation.

[0094] In embodiments of the invention, the Fc domains lack Fc gamma receptor binding activity.

[0095] In embodiments of the invention, the Fc domains comprise a P329G mutation, a L234A mutation and a L235A mutation (PGLALA).

[0096] In embodiments of the invention, the Fc domains comprise a mutation which enhances FcRn activity and/or half-life. In embodiments of the invention, such mutations are selected from any of the following combinations of mutations:

- a. M252Y/S254T/T256E (YTE),
- b. L309D/Q311H/N434S (DHS), or
- c. M428L/N434S (LS).

[0097] In embodiments of the invention, the Fc domains comprise a mutation that ablates their Protein A binding site, preferably wherein such mutation is H435R/Y436F (HY/RF).

[0098] In embodiments of the invention, the Fab domains are chimeric Fab domains comprising a murine variable region.

[0099] In embodiments of the invention:

- a. the fifth domain, third dimerizing polypeptide, and second polypeptide chain of the first domain is a stretch of consecutive amino acids,
 - b. the third domain, first dimerizing polypeptide, and first polypeptide chain of the first domain is a stretch of consecutive amino acids,
 - c. the fourth domain, second dimerizing polypeptide, and first polypeptide chain of the second domain is a stretch of consecutive amino acids, and
 - d. the sixth domain, fourth dimerizing polypeptide, and second polypeptide chain of the second domain is a stretch of consecutive amino acids,
- wherein each stretch of consecutive amino acids consists of the sequence of amino acids selected from the group consisting of SEQ ID NOs: 74-119.

Octahedral Antibodies

[0100] This invention also provides an octahedral antibody comprising a first, second, third, fourth, fifth and sixth domain, wherein:

- a. each of the first, second and third domains are selected from the group consisting of a Fab domain and an Fc domain,
- b. each of the first, second and third domains comprise:
 - i. a first polypeptide chain comprising a first N-terminus of the domain, and
 - ii. a second polypeptide chain comprising a second N-terminus of the domain,
- c. the first N-terminus of the first domain, the first N-terminus of the second domain and the first N-terminus of the third domain are joined to each other by a non-peptidyl linkage wherein the non-peptidyl linkage is:
 - i. a branched covalent linkage, or
 - ii. a non-covalent linkage between
 1. a first trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the first domain,
 2. a second trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the second domain, and

3. a third trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the third domain, wherein the first, second, and third trimerizing polypeptides are not immunoglobulin polypeptides,
- d. the fourth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the first domain, or
 - ii. the N-terminus terminus of the first trimerizing polypeptide,
- e. the fifth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the second domain, or
 - ii. the N-terminus of the second trimerizing polypeptide, and
- f. the sixth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i. the second N-terminus of the third domain, or
 - ii. the N-terminus of the third trimerizing polypeptide.

[0101] In embodiments of the octahedral antibody of the invention, the non-peptidyl linkage is a non-covalent linkage between a first trimerizing polypeptide attached to the first N-terminus of the first domain, a second trimerizing polypeptide attached to the first N-terminus of the second domain, and a third trimerizing polypeptide attached to the first N-terminus of the third domain, wherein the first, second, and third trimerizing polypeptides are selected from the group consisting of TNF ligand superfamily members OX40L/TNFLSF4, CD40L/TNFLSF5, FASL/TNFLSF6, CD70L/TNFLSF7, CD30L/TNFLSF8, 4-1BBL/TNFLSF9, TRAIL/TNFLSF10, RANKL/TNFLSF11, TWEAK/TNFLSF12, APRIL/TNFLSF13, BAFF/TNFLSF13B, LIGHT/TNFLSF14, VEGI/TNFLSF15, GITRL/TNFLSF18, EctodysplasinA/TNFLSF19, TNF/TNFLSF2, lymphotoxin alpha/TNFLSF1, and lymphotoxin beta/TNFLSF3.

[0102] In embodiments of the invention, the octahedral antibody further comprises a seventh, eighth, and/or ninth domain, wherein:

- a. the seventh domain is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain,

- b. the eighth domain is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the second domain, and/or
- c. the ninth domain is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the third domain.

[0103] In embodiments of the invention, the octahedral antibody further comprises a tenth, eleventh, and/or twelfth domain, wherein:

- a. the tenth domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i. the first C-terminus of the first domain, or
 - ii. the second C-terminus of the first domain,
- b. the eleventh domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i. the first C-terminus of the second domain, or
 - ii. the second C-terminus of the second domain,
- c. the twelfth domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i. the first C-terminus of the third domain, or
 - ii. the second C-terminus of the third domain.

[0104] In embodiments, the first, second, and third domains of octahedral antibodies of the invention may be any of the domains described herein as the first and second domains of a tetrahedral antibody of the invention. In embodiments, the fourth, fifth and sixth domains of octahedral antibodies of the invention may be any of the domains described herein as the third and fourth domains of a tetrahedral antibody of the invention. In embodiments, the seventh, eighth, and ninth domains of octahedral antibodies of the invention may be any of the domains described herein as the fifth and sixth domains of a tetrahedral antibody of the invention. In embodiments, the tenth, eleventh and twelfth domains of octahedral antibodies of the invention may be any of the domains described herein as the seventh and eighth domains of a tetrahedral antibody of the invention. Further, all such domains may comprise any of the features (such as mutations) described with respect to domains of tetrahedral antibodies of the invention.

Tetrahedral and Octahedral Antibodies

[0105] In embodiments of the invention:

- a. one or more of the first, second, third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral antibody, or one or more of the first, second, third, fourth, fifth, sixth domains, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody are Fc domains, wherein the one or more Fc domains are independently selected from any of the Fc domains disclosed herein,
- b. one or more of the first, second, third, fourth, fifth, sixth, seventh and eighth domains of the tetrahedral antibody, or one or more of the first, second, third, fourth, fifth, sixth domains, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody are Fab domains, wherein the one or more Fab domains are independently selected from any of the Fab domains disclosed herein,
- c. one or more of the third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral antibody, or one or more of the fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody are a secreted protein, wherein the one or more secreted protein are independently selected from any of the secreted proteins disclosed herein,
- d. one or more of the third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral antibody, or one or more of the fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody are extracellular domains of a transmembrane protein, wherein the one or more extracellular domains of a transmembrane protein are independently selected from any of the extracellular domains of a transmembrane protein disclosed herein,
- e. one or more of the third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral or one or more of the fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody:
 - i. comprise the structure of a compound that is a drug approved for treating a subject afflicted with a disease;
 - ii. comprise the structure of an organic compound having a molecular weight less than 1000 Daltons, a DNA aptamer, an RNA aptamer, an oligonucleotide, or a protein that is biologically active;

- iii. comprise a primary or a secondary amine;
- iv. are aripiprazole or oseltamivir;
- v. are a respiratory drug, an antiasthmatic agent, an analgesic agent, an antidepressant, an antianginal agent, an antiarrhythmic agent, an antihypertensive agent, an antidiabetic agent, an antihistamine, an anti-infective agent, an antibiotic, an antiinflammatory agent, an antiparkinsonism drug, an antipsychotics, an antipyretic agent, an antiulcer agent, an attention deficit hyperactivity disorder (ADHD) drug, a central nervous system stimulant, a decongestant, or a psychostimulant;
- vi. are alprenolol, acebutolol, amidephrine, amineptine, amosulalol, amoxapine, amphetaminil, atenolol, atomoxetine, balofloxacin, bamethan, befunolol, benazepril, benfluorex, benzoctamine, betahistine, betaxolol, bevantolol, bifemelane, bisoprolol, brinzolamide, bufeniodol, butethamine, camylofine, carazolol, carticaine, carvedilol, cephaeline, ciprofloxacin, cloZapine, clobenZorex, clorprenaline, cyclopentamine, delapril, demexiptiline, denopamine, desipramine, desloratadine, diclofenac, dimetofrine, dioxadrol, dobutamine, dopexamine, doripenem, dorzolamide, droprenilamine, duloxetine, eltopraZine, enalapril, enoxacin, epinephrine, ertapenem, esapraZole, esmolol, etoxadrol, fasudil, fendiline, fenethylline, fenfluramine, fenoldopam, fenoterol, fenproporex, flecamide, fluoxetine, formoterol, frovatriptan, gaboxadol, garenoxacin, gatifloxacin, grepafloxacin, hexoprenaline, imidapril, indalpine, indecainide, indeloxazine hydrochloride, isoxsuprine, ispronicline, labetalol, landiolol, lapatinib, levophacetoperane, lisinopril, lomefloxacin, lotrafiban, maprotiline, mecamlamine, mefloquine, mepindolol, meropenem, metapramine, metaproterenol, methoxyphenamine, dextrorotary methylphenidate, methylphenidate, metipranolol, metoprolol, mitoxantrone, mivazerol, moexipril, moprolol, moxifloxacin, nebivolol, nifenalol, nipradilol, norfloxacin, nortriptyline, nyldrin, olanZapine, oxamniquine, oxprenolol, oxyfedrine, paroxetine, perhexyline, phenmetrazine, phenylephrine, phenylpropylmethylamine, pholedrine,

- picilorex, pimethylline, pindolol, pipemidic acid, piridocaine, practolol, pradofloxacin, pramipexole, pramiverin, prenalterol, prenylamine, prilocalne, procaterol, pronethalol, propafenone, propranolol, propylhexedrine, protokylol, protriptyline, pseudoephedrine, reboxetine, rasagiline, (r)-rasagiline, repinotan, reproterol, rimiterol, ritodrine, safinamide, salbutamol/albuterol, salmeterol, sarizotan, sertraline, silodosin, sotalol, soterenol, sparfloxacin, spirapril, sulfinalol, synephrine, tamsulosin, tebanicline, tianeptine, tirofiban, tretoquinol, trimetazidine, troxipide, varenicline, vildagliptin, viloxazine, viquidil or xamoterol;
- vii. comprise a protein that is biologically active;
 - viii. are biologically active such that it has target-binding activity;
 - ix. are an independently-folding protein or a portion thereof;
 - x. are a glycosylated protein;
 - xi. comprise intra-chain disulfide bonds;
 - xii. binds a cytokine;
 - xiii. binds to a cytokine, wherein the cytokine is TNF α ;
 - xiv. comprise Atrial Natriuretic Peptide (ANP), Calcitonin, Corticotropin Releasing Hormone (CRH), Endothelin, Exenatide, Gastric Inhibitory Peptide (GIP), Glucagon-Like Peptide-1 (GLP-1), Glucagon-Like Peptide-2 (GLP-2), an analog of GLP-1 or GLP-2, Glucagon Vasoactive Intestinal Peptide (GVIP), Ghrelin, Peptide YY or Secretin, or a portion thereof;
 - xv. comprise a stretch of consecutive amino acids in the sequence HGEFTFTSDVSSYLEEQAAKEFIAWLVKGRG (SEQ ID NO: 4657);
 - xvi. comprise at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in the heavy chain of a Fab or a Fab' of an antibody;
 - xvii. comprise at least one at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in the light chain of a Fab or a Fab' of an antibody;
 - xviii. comprise at least one Fab or Fab' of an antibody, or a portion of at least one Fab or Fab';

- xix. comprise Fab-1 or Fab'1, or a portion thereof of an antibody;
 - xx. comprise Fab-2 or Fab'2, or a portion thereof of an antibody;
 - xxi. comprise two Fab or Fab' hands of an antibody;
 - xxii. comprise at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in a single chain antibody;
or
 - xxiii. comprise at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in a TNF α receptor.
- f. the tetrahedral or octahedral antibody comprises a covalent linkage, wherein the covalent linkage is selected from any covalent linkage disclosed herein, or comprises any heterobifunctional crosslinker disclosed herein; and/or
- g. the tetrahedral or octahedral antibody comprises one or more peptide linkers, wherein the one or more peptide linkers are independently selected from:
- i. a stretch of consecutive amino acids which is, or is present in, the sequence TSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPSPPAEGSTGD (SEQ ID NO: 3227);
 - ii. a stretch of consecutive amino acids which is, or is present in, the sequence GGGGAGGGGAGGGGAGGGGAGGGGAGGGG (SEQ ID NO: 226), or
 - iii. any peptide linker disclosed herein.

[0106] Figures 1-14 and 29A to 42 schematically describe various non-limiting examples of tetrahedral and octahedral antibodies of the invention. A description of the various types of domains of these tetrahedral and octahedral antibodies (as represented by ovals or groups of ovals in various colors and/or patterns) is provided in the Brief Description of the Drawings. Each figure represents an embodiment of the invention.

[0107] This invention also provides a composition comprising at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, at least 20%, at least 21%, at least 22%, at least 23%, at least 24%, at least 25%, at least 26%, at least 27%, at least 28%, at least 29%, at least 30%, at least 31%, at least 32%, at least 33%, at least 34%, at least 35%, at least 36%, at least 37%, at least 38%, at least 39%, at least 40%, at least 41%, at least 42%, at least 43%, at least 44%, at least 45%, at least 46%, at least 47%, at least 48%, at

least 49%, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, 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% of the tetrahedral or octahedral antibody of the invention, as a proportion (w/w) of peptide-containing molecules in the composition.

Domains

[0108] The domains of the tetrahedral and octahedral antibodies are numbered herein to facilitate description and identification of each domain. The first domain may be referred to herein as “D1,” or “domain 1.” The same principle applies to all other domains of the inventions (i.e. the second domain may be referred to herein as “D2” or “domain 2,” etc.). Further, unless specified otherwise, the tetrahedral antibodies of the invention may lack one or more domains without affecting the numbering of the other domains. For example, a tetrahedral antibody of the invention may comprise domains 1, 2, 3, 4, and 6 without comprising a domain 5. As another example, a tetrahedral antibody of the invention may comprise domains 1, 2, 3, 4, 7, and 8 without comprising domains 5 and 6 (see, e.g., Figure 42).

[0109] In principle, the first and second domains of the tetrahedral antibodies of the invention are interchangeable, i.e. a feature that is described as applying to the first domain can equally apply to the second domain in some embodiments. Similarly, the third and fourth domains of the tetrahedral antibodies of the invention are interchangeable, the fifth and sixth domains of the invention are interchangeable and the seventh and eighth domains are interchangeable.

[0110] Similarly, with respect to octahedral antibodies of the invention, the first, second, and third domains of the invention are interchangeable, the fourth, fifth, and sixth domains are interchangeable, the seventh, eighth, and ninth domains are interchangeable, and the tenth, eleventh and twelfth domains are interchangeable. As discussed above, in this context interchangeable means that any feature described herein as applying to one of the domains, may also apply to any of the other domains.

[0111] In the same way, a person of skill in the art will appreciate that domains 1 and 2 of the tetrahedral antibodies of the invention and domains 1, 2 and 3 of the octahedral antibodies of the invention serve a similar structural purpose and are therefore equivalent insofar as any description applying to domains 1 and 2 of tetrahedral antibodies of the invention may also apply to domains 1, 2, and 3 of octahedral antibodies of the invention. A similar equivalence applies as between domains 3 and 4 of the tetrahedral antibodies of the invention and domains 4, 5, and 6 of the octahedral antibodies of the invention. Further, a similar equivalence applies as between domains 5 and 6 of the tetrahedral antibodies of the invention and domains 7, 8 and 9 of the octahedral antibodies of the invention. Still further, a similar equivalence applies as between domains 7 and 8 of the tetrahedral antibodies of the invention and domains 10, 11 and 12 of the octahedral antibodies of the invention.

Chains

[0112] Various embodiments of the invention comprise domains which are made up of “heavy chains” and “light chains.” Heavy chains of the invention may be referred to herein as “H Chain,” or “H1,” “H2,” “H3,” etc. Similarly, light chains of the invention may be referred to herein as “L Chain,” or “L1,” “L2,” “L3,” etc.

[0113] In embodiments of the tetrahedral antibody of the invention comprise a first heavy chain (“H1”) and a second heavy chain (“H2”). H1 and H2 chains may pair with one another to form domains 1 and 2. H1 may also pair with a light chain to form domains 3 and 4, while H2 may also pair with a light chain to form domains 5 and 6. H1 and H2 chains may also homodimerize. In such cases, a dimerizing polypeptide on the H1 chain heterodimerizes with a dimerizing polypeptide on the H2 chain. The H1 or H2 chains may also comprise additional domains 7 and 8 as described herein. The H1 and/or H2 chains may also comprise a dimerizing polypeptide between domains 1 and 3 and/or between domains 2 and 4. In each of the tetrahedral antibodies of the invention, the H1 and H2 chains may comprise one or more peptide linkers. If the H1 and/or H2 chains comprise a dimerizing polypeptide the peptide linker may be present between either domain 1 or 2 and the dimerizing polypeptide, between the dimerizing polypeptide and domain 3 or 4, or both. A peptide linker may also be present between either domain 1 or 2 and the dimerizing polypeptide, between the dimerizing polypeptide and domain 5 or 6, or both.

[0114] A similar principle applies to octahedral antibody of invention. H1 and H2 chains pair with one another to form domains 1, 2 and 3. H1 may also pair with a light chain to form domains 4, 5, and 6, while H2 may pair with a light chain to form domains 7, 8, and 9. The H1 or H2 chains may also comprise additional domains 10, 11 and 12 as described herein.

[0115] Various embodiments of the invention comprise Fc fusion proteins. In such embodiments, part of an “Fc fusion chain” may pair with an “Fc chain” to form an Fc domain. Fc domains of the invention are typically domains 1 and/or 2 of the tetrahedral antibodies of the invention and domains 1, 2, and/or 3 of the octahedral antibodies of the invention. The “Fc fusion chains” typically also comprise a portion which forms domains 3, 4, 5, and/or 6 of the tetrahedral antibodies of the invention and domains 4, 5, 6, 7, 8, 9, 10, 11, and/or 12 of the octahedral antibodies of the invention.

[0116] In tetrahedral antibodies of the invention, the “Fc fusion chain” may include a dimerizing polypeptide between the portions forming part of domains 1 and 2 and the portions forming domains 3 and 4. In an alternative embodiment, the “Fc fusion chain” can lack a dimerizing polypeptide because the “Fc chain” of domains 1 and 2 each include a dimerizing polypeptide at their respective N-termini. In an alternative of this embodiment, the “Fc chain” of domains 1 and 2 are linked at their respective N-termini by a covalent linkage.

[0117] This invention specifically contemplates tetrahedral and octahedral antibodies wherein (1) the “Fc fusion chain” includes a dimerizing polypeptide between the portions forming part of domains 1 and 2 and the portions forming domains 3 and 4 and (2) domains 3 and 4 are not an ACE2 peptidase domain.

[0118] In octahedral antibodies of the invention, the “Fc fusion chain” may comprise a trimerizing polypeptide between the portions forming part of domains 1, 2, and 3, and the portion forming domains 4, 5, and 6.

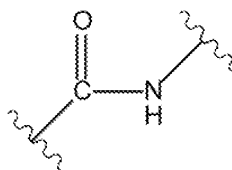
[0119] In each of the tetrahedral antibodies of the invention, the “Fc fusion chain” may comprise one or more peptide linkers between the portion forming part of domain 1 and the portion forming domains 3 or 5. If the “Fc fusion chain” comprises a dimerizing polypeptide the peptide linker may be present between either domain 1 and the dimerizing polypeptide, between the dimerizing polypeptide and domain 3 or 5, or both. Similarly, the “Fc fusion chain” may comprise a peptide linker between the portion forming part of domain 2 and the portion forming domain 4, or 6. If the

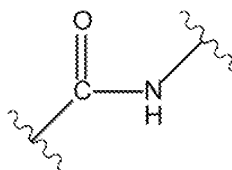
“Fc fusion chain” comprises a dimerizing polypeptide the peptide linker may be present between either domain 2 and the dimerizing polypeptide, between the dimerizing polypeptide and domain 4 or 6, or both.

[0120] In each of the octahedral antibodies of the invention, the “Fc fusion chain” may comprise a peptide linker between the portion forming part of domain 1 and the portion forming domain 4 or 7, between the portion forming part of domain 2 and the portion forming domain 5 or 8, and between the portion forming part of domain 3 and the portion forming domain 6 or 9. In each such case, if the “Fc fusion chain” comprises a trimerizing polypeptide, then the “Fc fusion chain” may comprise a peptide linker either between the portion forming part of domains 1, 2, and 3 and the trimerizing polypeptide, between the trimerizing polypeptide and the portion forming domains 4, 5, and 6, or both.

Terms

[0121] As used herein, and unless stated otherwise, each of the following terms shall have the definition set forth below.



[0122] Peptidyl linkage: the structure . A peptidyl linkage may be a peptide bond.

[0123] Stretch of consecutive amino acids: a plurality of amino acids arranged in a chain, each of which is joined to a preceding amino acid by a peptide bond, excepting that the first amino acid in the chain may optionally not be joined to a preceding amino acid. The amino acids of the chain may be naturally or non-naturally occurring, or may comprise a mixture thereof. The amino acids, unless otherwise indicated, may be genetically encoded, naturally-occurring but not genetically encoded, or non-naturally occurring, and any selection thereof.

[0124] N-terminal amino acid residue: the terminal residue of a stretch of two or more consecutive amino acids having a free α -amino (NH_2) functional group, or a derivative of an α -amino (NH_2) functional group.

[0125] N-terminus: the free α -amino (NH_2) group (or derivative thereof) of a N-terminal amino acid residue.

[0126] C-terminal amino acid residue: the terminal residue of a stretch of two or more consecutive amino acids having a free α -carboxyl (COOH) functional group, or a derivative of a α -carboxyl (COOH) functional group.

[0127] C-terminus: the free α -carboxyl (COOH) group (or derivative thereof) of a C-terminal amino acid residue.

[0128] A “biologically active structure”, as used herein, means a structure of a molecule or fragment thereof, capable of treating a disease or condition or localizing or targeting a compound of the invention to a site of a disease or condition in the body by performing a function or an action, or stimulating or responding to a function, an action or a reaction, in a biological context (e.g. in an organism, a cell, or an in vitro model thereof). Biologically active structures may comprise a structure of at least one of polypeptides, nucleic acids, small molecules such as small organic or inorganic molecules.

[0129] A “bond”, unless otherwise specified, or contrary to context, is understood to include a covalent bond, a dipole-dipole interaction such as a hydrogen bond, and intermolecular interactions such as van der Waals forces.

[0130] A “Signal Sequence” is a short (3-60 amino acids long) peptide chain that directs the post-translational transport of a polypeptide.

[0131] “Amino acid” as used herein, in one embodiment, means a L or D isomer of the genetically encoded amino acids, i.e. isoleucine, alanine, leucine, asparagine, lysine, aspartate, methionine, cysteine, phenylalanine, glutamate, threonine, glutamine, tryptophan, glycine, valine, proline, arginine, serine, histidine, tyrosine, selenocysteine, pyrrolysine and also includes homocysteine and homoselenocysteine.

[0132] Other examples of amino acids include an L or D isomer of taurine, gaba, dopamine, lanthionine, 2-aminoisobutyric acid, dehydroalanine, ornithine and citrulline, as well as non-natural homologues and synthetically modified forms thereof including amino acids having alkylene chains shortened or lengthened by up to two carbon atoms, amino acids comprising

optionally substituted aryl groups, and amino acids comprising halogenated groups, including halogenated alkyl and aryl groups as well as beta or gamma amino acids, and cyclic analogs.

[0133] Due to the presence of ionizable amino and carboxyl groups, the amino acids in these embodiments may be in the form of acidic or basic salts, or may be in neutral forms. Individual amino acid residues may also be modified by oxidation or reduction. Other contemplated modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, and methylation of the alpha-amino groups of lysine, arginine, and histidine side chains.

[0134] Covalent derivatives may be prepared by linking particular functional groups to the amino acid side chains or at the N- or C-termini.

[0135] Compounds comprising amino acids with R-group substitutions are within the scope of the invention. It is understood that substituents and substitution patterns on the compounds of the instant invention can be selected by one of ordinary skill in the art to provide compounds that are chemically stable from readily available starting materials.

[0136] "Natural amino acid" as used herein means a L or D isomer of the genetically encoded amino acids, i.e. isoleucine, alanine, leucine, asparagine, lysine, aspartate, methionine, cysteine, phenylalanine, glutamate, threonine, glutamine, tryptophan, glycine, valine, proline, arginine, serine, histidine, tyrosine, selenocysteine, pyrrolysine and homocysteine and homoselenocysteine.

[0137] "Non-natural amino acid" as used herein means a chemically modified L or D isomer of isoleucine, alanine, leucine, asparagine, lysine, aspartate, methionine, cysteine, phenylalanine, glutamate, threonine, glutamine, tryptophan, glycine, valine, proline, arginine, serine, histidine, tyrosine, selenocysteine, pyrrolysine, homocysteine, homoselenocysteine, taurine, gaba, dopamine, lanthionine, 2-aminoisobutyric acid, dehydroalanine, ornithine or citrulline, including cysteine and selenocysteine derivatives having C3-C10 aliphatic side chains between the alpha carbon and the S or Se. In one embodiment the aliphatic side chain is an alkylene. In another embodiment, the aliphatic side chain is an alkenylene or alkynylene.

[0138] In addition to the stretches of consecutive amino acid sequences described herein, it is contemplated that variants thereof can be prepared by introducing appropriate nucleotide changes into the encoding DNA, and/or by synthesis of the desired consecutive amino acid sequences.

Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the stretches of consecutive amino acids described herein when expression is the chosen method of synthesis (rather than chemical synthesis for example), such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

[0139] Variations in the sequences described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the consecutive amino acid sequence of interest that results in a change in the amino acid sequence as compared with the native sequence. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of about 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the full-length or mature native sequence. It is understood that any terminal variations are made within the context of the invention disclosed herein.

[0140] Amino acid sequence variants of the binding partner are prepared with various objectives in mind, including increasing the affinity of the binding partner for its ligand, facilitating the stability, purification and preparation of the binding partner, modifying its plasma half life, improving therapeutic efficacy, and lessening the severity or occurrence of side effects during therapeutic use of the binding partner.

[0141] Amino acid sequence variants of these sequences are also contemplated herein including insertional, substitutional, or deletional variants. Such variants ordinarily can be prepared by site-specific mutagenesis of nucleotides in the DNA encoding the target-binding monomer, by which DNA encoding the variant is obtained, and thereafter expressing the DNA in recombinant cell

culture. Fragments having up to about 100-150 amino acid residues can also be prepared conveniently by in vitro synthesis. Such amino acid sequence variants are predetermined variants and are not found in nature. The variants exhibit the qualitative biological activity (including target-binding) of the nonvariant form, though not necessarily of the same quantitative value. While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random or saturation mutagenesis (where all 20 possible residues are inserted) is conducted at the target codon and the expressed variant is screened for the optimal combination of desired activities. Such screening is within the ordinary skill in the art.

[0142] Amino acid insertions usually will be on the order of about from 1 to 10 amino acid residues; substitutions are typically introduced for single residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. It will be amply apparent from the following discussion that substitutions, deletions, insertions or any combination thereof are introduced or combined to arrive at a final construct.

[0143] As used herein, “modification” means an amino acid substitution, insertion, and/or deletion in a polypeptide sequence or an alteration to a moiety chemically linked to a protein. For example, a modification may be an altered carbohydrate or PEG structure attached to a protein. As used herein, “amino acid modification” means an amino acid substitution, insertion, and/or deletion in a polypeptide sequence. For clarity, unless otherwise noted, the amino acid modification is always to an amino acid coded for by DNA, e.g. the 20 amino acids that have codons in DNA and RNA.

[0144] As used herein, “amino acid substitution” or “substitution” means the replacement of an amino acid at a particular position in a parent polypeptide sequence with a different amino acid. In particular, in some embodiments, the substitution is to an amino acid that is not naturally occurring at the particular position, either not naturally occurring within the organism or in any organism. For example, the substitution E272Y refers to a variant polypeptide, in this case an Fc variant, in which the glutamic acid at position 272 is replaced with tyrosine. For clarity, a protein which has been engineered to change the nucleic acid coding sequence but not change the starting amino acid (for example exchanging CGG (encoding arginine) to CGA (still encoding arginine) to increase host organism expression levels) is not an “amino acid substitution”; that is, despite the

creation of a new gene encoding the same protein, if the protein has the same amino acid at the particular position that it started with, it is not an amino acid substitution.

[0145] As used herein, “amino acid insertion” or “insertion” means the addition of an amino acid sequence at a particular position in a parent polypeptide sequence. For example, -233E or 233E designates an insertion of glutamic acid after position 233 and before position 234. Additionally, -233ADE or A233ADE designates an insertion of AlaAspGlu after position 233 and before position 234.

[0146] As used herein, “amino acid deletion” or “deletion” means the removal of an amino acid sequence at a particular position in a parent polypeptide sequence. For example, E233- or E233# or E233() designates a deletion of glutamic acid at position 233. Additionally, EDA233- or EDA233# designates a deletion of the sequence GluAspAla that begins at position 233.

[0147] The terms “variant protein” or “protein variant”, or “variant” as used herein mean a protein that differs from that of a parent protein by virtue of at least one amino acid modification. Protein variant may refer to the protein itself, a composition comprising the protein, or the amino sequence that encodes it. Preferably, the protein variant has at least one amino acid modification compared to the parent protein, e.g. from about one to about seventy amino acid modifications, and preferably from about one to about five amino acid modifications compared to the parent. As described below, in some embodiments the parent polypeptide, for example an Fc parent polypeptide, is a human wild type sequence, such as the Fc region from IgG1, IgG2, IgG3 or IgG4, although human sequences with variants can also serve as “parent polypeptides”, for example the IgG1/2 hybrid of FIG. 13. The protein variant sequence herein will preferably possess at least about 80% identity with a parent protein sequence, and most preferably at least about 90% identity, more preferably at least about 95-98-99% identity. Variant protein can refer to the variant protein itself, compositions comprising the protein variant, or the DNA sequence that encodes it. Accordingly, the terms “antibody variant” or “variant antibody” as used herein mean an antibody that differs from a parent antibody by virtue of at least one amino acid modification, “IgG variant” or “variant IgG” as used herein is meant an antibody that differs from a parent IgG (again, in many cases, from a human IgG sequence) by virtue of at least one amino acid modification, and “immunoglobulin variant” or “variant immunoglobulin” as used herein mean an immunoglobulin sequence that differs from that of a parent immunoglobulin sequence by virtue of at least one

amino acid modification. “Fc variant” or “variant Fc” as used herein means a protein comprising an amino acid modification in an Fc domain. The Fc variants of the present invention are defined according to the amino acid modifications that compose them. Thus, for example, N434S or 434S is an Fc variant with the substitution serine at position 434 relative to the parent Fc polypeptide, wherein the numbering is according to the EU index. Likewise, M428L/N434S defines an Fc variant with the substitutions M428L and N434S relative to the parent Fc polypeptide. The identity of the WT amino acid may be unspecified, in which case the aforementioned variant is referred to as 428L/434S. It is noted that the order in which substitutions are provided is arbitrary, that is to say that, for example, 428L/434S is the same Fc variant as M428L/N434S, and so on. For all positions discussed in the present invention that relate to antibodies, unless otherwise noted, amino acid position numbering is according to the EU index. The EU index or EU index as in Kabat or EU numbering scheme refers to the numbering of the EU antibody (Edelman et al., 1969, *Proc Natl Acad Sci USA* 63:78-85, hereby entirely incorporated by reference.) The modification can be an addition, deletion, or substitution. Substitutions can include naturally occurring amino acids and, in some cases, synthetic amino acids. Examples include U.S. Pat. No. 6,586,207; WO 98/48032; WO 03/073238; US2004-0214988A1; WO 05/35727A2; WO 05/74524A2; J. W. Chin et al., (2002), *Journal of the American Chemical Society* 124:9026-9027; J. W. Chin, & P. G. Schultz, (2002), *ChemBioChem* 11:1135-1137; J. W. Chin, et al., (2002), *PICAS United States of America* 99:11020-11024; and, L. Wang, & P. G. Schultz, (2002), *Chem.* 1-10, all entirely incorporated by reference.

[0148] As used herein, “protein” means at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The terms “protein” and “stretch of consecutive amino acids” are used interchangeably herein. The peptidyl group may comprise naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures, i.e. “analogs”, such as peptoids (see Simon et al., *PNAS USA* 89(20):9367 (1992), entirely incorporated by reference). The amino acids may either be naturally occurring or synthetic (e.g. not an amino acid that is coded for by DNA); as will be appreciated by those in the art. For example, homo-phenylalanine, citrulline, ornithine and noreleucine are considered synthetic amino acids for the purposes of the invention, and both D- and L-(R or S) configured amino acids may be utilized. The variants of the present invention may comprise modifications that include the use of synthetic amino acids incorporated using, for example, the technologies developed by

Schultz and colleagues, including but not limited to methods described by Cropp & Shultz, 2004, Trends Genet. 20(12):625-30, Anderson et al., 2004, Proc Natl Acad Sci USA 101 (2):7566-71, Zhang et al., 2003, 303(5656):371-3, and Chin et al., 2003, Science 301(5635):964-7, all entirely incorporated by reference. In addition, polypeptides may include synthetic derivatization of one or more side chains or termini, glycosylation, PEGylation, circular permutation, cyclization, linkers to other molecules, fusion to proteins or protein domains, and addition of peptide tags or labels.

[0149] The term “residue” as used herein means a position in a protein and its associated amino acid identity. For example, Asparagine 297 (also referred to as Asn297 or N297) is a residue at position 297 in the human antibody IgG1.

[0150] As used herein “Fab” or “Fab region” means the polypeptide that comprises the VH, CH1, VL, and CL immunoglobulin domains. Fab may refer to this region in isolation, or this region in the context of a full length antibody, antibody fragment or Fab fusion protein. “Fv” or “Fv fragment” or “Fv region” as used herein mean a polypeptide that comprises the VL and VH domains of a single antibody.

[0151] “IgG subclass modification” or “isotype modification” as used herein mean an amino acid modification that converts one amino acid of one IgG isotype to the corresponding amino acid in a different, aligned IgG isotype. For example, because IgG1 comprises a tyrosine and IgG2 a phenylalanine at EU position 296, a F296Y substitution in IgG2 is considered an IgG subclass modification.

[0152] The term “non-naturally occurring modification” means an amino acid modification that is not isotypic. For example, because none of the IgGs comprise a serine at position 434, the substitution 434S in IgG1, IgG2, IgG3, or IgG4 (or hybrids thereof) is considered a non-naturally occurring modification.

[0153] The term “effector function” as used herein means a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Effector functions include but are not limited to ADCC, ADCP, and CDC.

[0154] The term “IgG Fc ligand” as used herein means a molecule, preferably a polypeptide, from any organism that binds to the Fc region of an IgG antibody to form an Fc/Fc ligand complex. Fc ligands include but are not limited to FcγRIs, FcγRIIs, FcγRIIIs, FcRn, C1q, C3, mannan binding

lectin, mannose receptor, staphylococcal protein A, streptococcal protein G, and viral FcγR. Fc ligands also include Fc receptor homologs (FcRH), which are a family of Fc receptors that are homologous to the FcγRs (Davis et al., 2002, Immunological Reviews 190:123-136, entirely incorporated by reference). Fc ligands may include undiscovered molecules that bind Fc. Particular IgG Fc ligands are FcRn and Fc gamma receptors. “Fc ligand” as used herein means a molecule, preferably a polypeptide, from any organism that binds to the Fc region of an antibody to form an Fc/Fc ligand complex.

[0155] The terms “Fc gamma receptor”, “FcγR”, “FcγR” or “FcγR” as used herein mean any member of the family of proteins that bind the IgG antibody Fc region and is encoded by an FcγR gene. In humans this family includes but is not limited to FcγRI (CD64), including isoforms FcγRIa, FcγRIb, and FcγRIc; FcγRII (CD32), including isoforms FcγRIIa (including allotypes H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16), including isoforms FcγRIIIa (including allotypes V158 and F158) and FcγRIIIb (including allotypes FcγRIIb-NA1 and FcγRIIb-NA2) (Jefferis et al., 2002, Immunol Lett 82:57-65, entirely incorporated by reference), as well as any undiscovered human FcγRs or FcγR isoforms or allotypes. An FcγR may be from any organism, including but not limited to humans, mice, rats, rabbits, and monkeys. Mouse FcγRs include but are not limited to FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIII-2 (CD16-2), as well as any undiscovered mouse FcγRs or FcγR isoforms or allotypes.

[0156] The terms “FcRn” or “neonatal Fc Receptor” as used herein is meant a protein that binds the IgG antibody Fc region and is encoded at least in part by an FcRn gene. The FcRn may be from any organism, including but not limited to humans, mice, rats, rabbits, and monkeys. As is known in the art, the functional FcRn protein comprises two polypeptides, often referred to as the heavy chain and light chain. The light chain is beta-2-microglobulin and the heavy chain is encoded by the FcRn gene. Unless otherwise noted herein, FcRn or an FcRn protein refers to the complex of FcRn heavy chain with beta-2-microglobulin. A variety of FcRn variants used to increase binding to the FcRn receptor, and in some cases, to increase serum half-life, are shown in the Figure Legend of Figure 11.

[0157] The terms “parent polypeptide” as used herein mean a starting polypeptide that is subsequently modified to generate a variant. The parent polypeptide may be a naturally occurring

polypeptide, or a variant or engineered version of a naturally occurring polypeptide. Parent polypeptide may refer to the polypeptide itself, compositions that comprise the parent polypeptide, or the amino acid sequence that encodes it. Accordingly, “parent immunoglobulin” as used herein means an unmodified immunoglobulin polypeptide that is modified to generate a variant, and “parent antibody” as used herein means an unmodified antibody that is modified to generate a variant antibody. It should be noted that “parent antibody” includes known commercial, recombinantly produced antibodies as outlined below.

[0158] The terms “Fc fusion protein” or “immunoadhesin” as used herein means a protein comprising an Fc region, generally linked (optionally through a linker moiety, as described herein) to a different protein, such as a binding moiety to a target protein, as described herein. In some cases, one monomer of the heterodimeric protein comprises an antibody heavy chain (either including an scFv or further including a light chain) and the other monomer is a Fc fusion, comprising a variant Fc domain and a ligand.

[0159] The term “position” as used herein is meant a location in the sequence of a protein. Positions may be numbered sequentially, or according to an established format, for example the EU index for antibody numbering.

[0160] The term “target antigen” as used herein means the molecule that is bound specifically by the variable region of a given antibody. A target antigen may be a protein, carbohydrate, lipid, or other chemical compound. A wide number of suitable target antigens are described below.

[0161] The term “target cell” as used herein means a cell that expresses a target antigen.

[0162] The term “variable region” as used herein means the region of an immunoglobulin that comprises one or more Ig domains substantially encoded by any of the V κ , V λ , and/or V H genes that make up the kappa, lambda, and heavy chain immunoglobulin genetic loci respectively.

[0163] The term “wild type or wt” as used herein means an amino acid sequence or a nucleotide sequence that is found in nature, including allelic variations. A WT protein has an amino acid sequence or a nucleotide sequence that has not been intentionally modified.

[0164] The antibodies of the present invention are generally isolated or recombinant. “Isolated,” when used to describe the various polypeptides disclosed herein, means a polypeptide that has been identified and separated and/or recovered from a cell or cell culture from which it was

expressed. Ordinarily, an isolated polypeptide will be prepared by at least one purification step. An "isolated antibody," refers to an antibody which is substantially free of other antibodies having different antigenic specificities.

[0165] "Specific binding" or "specifically binds to" or is "specific for" a particular antigen or an epitope means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target.

[0166] Specific binding for a particular antigen or an epitope can be exhibited, for example, by an antibody having a KD for an antigen or epitope of at least about 10^{-4} M, at least about 10^{-5} M, at least about 10^{-6} M, at least about 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9} M, alternatively at least about 10^{-10} M, at least about 10^{-11} M, at least about 10^{-12} M, or greater, where KD refers to a dissociation rate of a particular antibody-antigen interaction. Typically, an antibody that specifically binds an antigen will have a KD that is 20-, 50-, 100-, 500-, 1000-, 5,000-, 10,000- or more times greater for a control molecule relative to the antigen or epitope.

[0167] Also, specific binding for a particular antigen or an epitope can be exhibited, for example, by an antibody having a KA or Ka for an antigen or epitope of at least 20-, 50-, 100-, 500-, 1000-, 5,000-, 10,000- or more times greater for the epitope relative to a control, where KA or Ka refers to an association rate of a particular antibody-antigen interaction.

[0168] As used herein "ablation" means a decrease or removal of activity. Thus, for example, "ablating Fc γ R binding" means the Fc region amino acid variant has less than 50% starting binding as compared to an Fc region not containing the specific variant, with less than 70-80-90-95-98% loss of activity being preferred, and in general, with the activity being below the level of detectable binding in a Carterra assay.

[0169] As used herein, "ADCC" or "antibody dependent cell-mediated cytotoxicity" means cell-mediated reaction wherein nonspecific cytotoxic cells that express Fc γ Rs recognize bound antibody on a target cell and subsequently cause lysis of the target cell. ADCC is correlated with binding to Fc γ RIIIa; increased binding to Fc γ RIIIa leads to an increase in ADCC activity.

[0170] As used herein "ADCP" or antibody dependent cell-mediated phagocytosis mean the cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell.

[0171] In an aspect, the invention provides a stretch of consecutive amino acids (i.e. a protein) having at least about 80% sequence identity, preferably at least about 81% sequence identity, more preferably at least about 82% sequence identity, yet more preferably at least about 83% sequence identity, yet more preferably at least about 84% sequence identity, yet more preferably at least about 85% sequence identity, yet more preferably at least about 86% sequence identity, yet more preferably at least about 87% sequence identity, yet more preferably at least about 88% sequence identity, yet more preferably at least about 89% sequence identity, yet more preferably at least about 90% sequence identity, yet more preferably at least about 91% sequence identity, yet more preferably at least about 92% sequence identity, yet more preferably at least about 93% sequence identity, yet more preferably at least about 94% sequence identity, yet more preferably at least about 95% sequence identity, yet more preferably at least about 96% sequence identity, yet more preferably at least about 97% sequence identity, yet more preferably at least about 98% sequence identity and yet more preferably at least about 99% sequence identity to an amino acid sequence disclosed in the specification, a figure, a SEQ ID NO. or a sequence listing of the present application. Similarly, the invention provides dimers and trimers comprising such proteins, including those described in the Examples of the invention.

[0172] The % amino acid sequence identity values can be readily obtained using, for example, the WU-BLAST-2 computer program (Altschul et al., Methods in Enzymology 266:460-480 (1996)).

[0173] Fragments of native sequences are provided herein. Such fragments may be truncated at the N-terminus or C-terminus, or may lack internal residues, for example, when compared with a full-length native protein. Again, it is understood that any terminal variations are made within the context of the invention disclosed herein.

[0174] Certain fragments lack amino acid residues that are not essential for a desired biological activity of the sequence of interest.

[0175] Any of a number of conventional techniques may be used. Desired peptide fragments or fragments of stretches of consecutive amino acids may be chemically synthesized. An alternative approach involves generating fragments by enzymatic digestion, e.g. by treating the protein with

an enzyme known to cleave proteins at sites defined by particular amino acid residues, or by digesting the DNA with suitable restriction enzymes and isolating the desired fragment. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired polypeptide/sequence fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed at the 5' and 3' primers in the PCR.

[0176] In particular embodiments, conservative substitutions of interest are shown in Table A under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table A, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table A – Conservative amino acid substitutions

Original	Exemplary	Preferred
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro; ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	leu
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr

Original	Exemplary	Preferred
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

[0177] Substantial modifications in function or immunological identity of the sequence are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- a. hydrophobic: norleucine, met, ala, val, leu, ile;
- b. neutral hydrophilic: cys, ser, thr;
- c. acidic: asp, glu;
- d. basic: asn, gln, his, lys, arg;
- e. residues that influence chain orientation: gly, pro;
- f. aromatic: trp, tyr, phe.

[0178] Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

[0179] The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)), cassette mutagenesis (Wells et al., Gene, 34:315 (1985)), restriction selection mutagenesis (Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)) or other known techniques can be performed on the cloned DNA to produce the variant DNA.

[0180] Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is

typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant (Cunningham and Wells, *Science*, 244:1081-1085 (1989)). Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions (Creighton, *The Proteins*, (W.H. Freeman & Co., N.Y.); Chothia, *J. Mol. Biol.*, 150:1 (1976)). If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

[0181] Covalent modifications: The stretch of consecutive amino acids may be covalently modified. One type of covalent modification includes reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues that are not involved in an $-x-x-$ bond. Derivatization with bifunctional agents is useful, for instance, for crosslinking to a water-insoluble support matrix or surface for use in the method for purifying anti-sequence of interest antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-((p-azidophenyl)dithio)propioimide.

[0182] Other modifications include deamidation of glutamyl and asparaginy residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[0183] Another type of covalent modification comprises altering the native glycosylation pattern of the stretch of consecutive amino acids. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in amino acid sequences (either by removing the underlying glycosylation site or by deleting the glycosylation by chemical and/or enzymatic means), and/or adding one or more glycosylation sites that are not present in the native sequence. In addition, the phrase includes qualitative changes in the

glycosylation of the native proteins, involving a change in the nature and proportions of the various carbohydrate moieties present.

[0184] Addition of glycosylation sites to the amino acid sequence may be accomplished by altering the amino acid sequence. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence (for O-linked glycosylation sites). The amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the amino acid sequence at preselected bases such that codons are generated that will translate into the desired amino acids.

[0185] Another means of increasing the number of carbohydrate moieties on the amino acid sequence is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

[0186] Removal of carbohydrate moieties present on the amino acid sequence may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., *Arch. Biochem. Biophys.*, 259:52 (1987) and by Edge et al., *Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., *Meth. Enzymol.*, 138:350 (1987).

[0187] Another type of covalent modification comprises linking the amino acid sequence to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

[0188] The term “substitution”, “substituted” and “substituent” refers to a functional group in which one or more bonds to a hydrogen atom contained therein are replaced by a bond to non-hydrogen atoms, provided that normal valencies are maintained and that the substitution results in a stable compound. Substituted groups also include groups in which one or more bonds to a carbon(s) or hydrogen(s) atom are replaced by one or more bonds, including double or triple bonds, to a heteroatom. Examples of substituent groups include halogens (i.e., F, Cl, Br, and I); alkyl groups, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, tert-butyl, and trifluoromethyl; aryl

groups, such as phenyl; heteroaryl groups, such as triazole, dihydropyridazine and tetrazole; hydroxyl; alkoxy groups, such as methoxy, ethoxy, n-propoxy, and isopropoxy; aryloxy groups, such as phenoxy; arylalkyloxy, such as benzyloxy (phenylmethoxy) and p-trifluoromethylbenzyloxy (4-trifluoromethylphenylmethoxy); heteroaryloxy groups; sulfonyl groups, such as sulfonate, trifluoromethanesulfonyl, methanesulfonyl, and p-toluenesulfonyl; sulfinitro, nitrosyl; mercapto; sulfanyl groups, such as methylsulfanyl, ethylsulfanyl and propylsulfanyl; cyano; amino groups, such as amino, methylamino, dimethylamino, ethylamino, and diethylamino; and carboxyl. Where multiple substituent moieties are disclosed or claimed, the substituted compound can be independently substituted by one or more of the disclosed or claimed substituent moieties, singly or plurally. By independently substituted, it is meant that the (two or more) substituents can be the same or different. In the compounds used in the method of the present invention, alkyl, heteroalkyl, monocycle, bicycle, aryl, heteroaryl and heterocycle groups can be substituted by replacing one or more hydrogen atoms with alternative non-hydrogen groups. These include, but are not limited to, halo, hydroxy, mercapto, amino, carboxy, cyano and carbamoyl.

[0189] It is understood that substituents and substitution patterns on the compounds used in the method of the present invention can be selected by one of ordinary skill in the art to provide compounds that are chemically stable and that can be readily synthesized by techniques known in the art from readily available starting materials. If a substituent is itself substituted with more than one group, it is understood that these multiple groups may be on the same carbon or on different carbons, so long as a stable structure results.

[0190] In choosing the compounds used in the method of the present invention, one of ordinary skill in the art will recognize that the various substituents, i.e. R1, R2, etc. are to be chosen in conformity with well-known principles of chemical structure connectivity.

[0191] As used herein, "alkyl" includes both branched and straight-chain saturated aliphatic hydrocarbon groups having the specified number of carbon atoms and may be unsubstituted or substituted. Thus, C₁-C_n as in "C₁-C_n alkyl" is defined to include groups having 1, 2, ..., n-1 or n carbons in a linear or branched arrangement. For example, C₁-C₆, as in "C₁-C₆ alkyl" is defined to include groups having 1, 2, 3, 4, 5, or 6 carbons in a linear or branched arrangement, and specifically includes methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, pentyl, and hexyl. Unless otherwise specified contains one to twelve carbons. Alkyl groups can be unsubstituted or

substituted with one or more substituents, including but not limited to halogen, alkoxy, alkylthio, trifluoromethyl, difluoromethyl, methoxy, and hydroxyl. An embodiment can be C₁-C₁₂ alkyl, C₂-C₁₂ alkyl, C₃-C₁₂ alkyl, C₄-C₁₂ alkyl and so on. An embodiment can be C₁-C₈ alkyl, C₂-C₈ alkyl, C₃-C₈ alkyl, C₄-C₈ alkyl and so on. Alkyl is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0192] As used herein, “C₁-C₄ alkyl” includes both branched and straight-chain C₁-C₄ alkyl.

