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# DESCRIPTION

## Description

### Background of the Invention

**[0001]** Angiogenesis is implicated in the pathogenesis of a variety of disorders including solid tumors, intraocular neovascular syndromes such as proliferative retinopathies or age-related macular degeneration (AMD), rheumatoid arthritis, and psoriasis (Folkman et al. *J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun et al. *Annu. Rev. Physiol.* 53:217-239 (1991); and Gamer A, *Vascular diseases*. In: *Pathobiology of ocular disease. A dynamic approach*. Gamer A, Klintworth G K, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)). In solid tumors, angiogenesis and growth of new vasculature permits survival of the tumor, and a correlation has been demonstrated between the density of microvessels in tumor sections and patient survival in breast and other cancers (Weidner et al. *N Engl J Med* 324:1-6 (1991); Horak et al. *Lancet* 340:1120-1124 (1992); and Macchiarini et al. *Lancet* 340:145-146 (1992)).

**[0002]** Vascular endothelial growth factor (VEGF) is a known regulator of angiogenesis and neovascularization, and has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara et al. *Endocr. Rev.* 18:4-25 (1997)). The VEGF mRNA is overexpressed in many human tumors, and the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies (Berkman et al., *J Clin Invest* 91:153-159 (1993); Brown et al. *Human Pathol.* 26:86-91 (1995); Brown et al. *Cancer Res.* 53:4727-4735 (1993); Mattern et al. *Brit. J. Cancer* 73:931-934 (1996); and Dvorak et al. *Am J. Pathol.* 146:1029-1039 (1995); Aiello et al. *N. Engl. J. Med.* 331:1480-1487 (1994)). In addition, recent studies have shown the presence of localized VEGF in choroidal neovascular membranes in patients affected by AMD (Lopez et al. *Invest. Ophtalmo. Vis. Sci.* 37:855-868 (1996)). Anti-VEGF neutralizing antibodies can be used to suppress the growth of a variety of human tumor cell lines in nude mice and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Kim et al. *Nature* 362:841-844 (1993); Warren et al. *J. Clin. Invest* 95:1789-1797 (1995); Borgstrom et al. *Cancer Res.* 56:4032-4039 (1996); and Melnyk et al. *Cancer Res.* 56:921-924 (1996)) (Adamis et al. *Arch. Ophthalmol.* 114:66-71 (1996)).

**[0003]** Liang, W-C et al.: "Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF", *JBC* (2006), 281(2):951-961 discloses human VEGF specific antibodies.

**[0004]** Thus, there is a need for anti-VEGF monoclonal antibodies capable of being used for the treatment of solid tumors and various neovascular intraocular diseases.

### Summary of the Invention

**[0005]** The invention is defined by the appendant claims.

**[0006]** The invention provides soluble and stable anti-VEGF immunobinders comprising CDRs from rabbit monoclonal antibodies. Said antibodies are designed for the diagnosis and/or treatment of VEGF-mediated disorders. The hybridomas, nucleic acids, vectors and host cells for expression of the recombinant antibodies of the invention, methods for isolating them and the use of said antibodies in medicine are also disclosed but not claimed.

### Brief Description of the Drawings

**[0007]** *Figure 1* illustrates the binding kinetics of selected scFvs to hVEGF<sub>165</sub> using Biacore (hVEGF165). Fig. 1a shows the data obtained for 511max: Ka (1/Ms): 6,59E+05; SE (ka): 1,10E+03; kd(l/s):4,40E-05; SE(kd):6,30E-07; KD(M):

6,67E-11. Fig. 1b shows the data obtained for 578max: Ka (1/Ms): 7,00E+05; SE (ka): 1,40E+03; kd(1/s): 3,07E-04; SE(kd): 8,50E-07; KD(M): 4,39E-10.

**[0008]** *Figure 2 illustrates the species specificity by showing binding kinetics of 578max to human, mouse and rat VEGF. Fig. 2a shows the data obtained for human VEGF 165: Ka (1/Ms): 7,00E+05; SE (ka): 1,40E+03; kd(1/s): 3,07E-04; SE(kd): 8,50E-07; KD(M): 4,39E-10. Fig. 2b shows the data obtained for mouse VEGF164: Ka (1/Ms): 1,03E+06; SE (ka): 2,30E+03; kd(1/s): 4,40E-04; SE(kd): 9,40E-07; KD(M): 4,29E-10. Fig. 2c shows the data obtained for rat VEGF 164: Ka (1/Ms): 8,83E+05; SE (ka): 2,50E+03; kd(1/s): 5,28E-04; SE(kd): 1,20E-06; KD(M): 5,98E-10.*