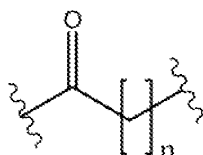
[0193] As used herein, the term “cycloalkane” refers to a monocyclic or bicyclic ring system, which may be unsaturated or partially unsaturated, i.e. possesses one or more double bonds. Monocyclic ring systems are exemplified by a saturated cyclic hydrocarbon group containing from 3 to 8 carbon atoms. Examples of monocyclic ring systems include cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl and cyclooctyl. Bicyclic fused ring systems are exemplified by a cycloalkyl ring fused to another cycloalkyl ring. Examples of bicyclic fused ring systems include, but are not limited to, decalin, 1,2,3,7,8,8a-hexahydronaphthalene, and the like. Thus, C₃-C₁₀ cycloalkane includes cyclic rings of alkanes of three to eight total carbon atoms, (e.g., cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl or cyclooctyl and so on). Cycloalkane groups can be unsubstituted or substituted with one or more substituents, including but not limited to halogen, alkoxy, alkylthio, trifluoromethyl, difluoromethyl, methoxy, and hydroxyl. Cycloalkane is intended to include moieties that are monovalent, divalent, trivalent, etc.

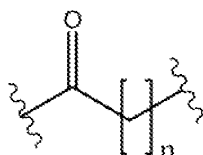
[0194] As used herein, the term “cycloalkene” refers to a cycloalkane which possesses one or more double bonds. Thus, C₅-C₁₀ cycloalkene includes cyclic rings of alkanes of five to ten total carbon atoms, (e.g., cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclohexadienyl, cyclooctenyl or cyclooctadienyl and so on). Cycloalkene is intended to include moieties that are monovalent, divalent, trivalent, etc. Cycloalkene is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0195] As used herein, “alkene” includes both branched and straight-chain aliphatic hydrocarbon groups having one or more double bond and the specified number of carbon atoms and may be unsubstituted or substituted. Thus, C₂-C_n as in “C₂-C_n alkene” is defined to include groups having 2, 3, ..., n-1 or n carbons in a linear or branched arrangement. For example, C₂-C₁₀, as in “C₂-C₁₀ alkene” is defined to include groups having 2, 3, 4, 5...10 carbons in a linear or branched

arrangement, and specifically includes vinyl, allyl, 1-butene, 2-butene, iso-butene, 1-pentene, 2-pentene, etc. Alkyne groups can be unsubstituted or substituted with one or more substituents, including but not limited to halogen, alkoxy, alkylthio, trifluoromethyl, difluoromethyl, methoxy, and hydroxyl. An embodiment can be C₂-C₃ alkene, C₂-C₄ alkene, C₂-C₅ alkene, and so on. Alkene is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0196] As used herein, an “acyl” refers to an alkyl group having a ketone at the first position. For example, an “acyl” embodiment can be acetyl, propionyl, butyryl and valeryl. As another example,



an “acyl” embodiment can be: , wherein n is 1-10. In another embodiment, n is 1-4.

[0197] Thus, a “C₂-C₅ acyl” can be acetyl, propionyl, butyryl, or and valeryl. Acyl is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0198] C₂-C₅ acylamino is an acyl group as defined above further substituted with an amine. The amine may be linked to the carbonyl portion of the acyl group so as to form an amide or the amine may be linked to a non-carbonyl portion of the acyl group. For example, the amino group may be at the alpha-position, the beta-position, the gamma-position, the delta-position, etc. As further examples, acylamino includes both alpha-aminoacetyl and acetamido groups. Acylamino includes beta-aminopropionyl).

[0199] C₂-C₅ acyloxy is an acyl group as defined above further substituted with an oxygen. The oxygen may be linked to the carbonyl portion of the acyl group so as to form an amide or the oxygen may be linked to a non-carbonyl portion of the acyl group. For example, the oxygen group may be at the alpha-position, the beta-position, the gamma-position, the delta-position, etc. As further examples, acyloxy includes both alpha-oxyacetyl and acetate groups. Acyloxy includes beta-oxypropionyl).

[0200] As used herein, “amino” includes primary, secondary, tertiary and quaternary amines. Thus, amino includes a -NH- group, a -NH₂ group, a -NR- group, a -NR₂⁺ group, a -NRH⁺ group, a -NH₂⁺ group, a -NH₃⁺ group and a -NR₃⁺ group, wherein R is alkyl or aryl. Amino is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0201] As used herein, “sulfur” includes a -S- group and a -SH group. The term sulfur is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0202] As used herein, “oxygen” includes a -O- group and a -OH group. The term sulfur is intended to moieties that are monovalent and divalent.

[0203] As used herein, “succinyl” is derived from succinic acid by removal of one or both hydroxyl groups. An embodiment can be -C(O)-CH₂-CH₂-C(O)-. Succinyl is intended to include moieties that are monovalent, divalent, trivalent, etc.

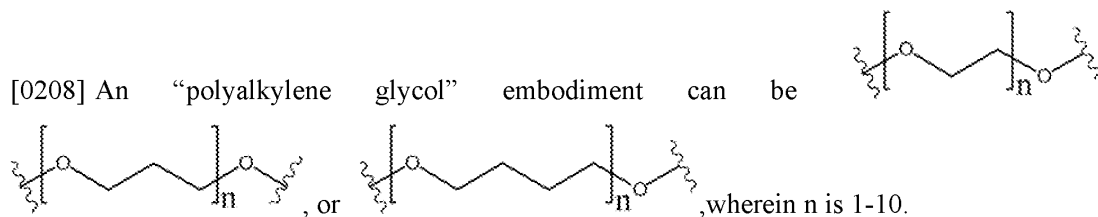
[0204] As used herein, a “malonyl” is derived from malonic acid by removal of one or both hydroxyl groups. An embodiment can be -C(O)-CH₂-C(O)-. Malonyl is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0205] As used herein, a “glutaryl” is derived from glutaric acid by removal of one or both hydroxyl groups. An embodiment can be -C(O)-CH₂-CH₂-CH₂-C(O)-. Glutaryl is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0206] As used herein, an “adipoyl” is derived from adipic acid by removal of one or both hydroxyl groups. An embodiment can be -C(O)-CH₂-CH₂-CH₂-CH₂-(O)-. Adipoyl is intended to include moieties that are monovalent, divalent, trivalent, etc.

[0207] A “polyalkylene glycol” is derived from polyalkylene glycol by removal of both hydrogens from the hydroxyl groups. An embodiment can be derived from polyethylene glycol, polypropylene glycol, or polybutylene glycol.

[0208] An “polyalkylene glycol” embodiment can be



[0209] As used herein, “aryl” is intended to mean any stable monocyclic, bicyclic or polycyclic carbon ring of up to 10 atoms in each ring, wherein at least one ring is aromatic, and may be unsubstituted or substituted. Examples of such aryl elements include but are not limited to: phenyl, p-toluenyl (4-methylphenyl), naphthyl, tetrahydro-naphthyl, indanyl, phenanthryl, anthryl or

acenaphthyl. In cases where the aryl substituent is bicyclic and one ring is non-aromatic, it is understood that attachment is via the aromatic ring.

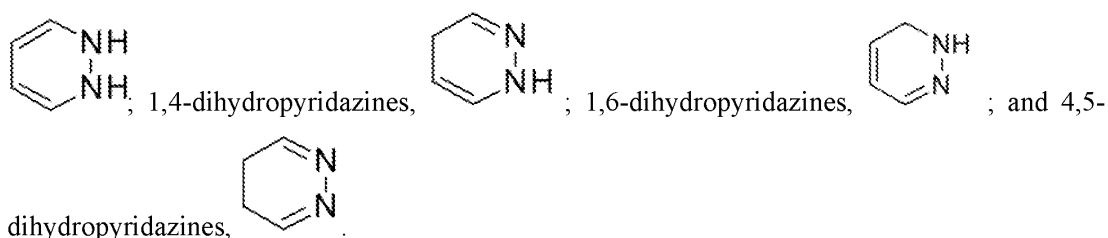
[0210] The term “heteroaryl”, as used herein, represents a stable monocyclic, bicyclic or polycyclic ring of up to 10 atoms in each ring, wherein at least one ring is aromatic and contains from 1 to 4 heteroatoms selected from the group consisting of O, N and S. Bicyclic aromatic heteroaryl groups include phenyl, pyridine, pyrimidine or pyridazine rings that are (a) fused to a 6-membered aromatic (unsaturated) heterocyclic ring having one nitrogen atom; (b) fused to a 5- or 6-membered aromatic (unsaturated) heterocyclic ring having two nitrogen atoms; (c) fused to a 5-membered aromatic (unsaturated) heterocyclic ring having one nitrogen atom together with either one oxygen or one sulfur atom; or (d) fused to a 5-membered aromatic (unsaturated) heterocyclic ring having one heteroatom selected from O, N or S. Heteroaryl groups within the scope of this definition include but are not limited to: benzoimidazolyl, benzofuranyl, benzofurazanyl, benzopyrazolyl, benzotriazolyl, benzothiophenyl, benzoxazolyl, carbazolyl, carbolinyl, cinnolinyl, dihydropyridizine, furanyl, indolinyl, indolyl, indolaziny, indazolyl, isobenzofuranyl, isoindolyl, isoquinolyl, isothiazolyl, isoxazolyl, naphthpyridinyl, oxadiazolyl, oxazolyl, oxazoline, isoxazoline, oxetanyl, pyranyl, pyrazinyl, pyrazolyl, pyridazinyl, pyridopyridinyl, pyridazinyl, pyridyl, pyrimidyl, pyrrolyl, quinazolinyl, quinolyl, quinoxalinyl, tetrazolyl, tetrazolopyridyl, thiadiazolyl, thiazolyl, thienyl, triazolyl, azetidiny, aziridinyl, 1,4-dioxanyl, hexahydroazepinyl, dihydrobenzoimidazolyl, dihydrobenzofuranyl, dihydrobenzothiophenyl, dihydrobenzoxazolyl, dihydrofuranyl, dihydroimidazolyl, dihydroindolyl, dihydroisooxazolyl, dihydroisothiazolyl, dihydrooxadiazolyl, dihydrooxazolyl, dihydropyrazinyl, dihydropyrazolyl, dihydropyridinyl, dihydropyrimidinyl, dihydropyrrolyl, dihydroquinolinyl, dihydrotetrazolyl, dihydrothiadiazolyl, dihydrothiazolyl, dihydrothienyl, dihydrotriazolyl, dihydroazetidiny, methylenedioxybenzoyl, tetrahydrofuranyl, tetrahydrothienyl, acridinyl, carbazolyl, cinnolinyl, quinoxalinyl, pyrrazolyl, indolyl, benzotriazolyl, benzothiazolyl, benzoxazolyl, isoxazolyl, isothiazolyl, furanyl, thienyl, benzothienyl, benzofuranyl, quinolinyl, isoquinolinyl, oxazolyl, isoxazolyl, indolyl, pyrazinyl, pyridazinyl, pyridinyl, pyrimidinyl, pyrrolyl, tetra-hydroquinoline. In cases where the heteroaryl substituent is bicyclic and one ring is non-aromatic or contains no heteroatoms, it is understood that attachment is via the aromatic ring or via the heteroatom containing ring, respectively. If the heteroaryl contains nitrogen atoms, it is understood that the corresponding N-oxides thereof are also encompassed by this definition.

[0211] The term “phenyl” is intended to mean an aromatic six membered ring containing six carbons, and any substituted derivative thereof.

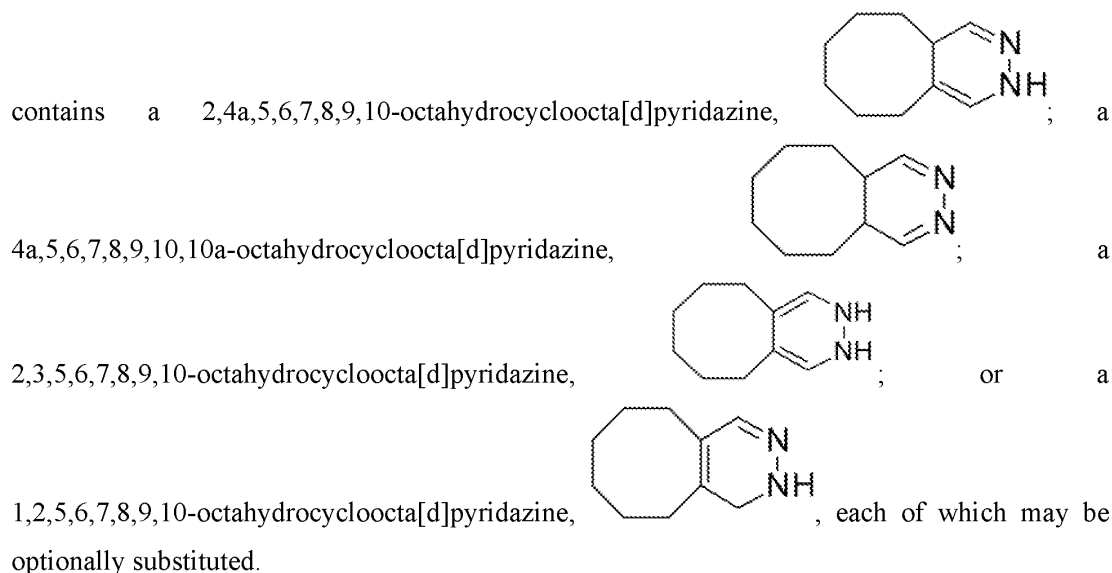
[0212] The term “benzyl” is intended to mean a methylene attached directly to a benzene ring. A benzyl group is a methyl group wherein a hydrogen is replaced with a phenyl group, and any substituted derivative thereof.

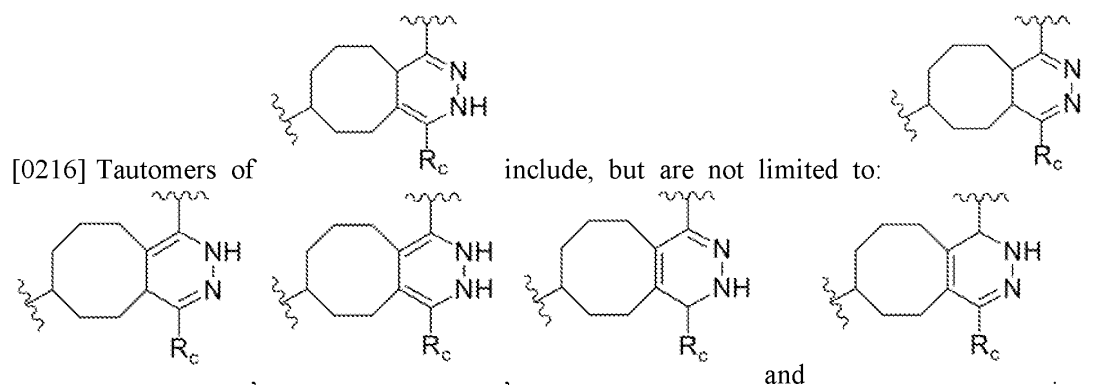
[0213] The term “triazole” is intended to mean a heteraryl having a five-membered ring containing two carbon atoms and three nitrogen atoms, and any substituted derivative thereof.

[0214] Dihydropyridazine is optionally substituted and includes 1,2-dihydropyridazines,



[0215] A chemical structure containing a cyclooctane fused to a dihydropyridazine includes, but is not limited to, a chemical structure which contains a cyclooctane fused to the 3rd and 4th position of a dihydropyridazine or a chemical structure which contains a saturated cycloocta[d]pyridazine, any of which are optionally substituted. For example, the chemical structure containing a cyclooctane fused to a dihydropyridazine includes, but is not limited to, a chemical structure which





[0217] In some embodiments, the dihydropyridazine is oxidized to a pyridazine.

[0218] In some embodiments, the dihydropyridazine is reduced to result in an open ring structure having a 1,4-dicarbonyl compound.

[0219] The compounds used in the method of the present invention may be prepared by techniques well known in organic synthesis and familiar to a practitioner ordinarily skilled in the art. However, these may not be the only means by which to synthesize or obtain the desired compounds.

[0220] Compounds of the subject invention can be converted to prodrugs to optimize absorption and bioavailability. Formation of a prodrug include, but is not limited to, reaction of a free hydroxyl group with a carboxylic acid to form an ester, reaction of a free hydroxyl group with a phosphorus oxychloride followed by hydrolysis to form a phosphate, or reaction of a free hydroxyl group with an amino acid to form an amino acid ester, the process of which has been described previously by Chandran in WO 2005/046575. The substituents are chosen and resulting analogs are evaluated according to principles well known in the art of medicinal and pharmaceutical chemistry, such as quantification of structure-activity relationships, optimization of biological activity and ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties.

[0221] The various R groups attached to the aromatic rings of the compounds disclosed herein may be added to the rings by standard procedures, for example those set forth in *Advanced Organic Chemistry: Part B: Reaction and Synthesis*, Francis Carey and Richard Sundberg, (Springer) 5th ed. Edition. (2007), the content of which is hereby incorporated by reference.

[0222] The compounds of present invention may be prepared by techniques described in *Vogel's Textbook of Practical Organic Chemistry*, A.I. Vogel, A.R. Tatchell, B.S. Furnis, A.J. Hannaford,

P.W.G. Smith, (Prentice Hall) 5th Edition (1996), March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, Michael B. Smith, Jerry March, (Wiley-Interscience) 5th Edition (2007), and references therein, which are incorporated by reference herein. However, these may not be the only means by which to synthesize or obtain the desired compounds.

[0223] A person having ordinary skill in the art will immediately understand that the definitions of the substituents and moieties provided herein are intended to obey the standard rules of chemical valency. For example, where a structure provided herein requires a particular substituent or moiety to be divalent, (e.g. a moiety in a linear chain of moieties) a person having ordinary skill in the art will immediately understand that the definitions of that substituent or moiety are divalent in order to obey the standard rules of chemical valency.

[0224] A person having ordinary skill in the art will immediately understand that some divalent moieties depicted in the present invention may be linked to other chemical structures in more than one way, e.g., the depicted structures may be linked to other chemical structures when rotated or flipped.

[0225] In some embodiments of the present invention, a compound comprises a nonproteinaceous polymer. In some embodiments, the nonproteinaceous polymer may be is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or in vitro methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol, polypropylene glycol, polyoxyethylene esters or methoxy polyethylene glycol; polyoxyalkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextran sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides,

e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; and heparin or heparon.

Salts

[0226] Salts of the compounds disclosed herein are within the scope of the invention. As used herein, a “salt” is salt of the instant compounds which has been modified by making acid or base salts of the compounds.

Stretches of Consecutive Amino Acids

[0227] Examples of stretches of consecutive amino acids as referred to herein include, but are not limited to, consecutive amino acids including binding domains such as secreted or transmembrane proteins, intracellular binding domains and antibodies (whole or portions thereof) and modified versions thereof. The following are some non-limiting examples:

Immunoglobulins

[0228] The term “antibody” is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), monovalent antibodies, multivalent antibodies, and antibody fragments so long as they exhibit the desired biological activity (e.g., Fab and/or single-armed antibodies).

[0229] 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. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively.

[0230] 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 multispecific antibodies formed from antibody fragments.

[0231] The terms “full length antibody,” “intact antibody,” and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

[0232] A “blocking” antibody or an “antagonist” antibody is one which significantly inhibits (either partially or completely) a biological activity of the antigen it binds.

[0233] An “antibody that binds to the same epitope” as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more. An exemplary competition assay is provided herein.

[0234] The term “variable region” or “variable domain” refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). (See, e.g., Kindt et al. *Kuby Immunology*, 6th ed., W.H. Freeman and Co., page 91 (2007).) A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano et al., *J. Immunol.* 150:880-887 (1993); Clarkson et al., *Nature* 352:624-628 (1991).

[0235] 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/or form structurally defined loops (“hypervariable loops”). Generally, native four-chain antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the “complementarity determining regions” (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition. Exemplary hypervariable loops occur 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).) Exemplary CDRs (CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2, and CDR-H3) occur at amino acid residues 24-34 of L1, 50-56 of L2, 89-97 of L3, 31-35B of H1, 50-65 of H2, and 95-102 of H3. (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health

Service, National Institutes of Health, Bethesda, Md. (1991).) With the exception of CDR1 in VH, CDRs generally comprise the amino acid residues that form the hypervariable loops. CDRs also comprise “specificity determining residues,” or “SDRs,” which are residues that contact antigen. SDRs are contained within regions of the CDRs called abbreviated-CDRs, or a-CDRs. Exemplary a-CDRs (a-CDR-L1, a-CDR-L2, a-CDR-L3, a-CDR-H1, a-CDR-H2, and a-CDR-H3) occur at amino acid residues 31-34 of L1, 50-55 of L2, 89-96 of L3, 31-35B of H1, 50-58 of H2, and 95-102 of H3. (See Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008).) Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*.

[0236] “Framework” or “FR” refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

[0237] The phrase “N-terminally truncated heavy chain”, as used herein, refers to a polypeptide comprising parts but not all of a full length immunoglobulin heavy chain, wherein the missing parts are those normally located on the N terminal region of the heavy chain. Missing parts may include, but are not limited to, the variable domain, CH1, and part or all of a hinge sequence. Generally, if the wild type hinge sequence is not present, the remaining constant domain(s) in the N-terminally truncated heavy chain would comprise a component that is capable of linkage to another Fc sequence (i.e., the “first” Fc polypeptide as described herein). For example, said component can be a modified residue or an added cysteine residue capable of forming a disulfide linkage.

[0238] “Fc receptor” or “FcR” describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an “activating receptor”) and FcγRIIB (an “inhibiting receptor”), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an

immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, e.g., Daeron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term “FcR” herein.

[0239] The term “Fc receptor” or “FcR” also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward., *Immunol. Today* 18(12):592-598 (1997); Ghetie et al., *Nature Biotechnology*, 15(7):637-640 (1997); Hinton et al., *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).

[0240] Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, e.g., Shields et al. *J. Biol. Chem.* 9(2):6591-6604 (2001).

[0241] The “hinge region,” “hinge sequence”, and variations thereof, as used herein, includes the meaning known in the art, which is illustrated in, for example, Janeway et al., *Immuno Biology: the immune system in health and disease*, (Elsevier Science Ltd., NY) (4th ed., 1999); Bloom et al., *Protein Science* (1997), 6:407-415; Humphreys et al., *J. Immunol. Methods* (1997), 209:193-202.

[0242] Unless indicated otherwise, the expression “multivalent antibody” is used throughout this specification to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is preferably engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

[0243] An “Fv” fragment is an antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight association, which can be covalent in nature, for example in scFv. It is in this configuration that the three HVRs of each variable domain interact to define an antigen binding site on the surface of the VH-VL dimer. Collectively, the six HVRs or a subset thereof confer antigen binding

specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although usually at a lower affinity than the entire binding site.

[0244] The “Fab” fragment contains a variable and constant domain of the light chain and a variable domain and the first constant domain (CH1) of the heavy chain. F(ab')₂ antibody fragments comprise a pair of Fab fragments which are generally covalently linked near their carboxy termini by hinge cysteines between them. Other chemical couplings of antibody fragments are also known in the art.

[0245] The phrase “antigen binding arm”, as used herein, refers to a component part of an antibody fragment that has an ability to specifically bind a target molecule of interest. Generally and preferably, the antigen binding arm is a complex of immunoglobulin polypeptide sequences, e.g., HVR and/or variable domain sequences of an immunoglobulin light and heavy chain.

[0246] “Single-chain Fv” or “scFv” antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains, which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, Vol 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

[0247] The term “diabodies” refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH and VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

[0248] The expression “linear antibodies” refers to the antibodies described in Zapata et al., *Protein Eng.*, 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-CH₁-V_H-CH₁) which, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[0249] The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

[0250] The term “chimeric” antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

[0251] A “humanized” antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., 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.

[0252] 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.

[0253] A “naked antibody” refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

[0254] “Native antibodies” refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 Daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. 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.

[0255] “Affinity” refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, “binding affinity” refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

[0256] An “affinity matured” antibody refers to an antibody with one or more alterations in one or more HVRs, compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

[0257] An antibody having a “biological characteristic” of a designated antibody is one which possesses one or more of the biological characteristics of that antibody which distinguish it from other antibodies that bind to the same antigen.

[0258] A “functional antigen binding site” of an antibody is one which is capable of binding a target antigen. The antigen binding affinity of the antigen binding site is not necessarily as strong as the parent antibody from which the antigen binding site is derived, but the ability to bind antigen must be measurable using any one of a variety of methods known for evaluating antibody binding to an antigen. Moreover, the antigen binding affinity of each of the antigen binding sites of a multivalent antibody herein need not be quantitatively the same. For the multimeric antibodies herein, the number of functional antigen binding sites can be evaluated using ultracentrifugation analysis as described in Example 2 of U.S. Patent Application Publication No. 2005/0186208 A1. According to this method of analysis, different ratios of target antigen to multimeric antibody are combined and the average molecular weight of the complexes is calculated assuming differing numbers of functional binding sites. These theoretical values are compared to the actual experimental values obtained in order to evaluate the number of functional binding sites.

[0259] A “species-dependent antibody” is one which has a stronger binding affinity for an antigen from a first mammalian species than it has for a homologue of that antigen from a second mammalian species. Normally, the species-dependent antibody “binds specifically” to a human antigen (i.e. has a binding affinity (K_d) value of no more than about 1×10^{-7} M, preferably no more than about 1×10^{-8} M and most preferably no more than about 1×10^{-9} M) but has a binding affinity for a homologue of the antigen from a second nonhuman mammalian species which is at least about 50 fold, or at least about 500 fold, or at least about 1000 fold, weaker than its binding affinity for the human antigen. The species-dependent antibody can be any of the various types of antibodies as defined above. In some embodiments, the species-dependent antibody is a humanized or human antibody.

[0260] An “isolated” antibody is one which has been separated from a component of its natural environment. In some embodiments, 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). For review of methods for assessment of antibody purity, see, e.g., Flatman et al., J. Chromatogr. B 848:79-87 (2007).

Fc domains

[0261] The term “Fc domain”, as used herein, generally refers to a monomer or dimer complex, comprising the C-terminal polypeptide sequences of an immunoglobulin heavy chain. The Fc domain may comprise native or variant Fc sequences. Although the boundaries of the Fc domain of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc domain is usually defined to stretch from an amino acid residue in the hinge region to the carboxyl terminus of the Fc sequence. The Fc sequence of an immunoglobulin generally comprises two constant regions, a CH2 region and a CH3 region, and optionally comprises a CH4 region. A human Fc domain may be obtained from any suitable immunoglobulin, such as the IgG1, IgG2, IgG3, or IgG4 subtypes, IgA, IgE, IgD or IgM.

[0262] Suitable Fc domains are prepared by recombinant DNA expression of pre-Fc chimeric polypeptides comprising 1) a signal peptide, obtained from a secreted or transmembrane protein, that is cleaved in front of a mature polypeptide having an N-terminal cysteine residue, contiguous with 2) an Fc domain polypeptide having an N-terminal cysteine residue.

[0263] Suitable examples of signal peptides are sonic hedgehog (SHH) (GenBank Acc. No. NM000193), IFNalpha-2 (IFN) (GenBank Acc. No. NP000596), and cholesterol ester transferase (CETP) (GenBank Accession No. NM000078). Other suitable examples include Indian hedgehog (Genbank Acc. No. NM002181), desert hedgehog (Genbank Acc. No. NM021044), IFNalpha-1 (Genbank Acc. No. NP076918), IFNalpha-4 (Genbank Acc. No. NM021068), IFNalpha-5 (Genbank Acc. No. NM002169), IFNalpha-6 (Genbank Acc. No. NM021002), IFNalpha-7 (Genbank Acc. No. NM021057), IFNalpha-8 (Genbank Acc. No. NM002170), IFNalpha-10 (Genbank Acc. No. NM002171), IFNalpha-13 (Genbank Acc. No. NM006900), IFNalpha-14 (Genbank Acc. No. NM002172), IFNalpha-16 (Genbank Acc. No. NM002173), IFNalpha-17 (Genbank Acc. No. NM021268) and IFNalpha-21 (Genbank Acc. No. NM002175).

[0264] Suitable examples of Fc domains and their pre-Fc chimeric polypeptides are shown in SEQ ID NO: 120 through SEQ ID NO: 215. The Fc domains are obtained by expressing the pre-Fc chimeric polypeptides in cells under conditions leading to their secretion and cleavage of the signal peptide. The pre-Fc polypeptides may be expressed in either prokaryotic or eukaryotic host cells. Preferably, mammalian host cells are transfected with expression vectors encoding the pre-Fc polypeptides.

[0265] Human IgG1 Fc domains having the N-terminal sequence CDKTHTCPPCPAPE, CPPCPAPE, and CPAPE are shown in SEQ ID NO: 120, SEQ ID NO: 128, and SEQ ID NO: 136, respectively, and the DNA sequences encoding them are shown in SEQ ID NO: 121, SEQ ID NO: 129, and SEQ ID NO: 137, respectively. The IgG1 domain of SEQ ID NO: 120 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 122 (SHH signal peptide), SEQ ID NO: 124 (IFN signal peptide), and SEQ ID NO: 126 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 123, SEQ ID NO: 125, and SEQ ID NO: 127, respectively. The IgG1 domain of SEQ ID NO: 128 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 130 (SHH signal peptide), SEQ ID NO: 132 (IFN signal peptide), and SEQ ID NO: 134 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 131, SEQ ID NO: 133, and SEQ ID NO: 135, respectively. The IgG1 domain of SEQ ID NO: 136 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 138 (SHH signal peptide), SEQ ID NO: 140 (IFN signal peptide), and SEQ ID NO: 142 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143, respectively.

[0266] Human IgG2 Fc domains having the N-terminal sequence CCVECPCPPAPE, CVECPCPPAPE, CPPCPAPE, and CPAPE are shown in SEQ ID NO: 144, SEQ ID NO: 152, SEQ ID NO: 160, and SEQ ID NO: 168, respectively, and the DNA sequences encoding them are shown in SEQ ID NO: 145, SEQ ID NO: 153, SEQ ID NO: 161, and SEQ ID NO: 169, respectively. The IgG2 domain of SEQ ID NO: 144 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 146 (SHH signal peptide), SEQ ID NO: 148 (IFN signal peptide), and SEQ ID NO: 150 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 147, SEQ ID NO: 149, and SEQ ID NO: 151, respectively. The IgG2 domain of SEQ ID NO: 152 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 154 (SHH signal peptide), SEQ ID NO: 156 (IFN signal peptide), and SEQ ID NO: 158 (CETP signal peptide) using the DNA sequences shown in SEQ ID NO: 155, SEQ ID NO: 157, and SEQ ID NO: 159, respectively. The IgG2 domain of SEQ ID NO: 160 is obtained from the pre-Fc chimeric polypeptides shown in SEQ ID NO: 162 (SHH signal peptide), SEQ ID NO: 164 (IFN signal peptide), and SEQ ID NO: 166 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 163, SEQ ID NO: 165, and SEQ ID NO: 167, respectively. The IgG2 domain of SEQ ID NO: 168 is obtained from the pre-Fc chimeric polypeptides shown in SEQ ID NO: 170 (SHH signal peptide), SEQ ID NO: 172 (IFN signal peptide), and SEQ ID NO: 174 (CETP signal

peptide), using the DNA sequences shown in SEQ ID NO: 171, SEQ ID NO: 173, and SEQ ID NO: 175, respectively.

[0267] Human IgG3 Fc domains having the N-terminal sequence (CPRCPEPKSDTPPP)3-CPRCPAPE, CPRCPAPE, and CPAPE are shown in SEQ ID NO: 176, SEQ ID NO: 184, and SEQ ID NO: 192, respectively, and the DNA sequences encoding them are shown in SEQ ID NO: 177, SEQ ID NO: 185, SEQ ID NO: 161, and SEQ ID NO: 193, respectively. The IgG3 domain of SEQ ID NO: 176 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 178 (SHH signal peptide), SEQ ID NO: 180 (IFN signal peptide), and SEQ ID NO: 182 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 179, SEQ ID NO: 181, and SEQ ID NO: 183, respectively. The IgG3 domain of SEQ ID NO: 184 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 186 (SHH signal peptide), SEQ ID NO: 188 (IFN signal peptide), and SEQ ID NO: 190 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 187, SEQ ID NO: 189, and SEQ ID NO: 191, respectively. The IgG3 domain of SEQ ID NO: 192 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 194 (SHH signal peptide), SEQ ID NO: 196 (IFN signal peptide), and SEQ ID NO: 198 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 195, SEQ ID NO: 197, and SEQ ID NO: 199, respectively.

[0268] The sequences of human IgG4 Fc domains having the N-terminal sequence CPSCPAPE and CPAPE are shown in SEQ ID NO: 200 and SEQ ID NO: 208, respectively, and the DNA sequences encoding them are shown in SEQ ID NO: 201 and SEQ ID NO: 209, respectively. The IgG4 domain of SEQ ID NO: 200 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 202 (SHH signal peptide), SEQ ID NO: 204 (IFN signal peptide), and SEQ ID NO: 206 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 203, SEQ ID NO: 205, and SEQ ID NO: 207, respectively. The IgG4 domain of SEQ ID NO: 208 is obtained by expressing the pre-Fc chimeric polypeptides shown in SEQ ID NO: 210 (SHH signal peptide), SEQ ID NO: 212 (IFN signal peptide), and SEQ ID NO: 214 (CETP signal peptide), using the DNA sequences shown in SEQ ID NO: 211, SEQ ID NO: 213, and SEQ ID NO: 215, respectively.

[0269] Suitable antibody variants having at their heavy chain N-terminus a cysteine residue are prepared by recombinant DNA expression of pre-heavy chain chimeric polypeptides comprising 1) a signal peptide, obtained from a secreted or transmembrane protein, that is cleaved in front of

a mature polypeptide having an N-terminal cysteine residue, contiguous with 2) a antibody heavy chain polypeptide having an N-terminal cysteine residue.

[0270] Suitable antibody variants having at their light chain N-terminus a cysteine residue are prepared by recombinant DNA expression of pre-light chain chimeric polypeptides comprising 1) a signal peptide, obtained from a secreted or transmembrane protein, that is cleaved in front of a mature polypeptide having an N-terminal cysteine residue, contiguous with 2) a antibody light chain polypeptide having an N-terminal cysteine residue.

[0271] Trastuzumab heavy and light chains are obtained by expressing the pre-heavy and pre-light chimeric polypeptides in cells under conditions leading to their secretion and cleavage of the signal peptide. The pre-heavy chain and pre-light chain polypeptides may be expressed in either prokaryotic or eukaryotic host cells. Preferably, mammalian host cells are transfected with expression vectors encoding the pre-heavy chain and pre-light chain polypeptides.

[0272] Protein sequences added to the N-terminus of the aforementioned antibody heavy chain, pre-heavy chain, light chain, and pre-light chain variants are illustrated herein for the recombinant antibody trastuzumab, but are generally applicable to any recombinant antibody. DNA sequences encoding trastuzumab and its variants may be constructed and expressed in mammalian cells by cotransfecting DNA vectors for its heavy and light chains, and variants derived thereof, as described in U.S. Patent No. 5,821,337 ("Immunoglobulin Variants") which is hereby incorporated by reference. The amino acid sequence of the wild-type trastuzumab light and heavy chains are shown in SEQ ID NO: 247 and SEQ ID NO: 248, respectively.

[0273] Suitable examples of trastuzumab light chains with N-terminal cysteine residues and their pre-Fc chimeric polypeptides are shown in SEQ ID NO: 249 through SEQ ID NO: 284. Suitable examples of trastuzumab heavy chains with N-terminal cysteine residues and their pre-Fc chimeric polypeptides are shown in SEQ ID NO: 285 through SEQ ID NO: 320.

[0274] Trastuzumab light chains having the N-terminal sequence C, CP, CPP, CPR, CPS, CDKT, CDKTHT, CVE, and CDTPPP are shown in SEQ ID NO: 249, SEQ ID NO: 253, SEQ ID NO: 257, SEQ ID NO: 261, SEQ ID NO: 265, SEQ ID NO: 269, SEQ ID NO: 273, SEQ ID NO: 277, and SEQ ID NO: 281, respectively. The light chain of SEQ ID NO: 249 is obtained by expressing the pre- light chimeric polypeptides shown in SEQ ID NO: 250 (SHH signal peptide), SEQ ID NO: 251 (IFN signal peptide), and SEQ ID NO: 252 (CETP signal peptide). The light chain of

SEQ ID NO: 253 is obtained by expressing the pre- light chain chimeric polypeptides shown in SEQ ID NO: 254 (SHH signal peptide), SEQ ID NO: 255 (IFN signal peptide), and SEQ ID NO: 256 (CETP signal peptide). The light chain of SEQ ID NO: 257 is obtained by expressing the pre- light chimeric polypeptides shown in SEQ ID NO: 258 (SHH signal peptide), SEQ ID NO: 259 (IFN signal peptide), and SEQ ID NO: 260 (CETP signal peptide). The light chain of SEQ ID NO: 261 is obtained by expressing the pre- light chimeric polypeptides shown in SEQ ID NO: 262 (SHH signal peptide), SEQ ID NO: 263 (IFN signal peptide), and SEQ ID NO: 264 (CETP signal peptide). The light chain of SEQ ID NO: 265 is obtained by expressing the pre-heavy light chimeric polypeptides shown in SEQ ID NO: 266 (SHH signal peptide), SEQ ID NO: 267 (IFN signal peptide), and SEQ ID NO: 268 (CETP signal peptide). The light chain of SEQ ID NO: 269 is obtained by expressing the pre- light chimeric polypeptides shown in SEQ ID NO: 270 (SHH signal peptide), SEQ ID NO: 271 (IFN signal peptide), and SEQ ID NO: 272 (CETP signal peptide). The light chain of SEQ ID NO: 273 is obtained by expressing the pre- light chimeric polypeptides shown in SEQ ID NO: 274 (SHH signal peptide), SEQ ID NO: 275 (IFN signal peptide), and SEQ ID NO: 276 (CETP signal peptide). The light chain of SEQ ID NO: 277 is obtained by expressing the pre- light chimeric polypeptides shown in SEQ ID NO: 278 (SHH signal peptide), SEQ ID NO: 279 (IFN signal peptide), and SEQ ID NO: 280 (CETP signal peptide). The light chain of SEQ ID NO: 281 is obtained by expressing the pre- light chimeric polypeptides shown in SEQ ID NO: 282 (SHH signal peptide), SEQ ID NO: 283 (IFN signal peptide), and SEQ ID NO: 284 (CETP signal peptide).

[0275] Trastuzumab heavy chains having the N-terminal sequence C, CP, CPP, CPR, CPS, CDKT, CDKTHT, CVE, and CDTPPP are shown in SEQ ID NO: 285, SEQ ID NO: 289, SEQ ID NO: 293, SEQ ID NO: 297, SEQ ID NO: 301, SEQ ID NO: 305, SEQ ID NO: 309, SEQ ID NO: 313, and SEQ ID NO: 317, respectively. The heavy chain of SEQ ID NO: 285 is obtained by expressing the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 286 (SHH signal peptide), SEQ ID NO: 287 (IFN signal peptide), and SEQ ID NO: 288 (CETP signal peptide). The heavy chain of SEQ ID NO: 289 is obtained by expressing the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 290 (SHH signal peptide), SEQ ID NO: 291 (IFN signal peptide), and SEQ ID NO: 292 (CETP signal peptide). The heavy chain of SEQ ID NO: 293 is obtained from the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 294 (SHH signal peptide), SEQ ID NO: 295 (IFN signal peptide), and SEQ ID NO: 296 (CETP signal peptide). The heavy chain of SEQ

ID NO: 297 is obtained from the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 298 (SHH signal peptide), SEQ ID NO: 299 (IFN signal peptide), and SEQ ID NO: 300 (CETP signal peptide). The heavy chain of SEQ ID NO: 301 is obtained by expressing the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 302 (SHH signal peptide), SEQ ID NO: 303 (IFN signal peptide), and SEQ ID NO: 304 (CETP signal peptide). The heavy chain of SEQ ID NO: 305 is obtained by expressing the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 306 (SHH signal peptide), SEQ ID NO: 307 (IFN signal peptide), and SEQ ID NO: 308 (CETP signal peptide). The heavy chain of SEQ ID NO: 309 is obtained from the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 310 (SHH signal peptide), SEQ ID NO: 311 (IFN signal peptide), and SEQ ID NO: 312 (CETP signal peptide). The heavy chain of SEQ ID NO: 313 is obtained from the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 314 (SHH signal peptide), SEQ ID NO: 315 (IFN signal peptide), and SEQ ID NO: 316 (CETP signal peptide). The heavy chain of SEQ ID NO: 317 is obtained from the pre-heavy chain chimeric polypeptides shown in SEQ ID NO: 318 (SHH signal peptide), SEQ ID NO: 319 (IFN signal peptide), and SEQ ID NO: 320 (CETP signal peptide).

[0276] Suitable host cells include 293 human embryonic cells (ATCC CRL-1573) and CHO-K1 hamster ovary cells (ATCC CCL-61) obtained from the American Type Culture Collection (Rockville, Md.). Cells are grown at 37°C. in an atmosphere of air, 95%; carbon dioxide, 5%. 293 cells are maintained in Minimal essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10%. CHO-K1 cells are maintained in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90%; fetal bovine serum, 10%. Other suitable host cells include CV1 monkey kidney cells (ATCC CCL-70), COS-7 monkey kidney cells (ATCC CRL-1651), VERO-76 monkey kidney cells (ATCC CRL-1587), HELA human cervical cells (ATCC CCL-2), W138 human lung cells (ATCC CCL-75), MDCK canine kidney cells (ATCC CCL-34), BRL3A rat liver cells (ATCC CRL-1442), BHK hamster kidney cells (ATCC CCL-10), MMT060562 mouse mammary cells (ATCC CCL-51), and human CD8⁺ T lymphocytes (described in U.S. Ser. No. 08/258,152 incorporated herein in its entirety by reference).

[0277] Examples of a suitable expression vectors are pCDNA3.1(+) shown in SEQ ID NO: 216 and pSA shown in SEQ ID NO: 217. Plasmid pSA contains the following DNA sequence elements:

1) pBluescriptIIKS(+) (nucleotides 912-2941/1-619, GenBank Accession No. X52327), 2) a human cytomegalovirus promoter, enhancer, and first exon splice donor (nucleotides 63-912, GenBank Accession No. K03104), 3) a human alpha1-globin second exon splice acceptor (nucleotides 6808-6919, GenBank Accession No. J00153), 4) an SV40 T antigen polyadenylation site (nucleotides 2770-2533, Reddy et al. (1978) Science 200, 494-502), and 5) an SV40 origin of replication (nucleotides 5725-5578, Reddy et al., *ibid*). Other suitable expression vectors include plasmids pSveCD4DHFR and pRKCD4 (U.S. Patent No. 5,336,603), plasmid pIK.1.1 (U.S. Patent No. 5,359,046), plasmid pVL-2 (U.S. Patent No. 5,838,464), plasmid pRT43.2F3 (described in U.S. Ser. No. 08/258,152 incorporated herein in its entirety by reference).

[0278] Suitable expression vectors for human IgG pre-Fc polypeptides may be constructed by the ligation of a HindIII-PspOM1 vector fragment prepared from SEQ ID NO: 217, with a HindIII-EagI insert fragment prepared from SEQ ID NOs: 123, 125, 127, 131, 133, 135, 139, 141, 143, 147, 149, 151, 155, 157, 159, 163, 165, 167, 171, 173, 175, 179, 181, 183, 187, 189, 191, 195, 197, 199, 203, 205, 207, 211, 213, and 215.

[0279] Suitable selectable markers include the Tn5 transposon neomycin phosphotransferase (NEO) gene (Southern and Berg (1982) J. Mol. Appl. Gen. 1, 327-341), and the dihydrofolate reductase (DHFR) cDNA (Lucas et al. (1996) Nucl. Acids Res. 24, 1774-1779). One example of a suitable expression vector that incorporates a NEO gene is plasmid pSA-NEO, which is constructed by ligating a first DNA fragment, prepared by digesting SEQ ID NO: 218 with EcoRI and BglII, with a second DNA fragment, prepared by digesting SEQ ID NO: 217 with EcoRI and BglII. SEQ ID NO: 218 incorporates a NEO gene (nucleotides 1551 to 2345, Genbank Accession No. U00004) preceded by a sequence for translational initiation (Kozak (1991) J. Biol. Chem, 266, 19867-19870). Another example of a suitable expression vector that incorporates a NEO gene and a DHFR cDNA is plasmid pSve-NEO-DHFR, which is constructed by ligating a first DNA fragment, prepared by digesting SEQ ID NO: 218 with EcoRI and BglII, with a second DNA fragment, prepared by digesting pSveCD4DHFR with EcoRI and BglII. Plasmid pSve-NEO-DHFR uses SV40 early promoter/enhancers to drive expression of the NEO gene and the DHFR cDNA. Other suitable selectable markers include the XPGT gene (Mulligan and Berg (1980) Science 209, 1422-1427) and the hygromycin resistance gene (Sugden et al. (1985) Mol. Cell. Biol. 5, 410-413).

[0280] In one embodiment, cells are transfected by the calcium phosphate method of Graham et al. (1977) *J. Gen. Virol.* 36, 59-74. A DNA mixture (10 ug) is dissolved in 0.5 ml of 1 mM Tris-HCl, 0.1 mM EDTA, and 227 mM CaCl₂. The DNA mixture contains (in a ratio of 10:1:1) the expression vector DNA, the selectable marker DNA, and a DNA encoding the VA RNA gene (Thimmappaya et al. (1982) *Cell* 31, 543-551). To this mixture is added, dropwise, 0.5 mL of 50 mM Hepes (pH 7.35), 280 mM NaCl, and 1.5 mM NaPO₄. The DNA precipitate is allowed to form for 10 minutes at 25°C, then suspended and added to cells grown to confluence on 100 mm plastic tissue culture dishes. After 4 hours at 37°C, the culture medium is aspirated and 2 ml of 20% glycerol in PBS is added for 0.5 minutes. The cells are then washed with serum-free medium, fresh culture medium is added, and the cells are incubated for 5 days.

[0281] In another embodiment, cells are transiently transfected by the dextran sulfate method of Sompariyac et al. (1981) *Proc. Nat. Acad. Sci.* 12, 7575-7579. Cells are grown to maximal density in spinner flasks, concentrated by centrifugation, and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet. After 4 hours at 37°C, the DEAE-dextran is aspirated and 20% glycerol in PBS is added for 1.5 minutes. The cells are then washed with serum-free medium, re-introduced into spinner flasks containing fresh culture medium with 5 micrograms/ml bovine insulin and 0.1 micrograms/ml bovine transferrin, and incubated for 4 days.

[0282] Following transfection by either method, the conditioned media is centrifuged and filtered to remove the host cells and debris. The sample containing the Fc domain is then concentrated and purified by any selected method, such as dialysis and/or column chromatography (see below). To identify the Fc domain in the cell culture supernatant, the culture medium is removed 24 to 96 hours after transfection, concentrated, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence or absence of a reducing agent such as dithiothreitol.

[0283] For unamplified expression, plasmids are transfected into human 293 cells (Graham et al., *J. Gen. Virol.* 36:59-74 (1977)), using a high efficiency procedure (Gorman et al., *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Media is changed to serum-free and harvested daily for up to five days. For amplified expression, plasmids are transfected into human 293 cells (Graham et al., *J. Gen. Virol.* 36:59-74 (1977)), using a high efficiency procedure (Gorman et al., *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Media is changed to serum-free and harvested daily for up to five days. The Fc domains are purified from the cell culture supernatant using HiTrap Protein A HP (Pharmacia).

The eluted Fc domains are buffer-exchanged into PBS using a Centricon-30 (Amicon), concentrated to 0.5 ml, sterile filtered using a Millex-GV (Millipore) at 4°C.

Fc Domain Modifications Altering Fc Receptor Binding and/or Effector Function

[0284] In certain embodiments, the Fc domain is engineered to have altered binding affinity to an Fc receptor and/or altered effector function, as compared to a non-engineered Fc domain. Embodiments of said modifications are described in U.S. Patent Application Publication No. US 20130058937 A1 and in the paragraphs that follow.

[0285] Binding to Fc receptors can be easily determined e.g. by ELISA, or by Surface Plasmon Resonance (SPR) using standard instrumentation such as a BIAcore instrument (GE Healthcare), and Fc receptors such as may be obtained by recombinant expression. A suitable such binding assay is described herein. Alternatively, binding affinity of Fc domains or tetrahedral antibodies comprising an Fc domain for Fc receptors may be evaluated using cell lines known to express particular Fc receptors, such as NK cells expressing FcγIIIa receptor.

[0286] Effector function of an Fc domain, can be measured by methods known in the art. Suitable in vitro assays to assess ADCC activity of a molecule of interest are described in PCT publication no. WO 2006/082515 or PCT patent application no. PCT/EP2012/055393, incorporated herein by reference in their entirety. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g. in a animal model such as that disclosed in Clynes et al., Proc Natl Acad Sci USA 95, 652-656 (1998).

[0287] In some embodiments binding of the Fc domain to a complement component, specifically to C1q, is altered. Accordingly, in some embodiments wherein the Fc domain is engineered to have altered effector function, said altered effector function includes altered CDC. C1q binding assays may be carried out to determine whether the Fc domain is able to bind C1q and hence has CDC activity. See e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro et al., J Immunol Methods 202, 163 (1996); Cragg et al., Blood 101, 1045-1052 (2003); and Cragg and Glennie, Blood 103, 2738-2743 (2004))

Fc Variants

[0288] The present invention is directed to the generation of multispecific, particularly bispecific binding proteins, and in particular, multispecific antibodies. The present invention generally relies on the use of engineered or variant Fc domains that can self-assemble in production cells to produce heterodimeric proteins, and methods to generate and purify such heterodimeric proteins.

[0289] Furthermore, as outlined herein, additional amino acid variants may be introduced into the Fc domains of the invention, to add additional functionalities. For example, amino acid changes within the Fc region can be added (either to one monomer or both) to facilitate increased ADCC or CDC (e.g. altered binding to Fc γ receptors); to allow or increase yield of the addition of toxins and drugs (e.g. for ADC), as well as to increase binding to FcRn and/or increase serum half-life of the resulting molecules. As is further described herein and as will be appreciated by those in the art, any and all of the variants outlined herein can be optionally and independently combined with other variants. Similarly, another category of functional variants are "Fc γ ablation variants" or "Fc γ silencing variants". In these embodiments, for some therapeutic applications, it is desirable to reduce or remove the normal binding of the Fc domain to one or more or all of the Fc γ receptors (e.g. Fc γ RI, Fc γ RIIa, Fc γ RIIb, Fc γ RIIIa, etc.) to avoid additional mechanisms of action. That is, for example, in many embodiments, particularly in the use of bispecific antibodies that bind CD3 monovalently and a tumor antigen on the other (e.g. CD19, her2/neu, etc.), it is generally desirable to ablate Fc γ RIIIa binding to eliminate or significantly reduce ADCC activity.

Additional Fc Variants for Additional Functionality

[0290] Accordingly, there are a number of useful Fc substitutions that can be made to alter binding to one or more of the Fc γ R receptors. Substitutions that result in increased binding as well as decreased binding can be useful. For example, it is known that increased binding to Fc γ RIIIa generally results in increased ADCC (antibody dependent cell-mediated cytotoxicity; the cell-mediated reaction wherein nonspecific cytotoxic cells that express Fc γ Rs recognize bound antibody on a target cell and subsequently cause lysis of the target cell). Similarly, decreased binding to Fc γ RIIb (an inhibitory receptor) can be beneficial as well in some circumstances. Amino acid substitutions that find use in the present invention include those listed in U.S. Ser. No. 11/124,620 (particularly FIG. 41), Ser. Nos. 11/174,287, 11/396,495, 11/538,406, all of which are expressly incorporated herein by reference in their entirety and specifically for the variants

disclosed therein. Particular variants that find use include, but are not limited to, Kabat positions 236A, 239D, 239E, 332E, 332D, 239D/332E, 267D, 267E, 328F, 267E/328F, 236A/332E, 239D/332E/330Y, 239D, 332E/330L, 243A, 243L, 264A, 264V and 299T.

[0291] In addition, there are additional Fc substitutions that find use in increased binding to the FcRn receptor and increased serum half-life, as specifically disclosed in U.S. Ser. No. 12/341,769, hereby incorporated by reference in its entirety, including, but not limited to, 434S, 434A, 428L, 308F, 259I, 428L/434S, 259I/308F, 436I/428L, 436I or V/434S, 436V/428L and 259I/308F/428L.

Antigen Binding Domains

[0292] As will be appreciated by a skilled artisan, there are two basic types of antigen binding domains, those that resemble antibody and T cell antigen binding domains (e.g. comprising a set of six CDRs, or in the case of single domain antibodies, single domain T cell receptors and the like, three CDRs) and those that can be otherwise be “ligand binding partners” (i.e. ligands or receptors, enzymes or substrates, or their equivalents), for example, that bind to targets without the use of CDRs.

Fab domains

[0293] A challenge in the production of multispecific antibodies is formation of various undesired side products apart from the desired functional molecule. When two or more distinct Fab domains are present, mispairing generally results from the pairing of wrong heavy chains with each other as well as pairing of a light chain with a wrong heavy chain counterpart or undesired pairing of light chains. The light chain mispairing problem is particularly challenging, and to date, no single approach by itself has proven consistent in preventing improper association of light chains with heavy chains and/or the formation of other side-products.

[0294] One approach that has been useful in forcing the pairing of a light chain polypeptide with its correct heavy chain counterpart utilizes chimeric heavy and light chains. Originally described as “variable region domain exchanged IgG”, or “inside-out (io) antibodies”, this approach is based upon intradomain crossovers between heavy and light chains involving the exchange of the heavy and light variable regions within a particular Fab domain (Chan et al, Molecular Immunology 421, 527-538 (2004)).

[0295] This approach, also known as "CrossMab technology", has been used to make distinct domain crossovers between heavy and light chains thereby creating different domain arrangements for heavy chains and light chains of different specificity. WO 2009/080251, WO 2009/080252, WO 2009/080253, WO 2009/080254 relate to bivalent, bispecific IgG antibodies with such domain crossovers. WO 2010/145792 and WO 2010/145792 relate to tetravalent antigen binding proteins with such domain crossovers. Multispecific antibodies with such a domain exchange in one binding arm (CrossMabVH-VL) are described in WO2009/080252 and Schaefer et al, PNAS, 108 (2011) 11187-1191. (All of the aforementioned citations are incorporated herein by reference in their entirety).

[0296] Variable region domain exchanges generally reduce but do not eliminate the byproducts due to the mismatch of a light chain against a first antigen with the wrong heavy chain against the second antigen. Moreover, the preparations are not completely free of other side products. The main side product is based on a Bence-Jones-type interaction (see also Schaefer, W. et al, PNAS, 108 (2011) 11187-1191; in Fig. SII of the Supplement).

[0297] Further reduction of side products and the associated aggregation behavior to improve the yield of such multispecific antibodies has been aided by the introduction of substitutions of charged amino acids with the opposite charge at specific amino acid positions in the VH and CL domains, as well as the CH1 and CL domains. The resulting "charge pair" modifications mediate electrostatic steering effects are complementary to the effect of variable region domain exchanges in helping to force the correct pairing of a light chain with its correct heavy chain.

[0298] Amino acid substitutions that find use in the present invention as constituents of such charge pairs include those described in Kannan et al (WO2014/081955), Shaefer et al (WO2015/150447), Ast et al (WO/2016/020309, and Carter et al (WO2016/172485), hereby incorporated by reference in their entirety. Preferred variants that find use include, but are not limited to, EU positions E123, Q124 and V133 in kappa and lambda light chain constant regions, K147, S183 and K213 in heavy chain constant regions, Q38 in light chain variable regions, and Q39 in the heavy chain variable regions. Specific examples of amino acid substitutions include, but are not limited to, substitution at position 123 or 124 in light chain constant regions by K, R or H, at position 147 or 213 in heavy chain constant regions by E or D, substitution at position 133 in light chain constant regions by K, R, H, E or D, substitution at position 183 in heavy chain

constant regions by K, R, H, E or D, at position 38 in light chain variable regions by K, R, H, E or D, and at position 39 in heavy chain variable regions by K, R, H, E or D.

[0299] This invention provides tetrahedral antibodies comprising two types of heavy chains and two types of light chains. In such antibodies, the third and fourth domains may comprise one or more charge pairs, while the fifth and sixth domains are a “CrossMab.” Conversely, the third and fourth domains may be a “CrossMab,” while the fifth and sixth domains comprise one or more charge pairs. Further, the third and fourth domains may be a “CrossMab” and comprise one or more charge pairs, while the fifth and sixth domains comprise neither such modifications. Conversely, the fifth and sixth domains may be a “CrossMab” and comprise one or more charge pairs, while the third and fourth domains comprise neither such modifications. In each case, such modifications facilitate correct pairing between the two types of heavy chains with their corresponding light chains. Such tetrahedral antibodies may be bispecific and tetravalent.

[0300] Similarly, this invention provides octahedral antibodies comprising two types of heavy chains and two types of light chains. In such antibodies, the fourth, fifth, and sixth domains may comprise one or more charge pairs, while the seventh, eighth, and ninth domains are a “CrossMab.” Conversely, the fourth, fifth, and sixth domains may be a “CrossMab,” while the seventh, eighth, and ninth comprise one or more charge pairs. Further, the fourth, fifth, and sixth domains may be a “CrossMab” **and** comprise one or more charge pairs, while the seventh, eighth, and ninth domains comprise neither such modifications. Conversely, the seventh, eighth, and ninth domains may be a “CrossMab” **and** comprise one or more charge pairs, while the fourth, fifth, and sixth domains comprise neither such modifications. In each case, such modifications facilitate correct pairing between the two types of heavy chains with their corresponding light chains. Such antibodies may be bispecific and octavalent.

Extracellular Proteins

[0301] Extracellular proteins play important roles in, among other things, the formation, differentiation and maintenance of multicellular organisms. A discussion of various intracellular proteins of interest is set forth in U.S. Patent No. 6,723,535, Ashkenazi et al., issued April 20, 2004, hereby incorporated by reference. Extracellular proteins include secreted proteins and the extracellular domains of transmembrane proteins.

Secreted proteins

[0302] The fate of many individual cells, e.g., proliferation, migration, differentiation, or interaction with other cells, is typically governed by information received from other cells and/or the immediate environment. This information is often transmitted by secreted polypeptides (for instance, mitogenic factors, survival factors, cytotoxic factors, differentiation factors, neuropeptides, and hormones) which are, in turn, received and interpreted by diverse cell receptors or membrane-bound proteins. These secreted polypeptides or signaling molecules normally pass through the cellular secretory pathway to reach their site of action in the extracellular environment.

[0303] Secreted proteins have various industrial applications, including as pharmaceuticals, diagnostics, biosensors and bioreactors. Most protein drugs available at present, such as thrombolytic agents, interferons, interleukins, erythropoietins, colony stimulating factors, and various other cytokines, are secretory proteins. Their receptors, which are membrane proteins, also have potential as therapeutic or diagnostic agents. Efforts are being undertaken by both industry and academia to identify new, native secreted proteins. Many efforts are focused on the screening of mammalian recombinant DNA libraries to identify the coding sequences for novel secreted proteins. Examples of screening methods and techniques are described in the literature (see, for example, Klein et al., Proc. Natl. Acad. Sci. 93:7108-7113 (1996); U.S. Patent No. 5,536,637)).

[0304] Interleukin-15 (IL-15) is a member of the gamma cytokine family that stimulates the proliferation of T, B, and natural killer (NK) cells and induces stem, central and effector memory CD8 T cells. The sequence of IL-15 is described in Grabstein et al., Science. 1994 May 13;264(5161):965-8. The biology of IL-15 and its therapeutic implications are discussed, for example, in Steel et al. Trends Pharmacol Sci. 2012 Jan; 33(1): 35–41, Perera et al. Microbes Infect. 2012 March; 14(3): 247–261, and Waldmann et al. Nature Reviews Immunology volume 6, pages 595–601(2006).

Extracellular domains of transmembrane proteins

[0305] Membrane-bound proteins and receptors can play important roles in, among other things, the formation, differentiation, and maintenance of multicellular organisms. The fate of many individual cells, e.g., proliferation, migration, differentiation, or interaction with other cells, is typically governed by information received from other cells and/or the immediate environment. This information is often transmitted by secreted polypeptides (for instance, mitogenic factors,

survival factors, cytotoxic factors, differentiation factors, neuropeptides, and hormones) which are, in turn, received and interpreted by diverse cell receptors or membrane-bound proteins. Such membrane-bound proteins and cell receptors include, but are not limited to, cytokine receptors, receptor kinases, receptor phosphatases, receptors involved in cell-cell interactions, and cellular adhesion molecules like selectins and integrins. For instance, transduction of signals that regulate cell growth and differentiation is regulated in part by phosphorylation of various cellular proteins. Protein tyrosine kinases, enzymes that catalyze that process, can also act as growth factor receptors. Examples include fibroblast growth factor receptor and nerve growth factor receptor.

[0306] Membrane-bound proteins and receptor molecules have various industrial applications, including as pharmaceutical and diagnostic agents. Receptor immunoadhesins, for instance, can be employed as therapeutic agents to block receptor-ligand interactions. The membrane-bound proteins can also be employed for screening of potential peptide or small molecule inhibitors of the relevant receptor/ligand interaction.

[0307] The tumor necrosis factor receptor superfamily (TNFRSF) is a protein superfamily of cytokine receptors characterized by the ability to bind tumor necrosis factors (TNFs) via an extracellular cysteine-rich domain. Members of the TNFRSF are described in Locksley et al., *Cell*. 2001 Feb 23;104(4):487-501 and the table reproduced below. *See also* Hehlhans et al., *Immunology*. 2005 May; 115(1): 1–20.

Table B-1 – Members of the TNFR Superfamily

Receptor	Standardized	Other Names	Accession	Human Chromosome	Mouse Chromosome
NGFR	TNFRSF16	p75	M14764	17q21-q22	11, 55.6 cM
Troy	TNFRSF19	Taj	AF167555	13q12.11-12.3	14
EDAR			AF130988	2q11-q13	10, 29.0 cM
XEDAR		EDA-A2R		AF298812	X
CD40	TNFRSF5	p50, Bp50	X60592	20q12-q13.2	2, 97.0 cM
DcR3	TNFRSF6B		AF104419	20q13	
FAS	TNFRSF6	CD95, APO-1, APT1	M67454	10q24.1	19, 23.0 cM
OX40	TNFRSF4	CD134, ACT35, TXGP1L	X75962	1p36	4, 79.4 cM
AITR	TNFRSF18	GITR	AF125304	1p36.3	4
CD30	TNFRSF8	Ki-1, DIS166E	M83554	1p36	4, 75.5 cM
HveA	TNFRSF14	HVEM, ATAR, TR2, LIGHTR	U70321	1p36.3-p36.2	
4-1BB	TNFRSF9	CD137, ILA	L12964	1p36	4, 75.5 cM

Receptor	Standardized	Other Names	Accession	Human Chromosome	Mouse Chromosome
TNFR2	TNFRSF1B	CD120b, p75, TNFBR, TNFR80, TNF-R-II	M32315	1p36.3-p36.2	4, 75.5 cM
DR3	TNFRSF12	TRAMP, WSL-1, LARD, WSL-LR, DDR3, TR3, APO-3	U72763	1p36.2	
CD27	TNFRSF7	Tp55, S152	M63928	12p13	6, 60.35 cM
TNFR1	TNFRSF1A	CD120a p55-R, TNFAR, TNFR60 TNF-R-I	M75866	12p13.2	6, 60.55 cM
LT β R	TNFRSF3	TNFR2-RP, TNFCR, TNF-R-III	L04270	12p13	6, 60.4 cM
RANK	TNFRSF11A	TRANCE-R	AF018253	18q22.1	
TACI		CAML interactor		AF023614	17p11
BCMA	TNFRSF17	BCM	Z29574	16p13.1	
DR6		TR7	NM_014452	6p21.1-12.2	
OPG	TNFRSF11B	OCIF, TR1 osteoprotegerin	U94332	8Q24	
DR4	TNFRSF10A	Apo2, TRAILR-1	U90875	8p21	
DR5	TNFRSF10B	KILLER, TRICK2A, TRAIL-R2, TRICKB	AF012628	8p22-p21	
DcR1	TNFRSF10C	TRAILR3, LIT, TRID	AF012536	8p22-p21	
DcR2	TNFRSF10D	TRUNDD, TRAILR4	AF029761	8p21	

[0308] The immunoglobulin superfamily (IgSF) is a large protein superfamily of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. The IgSF is discussed in Natarajan et al. (April 2015) Immunoglobulin Superfamily. In: eLS. John Wiley & Sons, Ltd: Chichester.

[0309] Angiotensin-converting enzyme 2 (ACE2) is an enzyme attached to the cell membranes of cells in the lungs, arteries, heart, kidney, and intestines. ACE2 is discussed in Donoghue, et al., 2000. Circulation research, 87(5), pp. e1-e9.

[0310] Extracellular proteins of the invention are provided in SEQ ID NOs: 847-890 and 935-2038. SEQ ID NOs: 891-934 and 2039-3142 provide the sequences of extracellular domains that also include the sequence of the signal peptide (SP) and pre-protein (PP) sequence.

Covalent Linkages

[0311] Covalent linkages of the invention may comprise, or consist of, any “non-peptidyl linkage” described in U.S. Patent Application Publication No. US 20170008950 A1, published January 12, 2017, the contents of which are hereby incorporated-by-reference.

Peptide Linkers

[0312] Peptide linkers are stretches of consecutive amino acids which link two domains.

[0313] In one embodiment, the peptide linker has a length of at least 5 amino acids. In one embodiment, the peptide linker has a length of 5-100 amino acids.

[0314] In one embodiment, the peptide linker has a length of 10-50 amino acids.

[0315] In one embodiment, peptide linkers have a length of 23 amino acids. In one embodiment, the peptide linker connecting domain 1 and a dimerizing polypeptide and the peptide linker connecting domains 2 and a dimerizing polypeptide each have a length of 23 amino acids. In one embodiment, such peptide linkers each have a length of 23 amino acids and are derived from the stalk region of a TNF receptor, preferably wherein the TNF receptor is TNF receptor 1B, still more preferably wherein the peptide linker consists of the amino acid sequence set forth in SEQ ID NO: 4468.

[0316] In one embodiment, the peptide linker is a stretch of consecutive amino acids found in an immunoglobulin hinge region or portion thereof.

[0317] In one embodiment, the peptide linker is a stretch of 5 to 57 consecutive amino acids found in the sequence:

TSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLPMGPPPAEGSTGD (SEQ ID NO: 3227)

[0318] In one embodiment, the peptide linker is a glycine-serine or glycine-alanine linker.

[0319] In one embodiment, the peptide linker is

- a. (GxS)_n or (GxS)_nGm or (GxA)_n or (GxA)_nGm

- b. with G=glycine, S=serine, A=alanine, and
- c. $x=3$, $n=2, 3, 4, 5$ or 6 , $m=0, 1, 2$ or 3 ; or
- d. $x=4$, $n=1, 2, 3, 4$ or 5 , $m=0, 1, 2$ or 3 .

[0320] Exemplary linkers of the invention are provided in SEQ ID NOs: 3143-4656.

Intra-homodimerization and intra-heterodimerization within domains

Fc Domain Modifications Promoting Heterodimerization

[0321] The invention provides tetrahedral antibodies, wherein the first and second domains each comprise two polypeptide chains, such that there are two N-termini and two C-termini upon which additional domains may be attached either directly by a peptide bond or via a peptide linker). Similarly, the invention provides octahedral antibodies, wherein the first, second and third domains each comprise two polypeptide chains, such that there are two N-termini and two C-termini upon which additional domains may be attached either directly by a peptide bond or via a peptide linker). Other domains of the invention (such as the third, fourth, fifth, sixth, seventh, and eighth domains of a tetrahedral antibody, and the fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of an octahedral antibody) may also comprise two polypeptide chains.

[0322] In particular embodiments, such domains are Fc domains comprising a modification promoting association of two different Fc domain subunits, i.e. intra-heterodimerization. A modification may be present in the first Fc domain subunit and/or the second Fc domain subunit. Embodiments of said modifications are described in U.S. Patent Application Publication No. US 20130058937 A1 and in the paragraphs that follow.

[0323] The site of most extensive protein-protein interaction between the two subunits of a human IgG Fc domain is in the CH3 domain of the Fc domain. Thus, in one embodiment said modification is in the CH3 domain of the Fc domain.

[0324] In a specific embodiment said modification is a so-called “knob-into-hole” modification, comprising a “knob” modification in one of the two subunits of the Fc domain and a “hole” modification in the other one of the two subunits of the Fc domain.

[0325] The knob-into-hole technology is described e.g. in U.S. Pat. No. 5,731,168; U.S. Pat. No. 7,695,936; Ridgway et al., Prot Eng 9, 617-621 (1996) and Carter, J Immunol Meth 248, 7-15

(2001). Generally, the method involves introducing a protuberance (“knob”) at the interface of a first polypeptide and a corresponding cavity (“hole”) in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity so as to promote heterodimer formation and hinder homodimer formation. Protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine).

[0326] Accordingly, in a particular embodiment, in the CH3 domain of the first Fc domain subunit of an Fc domain an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the CH3 domain of the first subunit which is positionable in a cavity within the CH3 domain of the second subunit, and in the CH3 domain of the second Fc domain subunit an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the CH3 domain of the second subunit within which the protuberance within the CH3 domain of the first subunit is positionable.

[0327] The protuberance and cavity can be made by altering the nucleic acid encoding the polypeptides, e.g. by site-specific mutagenesis, or by peptide synthesis.

[0328] In a specific embodiment, in the CH3 domain of the first subunit of the Fc domain the threonine residue at position 366 is replaced with a tryptophan residue (T366W), and in the CH3 domain of the second subunit of the Fc domain the tyrosine residue at position 407 is replaced with a valine residue (Y407V). In one embodiment, in the second subunit of the Fc domain additionally the threonine residue at position 366 is replaced with a serine residue (T366S) and the leucine residue at position 368 is replaced with an alanine residue (L368A). In yet a further embodiment, in the first subunit of the Fc domain additionally the serine residue at position 354 is replaced with a cysteine residue (S354C), and in the second subunit of the Fc domain additionally the tyrosine residue at position 349 is replaced by a cysteine residue (Y349C). Introduction of these two cysteine residues results in formation of a disulfide bridge between the two subunits of the Fc domain, further stabilizing the dimer (Carter, J Immunol Methods 248, 7-15 (2001)).

[0329] In an alternative embodiment a modification promoting association of the first and the second subunit of the Fc domain comprises a modification mediating electrostatic steering effects, e.g. as described in PCT publication WO 2009/089004. Generally, this method involves replacement of one or more amino acid residues at the interface of the two Fc domain subunits by charged amino acid residues so that homodimer formation becomes electrostatically unfavorable but heterodimerization electrostatically favorable.

[0330] Illustrative examples include but are not limited to, for example, US Patent Application 20030078385 (Arathoon et al.—Genentech; describing knob into holes); WO2007147901 (Kjærgaard et al.—Novo Nordisk; describing ionic interactions); WO 2009089004 (Kannan et al.—Amgen; describing electrostatic steering effects); U.S. Provisional Patent Application 61/243,105 (Christensen et al.—Genentech; describing coiled coils).

[0331] In particular embodiments, domains 1 and 2 of a tetrahedral antibody of the invention, or domains 1, 2 and 3 of an octahedral antibody of the invention, are two or three distinct heterodimeric Fc domains comprising distinct sets of modifications promoting intra-heterodimerization of their respective Fc domain subunits. Illustrative examples of distinct sets of mutations in the antibody heavy chain constant region that can be used to promote the preferential assembly of distinct Fc heterodimers include but are not limited to, for example, U.S. Ser. Nos. 11/533,709, 13/494,870, 12/875,015, 13/289,934, 14/773418, 12/811,207, 13/866,756, 14/647,480, and 14/830,336. For example, mutations can be made in the CH3 domain based on human IgG1 and incorporating distinct pairs of amino acid substitutions within a first polypeptide and a second polypeptide that allow these two chains to selectively heterodimerize with each other. The positions of amino acid substitutions illustrated below are all numbered according to the EU index as in Kabat.

[0332] For example, one or more mutations can be incorporated into the constant region as compared to human IgG1 constant region, for example at Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411 and/or K439. Exemplary substitutions include, for example, Q347E, Q347R, Y349S, Y349K, Y349T, Y349D, Y349E, Y349C, T350V, L351K, L351D, L351Y, S354C, E356K, E357Q, E357L, E357W, K360E, K360W, Q362E, S364K, S364E, S364H, S364D, T366V, T366I, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E,

K392L, K392M, K392V, K392F, K392D, K392E, T394F, T394W, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y407I, Y407V, K409F, K409W, K409D, T411D, T411E, K439D, and K439E.

[0333] Alternatively, amino acid substitutions could be selected from the following sets of substitutions shown in the tables below:

Table C-1 – Sets of substitutions

	First Polypeptide	Second Polypeptide
Set 1	S364E/F405A	Y349K/T394F
Set 2	S364H/D401K	Y349T/T411E
Set 3	S364H/T394F	Y349T/F405A
Set 4	S364E/T394F	Y349K/F405A
Set 5	S364E/T411E	Y349K/D401K
Set 6	S364D/T394F	Y349K/F405A
Set 7	S364H/F405A	Y349T/T394F
Set 8	S364K/E357Q	L368D/K370S
Set 9	L368D/K370S	S364K
Set 10	L368E/K370S	S364K
Set 11	K360E/Q362E	D401K
Set 12	L368D/K370S	S364K/E357L
Set 13	K370S	S364K/E357Q
Set 14	F405L	K409R
Set 15	K409R	F405L

Table C-2 – Sets of substitutions

	First Polypeptide	Second Polypeptide
Set 1	K409W	D399V/F405T
Set 2	Y349S	E357W
Set 3	K360E	Q347R
Set 4	K360E/K409W	Q347R/D399V/F405T
Set 5	Q347E/K360E/K409W	Q347R/D399V/F405T
Set 6	Y349S/K409W	E357W/D399V/F405T

Table C-3 – Sets of substitutions

	First Polypeptide	Second Polypeptide
Set 1	T366K/L351K	L351D/L368E
Set 2	T366K/L351K	L351D/Y349E
Set 3	T366K/L351K	L351D/Y349D
Set 4	T366K/L351K	L351D/Y349E/L368E
Set 5	T366K/L351K	L351D/Y349D/L368E
Set 6	E356K/D399K	K392D/K409D

Table C-4 – Sets of substitutions

First Polypeptide	Second Polypeptide
L351Y, D399R, D399K,	T366V, T366I, T366L, T366M, N390D,
S400K, S400R, Y407A,	N390E, K392L, K392M, K392V, K392F
Y407I, Y407V	K392D, K392E, K409F, K409W, T411D and T411E

[0334] Alternatively, at least one amino acid substitutions could be selected from the following set of substitutions in Table C-5, where the position(s) indicated in the First Polypeptide column is replaced by any known negatively-charged amino acid, and the position(s) indicated in the Second Polypeptide Column is replaced by any known positively-charged amino acid.

Table C-5 – Sets of substitutions

First Polypeptide	Second Polypeptide
K392, K370, K409, or K439	D399, E356, or E357

[0335] Alternatively, at least one amino acid substitutions could be selected from the following set of in Table C-6, where the position(s) indicated in the First Polypeptide column is replaced by any known positively-charged amino acid, and the position(s) indicated in the Second Polypeptide Column is replaced by any known negatively-charged amino acid.

Table C-6 – Sets of substitutions

First Polypeptide	Second Polypeptide
D399, E356, or E357	K409, K439, K370, or K392

[0336] Alternatively, amino acid substitutions could be selected from the following set of in Table C-7.

Table C-7 – Sets of substitutions

First Polypeptide	Second Polypeptide
T350V, L351Y, F405A, and Y407V	T350V, T366L, K392L, and T394W

[0337] Alternatively, or in addition, the structural stability of a heteromultimer protein may be increased by introducing S354C on either of the first or second polypeptide chain, and Y349C on the opposing polypeptide chain, which forms an artificial disulfide bridge within the interface of the two polypeptides.

Extra-homodimerization and extra-heterodimerization between domain 1 and domain 2

[0338] In an embodiment, homodimerization between domain 1 and domain 2 (i.e. extra-homodimerization) is achieved by use of a first and a second dimerizing polypeptide which homodimerize. One example of a homodimerization domain is the ACE2 collectrin-like domain (CLD).

[0339] In an embodiment, heterodimerization between domain 1 and domain 2 (i.e. extra-heterodimerization) is achieved by use of a first and a second dimerizing polypeptide which favor the formation of a heterodimer over a homodimer. Any non-immunoglobulin dimerization polypeptide having a strong preference for forming heterodimers over homodimers is within the scope of the invention.

Dimerizing polypeptides*Leucine Zipper domains*

[0340] Leucine zippers are well known in the art (see Hakoshima; Encyclopedia of Life Sciences; 2005, for example). The leucine zipper is a super-secondary structure that may function as a dimerization domain. Its presence generates adhesion forces in parallel alpha helices. A single leucine zipper consists of multiple leucine residues at approximately 7-residue intervals, which forms an amphipathic alpha helix with a hydrophobic region running along one side. This hydrophobic region provides an area for dimerization, allowing the motifs to “zip” together. Leucine zippers are typically 20 to 40 amino acids in length, for example approximately 30 amino acids. *See also* Pack, P. & Plueckthun, A., Biochemistry 31, 1579-1584 (1992).

[0341] Leucine zipper domains may be such that the formation of heterodimers is more favorable than the formation of homodimers of the helices. Leucine zippers may be synthetic or naturally occurring. Synthetic leucines can be designed to have a much higher binding affinity. Alternatively, naturally occurring leucine zippers or leucine zippers with a similar binding affinity may be used. Leucine zippers from the c-jun and c-fos protein are an example of leucine zippers. Other leucine zippers include those from the myc and max proteins (Amati, B., S. Dalton, et al. (1992). Nature 359(6394): 423-6.). Other leucine zippers with suitable properties may be designed, for example as described in O'Shea, E. K., Lumb, K. J. and Kim, P. S. (1993) Curr. Biol. 3: 658-667. *See also* O'Shea, E. K., Rutkowski, R., et al. (1989) Science 245: 646-648 and O'Shea, E. K.,

R. Rutkowski, et al. (1992). Cell 68(4): 699-708 describe leucine zippers from c-jun (α chain) and c-fos (β chain).

[0342] As discussed in U.S. Patent Application Publication No. US 20020119149 A1 (Jakobsen), leucine zippers can be designed and engineered by those skilled in the art to form homodimers, heterodimers or trimeric complexes. See Lumb, K. J. and P. S. Kim (1995). Biochemistry 34(27): 8642-8; Nautiyal, S., D. N. Woolfson, et al. (1995). Biochemistry 34(37): 11645-51.; Boice, J. A., G. R. Dieckmann, et al. (1996). Biochemistry 35(46): 14480-5; and Chao, H., M. E. Houston, Jr., et al. (1996). Biochemistry 35(37): 12175-85.

Collectrin-like domains

[0343] Full-length ACE2 consists of an N-terminal Peptidase Domain (PD) and a C-terminal collectrin-like domain (CLD) that ends with a single transmembrane helix and a ~40-residue intracellular segment. The CLD (residues 616 to 768 of ACE2), consists of a small extracellular domain, a long linker, and the single transmembrane (TM) helix. The CLD comprises the “neck domain” of ACE2 (residues 616 to 726), which is the primary mediator of dimerization of ACE2. (Yan et al., Science 367, 1444–1448 (2020))

Collectrin domains

[0344] Collectrin is a homolog of ACE2 and is a transmembrane glycoprotein specifically expressed in collecting tubules of the kidney. Based on its homology to the ACE2 CLD, it is expected that Collectrin domains may be capable of forming homodimers (Zhang et al. J Biol. Chem. Vol. 276, No. 20, Issue of May 18, pp. 17132–17139, 2001)

Trimerizing domains

TNF ligand superfamily members

[0345] Members of the TNF ligand superfamily may be used as trimerizing domains of the inventions. They are described in Locksley et al., Cell. 2001 Feb 23;104(4):487-501 and the table reproduced below.

Table B-2 – Members of the TNF Superfamily

Ligand	Standardized	Other Names	Accession	Human Chromosome	Mouse Chromosome
EDA		EDA1	NM 001399	Xq12-q13.1	X, 37.0 cM

CD40L	TNFSF5	IMD3, HIGM1, TRAP, CD154, gp39	X67878	Xq26	X, 18.0 cM
FasL	TNFSF6	APT1LG1	U11821	1q23	1, 85.0 cM
OX40L	TNFSF4	gp34 TXGP1	D90224	1q25	1, 84.9 cM
AITRL	TNFSF18	TL6, hGITRL	AF125303	1q23	
CD30L	TNFSF8		L09753	9q33	4, 32.2 cM
VEGI	TNFSF15	TL1	AF039390		
LIGHT	TNFSF14	LT_, HVEM-L	AF036581	19 (probable)	17
4-1BBL	TNFSF9		U03398	19p13.3	17
CD27L	TNFSF7	CD70	L08096	19p13	17, 20.0 cM
LT α	TNFSF1	TNFB, LT	X01393	6p21.3	17, 19.06 cM
TNF	TNFSF2	tumor necrosis factor; cachectin, TNFA, DIF	X01394	6p21.3	17, 19.06
LT β	TNFSF3	TNFC, p33	L11015	6p21.3	17, 19.061
TWEAK	TNFSF12	DR3L APO3L	AF030099	17p13	11?
APRIL	TNFSF13		NM_003808	17p13.1	11?
BLYS	TNFSF13B	BAFF, THANK, TALL1	AF132600	13q32-34	
RANKL	TNFSF11	TRANCE, OPGL, ODF	AF013171	13q14	14, 45.0
TRAIL	TNFSF10	Apo-2L TL2	U37518	3q26	

[0346] Suitable examples of the trimerizing polypeptides of the invention are the complete extracellular regions or portions thereof of TNFSF1 (UniProtKB TNFB_HUMAN, GenBank Accession No. P01374), TNFSF2 (UniProtKB TNFA_HUMAN, GenBank Accession No. P01375), TNFSF3 (UniProtKB TNFC_HUMAN, GenBank Accession No. Q06643), TNFSF4 (UniProtKB TNFL4_HUMAN, GenBank Accession No. P23510), TNFSF5 (UniProtKB CD40L_HUMAN, GenBank Accession No. P29965), TNFSF6 (UniProtKB TNFL6_HUMAN, GenBank Accession No. P48023), TNFSF7 (UniProtKB CD70_HUMAN, GenBank Accession No. P32970), TNFSF8 (UniProtKB TNFL8_HUMAN, GenBank Accession No. P32971), TNFSF9 (UniProtKB TNFL9_HUMAN, GenBank Accession No. P41273), TNFSF10 (UniProtKB TNF10_HUMAN, GenBank Accession No. P50591), TNFSF11 (UniProtKB TNF11_HUMAN, GenBank Accession No. O14788), TNFSF12 (UniProtKB TNF12_HUMAN, GenBank Accession No. O43508), TNFSF13 (UniProtKB TNF13_HUMAN, GenBank Accession No. O75888), TNFSF13B (UniProtKB TN13B_HUMAN, GenBank Accession No. Q9Y275), TNFSF14 (UniProtKB TNF14_HUMAN; GenBank Accession No. O43557), TNFSF15

(UniProtKB TNF15_HUMAN, GenBank Accession No. O95150), and TNFSF18 (UniProtKB TNF18_HUMAN; GenBank Accession No. Q9UNG2).

[0347] Preferred trimerizing polypeptides are shown in SEQ ID NOs: 787-790 (TNFSF1), SEQ ID NOs: 791-792 (TNFSF2), SEQ ID NOs: 793-795 (TNFSF3), SEQ ID NOs: 796-798 (TNFSF4), SEQ ID NOs: 799-802 (TNFSF5), SEQ ID NOs: 803-806, SEQ ID NOs: 807-809 (TNFSF7), SEQ ID NOs: 810-813 (TNFSF8), SEQ ID NOs: 814-816 (TNFSF9), SEQ ID NOs: 817-820 (TNFSF10), SEQ ID NOs: 821-822 (TNFSF11), SEQ ID NOs: 823-827 (TNFSF12), SEQ ID NOs: 828-831 (TNFSF13), SEQ ID NOs: 832-833 (TNFSF13B), SEQ ID NOs: 834-837 (TNFSF14), SEQ ID NOs: 838-841 (TNFSF15), and SEQ ID NOs: 842-843 (TNFSF18).

Kits

[0348] Another aspect of the present invention provides kits comprising the compounds disclosed herein and the pharmaceutical compositions comprising these compounds. A kit may include, in addition to the compound or pharmaceutical composition, diagnostic or therapeutic agents. A kit may also include instructions for use in a diagnostic or therapeutic method. In a diagnostic embodiment, the kit includes the compound or a pharmaceutical composition thereof and a diagnostic agent. In a therapeutic embodiment, the kit includes the antibody or a pharmaceutical composition thereof and one or more therapeutic agents, such as an additional antineoplastic agent, anti-tumor agent or chemotherapeutic agent.

General techniques

[0349] The description below relates primarily to production of stretches of consecutive amino acids or polypeptides of interest by culturing cells transformed or transfected with a vector containing an encoding nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed. For instance, the amino acid sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques (see, e.g., Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, Calif. (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)). In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, Calif.) using manufacturer's instructions. Various portions of the stretches of consecutive amino acids or polypeptides of interest may be chemically synthesized separately and combined using chemical

or enzymatic methods to produce the full-length stretches of consecutive amino acids or polypeptides of interest.

Selection and Transformation of Host Cells

[0350] Host cells are transfected or transformed with expression or cloning vectors described herein for production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in *Mammalian Cell Biotechnology: a Practical Approach*, M. Butler, ed. (IRL Press, 1991) and Sambrook et al., *supra*.

[0351] Methods of eukaryotic cell transfection and prokaryotic cell transformation are known to the ordinarily skilled artisan, for example, CaCl_2 , CaPO_4 , liposome-mediated and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., *supra*, or electroporation is generally used for prokaryotes. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene*, 23:315 (1983) and WO 89/05859 published Jun. 29, 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology*, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transfections have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.*, 130:946(1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA)*, 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., *Methods in Enzymology*, 185:527-537 (1990) and Mansour et al., *Nature*, 336:348-352 (1988).

[0352] Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *E. coli*. Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294

(ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5772 (ATCC 53,635). Other suitable prokaryotic host cells include Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescens*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. These examples are illustrative rather than limiting. Strain W3110 is one particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell secretes minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins endogenous to the host, with examples of such hosts including *E. coli* W3110 strain 1A2, which has the complete genotype *tonA*; *E. coli* W3110 strain 9E4, which has the complete genotype *tonA ptr3*; *E. coli* W3110 strain 27C7 (ATCC 55,244), which has the complete genotype *tonAptr3phoA E15 (argF-lac)169 degP ompT kan^r*; *E. coli* W3110 strain 37D6, which has the complete genotype *tonA ptr3 phoA E15 (argF-lac)169 degP ompT rbs7 ilvG kan^r*; *E. coli* W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant *degP* deletion mutation; and an *E. coli* strain having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued Aug. 7, 1990. Alternatively, in vitro methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

[0353] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism. Others include *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, 290:140 (1981); EP 139,383 published May 2, 1985); *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529; Fleer et al., *Bio/Technology*, 9:968-975 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., *J. Bacteriol.*, 737 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilum* (ATCC 36,906; Van den Berg et al., *Bio/Technology*, 8:135 (1990)), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., *J. Basic Microbiol.*, 28:265-278 (1988)); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa* (Case et al., *Proc. Natl. Acad. Sci. USA*, 76:5259-5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published Oct. 31, 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357

published Jan. 10, 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., *Biochem. Biophys. Res. Commun.*, 112:284-289 (1983); Tilburn et al., *Gene*, 26:205-221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA*, 81:1470-1474 (1984)) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4:475479 (1985)). Methylotropic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula*, *Candida*, *Kloeckera*, *Pichia*, *Saccharomyces*, *Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, *The Biochemistry of Methylotrophs*, 269 (1982).

[0354] Suitable host cells for the expression of glycosylated stretches of consecutive amino acids or polypeptides of interest are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as *Drosophila* S2 and *Spodoptera* Sf9, as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen. Virol.*, 36:59 (1977)); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

Selection and Use of a Replicable Vector

[0355] The nucleic acid (e.g., cDNA or genomic DNA) encoding the stretch of consecutive amino acids or polypeptides of interest may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

[0356] The stretches of consecutive amino acids or polypeptides of interest may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the encoding DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* alpha-factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published Apr. 4, 1990), or the signal described in WO 90/13646 published Nov. 15, 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders.

[0357] Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2mu plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

[0358] Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

[0359] An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141

(1979); Tschemper et al., *Gene*, 10:157 (1980)). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85:12 (1977)).

[0360] Expression and cloning vectors usually contain a promoter operably linked to the encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems (Chang et al., *Nature*, 275:615 (1978); Goeddel et al., *Nature*, 281:544 (1979)), alkaline phosphatase, a tryptophan (*trp*) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057 (1980); EP 36,776), and hybrid promoters such as the *tac* promoter (deBoer et al., *Proc. Natl. Acad. Sci. USA*, 80:21-25 (1983)). Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the encoding DNA.

[0361] Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.*, 255:2073 (1980)) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Re.g.*, 7:149 (1968); Holland, *Biochemistry*, 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[0362] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

[0363] Transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published Jul. 5, 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin

promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

[0364] Transcription of a DNA encoding the stretches of consecutive amino acids or polypeptides of interest by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, alpha-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the coding sequence, but is preferably located at a site 5' from the promoter.

[0365] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding stretches of consecutive amino acids or polypeptides of interest.

[0366] Still other methods, vectors, and host cells suitable for adaptation to the synthesis of stretches of consecutive amino acids or polypeptides in recombinant vertebrate cell culture are described in Gething et al., *Nature* 293:620-625 (1981); Mantei et al., *Nature*, 281:4046 (1979); EP 117,060; and EP 117,058.

Detecting Gene Amplification/Expression

[0367] Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77:5201-5205 (1980)), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The

antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

[0368] Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence stretches of consecutive amino acids or polypeptides of interest or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to DNA encoding a stretch of consecutive amino acids or polypeptide of interest and encoding a specific antibody epitope.

Purification of Polypeptide

[0369] Forms of the stretches of consecutive amino acids or polypeptides of interest may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or by enzymatic cleavage. Cells employed in expression of the stretches of consecutive amino acids or polypeptides of interest can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

[0370] It may be desired to purify the stretches of consecutive amino acids or polypeptides of interest from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, *Methods in Enzymology*, 182 (1990); Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on

the nature of the production process used and the particular stretches of consecutive amino acids or polypeptides of interest produced.

Intein-Based C-Terminal Syntheses

[0371] As described, for example, in U.S. Patent No. 6,849,428, issued Feb. 1, 2005, inteins are the protein equivalent of the self-splicing RNA introns (see Perler et al., *Nucleic Acids Res.* 22:1125-1127 (1994)), which catalyze their own excision from a precursor protein with the concomitant fusion of the flanking protein sequences, known as exteins (reviewed in Perler et al., *Curr. Opin. Chem. Biol.* 1:292-299 (1997); Perler, F. B. *Cell* 92(1):1-4 (1998); Xu et al., *EMBO J.* 15(19):5146-5153 (1996)).

[0372] Studies into the mechanism of intein splicing led to the development of a protein purification system that utilized thiol-induced cleavage of the peptide bond at the N-terminus of the Sce VMA intein (Chong et al., *Gene* 192(2):271-281 (1997)). Purification with this intein-mediated system generates a bacterially-expressed protein with a C-terminal thioester (Chong et al., (1997)). In one application, where it is described to isolate a cytotoxic protein, the bacterially expressed protein with the C-terminal thioester is then fused to a chemically-synthesized peptide with an N-terminal cysteine using the chemistry described for “native chemical ligation” (Evans et al., *Protein Sci.* 7:2256-2264 (1998); Muir et al., *Proc. Natl. Acad. Sci. USA* 95:6705-6710 (1998)).

[0373] This technique, referred to as “intein-mediated protein ligation” (IPL), represents an important advance in protein semi-synthetic techniques. However, because chemically-synthesized peptides of larger than about 100 residues are difficult to obtain, the general application of IPL was limited by the requirement of a chemically-synthesized peptide as a ligation partner.

[0374] IPL technology was significantly expanded when an expressed protein with a predetermined N-terminus, such as cysteine, was generated, as described for example in U.S. Patent No. 6,849,428. This allows the fusion of one or more expressed proteins from a host cell, such as bacterial, yeast or mammalian cells. In one non-limiting example the intein a modified RIR1 *Methanobacterium thermoautotrophicum* is that cleaves at either the C-terminus or N-terminus is used which allows for the release of a bacterially expressed protein during a one-column purification, thus eliminating the need proteases entirely.

[0375] Intein technology is one example of one route to obtain components. In one embodiment, the subunits of the compounds of the invention are obtained by transfecting suitable cells, capable of expressing and secreting mature chimeric polypeptides, wherein such polypeptides comprise, for example, an adhesin domain contiguous with an isolatable c-terminal intein domain (see U.S. Patent No. 6,849,428, Evans et al., issued February 1, 2005, hereby incorporated by reference). The cells, such as mammalian cells or bacterial cells, are transfected using known recombinant DNA techniques. The secreted chimeric polypeptide can then be isolated, e.g. using a chitin-derivatized resin in the case of an intein-chitin binding domain (see U.S. Patent No. 6,897,285, Xu et al., issued May 24, 2005, hereby incorporated by reference), and is then treated under conditions permitting thiol-mediated cleavage and release of the now C-terminal thioester-terminated subunit. The thioester-terminated adhesion subunit is readily converted to a C-terminal cysteine terminated subunit.

[0376] For example, following an intein autocleavage reaction, a thioester intermediate is generated that permits the facile addition of cysteine, selenocysteine, homocysteine, or homoselenocysteine, or a derivative of cysteine, selenocysteine, homocysteine, homoselenocysteine, to the C-terminus by native chemical ligation. Methods of adding a cysteine, selenocysteine, homocysteine, or homoselenocysteine, or a derivative of cysteine, selenocysteine, homocysteine, homoselenocysteine, to the C-terminus by native chemical ligation which are useful in aspects of the present invention are described in U.S. Patent Application No. 2008/0254512, Capon, published October 16, 2008, the entire contents of which are hereby incorporated herein by reference.

Examples of Expression of Stretches of Consecutive Amino Acids or Polypeptide Components of Interest in various cells

In E. coli

[0377] The DNA sequence encoding the desired amino acid sequence of interest or polypeptide is initially amplified using selected PCR primers. The primers should contain restriction enzyme sites which correspond to the restriction enzyme sites on the selected expression vector. A variety of expression vectors may be employed. An example of a suitable vector is pBR322 (derived from *E. coli*; see Bolivar et al., *Gene*, 2:95 (1977)) which contains genes for ampicillin and tetracycline resistance. The vector is digested with restriction enzyme and dephosphorylated. The PCR

amplified sequences are then ligated into the vector. The vector will preferably include sequences which encode for an antibiotic resistance gene, a trp promoter, a polyhis leader (including the first six STII codons, polyhis sequence, and enterokinase cleavage site), the specific amino acid sequence of interest / polypeptide coding region, lambda transcriptional terminator, and an argU gene.

[0378] The ligation mixture is then used to transform a selected E. coli strain using the methods described in Sambrook et al., supra. Transformants are identified by their ability to grow on LB plates and antibiotic resistant colonies are then selected. Plasmid DNA can be isolated and confirmed by restriction analysis and DNA sequencing.

[0379] Selected clones can be grown overnight in liquid culture medium such as LB broth supplemented with antibiotics. The overnight culture may subsequently be used to inoculate a larger scale culture. The cells are then grown to a desired optical density, during which the expression promoter is turned on.

[0380] After culturing the cells for several more hours, the cells can be harvested by centrifugation. The cell pellet obtained by the centrifugation can be solubilized using various agents known in the art, and the solubilized amino acid sequence of interest or polypeptide can then be purified using a metal chelating column under conditions that allow tight binding of the protein.

[0381] The primers can contain restriction enzyme sites which correspond to the restriction enzyme sites on the selected expression vector, and other useful sequences providing for efficient and reliable translation initiation, rapid purification on a metal chelation column, and proteolytic removal with enterokinase. The PCR-amplified, poly-His tagged sequences can be ligated into an expression vector used to transform an E. coli host based on, for example, strain 52 (W3110 fuhA(tonA) Ion galE rpoHts(htpRts) clpP(lacIq). Transformants can first be grown in LB containing 50 mg/ml carbenicillin at 30°C with shaking until an O.D.600 of 3-5 is reached. Cultures are then diluted 50-100 fold into C RAP media (prepared by mixing 3.57 g (NH₄)₂ SO₄, 0.71 g sodium citrate-2H₂O, 1.07 g KCl, 5.36 g Difco yeast extract, 5.36 g Sheffield hycase SF in 500 mL water, as well as 110 mM MPOS, pH 7.3, 0.55% (w/v) glucose and 7 mM MgSO₄) and grown for approximately 20-30 hours at 30°C. with shaking. Samples were removed to verify expression by SDS-PAGE analysis, and the bulk culture is centrifuged to pellet the cells. Cell pellets were frozen until purification and refolding.

[0382] *E. coli* paste from 0.5 to 1 L fermentations (6-10 g pellets) was resuspended in 10 volumes (w/v) in 7 M guanidine, 20 mM Tris, pH 8 buffer. Solid sodium sulfite and sodium tetrathionate is added to make final concentrations of 0.1M and 0.02 M, respectively, and the solution was stirred overnight at 4°C. This step results in a denatured protein with all cysteine residues blocked by sulfitolization. The solution was centrifuged at 40,000 rpm in a Beckman Ultracentrifuge for 30 min. The supernatant was diluted with 3-5 volumes of metal chelate column buffer (6 M guanidine, 20 mM Tris, pH 7.4) and filtered through 0.22 micron filters to clarify. Depending the clarified extract was loaded onto a 5 mil Qiagen Ni-NTA metal chelate column equilibrated in the metal chelate column buffer. The column was washed with additional buffer containing 50 mM imidazole (Calbiochem, Utrol grade), pH 7.4. The protein was eluted with buffer containing 250 mM imidazole. Fractions containing the desired protein were pooled and stored at 4.degree. C. Protein concentration was estimated by its absorbance at 280 nm using the calculated extinction coefficient based on its amino acid sequence.

In Mammalian Cells

[0383] This general example illustrates a preparation of a glycosylated form of a desired amino acid sequence of interest or polypeptide component by recombinant expression in mammalian cells.

[0384] The vector pRK5 (see EP 307,247, published Mar. 15, 1989) can be employed as the expression vector. Optionally, the encoding DNA is ligated into pRK5 with selected restriction enzymes to allow insertion of the DNA using ligation methods such as described in Sambrook et al., supra.