**[0009]** *Figure 3 illustrates the binding kinetics of 578max to VEGF isoforms (hVEGF 121 and hVEGF 110). Fig. 3a shows the data obtained for human VEGF 165: Ka (1/Ms): 7,00E+05; SE (ka): 1,4E+03; kd(1/s): 3,07E-04; SE(kd): 8,50E-07; KD(M): 4,39E-10. Fig. 3b shows the data obtained for human VEGF121: Ka (1/Ms): 5,87E+05; SE (ka): 1,20E+03; kd(1/s): 5,58E-04; SE(kd): 9,60E-07; KD(M): 9,50E-11. Fig. 3c shows the data obtained for human VEGF 110: Ka (1/Ms): 5,23E+05; SE (ka): 1,30E+03; kd(1/s): 7,22E-04; SE(kd): 8,10E-07; KD(M): 1,38E-09.*

**[0010]** *Figure 4 depicts the binding kinetics of 578max, 578minmax and 578wt to hVEGF165. Fig. 4a shows the data obtained for 578max: Ka (1/Ms): 7,00E+05; SE (ka): 1,40E+03; kd(1/s): 3,07E-04; SE(kd): 8,50E-07; KD(M): 4,39E-10. Fig. 4b shows the data obtained for 578minmax: Ka (1/Ms): 8,06E+05; SE (ka): 2,10E+03; kd(1/s): 5,04E-04; SE(kd): 1,10E-06; KD(M): 6,25E-10. Fig. 4c shows the data obtained for 578wt-His: Ka (1/Ms): 8,45E+05; SE (ka): 1,60E+03; kd(1/s): 1,69E-04; SE(kd): 7,60E-07; KD(M): 2,00E-10.*

**[0011]** *Figure 5 illustrates thermal stability of 578max, 578minmax and 578minmax\_DHP (unfolding measured by FT-IR). Fig. 5a: 578minmax (ESBA903): Tm = 71,1°C; Fig. 5b: 578minmax\_DHP (#961): Tm=70,2°C; Fig. 5c: 578max (#821): Tm = 70,4°C.*

**[0012]** *Figure 6 illustrates denaturation and precipitation of 578 derivatives after thermal stress (Fig 6a: 50°C, Fig 6b: 60°C, Fig 6c: 70°C) for 30 min.*

**[0013]** *Figure 7 illustrates solubility of 578max, 578minmax and 578minmax\_DHP (determined by ammonium sulfate precipitation). Fig 7a: 578max (#821). The V50 was 27,24 %. Fig. 7b: 578minmax (ESBA903). The V50 was 28,13. Fig.: 7c: 578minmax\_DHP (#961). The V50was 32,36 %.*

**[0014]** *Figure 8 illustrates VEGFR2 competition ELISA versus HUVEC assay as methods to measure potency. Fig. 8a: Comparison of Lucentis and 511max (#802) in VEGFR2 competition ELISA. R<sup>2</sup> of Lucentis: 0,9417; R<sup>2</sup> of ESBA802: 0,9700. EC50 of Lucentis: 7,137 nM; EC50 of #802: 0,8221 nM. Fig 8b: Comparison of Lucentis and 578max (#821). in VEGFR2 competition ELISA. Fig 8c: Comparison of Lucentis, 511maxC-his and 534max in HUVEC assay. R<sup>2</sup> of Lucentis 0,9399; R<sup>2</sup> of EP511maxC-his: 0,9313, R<sup>2</sup> of EP534max: 0,7391. EC50 of Lucentis: 0,08825 nM, EC50 of 511maxC-his: 0,7646 nM, EC50 of 534max: 63,49 nM. Fig. 8d: Comparison of Lucentis, 578min and 578max in HUVEC assay. R<sup>2</sup> of Lucentis: 0,9419, R<sup>2</sup> of EP578min: 0,8886, R<sup>2</sup> of EP578max: 0,9274. EC50 of Lucentis: 0,1529 nM, EC50 of 578min: 1,528 nM, EC50 of 578max: 0,1031 nM.*

**[0015]** *Figure 9 illustrates the effects of 578minmax on HUVEC proliferation induced by hVEGF165. The parameters of the assay were the following: h VEGF 165 concentration: 0,08nM (3ng/ml); incubation with VEGF and test item: 96h. The EC50 was 0,08959 nM for Lucentis and 0,05516 nM for 578minmax, whereas the R<sup>2</sup> was 0,9066 for Lucentis and 0,9622 for 578minmax.*