[0385] In one embodiment, the selected host cells may be 293 cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in tissue culture plates in medium such as DMEM supplemented with fetal calf serum and optionally, nutrient components and/or antibiotics. About 10 µg of the ligated vector DNA is mixed with about 1 µg DNA encoding the VA RNA gene [Thimmappaya et al., Cell 31:543 (1982)] and dissolved in 500 µl of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M CaCl₂. To this mixture is added, dropwise, 500 µl of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM NaPO₄, and a precipitate is allowed to form for 10 minutes at 25°C. The precipitate is suspended and added to the 293 cells and allowed to settle for about four hours at 37°C. The culture medium is aspirated off and 2 ml of 20% glycerol in PBS is added for 30 seconds. The 293 cells

are then washed with serum free medium, fresh medium is added and the cells are incubated for about 5 days.

[0386] Approximately 24 hours after the transfections, the culture medium is removed and replaced with culture medium (alone) or culture medium containing 200 $\mu\text{Ci/ml}$ ^{35}S -cysteine and 200 $\mu\text{Ci/ml}$ ^{35}S -methionine. After a 12 hour incubation, the conditioned medium is collected, concentrated on a spin filter, and loaded onto a 15% SDS gel. The processed gel may be dried and exposed to film for a selected period of time to reveal the presence of amino acid sequence of interest or polypeptide component. The cultures containing transfected cells may undergo further incubation (in serum free medium) and the medium is tested in selected bioassays.

[0387] In an alternative technique, the nucleic acid amino acid sequence of interest or polypeptide component may be introduced into 293 cells transiently using the dextran sulfate method described by Somparyrac et al., *Proc. Natl. Acad. Sci.*, 12:7575 (1981). 293 cells are grown to maximal density in a spinner flask and 700 μg of the ligated vector is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds, washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture medium, 5 $\mu\text{g/ml}$ bovine insulin and 0.1 $\mu\text{g/ml}$ bovine transferrin. After about four days, the conditioned media is centrifuged and filtered to remove cells and debris. The sample containing expressed amino acid sequence of interest or polypeptide component can then be concentrated and purified by any selected method, such as dialysis and/or column chromatography.

[0388] In another embodiment, the amino acid sequence of interest or polypeptide component can be expressed in CHO cells. The amino acid sequence of interest or polypeptide component can be transfected into CHO cells using known reagents such as CaPO_4 or DEAE-dextran. As described above, the cell cultures can be incubated, and the medium replaced with culture medium (alone) or medium containing a radiolabel such as ^{35}S -methionine. After determining the presence of amino acid sequence of interest or polypeptide component, the culture medium may be replaced with serum free medium. Preferably, the cultures are incubated for about 6 days, and then the conditioned medium is harvested. The medium containing the expressed amino acid sequence of interest or polypeptide component can then be concentrated and purified by any selected method.

[0389] Epitope-tagged amino acid sequence of interest or polypeptide component may also be expressed in host CHO cells. The amino acid sequence of interest or polypeptide component may be subcloned out of a pRK5 vector. The subclone insert can undergo PCR to fuse in frame with a selected epitope tag such as a poly-his tag into a Baculovirus expression vector. The poly-his tagged amino acid sequence of interest or polypeptide component insert can then be subcloned into a SV40 driven vector containing a selection marker such as DHFR for selection of stable clones. Finally, the CHO cells can be transfected (as described above) with the SV40 driven vector. Labeling may be performed, as described above, to verify expression. The culture medium containing the expressed poly-His tagged amino acid sequence of interest or polypeptide component can then be concentrated and purified by any selected method, such as by Ni²⁺-chelate affinity chromatography.

[0390] In an embodiment the amino acid sequence of interest or polypeptide component are expressed as an IgG construct (immunoadhesin), in which the coding sequences for the soluble forms (e.g. extracellular domains) of the respective proteins are fused to an IgG1 constant region sequence containing the hinge, CH2 and CH2 domains and/or is a poly-His tagged form.

[0391] Following PCR amplification, the respective DNAs are subcloned in a CHO expression vector using standard techniques as described in Ausubel et al., Current Protocols of Molecular Biology, Unit 3.16, John Wiley and Sons (1997). CHO expression vectors are constructed to have compatible restriction sites 5' and 3' of the DNA of interest to allow the convenient shuttling of cDNA's. The vector used in expression in CHO cells is as described in Lucas et al., Nucl. Acids Res. 24:9 (1774-1779 (1996), and uses the SV40 early promoter/enhancer to drive expression of the cDNA of interest and dihydrofolate reductase (DHFR). DHFR expression permits selection for stable maintenance of the plasmid following transfection.

In Yeast

[0392] The following method describes recombinant expression of a desired amino acid sequence of interest or polypeptide component in yeast.

[0393] First, yeast expression vectors are constructed for intracellular production or secretion of a stretch of consecutive amino acids from the ADH2/GAPDH promoter. DNA encoding a desired amino acid sequence of interest or polypeptide component, a selected signal peptide and the promoter is inserted into suitable restriction enzyme sites in the selected plasmid to direct

intracellular expression of the amino acid sequence of interest or polypeptide component. For secretion, DNA encoding the stretch of consecutive amino acids can be cloned into the selected plasmid, together with DNA encoding the ADH2/GAPDH promoter, the yeast alpha-factor secretory signal/leader sequence, and linker sequences (if needed) for expression of the stretch of consecutive amino acids.

[0394] Yeast cells, such as yeast strain AB110, can then be transformed with the expression plasmids described above and cultured in selected fermentation media. The transformed yeast supernatants can be analyzed by precipitation with 10% trichloroacetic acid and separation by SDS-PAGE, followed by staining of the gels with Coomassie Blue stain.

[0395] Recombinant amino acid sequence of interest or polypeptide component can subsequently be isolated and purified by removing the yeast cells from the fermentation medium by centrifugation and then concentrating the medium using selected cartridge filters. The concentrate containing the amino acid sequence of interest or polypeptide component may further be purified using selected column chromatography resins.

In Baculovirus-Infected Insect Cells

[0396] The following method describes recombinant expression of stretches of consecutive amino acids in Baculovirus-infected insect cells.

[0397] The desired nucleic acid encoding the stretch of consecutive amino acids is fused upstream of an epitope tag contained with a baculovirus expression vector. Such epitope tags include poly-his tags and immunoglobulin tags (like Fc regions of IgG). A variety of plasmids may be employed, including plasmids derived from commercially available plasmids such as pVL1393 (Novagen). Briefly, the amino acid sequence of interest or polypeptide component or the desired portion of the amino acid sequence of interest or polypeptide component (such as the sequence encoding the extracellular domain of a transmembrane protein) is amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer may incorporate flanking (selected) restriction enzyme sites. The product is then digested with those selected restriction enzymes and subcloned into the expression vector.

[0398] Recombinant baculovirus is generated by co-transfecting the above plasmid and BaculoGoldTM virus DNA (Pharming) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL

1711) using lipofectin (commercially available from GIBCO-BRL). After 4-5 days of incubation at 28°C, the released viruses are harvested and used for further amplifications. Viral infection and protein expression is performed as described by O'Reilley et al., *Baculovirus expression vectors: A laboratory Manual*, Oxford: Oxford University Press (1994).

[0399] Expressed poly-his tagged amino acid sequence of interest or polypeptide component can then be purified, for example, by Ni^{2+} -chelate affinity chromatography as follows. Extracts are prepared from recombinant virus-infected Sf9 cells as described by Rupert et al., *Nature*, 362:175-179 (1993). Briefly, Sf9 cells are washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM MgCl_2 ; 0.1 mM EDTA; 10% Glycerol; 0.1% NP40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The sonicates are cleared by centrifugation, and the supernatant is diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% Glycerol, pH 7.8) and filtered through a 0.45 μm filter. A Ni^{2+} -NTA agarose column (commercially available from Qiagen) is prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated with 25 mL of loading buffer. The filtered cell extract is loaded onto the column at 0.5 mL per minute. The column is washed to baseline A_{280} with loading buffer, at which point fraction collection is started. Next, the column is washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% Glycerol, pH 6.0), which elutes nonspecifically bound protein. After reaching A_{280} baseline again, the column is developed with a 0 to 500 mM Imidazole gradient in the secondary wash buffer. One mL fractions are collected and analyzed by SDS-PAGE and silver staining or western blot with Ni^{2+} -NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing the eluted His₁₀-tagged sequence are pooled and dialyzed against loading buffer.

[0400] Alternatively, purification of the IgG tagged (or Fc tagged) amino acid sequence can be performed using known chromatography techniques, including for instance, Protein A or Protein G column chromatography.

[0401] Fc containing constructs of proteins can be purified from conditioned media as follows. The conditioned media is pumped onto a 5 ml Protein A column (Pharmacia) which is equilibrated in 20 mM Na phosphate buffer, pH 6.8. After loading, the column is washed extensively with equilibration buffer before elution with 100 mM citric acid, pH 3.5. The eluted protein is immediately neutralized by collecting 1 ml fractions into tubes containing 275 mL of 1 M Tris buffer, pH 9. The highly purified protein is subsequently desalted into storage buffer as described

above for the poly-His tagged proteins. The homogeneity of the proteins is verified by SDS polyacrylamide gel (PEG) electrophoresis and N-terminal amino acid sequencing by Edman degradation.

Examples of Pharmaceutical Compositions

[0402] Non-limiting examples of such compositions and dosages are set forth as follows:

Etanercept and related compositions

[0403] Compositions comprising tetrahedral or octahedral antibodies wherein one or more domains of tetrahedral or octahedral antibodies comprise consecutive amino acids having the sequence of etanercept (e.g. Enbrel) or the sequence of one or more domains thereof, may comprise mannitol, sucrose, and tromethamine. In an embodiment, the composition is in the form of a lyophilizate. In an embodiment, the composition is reconstituted with, for example, Sterile Bacteriostatic Water for Injection (BWFI), USP (containing 0.9% benzyl alcohol). In an embodiment the compound is administered to a subject for reducing signs and symptoms, inducing major clinical response, inhibiting the progression of structural damage, and improving physical function in subjects with moderately to severely active rheumatoid arthritis. The compound may be initiated in combination with methotrexate (MTX) or used alone. In an embodiment the compound is administered to a subject for reducing signs and symptoms of moderately to severely active polyarticular-course juvenile rheumatoid arthritis in subjects who have had an inadequate response to one or more DMARDs. In an embodiment the compound is administered to a subject for reducing signs and symptoms, inhibiting the progression of structural damage of active arthritis, and improving physical function in subjects with psoriatic arthritis. In an embodiment the compound is administered to a subject for reducing signs and symptoms in subjects with active ankylosing spondylitis. In an embodiment the compound is administered to a subject for the treatment of chronic moderate to severe plaque psoriasis. In an embodiment wherein the subject has rheumatoid arthritis, psoriatic arthritis, or ankylosing spondylitis the compound is administered at 25-75mg per week given as one or more subcutaneous (SC) injections. In a further embodiment the compound is administered at 50mg per week in a single SC injection. In an embodiment wherein the subject has plaque psoriasis the compound is administered at 25-75mg twice weekly or 4 days apart for 3 months followed by a reduction to a maintenance dose of 25-75mg per week. In a further embodiment the compound is administered at a dose of at 50 mg twice

weekly or 4 days apart for 3 months followed by a reduction to a maintenance dose of 50mg per week. In an embodiment the dose is between 2x and 100x less than the doses set forth herein. In an embodiment wherein the subject has active polyarticular-course JRA the compound may be administered at a dose of 0.2-1.2 mg/kg per week (up to a maximum of 75 mg per week). In a further embodiment the compound is administered at a dose of 0.8 mg/kg per week (up to a maximum of 50 mg per week). In some embodiments the dose is between 2x and 100x less than the doses set forth hereinabove.

Infliximab, adalimumab, and related compositions

[0404] Compositions comprising tetrahedral or octahedral antibodies wherein one or more domains of tetrahedral or octahedral antibodies comprise consecutive amino acids having the sequence of infliximab (e.g. Remicade) or the sequence of one or more domains thereof, may comprise sucrose, polysorbate 80, monobasic sodium phosphate, monohydrate, and dibasic sodium phosphate, dihydrate. Preservatives are not present in one embodiment. In an embodiment, the composition is in the form of a lyophilizate. In an embodiment, the composition is reconstituted with, for example, Water for Injection (BWFJ), USP. In an embodiment the pH of the composition is 7.2 or is about 7.2. In one embodiment the compound is administered to a subject with rheumatoid arthritis in a dose of 2-4 mg/kg given as an intravenous infusion followed with additional similar doses at 2 and 6 weeks after the first infusion then every 8 weeks thereafter. In a further embodiment the compound is administered in a dose of 3 mg/kg given as an intravenous infusion followed with additional similar doses at 2 and 6 weeks after the first infusion then every 8 weeks thereafter. In an embodiment the dose is adjusted up to 10 mg/kg or treating as often as every 4 weeks. In an embodiment the compound is administered in combination with methotrexate. In one embodiment the compound is administered to a subject with Crohn's disease or fistulizing Crohn's disease at dose of 2-7 mg/kg given as an induction regimen at 0, 2 and 6 weeks followed by a maintenance regimen of 4-6 mg/kg every 8 weeks thereafter for the treatment of moderately to severely active Crohn's disease or fistulizing disease. In a further embodiment the compound is administered at a dose of 5 mg/kg given as an induction regimen at 0, 2 and 6 weeks followed by a maintenance regimen of 5 mg/kg every 8 weeks thereafter for the treatment of moderately to severely active Crohn's disease or fistulizing disease. In an embodiment the dose is adjusted up to 10 mg/kg. In one embodiment the compound is administered to a subject with ankylosing spondylitis at a dose of 2-7 mg/kg given as an intravenous infusion followed with additional similar

doses at 2 and 6 weeks after the first infusion, then every 6 weeks thereafter. In a further embodiment the compound is administered at a dose of 5 mg/kg given as an intravenous infusion followed with additional similar doses at 2 and 6 weeks after the first infusion, then every 6 weeks thereafter. In one embodiment the compound is administered to a subject with psoriatic arthritis at a dose of 2-7 mg/kg given as an intravenous infusion followed with additional similar doses at 2 and 6 weeks after the first infusion then every 8 weeks thereafter. In a further embodiment the compound is administered at a dose of 5 mg/kg given as an intravenous infusion followed with additional similar doses at 2 and 6 weeks after the first infusion then every 8 weeks thereafter. In an embodiment the compound is administered with methotrexate. In one embodiment the compound is administered to a subject with ulcerative colitis at a dose of 2-7 mg/kg given as an induction regimen at 0, 2 and 6 weeks followed by a maintenance regimen of 2-7 mg/kg every 8 weeks thereafter for the treatment of moderately to severely active ulcerative colitis. In a further embodiment the compound is administered to a subject with ulcerative colitis at a dose of 5 mg/kg given as an induction regimen at 0, 2 and 6 weeks followed by a maintenance regimen of 5 mg/kg every 8 weeks thereafter. In some embodiments the dose is between 2x and 100x less than the doses set forth hereinabove for treating the individual diseases.

Rituximab, ocrelizumab, and related compositions

[0405] Compositions comprising tetrahedral or octahedral antibodies wherein one or more domains of tetrahedral or octahedral antibodies comprise consecutive amino acids having the sequence of rituximab, ocrelizumab, or the sequence of one or more domains thereof, may comprise sodium chloride, sodium citrate dihydrate, sodium citrate dihydrate, and Sterile Water for Injection. In an embodiment, the composition is provided in a sterile, clear, colourless, preservative-free liquid concentrate for intravenous (IV) administration. In an embodiment, the composition is supplied at a concentration of 10 mg/ml. In an embodiment, the product is formulated for intravenous administration in 9.0 mg/ml sodium chloride, 7.35 mg/ml sodium citrate dihydrate, 0.7 mg/ml polysorbate 80, and Sterile Water for Injection. In an embodiment the pH of the composition is 6.5 or about 6.5. In an embodiment, the composition comprises the tetrahedral or octahedral antibody at a concentration of 30 mg/mL in 20 mM sodium acetate, 106 mM trehalose dihydrate, 0.02% (w/v) polysorbate 20, at pH 5.3.

Blinatumomab, and related compositions

[0406] Compositions comprising tetrahedral or octahedral antibodies wherein one or more domains of tetrahedral or octahedral antibodies comprise consecutive amino acids having the sequence of blinatumomab, or the sequence of one or more domains thereof, may comprise citric acid monohydrate (E330), trehalose dihydrate, lysine hydrochloride, polysorbate 80, sodium hydroxide (for pH-adjustment), and water for injections. In an embodiment, the pH is 7 or about 8. For treatment of relapsed or refractory B-precursor ALL, the dose depends on the patient's bodyweight. The composition is infused continuously during a treatment cycle of 4 weeks. Each cycle of treatment is separated by a 2-week treatment-free interval. Patients who have no signs of cancer after 2 cycles may be treated with up to 3 additional cycles of treatment. For treatment of patients with minimal residual disease, the dose depends on the patient's bodyweight. The composition is infused continuously during a treatment cycle of 4 weeks. Patients may be treated for up to 3 additional treatment cycles, each one given after a 2-week treatment-free interval.

ACE2 and related compositions

[0407] Compositions comprising tetrahedral or octahedral antibodies wherein one or more domains of tetrahedral or octahedral antibodies comprise consecutive amino acids having the sequence of ACE2 or portions thereof, may comprise known excipients such as those discussed in the sections above. Such compositions may be used, for example, for treatment of SARS-CoV-2.

[0408] In each of the embodiments of the compositions described herein, the compositions, when in the form of a lyophilizate, may be reconstituted with, for example, sterile aqueous solutions, sterile water, Sterile Water for Injections (USP), Sterile Bacteriostatic Water for Injections (USP), and equivalents thereof known to those skilled in the art.

[0409] It is understood that in administration of any of the instant compounds, the compound may be administered in isolation, in a carrier, as part of a pharmaceutical composition, or in any appropriate vehicle.

Dosage

[0410] It is understood that where a dosage range is stated herein, e.g. 1-10mg/kg per week, the invention disclosed herein also contemplates each integer dose, and tenth thereof, between the

upper and lower limits. In the case of the example given, therefore, the invention contemplates 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4 etc. mg/kg up to 10mg/kg.

[0411] In embodiments, the compounds of the present invention can be administered as a single dose or may be administered as multiple doses.

[0412] In general, the daily dosage for treating a disorder or condition according to the methods described above will generally range from about 0.01 to about 10.0 mg/kg body weight of the subject to be treated.

[0413] Variations based on the aforementioned dosage ranges may be made by a physician of ordinary skill taking into account known considerations such as the weight, age, and condition of the person being treated, the severity of the affliction, and the particular route of administration chosen.

[0414] It is also expected that the compounds disclosed will effect cooperative binding with attendant consequences on effective dosages required.

Pharmaceuticals

[0415] The term “pharmaceutically acceptable carrier” is understood to include excipients, carriers or diluents. The particular carrier, diluent or excipient used will depend upon the means and purpose for which the active ingredient is being applied.

[0416] For parenteral administration, solutions containing a compound of this invention or a pharmaceutically acceptable salt thereof in sterile aqueous solution may be employed. Such aqueous solutions should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. The sterile aqueous media employed are all readily available by standard techniques known to those skilled in the art.

[0417] The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions. The preferred form depends on the intended mode of administration and therapeutic application. Some compositions are in the form of injectable or infusible solutions. A mode of administration is parenteral (e.g., intravenous, subcutaneous,

intraperitoneal, intramuscular). In an embodiment, the compound is administered by intravenous infusion or injection. In another embodiment, the compound is administered by intramuscular or subcutaneous injection. In another embodiment, the compound is administered intranasally.

[0418] For therapeutic use, the compositions disclosed here can be administered in various manners, including soluble form by bolus injection, continuous infusion, sustained release from implants, oral ingestion, local injection (e.g. intracardiac, intramuscular), systemic injection, or other suitable techniques well known in the pharmaceutical arts. Other methods of pharmaceutical administration include, but are not limited to oral, subcutaneously, transdermal, intravenous, intramuscular and parenteral methods of administration. Typically, a soluble composition will comprise a purified compound in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. The preparation of such compositions can entail combining a compound with buffers, antioxidants, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. The product can be formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents.

[0419] Other derivatives comprise the compounds/compositions of this invention covalently bonded to a nonproteinaceous polymer. The bonding to the polymer is generally conducted so as not to interfere with the preferred biological activity of the compound, e.g. the binding activity of the compound to a target. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or in vitro methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol, polypropylene glycol, polyoxyethylene esters or methoxy polyethylene glycol; polyoxyalkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronic); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and

heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextran sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; as well as heparin or heparon.

[0420] The pharmaceutical compositions of the invention may include a “therapeutically effective amount” or a “prophylactically effective amount” of a compound of the invention. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the compound may vary according to factors such as the disease state, age, sex, and weight of the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the compound are outweighed by the therapeutically beneficial effects. A “prophylactically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

General

[0421] All combinations of the various elements disclosed herein are within the scope of the invention.

[0422] As used herein, all headings are simply for organization and are not intended to limit the disclosure in any manner. The content of any individual section may be equally applicable to all sections. All combinations of the various elements disclosed herein are within the scope of the invention.

[0423] Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

[0424] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single

embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

[0425] This invention will be better understood by reference to the Examples which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

EXAMPLES

Example 1 - Methods and Materials

Recombinant Proteins

[0426] Recombinant proteins were expressed in Chinese hamster ovary (CHO) cells using a mammalian expression vector and purified by Protein A affinity chromatography and size-exclusion chromatography (SEC). Production was carried out by transient expression in CHO-K1 cells adapted to serum-free suspension culture (TunaCHO, LakePharma Inc., Belmont, CA). Suspension CHO cells were seeded in a shake flask and expanded using a serum-free and chemically defined medium. On the day of transfection, the expanded cells were seeded into a new vessel with fresh medium. Transient transfections were done with the addition of transfection reagents complexed with DNA under high density conditions. Transfections were carried out in cultures of 0.1 to 2.0 liters. After transfection, the cells were maintained as a batch-fed culture in a shake flask until the end of the production run. The conditioned cell culture fluid was harvested after 7 to 14 days, clarified by centrifugation, and sterile-filtered prior to purification.

[0427] Protein A affinity chromatography was carried out by applying the culture supernatant to a column packed with CaptivA® Protein A Affinity Resin (Repligen, Massachusetts, USA) pre-equilibrated with 137 mM NaCl 2.7 mM KCl 10 mM Na₂HPO₄ 2 mM KH₂PO₄ pH 7.4 (PBS). The column was washed with PBS buffer until the OD₂₈₀ value returned to baseline. The target protein was then eluted with 0.25% acetic acid buffer at pH 3.5. Fractions were collected, buffered with 1 M HEPES, and the OD₂₈₀ value of each fraction was recorded. Fractions containing the target protein were pooled, buffer exchanged into 100 mM HEPES, 100 mM NaCl, 50 mM

NaOAc, pH 6.0, and filtered through a 0.2 μ m membrane filter and stored at 4°C prior to use. The protein concentration was calculated from the OD280 value and the calculated extinction coefficient.

[0428] Preparative SEC was carried out using an AKTA Avant 25 FPLC system (GE Healthcare, Uppsala, Sweden). Proteins were first concentrated to 10-15mg/ml using an Amicon Ultra-15, 3MWCO, ultracentrifugal filter unit, catalog #UFC900324 (Millipore, Burlington, MA), then loaded onto a HiLoad 26/600 Superdex 200 prep grade column (GE Healthcare). Elution was carried out with Dulbecco's phosphate-buffered saline without calcium or magnesium salts (PBS) (UCSF Cell Culture Facility, San Francisco, CA) containing 10mM EDTA. Fractions corresponding to the protein of interest were identified based upon analytical SEC and analysis by reducing and non-reducing SDS-PAGE, and pooled, concentrated by ultrafiltration, and stored at 4°C.

Heterobifunctional Crosslinkers

Table 1: Heterobifunctional Crosslinkers

Name	Formula	Mol Wt	Sequence	Manufacture	Item #
Az-thioester	C ₃₂ H ₄₅ O ₁₀ N ₁₁ S	775.83	Azidoacetyl-DKTHT-thiophenol	CPC Scientific Inc	860245
Az-P12-thioester	C ₅₉ H ₉₈ O ₂₃ N ₁₂ S	1375.54	Azide-PEG ₁₂ -DHTHT-thiophenol	CPC Scientific Inc	852119
Az-P24-thioester	C ₈₃ H ₁₄₆ O ₃₅ N ₁₂ S	1904.17	Azide-PEG ₂₄ -DHTHT-thiophenol	CPC Scientific Inc	834144
Az-P36-thioester	C ₁₀₇ H ₁₉₄ O ₄₇ N ₁₂ S	2432.79	Azide-PEG ₃₆ -DHTHT-thiophenol	CPC Scientific Inc	860249
Az-P48-thioester	C ₁₃₄ H ₂₄₇ O ₆₀ N ₁₃ S	3032.50	Azidoacetyl-PEG ₃₆ PEG ₁₂ -DKTHT-thiophenol	CPC Scientific Inc	869441
Tet-DBCO	C ₃₄ H ₃₃ N ₇ O ₇ S	683.70	Methyltetrazine-Sulfo-DBCO	Click Chemistry Tools	1022
TCO-P4-DBCO	C ₄₁ H ₅₄ N ₄ O ₁₂ S	826.95	Trans-Cyclooctene-Sulfo-PEG ₄ -DBCO	Click Chemistry Tools	1005
Tet-P4-Mal	C ₂₄ H ₃₀ N ₆ O ₇	514.53	Methyltetrazine-PEG ₄ -Maleimide	Click Chemistry Tools	1068
TCO-P3-Mal	C ₂₆ H ₄₁ N ₃ O ₈	523.62	Trans-Cyclooctene-PEG ₃ -Maleimide	Click Chemistry Tools	1002
TCO-P12-Mal	C ₄₆ H ₈₀ N ₄ O ₁₈	977.14	Trans-Cyclooctene-PEG ₁₂ -Maleimide	AnaSpec Inc	659252
TCO-P24-Mal	C ₇₀ H ₁₂₈ N ₄ O ₃₀	1505.77	Trans-Cyclooctene-PEG ₂₄ -Maleimide	AnaSpec Inc	659254
TCO-P36-Mal	C ₉₄ H ₁₇₆ N ₄ O ₄₂	2034.40	Trans-Cyclooctene-PEG ₃₆ -Maleimide	AnaSpec Inc	659256

Electrospray Ionization Mass Spectrometry (ESI-MS)

[0429] Intact proteins were analyzed by ESI-MS using a Model 1260 HPLC system (Agilent Technologies, Santa Clara, CA) and MicroTOF-QII MS system (Bruker Corporation, Billerica, MA). A BioResolve RP mAb Polyphenyl column with a 2.7 micron particle size, 450 angstrom pore size, and dimensions of 2.1 x 100 mm (Waters Corporation, Milford, MA) was used at 50°C with a flow rate of 0.3ml/min, and injection volume of 2-5 uL. The mobile phase was a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) as follows: 0 min, 95%:5%; 2 min, 65%:35%; 10 min, 54%:46%; 11 min, 5%:95% held for 3 minutes. Data was collected in full scan MS mode with a mass range of 600-4000 Da. The collision RF setting was 800 Vpp. For deglycosylation analysis, samples were reduced and deglycosylated with Rapid PNGase F, P0710S (New England BioLabs Inc, Ipswich, MA) according to the manufacturer's instructions. Bruker DataAnalysis Version 4.0 SP4 software was used for mass spectral display and deconvolution.

Analytical size-exclusion HPLC (SE-HPLC)

[0430] Analytical SE-HPLC was carried out using a Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan). Zenix-C columns with a 3 micron particle size, 300 angstrom pore size, and dimensions of 4.6 x 300mm (Sepax Technologies, Newark, Delaware) were used individually or as a pair of columns connected in series. Mobile phase, flow rate, column temperature, and detection wavelength used were 50mM sodium phosphate pH 7.4 and 300mM NaCl, 0.2 mL/min, 25°C, and 280/214 nm, respectively. LabSolutions v5.9 software (Shimadzu Corporation) was used for UV data acquisition and processing.

Multi-angle light scattering analysis (MALS)

[0431] The molar mass of proteins was determined by combining SE-HPLC with measurement of refractive index (RI) and multi-angle light scattering (SEC-MALS) using an Optilab T-rEX RI detector and Treos MALS detector in-line (Wyatt Technology, Goleta, CA). The temperature of detectors was maintained at 25°C. The signal obtained for the monomeric form of bovine serum albumin (BSA) was used to normalize the detectors and to correct for band broadening between detectors. Astra v7.3 software (Wyatt Technology) was used for light scattering and refractive index data acquisition and processing. A value of 0.185 mL/g was used for the dn/dc ratio of proteins.

Stoichiometric binding measurements by SE-HPLC

[0432] The relative binding affinities of two distinct proteins to a common ligand-binding partner was determined by stoichiometric binding analysis using SE-HPLC. Partially purified mixtures of the two proteins, or mixtures prepared by combining two purified proteins, were assessed for the relative binding affinity of their constituents in a binding reaction containing a molar ratio of 0.2 to 2.0 of the ligand-binding partner. Following incubation for two hours at 25°C, binding reaction were analyzed by SE-HPLC to determine the remaining unbound fraction of each of the two proteins.

Kinetic Exclusion Assay (KinExA®)

[0433] Equilibrium binding affinity and kinetic binding measurements were made between unmodified molecules in solution using a KinExA 3200 instrument (Sapidyne Instruments, Boise, Idaho). For K_d analysis of ACE2 binding to SARS-CoV-2 S protein, PMMA beads were adsorption-coated with recombinant SARS-CoV-2 spike protein, Reference No. 46328 (LakePharma Inc, Belmont, CA); then used as the solid-phase to capture the ACE2 protein (the constant binding partner (CBP)). For each experiment, S protein was titrated in a background of the ACE2 protein and allowed to reach equilibrium. The binding reactions were then briefly exposed to the solid phase and a portion of free ACE2 protein was captured and then detected with a fluorescent secondary molecule. The short contact time with the solid phase is less than the time needed for dissociation of the pre-formed complex in solution, thus competition between the solution and the solid phase titrated binding partner is “kinetically excluded.” Since the solid phase is only used as a probe for the free CBP in each sample, the solution equilibrium is not altered during such KinExA measurements.

Octet

Kinetic assay on Fc gamma receptors

[0434] The kinetic characterization of antibody binding on Fc gamma receptors were performed on a Octet Red384 (Sartorius) using 384-well plates.

[0435] Various recombinant human Fc gamma receptors containing polyhistidine tag at C-terminus were purchased from R&D systems. The Fc gamma receptors with concentration of 5 µg/ml were immobilized on Anti-Penta-His biosensors (Sartorius) in PBS-B (PBS with 1 mg/mL

BSA, pH 7.4) at 26 °C with the orbital shake speed of 1000 rpm. After washing with PBS-B, the Fc gamma receptors captured biosensors were submerged in different concentrations of testing antibodies for 15 seconds. The biosensors were then submerged in PBS-B for 60 seconds during dissociation. Biosensors were regenerated by stripping with glycine pH1.5 at the end of each cycle. Data analysis was conducted using a standard 1:1 binding model.

Example 2 - Tetrahedral Antibodies with the structures shown in Figure 1 (Panel A), Figure 2, Figure 11 (Panels A-H), Figure 12

[0436] Panel A of Figure 1, Figure 2, and panels A-H of Figure 11 show the schematic structure of tetrahedral antibodies of this example.

[0437] Figure 12 describes the preparation of tetrahedral antibody Rc6-P4-Rc6.

Rc6-Rc6

[0438] A tetrahedral antibody with the structure shown in Figure 1, Panel A was constructed with the following domains:

- a. Domain 1: Fc
- b. Domain 2: Fc
- c. Domain 3: anti-CD20Fab
- d. Domain 4: anti-CD20Fab

Rc6-B19

[0439] A tetrahedral antibody with the structure shown in Figure 1, Panel A was constructed with the following domains:

- a. Domain 1: Fc
- b. Domain 2: Fc
- c. Domain 3: anti-CD20Fab
- d. Domain 4: anti-CD19Fab

Rc66SIDE-Rc66SIDE

[0440] A tetrahedral antibody with the structure shown in Figure 1A was constructed with the following domains:

- a. Domain 1: FcSIDE
- b. Domain 2: FcSIDE

- c. Domain 3: anti-CD20Fab
- d. Domain 4: anti-CD20Fab

HA9AAC9-HA9AAC9

[0441] A tetrahedral antibody with the structure shown in Figure 1, Panel A was constructed with the following domains:

- a. Domain 1: FcAAC9
- b. Domain 2: FcAAC9
- c. Domain 3: anti-CD16Fab
- d. Domain 4: anti-CD26Fab

6Ec66-Soc66

[0442] A tetrahedral antibody with the structure shown in Figure 1, Panel A was constructed with the following domains:

- a. Domain 1: Fc
- b. Domain 2: Fc
- c. Domain 3: anti-betaAmyloidFab
- d. Domain 4: anti-betaAmyloid Fab

Ace2-Ace2

[0443] A tetrahedral antibody with the structure shown in Figure 1, Panel A was constructed with the following domains:

- a. Domain 1: Fc
- b. Domain 2: Fc
- c. Domain 3: Ace2-615
- d. Domain 4: Ace2-615

Table 2: SEQ IDs of Proteins Used for the Preparation of Tetrahedral Antibodies

Protein	Light chain 1	Light Chain 2	Heavy chain	Fc fusion chain	Fc chain
Rc6	SEQ ID NO 1		SEQ ID NO 2		SEQ ID NO 3
Rc6HYRF	SEQ ID NO 1		SEQ ID NO 4		SEQ ID NO 3
Rc60	SEQ ID NO 1		SEQ ID NO 5		SEQ ID NO 6
Rc66	SEQ ID NO 1		SEQ ID NO 7		SEQ ID NO 8
Rc66SIDE	SEQ ID NO 1		SEQ ID NO 9		SEQ ID NO 10
Rc66AAC9	SEQ ID NO 1		SEQ ID NO 11		SEQ ID NO 12
HA9c66AAC9	SEQ ID NO 16		SEQ ID NO 17		SEQ ID NO 12
HRc66	SEQ ID NO 18		SEQ ID NO 19		SEQ ID NO 8
B19c66	SEQ ID NO 20		SEQ ID NO 21		SEQ ID NO 8
B19c66AAC9	SEQ ID NO 20		SEQ ID NO 22		SEQ ID NO 12
Blc6AAC9				SEQ ID NO 23	SEQ ID NO 12
Drc66	SEQ ID NO 24		SEQ ID NO 25		SEQ ID NO 8
6Ec66	SEQ ID NO 32		SEQ ID NO 33		SEQ ID NO 8
Soc66	SEQ ID NO 35		SEQ ID NO 36		SEQ ID NO 8
IL15c6AAC9				SEQ ID NO 26	SEQ ID NO 12
IL15c6AAC9N79Q				SEQ ID NO 31	SEQ ID NO 12
IL15Rc6AAC9				SEQ ID NO 27	SEQ ID NO 12
ObSpc60PG	SEQ ID NO 43	SEQ ID NO 44	SEQ ID NO 45		SEQ ID NO 42
Obc60PG	SEQ ID NO 46		SEQ ID NO 47		SEQ ID NO 42
ObSP41BBLc60PG	SEQ ID NO 43	SEQ ID NO 44	SEQ ID NO 48		SEQ ID NO 42
Ob41BBLc60PG	SEQ ID NO 46		SEQ ID NO 49		SEQ ID NO 42
ObSpc60PG41BBL-RF	SEQ ID NO 43	SEQ ID NO 44	SEQ ID NO 45		SEQ ID NO 50
Obc60PG41BBL-RF	SEQ ID NO 46		SEQ ID NO 47		SEQ ID NO 50
Glc60PG	SEQ ID NO 38	SEQ ID NO 39	SEQ ID NO 40		SEQ ID NO 42
Cic60PG	SEQ ID NO 51	SEQ ID NO 52	SEQ ID NO 53		SEQ ID NO 42
ACE2RQ615c60PG				SEQ ID NO 62	SEQ ID NO 42
ACE2RQ615c61PG				SEQ ID NO 63	SEQ ID NO 64
ACE2RQ106c60PG				SEQ ID NO 82	SEQ ID NO 42
ACE2RQ106x6c60PG				SEQ ID NO 84	SEQ ID NO 42
CoV2spike680c60PG				SEQ ID NO 86	SEQ ID NO 42
SARSspike666c60PG				SEQ ID NO 88	SEQ ID NO 42
RaTG13spike680c60PG				SEQ ID NO 89	SEQ ID NO 42
CD3Ec60PG				SEQ ID NO 55	SEQ ID NO 42
41BBc60PG				SEQ ID NO 56	SEQ ID NO 42
CD19c60PG				SEQ ID NO 58	SEQ ID NO 42
CEACAM5c60PG				SEQ ID NO 60	SEQ ID NO 42

Table 3: Properties of Proteins Used for the Preparation of Tetrahedral Antibodies

Protein	D1 domain type	D1 binding specificity	D3 domain type	D3 binding specificity
Rc6	Fc homodimer	FcRn, FcR γ	Fab	CD20
Rc6HYRF	Fc heterodimer (HYRF)	FcRn, FcR γ (SpA-low)	Fab	CD20
Rc60	Fc heterodimer (KiH)	FcRn, FcR γ	Fab	CD20
Rc66	Fc heterodimer (ZW1)	FcRn, FcR γ	Fab	CD20
Rc66SIDE	Fc heterodimer (ZW1)	FcRn, FcR γ -high	Fab	CD20
Rc66AAC9	Fc heterodimer (ZW1)	FcRn, FcR γ -low	Fab	CD20
HA9c66AAC9	Fc heterodimer (ZW1)	FcRn, FcR γ -low	Fab	CD16
HRc66	Fc heterodimer (ZW1)	FcRn, FcR γ	Fab	CD20
B19c66	Fc heterodimer (ZW1)	FcRn, FcR γ	Fab	CD19
B19c66AAC9	Fc heterodimer (ZW1)	FcRn, FcR γ -low	Fab	CD19
Blc6AAC9	Fc heterodimer (ZW1)	FcRn, FcR γ -low	ScFv-ScFv	CD19, CD3e
Drc66	Fc heterodimer (ZW1)	FcRn, FcR γ	Fab	DR5
6Ec66	Fc heterodimer (ZW1)	FcRn, FcR γ	Fab	amyloid beta (3-8)
Soc66	Fc heterodimer (ZW1)	FcRn, FcR γ	Fab	amyloid beta (17-24)
IL15c6AAC9	Fc heterodimer (ZW1)	FcRn, FcR γ -low	IL-15	IL-15R alpha
IL15c6AAC9N79Q	Fc heterodimer (ZW1)	FcRn, FcR γ -low	IL-15	IL-15R alpha
IL15Rc6AAC9	Fc heterodimer (ZW1)	FcRn, FcR γ -low	IL-15R α	IL-15
ObSpc60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	Fab-Fab	CD20, CD3e
Obc60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	Fab	CD20
ObSP41BBLc60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low, 4-1BB	Fab-Fab	CD20, CD3e
Ob41BBLc60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low, 4-1BB	Fab	CD20
ObSpc60PG41BBL-RF	Fc heterodimer (KiH)	FcRn, FcR γ -low, 4-1BB	Fab-Fab	CD20, CD3e
Obc60PG41BBL-RF	Fc heterodimer (KiH)	FcRn, FcR γ -low, 4-1BB	Fab	CD20
Glc60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	Fab-CrossFab(VH-VL)	CD20, CD3e
Cic60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	Fab-CrossFab(CH1-CL)	CEACAM5, CD3e

Protein	D1 domain type	D1 binding specificity	D3 domain type	D3 binding specificity
ACE2RQ615c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	ACE2	SARS-CoV-2 Spike protein
ACE2RQ615c61PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	ACE2	SARS-CoV-2 Spike protein
ACE2RQ106c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	ACE2	SARS-CoV-2 Spike protein
ACE2RQ106x6c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	ACE2	SARS-CoV-2 Spike protein
CoV2spike680c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	SARS-CoV-2 Spike	ACE2
SARSSpike666c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	SARS-CoV-1 Spike	ACE2
RaTG13spike680c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	RaTG13 Spike	ACE2
CD3Ec60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	CD3e	anti-CD3e
41BBc60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	4-1BB	anti-4-1BB, 4-1BBL
CD19c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	CD19	anti-CD19
CEACAM5c60PG	Fc heterodimer (KiH)	FcRn, FcR γ -low	CEACAM5	anti-CEACAM5

Table 4: Preparation of Tetrahedral Antibodies

Name	Protein 1	Protein 2	Crosslinker 1a	Crosslinker 1b	Crosslinker 2a	Crosslinker 2b
Rc6-P4-Rc6	Rc6	Rc6	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Rc66SIDE-P4-Rc66SIDE	Rc66SIDE	Rc66SIDE	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Rc66SIDE-P16-Rc66SIDE	Rc66SIDE	Rc66SIDE	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-P12-Thioester
Rc66SIDE-P28-Rc66SIDE	Rc66SIDE	Rc66SIDE	Az-P12-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-P12-Thioester
B19c66-P4-Rc6	B19c66	Rc6	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Soc66-P28-6Ec66	Soc66	6Ec66	Az-P12-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-P12-Thioester
HA9c66AAC9-P4-HA9c66AAC9	HA9c66AAC9	HA9c66AAC9	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
HA9c66AAC9-P4-IL15c6AAC9	HA9c66AAC9	IL15c6AAC9	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Obc60PG-P4-Obc60PG	Obc60PG	Obc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Obc60PG-P4-Ob41BBc60PG	Obc60PG	Ob41BBc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Obc60PG-P4-Obc60PG41BB	Obc60PG	Obc60PG41BB	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Ob41BBc60PG-P4-Ob41BBc60PG	Ob41BBc60PG	Ob41BBc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester

Name	Protein 1	Protein 2	Crosslinker 1a	Crosslinker 1b	Crosslinker 2a	Crosslinker 2b
Ob41BBc60PG-P4-Obc60PG41BB	Ob41BBc60PG	Obc60PG41BB	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Obc60PG41BB-P4-Obc60PG41BB	Obc60PG41BB	Obc60PG41BB	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ObSpc60PG-P4-Obc60PG	ObSpc60PG	Obc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ObSpc60PG-P4-Ob41BBc60PG	ObSpc60PG	Ob41BBc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ObSpc60PG-P4-Obc60PG41BB	ObSpc60PG	Obc60PG41BB	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ObSP41BBLc60PG-P4-Ob41BBLc60PG	ObSP41BBLc60PG	Ob41BBLc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ObSP41BBLc60PG-P4-Obc60PG41BBL	ObSP41BBLc60PG	Obc60PG41BBL	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ObSpc60PG41BBL-P4-Obc60PG41BBL	ObSpc60PG41BBL	Obc60PG41BBL	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Glc60PG-P4-Obc60PG	Glc60PG	Obc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Glc60PG-P4-Ob41BBc60PG	Glc60PG	Ob41BBc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
Glc60PG-P4-Obc60PG41BB	Glc60PG	Obc60PG41BB	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-IL15Rc6AAC9	IL15c6AAC9	IL15Rc6AAC9	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-Rc66AAC9	IL15c6AAC9	Rc66AAC9	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-B19c66AAC9	IL15c6AAC9	B19c66AAC9	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-Glc60PG	IL15c6AAC9	Glc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-Cic60PG	IL15c6AAC9	Cic60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-Obc60PG	IL15c6AAC9	Obc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-Ob41BBc60PG	IL15c6AAC9	Ob41BBc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-Obc60PG41BB	IL15c6AAC9	Obc60PG41BB	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-ObSpc60PG	IL15c6AAC9	ObSpc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-ObSP41BBLc60PG	IL15c6AAC9	ObSP41BBLc60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
IL15c6AAC9-P4-ObSpc60PG41BBL	IL15c6AAC9	ObSpc60PG41BBL	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ACE2RQ615c60PG-P4-	ACE2RQ615c60PG	ACE2RQ615c60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester

Name	Protein 1	Protein 2	Crosslinker 1a	Crosslinker 1b	Crosslinker 2a	Crosslinker 2b
ACE2RQ615c60PG						
ACE2RQ615c61PG-P7-ACE2RQ615c61PG	ACE2RQ615c61PG	ACE2RQ615c61PG	Tet-P4-Mal	NA	Tco-P3-Mal	NA
ACE2RQ106c60PG-P4-ACE2RQ106c60PG	ACE2RQ106c60PG	ACE2RQ106c60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
ACE2RQ106x6c60PG-P4-ACE2RQ106c60PG	ACE2RQ106x6c60PG	ACE2RQ106x6c60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
CoV2spike680c60PG-P4-CoV2spike680c60PG	CoV2spike680c60PG	CoV2spike680c60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
SARSspike666c60PG-P4-SARSspike666c60PG	SARSspike666c60PG	SARSspike666c60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester
RaTG13spike680c60PG-P4-RaTG13spike680c60PG	RaTG13spike680c60PG	RaTG13spike680c60PG	Az-Thioester	Tet-DBCO	TCO-P4-DBCO	Az-Thioester

NA, not applicable (only two crosslinkers are required)

Table 5: Yields of Tetrahedral Antibodies

Tetrahedral Antibody	Reactant 1	Reactant 2	Reactant 1 (mg)	Reactant 2 (mg)	Product (AUC)	Unreacted (AUC)	Yield %
Rc6-P4-Rc6	Rc6-Tet	Rc6-P4-TCO	3.03	3.10	885.87	626.26	58.6
Rc66SIDE-P4-Rc66SIDE	Rc66SIDE-Tet	Rc66SIDE-P4-TCO	2.15	2.50	785.13	674.60	59.9
Rc66SIDE-P16-Rc66SIDE	Rc66SIDE-Tet	Rc66SIDE-P16-TCO	2.15	2.50	675.70	513.68	56.8
Rc66SIDE-P28-Rc66SIDE	Rc66SIDE-P12-Tet	Rc66SIDE-P16-TCO	2.61	2.90	760.10	679.84	52.8
B19c66-P4-Rc6	B19c66-Tet	Rc6-P4-TCO	4.02	2.87	913.82	1164.97	44.0
Soc66-P28-6Ec66	Soc66-P12-Tet	6Ec66-P16-TCO	4.96	3.55	756.17	2083.34	26.6
HA9c66AAC9-P4-HA9c66AAC9	HA9c66AAC9-Tet	HA9c66AAC9-P4-TCO	11.52	8.37	1134.69	2510.24	31.1
HA9c6AAC9-P4-IL15c6AAC9	HA9c66AAC9-Tet	IL15c6AAC9-P4-TCO	1.55	1.33	288.00	471.32	37.9

Yield (%) = (Product)/(Product+Unreacted)

Table 6: Intact Mass Measurements of Tetrahedral Antibodies by ESI-MS

Control Antibody	Tetrahedral Antibody	Intact Mass (non-reducing)	L1 chain (reducing)	H1 chain (reducing)	Fc chain (reducing)	Fc-Px-Fc (reducing)	Fc fusion chain (reducing)
Rituxan*		147,077.1 (147,077.0)	23,039.3 (23,057.7)	50,514.2 (50,530.8)			
Rc6*		99,815.6 (99,814.4)	23,039.4 (23,057.7)	50,513.9 (50,530.8)	26,281.9 (26,281.9)		
	Rc6-P4-Rc6*	202,450.4 (202,445.2)	23,035.9 (23,057.7)	50,510.4 (50,530.8)		55,374.3 (55,380.2)	

* The heavy and light chains undergo an N-terminal glutamine to pyroglutamate conversion (17Da decrease)

Table 7: Predicted Binding Domains of Tetrahedral Antibodies

Tetrahedral Antibody	D1 binding specificity	D2 binding specificity	D3 binding specificity	D4 binding specificity
Rc6-P4-Rc6	FcRn, FcRγ	FcRn, FcRγ	CD20	CD20
Rc66SIDE-P4-Rc66SIDE	FcRn, FcRγ-high	FcRn, FcRγ-high	CD20	CD20
Rc66SIDE-P16-Rc66SIDE	FcRn, FcRγ-high	FcRn, FcRγ-high	CD20	CD20
Rc66SIDE-P28-Rc66SIDE	FcRn, FcRγ-high	FcRn, FcRγ-high	CD20	CD20
B19c66-P4-Rc6	FcRn, FcRγ	FcRn, FcRγ	CD19	CD20
Soc66-P28-S6Ec66	FcRn, FcRγ	FcRn, FcRγ	amyloid beta (17-24)	amyloid beta (3-8)
HA9c66AAC9-P4-HA9c66AAC9	FcRn, FcRγ-low	FcRn, FcRγ-low	CD16	CD16
HA9c66AAC9-P4-IL15c6AAC9	FcRn, FcRγ-low	FcRn, FcRγ-low	CD16	IL-15R alpha
Obc60PG-P4-Obc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low	CD20	CD20
Obc60PG-P4-Ob41BBc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	CD20	CD20
Obc60PG-P4-Obc60PG41BB	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	CD20	CD20
Ob41BBc60PG-P4-Ob41BBc60PG	FcRn, FcRγ-low, 4-1BB	FcRn, FcRγ-low, 4-1BB	CD20	CD20
Ob41BBc60PG-P4-Obc60PG41BB	FcRn, FcRγ-low, 4-1BB	FcRn, FcRγ-low, 4-1BB	CD20	CD20
Obc60PG41BB-P4-Obc60PG41BB	FcRn, FcRγ-low, 4-1BB	FcRn, FcRγ-low, 4-1BB	CD20	CD20
ObSpc60PG-P4-Obc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low	CD20, CD3	CD20
ObSpc60PG-P4-Ob41BBc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	CD20, CD3	CD20
ObSpc60PG-P4-Obc60PG41BB	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	CD20, CD3	CD20
ObSP41BBLc60PG-P4-Ob41BBLc60PG	FcRn, FcRγ-low, 4-1BB	FcRn, FcRγ-low, 4-1BB	CD20, CD3	CD20
ObSP41BBLc60PG-P4-Obc60PG41BBL	FcRn, FcRγ-low, 4-1BB	FcRn, FcRγ-low, 4-1BB	CD20, CD3	CD20
ObSpc60PG41BBL-P4-Obc60PG41BBL	FcRn, FcRγ-low, 4-1BB	FcRn, FcRγ-low, 4-1BB	CD20, CD3	CD20
Glc60PG-P4-Obc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low	CD20, CD3	CD20
Glc60PG-P4-Ob41BBc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	CD20, CD3	CD20
Glc60PG-P4-Obc60PG41BB	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	CD20, CD3	CD20
IL15c6AAC9-P4-IL15Rc6AAC9	FcRn, FcRγ-low	FcRn, FcRγ-low	IL-15R alpha	IL-15
IL15c6AAC9-P4-Rc66AAC9	FcRn, FcRγ-low	FcRn, FcRγ-low	IL-15R alpha	CD20
IL15c6AAC9-P4-B19c66AAC9	FcRn, FcRγ-low	FcRn, FcRγ-low	IL-15R alpha	CD19
IL15c6AAC9-P4-Obc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low	IL-15R alpha	CD20
IL15c6AAC9-P4-Ob41BBc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	IL-15R alpha	CD20
IL15c6AAC9-P4-Obc60PG41BB	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	IL-15R alpha	CD20
IL15c6AAC9-P4-ObSpc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low	IL-15R alpha	CD20, CD3
IL15c6AAC9-P4-ObSP41BBLc60PG	FcRn, FcRγ-low	FcRn, FcRγ-low, 4-1BB	IL-15R alpha	CD20, CD3

Tetrahedral Antibody	D1 binding specificity	D2 binding specificity	D3 binding specificity	D4 binding specificity
IL15c6AAC9-P4-ObSpc60PG41BBL	FcRn, FcR γ -low	FcRn, FcR γ -low, 4-1BB	IL-15R alpha	CD20, CD3
IL15c6AAC9-P4-Glc60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	IL-15R alpha	CD20, CD3
IL15c6AAC9-P4-Cic60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	IL-15R alpha	CEACAM5, CD3
ACE2RQ615c60PG-P4-ACE2RQ615c60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 S protein	SARS-CoV-2 S protein
ACE2RQ615c61PG-P7-ACE2RQ615c61PG	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 S protein	SARS-CoV-2 S protein
ACE2RQ106c60PG-P4-ACE2RQ106c60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 S protein	SARS-CoV-2 S protein
ACE2RQ106x6c60PG-P4-ACE2RQ106c60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 S protein	SARS-CoV-2 S protein
CoV2spike680c60PG-P4-CoV2spike680c60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	ACE2	ACE2
SARSSpike666c60PG-P4-SARSSpike666c60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	ACE2	ACE2
RaTG13spike680c60PG-P4-RaTG13spike680c60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	ACE2	ACE2

Table 8: Observed IgG-FcR γ Binding of Tetrahedral Antibodies

Tetrahedral Antibody	Control Antibody	CD16a 158F FcRγIIIa	CD16a 158V FcRγIIIa	CD16b FcRγIIIb	CD32a 131H FcRγIIa	CD32a 131R FcRγIIa	CD32b/c FcRγIIb	CD64 FcRγI
	Rituxan (antibody)	1.10E-06 (1.0)	7.15E-07 (1.0)	1.53E-06 (1.0)	8.92E-07 (1.0)	1.25E-06 (1.0)	1.52E-06 (1.0)	5.28E-08 (1.0)
	Rituximab-SIDE (antibody)	1.18E-07 (9.3)	6.08E-08 (11.8)	1.94E-07 (7.9)	4.45E-07 (2.0)	3.88E-07 (3.2)	4.52E-07 (3.3)	9.41E-09 (5.6)
	Rc66SIDE E (One-arm antibody)	7.68E-08 (14.3)	4.62E-08 (15.5)	1.63E-07 (9.4)	2.55E-07 (3.5)	2.02E-07 (6.2)	2.53E-07 (6.0)	1.20E-08 (4.4)
Rc66SIDE-P4-Rc66SIDE		3.87E-09 (283.8)	1.14E-09 (628.9)	1.05E-09 (1456.1)	2.54E-09 (351.3)	4.93E-09 (252.9)	3.17E-09 (478.5)	<1.0E-12 (>10,000)
Rc66SIDE-P16-Rc66SIDE		2.71E-09 (405.6)	2.57E-10 (2785.7)	<1.0E-12 (>10,000)	8.08E-10 (1103.3)	1.83E-09 (682.0)	5.56E-10 (2727.3)	<1.0E-12 (>10,000)
Rc66SIDE-P28-Rc66SIDE		6.62E-09 (166.0)	4.98E-09 (143.7)	5.64E-09 (270.7)	5.42E-09 (164.4)	8.11E-09 (153.7)	7.28E-09 (208.1)	1.14E-09 (46.3)

Table 9: Observed Binding by Tetrahedral Antibodies to CD20, CD10 and CD16a

Tetrahedral Antibody	Control Antibody	CD20 Lipoparticles Expt #1	CD20 Lipoparticles Expt #2	CD19	CD16a 158V FcRγIIIa
	Rituxan (antibody)	<1.0E-12	<1.0E-12	No binding	2.62E-07

Tetrahedral Antibody	Control Antibody	CD20 Lipoparticles Expt #1	CD20 Lipoparticles Expt #2	CD19	CD16a 158V FcRγIIa
	Rc6 (one-arm antibody)	5.23E-08	1.71E-08	No binding	3.03E-07
	Rc66-SIDE (one-arm antibody)	2.14E-08	ND	ND	ND
Rc6-P4-Rc6		<1.0E-12	ND	ND	ND
Rc66SIDE-P4-Rc66SIDE		<1.0E-12	ND	ND	ND
Rc66SIDE-P16-Rc66SIDE		<1.0E-12	ND	ND	ND
Rc66SIDE-P28-Rc66SIDE		<1.0E-12	ND	ND	ND
	B19c66 (one-arm antibody)	No binding	No binding	2.18E-08	2.34E-07
	B19c66AAC9 (one-arm antibody)	ND	ND	2.10E-08	No binding
B19c66-P4-Rc6		ND	3.99E-08	1.81E-08	2.45E-08

ND, not done

Example 3 - Tetrahedral Antibodies with the structures shown in Panel B of Figure 1, Figure 3, and Figure 13

[0444] Panel B of Figure 1 and Figure 3 show the schematic structure of tetrahedral antibodies of this example.

[0445] Figure 13 describes the preparation of tetrahedral antibodies with non-covalent linkages as shown in Panel B of Figure 1.

Example 4 – Tetrahedral Antibodies with the structures shown in Panel C of Figure 1, Figure 4, and Figure 14-18

[0446] Panel C of Figure C and Figure 4 show the schematic structure of tetrahedral antibodies of this example.

[0447] Figure 14 describes the preparation of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG.

[0448] Figure 15 shows analysis of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG by SE-HPLC.

[0449] Figure 16 shows analysis of tetrahedral antibodies by SE-HPLC with different peptide linkers (L-1) ACE2RQ740c60PGRF-ACE2RQ740c60PGRF, (L-185) ACE2RQ740c60PGRF185-ACE2RQ740c60PGRF185, (L-198) ACE2RQ740c60PGRF198-

ACE2RQ740c60PGRF198, (L-208) ACE2RQ740c60PGRF208-ACE2RQ740c60PGRF208, (L-212) ACE2RQ740c60PGRF235-ACE2RQ740c60PGRF235, (L-235) ACE2RQ740c60PGRF235-ACE2RQ740c60PGRF235, (L-240) ACE2RQ740c60PGRF240-ACE2RQ740c60PGRF240.

[0450] Figure 17 shows analysis of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG by SE-HPLC/MALS.

[0451] Figure 18 describes the stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2RQ740c60PG-ACE2RQ740c60PG and ACE monomer ACE2RQ615c60PG.

Table 10: SEQ IDs of Proteins Used for the Preparation of Tetrahedral Antibodies

Protein name	Fc fusion chain	Linker length	Linker Sequence	Fc chain
ACE2RQ740c60PG	SEQ ID NO 65	5	EPKSS (Residues 724-728 of SEQ ID NO 65)	SEQ ID NO 42
ACE2RQ740c60PGRF-240	SEQ ID NO 66	62	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPTPEPST APSTSFLPMGSPSPAEG STGDEPKSS (Residues 724-785 of SEQ ID NO 66)	SEQ ID NO 73
ACE2RQ740c60PGRF-235	SEQ ID NO 67	57	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPTPEPST APSTSFLPMGSPSPAEG STGD (Residues 724-780 of SEQ ID NO 67)	SEQ ID NO 73
ACE2RQ740c60PGRF-212	SEQ ID NO 68	34	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPTPEPS (Residues 724-757 of SEQ ID NO 68)	SEQ ID NO 73
ACE2RQ740c60PGRF-208	SEQ ID NO 69	30	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPT (Residues 724-753 of SEQ ID NO 69)	SEQ ID NO 73
ACE2RQ740c60PGRF-198	SEQ ID NO 70	20	TSTSPTRSMAPGAVHLP QPV (Residues 724-743 of SEQ ID NO 70)	SEQ ID NO 73
ACE2RQ740c60PGRF-185	SEQ ID NO 71	7	TSTSPTR (Residues 724-730 of SEQ ID NO 71)	SEQ ID NO 73
ACE2RQ740c60PGRF	SEQ ID NO 72	5	EPKSS (Residues 724-728 of SEQ ID NO 72)	SEQ ID NO 73

Table 11: Tetrahedral Antibody formation by dimerization of the ACE2RQ740c60 protein

Protein	ACE2 Tetrahedral Antibody retention time (minutes)	ACE2 Tetrahedral Antibody (%)	ACE2 Monomer retention time (minutes)	ACE2 Monomer (%)	HMW retention time (minutes)	HMW (%)
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ACE2RQ615c60PG	ND	ND	27.169	54.2	22.004-23.975	45.8
ACE2RQ740c60PG	23.912	76.1	ND	ND	21.642	23.9

ND; not detected; HMW, high molecular weight

Table 12: Molar Mass of the SEC-purified ACE2RQ740c60 Tetrahedral Antibody by SE-HPLC/MALS

ACE2 Tetrahedral Antibody	ACE2 Monomer	Tetrahedral Antibody retention time (minutes)	ACE2 Monomer retention time (minutes)	Molar Mass (kDa)
ACE2RQ740c60PG- ACE2RQ740c60PG		24.206	ND	297.4 ($\pm$ 1.9%)
	ACE2RQ615c60PG	ND	27.567	143.4 ($\pm$ 1.7%)

ND; not detected; HMW, high molecular weight

Table 13: Tetrahedral Antibody Formation by Non-covalent Linkage of ACE2RQ740c60 Protein with Different Peptide Linkers

Tetrahedral Antibody	Linker length	Linker sequence	Dimer retention time (minutes)	Dimer (%)	Mono mer (%)	HMW (%)
ACE2RQ740c60PGRF240- ACE2RQ740c60PGRF240	62	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPTPEPS TAPSTSFLPMGPSPPA EGSTGDEPKSS (Residues 724-785 of SEQ ID NO 66)	22.566	87.7	ND	11.0
ACE2RQ740c60PGRF235- ACE2RQ740c60PGRF235	57	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPTPEPS TAPSTSFLPMGPSPPA EGSTGD (Residues 724- 780 of SEQ ID NO 67)	22.634	94.7	ND	4.0
ACE2RQ740c60PGRF212- ACE2RQ740c60PGRF212	34	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPTPEPS (Residues 724-757 of SEQ ID NO 68)	23.138	93.4	ND	5.4
ACE2RQ740c60PGRF208- ACE2RQ740c60PGRF208	30	TSTSPTRSMAPGAVHLP QPVSTRSQHTQPT (Residues 724-753 of SEQ ID NO 69)	23.227	96.3	ND	2.4
ACE2RQ740c60PGRF198- ACE2RQ740c60PGRF198	20	TSTSPTRSMAPGAVHLP QPV (Residues 724-743 of SEQ ID NO 70)	23.544	64.8	ND	32.8
ACE2RQ740c60PGRF185- ACE2RQ740c60PGRF185	7	TSTSPTR (Residues 724- 730 of SEQ ID NO 71)	23.817	87.6	ND	11.0
ACE2RQ740c60PGRF- ACE2RQ740c60PGRF	5	EPKSS (Residues 724-728 of SEQ ID NO 72)	23.995	88.0	ND	11.1

ND, not detected; HMW, high molecular weight

Table 14: Predicted Binding Domains of ACE2 Tetrahedral Antibodies

Tetrahedral Antibody	D1 binding specificity	D2 binding specificity	D3 binding specificity	D4 binding specificity
ACE2RQ740c60PG- ACE2RQ740c60PG	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740c60PGRF240- ACE2RQ740c60PGRF240	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740c60PGRF235- ACE2RQ740c60PGRF235	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740c60PGRF212- ACE2RQ740c60PGRF212	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740c60PGRF208- ACE2RQ740c60PGRF208	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740c60PGRF198- ACE2RQ740c60PGRF198	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740c60PGRF185- ACE2RQ740c60PGRF185	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740c60PGRF- ACE2RQ740c60PGRF	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein

Example 5 - Tetrahedral Antibodies with the structure shown in Panel D of Figure 1 and Figures 5-10, 19-28

[0452] Panel D of Figure 1 and Figures 5-10 show the schematic structure of tetrahedral antibodies of this example.

[0453] Figure 19 describes preparation of tetrahedral antibody ACE2740FcG9-ACE2740FcG9.

[0454] Figure 20 shows analysis of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 by SE-HPLC.

[0455] Figure 21 shows analysis of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 by SE-HPLC/MALS.

[0456] Figure 22 shows analysis of tetrahedral antibody ACE2RQ740FcPG-ACE2RQ740FcPG by SE-HPLC/MALS.

[0457] Figure 23 shows stoichiometric binding analysis of an impure preparation of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 and ACE2 dimer ACE2740FcG9.

[0458] Figure 24 shows stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 and ACE2 dimer ACE2-740Fc-G9.

[0459] Figure 25 shows stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2RQ740FcPG-ACE2RQ740FcPG and ACE2 dimer ACE2-740Fc-G9.

[0460] Figure 26 shows stoichiometric binding analysis of a mixture of tetrahedral antibody ACE2740FcG9-ACE2740FcG9 and ACE2 dimer ACE2-615Fc-G9.

[0461] Figure 27 shows inhibition of SARS-CoV-2-VSV pseudotype virus infection by ACE2 tetrahedral antibodies ACE2740FcG9-ACE2740FcG9 and ACE2RQ740FcPG-ACE2RQ740FcPG, and by ACE2 dimers ACE2-615Fc-G9 and ACE2RQ615FcPG.

[0462] Figure 28 shows inhibition of SARS-CoV-2-VSV pseudotype virus infection by ACE2 tetrahedral antibodies ACE2740FcG9-ACE2740FcG9 and ACE2RQ740FcPG-ACE2RQ740FcPG, and by ACE2 dimers ACE2-740Fc-G9 and ACE2RQ740FcPG.

Table 15: SEQ IDs of Proteins Used for the Preparation of Tetrahedral Antibodies

Protein name	Fc fusion chain	Linker length	Linker Sequence
ACE2-615Fc-G9	SEQ ID NO 74	9	GGGGAGGGG (Residues 598-607 of SEQ ID NO: 74)
ACE2-740Fc-G9	SEQ ID NO 75	9	GGGGAGGGG (Residues 724-732 of SEQ ID NO: 75)
ACE2RQ615FcPG	SEQ ID NO 76	5	EPKSS (Residues 598-603 of SEQ ID NO: 76)
ACE2RQ740FcPG	SEQ ID NO 77	5	EPKSS (Residues 724-728 of SEQ ID NO: 77)
ACE2RQ740FcPG-G9	SEQ ID NO 78	9	GGGGAGGGG (Residues 724-732 of SEQ ID NO: 78)
ACE2-740FcPG-G9	SEQ ID NO 79	9	GGGGAGGGG (Residues 724-732 of SEQ ID NO: 79)
ACE2RQ740Fc-G9	SEQ ID NO 80	9	GGGGAGGGG (Residues 724-732 of SEQ ID NO: 80)
ACE2-740Fc-G9	SEQ ID NO 81	9	GGGGAGGGG (Residues 724-732 of SEQ ID NO: 81)
ACE2RQ740FcPG-235	SEQ ID NO 90	57	TSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPS TAPSTSFLPMGPSPPAEGSTGD (Residues 724-780 of SEQ ID NO: 90)
ACE2RQ740FcPG-212	SEQ ID NO 91	34	TSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPS (Residues 724-757 of SEQ ID NO: 91)
ACE2RQ740FcPG-208	SEQ ID NO 92	30	TSTSPTRSMAPGAVHLPQPVSTRSQHTQPT (Residues 724-753 of SEQ ID NO: 92)
ACE2RQ740FcPG-202	SEQ ID NO 93	24	TSTSPTRSMAPGAVHLPQPVSTRS (Residues 724-747 of SEQ ID NO: 93)
ACE2RQ740FcPG-201	SEQ ID NO 94	23	TSTSPTRSMAPGAVHLPQPVSTR (Residues 724-746 of SEQ ID NO: 94)
ACE2RQ740FcPG-200	SEQ ID NO 95	22	TSTSPTRSMAPGAVHLPQPVST (Residues 724-745 of SEQ ID NO: 95)
ACE2RQ740FcPG-199	SEQ ID NO 96	21	TSTSPTRSMAPGAVHLPQPV (Residues 724-744 of SEQ ID NO: 96)
ACE2RQ740FcPG-198	SEQ ID NO 97	20	TSTSPTRSMAPGAVHLPQPV (Residues 724-743 of SEQ ID NO: 97)

Protein name	Fc fusion chain	Linker length	Linker Sequence
ACE2RQ740FcPG-197	SEQ ID NO 98	19	TSTSPTRSMAPGAVHLPQP (Residues 724-742 of SEQ ID NO: 98)
ACE2RQ740FcPG-196	SEQ ID NO 99	18	TSTSPTRSMAPGAVHLPQ (Residues 724-741 of SEQ ID NO: 99)
ACE2RQ740FcPG-195	SEQ ID NO 100	17	TSTSPTRSMAPGAVHLP (Residues 724-740 of SEQ ID NO: 100)
ACE2RQ740FcPG-194	SEQ ID NO 101	16	TSTSPTRSMAPGAVHL (Residues 724-739 of SEQ ID NO: 101)
ACE2RQ740FcPG-193	SEQ ID NO 102	15	TSTSPTRSMAPGAVH (Residues 724-738 of SEQ ID NO: 102)
ACE2RQ740FcPG-192	SEQ ID NO 103	14	TSTSPTRSMAPGAV (Residues 724-737 of SEQ ID NO: 103)
ACE2RQ740FcPG-191	SEQ ID NO 104	13	TSTSPTRSMAPGA (Residues 724-736 of SEQ ID NO: 104)
ACE2RQ740FcPG-190	SEQ ID NO 105	12	TSTSPTRSMAPG (Residues 724-735 of SEQ ID NO: 105)
ACE2RQ740FcPG-189	SEQ ID NO 106	11	TSTSPTRSMAP (Residues 724-734 of SEQ ID NO: 106)
ACE2RQ740FcPG-188	SEQ ID NO 107	10	TSTSPTRSMA (Residues 724-733 of SEQ ID NO: 107)
ACE2RQ740FcPG-187	SEQ ID NO 108	9	TSTSPTRSM (Residues 724-732 of SEQ ID NO: 108)
ACE2RQ740FcPG-186	SEQ ID NO 109	8	TSTSPTRS (Residues 724-731 of SEQ ID NO: 109)
ACE2RQ740FcPG-185	SEQ ID NO 110	7	TSTSPTR (Residues 724-730 of SEQ ID NO: 110)
ACE2RQ740FcPG-184	SEQ ID NO 111	6	TSTSP (Residues 724-729 of SEQ ID NO: 111)
ACE2RQ740FcPG-183	SEQ ID NO 112	5	TSTSP (Residues 724-728 of SEQ ID NO: 105)
ACE2RQ740FcPG-G14	SEQ ID NO 113	14	GGGGAGGGGAGGGG (Residues 724-737 of SEQ ID NO: 113)
ACE2RQ740FcPG-G12	SEQ ID NO 114	12	GGAGGGGAGGGG (Residues 724-735 of SEQ ID NO: 114)
ACE2RQ740FcPG-G10	SEQ ID NO 115	10	AGGGGAGGGG (Residues 724-733 of SEQ ID NO: 115)
ACE2RQ740FcPG-G8	SEQ ID NO 116	8	GGAGGGG (Residues 724-731 of SEQ ID NO: 116)
ACE2RQ740FcPG-G7	SEQ ID NO 117	7	GGAGGGG (Residues 724-730 of SEQ ID NO: 117)
ACE2RQ740FcPG-G6	SEQ ID NO 118	6	GAGGGG (Residues 724-729 of SEQ ID NO: 118)
ACE2RQ740FcPG-G5	SEQ ID NO 119	5	AGGGG (Residues 724-728 of SEQ ID NO: 119)

Table 16: Tetrahedral Antibody formation by dimerization of the ACE2RQ740c60 and ACE2-740Fc-G9 proteins

Protein name	ACE2 Tetrahedral Antibody retention time (minutes)	ACE2 Tetrahedral Antibody (%)	ACE2 Dimer retention time (minutes)	ACE2 Dimer (%)	High Mol Wt retention time (minutes)	High Molecular Weight (%)
ACE2-615Fc-G9	ND	ND	25.255	90.1	21.956-22.857	8.6
ACE2-740Fc-G9	22.674	26.7	24.960	63.8	21.679	8.9

Table 17: Molar Mass of SEC-purified ACE2 Tetrahedral Antibodies by SE-HLPC/MALS

ACE2 Tetrahedral Antibody	ACE2 DIMER	Retention time (minutes)	Molar Mass (kDa)
ACE2740FcG9- ACE2740FcG9		22.621	510.1 ($\pm$ 2.3%)
	ACE2-740Fc-G9	24.919	256.6 ($\pm$ 2.9%)
ACE2RQ740FcPG- ACE2RQ740FcPG		22.454	493.8 ($\pm$ 2.0%)
	ACE2RQ740FcPG	24.831	242.4 ($\pm$ 3.6%)

Table 18: Binding domains of Tetrahedral Antibodies

Tetrahedral Antibody	D1/D2 binding specificity	D3/D4 binding specificity	D5/D6 binding specificity
ACE2615FcG9- ACE2615FcG9	NA	NA	NA
ACE2740FcG9- ACE2740FcG9	FcRn, FcR γ -low	FcRn, FcR γ -low	SARS-CoV-2 Spike protein
ACE2RQ615FcPG- ACE2RQ615FcPG	NA	NA	NA
ACE2RQ740FcPG- ACE2RQ740FcPG	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG9- ACE2RQ740FcPGG9	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2740FcPGG9- ACE2740FcPGG9	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcG9- ACE2RQ740FcG9	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2740FcG9- ACE2740FcG9	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG235- ACE2RQ740FcPG235	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG212- ACE2RQ740FcPG212	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG208- ACE2RQ740FcPG208	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG202- ACE2RQ740FcPG202	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG201- ACE2RQ740FcPG201	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG200- ACE2RQ740FcPG200	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG199- ACE2RQ740FcPG199	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG198- ACE2RQ740FcPG198	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG197- ACE2RQ740FcPG197	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG196- ACE2RQ740FcPG196	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG195- ACE2RQ740FcPG195	FcRn, FcR γ -low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein

Tetrahedral Antibody	D1/D2 binding specificity	D3/D4 binding specificity	D5/D6 binding specificity
ACE2RQ740FcPG194- ACE2RQ740FcPG194	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG193- ACE2RQ740FcPG193	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG192- ACE2RQ740FcPG192	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG191- ACE2RQ740FcPG191	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG190- ACE2RQ740FcPG190	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG189- ACE2RQ740FcPG189	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG188- ACE2RQ740FcPG188	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG187- ACE2RQ740FcPG187	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG186- ACE2RQ740FcPG186	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG185- ACE2RQ740FcPG185	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG184- ACE2RQ740FcPG184	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPG183- ACE2RQ740FcPG183	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG14- ACE2RQ740FcPGG14	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG12- ACE2RQ740FcPGG12	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG10- ACE2RQ740FcPGG10	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG8- ACE2RQ740FcPGG8	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG7- ACE2RQ740FcPGG7	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG6- ACE2RQ740FcPGG6	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein
ACE2RQ740FcPGG5- ACE2RQ740FcPGG5	FcRn, FcRγ-low	SARS-CoV-2 Spike protein	SARS-CoV-2 Spike protein

NA, not applicable (no appreciable formation)

Table 19: SARS-CoV-2-VSV Virus Inhibition by ACE2 Tetrahedral Antibodies

ACE2 Tetrahedral Antibody	ACE2 DIMER	SARS-CoV-2 (VSV pseudotype) NT₅₀ (ug/mL)	VSV-G (VSV contro)l NT₅₀ (ug/mL)
ACE2740FcG9-ACE2740FcG9		0.060	> 66.6
	ACE2-740Fc-G9	3.12	> 66.6
	ACE2-615Fc-G9	13.05	> 66.6
ACE2RQ740FcPG- ACE2RQ740FcPG		0.11	> 66.6
	ACE2RQ740FcPG	3.10	> 66.6
	ACE2RQ615FcPG	17.56	> 66.6

Example 6 – Structural Studies of the Superdimeric ACE2 Tetrahedral Antibody

[0463] The experiment of Figure 19 demonstrated the production of a tetrahedral antibody comprising four ACE 2 domains via the superdimerization of four identical ACE2-Fc fusion polypeptide chains, construct ACE2-740FcPG-G9 (SEQ ID NO:81). The superdimerization reaction required the presence of the collectrin-like domain acting as a dimerizing polypeptide (Panel B of Figure 19); in its absence no tetrahedral antibody was detected (Panel A of Figure 19). The yield of tetrahedral antibody in this experiment was 27%; in addition, a dimeric form of ACE2-740FcPG-G9 was produced at a level of 64% (Figure 20, Table 16). SEC-MALS analysis indicated a molar mass of 510 kDa and 257 kDa for the superdimeric and dimeric forms in good agreement with their predicted molecular weights of 438 kDa and 219 kDa, respectively, especially in view of the extensive glycosylation of the ACE2 molecule (Figure 21, Table 17).

[0464] The quaternary structure predicted for the superdimeric ACE2 tetrahedral antibody is shown in part C of Figure 43. Confirmation of this structure was provided by fragmentation studies carried out with the IdeZ protease which cuts the IgG1 molecule just below the hinge region. Upon digestion with IdeZ (Genovis, Lund, Sweden), followed by reduction of the hinge disulfides with TCEP, both the superdimeric form (part C of Figure 43) and dimeric form (Part B of Figure 43) yielded ACE2 dimer as predicted, whereas the monomeric ACE2-Fc fusion protein shown in part A of Figure 43 (SEQ ID NO:74) yielded only the expected ACE2 monomer.

[0465] The superdimer and dimer forms of the ACE2 tetrahedral antibody were readily separated by size exclusion chromatography (Figures 20, 21). Following purification, both forms were apparently stable and did not noticeably interconvert over a period of a month, suggesting that the relative formation of the two species had occurred prior to transit from the cell. To determine whether the linker connecting the dimerizing polypeptide to the Fc domain plays a role in the relative abundance of the superdimeric and dimeric forms obtained from the cell supernatant, a series of tetrahedral antibodies was prepared using a series of linkers of varying lengths (Table 15) and the relative yield of superdimer and dimer determined. As summarized in Table 20, the 201 linker consisting of 23 amino acid sequence derived from the stalk region of TNF receptor 1B gave rise to the highest ratio of the superdimeric and dimeric forms, 38%, as well as the lowest levels of high molecular weight (HMW) species, 5.1% (Table 20).

Table 20

Protein ID	Protein name	SEQ ID	Linker length	HMW	SD	D	% SD: D
6-01	ACE2RQ740FcPG-235	SEQ ID NO 90	57	6.2	25.4	68.0	27.2
6-02	ACE2RQ740FcPG-212	SEQ ID NO 91	34	5.1	29.6	65.1	31.2
6-03	ACE2RQ740FcPG-208	SEQ ID NO 92	30	3.8	33.8	62.1	35.3
6-04	ACE2RQ740FcPG-202	SEQ ID NO 93	24	5.3	35.8	58.7	37.8
6-05	ACE2RQ740FcPG-201	SEQ ID NO 94	23	5.1	36.2	58.7	38.1
6-06	ACE2RQ740FcPG-200	SEQ ID NO 95	22	7.8	34.5	57.4	37.6
6-07	ACE2RQ740FcPG-199	SEQ ID NO 96	21	6.2	35.2	58.4	37.6
6-08	ACE2RQ740FcPG-198	SEQ ID NO 97	20	8.0	34.1	57.6	37.2
6-09	ACE2RQ740FcPG-197	SEQ ID NO 98	19	8.0	33.9	57.7	37.0
6-10	ACE2RQ740FcPG-196	SEQ ID NO 99	18	9.5	33.4	56.9	37.0
6-11	ACE2RQ740FcPG-195	SEQ ID NO 100	17	8.6	32.8	58.4	36.0
6-12	ACE2RQ740FcPG-194	SEQ ID NO 101	16	6.3	34.3	59.2	36.7
6-13	ACE2RQ740FcPG-193	SEQ ID NO 102	15	7.9	32.1	59.7	35.0
6-14	ACE2RQ740FcPG-192	SEQ ID NO 103	14	8.2	31.8	59.7	34.7
6-15	ACE2RQ740FcPG-191	SEQ ID NO 104	13	7.1	33.3	59.5	35.9
6-16	ACE2RQ740FcPG-190	SEQ ID NO 105	12	7.5	30.4	61.8	33.0
6-17	ACE2RQ740FcPG-189	SEQ ID NO 106	11	8.1	30.2	61.6	32.9
6-18	ACE2RQ740FcPG-188	SEQ ID NO 107	10	7.5	30.9	61.5	33.4
6-19	ACE2RQ740FcPG-187	SEQ ID NO 108	9	9.3	28.8	61.7	31.9
6-20	ACE2RQ740FcPG-186	SEQ ID NO 109	8	6.6	30.2	63.0	32.4
6-21	ACE2RQ740FcPG-185	SEQ ID NO 110	7	6.7	27.7	65.4	29.7
6-22	ACE2RQ740FcPG-184	SEQ ID NO 111	6	7.4	28.2	64.1	30.6
6-23	ACE2RQ740FcPG-183	SEQ ID NO 112	5	8.2	26.3	65.5	28.7
6-24	ACE2RQ740FcPG-G14	SEQ ID NO 113	14	6.5	27.8	65.4	29.8
6-25	ACE2RQ740FcPG-G12	SEQ ID NO 114	12	15.0	29.8	54.7	35.3
6-26	ACE2RQ740FcPG-G10	SEQ ID NO 115	10	8.8	27.5	63.6	30.2
	ACE2RQ740FcPG-G9	SEQ ID NO 78	9	9.3	26.3	63.4	29.3
	ACE2RQ740FcPG-G9	SEQ ID NO 78	9	9.3	26.3	63.4	29.3
	ACE2RQ740Fc-G9	SEQ ID NO 80	9	7.8	27.0	64.1	29.6
	ACE2-740FcPG-G9	SEQ ID NO 79	9	10.8	25.6	62.2	29.2
	ACE2-740Fc-G9	SEQ ID NO 81	9	6.8	25.7	66.3	27.9
6-27	ACE2RQ740FcPG-G8	SEQ ID NO 116	8	11.3	28.8	59.7	32.6
6-28	ACE2RQ740FcPG-G7	SEQ ID NO 117	7	11.4	29.0	59.6	32.7

Protein ID	Protein name	SEQ ID	Linker length	HMW	SD	D	% SD: D
6-29	ACE2RQ740FcPG-G6	SEQ ID NO 118	6	6.7	26.8	65.8	28.9
6-30	ACE2RQ740FcPG-G5	SEQ ID NO 119	5	8.5	25.6	65.7	28.1

Example 7 – Functional Studies of the Superdimeric ACE2 Tetrahedral Antibody

[0466] The stoichiometric binding experiments of Figures 23-25 demonstrated that when SARS-CoV-2 spike protein is added to mixtures of the ACE2 superdimer and dimer, essentially all of the superdimer is consumed before the dimer begins to bind. This was observed whether the experiment was carried out with the superdimer/dimer mixture produced by cells (Figure 23) or with a mixture that was prepared using purified and separated superdimer and dimer obtained by size exclusion chromatography (Figure 24). Similar order-of-binding results were obtained whether the wild-type ACE2 protein or a catalytically inactive ACE2 mutant protein (R273Q) was used in the experiment (compare Figures 24 and 25). These results suggested that the ACE2 superdimer binds with much higher affinity than the dimer. SARS-CoV-2 pseudovirus neutralization experiments (Figures 27, 28) consistently demonstrated that the ACE2 superdimer is approximately two orders of magnitude more potent than either the ACE2 dimer found in superdimer preparations (Figure 43, panel b) or an ACE2-Fc dimer lacking the collectrin-like dimerizing polypeptide (Figure 43, panel a).

[0467] To confirm the biological relevance of the stoichiometric binding and pseudovirus experiments, neutralization assays were carried out with live SARS-CoV-2 virus. These experiments confirmed that the ACE2 is approximately two orders of magnitude more potent in neutralizing virus than the ACE2 dimer, and approximately three orders of magnitude more potent than the ACE2-Fc dimer (Figure 44). Similar results were obtained in neutralization assay carried out with the NL63 alpha coronavirus which also infects cells through the ACE2 receptor.

[0468] Method: Live SARS-Cov-2 neutralization assay. USA-WA1/2020 (NR-52281) and Germany/BavPat1/2020 (NR-52370) were obtained from BEI Resources, Manassas, VA and expanded using a single passage in Vero/TMPRSS2. Virus was end-point titrated in Vero/TMPRSS2 and 100 TCID₅₀ used per well. Virus was pre-incubated for 1 hour at 37 °C with serial dilutions of antibody and then plated in replicates of 8 on Vero/TMPRSS2 cells. After 7 days wells were scored for cytopathic effect.

Table 21

Protein ID	EC50 (nM) Live Virus Neutralization			
	SARS-CoV-2	Fold Increase	NL63	Fold Increase
6-05 Superdimer	0.233	403.7	1.58	86.5
6-05 Dimer	0.970	96.9	10.43	13.1
6-05 Impure	14.87	6.3	29.94	4.6
ACE2Fc615	93.95	1.0	136.50	1.0

Example 8 – Engineering ACE 2 Super-Heterodimeric Tetrahedral Antibodies

[0469] As linker optimization alone did not overcome the apparent structural constraints that limited the yield of superdimeric ACE2 tetrahedral antibody, a series of constructs was generated with the predicted structure shown in part E of Figure 43 (Table 22). This structure, termed “super-heterodimer”, differs from the “super-homodimer shown in Part C of Figure 43 by having one pair instead of two pair of dimerizing polypeptides. It was reasoned that the super-heterodimer would have the advantage of greater conformational flexibility by limiting superdimerization to one of the heavy chains. This prediction was borne out; by independently varying the linker length on the H1 and H2 chains, the ACE2 super-heterodimer was obtained in yields as high as 98.8% from culture supernatant (Table 23).

Table 22: SEQ IDs Used for the Preparation of Hetero-Superdimeric Tetrahedral Antibodies

Protein ID	Protein name	Domain Type D3/D4/D5/D6	H1 Chain	H2 Chain
5-01	ACE2FcPGRF-740RQ186/615RQ201	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 342
5-02	ACE2FcPGRF-740RQ186/615RQ202	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 343
5-03	ACE2FcPGRF-740RQ186/615RQ208	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 344
5-04	ACE2FcPGRF-740RQ186/615RQ212	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 345
5-05	ACE2FcPGRF-740RQ186/615RQ213	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 346
5-06	ACE2FcPGRF-740RQ186/615RQ216	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 347
5-07	ACE2FcPGRF-740RQ186/615RQ217	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 348
5-08	ACE2FcPGRF-740RQ186/615RQ218	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 349
5-09	ACE2FcPGRF-740RQ186/615RQ224	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 350
5-10	ACE2FcPGRF-740RQ186/615RQ235	ACE2 R273Q	SEQ ID NO: 338	SEQ ID NO: 351
5-11	ACE2FcPGRF-740RQ194/615RQ201	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 342
5-12	ACE2FcPGRF-740RQ194/615RQ202	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 343
5-13	ACE2FcPGRF-740RQ194/615RQ208	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 344
5-14	ACE2FcPGRF-740RQ194/615RQ212	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 345
5-15	ACE2FcPGRF-740RQ194/615RQ213	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 346
5-16	ACE2FcPGRF-740RQ194/615RQ216	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 347
5-17	ACE2FcPGRF-740RQ194/615RQ217	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 348
5-18	ACE2FcPGRF-740RQ194/615RQ218	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 349
5-19	ACE2FcPGRF-740RQ194/615RQ224	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 350
5-20	ACE2FcPGRF-740RQ194/615RQ235	ACE2 R273Q	SEQ ID NO: 339	SEQ ID NO: 351
5-21	ACE2FcPGRF-740RQ201/615RQ201	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 342
5-22	ACE2FcPGRF-740RQ201/615RQ202	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 343
5-23	ACE2FcPGRF-740RQ201/615RQ208	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 344
5-24	ACE2FcPGRF-740RQ201/615RQ212	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 345
5-25	ACE2FcPGRF-740RQ201/615RQ213	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 346
5-26	ACE2FcPGRF-740RQ201/615RQ216	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 347
5-27	ACE2FcPGRF-740RQ201/615RQ217	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 348
5-28	ACE2FcPGRF-740RQ201/615RQ218	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 349
5-29	ACE2FcPGRF-740RQ201/615RQ224	ACE2 R273Q	SEQ ID NO: 340	SEQ ID NO: 350
5-30	ACE2FcPGRF-740RQ201/615RQ235	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 351
5-31	ACE2FcPGRF-740RQ208/615RQ201	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 342
5-32	ACE2FcPGRF-740RQ208/615RQ202	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 343

Protein ID	Protein name	Domain Type D3/D4/D5/D6	H1 Chain	H2 Chain
5-33	ACE2FcPGRF-740RQ208/615RQ208	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 344
5-34	ACE2FcPGRF-740RQ208/615RQ212	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 345
5-35	ACE2FcPGRF-740RQ208/615RQ213	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 346
5-36	ACE2FcPGRF-740RQ208/615RQ216	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 347
5-37	ACE2FcPGRF-740RQ208/615RQ217	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 348
5-38	ACE2FcPGRF-740RQ208/615RQ218	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 349
5-39	ACE2FcPGRF-740RQ208/615RQ224	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 350
5-40	ACE2FcPGRF-740RQ208/615RQ235	ACE2 R273Q	SEQ ID NO: 341	SEQ ID NO: 351

Table 23

Protein ID	H1 Chain Linker	H2 Chain Linker	Dimerizing Polypeptide Pair D3/D4	HMW (%)	Main Peak (%)	Retention Time (min)
5-01	186	201	Collectrin-like	28.7	71.3	21.41
5-02	186	202	Collectrin-like	30.9	69.1	21.37
5-03	186	208	Collectrin-like	5.1	81.6	21.25
5-04	186	212	Collectrin-like	20.3	79.7	21.22
5-05	186	213	Collectrin-like	21.2	78.8	21.19
5-06	186	216	Collectrin-like	17.9	82.1	21.16
5-07	186	217	Collectrin-like	24.3	75.7	21.11
5-08	186	218	Collectrin-like	25.1	74.9	21.11
5-09	186	224	Collectrin-like	42.0	58.0	21.04
5-10	186	235	Collectrin-like	26.3	73.7	20.98
5-11	194	201	Collectrin-like	24.7	75.3	21.29
5-12	194	202	Collectrin-like	16.2	83.8	21.29
5-13	194	208	Collectrin-like	8.5	91.5	21.19
5-14	194	212	Collectrin-like	19.0	81.0	20.95
5-15	194	213	Collectrin-like	18.3	81.7	21.00
5-16	194	216	Collectrin-like	32.0	68.0	20.91
5-17	194	217	Collectrin-like	27.4	72.6	20.92
5-18	194	218	Collectrin-like	45.8	53.9	20.84
5-19	194	224	Collectrin-like	21.6	78.4	20.83
5-20	194	235	Collectrin-like	46.3	53.6	20.67
5-21	201	201	Collectrin-like	1.2	98.8	21.55
5-22	201	202	Collectrin-like	4.1	95.9	21.47
5-23	201	208	Collectrin-like	4.0	95.9	21.39
5-24	201	212	Collectrin-like	3.3	96.7	21.34
5-25	201	213	Collectrin-like	4.7	95.3	21.32
5-26	201	216	Collectrin-like	4.7	95.3	21.28
5-27	201	217	Collectrin-like	4.0	96.0	20.96
5-28	201	218	Collectrin-like	7.5	92.5	20.96
5-29	201	224	Collectrin-like	11.1	88.7	21.30
5-30	201	235	Collectrin-like	4.9	95.1	21.16
5-31	208	201	Collectrin-like	4.0	95.8	21.39
5-32	208	202	Collectrin-like	10.7	89.2	21.36
5-33	208	208	Collectrin-like	11.0	89.0	21.32
5-34	208	212	Collectrin-like	4.6	95.4	21.25
5-35	208	213	Collectrin-like	3.2	96.7	21.26
5-36	208	216	Collectrin-like	19.3	73.6	21.25
5-37	208	217	Collectrin-like	22.9	76.3	21.21

Protein ID	H1 Chain Linker	H2 Chain Linker	Dimerizing Polypeptide Pair D3/D4	HMW (%)	Main Peak (%)	Retention Time (min)
5-38	208	218	Collectrin-like	14.5	85.5	21.18
5-39	208	224	Collectrin-like	15.0	84.9	21.16
5-40	208	235	Collectrin-like	16.7	75.5	21.10

Example 9 – Viral neutralizing activity of ACE2 super-heterodimeric tetrahedral antibody

[0470] Live virus neutralization studies confirmed that the super-heterodimer retained the exquisite potency of the super-homodimer in neutralizing both the SARS-CoV-2 beta coronavirus and the NL63 alpha coronavirus.(Table 24).

Table 24

Protein ID	Tetrahedral Antibody Type	No. of Dimerizing Polypeptide Pairs	H Chain	H1 Chain	H2 Chain	EC50 (nM)	
						SARS-CoV-2	NL63
6-05	Super-homodimer	2	201	N/A	N/A	0.4610	10.20
5-21	Super-heterodimer	1	N/A	201	201	0.9421	7.688
5-27	Super-heterodimer	1	N/A	201	217	0.9556	4.160
5-28	Super-heterodimer	1	N/A	201	218	1.276	10.05
5-34	Super-heterodimer	1	N/A	208	212	0.6933	3.687
5-37	Super-heterodimer	1	N/A	208	217	0.8348	5.400

Example 10 – ACE2 Mutants

[0471] In the course of these investigations, the R273Q mutation was evaluated as a means to render ACE2 enzymatically active to avoid possible complications associated with clinical use of the active enzyme in SARS Co-V-2 patients. We discovered that R273Q had a severe effect on the plasma half-life of ACE2. Accordingly, five new mutations, R273A, R293G, R273C, H378A and E402A, were generated and evaluated in several ACE2 constructs (Tables 25A, 25B and 26A). The effect of the mutations on carboxypeptidase was consistent among different was consistent among the various constructs. It was determined that H378A was the best tolerated with respect to half-life and provided for the greatest reduction in enzyme activity.

[0472] The H378A mutation decreased the level of ACE2 carboxypeptidase activity by 99.95% with angiotensin-II, 99.93% with bradykinin and 99.85% with apelin-13 (in Table 26B).

[0473] Materials: Angiotensin II peptide (AS-20633), Des-Arg9-bradykinin peptide (AS-65642) (AnaSpec, Fremont, CA), Apelin-13 peptide (APEL-003) (CPC Scientific, San Jose, CA). Recombinant human ACE2 (79200) (BioLegend, San Diego, CA) was used as a positive control. Phenylalanine assay kits (ab83376) (Abcam, Cambridge, UK).

[0474] Methods: ACE2 carboxypeptidase activity was quantified using the presence of phenylalanine. The carboxypeptidase reaction was initiated when 0.025 µg (wild-type) or 15 µg (ACE2 mutant) of COVICEPT was added to 0.2 µM angiotensin II, bradykinin or apelin-13 in the presence of the reaction buffer (PBS with 10 µM ZnCl₂) at 37 °C. Twenty microliter aliquots were taken out from the reaction mixture every 2 minutes up to 20 minutes and heat inactivated at 80 °C for 5 minutes. The amount of phenylalanine in the heat inactivated aliquots was quantified using the phenylalanine assay kit.