**[0016]** *Figure 10 illustrates the effects of 578minmax on HUVEC Proliferation induced by mouse VEGF 164 and rat VEGF164. The parameters of the assay were the following: mouse VEGF164 concentration: 0,08nM (3ng/ml); rat VEGF164 concentration: 0,3nM (11,3ng/ml). Both concentrations were selected at EC90 for VEGF induced HUVEC proliferation; incubation with VEGF and test item: 96h. Fig. 10a illustrates the data obtained for mouse VEGF. The EC50 was 0,1196 nM for V1253 and 0,06309 nM for 578minmax, whereas the R<sup>2</sup> was 0,02744 for Lucentis, 0,9348 for V1253 and 0,9767 for EP578minmax. Lucentis did not inhibit HUVEC proliferation induced by mouse VEGF. Fig. 10b illustrates the data obtained for rat VEGF. The EC50 was 1,597 nM for V1253 and 0,06974 nM for 578minmax, whereas the R<sup>2</sup> was 0,7664 for V1253 and 0,6635 for 578minmax.*

**[0017]** *Figure 11 illustrates efficacy studies using Miles assay in nude guinea pigs (part I). The dye almar blue 1 was administered intravenously to nude guinea pigs. One hour after dye injection, a premixture 2 of hVEGF (2,61nM) and Lucentis, ESBA903 or #802, respectively, was injected into the skin of the animal 3. One hour after injection of the solutions, the animals 3 were euthanized and the pelts were collected, cleaned and photographed digitally using incident and transmitted light. The area of Evans Blue dye that extravasated into the injection sites was evaluated using Image J and the dose-area retention was plotted.*

**[0018]** *Figure 12 illustrates efficacy studies using Miles assay in nude guinea pigs (part II). Fig. 12a shows the results obtained for #803 (511max). The EC50 was 5,990nM and had a statistical spread between 2,060 and 17,41 nM whereas the R<sup>2</sup> was 0,5800. Fig. 12b shows the results obtained for ESBA903 (578minmax). The EC50 was 3,989 and had a statistical spread between 1,456 and 10,93 nM whereas the R<sup>2</sup> was 0,3920. Fig. 12c shows the area of dye leakage for Lucentis. The EC50 could not be calculated for Lucentis due to the poor fit of the curve.*

**[0019]** *Figure 13 illustrates efficacy studies using modified miles assay in rats (premixed hVEGF165 and 578minmax (ESBA903)). Fig. 13a illustrates the anti-permeability efficacy of Avastin upon VEGF induced retinal vascular leakage in rats - dose response. Avastin inhibits hVEGF-induced retinal vascular permeability. Premixed before injection. Approximately equimolar, 3fold, or 10 fold excess. \*p<0,05 (VEGF s. BSA), \*\* p<0,05 (Avastin treated vs. VEGF). Fig 13b shows the anti-permeability efficacy of ESBA903 upon VEGF induced retinal vascular leakage in rats. Dose response (premixed, ivt). Complete inhibition of hVEGF-induced retinal vascular permeability by ESBA903. Premixed before injection. Approximately equimolar, 3fold, or 10 fold excess. \*p<0,05 (VEGF s. BSA), \*\* p<0,05 (ESBA903 treated vs. VEGF).*

**[0020]** *Figure 14 illustrates efficacy studies using modified miles assay in rats (topical administration of 578minmax (ESBA903)). The anti-permeability efficacy of AL-51287 (ESBA903) upon VEGF induced retinal vascular leakage in rats was tested upon topical administration. Five days pretreatment, 4 drops/day with a 10 ng/ml ESBA903 formulation. \*p<0,05 (VEGF s. BSA), \*\* p<0,05 (VEGF vs. AL-51287), \*\*\*p=0,060 (AL-51287 vs. AL-52667), \*\*\*\*(VEGF vs. AL-39324); p<0,05 (AL-39324 vs. vehicle ref ctrl). AL-51287: ESBA903; AL-52657: topical vehicle reference control; AL-39324: small molecule RTK inhibitor.*

**[0021]** *Figure 15 illustrates the definition of CDR1 of VH as used herein.*

#### Detailed Description

**[0022]** *The invention provides soluble and stable anti-VEGF immunobinders comprising CDRs from rabbit monoclonal antibodies. Said immunobinders are designed for the diagnosis and/or treatment of VEGF-mediated disorders. The hybridomas, nucleic acids, vectors and host cells for expression of the recombinant antibodies of the invention, methods for isolating them and the use of said antibodies in medicine are also disclosed but not claimed.*