Table 25A

Protein ID	Protein Name	H1 Chain	H2 Chain
10-01	ACE2FcPGRF-740wt201/615wt201	SEQ ID NO: 354	SEQ ID NO: 366
10-02	ACE2FcPGRF-740RA201/615RA201	SEQ ID NO: 355	SEQ ID NO: 367
10-03	ACE2FcPGRF-740RG201/615RG201	SEQ ID NO: 356	SEQ ID NO: 368
10-04	ACE2FcPGRF-740RV201/615RV201	SEQ ID NO: 357	SEQ ID NO: 369
10-05	ACE2FcPGRF-740HA201/615HA201	SEQ ID NO: 358	SEQ ID NO: 370
10-06	ACE2FcPGRF-740EA201/615EA201	SEQ ID NO: 359	SEQ ID NO: 371
10-07	ACE2FcPGRF-740wt201/615wt208	SEQ ID NO: 354	SEQ ID NO: 372
10-08	ACE2FcPGRF-740RA201/615RA208	SEQ ID NO: 355	SEQ ID NO: 373
10-09	ACE2FcPGRF-740RG201/615RG208	SEQ ID NO: 356	SEQ ID NO: 374

Protein ID	Protein Name	H1 Chain	H2 Chain
10-10	ACE2FcPGRF-740RV201/615RV208	SEQ ID NO: 357	SEQ ID NO: 375
10-11	ACE2FcPGRF-740HA201/615HA208	SEQ ID NO: 358	SEQ ID NO: 376
10-12	ACE2FcPGRF-740EA201/615EA208	SEQ ID NO: 359	SEQ ID NO: 377
10-13	ACE2FcPGRF-740wt208/615wt201	SEQ ID NO: 360	SEQ ID NO: 366
10-14	ACE2FcPGRF-740RA208/615RA201	SEQ ID NO: 361	SEQ ID NO: 367
10-15	ACE2FcPGRF-740RG208/615RG201	SEQ ID NO: 362	SEQ ID NO: 368
10-16	ACE2FcPGRF-740RV208/615RV201	SEQ ID NO: 363	SEQ ID NO: 369
10-17	ACE2FcPGRF-740HA208/615HA201	SEQ ID NO: 364	SEQ ID NO: 370
10-18	ACE2FcPGRF-740EA208/615EA201	SEQ ID NO: 365	SEQ ID NO: 371
10-19	ACE2FcPGRF-740wt208/615wt212	SEQ ID NO: 360	SEQ ID NO: 378
10-20	ACE2FcPGRF-740RA208/615RA212	SEQ ID NO: 361	SEQ ID NO: 379
10-21	ACE2FcPGRF-740RG208/615RG212	SEQ ID NO: 362	SEQ ID NO: 380
10-22	ACE2FcPGRF-740RV208/615RV212	SEQ ID NO: 363	SEQ ID NO: 381
10-23	ACE2FcPGRF-740HA208/615HA212	SEQ ID NO: 364	SEQ ID NO: 382
10-24	ACE2FcPGRF-740EA208/615EA212	SEQ ID NO: 365	SEQ ID NO: 383
10-25	ACE2FcPGRF-740wt201/615wt201/TMEM27CA	SEQ ID NO: 354	SEQ ID NO: 384
10-26	ACE2FcPGRF-740RA201/615RA201/TMEM27CA	SEQ ID NO: 355	SEQ ID NO: 385
10-27	ACE2FcPGRF-740RG201/615RG201/TMEM27CA	SEQ ID NO: 356	SEQ ID NO: 386
10-28	ACE2FcPGRF-740RV201/615RV201/TMEM27CA	SEQ ID NO: 357	SEQ ID NO: 387
10-29	ACE2FcPGRF-740HA201/615HA201/TMEM27CA	SEQ ID NO: 358	SEQ ID NO: 388
10-30	ACE2FcPGRF-740EA201/615EA201/TMEM27CA	SEQ ID NO: 359	SEQ ID NO: 389
10-31	ACE2FcPGRF-740wt201/607wt201/TMEM27	SEQ ID NO: 354	SEQ ID NO: 390
10-32	ACE2FcPGRF-740RA201/607RA201/TMEM27	SEQ ID NO: 355	SEQ ID NO: 391
10-33	ACE2FcPGRF-740RG201/607RG201/TMEM27	SEQ ID NO: 356	SEQ ID NO: 392
10-34	ACE2FcPGRF-740RV201/607RV201/TMEM27	SEQ ID NO: 357	SEQ ID NO: 393
10-35	ACE2FcPGRF-740HA201/607HA201/TMEM27	SEQ ID NO: 358	SEQ ID NO: 394
10-36	ACE2FcPGRF-740EA201/607EA201/TMEM27	SEQ ID NO: 359	SEQ ID NO: 395
10-37	ACE2FcPGRF-740wt201/611wt201/TMEM27	SEQ ID NO: 354	SEQ ID NO: 396
10-38	ACE2FcPGRF-740RA201/611RA201/TMEM27	SEQ ID NO: 355	SEQ ID NO: 397
10-39	ACE2FcPGRF-740RG201/611RG201/TMEM27	SEQ ID NO: 356	SEQ ID NO: 398
10-40	ACE2FcPGRF-740RV201/611RV201/TMEM27	SEQ ID NO: 357	SEQ ID NO: 399
10-41	ACE2FcPGRF-740HA201/611HA201/TMEM27	SEQ ID NO: 358	SEQ ID NO: 400
10-42	ACE2FcPGRF-740EA201/611EA201/TMEM27	SEQ ID NO: 359	SEQ ID NO: 401

Table 25B

Protein ID	Protein Name	H1 Chain	Fc Chain	ACE2 Mutations
10-43	ACE2FcPGRF-740wt201	SEQ ID NO: 402		
10-44	ACE2FcPGRF-740RA201	SEQ ID NO: 403		
10-45	ACE2FcPGRF-740RG201	SEQ ID NO: 404		
10-46	ACE2FcPGRF-740RV201	SEQ ID NO: 405		
10-47	ACE2FcPGRF-740HA201	SEQ ID NO: 406		
10-48	ACE2FcPGRF-740EA201	SEQ ID NO: 407		
10-49	ACE2FcPGRF-740wt201/313	SEQ ID NO: 408		K31F/N33D/H34S/E35Q
10-50	ACE2FcPGRF-740RA201/313	SEQ ID NO: 409		K31F/N33D/H34S/E35Q
10-51	ACE2FcPGRF-740RG201/313	SEQ ID NO: 410		K31F/N33D/H34S/E35Q
10-52	ACE2FcPGRF-740RV201/313	SEQ ID NO: 411		K31F/N33D/H34S/E35Q
10-53	ACE2FcPGRF-740HA201/313	SEQ ID NO: 412		K31F/N33D/H34S/E35Q
10-54	ACE2FcPGRF-740EA201/313	SEQ ID NO: 413		K31F/N33D/H34S/E35Q
10-55	ACE2c60PGRF-740wt208	SEQ ID NO: 360	SEQ ID NO: 420	
10-56	ACE2c60PGRF-740RA208	SEQ ID NO: 361	SEQ ID NO: 420	

Protein ID	Protein Name	H1 Chain	Fc Chain	ACE2 Mutations
10-57	ACE2c60PGRF-740RG208	SEQ ID NO: 362	SEQ ID NO: 420	
10-58	ACE2c60PGRF-740RV208	SEQ ID NO: 363	SEQ ID NO: 420	
10-59	ACE2c60PGRF-740HA208	SEQ ID NO: 364	SEQ ID NO: 420	
10-60	ACE2c60PGRF-740EA208	SEQ ID NO: 365	SEQ ID NO: 420	
10-61	ACE2c60PGRF-740wt208/313	SEQ ID NO: 414	SEQ ID NO: 420	K31F/N33D/H34S/E35Q
10-62	ACE2c60PGRF-740RA208/313	SEQ ID NO: 415	SEQ ID NO: 420	K31F/N33D/H34S/E35Q
10-63	ACE2c60PGRF-740RG208/313	SEQ ID NO: 416	SEQ ID NO: 420	K31F/N33D/H34S/E35Q
10-64	ACE2c60PGRF-740RV208/313	SEQ ID NO: 417	SEQ ID NO: 420	K31F/N33D/H34S/E35Q
10-65	ACE2c60PGRF-740HA208/313	SEQ ID NO: 418	SEQ ID NO: 420	K31F/N33D/H34S/E35Q
10-66	ACE2c60PGRF-740EA208/313	SEQ ID NO: 419	SEQ ID NO: 420	K31F/N33D/H34S/E35Q

Table 26A

Protein ID	Domain Type D3/D4	Domain Type D5/D6	First Dimerizing Polypeptide Pair D3/D4	First Dimerizing Polypeptide Sequence	Second Dimerizing Polypeptide Pair D5/D6	Second Dimerizing Polypeptide Sequence
10-01	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782		
10-02	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782		
10-03	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782		
10-04	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782		
10-05	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782		
10-06	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782		
10-07	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782		
10-08	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782		
10-09	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782		
10-10	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782		
10-11	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782		
10-12	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782		
10-13	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782		
10-14	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782		
10-15	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782		
10-16	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782		
10-17	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782		
10-18	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782		
10-19	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782		
10-20	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782		
10-21	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782		
10-22	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782		
10-23	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782		
10-24	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782		
10-25	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 784
10-26	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 784
10-27	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 784
10-28	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 784
10-29	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 784
10-30	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 784
10-31	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 783
10-32	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 783
10-33	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 783
10-34	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 783
10-35	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 783
10-36	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 783
10-37	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 786
10-38	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 786
10-39	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 786
10-40	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 786
10-41	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 786
10-42	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782	Collectrin	SEQ ID NO: 786
10-43	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-44	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-45	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-46	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-47	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-48	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-49	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-50	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-51	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-52	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-53	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782

Protein ID	Domain Type D3/D4	Domain Type D5/D6	First Dimerizing Polypeptide Pair D3/D4	First Dimerizing Polypeptide Sequence	Second Dimerizing Polypeptide Pair D5/D6	Second Dimerizing Polypeptide Sequence
10-54	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782	Collectrin-like	SEQ ID NO: 782
10-55	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782		
10-56	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782		
10-57	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782		
10-58	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782		
10-59	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782		
10-60	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782		
10-61	ACE2 wt	ACE2 wt	Collectrin-like	SEQ ID NO: 782		
10-62	ACE2 R273A	ACE2 R273A	Collectrin-like	SEQ ID NO: 782		
10-63	ACE2 R273G	ACE2 R273G	Collectrin-like	SEQ ID NO: 782		
10-64	ACE2 R273V	ACE2 R273V	Collectrin-like	SEQ ID NO: 782		
10-65	ACE2 H378A	ACE2 H378A	Collectrin-like	SEQ ID NO: 782		
10-66	ACE2 E201A	ACE2 E201A	Collectrin-like	SEQ ID NO: 782		

Table 26B

Protein ID	Specific Activity (pmol/min/ug)			
	ACE2	Angiotensin II	Bradykinin	Apelin-13
13-17	wt	1655.2	1724.8	688
13-18	H378A	0.76	1.14	1.01

Example 11 – SARS-CoV-2 Neutralizing Antibodies

[0475] Biosimilar versions of eight antibodies that have received Emergency Use Authorization or are in late stage trials, REGN10897, REGN10933, Bamlanivimab, Etesevimab, AZD1061, AZD 8895, VIR-7841, and CT-P59, were produced according to Table 27 to evaluate the relative effectiveness of ACE2 superdimeric tetrahedral antibodies against SARS-CoV-2 variants.

Table 27

Protein ID	Antibody Name	Other Name	Fc Domain Mutations	L Chain	H Chain
12-01	Imdevimab	REGN10987		SEQ ID NO: 423	SEQ ID NO: 424
12-02	Casirivimab	REGN10933		SEQ ID NO: 429	SEQ ID NO: 430
12-03	Bamlanivimab	LY-CoV555		SEQ ID NO: 441	SEQ ID NO: 442
12-04	Etesevimab	LY-CoV016	L234A/L235A	SEQ ID NO: 445	SEQ ID NO: 446
12-05	Cilgavimab	AZD1061	L234F/L235E/P331S M252Y/S254T/T256E	SEQ ID NO: 449	SEQ ID NO: 450
12-06	Tixagevimab	AZD8895	L234F/L235E/P331S M252Y/S254T/T256E	SEQ ID NO: 453	SEQ ID NO: 454
12-07	Sotrovimab	VIR-7831	M428L/N434S	SEQ ID NO: 457	SEQ ID NO: 458
12-08	Regdanvimab	CT-P59		SEQ ID NO: 461	SEQ ID NO: 462
12-09	B13A-PG		L234A/L235A/P329G	SEQ ID NO: 465	SEQ ID NO: 466
12-10	O24A-PG		L234A/L235A/P329G	SEQ ID NO: 469	SEQ ID NO: 470
16-01	B13A			SEQ ID NO: 465	SEQ ID NO: 554
16-02	O24A			SEQ ID NO: 469	SEQ ID NO: 555

Example 12

[0476] To provide proof-of-principle demonstrating tetrahedral antibodies comprising two distinct types of domain that specifically bind two different targets, two series of constructs were generated, a first series with the predicted structure shown in Panel B of Figure 31, according to Tables 28 and 29, and a second series with the predicted structure shown in Panel C of Figure 32, according to Tables 30 and 31, then evaluated for their structural and functional bispecificity.

Table 28

Protein ID	Protein Name	H1 Chain	L2 Chain	H2 Chain
13-01	ACE2FcPG-740wt201RF/REGN10987	SEQ ID NO: 471	SEQ ID NO: 423	SEQ ID NO: 472
13-02	ACE2FcPG-740HA201RF/REGN10987	SEQ ID NO: 473	SEQ ID NO: 423	SEQ ID NO: 472
13-03	ACE2FcPG-740wt201RF/REGN10933	SEQ ID NO: 471	SEQ ID NO: 429	SEQ ID NO: 474
13-04	ACE2FcPG-740HA201RF/REGN10933	SEQ ID NO: 473	SEQ ID NO: 429	SEQ ID NO: 474
13-05	ACE2FcPG-740wt201RF/Bamlanivimab	SEQ ID NO: 471	SEQ ID NO: 441	SEQ ID NO: 475
13-06	ACE2FcPG-740HA201RF/Bamlanivimab	SEQ ID NO: 473	SEQ ID NO: 441	SEQ ID NO: 475
13-07	ACE2FcPG-740wt201RF/Etesevimab	SEQ ID NO: 471	SEQ ID NO: 445	SEQ ID NO: 476
13-08	ACE2FcPG-740HA201RF/Etesevimab	SEQ ID NO: 473	SEQ ID NO: 445	SEQ ID NO: 476
13-09	ACE2FcFES-740wt201RF/Cilgavimab-YTE	SEQ ID NO: 477	SEQ ID NO: 449	SEQ ID NO: 478
13-10	ACE2FcFES-740HA201RF/Cilgavimab-YTE	SEQ ID NO: 479	SEQ ID NO: 449	SEQ ID NO: 478
13-11	ACE2FcFES-740wt201RF/Tixagevimab-YTE	SEQ ID NO: 477	SEQ ID NO: 453	SEQ ID NO: 480
13-12	ACE2FcFES-740HA201RF/Tixagevimab-YTE	SEQ ID NO: 479	SEQ ID NO: 453	SEQ ID NO: 480
13-13	ACE2FcPG-740wt201RF/Sotrovimab	SEQ ID NO: 481	SEQ ID NO: 457	SEQ ID NO: 482
13-14	ACE2FcPG-740HA201RF/Sotrovimab	SEQ ID NO: 483	SEQ ID NO: 457	SEQ ID NO: 482
13-15	ACE2FcPG-740wt201RF/Regdanvimab	SEQ ID NO: 471	SEQ ID NO: 461	SEQ ID NO: 484
13-16	ACE2FcPG-740HA201RF/Regdanvimab	SEQ ID NO: 473	SEQ ID NO: 461	SEQ ID NO: 484
13-17	ACE2FcPG-740wt201RF/B13A	SEQ ID NO: 471	SEQ ID NO: 465	SEQ ID NO: 485
13-18	ACE2FcPG-740HA201RF/B13A	SEQ ID NO: 473	SEQ ID NO: 465	SEQ ID NO: 485
13-19	ACE2FcPG-740wt201RF/O24A	SEQ ID NO: 471	SEQ ID NO: 469	SEQ ID NO: 486
13-20	ACE2FcPG-740HA201RF/O24A	SEQ ID NO: 473	SEQ ID NO: 469	SEQ ID NO: 486
13-21	ACE2FcFES-740wt201RF/B13A-YTE	SEQ ID NO: 477	SEQ ID NO: 465	SEQ ID NO: 487
13-22	ACE2FcFES-740HA201RF/B13A-YTE	SEQ ID NO: 479	SEQ ID NO: 465	SEQ ID NO: 487
13-23	ACE2FcFES-740wt201RF/O24A-YTE	SEQ ID NO: 477	SEQ ID NO: 469	SEQ ID NO: 488
13-24	ACE2FcFES-740HA201RF/O24A-YTE	SEQ ID NO: 479	SEQ ID NO: 469	SEQ ID NO: 488

Table 29

Protein ID	Domain Type D3/D4	Domain Type D5/D6	First Dimerizing Polypeptide Pair D3/D4	First Dimerizing Polypeptide Sequence	Fc Domain Type D1/D2
13-01	ACE2 wt	REGN10987	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-02	ACE2 H378A	REGN10987	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-03	ACE2 wt	REGN10933	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-04	ACE2 H378A	REGN10933	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-05	ACE2 wt	LY-CoV555	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-06	ACE2 H378A	LY-CoV555	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-07	ACE2 wt	LY-CoV016	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-08	ACE2 H378A	LY-CoV016	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-09	ACE2 wt	AZD1061	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E
13-10	ACE2 H378A	AZD1061	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E
13-11	ACE2 wt	AZD8895	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E
13-12	ACE2 H378A	AZD8895	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E
13-13	ACE2 wt	VIR-7831	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G M428L/N434S
13-14	ACE2 H378A	VIR-7831	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G M428L/N434S
13-15	ACE2 wt	CT-P59	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-16	ACE2 H378A	CT-P59	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-17	ACE2 wt	B13A	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-18	ACE2 H378A	B13A	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-19	ACE2 wt	O24A	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-20	ACE2 H378A	O24A	Collectrin-like	SEQ ID NO:782	L234A/L235A/P329G
13-21	ACE2 wt	B13A	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E
13-22	ACE2 H378A	B13A	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E
13-23	ACE2 wt	O24A	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E
13-24	ACE2 H378A	O24A	Collectrin-like	SEQ ID NO:782	L234F/L235E/P331S M252Y/S254T/T256E

Example 13Table 30

Protein ID	Protein Name	L1 Chain	H1 Chain	L2 Chain	H2 Chain
14-01	REGN10987-CLD201RF-N84S/REGN10987	SEQ ID NO:423	SEQ ID NO:489	SEQ ID NO:423	SEQ ID NO:472
14-02	REGN10987(x)CLD201RF-N84S/REGN10933	SEQ ID NO:503	SEQ ID NO:490	SEQ ID NO:429	SEQ ID NO:474
14-03	REGN10933-CLD201RF-N84S/REGN10933	SEQ ID NO:429	SEQ ID NO:491	SEQ ID NO:429	SEQ ID NO:474
14-04	REGN10933(x)CLD201RF-N84S/REGN10987	SEQ ID NO:504	SEQ ID NO:492	SEQ ID NO:423	SEQ ID NO:472
14-05	Bamlanivimab-CLD201RF/Bamlanivimab	SEQ ID NO:441	SEQ ID NO:493	SEQ ID NO:441	SEQ ID NO:475
14-06	Bamlanivimab(x)CLD201RF/Etesevimab	SEQ ID NO:505	SEQ ID NO:494	SEQ ID NO:445	SEQ ID NO:476
14-07	Etesevimab-CLD201RF-N83S/Etesevimab	SEQ ID NO:445	SEQ ID NO:495	SEQ ID NO:445	SEQ ID NO:476
14-08	Etesevimab(x)CLD201RF-N83S/Bamlanivimab	SEQ ID NO:506	SEQ ID NO:496	SEQ ID NO:441	SEQ ID NO:475
14-09	Cilgavimab-CLD201RF-N86S/Cilgavimab-YTE	SEQ ID NO:449	SEQ ID NO:497	SEQ ID NO:449	SEQ ID NO:478
14-10	Cilgavimab(x)CLD201RF-N86S/Tixagevimab-YTE	SEQ ID NO:507	SEQ ID NO:498	SEQ ID NO:453	SEQ ID NO:480
14-11	Tixagevimab-CLD201RF/Tixagevimab	SEQ ID NO:453	SEQ ID NO:499	SEQ ID NO:453	SEQ ID NO:480
14-12	Tixagevimab(x)CLD201RF/Cilgavimab	SEQ ID NO:508	SEQ ID NO:500	SEQ ID NO:449	SEQ ID NO:478
14-13	Sotrovimab-CLD201RF/Sotrovimab	SEQ ID NO:457	SEQ ID NO:501	SEQ ID NO:457	SEQ ID NO:482
14-14	Regdanvimab-CLD201RF/Regdanvimab	SEQ ID NO:461	SEQ ID NO:502	SEQ ID NO:461	SEQ ID NO:484

Table 31

Protein ID	Domain Type D3/D4	Fab Domain Configuration D3/D4	Domain Type D5/D6	Fab Domain Configuration D5/D6	Fc Domain Type D1/D2
14-01	REGN10987	VL-CL/VH-CH1	REGN10987	VL-CL/VH-CH1	L234A/L235A/P329G
14-02	REGN10987	VH-CH1/VL-CL	REGN10933	VK-CK/VH-CH1	L234A/L235A/P329G
14-03	REGN10933	VK-CK/VH-CH1	REGN10933	VK-CK/VH-CH1	L234A/L235A/P329G
14-04	REGN10933	VH-CH1/VK-CK	REGN10987	VL-CL/VH-CH1	L234A/L235A/P329G
14-05	LY-CoV555	VK-CK/VH-CH1	LY-CoV555	VK-CK/VH-CH1	L234A/L235A/P329G
14-06	LY-CoV555	VH-CH1/VK-CK	LY-CoV016	VK-CK/VH-CH1	L234A/L235A/P329G
14-07	LY-CoV016	VK-CK/VH-CH1	LY-CoV016	VK-CK/VH-CH1	L234A/L235A/P329G
14-08	LY-CoV016	VH-CH1/VK-CK	LY-CoV555	VK-CK/VH-CH1	L234A/L235A/P329G
14-09	AZD1061	VK-CK/VH-CH1	AZD1061	VK-CK/VH-CH1	L234F/L235E/P331S M252Y/S254T/T256E
14-10	AZD1061	VH-CH1/VK-CK	AZD8895	VK-CK/VH-CH1	L234F/L235E/P331S M252Y/S254T/T256E
14-11	AZD8895	VK-CK/VH-CH1	AZD8895	VK-CK/VH-CH1	L234F/L235E/P331S M252Y/S254T/T256E
14-12	AZD8895	VH-CH1/VK-CK	AZD1061	VK-CK/VH-CH1	L234F/L235E/P331S M252Y/S254T/T256E
14-13	VIR-7831	VK-CK/VH-CH1	VIR-7831	VK-CK/VH-CH1	L234A/L235A/P329G M428L/N434S
14-14	CT-P59	VL-CL/VH-CH1	CT-P59	VL-CL/VH-CH1	L234A/L235A/P329G

Example 14 – Pharmacokinetics

[0477] Pharmacokinetic studies were carried out with two series of constructs designed to evaluate the effect of mutations in the Fc region that affect FcγR and FcRn binding. The first series is described in Tables 28 and 29, The second series is described in Tables 33 and 34.

[0478] Methods: The study was conducted by The Jackson Laboratory, Bar Harbor, ME. 6-8 week old male B6.Cg-*Fcgrt^{tm1Dcr}* Tg(FCGRT)^{32Dcr/DcrJ} mice were homozygous for the human FcRn transgene. Body weights were measured within 1 day of each test article administration. At 0 hours on Day 0, COVICEPT was administered by IV injection at 10 mg/kg in a volume of 5 ml/kg. 25μL blood samples were collected from each mouse at 5m, 6h, 1d, 4d, 7d, 10d, 14d, 17d, 21d, and 28d. The blood samples were collected into 1uL of K₃EDTA, processed to plasma, diluted 1/10 in 50% glycerol in PBS, frozen in specialized 96 well storage pates and stored at -20°C. Plasma samples were assessed by electrochemiluminescent immunoassay (Meso Scale Diagnostics LLC, Rockville, MD) to detect the level of COVICEPT.

[0479] Electrochemiluminescent immunoassay: Wild-type spike protein (LakePharma, Inc., San Carlos, CA) was diluted to 20 nM with PBS and coated on QuickPlex 96-Well plates (Meso Scale Diagnostics) with 25 μL per well. The plates were sealed and incubated overnight at 4°C. The plates were then blocked by PBS-B (PBS with 1% BSA) at room temperature for 30 minutes. The plasma samples were diluted 250- to 4000-fold with PBS-B. Twenty five microliters of the diluted plasma samples were added to the coated MSD plates using Biomek I5 liquid handler (Beckman Coulter Inc., Brea, CA). Plasma samples were incubated with shaking (700 rpm) at room temperature for 60 mins. Twenty five microliters of biotinylated goat anti human-Fab antibody (Abcam), or biotinylated goat Anti-Human ACE-2 detection antibody (R&D Systems) was used as a detection antibody. Detection antibody was incubated with shaking (700 rpm) at room temperature for 60 mins. Twenty five microliters of sulfo-tag streptavidin and Read buffer A (Meso Scale Diagnostics LLC, Rockville, MD) were then added to generate the electrochemiluminescent signal. Data were analyzed with MSD discovery workbench 4.0.13 (Meso Scale Diagnostics LLC, Rockville, MD). Pharmacokinetic analysis was performed using PKSolver22.

Table 32A

Protein ID	Protein Name	Detection	Half-life (days)
1317	B13-ACE2 WT	ACE2	4.6
		Fab	4.9
1318	B13-ACE2 HA	ACE2	5.4
		Fab	5.4
1321	B13-ACE2 WT_YTE	ACE2	9.0
		Fab	9.4
1322	B13-ACE2 HA_YTE	ACE2	7.9
		Fab	8.3

Table 32C

Protein ID	Domain Type D3/D4	Domain Type D5/D6	FcR Domain Type D1/D2	FcRn Domain Type D1/D2	Half-life (days)
15-01	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G		1.3
15-02	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G		1.1
15-03	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M252Y/S254T/T256E	1.7
15-04	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M252Y/S254T/T256E	1.1
15-05	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M428L/N434S	1.4
15-06	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M428L/N434S	1.4
15-07	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	FCIL309D/Q311H/N434S	1.1
15-08	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	FCIL309D/Q311H/N434S	1.3
15-09	ACE2 H378A	B13A			2.8
15-10	ACE2 H378A	B13A	L234A/L235A/P329G		3.5
15-11	ACE2 H378A	B13A		M252Y/S254T/T256E	6.2
15-12	ACE2 H378A	B13A	L234A/L235A/P329G	M252Y/S254T/T256E	3.9
15-13	ACE2 H378A	B13A		M428L/N434S	2.8
15-14	ACE2 H378A	B13A	L234A/L235A/P329G	M428L/N434S	4.5
15-15	ACE2 H378A	B13A		FCIL309D/Q311H/N434S	4.4
15-16	ACE2 H378A	B13A	L234A/L235A/P329G	FCIL309D/Q311H/N434S	6.0

Example 15

[0480] A series of constructs were generated according to Tables 33 and 34 to evaluate the effect of mutations in the Fc region that affect FcγR and FcγRn binding and pharmacokinetics.

[0481] The results of the binding measurements are presented in Tables 35A, 35B, 35C, 35D.

[0482] Methods: Surface plasmon resonance capture kinetic experiments were performed on a Carterra LSA instrument purchased from Carterra, Inc. Salt Lake City, UT. COVICEPT and its controls (5 μg/ml) were immobilized on the Carterra CMD-P chip using amine coupling. Recombinant Fc gamma receptors (R&D Systems) were diluted with running buffer (PBS with 0.05 % TWEEN) to a concentration of 2000 nM, followed by 7 times 3-fold serial dilution to 0.9 nM. Fc gamma receptors were injected onto the CMD-P chip for 5 minutes during the association phase followed by running buffer injection for another 5 minutes during the dissociation phase.

The CMD-P chip was regenerated by flowing Pierce™ IgG Elution Buffer for 1 minute (Thermo Fisher Scientific Inc., Waltham, MA) after each round of Fc-gamma receptors injection. Data were analyzed using the Catterra's Kinetic tool.

Table 33

Protein ID	Protein Name	H1 Chain	L2 Chain	H2 Chain
15-01	ACE2FcPG-740HA201/615HA201RF	SEQ ID NO: 509		SEQ ID NO: 510
15-02	ACE2FcPG-740HA201RF/615HA201	SEQ ID NO: 473		SEQ ID NO: 511
15-03	ACE2FcPG-740HA201/615HA201RF-YTE	SEQ ID NO: 512		SEQ ID NO: 513
15-04	ACE2FcPG-740HA201RF/615HA201-YTE	SEQ ID NO: 514		SEQ ID NO: 515
15-05	ACE2FcPG-740HA201/615HA201RF-LS	SEQ ID NO: 516		SEQ ID NO: 517
15-06	ACE2FcPG-740HA201RF/615HA201-LS	SEQ ID NO: 518		SEQ ID NO: 519
15-07	ACE2FcPG-740HA201/615HA201RF-DHS	SEQ ID NO: 520		SEQ ID NO: 521
15-08	ACE2FcPG-740HA201RF/615HA201-DHS	SEQ ID NO: 522		SEQ ID NO: 523
15-09	ACE2Fc-740HA201RF/B13A	SEQ ID NO: 524	SEQ ID NO: 465	SEQ ID NO: 525
15-10	ACE2FcPG-740HA201RF/B13A	SEQ ID NO: 473	SEQ ID NO: 465	SEQ ID NO: 485
15-11	ACE2Fc-740HA201RF/B13A-YTE	SEQ ID NO: 526	SEQ ID NO: 465	SEQ ID NO: 527
15-12	ACE2FcPG-740HA201RF/B13A-YTE	SEQ ID NO: 514	SEQ ID NO: 465	SEQ ID NO: 528
15-13	ACE2Fc-740HA201RF/B13A-LS	SEQ ID NO: 529	SEQ ID NO: 465	SEQ ID NO: 530
15-14	ACE2FcPG-740HA201RF/B13A-LS	SEQ ID NO: 518	SEQ ID NO: 465	SEQ ID NO: 531
15-15	ACE2Fc-740HA201RF/B13A-DHS	SEQ ID NO: 532	SEQ ID NO: 465	SEQ ID NO: 533
15-16	ACE2FcPG-740HA201RF/B13A-DHS	SEQ ID NO: 522	SEQ ID NO: 465	SEQ ID NO: 534
15-17	ACE2Fc-740HA201RF/O24A	SEQ ID NO: 524	SEQ ID NO: 469	SEQ ID NO: 535
15-18	ACE2FcPG-740HA201RF/O24A	SEQ ID NO: 473	SEQ ID NO: 469	SEQ ID NO: 486
15-19	ACE2Fc-740HA201RF/O24A-YTE	SEQ ID NO: 526	SEQ ID NO: 469	SEQ ID NO: 536
15-20	ACE2FcPG-740HA201RF/O24A-YTE	SEQ ID NO: 514	SEQ ID NO: 469	SEQ ID NO: 537
15-21	ACE2Fc-740HA201RF/O24A-LS	SEQ ID NO: 529	SEQ ID NO: 469	SEQ ID NO: 538
15-22	ACE2FcPG-740HA201RF/O24A-LS	SEQ ID NO: 518	SEQ ID NO: 469	SEQ ID NO: 539
15-23	ACE2Fc-740HA201RF/O24A-DHS	SEQ ID NO: 532	SEQ ID NO: 469	SEQ ID NO: 540
15-24	ACE2FcPG-740HA201RF/O24A-DHS	SEQ ID NO: 522	SEQ ID NO: 469	SEQ ID NO: 541
15-49	ACE2Fc-740HA201/615HA201RF	SEQ ID NO: 542		SEQ ID NO: 543
15-50	ACE2Fc-740HA201RF/615HA201	SEQ ID NO: 524		SEQ ID NO: 544
15-51	ACE2Fc-740HA201/615HA201RF-YTE	SEQ ID NO: 545		SEQ ID NO: 546
15-52	ACE2Fc-740HA201RF/615HA201-YTE	SEQ ID NO: 526		SEQ ID NO: 547
15-53	ACE2Fc-740HA201/615HA201RF-LS	SEQ ID NO: 548		SEQ ID NO: 549
15-54	ACE2Fc-740HA201RF/615HA201-LS	SEQ ID NO: 529		SEQ ID NO: 550
15-55	ACE2Fc-740HA201/615HA201RF-DHS	SEQ ID NO: 551		SEQ ID NO: 552
15-56	ACE2Fc-740HA201RF/615HA201-DHS	SEQ ID NO: 532		SEQ ID NO: 553

Table 34

Protein ID	Domain Type D3/D4	Domain Type D5/D6	FcR Domain Type D1/D2	FcRn Domain Type D1/D2	Fc Domain Type Protein A D1/D2	
					H1 Chain	H2 Chain
15-01	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G		HY	RF
15-02	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G		RF	HY
15-03	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M252Y/S254T/T256E	HY	RF
15-04	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M252Y/S254T/T256E	RF	HY
15-05	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M428L/N434S	HY	RF
15-06	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	M428L/N434S	RF	HY
15-07	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	FCFL309D/Q311H/N434S	HY	RF
15-08	ACE2 H378A	ACE2 H378A	L234A/L235A/P329G	FCFL309D/Q311H/N434S	RF	HY
15-09	ACE2 H378A	B13A			RF	HY
15-10	ACE2 H378A	B13A	L234A/L235A/P329G		RF	HY
15-11	ACE2 H378A	B13A		M252Y/S254T/T256E	RF	HY
15-12	ACE2 H378A	B13A	L234A/L235A/P329G	M252Y/S254T/T256E	RF	HY
15-13	ACE2 H378A	B13A		M428L/N434S	RF	HY
15-14	ACE2 H378A	B13A	L234A/L235A/P329G	M428L/N434S	RF	HY
15-15	ACE2 H378A	B13A		FCFL309D/Q311H/N434S	RF	HY
15-16	ACE2 H378A	B13A	L234A/L235A/P329G	FCFL309D/Q311H/N434S	RF	HY
15-17	ACE2 H378A	O24A			RF	HY
15-18	ACE2 H378A	O24A	L234A/L235A/P329G		RF	HY
15-19	ACE2 H378A	O24A		M252Y/S254T/T256E	RF	HY
15-20	ACE2 H378A	O24A	L234A/L235A/P329G	M252Y/S254T/T256E	RF	HY
15-21	ACE2 H378A	O24A		M428L/N434S	RF	HY
15-22	ACE2 H378A	O24A	L234A/L235A/P329G	M428L/N434S	RF	HY
15-23	ACE2 H378A	O24A		FCFL309D/Q311H/N434S	RF	HY
15-24	ACE2 H378A	O24A	L234A/L235A/P329G	FCFL309D/Q311H/N434S	RF	HY
15-49	ACE2 H378A	ACE2 H378A			HY	RF
15-50	ACE2 H378A	ACE2 H378A			RF	HY
15-51	ACE2 H378A	ACE2 H378A		M252Y/S254T/T256E	HY	RF
15-52	ACE2 H378A	ACE2 H378A		M252Y/S254T/T256E	RF	HY
15-53	ACE2 H378A	ACE2 H378A		M428L/N434S	HY	RF
15-54	ACE2 H378A	ACE2 H378A		M428L/N434S	RF	HY
15-55	ACE2 H378A	ACE2 H378A		FCFL309D/Q311H/N434S	HY	RF
15-56	ACE2 H378A	ACE2 H378A		FCFL309D/Q311H/N434S	RF	HY

Table 35A

Protein ID	FcR Domain Type D1/D2	FcRn Domain Type D1/D2	FcγRI ₁ CD64			
			ka (M ⁻¹ s ⁻¹)	kd (s ⁻¹)	KD (M)	Rmax (RU)
15-01	L234A/L235A/P329G		NB	NB	NB	NB
15-02	L234A/L235A/P329G		NB	NB	NB	NB
15-03	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-04	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-05	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-06	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-07	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-08	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-09			9.71E+05	7.44E-04	7.67E-10	3.61E+01
15-10	L234A/L235A/P329G		NB	NB	NB	NB
15-11		M252Y/S254T/T256E	7.62E+05	1.20E-03	1.57E-09	3.64E+01
15-12	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-13		M428L/N434S	8.29E+05	5.83E-04	7.02E-10	5.34E+01
15-14	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-15		L309D/Q311H/N434S	7.40E+05	7.25E-04	9.80E-10	6.05E+01
15-16	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-17			6.94E+05	7.16E-04	1.03E-09	4.37E+01
15-18	L234A/L235A/P329G		NB	NB	NB	NB
15-19		M252Y/S254T/T256E	7.07E+05	1.57E-03	2.22E-09	2.90E+01
15-20	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-21		M428L/N434S	8.87E+05	6.72E-04	7.58E-10	2.97E+01
15-22	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-23		L309D/Q311H/N434S	9.08E+05	6.81E-04	7.50E-10	3.84E+01
15-24	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-49			9.04E+05	7.02E-04	7.76E-10	4.87E+01
15-50			1.01E+06	7.62E-04	7.52E-10	3.05E+01
15-51		M252Y/S254T/T256E	9.34E+05	1.10E-03	1.18E-09	2.28E+01
15-52		M252Y/S254T/T256E	8.54E+05	1.17E-03	1.37E-09	2.69E+01
15-53		M428L/N434S	9.99E+05	6.98E-04	6.98E-10	2.21E+01
15-54		M428L/N434S	NB	NB	NB	NB
15-56		L309D/Q311H/N434S	7.74E+05	9.16E-04	1.18E-09	3.76E+01

Table 35B

Protein ID	FcR Domain Type D1/D2	FcRn Domain Type D1/D2	FcγRIIa (131 R)_ CD 32a 131 R			
			ka (M ⁻¹ s ⁻¹)	kd (s ⁻¹)	KD (M)	Rmax (RU)
15-01	L234A/L235A/P329G		NB	NB	NB	NB
15-02	L234A/L235A/P329G		NB	NB	NB	NB
15-03	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-04	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-05	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-06	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-07	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-08	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-09			1.56E+06	3.97E-02	2.55E-08	3.33E+01
15-10	L234A/L235A/P329G		NB	NB	NB	NB
15-11		M252Y/S254T/T256E	7.26E+05	1.12E-01	1.54E-07	2.79E+01
15-12	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-13		M428L/N434S	1.37E+06	1.98E-02	1.44E-08	4.03E+01
15-14	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-15		L309D/Q311H/N434S	9.86E+05	2.31E-02	2.34E-08	4.57E+01
15-16	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-17			8.83E+05	4.35E-02	4.93E-08	1.37E+01
15-18	L234A/L235A/P329G		NB	NB	NB	NB
15-19		M252Y/S254T/T256E	7.19E+05	1.98E-01	2.76E-07	1.65E+01
15-20	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-21		M428L/N434S	1.55E+06	7.79E-02	5.03E-08	2.06E+01
15-22	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-23		L309D/Q311H/N434S	1.66E+06	8.06E-02	4.86E-08	1.98E+01
15-24	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-49			1.32E+06	3.65E-02	2.76E-08	3.33E+01
15-50			2.03E+06	7.99E-02	3.94E-08	1.91E+01
15-51		M252Y/S254T/T256E	NB	NB	NB	NB
15-52		M252Y/S254T/T256E	1.14E+06	2.08E-01	1.82E-07	1.67E+01
15-53		M428L/N434S	NB	NB	NB	NB
15-54		M428L/N434S	NB	NB	NB	NB
15-56		L309D/Q311H/N434S	1.13E+06	6.80E-02	6.00E-08	2.08E+01

Table 35C

Protein ID	FcR Domain Type D1/D2	FcRn Domain Type D1/D2	FcγRIIb/c_CD32b/c			
			ka (M ⁻¹ s ⁻¹)	kd (s ⁻¹)	KD (M)	Rmax (RU)
15-01	L234A/L235A/P329G		NB	NB	NB	NB
15-02	L234A/L235A/P329G		NB	NB	NB	NB
15-03	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-04	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-05	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-06	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-07	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-08	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-09			1.75E+06	2.87E-01	1.64E-07	2.27E+01
15-10	L234A/L235A/P329G		NB	NB	NB	NB
15-11		M252Y/S254T/T256E	NB	NB	NB	NB
15-12	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-13		M428L/N434S	1.63E+06	2.16E-01	1.32E-07	3.42E+01
15-14	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-15		L309D/Q311H/N434S	1.26E+06	1.99E-01	1.57E-07	3.67E+01
15-16	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-17			NB	NB	NB	NB
15-18	L234A/L235A/P329G		NB	NB	NB	NB
15-19		M252Y/S254T/T256E	NB	NB	NB	NB
15-20	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-21		M428L/N434S	1.55E+06	3.46E-01	2.23E-07	1.62E+01
15-22	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-23		L309D/Q311H/N434S	NB	NB	NB	NB
15-24	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-49			1.65E+06	2.89E-01	1.75E-07	2.27E+01
15-50			NB	NB	NB	NB
15-51		M252Y/S254T/T256E	NB	NB	NB	NB
15-52		M252Y/S254T/T256E	NB	NB	NB	NB
15-53		M428L/N434S	NB	NB	NB	NB
15-54		M428L/N434S	NB	NB	NB	NB
15-56		L309D/Q311H/N434S	NB	NB	NB	NB

Table 35D

Protein ID	FcR Domain Type D1/D2	FcRn Domain Type D1/D2	FcγRIIIa 158 F _ CD 16 a 158 F			
			ka (M ⁻¹ s ⁻¹)	kd (s ⁻¹)	KD (M)	Rmax (RU)
15-01	L234A/L235A/P329G		NB	NB	NB	NB
15-02	L234A/L235A/P329G		NB	NB	NB	NB
15-03	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-04	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-05	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-06	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-07	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-08	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-09			6.98E+04	1.13E-01	1.61E-06	4.14E+01
15-10	L234A/L235A/P329G		NB	NB	NB	NB
15-11		M252Y/S254T/T256E	NB	NB	NB	NB
15-12	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-13		M428L/N434S	5.92E+04	4.97E-02	8.40E-07	4.41E+01
15-14	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-15		L309D/Q311H/N434S	6.04E+04	6.92E-02	1.15E-06	4.81E+01
15-16	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-17			NB	NB	NB	NB
15-18	L234A/L235A/P329G		NB	NB	NB	NB
15-19		M252Y/S254T/T256E	NB	NB	NB	NB
15-20	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-21		M428L/N434S	NB	NB	NB	NB
15-22	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-23		L309D/Q311H/N434S	NB	NB	NB	NB
15-24	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-49			8.90E+04	1.04E-01	1.17E-06	3.38E+01
15-50			NB	NB	NB	NB
15-51		M252Y/S254T/T256E	NB	NB	NB	NB
15-52		M252Y/S254T/T256E	NB	NB	NB	NB
15-53		M428L/N434S	NB	NB	NB	NB
15-54		M428L/N434S	NB	NB	NB	NB
15-56		L309D/Q311H/N434S	NB	NB	NB	NB

Table 35E

Protein ID	FcR Domain Type D1/D2	FcRn Domain Type D1/D2	FcγRIIb/c_CD32b/c FcγRIIIa 158 V _ CD 16 a 158 V			
			ka (M ⁻¹ s ⁻¹)	kd (s ⁻¹)	KD (M)	Rmax (RU)
15-01	L234A/L235A/P329G		NB	NB	NB	NB
15-02	L234A/L235A/P329G		NB	NB	NB	NB
15-03	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-04	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-05	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-06	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-07	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-08	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-09			1.05E+05	3.33E-02	3.16E-07	3.79E+01
15-10	L234A/L235A/P329G		NB	NB	NB	NB
15-11		M252Y/S254T/T256E	NB	NB	NB	NB
15-12	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-13		M428L/N434S	9.51E+04	2.02E-02	2.13E-07	4.58E+01
15-14	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-15		L309D/Q311H/N434S	8.55E+04	2.52E-02	2.95E-07	5.12E+01
15-16	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-17			NB	NB	NB	NB
15-18	L234A/L235A/P329G		NB	NB	NB	NB
15-19		M252Y/S254T/T256E	NB	NB	NB	NB
15-20	L234A/L235A/P329G	M252Y/S254T/T256E	NB	NB	NB	NB
15-21		M428L/N434S	1.78E+05	3.90E-02	2.19E-07	2.00E+01
15-22	L234A/L235A/P329G	M428L/N434S	NB	NB	NB	NB
15-23		L309D/Q311H/N434S	NB	NB	NB	NB
15-24	L234A/L235A/P329G	L309D/Q311H/N434S	NB	NB	NB	NB
15-49			1.70E+05	3.62E-02	2.13E-07	3.40E+01
15-50			NB	NB	NB	NB
15-51		M252Y/S254T/T256E	NB	NB	NB	NB
15-52		M252Y/S254T/T256E	NB	NB	NB	NB
15-53		M428L/N434S	NB	NB	NB	NB
15-54		M428L/N434S	NB	NB	NB	NB
15-56		L309D/Q311H/N434S	NB	NB	NB	NB

Example 16 – SARS-CoV-2 Variant Neutralization Studies

[0483] Tetrahedral antibody constructs 15-15 and 15-16 (ACE2-B13A), 15-23 and 15-24 (ACE2-O24), 15-07 and 15-53 (ACE2-ACE2), and 10-61 and 10-65 (ACE2 core dimer as shown in Figure 43) were evaluated for activity against a range of antibody-resistant variants of SARS-CoV-2 using a pseudovirus neutralization assay (Integral Molecular, Philadelphia, PA). The 10-61 and 10-65 constructs incorporated the 313 affinity enhancing mutations described in Glasgow et al. PNAS 117:28046-28055 (2020)). Each construct was evaluated against eleven SARS-CoV-2 variants and one SARS-CoV-1 strain, and compared side-by-side with the parent antibodies B13A and O24, and biosimilar versions of eight antibodies that have received Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration (FDA) or are in late-stage clinical trials: REGN10897, REGN10933, Bamlanivimab, Etesevimab, AZD1061, AZD 8895, VIR-7841, and CT-P59.

[0484] The SARS-CoV-2 variants tested were D614G B.1 (RVP-702), Californian B.1.427/B.1.429 (RVP-713), Indian B.1.617.2 (RVP-763), South African B.1.351 (RVP-707), South African Δ B.1.351 (RVP-724), N439K (RVP-703), Brazilian P.1 (RVP-708), Nigerian/European B.1.525 (RVP-723), New York B.1.526 (RVP-726), UK B.1.1.7 (RVP-706), and UK B.1.1.7 with E484K (RVP-717). The SARS-CoV-1 variant tested was Urbani (RVP-801). The Integral Molecular catalog numbers are indicated in parentheses.

[0485] The results are shown in Figures 48A-48P. Notably, the tetrahedral antibody constructs HB1515 and HB1516 were effective against all eleven SARS-CoV-2 variants as well as SARS-CoV-1. By contrast, significant viral resistance was observed for all ten antibodies with at least two of the SARS-CoV-2 variants. In addition, HB1515 and HB1516 were at least as potent as all ten of the antibodies tested (with the exception of CT-P59 against the D614G variant).

[0486] Methods: SARS-CoV-2 pseudovirus neutralization assay. The neutralization assay was carried out according to the manufacturers' protocols. In brief, serially diluted antibodies were incubated with pseudotyped SARS-CoV-2-Renilla Luciferase for 1 hr. at 37 °C. At least nine concentrations were tested for each antibody. Pseudovirus in culture media without antibody was used as a negative control to determine 100% infectivity. The mixtures were then incubated with 293T-hsACE2 cells at 2.5×10^5 cells/ml in the 96-well plates. Infection took place over approximately 72 hrs. at 37 °C with 5% CO₂. The luciferase signal was measured using the

Renilla-Glo luciferase assay system (Promega, Cat# E2710) with the luminometer set at 1 ms integration time. The obtained relative luminescence signals (RLU) from the negative control wells were normalized and used to calculate the neutralization percentage for each concentration. These data were processed by Prism 9 (GraphPad) to fit a 4PL curve and calculate the log IC₅₀.

Example 17 - Efficacy of Tetrahedral Antibody HB1516 in the Hamster Covid-19 model

[0487] A leading animal model for the study of SARS-CoV-2 utilizes Golden Syrian Hamsters to study the pathogenicity and test therapeutics or vaccines. Prior studies using this model have found that SARS-CoV-2 infection of hamsters results in mild to moderate clinical signs (rapid breathing, weight loss, high titers of virus in the upper and lower respiratory tract and histological changes in the lungs) showing similar infectivity and pathology characteristics between hamsters and humans. HB1516 (protein 15-16) was evaluated in a prophylactic version of this model to determine its efficacy in protecting against challenge using the South African variant of SARS-CoV-2 and compared with REGN-CoV-2, a neutralizing antibody cocktail (Regeneron, Tarrytown, N.Y.) consisting of REGN10987 (protein 12-01) and REGN10933 (protein 12-01), which has received an Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration (FDA) and previously demonstrated efficacy in the hamsters (Baum et al, Science 370, 1110–1115 (2020)).

[0488] Materials. The South African variant of SARS-CoV-2 virus used for infection was sourced from BEI Resources, American Type Culture Collection (Manassas, VA): Catalog No. NR-54974 (hCoV-19/South Africa/KRISP-K005325/2020). This SARS-CoV-2 variant has the following amino acid mutations in its spike (S) protein with reference to the sequence of the Wuhan-1 isolate (NCBI Reference Sequence: NC_045512.2): L18F, D80A, D215A, L242/A243/L244 deletion, K417N, E484K, N501Y, D614G, A701V. The biosimilar versions of REGN10987 and REGN10933 used in this study were produced at Lakepharma Inc. (Belmont, CA).

[0489] Methods. The study was conducted at BIOQUAL, Inc. (Rockville, MD). A total of 24 male golden hamsters 6-8 weeks old were assigned to four groups (n=6). Animals were treated with the appropriate material for their group on study day minus one (-1) via the intraperitoneal (IP) route. Test articles were prepared in Dulbecco's Phosphate-Buffered Saline (ATCC No. 30-2020) and administered at the following concentrations: HB1516 (25mg/kg), REGN10933 (25mg/kg), and REGN-CoV-2 (25mg/kg each of REGN10987 and REGN10933).

[0490] On study day 0, animals were bled pre-challenge, followed by intranasal challenge with the South Africa SARS-CoV-2 strain. Animals were observed twice daily in the post-challenge phase and their weights were collected daily.

[0491] Results. As shown in Figure 49, the PBS negative control group lost weight steadily following infection and by day 7 had lost approximately 18% of their starting weight. The HB1516 treated group maintained their weight throughout the study and by day 7 had gained approximately 3% of their starting weight (HB1516 vs. PBS control: p value <0.0001), comparable to results for the REGN-CoV-2 treated group (HB1516 vs. REGN-CoV: p value 0.9019). By contrast, the REGN10933 treated group had lost about 7% of their weight through day 5.

Example 18 – Tetrahedral Antibodies Comprising Fab Domains of Two Distinct Binding Specificities for SARS-CoV-2 Spike Protein

[0492] To provide proof-of-principle demonstrating tetrahedral antibodies comprising two distinct types of Fab domain that specifically bind two different targets, two series of constructs were generated with the predicted structure shown in Panel C of Figure 32, according to Table 36, then evaluated for their structural and functional bispecificity. The first series of constructs employed VH and VL regions from REGN10987 (SEQ ID NOs: 421-422) and REGN10933 (SEQ ID NOs: 423-424); the second series of constructs employed VH and VL regions from B13A (SEQ ID NOs: 463-464) and O24A (SEQ ID NOs: 467-468) (Tables 36 and 37).

[0493] To optimize correct VH/VL pairing and to minimize VH/VL mispairing, V region exchange was combined with electrostatic steering (Table 38). Three sets of electrostatically matched mutations were employed. In each series of constructs V region exchange was evaluated in both orientations, e.g., REGN10987/REGN10933 and REGN10933/REGN10987. In each orientation the V regions of the D5/D6 domains were exchanged. Heterodimerization of the H1 and H2 chains was achieved using the Knob-into-Hole approach (knob: T366W/S354C, hole: T366/L368A/Y407V/Y349C). To further facilitate purification of the correctly paired product by Protein A chromatography, either of the H1 or the H2 chain incorporated the H435R/Y436F substitution (Table 37).

[0494] The constructs were evaluated for structural bispecificity by intact mass spectrometry. Following removal of N-glycan and O-glycan using Protein Deglycosylation Mix II (NEB, Ipswich, MA), samples were analyzed under non-reducing, denaturing conditions by LS/MS on a

Maxis II UHR QTOF instrument following size-exclusion chromatography (Bruker, Billerica, MA). One to five ug of each sample was injected at a flowrate of 20 uL in a mobile phase consisting of a 10 min gradient of water with 180 mM ammonium acetate at room temperature. A full scan MS acquisition method was used with a mass resolution of 10,000.

[0495] Following deconvolution, the intensity of the signal was determined for the correctly VH/VL paired tetrahedral antibody (L1L2H1H2) and the two mispaired side-products (L1L1H1H2, L2L2H1H2). Results were obtained for 16 of the constructs (Figure 50A-50C). No significant mispairing was detected for the H1/H2 chains. The percentage of the main product and side products was calculated and normalized to the total yield of the correct and incorrect products (Table 39). The yield of the correctly paired VH/VL construct (L1L2H1H2) ranged from 98.0 to 99.7%; the yield of the correctly paired VH/VL for four of the constructs was greater than 99.5%.

Table 36

Protein ID	L1 Chain	H1 Chain	L2 Chain	H2 Chain
17-01	SEQ ID NO: 576	SEQ ID NO: 556	SEQ ID NO: 577	SEQ ID NO: 557
17-02	SEQ ID NO: 578	SEQ ID NO: 558	SEQ ID NO: 579	SEQ ID NO: 559
17-03	SEQ ID NO: 578	SEQ ID NO: 558	SEQ ID NO: 580	SEQ ID NO: 560
17-04	SEQ ID NO: 581	SEQ ID NO: 561	SEQ ID NO: 582	SEQ ID NO: 562
17-05	SEQ ID NO: 583	SEQ ID NO: 563	SEQ ID NO: 584	SEQ ID NO: 564
17-06	SEQ ID NO: 583	SEQ ID NO: 563	SEQ ID NO: 585	SEQ ID NO: 565
17-07	SEQ ID NO: 586	SEQ ID NO: 556	SEQ ID NO: 577	SEQ ID NO: 557
17-08	SEQ ID NO: 587	SEQ ID NO: 558	SEQ ID NO: 579	SEQ ID NO: 559
17-09	SEQ ID NO: 587	SEQ ID NO: 558	SEQ ID NO: 580	SEQ ID NO: 560
17-10	SEQ ID NO: 581	SEQ ID NO: 561	SEQ ID NO: 588	SEQ ID NO: 562
17-11	SEQ ID NO: 583	SEQ ID NO: 563	SEQ ID NO: 589	SEQ ID NO: 564
17-12	SEQ ID NO: 583	SEQ ID NO: 563	SEQ ID NO: 590	SEQ ID NO: 565
17-13	SEQ ID NO: 576	SEQ ID NO: 566	SEQ ID NO: 577	SEQ ID NO: 567
17-14	SEQ ID NO: 578	SEQ ID NO: 568	SEQ ID NO: 579	SEQ ID NO: 569
17-15	SEQ ID NO: 578	SEQ ID NO: 568	SEQ ID NO: 580	SEQ ID NO: 570
17-16	SEQ ID NO: 581	SEQ ID NO: 571	SEQ ID NO: 582	SEQ ID NO: 572
17-17	SEQ ID NO: 583	SEQ ID NO: 573	SEQ ID NO: 584	SEQ ID NO: 574
17-18	SEQ ID NO: 583	SEQ ID NO: 573	SEQ ID NO: 585	SEQ ID NO: 575
17-19	SEQ ID NO: 586	SEQ ID NO: 566	SEQ ID NO: 577	SEQ ID NO: 567
17-20	SEQ ID NO: 587	SEQ ID NO: 568	SEQ ID NO: 579	SEQ ID NO: 569
17-21	SEQ ID NO: 587	SEQ ID NO: 568	SEQ ID NO: 580	SEQ ID NO: 570
17-22	SEQ ID NO: 581	SEQ ID NO: 571	SEQ ID NO: 588	SEQ ID NO: 572
17-23	SEQ ID NO: 583	SEQ ID NO: 573	SEQ ID NO: 589	SEQ ID NO: 574
17-24	SEQ ID NO: 583	SEQ ID NO: 573	SEQ ID NO: 590	SEQ ID NO: 575
17-25	SEQ ID NO: 631	SEQ ID NO: 591	SEQ ID NO: 632	SEQ ID NO: 592
17-26	SEQ ID NO: 633	SEQ ID NO: 593	SEQ ID NO: 634	SEQ ID NO: 594
17-27	SEQ ID NO: 633	SEQ ID NO: 593	SEQ ID NO: 635	SEQ ID NO: 595
17-28	SEQ ID NO: 631	SEQ ID NO: 596	SEQ ID NO: 632	SEQ ID NO: 597
17-29	SEQ ID NO: 633	SEQ ID NO: 598	SEQ ID NO: 634	SEQ ID NO: 599
17-30	SEQ ID NO: 633	SEQ ID NO: 598	SEQ ID NO: 635	SEQ ID NO: 600
17-31	SEQ ID NO: 636	SEQ ID NO: 601	SEQ ID NO: 637	SEQ ID NO: 602
17-32	SEQ ID NO: 638	SEQ ID NO: 603	SEQ ID NO: 639	SEQ ID NO: 604
17-33	SEQ ID NO: 638	SEQ ID NO: 603	SEQ ID NO: 640	SEQ ID NO: 605
17-34	SEQ ID NO: 636	SEQ ID NO: 606	SEQ ID NO: 637	SEQ ID NO: 607
17-35	SEQ ID NO: 638	SEQ ID NO: 608	SEQ ID NO: 639	SEQ ID NO: 609
17-36	SEQ ID NO: 638	SEQ ID NO: 608	SEQ ID NO: 640	SEQ ID NO: 610
17-37	SEQ ID NO: 631	SEQ ID NO: 611	SEQ ID NO: 632	SEQ ID NO: 612
17-38	SEQ ID NO: 633	SEQ ID NO: 613	SEQ ID NO: 634	SEQ ID NO: 614
17-39	SEQ ID NO: 633	SEQ ID NO: 613	SEQ ID NO: 635	SEQ ID NO: 615
17-40	SEQ ID NO: 631	SEQ ID NO: 616	SEQ ID NO: 632	SEQ ID NO: 617
17-41	SEQ ID NO: 633	SEQ ID NO: 618	SEQ ID NO: 634	SEQ ID NO: 619
17-42	SEQ ID NO: 633	SEQ ID NO: 618	SEQ ID NO: 635	SEQ ID NO: 620
17-43	SEQ ID NO: 636	SEQ ID NO: 621	SEQ ID NO: 637	SEQ ID NO: 622
17-44	SEQ ID NO: 638	SEQ ID NO: 623	SEQ ID NO: 639	SEQ ID NO: 624
17-45	SEQ ID NO: 638	SEQ ID NO: 623	SEQ ID NO: 640	SEQ ID NO: 625
17-46	SEQ ID NO: 636	SEQ ID NO: 626	SEQ ID NO: 637	SEQ ID NO: 627
17-47	SEQ ID NO: 638	SEQ ID NO: 628	SEQ ID NO: 639	SEQ ID NO: 629
17-48	SEQ ID NO: 638	SEQ ID NO: 628	SEQ ID NO: 640	SEQ ID NO: 630

Table 37

Protein ID	D1-Fc			D5-Fab	D4-Fab	D3-Fab	D6-Fab	D2-Fc		
	FcRn	H1	H2					FcRn	H1	H2
17-01	DHS	RF	HY	REGN10933	REGN10987	REGN10987	REGN10933	DHS	RF	HY
17-02	DHS	RF	HY	REGN10933	REGN10987	REGN10987	REGN10933	DHS	RF	HY
17-03	DHS	RF	HY	REGN10933	REGN10987	REGN10987	REGN10933	DHS	RF	HY
17-04	DHS	RF	HY	REGN10987	REGN10933	REGN10933	REGN10987	DHS	RF	HY
17-05	DHS	RF	HY	REGN10987	REGN10933	REGN10933	REGN10987	DHS	RF	HY
17-06	DHS	RF	HY	REGN10987	REGN10933	REGN10933	REGN10987	DHS	RF	HY
17-07	DHS	RF	HY	REGN10933	REGN10987	REGN10987	REGN10933	DHS	RF	HY
17-08	DHS	RF	HY	REGN10933	REGN10987	REGN10987	REGN10933	DHS	RF	HY
17-09	DHS	RF	HY	REGN10933	REGN10987	REGN10987	REGN10933	DHS	RF	HY
17-10	DHS	RF	HY	REGN10987	REGN10933	REGN10933	REGN10987	DHS	RF	HY
17-11	DHS	RF	HY	REGN10987	REGN10933	REGN10933	REGN10987	DHS	RF	HY
17-12	DHS	RF	HY	REGN10987	REGN10933	REGN10933	REGN10987	DHS	RF	HY
17-13	DHS	HY	RF	REGN10933	REGN10987	REGN10987	REGN10933	DHS	HY	RF
17-14	DHS	HY	RF	REGN10933	REGN10987	REGN10987	REGN10933	DHS	HY	RF
17-15	DHS	HY	RF	REGN10933	REGN10987	REGN10987	REGN10933	DHS	HY	RF
17-16	DHS	HY	RF	REGN10987	REGN10933	REGN10933	REGN10987	DHS	HY	RF
17-17	DHS	HY	RF	REGN10987	REGN10933	REGN10933	REGN10987	DHS	HY	RF
17-18	DHS	HY	RF	REGN10987	REGN10933	REGN10933	REGN10987	DHS	HY	RF
17-19	DHS	HY	RF	REGN10933	REGN10987	REGN10987	REGN10933	DHS	HY	RF
17-20	DHS	HY	RF	REGN10933	REGN10987	REGN10987	REGN10933	DHS	HY	RF
17-21	DHS	HY	RF	REGN10933	REGN10987	REGN10987	REGN10933	DHS	HY	RF
17-22	DHS	HY	RF	REGN10987	REGN10933	REGN10933	REGN10987	DHS	HY	RF
17-23	DHS	HY	RF	REGN10987	REGN10933	REGN10933	REGN10987	DHS	HY	RF
17-24	DHS	HY	RF	REGN10987	REGN10933	REGN10933	REGN10987	DHS	HY	RF
17-25	wt	RF	HY	O24A	B13A	B13A	O24A	wt	RF	HY
17-26	wt	RF	HY	O24A	B13A	B13A	O24A	wt	RF	HY
17-27	wt	RF	HY	O24A	B13A	B13A	O24A	wt	RF	HY
17-28	DHS	RF	HY	O24A	B13A	B13A	O24A	DHS	RF	HY
17-29	DHS	RF	HY	O24A	B13A	B13A	O24A	DHS	RF	HY
17-30	DHS	RF	HY	O24A	B13A	B13A	O24A	DHS	RF	HY
17-31	wt	RF	HY	B13A	O24A	O24A	B13A	wt	RF	HY
17-32	wt	RF	HY	B13A	O24A	O24A	B13A	wt	RF	HY
17-33	wt	RF	HY	B13A	O24A	O24A	B13A	wt	RF	HY
17-34	DHS	RF	HY	B13A	O24A	O24A	B13A	DHS	RF	HY
17-35	DHS	RF	HY	B13A	O24A	O24A	B13A	DHS	RF	HY
17-36	DHS	RF	HY	B13A	O24A	O24A	B13A	DHS	RF	HY
17-37	wt	HY	RF	O24A	B13A	B13A	O24A	wt	HY	RF
17-38	wt	HY	RF	O24A	B13A	B13A	O24A	wt	HY	RF
17-39	wt	HY	RF	O24A	B13A	B13A	O24A	wt	HY	RF
17-40	DHS	HY	RF	O24A	B13A	B13A	O24A	DHS	HY	RF
17-41	DHS	HY	RF	O24A	B13A	B13A	O24A	DHS	HY	RF
17-42	DHS	HY	RF	O24A	B13A	B13A	O24A	DHS	HY	RF
17-43	wt	HY	RF	B13A	O24A	O24A	B13A	wt	HY	RF
17-44	wt	HY	RF	B13A	O24A	O24A	B13A	wt	HY	RF
17-45	wt	HY	RF	B13A	O24A	O24A	B13A	wt	HY	RF
17-46	DHS	HY	RF	B13A	O24A	O24A	B13A	DHS	HY	RF
17-47	DHS	HY	RF	B13A	O24A	O24A	B13A	DHS	HY	RF
17-48	DHS	HY	RF	B13A	O24A	O24A	B13A	DHS	HY	RF

Table 38

Protein ID	L1 Chain		H1 Chain		L2 Chain		H2 Chain	
	V region type	Amino acid Substitutions	V region Type	Amino acid Substitutions	V region Type	Amino acid Substitutions	V region Type	Amino acid Substitutions
17-01	VL-CL	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-02	VL-CL	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-03	VL-CL	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-04	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CL		VL-CH1	
17-05	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CL	Q39K/V133E	VL-CH1	Q38E/S183K
17-06	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CL	Q39E/V133E	VL-CH1	Q38K/S183K
17-07	VL-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-08	VL-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-09	VL-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-10	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VL-CH1	
17-11	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VL-CH1	Q38E/S183K
17-12	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VL-CH1	Q38K/S183K
17-13	VL-CL	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-14	VL-CL	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-15	VL-CL	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-16	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CL		VL-CH1	
17-17	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CL	Q39K/V133E	VL-CH1	Q38E/S183K
17-18	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CL	Q39E/V133E	VL-CH1	Q38K/S183K
17-19	VL-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-20	VL-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-21	VL-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-22	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VL-CH1	
17-23	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VL-CH1	Q38E/S183K
17-24	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VL-CH1	Q38K/S183K
17-25	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-26	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-27	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-28	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-29	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-30	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-31	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-32	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-33	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-34	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-35	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-36	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-37	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-38	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-39	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-40	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-41	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-42	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-43	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-44	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-45	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
17-46	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
17-47	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
17-48	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K

Table 39

Protein ID	L1L1H1H2 (%)	L1L2H1H2 (%)	L2L2H1H2 (%)	Delta, Da	Theoretical Average Intact Mass, Da		
					L1L1H1H2	L1L2H1H2	L2L2H1H2
17-01	0.2	99.7	0.1	2027	160023	162050	164077
17-02	0.4	99.6	0.0	2053	160113	162166	164220
17-03	0.8	99.2	0.0	2054	160112	162166	164221
17-04	0.0	98.0	2.0	1021	161042	162063	163084
17-07	0.0	99.6	0.4	1498	161082	162580	164077

Protein ID	L1L1H1H2 (%)	L1L2H1H2 (%)	L2L2H1H2 (%)	Delta, Da	Theoretical Average Intact Mass, Da		
					L1L1H1H2	L1L2H1H2	L2L2H1H2
17-08	0.3	98.6	1.1	1525	161170	162695	163220
17-09	0.9	98.4	0.7	1526	161169	162695	164221
17-10	0.3	98.6	1.1	1481	161042	162523	164003
17-11	0.3	99.1	0.5	1535	161130	162665	164200
17-12	0.3	98.9	0.8	1536	161129	162655	164200
17-19	0.5	99.2	0.3	1498	161082	162580	164077
17-20	0.2	99.3	0.5	1525	161170	162695	164220
17-21	0.3	99.5	0.2	1526	161169	162695	164221
17-22	0.4	99.0	0.6	1481	161042	162523	164003
17-23	0.3	98.5	1.2	1535	161130	162665	164200
17-24	0.4	98.9	0.7	1536	161129	162665	164200

Example 19 – Neutralization of SARS-CoV-2 Pseudovirus by Bispecific Tetrahedral Antibodies

[0496] The tetrahedral antibody constructs of Example 18 were evaluated for their functional bispecificity using a SARS-CoV-2 pseudovirus neutralization assay (Integral Molecular, Philadelphia, PA) as described in Example 18. Each construct was evaluated against two SARS-CoV-2 variants: an N439L variant highly resistant to REGN10987 (genotype N439K, D614G; Catalog # RVP-703,) and a South African Δ3 B.1.351 variant highly resistant to REGN10933 (genotype L18F, D80A, D215G, ΔL242/L243/L244, R246I, K417N, N501Y, E484K, A701V; Catalog #RVP-724). The activity each of the bispecific constructs was directly compared with the parent antibodies REGN10987 and REN10933 as well as REGN-CoV-2, a 1:1 cocktail of REGN10987 and REGN10933. The biosimilar versions of REGN10987, REGN10933 and REGN-CoV-2 used in this study were produced at Lakepharma Inc. (Belmont, CA).

[0497] Results were obtained for 18 of the constructs (Figure 51A-51C, Table 40). With respect to the control constructs, REGN10987 was approximately 1,000-fold less potent than REGN10933 against the N439K variant, and REGN10933 was approximately 3,000-fold less potent than REGN10987 against the South African variant. The REGN-CoV-2 cocktail was approximately 1.5-fold more potent than REGN10933 against the N439 variant and approximately equal in potency to REGB10987 against the South African variant. All 18 of the tetravalent, bispecific tetrahedral antibody constructs potently neutralized both the N439K and South African variants; 15 of the 18 bispecific constructs were at least as potent as the REGN-CoV-2 cocktail against the N43K variant, and 14 of the 18 bispecific constructs were approximately two- to three-fold more potent than the REGN-CoV-2 cocktail against the South African variant.

Table 40

Protein ID	EC50 (pM) Neutralization		D5-Fab	D4-Fab	D3-Fab	D6-Fab
	N439K	South Africa B.1.351				
REGN10987	2,256.46	2.48				
REGN10987	2,216.82	2.37				
REGN10933	2.09	7,776.27				
REGN10933	1.80	8,548.99				
REGN-CoV2	1.18	1.87				
REGN-CoV2	1.44	2.91				
17-01	1.10	0.82	REGN10933	REGN10987	REGN10987	REGN10933
17-02	1.08	1.19	REGN10933	REGN10987	REGN10987	REGN10933
17-03	1.25	1.29	REGN10933	REGN10987	REGN10987	REGN10933
17-04	0.87	1.25	REGN10987	REGN10933	REGN10933	REGN10987
17-05	1.88	3.88	REGN10987	REGN10933	REGN10933	REGN10987
17-06	2.51	5.81	REGN10987	REGN10933	REGN10933	REGN10987
17-07	0.92	0.84	REGN10933	REGN10987	REGN10987	REGN10933
17-08	1.03	0.97	REGN10933	REGN10987	REGN10987	REGN10933
17-09	1.16	1.59	REGN10933	REGN10987	REGN10987	REGN10933
17-10	0.96	0.75	REGN10987	REGN10933	REGN10933	REGN10987
17-11	0.92	0.90	REGN10987	REGN10933	REGN10933	REGN10987
17-12	2.22	2.53	REGN10987	REGN10933	REGN10933	REGN10987
17-19	1.11	0.86	REGN10933	REGN10987	REGN10987	REGN10933
17-20	1.43	0.98	REGN10933	REGN10987	REGN10987	REGN10933
17-21	1.07	1.24	REGN10933	REGN10987	REGN10987	REGN10933
17-22	0.94	0.90	REGN10987	REGN10933	REGN10933	REGN10987
17-23	0.88	0.98	REGN10987	REGN10933	REGN10933	REGN10987
17-24	1.11	0.96	REGN10987	REGN10933	REGN10933	REGN10987

Example 20 – Tetravalent, Bispecific Tetrahedral Antibodies Comprising Two Distinct Fabs that Specifically Bind CD38, BCMA, CD16 or CD3

[0498] The exceptional activity of tetravalent, bispecific tetrahedral antibodies against SARS-CoV-2 suggests their broad applicability in other fields such as cancer in which there is intense interest among investigators in the potential of multispecific targeting agents. Accordingly, three series of constructs were generated with the predicted structures shown in panel D of Figure 31, panel C of Figure 32, and panel D of Figure 34, then evaluated for their bispecificity.

[0499] The first series of constructs employed VH and VL regions from daratumumab (Da), an FDA-approved anti-CD38 antibody used to target and treat multiple myeloma (SEQ ID Nos 641, 686), HA9, an anti-CD16 antibody that specifically targets and engages Natural Killer (NK) cells (SEQ ID Nos 648, 688), and SP34, an anti-CD3 antibody that specifically targets and engages T cells (SEQ ID Nos 657, 697). The second series of constructs additionally employed VH and VL regions from 4C8, an anti-BCMA antibody that targets myeloma cells and certain other cell types (SEQ ID Nos 663, 700). The third series of constructs additionally employed VH and VL regions from B34, an anti-BCMA antibody that targets myeloma cells and certain other cell types (SEQ ID Nos 676, 703) (Tables 41, 42, 43)

[0500] To optimize correct VH/VL pairing and to minimize VH/VL mispairing, V region exchange was combined with electrostatic steering as described in Example 18 (Table 44). Additionally, to further facilitate purification of the correctly paired product by Protein A chromatography, in some constructs, particularly those having the predicted structures shown in Figure 31, Panel D and Figure 32 Panel C, the H1 chain incorporated the H435R/Y436F substitution while the H2 chain had the H435/Y436 wild-type sequence. In other constructs, particularly those having the predicted structure shown in Figure 34, Panel D, the H1 and H2 chains incorporated the H435R/Y436F substitution while the Fc chain had the H435/Y436 wild-type sequence (Table 42).

[0501] The constructs were evaluated for structural bispecificity by intact mass spectrometry, following removal of N-glycan and O-glycan, as described in Example 18. Results were obtained for 18 of the constructs (Table 45). No significant mispairing was detected for the H1/H2 chains. The percentage of the main product and side products was calculated and normalized to the total yield of the correct and incorrect products (Table 45). The yield of the correctly paired VH/VL

construct (L1L2H1H2) ranged from 94.6 to 98.2% when daratumumab was combined with HA9, 94.3 to 97.7% when 4C8 was combined with HA9, and 96.8 to 99.5% when 4C8 was combined with daratumumab. For constructs 18-30, 18-31 and 18-32, two bispecific products were detected by LC/MS as expected for the predicted structure shown in Figure 34, Panel D, and consistent with the denaturing conditions of the LC/MS method that was employed. The first structure corresponds to the D2/D4/D6 portion of the tetrahedral antibody; the second structure corresponds to the D1/D3 portion of the tetrahedral antibody. The yield of the correctly paired VH/VL construct contained within the D2/D4/D6 portion (L1L2H1H2) ranged from 97.1 to 99.5%; the yield of the correctly paired VH/VL construct contained within the D1/D3 portion (L1H1Fc) ranged from 99.4 to 99.8%.

Table 41

Protein ID	L1 Chain	H1 Chain	L2 Chain	H2 Chain	Fc Chain
18-01	SEQ ID NO: 686	SEQ ID NO: 641			
18-02	SEQ ID NO: 686	SEQ ID NO: 642			
18-03	SEQ ID NO: 686	SEQ ID NO: 643	SEQ ID NO: 686	SEQ ID NO: 644	
18-04	SEQ ID NO: 686	SEQ ID NO: 645	SEQ ID NO: 686	SEQ ID NO: 646	
18-05	SEQ ID NO: 687	SEQ ID NO: 647	SEQ ID NO: 688	SEQ ID NO: 648	
18-06	SEQ ID NO: 689	SEQ ID NO: 649	SEQ ID NO: 690	SEQ ID NO: 650	
18-07	SEQ ID NO: 689	SEQ ID NO: 649	SEQ ID NO: 691	SEQ ID NO: 651	
18-08	SEQ ID NO: 692	SEQ ID NO: 652	SEQ ID NO: 693	SEQ ID NO: 653	
18-09	SEQ ID NO: 694	SEQ ID NO: 654	SEQ ID NO: 695	SEQ ID NO: 655	
18-10	SEQ ID NO: 694	SEQ ID NO: 654	SEQ ID NO: 696	SEQ ID NO: 656	
18-11	SEQ ID NO: 687	SEQ ID NO: 647	SEQ ID NO: 697	SEQ ID NO: 657	
18-12	SEQ ID NO: 689	SEQ ID NO: 649	SEQ ID NO: 698	SEQ ID NO: 658	
18-13	SEQ ID NO: 689	SEQ ID NO: 649	SEQ ID NO: 699	SEQ ID NO: 659	
18-14	SEQ ID NO: 687	SEQ ID NO: 647	SEQ ID NO: 697	SEQ ID NO: 660	SEQ ID NO: 706
18-15	SEQ ID NO: 689	SEQ ID NO: 649	SEQ ID NO: 698	SEQ ID NO: 661	SEQ ID NO: 706
18-16	SEQ ID NO: 689	SEQ ID NO: 649	SEQ ID NO: 699	SEQ ID NO: 662	SEQ ID NO: 706
18-17	SEQ ID NO: 700	SEQ ID NO: 663			
18-18	SEQ ID NO: 700	SEQ ID NO: 664			
18-19	SEQ ID NO: 700	SEQ ID NO: 665	SEQ ID NO: 700	SEQ ID NO: 666	
18-20	SEQ ID NO: 700	SEQ ID NO: 667	SEQ ID NO: 700	SEQ ID NO: 668	
18-21	SEQ ID NO: 701	SEQ ID NO: 669	SEQ ID NO: 688	SEQ ID NO: 648	
18-22	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 690	SEQ ID NO: 650	
18-23	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 691	SEQ ID NO: 651	
18-24	SEQ ID NO: 701	SEQ ID NO: 671	SEQ ID NO: 693	SEQ ID NO: 672	
18-25	SEQ ID NO: 702	SEQ ID NO: 673	SEQ ID NO: 695	SEQ ID NO: 674	
18-26	SEQ ID NO: 702	SEQ ID NO: 673	SEQ ID NO: 696	SEQ ID NO: 675	
18-27	SEQ ID NO: 701	SEQ ID NO: 669	SEQ ID NO: 693	SEQ ID NO: 653	
18-28	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 695	SEQ ID NO: 655	
18-29	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 696	SEQ ID NO: 656	
18-30	SEQ ID NO: 701	SEQ ID NO: 669	SEQ ID NO: 693	SEQ ID NO: 653	SEQ ID NO: 706
18-31	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 695	SEQ ID NO: 655	SEQ ID NO: 706
18-32	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 696	SEQ ID NO: 656	SEQ ID NO: 706
18-33	SEQ ID NO: 701	SEQ ID NO: 669	SEQ ID NO: 697	SEQ ID NO: 657	
18-34	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 698	SEQ ID NO: 658	

Protein ID	L1 Chain	H1 Chain	L2 Chain	H2 Chain	Fc Chain
18-35	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 699	SEQ ID NO: 659	
18-36	SEQ ID NO: 701	SEQ ID NO: 669	SEQ ID NO: 697	SEQ ID NO: 660	SEQ ID NO: 706
18-37	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 698	SEQ ID NO: 661	SEQ ID NO: 706
18-38	SEQ ID NO: 702	SEQ ID NO: 670	SEQ ID NO: 699	SEQ ID NO: 662	SEQ ID NO: 706
18-39	SEQ ID NO: 703	SEQ ID NO: 676			
18-40	SEQ ID NO: 703	SEQ ID NO: 677			
18-41	SEQ ID NO: 703	SEQ ID NO: 678	SEQ ID NO: 703	SEQ ID NO: 679	
18-42	SEQ ID NO: 703	SEQ ID NO: 680	SEQ ID NO: 703	SEQ ID NO: 681	
18-43	SEQ ID NO: 704	SEQ ID NO: 682	SEQ ID NO: 688	SEQ ID NO: 648	
18-44	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 690	SEQ ID NO: 650	
18-45	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 691	SEQ ID NO: 651	
18-46	SEQ ID NO: 704	SEQ ID NO: 684	SEQ ID NO: 693	SEQ ID NO: 672	
18-47	SEQ ID NO: 705	SEQ ID NO: 685	SEQ ID NO: 695	SEQ ID NO: 674	
18-48	SEQ ID NO: 705	SEQ ID NO: 685	SEQ ID NO: 696	SEQ ID NO: 675	
18-49	SEQ ID NO: 704	SEQ ID NO: 682	SEQ ID NO: 693	SEQ ID NO: 653	
18-50	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 695	SEQ ID NO: 655	
18-51	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 696	SEQ ID NO: 656	
18-52	SEQ ID NO: 704	SEQ ID NO: 682	SEQ ID NO: 693	SEQ ID NO: 653	SEQ ID NO: 706
18-53	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 695	SEQ ID NO: 655	SEQ ID NO: 706
18-54	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 696	SEQ ID NO: 656	SEQ ID NO: 706
18-55	SEQ ID NO: 704	SEQ ID NO: 682	SEQ ID NO: 697	SEQ ID NO: 657	
18-56	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 698	SEQ ID NO: 658	
18-57	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 699	SEQ ID NO: 659	
18-58	SEQ ID NO: 704	SEQ ID NO: 682	SEQ ID NO: 697	SEQ ID NO: 660	SEQ ID NO: 706
18-59	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 698	SEQ ID NO: 661	SEQ ID NO: 706
18-60	SEQ ID NO: 705	SEQ ID NO: 683	SEQ ID NO: 699	SEQ ID NO: 662	SEQ ID NO: 706

Table 42

Protein ID	D1-Fc			D5 Fab	D4 Fab	D3 Fab	D6 Fab	D2-Fc		
	Fc Receptor binding	H1	H2					Fc Receptor binding	H1	H2
18-01	wt									
18-02	L234A/L235A/P329G									
18-03	wt	RF	HY	Da	Da	Da	Da	wt	RF	HY
18-04	L234A/L235A/P329G	RF	HY	Da	Da	Da	Da	L234A/L235A/P329G	RF	HY
18-05	L234A/L235A/P329G	RF	HY	HA9	Da	Da	HA9	L234A/L235A/P329G	RF	HY
18-06	L234A/L235A/P329G	RF	HY	HA9	Da	Da	HA9	L234A/L235A/P329G	RF	HY
18-07	L234A/L235A/P329G	RF	HY	HA9	Da	Da	HA9	L234A/L235A/P329G	RF	HY
18-08	L234A/L235A/P329G	RF	HY	Da	HA9	HA9	Da	L234A/L235A/P329G	RF	HY
18-09	L234A/L235A/P329G	RF	HY	Da	HA9	HA9	Da	L234A/L235A/P329G	RF	HY
18-10	L234A/L235A/P329G	RF	HY	Da	HA9	HA9	Da	L234A/L235A/P329G	RF	HY
18-11	L234A/L235A/P329G	RF	HY	SP34	Da	Da	SP34	L234A/L235A/P329G	RF	HY
18-12	L234A/L235A/P329G	RF	HY	SP34	Da	Da	SP34	L234A/L235A/P329G	RF	HY
18-13	L234A/L235A/P329G	RF	HY	SP34	Da	Da	SP34	L234A/L235A/P329G	RF	HY
18-14	L234A/L235A/P329G	RF	HY		Da	Da	SP34	L234A/L235A/P329G	RF	RF
18-15	L234A/L235A/P329G	RF	HY		Da	Da	SP34	L234A/L235A/P329G	RF	RF
18-16	L234A/L235A/P329G	RF	HY		Da	Da	SP34	L234A/L235A/P329G	RF	RF
18-17	wt									
18-18	S239D/I332E									
18-19	wt	RF	HY	4C8	4C8	4C8	4C8	wt	RF	HY
18-20	S239D/I332E	RF	HY	4C8	4C8	4C8	4C8	S239D/I332E	RF	HY
18-21	L234A/L235A/P329G	RF	HY	HA9	4C8	4C8	HA9	L234A/L235A/P329G	RF	HY
18-22	L234A/L235A/P329G	RF	HY	HA9	4C8	4C8	HA9	L234A/L235A/P329G	RF	HY
18-23	L234A/L235A/P329G	RF	HY	HA9	4C8	4C8	HA9	L234A/L235A/P329G	RF	HY
18-24	wt	RF	HY	Da	4C8	4C8	Da	wt	RF	HY
18-25	wt	RF	HY	Da	4C8	4C8	Da	wt	RF	HY
18-26	wt	RF	HY	Da	4C8	4C8	Da	wt	RF	HY
18-27	L234A/L235A/P329G	RF	HY	Da	4C8	4C8	Da	L234A/L235A/P329G	RF	HY
18-28	L234A/L235A/P329G	RF	HY	Da	4C8	4C8	Da	L234A/L235A/P329G	RF	HY
18-29	L234A/L235A/P329G	RF	HY	Da	4C8	4C8	Da	L234A/L235A/P329G	RF	HY
18-30	L234A/L235A/P329G	RF	HY		4C8	4C8	Da	L234A/L235A/P329G	RF	RF
18-31	L234A/L235A/P329G	RF	HY		4C8	4C8	Da	L234A/L235A/P329G	RF	RF
18-32	L234A/L235A/P329G	RF	HY		4C8	4C8	Da	L234A/L235A/P329G	RF	RF
18-33	L234A/L235A/P329G	RF	HY	SP34	4C8	4C8	SP34	L234A/L235A/P329G	RF	HY
18-34	L234A/L235A/P329G	RF	HY	SP34	4C8	4C8	SP34	L234A/L235A/P329G	RF	HY
18-35	L234A/L235A/P329G	RF	HY	SP34	4C8	4C8	SP34	L234A/L235A/P329G	RF	HY
18-36	L234A/L235A/P329G	RF	HY		4C8	4C8	SP34	L234A/L235A/P329G	RF	RF
18-37	L234A/L235A/P329G	RF	HY		4C8	4C8	SP34	L234A/L235A/P329G	RF	RF
18-38	L234A/L235A/P329G	RF	HY		4C8	4C8	SP34	L234A/L235A/P329G	RF	RF
18-39	wt									
18-40	S239D/I332E									
18-41	wt	RF	HY	B34	B34	B34	B34	wt	RF	HY
18-42	S239D/I332E	RF	HY	B34	B34	B34	B34	S239D/I332E	RF	HY
18-43	L234A/L235A/P329G	RF	HY	HA9	B34	B34	HA9	L234A/L235A/P329G	RF	HY
18-44	L234A/L235A/P329G	RF	HY	HA9	B34	B34	HA9	L234A/L235A/P329G	RF	HY
18-45	L234A/L235A/P329G	RF	HY	HA9	B34	B34	HA9	L234A/L235A/P329G	RF	HY
18-46	wt	RF	HY	Da	B34	B34	Da	wt	RF	HY
18-47	wt	RF	HY	Da	B34	B34	Da	wt	RF	HY
18-48	wt	RF	HY	Da	B34	B34	Da	wt	RF	HY
18-49	L234A/L235A/P329G	RF	HY	Da	B34	B34	Da	L234A/L235A/P329G	RF	HY
18-50	L234A/L235A/P329G	RF	HY	Da	B34	B34	Da	L234A/L235A/P329G	RF	HY
18-51	L234A/L235A/P329G	RF	HY	Da	B34	B34	Da	L234A/L235A/P329G	RF	HY
18-52	L234A/L235A/P329G	RF	HY		B34	B34	Da	L234A/L235A/P329G	RF	RF
18-53	L234A/L235A/P329G	RF	HY		B34	B34	Da	L234A/L235A/P329G	RF	RF
18-54	L234A/L235A/P329G	RF	HY		B34	B34	Da	L234A/L235A/P329G	RF	RF
18-55	L234A/L235A/P329G	RF	HY	SP34	B34	B34	SP34	L234A/L235A/P329G	RF	HY
18-56	L234A/L235A/P329G	RF	HY	SP34	B34	B34	SP34	L234A/L235A/P329G	RF	HY
18-57	L234A/L235A/P329G	RF	HY	SP34	B34	B34	SP34	L234A/L235A/P329G	RF	HY
18-58	L234A/L235A/P329G	RF	HY		B34	B34	SP34	L234A/L235A/P329G	RF	RF
18-59	L234A/L235A/P329G	RF	HY		B34	B34	SP34	L234A/L235A/P329G	RF	RF
18-60	L234A/L235A/P329G	RF	HY		B34	B34	SP34	L234A/L235A/P329G	RF	RF

Table 43

Protein ID	D1 Target	D5 Target	D4 Target	D3 Target	D6 Target	D2-Fc	Other
18-01	FcγR						α-CD38 mAb
18-02	Fc silent						α-CD38 mAb
18-03	FcγR	CD38	CD38	CD38	CD38	FcgR	
18-04	Fc silent	CD38	CD38	CD38	CD38	Fc silent	
18-05	Fc silent	CD16	CD38	CD38	CD16	Fc silent	
18-06	Fc silent	CD16	CD38	CD38	CD16	Fc silent	
18-07	Fc silent	CD16	CD38	CD38	CD16	Fc silent	
18-08	Fc silent	CD38	CD16	CD16	CD38	Fc silent	
18-09	Fc silent	CD38	CD16	CD16	CD38	Fc silent	
18-10	Fc silent	CD38	CD16	CD16	CD38	Fc silent	
18-11	Fc silent	CD3	CD38	CD38	CD3	Fc silent	
18-12	Fc silent	CD3	CD38	CD38	CD3	Fc silent	
18-13	Fc silent	CD3	CD38	CD38	CD3	Fc silent	
18-14	Fc silent		CD38	CD38	CD3	Fc silent	
18-15	Fc silent		CD38	CD38	CD3	Fc silent	
18-16	Fc silent		CD38	CD38	CD3	Fc silent	
18-17	FcγR						α-BCMA mAb
18-18	FcγR-high						α-BCMA mAb
18-19	FcγR	BCMA	BCMA	BCMA	BCMA	FcgR	
18-20	FcγR-high	BCMA	BCMA	BCMA	BCMA	FcgR-high	
18-21	Fc silent	CD16	BCMA	BCMA	CD16	Fc silent	
18-22	Fc silent	CD16	BCMA	BCMA	CD16	Fc silent	
18-23	Fc silent	CD16	BCMA	BCMA	CD16	Fc silent	
18-24	FcγR	CD38	BCMA	BCMA	CD38	FcγR	
18-25	FcγR	CD38	BCMA	BCMA	CD38	FcγR	
18-26	FcγR	CD38	BCMA	BCMA	CD38	FcγR	
18-27	Fc silent	CD38	BCMA	BCMA	CD38	Fc silent	
18-28	Fc silent	CD38	BCMA	BCMA	CD38	Fc silent	
18-29	Fc silent	CD38	BCMA	BCMA	CD38	Fc silent	
18-30	Fc silent		BCMA	BCMA	CD38	Fc silent	
18-31	Fc silent		BCMA	BCMA	CD38	Fc silent	
18-32	Fc silent		BCMA	BCMA	CD38	Fc silent	
18-33	Fc silent	CD3	BCMA	BCMA	CD3	Fc silent	
18-34	Fc silent	CD3	BCMA	BCMA	CD3	Fc silent	
18-35	Fc silent	CD3	BCMA	BCMA	CD3	Fc silent	
18-36	Fc silent		BCMA	BCMA	CD3	Fc silent	
18-37	Fc silent		BCMA	BCMA	CD3	Fc silent	
18-38	Fc silent		BCMA	BCMA	CD3	Fc silent	
18-39	FcγR						α-BCMA mAb
18-40	FcγR-high						α-BCMA mAb
18-41	FcγR	BCMA	BCMA	BCMA	BCMA	FcgR	
18-42	FcγR-high	BCMA	BCMA	BCMA	BCMA	FcgR-high	
18-43	Fc silent	CD16	BCMA	BCMA	CD16	Fc silent	
18-44	Fc silent	CD16	BCMA	BCMA	CD16	Fc silent	
18-45	Fc silent	CD16	BCMA	BCMA	CD16	Fc silent	
18-46	FcγR	CD38	BCMA	BCMA	CD38	FcγR	
18-47	FcγR	CD38	BCMA	BCMA	CD38	FcγR	
18-48	FcγR	CD38	BCMA	BCMA	CD38	FcγR	
18-49	Fc silent	CD38	BCMA	BCMA	CD38	Fc silent	
18-50	Fc silent	CD38	BCMA	BCMA	CD38	Fc silent	
18-51	Fc silent	CD38	BCMA	BCMA	CD38	Fc silent	
18-52	Fc silent		BCMA	BCMA	CD38	Fc silent	
18-53	Fc silent		BCMA	BCMA	CD38	Fc silent	
18-54	Fc silent		BCMA	BCMA	CD38	Fc silent	
18-55	Fc silent	CD3	BCMA	BCMA	CD3	Fc silent	
18-56	Fc silent	CD3	BCMA	BCMA	CD3	Fc silent	
18-57	Fc silent	CD3	BCMA	BCMA	CD3	Fc silent	
18-58	Fc silent		BCMA	BCMA	CD3	Fc silent	
18-59	Fc silent		BCMA	BCMA	CD3	Fc silent	
18-60	Fc silent		BCMA	BCMA	CD3	Fc silent	

Table 44

Protein ID	L1 Chain		H1 Chain		L2 Chain		H2 Chain	
	V region type	Amino acid Substitutions	V region Type	Amino acid Substitutions	V region Type	Amino acid Substitutions	V region Type	Amino acid Substitutions
18-01	VK-CK		VH-CH1					
18-02	VK-CK		VH-CH1					
18-03	VK-CK		VH-CH1		VK-CK		VH-CH1	
18-04	VK-CK		VH-CH1		VK-CK		VH-CH1	
18-05	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-06	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-07	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-08	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-09	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-10	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-11	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-12	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-13	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-14	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-15	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-16	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-17	VK-CK		VH-CH1					
18-18	VK-CK		VH-CH1					
18-19	VK-CK		VH-CH1		VK-CK		VH-CH1	
18-20	VK-CK		VH-CH1		VK-CK		VH-CH1	
18-21	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-22	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-23	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-24	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-25	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-26	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-27	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-28	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-29	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-30	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-31	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-32	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-33	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-34	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-35	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-36	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-37	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-38	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-39	VK-CK		VH-CH1					
18-40	VK-CK		VH-CH1					
18-41	VK-CK		VH-CH1		VK-CK		VH-CH1	
18-42	VK-CK		VH-CH1		VK-CK		VH-CH1	
18-43	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VK-CK		VK-CH1	
18-44	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-45	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-46	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-47	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-48	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-49	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-50	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-51	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-52	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-53	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-54	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-55	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-56	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-57	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
18-58	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
18-59	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
18-60	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K

Table 45

Protein ID	L1L1H1H2 (%)	L1L2H1H2 (%)	L2L2H1H2 (%)	Delta, Da	Theoretical Average Intact Mass, Da			L1H1Fc	L2H1Fc
					L1L1H1H2	L1L2H1H2	L2L2H1H2		
18-05	0.4%	95.8%	*3.8%	1048	161216	162264	163312		
18-06	0.4%	95.3%	*4.3%	1075	161304	162379	163455		
18-07	0.4%	94.6%	*5.0%	1076	161303	162379	163455		
18-08	0.3%	97.9%	1.8%	1200	161064	162264	163465		
18-09	0.2%	98.2%	1.6%	1228	161099	162327	163554		
18-10	0.6%	97.9%	1.4%	1228	161151	162379	163608		
18-21	0.8%	94.3%	*4.9%	976	161150	162126	163102		
18-22	1.3%	97.7%	1.0%	1132	161238	162370	163503		
18-23	0.5%	96.7%	2.8%	1133	161237	162370	163503		
18-24	0.7%	97.3%	2.0%	1447	161210	162656	164103		
18-25	0.7%	97.8%	1.5%	1474	161298	162771	164245		
18-26	0.9%	98.7%	0.4%	1475	161297	162771	164246		
18-27	0.6%	96.8%	2.6%	1475	160961	162408	163854		
18-28	1.0%	96.8%	2.2%	1474	161049	162523	163997		
18-29	0.5%	98.0%	1.5%	1475	161048	162523	163998		
18-30	0.0%	97.1%	2.9%	1447	188158	189605	191051	99.4%	0.6%
18-31	0.0%	98.3%	1.7%	1474	188249	189723	191197	99.8%	0.2%
18-32	0.0%	99.5%	0.5%	1475	188248	189723	191198	99.7%	0.3%

Example 21 - Hexavalent, Trispecific Tetrahedral Antibodies Comprising Two Distinct Fabs that Specifically Bind CD19, CD20 and the 4-1BB Receptor

[0502] Tetrahedral antibodies offer a unique opportunity to exploit the contribution of dimensionality in creating multivalent, multispecific targeting agents for the treatment of cancer and other disease. Accordingly, a series of constructs was generated with the predicted structures shown in panel C of Figure 40, thus enabling the evaluation and application of their trispecificity.

[0503] This series of trispecific constructs employed VH and VL regions from FMC63, an FDA-approved anti-CD19 antibody used in the form of a chimeric antigen receptor (CAR-T) to treat B cell cancer (SEQ ID Nos 734, 708), and rituximab, an FDA-approved standalone anti-CD20 antibody used to treat B cell cancer and inflammatory disease (SEQ ID Nos 739, 713). Additionally, this series of trispecific constructs employed a single chain 4-1BB ligand (SEQ ID No 844) (Tables 46, 47, 48), and optionally employ a single chain OX40 ligand (SEQ ID No 845), or single chain GITR ligand (SEQ ID No 846). The targeting CD19 and CD20 by a single therapeutic agent offers a significant opportunity in the treatment and prevention of resistance and remission in B cell cancer, while the simultaneous delivery of 4-1BBL, OX40L or GITRL offers a significant opportunity to activate NK cells and T cells in the vicinity of the cancer.

[0504] To optimize correct VH/VL pairing and to minimize VH/VL mispairing, V region exchange was combined with electrostatic steering as described in Example 18 (Table 49). Additionally, to further facilitate purification of the correctly paired product by Protein A chromatography, the H1 chain incorporated the H435R/Y436F substitution. The constructs are then evaluated for their structural trispecificity by intact mass spectrometry, following removal of N-glycan and O-glycan, as described in Example 18. These constructs and those of Example 20 are further evaluated for their functional bi- and trispecificity using a variety of in vitro and in vivo cancer cell targeting and cytotoxicity models that are well known to one skilled in the art.

Table 46

Protein ID	L1 Chain	H1 Chain	L2 Chain	H2 Chain
19-01	SEQ ID NO: 733	SEQ ID NO: 707	SEQ ID NO: 734	SEQ ID NO: 708
19-02	SEQ ID NO: 735	SEQ ID NO: 709	SEQ ID NO: 736	SEQ ID NO: 710
19-03	SEQ ID NO: 735	SEQ ID NO: 709	SEQ ID NO: 737	SEQ ID NO: 711
19-04	SEQ ID NO: 738	SEQ ID NO: 712	SEQ ID NO: 739	SEQ ID NO: 713
19-05	SEQ ID NO: 740	SEQ ID NO: 714	SEQ ID NO: 741	SEQ ID NO: 715
19-06	SEQ ID NO: 740	SEQ ID NO: 714	SEQ ID NO: 742	SEQ ID NO: 716

Protein ID	L1 Chain	H1 Chain	L2 Chain	H2 Chain
19-07	SEQ ID NO: 733	SEQ ID NO: 707	SEQ ID NO: 734	SEQ ID NO: 717
19-08	SEQ ID NO: 735	SEQ ID NO: 709	SEQ ID NO: 736	SEQ ID NO: 718
19-09	SEQ ID NO: 735	SEQ ID NO: 709	SEQ ID NO: 737	SEQ ID NO: 719
19-10	SEQ ID NO: 738	SEQ ID NO: 712	SEQ ID NO: 739	SEQ ID NO: 720
19-11	SEQ ID NO: 740	SEQ ID NO: 714	SEQ ID NO: 741	SEQ ID NO: 721
19-12	SEQ ID NO: 740	SEQ ID NO: 714	SEQ ID NO: 742	SEQ ID NO: 722
19-13	SEQ ID NO: 733	SEQ ID NO: 723	SEQ ID NO: 734	SEQ ID NO: 724
19-14	SEQ ID NO: 735	SEQ ID NO: 725	SEQ ID NO: 736	SEQ ID NO: 726
19-15	SEQ ID NO: 735	SEQ ID NO: 725	SEQ ID NO: 737	SEQ ID NO: 727
19-16	SEQ ID NO: 738	SEQ ID NO: 728	SEQ ID NO: 739	SEQ ID NO: 729
19-17	SEQ ID NO: 740	SEQ ID NO: 730	SEQ ID NO: 741	SEQ ID NO: 731
19-18	SEQ ID NO: 740	SEQ ID NO: 730	SEQ ID NO: 742	SEQ ID NO: 732

Table 47

Protein ID	D1-Fc	D7	D5-Fab	D4-Fab	D3-Fab	D6-Fab	D8	D2-Fc
19-01			FMC63	Rituximab	Rituximab	FMC63		
19-02			FMC63	Rituximab	Rituximab	FMC63		
19-03			FMC63	Rituximab	Rituximab	FMC63		
19-04			Rituximab	FMC63	FMC63	Rituximab		
19-05			Rituximab	FMC63	FMC63	Rituximab		
19-06			Rituximab	FMC63	FMC63	Rituximab		
19-07		4-1BBL	FMC63	Rituximab	Rituximab	FMC63	4-1BBL	
19-08		4-1BBL	FMC63	Rituximab	Rituximab	FMC63	4-1BBL	
19-09		4-1BBL	FMC63	Rituximab	Rituximab	FMC63	4-1BBL	
19-10		4-1BBL	Rituximab	FMC63	FMC63	Rituximab	4-1BBL	
19-11		4-1BBL	Rituximab	FMC63	FMC63	Rituximab	4-1BBL	
19-12		4-1BBL	Rituximab	FMC63	FMC63	Rituximab	4-1BBL	
19-13	L234A/L235A/P329G	4-1BBL	FMC63	Rituximab	Rituximab	FMC63	4-1BBL	L234A/L235A/P329G
19-14	L234A/L235A/P329G	4-1BBL	FMC63	Rituximab	Rituximab	FMC63	4-1BBL	L234A/L235A/P329G
19-15	L234A/L235A/P329G	4-1BBL	FMC63	Rituximab	Rituximab	FMC63	4-1BBL	L234A/L235A/P329G
19-16	L234A/L235A/P329G	4-1BBL	Rituximab	FMC63	FMC63	Rituximab	4-1BBL	L234A/L235A/P329G
19-17	L234A/L235A/P329G	4-1BBL	Rituximab	FMC63	FMC63	Rituximab	4-1BBL	L234A/L235A/P329G
19-18	L234A/L235A/P329G	4-1BBL	Rituximab	FMC63	FMC63	Rituximab	4-1BBL	L234A/L235A/P329G

Table 48

Protein ID	D1 Target	D7 Target	D5 Target	D4 Target	D3 Target	D6 Target	D8 Target	D2 Target
19-01	FcgR		CD19	CD20	CD20	CD19		FcgR
19-02	FcgR		CD19	CD20	CD20	CD19		FcgR
19-03	FcgR		CD19	CD20	CD20	CD19		FcgR
19-04	FcgR		CD20	CD19	CD19	CD20		FcgR
19-05	FcgR		CD20	CD19	CD19	CD20		FcgR
19-06	FcgR		CD20	CD19	CD19	CD20		FcgR
19-07	FcgR	4-1BBR	CD19	CD20	CD20	CD19	4-1BBR	FcgR
19-08	FcgR	4-1BBR	CD19	CD20	CD20	CD19	4-1BBR	FcgR
19-09	FcgR	4-1BBR	CD19	CD20	CD20	CD19	4-1BBR	FcgR
19-10	FcgR	4-1BBR	CD20	CD19	CD19	CD20	4-1BBR	FcgR
19-11	FcgR	4-1BBR	CD20	CD19	CD19	CD20	4-1BBR	FcgR

19-12	FcgR	4-1BBR	CD20	CD19	CD19	CD20	4-1BBR	FcgR
19-13	Fc silent	4-1BBR	CD19	CD20	CD20	CD19	4-1BBR	Fc silent
19-14	Fc silent	4-1BBR	CD19	CD20	CD20	CD19	4-1BBR	Fc silent
19-15	Fc silent	4-1BBR	CD19	CD20	CD20	CD19	4-1BBR	Fc silent
19-16	Fc silent	4-1BBR	CD20	CD19	CD19	CD20	4-1BBR	Fc silent
19-17	Fc silent	4-1BBR	CD20	CD19	CD19	CD20	4-1BBR	Fc silent
19-18	Fc silent	4-1BBR	CD20	CD19	CD19	CD20	4-1BBR	Fc silent

Table 49

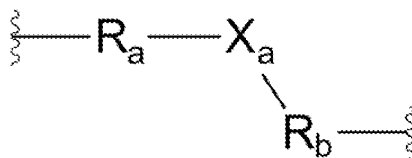
Protein ID	L1 Chain		H1 Chain		L2 Chain		H2 Chain	
	V region type	Amino acid Substitutions	V region Type	Amino acid Substitutions	V region Type	Amino acid Substitutions	V region Type	Amino acid Substitutions
19-01	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
19-02	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
19-03	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
19-04	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
19-05	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
19-06	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
19-07	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
19-08	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
19-09	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
19-10	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
19-11	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
19-12	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
19-13	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
19-14	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
19-15	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K
19-16	VK-CK	E123R/Q124R	VH-CH1	K147E/K213E	VH-CK		VK-CH1	
19-17	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39K/V133E	VK-CH1	Q38E/S183K
19-18	VK-CK	Q38E/V133K	VH-CH1	Q39K/S183E	VH-CK	Q39E/V133E	VK-CH1	Q38K/S183K

CLAIMS

What is claimed is:

1. A tetrahedral antibody comprising a first, second, third, and fourth domain, wherein:
 - a) each of the first and second domains are selected from the group consisting of a Fab domain and an Fc domain,
 - b) each of the first and second domains comprise:
 - i) a first polypeptide chain comprising a first N-terminus of the domain, and
 - ii) a second polypeptide chain comprising a second N-terminus of the domain,
 - c) the first N-terminus of the first domain and the first N-terminus of the second domain are joined to each other by a non-peptidyl linkage wherein the non-peptidyl linkage is:
 - i) a covalent linkage, or
 - ii) a non-covalent linkage between
 - (1) a first dimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the first domain, and
 - (2) a second dimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the second domain,wherein the first and second dimerizing polypeptides are not immunoglobulin polypeptides,
 - d) the third domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i) the second N-terminus of the first domain, or
 - ii) the N-terminus of the first dimerizing polypeptide, and
 - e) the fourth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i) the second N-terminus of the second domain, or
 - ii) the N-terminus of the second dimerizing polypeptide.
2. The tetrahedral antibody of claim 1, wherein:
 - a) the first domain is a Fc domain and the second domain is a Fab domain,
 - b) the first and second domains are Fc domains,

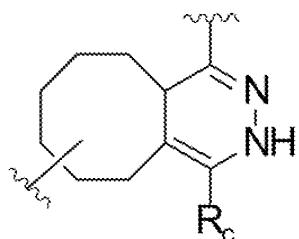
- c) the first and second domains are Fab domains,
 - d) the third and fourth domains are Fab domains,
 - e) the third and/or fourth domain are selected from the group consisting of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein,
 - f) the third domain is selected from the group of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein, and the fourth domain is a Fab,
 - g) the third domain is IL-15,
 - h) the third domain is IL-15 and the fourth domain is an IL-15R α sushi domain,
 - i) the third domain is IL-15 and the fourth domain is a Fab,
 - j) the third and fourth domains are each the ACE2 peptidase domain (PD),
 - k) the first and second domains are Fc domains, and the third and fourth domains are selected from the group consisting of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein,
 - l) the first and second domains are Fc domains, the third domain is selected from the group consisting of (i) a secreted protein, and (ii) the extracellular domain of a transmembrane protein, and the fourth domain is Fab,
 - m) the first and second domains are Fc domains and the third and fourth domains are Fab domains,
 - n) the first and second domains are Fc domains and the third and fourth domains are each the ACE2 peptidase domain (PD),
 - o) the first domain is an Fc domain and the second, third and fourth domains are Fab domains,
 - p) the first domain is an Fc domain, the second domain is a Fab domain, the third domain is IL-15, and the fourth domain is an IL-15R α sushi domain, or
 - q) the first domain is an Fc domain, the second domain is a Fab domain, the third domain is IL-15, and the fourth domain is a Fab.
3. The tetrahedral antibody of claim 1 or claim 2, wherein the non-peptidyl linkage between the first N-terminus of the first domain and the first N-terminus of the second domain is a covalent linkage.
4. The tetrahedral antibody of claim 3, wherein the covalent linkage comprises the structure:



wherein:

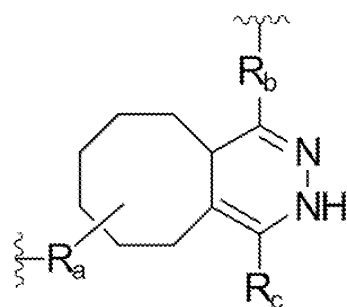
- a) X_a is a chemical structure selected from the group consisting of:
 - i) a chemical structure which comprises a cyclooctane fused to a dihydropyridazine,
 - ii) a chemical structure which comprises a cyclooctene fused to a pyridazine,
- b) R_a is a bond or a chemical structure which connects X_a to the first N-terminus of the first domain, and
- c) R_b is a bond or a chemical structure which connects X_a to the first N-terminus of the second domain.

5. The tetrahedral antibody of claim 4, wherein X_a comprises the structure



, wherein R_c is H, alkyl, or aryl, or a tautomer thereof.

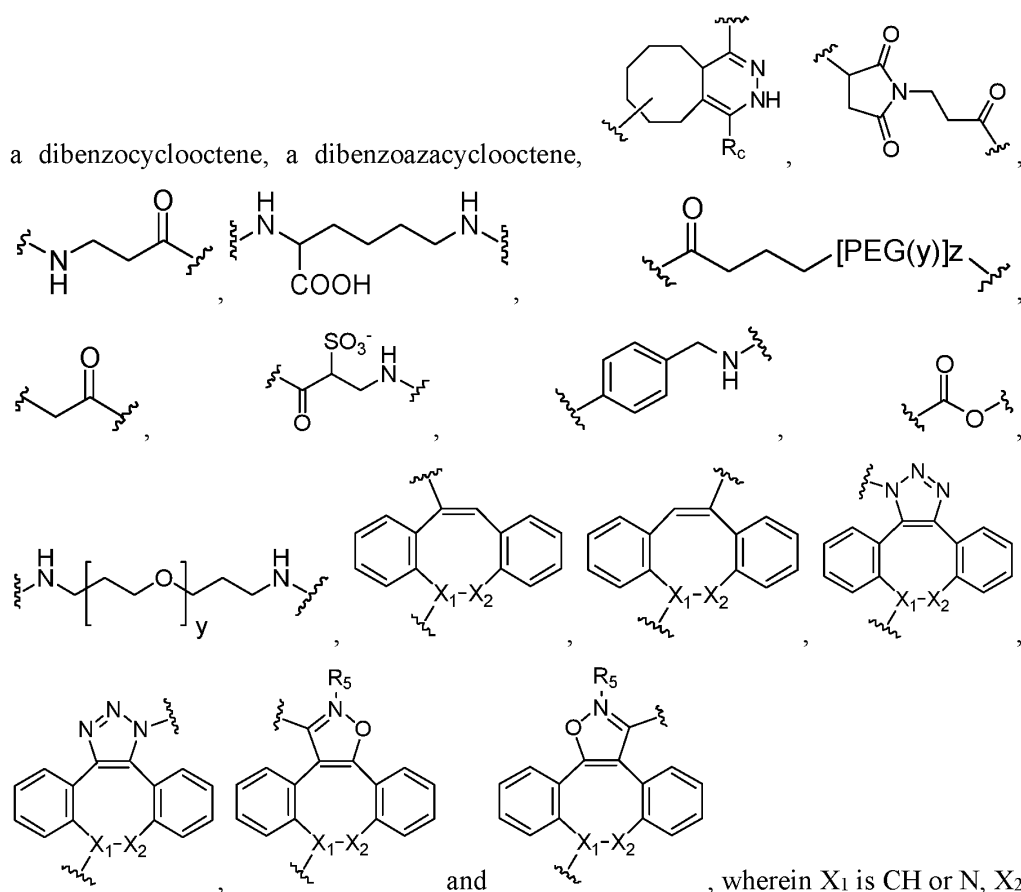
6. The tetrahedral antibody of any of claims 4-5, wherein the covalent linkage comprises the



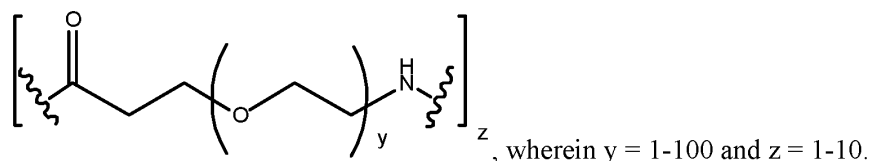
structure thereof.

, wherein R_c is H, alkyl, or aryl, or a tautomer thereof.

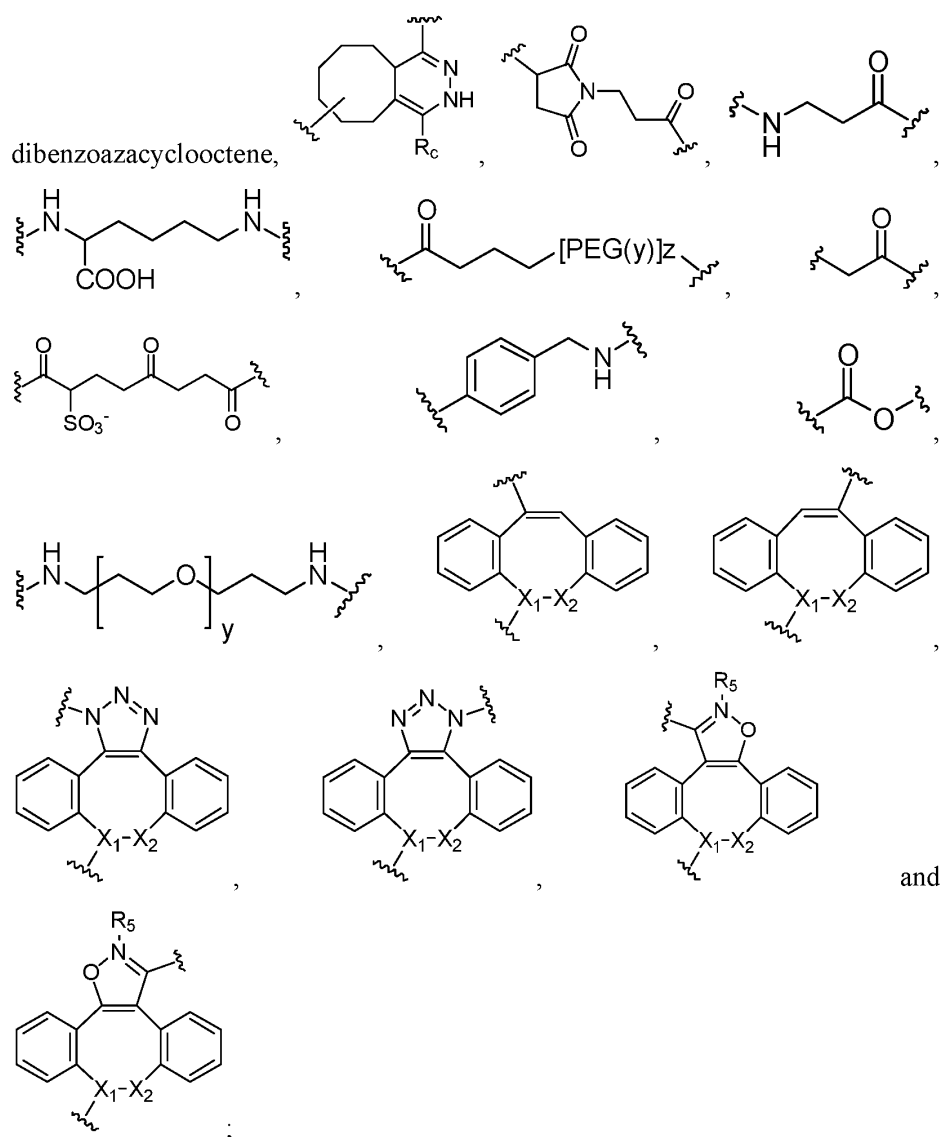
7. The tetrahedral antibody of any of claims 4-6, wherein R_a and R_b are, independently, a bond, or a chemical structure comprising or consisting of a chain of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more moieties, wherein each moiety is independently selected from the group consisting of [PEG(y)]_z, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C₁-C₁₀ alkyl, C₃-C₁₀ cycloalkane, C₂-C₁₀ alkene, C₅-C₁₀ cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C₂-C₅ acyl, C₂-C₅ acylamino, C₂-C₅ acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a isoxazolidine,



is CH₂ or a carbonyl group, and R₅ is an aryl or alkyl group, wherein [PEG(y)]_z is:

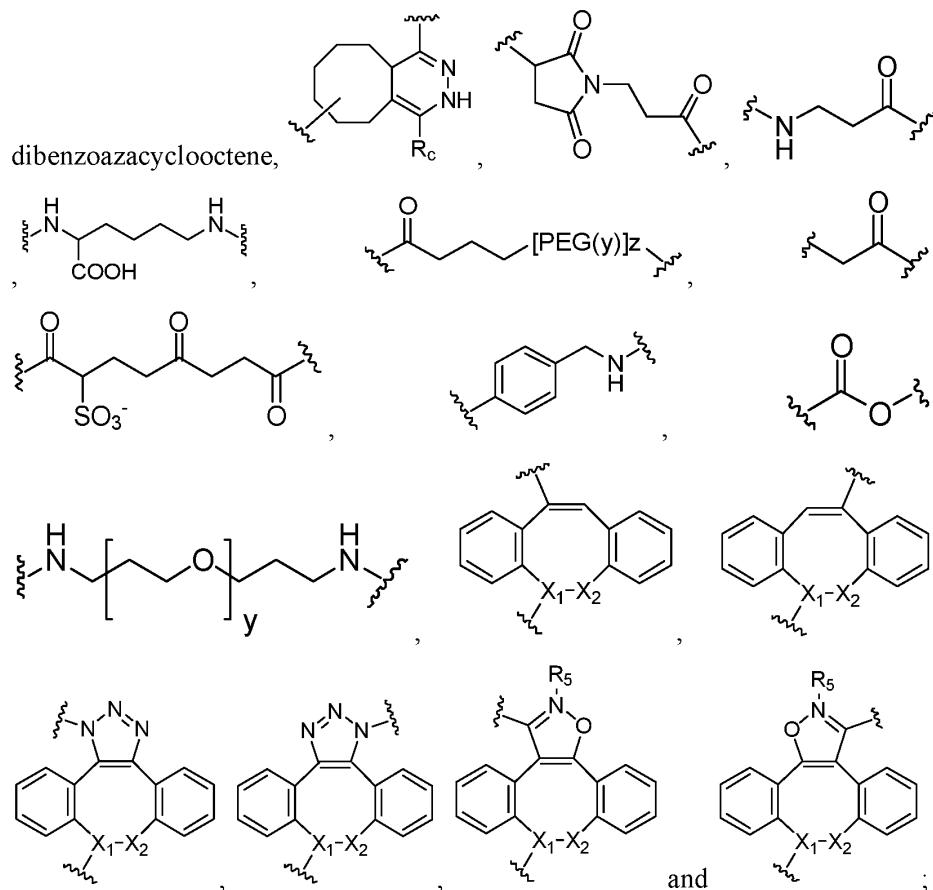


8. The tetrahedral antibody of claim 7, wherein R_a and/or R_b each independently:
- comprise a [PEG(y)]_z group;
 - comprise a polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), or polysaccharide group;
 - comprise a C₁-C₄ alkyl group;
 - comprise a succinimide;
 - comprise an amine;
 - comprise a succinyl, malonyl, glutaryl, phthalyl or adipoyl;
 - comprise a malonyl;
 - comprise an amino acid;
 - comprise a cysteine;
 - comprise a lysine;
 - consist of a chain of 3 moieties selected from the group consisting of [PEG(y)]_z, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C₁-C₁₀ alkyl, C₃-C₁₀ cycloalkane, C₂-C₁₀ alkene, C₅-C₁₀ cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C₂-C₅ acyl, C₂-C₅ acylamino, C₂-C₅ acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a isoxazolidine, a dibenzocyclooctene, a

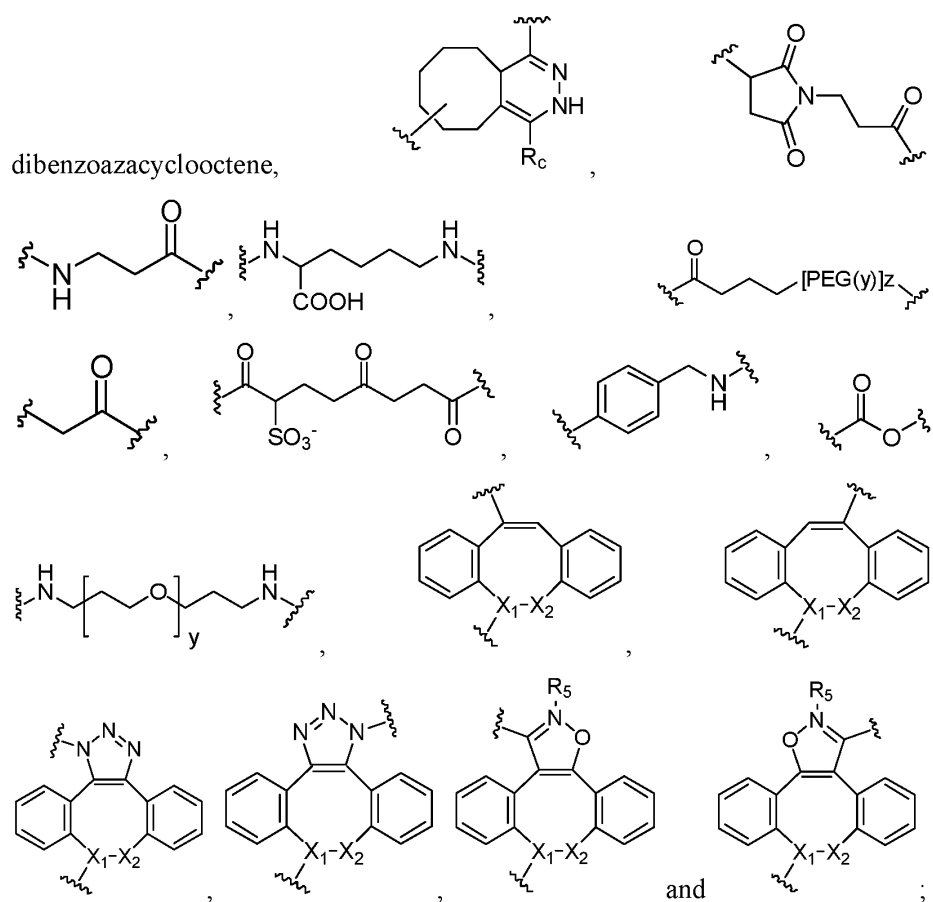


- 1) consist of a chain of 4 moieties selected from the group consisting of [PEG(y)]z, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C₁-C₁₀ alkyl, C₃-C₁₀ cycloalkane, C₂-C₁₀ alkene, C₅-C₁₀ cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C₂-C₅ acyl, C₂-C₅ acylamino, C₂-C₅ acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical

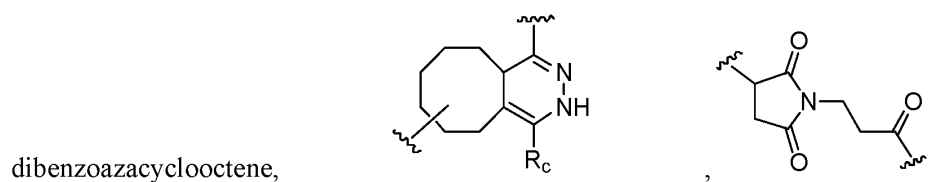
structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a isoxazolidine, a dibenzocyclooctene, a

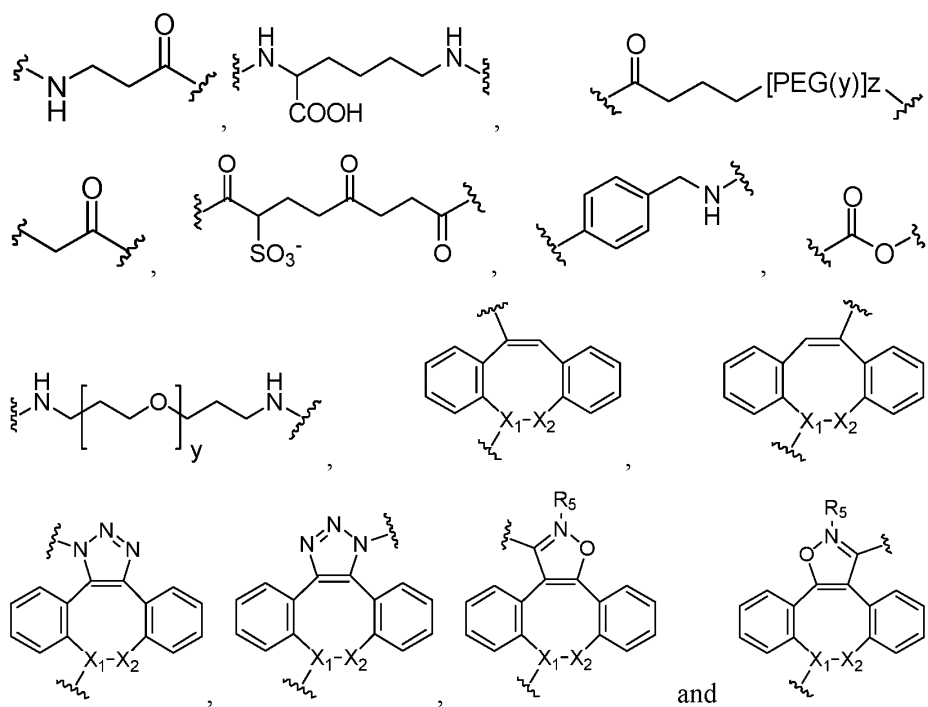


- consist of a chain of 5 moieties selected from the group consisting of [PEG(y)]_z, polyalkylene glycol, polyoxyalkylated polyol, polyvinyl alcohol, polyvinyl alkyl ether, poly(lactic acid), poly(lactic-glycolic acid), polysaccharide, a branched residue, C₁-C₁₀ alkyl, C₃-C₁₀ cycloalkane, C₂-C₁₀ alkene, C₅-C₁₀ cycloalkene, amine, sulfur, oxygen, succinimide, maleimide, glycerol, triazole, isoxazolidine, C₂-C₅ acyl, C₂-C₅ acylamino, C₂-C₅ acyloxy, succinyl, malonyl, glutaryl, phthalyl, adipoyl, an amino acid, an aryl group, a heteroaryl group, a carbamate, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to a isoxazolidine, a dibenzocyclooctene, a



- n) comprise a [PEG(y)]z group bonded to a lysine;
- o) comprise a C₁-C₄ acyl group bonded to a succinimide group;
- p) comprise a lysine bonded to a C₁-C₄ acyl
- q) comprise a [PEG(y)]z group, which is bonded to a glutaryl;
- r) consist of a chain of three, four or five moieties selected from the group consisting of [PEG(y)]z, C₂-C₅ acyl, succinyl, malonyl, glutaryl, an amino acid, a chemical structure containing a cyclooctane fused to a dihydropyridazine, a chemical structure containing a cyclooctene fused to a triazole, a chemical structure containing a cyclooctene fused to an isoxazolidine, a dibenzocyclooctene, a





wherein X_1 is CH or N, X_2 is CH_2 or a carbonyl group, and R_5 is an aryl or alkyl

group, wherein $[PEG(y)]_z$ is:

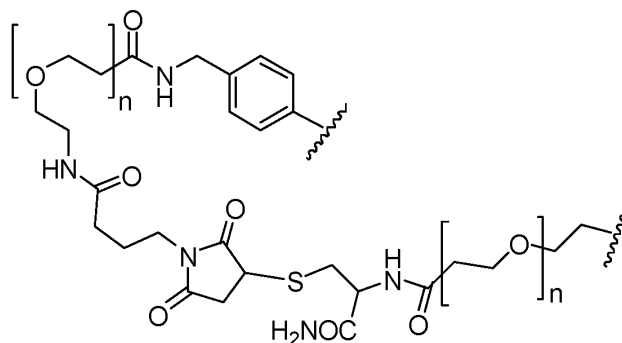
$$\left[\text{C}(=\text{O})\text{CH}_2\text{CH}_2\left(\text{OCH}_2\text{CH}_2\right)_y\text{NH} \right]_z$$

wherein $y = 1-100$ and $z = 1-10$;

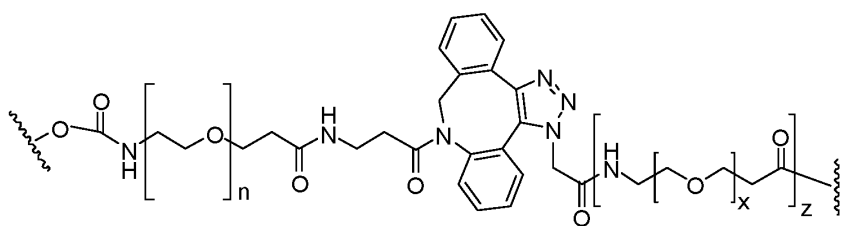
- s) is a bond;
- t) is a cysteine;
- u) has a linear structure; or
- v) has a branched structure;

w) has the structure:

;

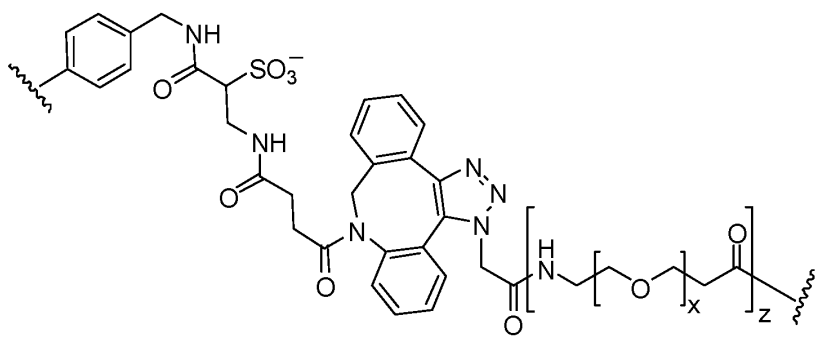


x) is: $\text{H}_2\text{NOC}(\text{O})_n$, wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50;



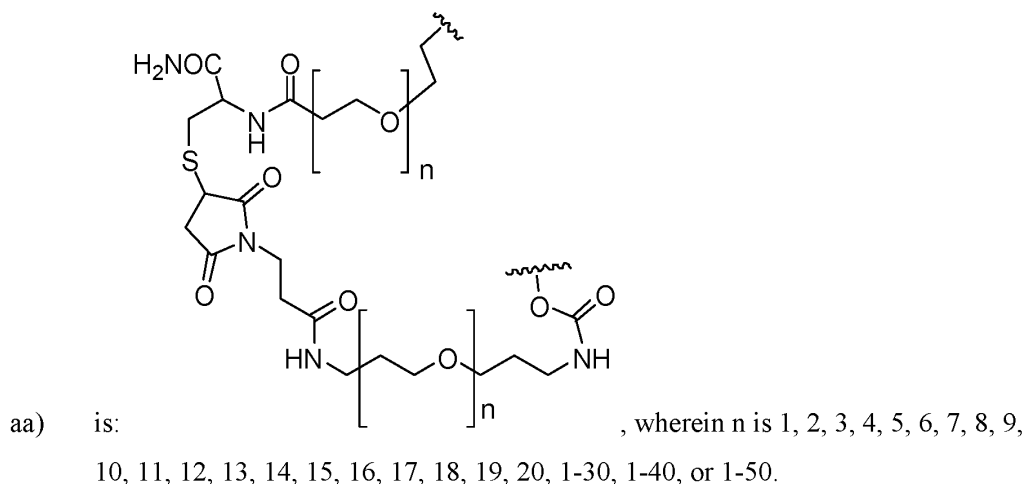
y) is:

wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50, x is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50 and z is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50;

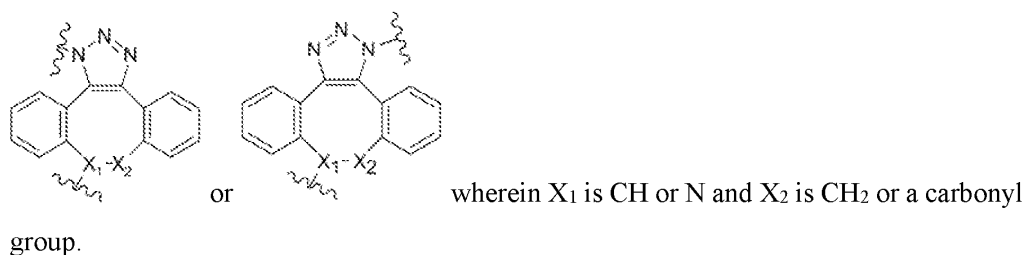


z) is:

wherein x is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50 and z is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-30, 1-40, or 1-50; or



9. The tetrahedral antibody of claims 7 or 8, wherein R_a and/or R_b comprise the moiety



10. The tetrahedral antibody of claim 1 or claim 2, wherein the non-peptidyl linkage is a non-covalent linkage between a first dimerizing polypeptide attached to the first N-terminus of the first domain and a second dimerizing polypeptide attached to the first N-terminus of the second domain.
11. The tetrahedral antibody of claim 10, wherein the first and second dimerizing polypeptides are selected from the group consisting of:
- a leucine zipper domain,
 - a collectrin-like domain (CLD), and
 - a collectrin domain (CD).
12. The tetrahedral antibody of claim 10 or 11, wherein:
- the first dimerizing polypeptide is the same as the second dimerizing polypeptide, and

- b) the dimerizing polypeptides form a homodimer.
13. The tetrahedral antibody of claim 10 or 11, wherein:
- a) the first dimerizing polypeptide is different than the second dimerizing polypeptide, and
 - b) the first and second dimerizing polypeptides form a heterodimer.
14. The tetrahedral antibody of claim 13, wherein the first and second dimerizing polypeptides, when in the presence of each other, form less than 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% homodimers.
15. The tetrahedral antibody of any of claims 10-12, wherein:
- a) the third and fourth domains are each the ACE2 peptidase domain (PD), and
 - b) the first and second dimerizing polypeptides are each the ACE2 collectrin-like domain (CLD).
16. The tetrahedral antibody of claim 15, wherein the first and second domains are Fc domains.
17. The tetrahedral antibody of any of claims 10-15, wherein:
- a) the first and second domains are Fc domains and the third domain is a first type of Fab domain,
 - b) the first and second domains are Fc domains and the third and fourth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
 - c) the first domain is an Fc domain, and the second and third domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain, or
 - d) the first domain is an Fc domain, and the second, third, and fourth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain.
18. The tetrahedral antibody of any of claims 10-14 and 17, additionally comprising a fifth domain, wherein the fifth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:

- a) the N-terminus of the first dimerizing polypeptide,
 - b) the second N-terminus of the first domain, or
 - c) the N-terminus of a third dimerizing polypeptide, wherein the third dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain.
19. The tetrahedral antibody of claim 18, wherein:
- a) the first and second domains are Fc domains and the fifth domain is a first type of Fab domain,
 - b) the first and second domains are Fc domains, and the third and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
 - c) the first and second domains are Fc domains, and the fourth and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
 - d) the first and second domains are Fc domains, and the third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
 - e) the first domain is an Fc domain, and the second and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
 - f) the first domain is an Fc domain, and the second, third, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
 - g) the first domain is an Fc domain, and the second, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain, or
 - h) the first domain is an Fc domain, and the second, third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, a third type of Fab domain, and a fourth type of Fab domain.

20. The tetrahedral antibody of any of claims 10-19, additionally comprising a fifth and/or sixth domain, wherein:
- a) the fifth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i) the N-terminus of the first dimerizing polypeptide,
 - ii) the second N-terminus of the first domain, or
 - iii) the N-terminus of a third dimerizing polypeptide, wherein the third dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain,
 - b) the sixth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i) the N-terminus of the second dimerizing polypeptide,
 - ii) the second N-terminus of the second domain, or
 - iii) the N-terminus of a fourth dimerizing polypeptide, wherein the fourth dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the second domain.
21. The tetrahedral antibody of claim 20, wherein:
- a) the first and second domains are Fc domains and the fifth domain is a first type of Fab domain,
 - b) the first and second domains are Fc domains, and the third and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
 - c) the first and second domains are Fc domains, and the fourth and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,
 - d) the first and second domains are Fc domains, and the third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
 - e) the first domain is an Fc domain, and the second and fifth domains are independently selected from the group consisting of a first type of Fab domain and a second type of Fab domain,

- f) the first domain is an Fc domain, and the second, third, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
 - g) the first domain is an Fc domain, and the second, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, and a third type of Fab domain,
 - h) the first domain is an Fc domain, and the second, third, fourth, and fifth domains are independently selected from the group consisting of a first type of Fab domain, a second type of Fab domain, a third type of Fab domain, and a fourth type of Fab domain, or
 - i) the first and second domains are Fc domains, the third and fourth domains are the ACE2 peptidase domain, and the fifth and sixth domains are Fab domains.
22. The tetrahedral antibody of any of claims 1 to 21, further comprising a seventh and/or eighth domain, wherein:
- a) the seventh domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i) the first C-terminus of the first domain, or
 - ii) the second C-terminus of the first domain,
 - b) the eighth domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i) the first C-terminus of the second domain, or
 - ii) the second C-terminus of the second domain.
23. The tetrahedral antibody of claim 20, wherein:
- a) the third, fourth, fifth and sixth domains are each the ACE2 PD and the dimerizing polypeptides are each the ACE2 CLD,
 - b) the third and fourth domains are each the ACE2 PD, the fifth and sixth domains are each Fab domains, and the dimerizing polypeptides are each the ACE2 CLD,
 - c) the third and fourth domains are each Fab domains, the fifth and sixth domains are each the ACE2 PD, and the dimerizing polypeptides are each the ACE2 CLD, or

- d) the first and second domains are Fc domains, the third and fourth domains are the ACE2 PD, the fifth and sixth domains are Fab domains, the dimerizing polypeptides are each the ACE2 CLD, and the first and second domains are each connected to their respective dimerizing polypeptides via a peptide linker, preferably wherein such domains and linkers are characterized by one or more or all of the following features:
- i) the Fc domains are characterized by one or more or all of the following features:
 - (1) are heterodimers,
 - (2) are IgG1 Fc domains,
 - (3) comprise a silencing mutation such that the Fc domain lacks Fc gamma receptor binding activity, preferably wherein such mutation is a combination of the following mutations: P329G/L234A/L235A (PGLALA),
 - (4) comprise a mutation that enhances FcRn activity, preferably wherein such mutation extends the half-life of the tetrahedral antibody, preferably wherein the mutation is a combination the following mutations: L309D/Q311H/N434S (DHS), and
 - (5) comprise a mutation that ablates their Protein A binding site, preferably wherein such mutation is H435R/Y436F (HY/RF),
 - ii) the ACE2 peptidase domains comprise a mutation which blocks its angiotensin converting enzyme activity, preferably wherein such mutation is an H378A mutation,
 - iii) the Fab domains are chimeric Fab domains comprising a murine variable region,
 - iv) the peptide linkers each have a length of 23 amino acids and are derived from the stalk region of a TNF receptor, preferably wherein the TNF receptor is TNF receptor 1B, still more preferably wherein the peptide linker consists of the amino acid sequence set forth in SEQ ID NO: 4468,
 - v) such domains and peptide linkers are formed by three different types of polypeptide chains, more preferably wherein the three different types of

polypeptide chains are denoted H1, L2, and H2, the H1 chain comprises the amino acid sequence set forth in SEQ ID NO: 522, the L2 chain comprises the amino acid sequence set forth in SEQ ID NO: 465, and the H2 chain comprises the amino acid sequence set forth in SEQ ID NO: 534.

24. The tetrahedral antibody of claim 20, wherein:
 - a) the third and fourth domains are each the ACE2 PD,
 - b) the fifth domain, if present, is attached at its C-terminus by a peptide bond or via a peptide linker to the N-terminus of a third dimerizing polypeptide, wherein the third dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain,
 - c) the sixth domain, if present, is attached at its C-terminus by a peptide bond or via a peptide linker to the N-terminus of a fourth dimerizing polypeptide, wherein the fourth dimerizing polypeptide is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the second domain, and
 - d) the first, second, third, and fourth dimerizing polypeptides are each the ACE2 CLD.
25. The tetrahedral antibody of any of claims 14-24, wherein the ACE2 PD comprises or consists of amino acids 18-615 of the ACE2 protein or a portion thereof.
26. The tetrahedral antibody of any of claims 14-24, wherein the ACE2 CLD comprises or consists of amino acids 616-740 of the ACE2 protein or a portion thereof.
27. The tetrahedral antibody of any of claims 14-26, wherein the ACE2 PD is catalytically active.
28. The tetrahedral antibody of any of claims 14-26, wherein ACE2 PD is catalytically inactive.
29. The tetrahedral antibody of claim 28, wherein ACE2 PD comprises a R273Q or a H378A mutation.
30. The tetrahedral antibody of any of claims 14-29, wherein the Fc domains lack Fc gamma receptor binding activity

31. The tetrahedral antibody of claim 30, wherein the Fc domains comprise a P329G mutation, a L234A mutation and a L235A mutation.
32. The tetrahedral antibody of claim 24, wherein:
- a) the fifth domain, third dimerizing polypeptide, and second polypeptide chain of the first domain is a stretch of consecutive amino acids,
 - b) the third domain, first dimerizing polypeptide, and first polypeptide chain of the first domain is a stretch of consecutive amino acids,
 - c) the fourth domain, second dimerizing polypeptide, and first polypeptide chain of the second domain is a stretch of consecutive amino acids, and
 - d) the sixth domain, fourth dimerizing polypeptide, and second polypeptide chain of the second domain is a stretch of consecutive amino acids,
- wherein each stretch of consecutive amino acids consists of the sequence of amino acids selected from the group consisting of SEQ ID NOs: 74-119.
33. An octahedral antibody comprising a first, second, third, fourth, fifth and sixth domain, wherein:
- a) each of the first, second and third domains are selected from the group consisting of a Fab domain and an Fc domain,
 - b) each of the first, second and third domains comprise:
 - i) a first polypeptide chain comprising a first N-terminus of the domain, and
 - ii) a second polypeptide chain comprising a second N-terminus of the domain,
 - c) the first N-terminus of the first domain, the first N-terminus of the second domain and the first N-terminus of the third domain are joined to each other by a non-peptidyl linkage wherein the non-peptidyl linkage is:
 - i) a branched covalent linkage, or
 - ii) a non-covalent linkage between
 - (1) a first trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the first domain,
 - (2) a second trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the second domain, and

- (3) a third trimerizing polypeptide attached by a peptide bond or via a peptide linker to the first N-terminus of the third domain, wherein the first, second, and third trimerizing polypeptides are not immunoglobulin polypeptides,
 - d) the fourth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i) the second N-terminus of the first domain, or
 - ii) the N-terminus terminus of the first trimerizing polypeptide,
 - e) the fifth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i) the second N-terminus of the second domain, or
 - ii) the N-terminus of the second trimerizing polypeptide, and
 - f) the sixth domain is attached at its C-terminus by a peptide bond or via a peptide linker to:
 - i) the second N-terminus of the third domain, or
 - ii) the N-terminus of the third trimerizing polypeptide.
34. The octahedral antibody of claim 33, wherein the non-peptidyl linkage is a non-covalent linkage between a first trimerizing polypeptide attached to the first N-terminus of the first domain, a second trimerizing polypeptide attached to the first N-terminus of the second domain, and a third trimerizing polypeptide attached to the first N-terminus of the third domain, wherein the first, second, and third trimerizing polypeptides are selected from the group consisting of TNF ligand superfamily members OX40L/TNFLSF4, CD40L/TNFLSF5, FASL/TNFLSF6, CD70L/TNFLSF7, CD30L/TNFLSF8, 4-1BBL/TNFLSF9, TRAIL/TNFLSF10, RANKL/TNFLSF11, TWEAK/TNFLSF12, APRIL/TNFLSF13, BAFF/TNFLSF13B, LIGHT/TNFLSF14, VEGI/TNFLSF15, GITRL/TNFLSF18, EctodysplasinA/TNFLSF19, TNF/TNFLSF2, lymphotoxin alpha/TNFLSF1, and lymphotoxin beta/TNFLSF3.
35. The octahedral antibody of claim 33 or 34, further comprising a seventh, eighth, and/or ninth domain, wherein:

- a) the seventh domain is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the first domain,
 - b) the eighth domain is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the second domain, and/or
 - c) the ninth domain is attached at its C-terminus by a peptide bond or via a peptide linker to the second N-terminus of the third domain.
36. The octahedral antibody of any one of claims 33 to 35, further comprising a tenth, eleventh, and/or twelfth domain, wherein:
- a) the tenth domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i) the first C-terminus of the first domain, or
 - ii) the second C-terminus of the first domain,
 - b) the eleventh domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i) the first C-terminus of the second domain, or
 - ii) the second C-terminus of the second domain,
 - c) the twelfth domain is attached at its N-terminus by a peptide bond or via a peptide linker to:
 - i) the first C-terminus of the third domain, or
 - ii) the second C-terminus of the third domain.
37. The tetrahedral or octahedral antibody of any of claims 1 to 36, wherein:
- a) one or more of the first, second, third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral antibody, or one or more of the first, second, third, fourth, fifth, sixth domains, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody are Fc domains, wherein the one or more Fc domains are independently selected from any of the Fc domains disclosed herein,
 - b) one or more of the first, second, third, fourth, fifth, sixth, seventh and eighth domains of the tetrahedral antibody, or one or more of the first, second, third, fourth, fifth, sixth domains, seventh, eighth, ninth, tenth, eleventh, and twelfth

- domains of the octahedral antibody are Fab domains, wherein the one or more Fab domains are independently selected from any of the Fab domains disclosed herein,
- c) one or more of the third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral antibody, or one or more of the fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody are a secreted protein, wherein the one or more secreted protein are independently selected from any of the secreted proteins disclosed herein,
 - d) one or more of the third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral antibody, or one or more of the fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody are extracellular domains of a transmembrane protein, wherein the one or more extracellular domains of a transmembrane protein are independently selected from any of the extracellular domains of a transmembrane protein disclosed herein,
 - e) one or more of the third, fourth, fifth, sixth, seventh, and eighth domains of the tetrahedral or one or more of the fourth, fifth, sixth, seventh, eighth, ninth, tenth, eleventh, and twelfth domains of the octahedral antibody:
 - i) comprise the structure of a compound that is a drug approved for treating a subject afflicted with a disease,
 - ii) comprise the structure of an organic compound having a molecular weight less than 1000 Daltons, a DNA aptamer, an RNA aptamer, an oligonucleotide, or a protein that is biologically active,
 - iii) comprise a primary or a secondary amine,
 - iv) are aripiprazole or oseltamivir,
 - v) are a respiratory drug, an antiasthmatic agent, an analgesic agent, an antidepressant, an antianginal agent, an antiarrhythmic agent, an antihypertensive agent, an antidiabetic agent, an antihistamine, an anti-infective agent, an antibiotic, an antiinflammatory agent, an antiparkinsonism drug, an antipsychotics, an antipyretic agent, an antiulcer agent, an attention deficit hyperactivity disorder (ADHD) drug, a central nervous system stimulant, a decongestant, or a psychostimulant,

- vi) are alprenolol, acebutolol, amidephrine, amineptine, amosulalol, amoxapine, amphetaminil, atenolol, atomoxetine, balofloxacin, bamethan, befunolol, benazepril, benfluorex, benzoctamine, betahistine, betaxolol, bevantolol, bifemelane, bisoprolol, brinzolamide, bufeniode, butethamine, camylofine, carazolol, carticaine, carvedilol, cephaeline, ciprofloxacin, cloZapine, clobenZorex, clorprenaline, cyclopentamine, delapril, demexiptiline, denopamine, desipramine, desloratadine, diclofenac, dimetofrine, dioxadrol, dobutamine, dopexamine, doripenem, dorzolamide, droprenilamine, duloxetine, eltopraZine, enalapril, enoxacin, epinephrine, ertapenem, esapraZole, esmolol, etoxadrol, fasudil, fendiline, fenethylline, fenfluramine, fenoldopam, fenoterol, fenproporex, flecamide, fluoxetine, formoterol, frovatriptan, gaboxadol, garenoxacin, gatifloxacin, grepafloxacin, hexoprenaline, imidapril, indalpine, indecainide, indeloxazine hydrochloride, isoxsuprine, ispronidine, labetalol, landiolol, lapatinib, levophacetoperane, lisinopril, lomefloxacin, lotrafiban, maprotiline, mecamlamine, mefloquine, mepindolol, meropenem, metapramine, metaproterenol, methoxyphenamine, dextrorotary methylphenidate, methylphenidate, metipranolol, metoprolol, mitoxantrone, mivazerol, moexipril, moprolol, moxifloxacin, nebivolol, nifenalol, nipradilol, norfloxacin, nortriptyline, nyldrin, olanZapine, oxamniquine, oxprenolol, oxyfedrine, paroxetine, perhexyline, phenmetrazine, phenylephrine, phenylpropylmethylamine, pholedrine, picilorex, pimethylamine, pindolol, pipemidic acid, piridocaine, practolol, pradofloxacin, pramipexole, pramiverin, prenalterol, prenylamine, prilocalne, procaterol, pronethalol, propafenone, propranolol, propylhexedrine, protokylol, protriptyline, pseudoephedrine, reboxetine, rasagiline, (r)-rasagiline, repinotan, reproterol, rimiterol, ritodrine, safinamide, salbutamol/albuterol, salmeterol, sarizotan, sertraline, silodosin, sotalol, soterenol, sparfloxacin, spirapril, sulfinalol, synephrine, tamsulosin, tebanicline, tianeptine, tirofiban, tretoquinol, trimetazidine, troxipide, varenicline, vildagliptin, viloxazine, viquidil or xamoterol,

- vii) comprise a protein that is biologically active,
- viii) are biologically active such that it has target-binding activity,
- ix) are an independently-folding protein or a portion thereof,
- x) are a glycosylated protein,
- xi) comprise intra-chain disulfide bonds,
- xii) binds a cytokine,
- xiii) binds to a cytokine, wherein the cytokine is $\text{TNF}\alpha$,
- xiv) comprise Atrial Natriuretic Peptide (ANP), Calcitonin, Corticotropin Releasing Hormone (CRH), Endothelin, Exenatide, Gastric Inhibitory Peptide (GIP), Glucagon-Like Peptide-1 (GLP-1), Glucagon-Like Peptide-2 (GLP-2), an analog of GLP-1 or GLP-2, Glucagon Vasoactive Intestinal Peptide (GVIP), Ghrelin, Peptide YY or Secretin, or a portion thereof,
- xv) comprise a stretch of consecutive amino acids in the sequence HGEFTFTSDVSSYLEEQAAKEFIAWLVKGRG (SEQ ID NO: 4657),
- xvi) comprise at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in the heavy chain of a Fab or a Fab' of an antibody,
- xvii) comprise at least one at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in the light chain of a Fab or a Fab' of an antibody,
- xviii) comprise at least one Fab or Fab' of an antibody, or a portion of at least one Fab or Fab',
- xix) comprise Fab-1 or Fab'1, or a portion thereof of an antibody,
- xx) comprise Fab-2 or Fab'2, or a portion thereof of an antibody,
- xxi) comprise two Fab or Fab' hands of an antibody,
- xxii) comprise at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in a single chain antibody, or
- xxiii) comprise at least one stretch of consecutive amino acids which are identical to a stretch of consecutive amino acids present in a $\text{TNF}\alpha$ receptor.

- f) the tetrahedral or octahedral antibody comprises a covalent linkage, wherein the covalent linkage is selected from any covalent linkage disclosed herein, or comprises any heterobifunctional crosslinker disclosed herein, and/or
 - g) the tetrahedral or octahedral antibody comprises one or more peptide linkers, wherein the one or more peptide linkers are independently selected from:
 - i) a stretch of consecutive amino acids which is, or is present in, the sequence TSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTFLLPMGPSPPAEGSTGD (SEQ ID NO: 3227),
 - ii) a stretch of consecutive amino acids which is, or is present in, the sequence GGGGAGGGGAGGGGAGGGGAGGGGAGGGG (SEQ ID NO: 3226), or
 - iii) any peptide linker disclosed herein.
38. A composition comprising at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, at least 20%, at least 21%, at least 22%, at least 23%, at least 24%, at least 25%, at least 26%, at least 27%, at least 28%, at least 29%, at least 30%, at least 31%, at least 32%, at least 33%, at least 34%, at least 35%, at least 36%, at least 37%, at least 38%, at least 39%, at least 40%, at least 41%, at least 42%, at least 43%, at least 44%, at least 45%, at least 46%, at least 47%, at least 48%, at least 49%, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, 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% of the tetrahedral or octahedral antibody of any of claims 1 to 37 as a proportion (w/w) of peptide-containing molecules in the composition.