#### *Definitions*

**[0023]** *In order that the present invention may be more readily understood, certain terms will be defined as follows. Additional definitions are set forth throughout the detailed description.*

**[0024]** *The term "VEGF" refers to the 165-amino acid vascular endothelial cell growth factor, and related 121-, 189-, and 206-amino acid vascular endothelial cell growth factors, as described by Leung et al., Science 246:1306 (1989), and Houck et al., Mol. Endocrin. 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.*

**[0025]** *The term "VEGF receptor" or "VEGFr" refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor is the fms-like tyrosine kinase (fit), a transmembrane receptor in the tyrosine kinase family. DeVries et al., Science 255:989 (1992); Shibuya et al., Oncogene 5:519 (1990). The fit receptor comprises an extracellular domain,*

a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a VEGF receptor is the flk-1 receptor (also referred to as KDR). Matthews et al., Proc. Natl. Acad. Sci. 88:9026 (1991); Terman et al., Oncogene 6:1677 (1991); Terman et al., Biochem. Biophys. Res. Commun. 187:1579 (1992). Binding of VEGF to the fit receptor results in the formation of at least two high molecular weight complexes, having an apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

**[0026]** The term "rabbit" as used herein refers to an animal belonging to the family of the leporidae.

**[0027]** The term "antibody" as used herein is a synonym for "immunoglobulin." Antibodies according to the present invention may be whole immunoglobulins or fragments thereof, comprising at least one variable domain of an immunoglobulin, such as single variable domains, Fv (Skerra A. and Pluckthun, A. (1988) Science 240:1038-41), scFv (Bird, R.E. et al. (1988) Science 242:423-26; Huston, J.S. et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-83), Fab, (Fab')2 or other fragments well known to a person skilled in the art.

**[0028]** The term "CDR" refers to one of the six hypervariable regions within the variable domains of an antibody that mainly contribute to antigen binding. One of the most commonly used definitions for the six CDRs was provided by Kabat E.A. et al., (1991) Sequences of proteins of immunological interest. NIH Publication 91-3242). As used herein, Kabat's definition of CDRs only apply for CDR1, CDR2 and CDR3 of the light chain variable domain (CDR L1, CDR L2, CDR L3, or L1, L2, L3), as well as for CDR2 and CDR3 of the heavy chain variable domain (CDR H2, CDR H3, or H2, H3). CDR1 of the heavy chain variable domain (CDR H1 or H1), however, as used herein is defined by the following residues (Kabat numbering): It starts with position 26 and ends prior to position 36. This is basically a fusion of CDR H1 as differently defined by Kabat and Chotia (see also Figure 15 for illustration).

**[0029]** The term "antibody framework", or sometimes only "framework", as used herein refers to the part of the variable domain, either VL or VH, which serves as a scaffold for the antigen binding loops (CDRs) of this variable domain. In essence it is the variable domain without the CDRs.

**[0030]** The term "single chain antibody", "single chain Fv" or "scFv" is intended to refer to a molecule comprising an antibody heavy chain variable domain (or region; VH) and an antibody light chain variable domain (or region; VL) connected by a linker. Such scFv molecules can have the general structures: NH<sub>2</sub>-VL-linker-VH-COOH or NH<sub>2</sub>-VH-linker-VL-COOH.

**[0031]** As used herein, "identity" refers to the sequence matching between two polypeptides, molecules or between two nucleic acids. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit (for instance, if a position in each of the two DNA molecules is occupied by adenine, or a position in each of two polypeptides is occupied by a lysine), then the respective molecules are identical at that position. The "percentage identity" between two sequences is a function of the number of matching positions shared by the two sequences divided by the number of positions compared x 100. For instance, if 6 of 10 of the positions in two sequences are matched, then the two sequences have 60% identity. By way of example, the DNA sequences CTGACT and CAGGTT share 50% identity (3 of the 6 total positions are matched). Generally, a comparison is made when two sequences are aligned to give maximum identity. Such alignment can be provided using, for instance, the method of Needleman et al. (1970) J. Mol. Biol. 48: 443-453, implemented conveniently by computer programs such as the Align program (DNAStar, Inc.). The percent identity between two amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG,software package (available at [www.gcg.com](http://www.gcg.com)), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

**[0032]** "Similar" sequences are those which, when aligned, share identical and similar amino acid residues, where similar residues are conservative substitutions for corresponding amino acid residues in an aligned reference sequence. In this regard, a "conservative substitution" of a residue in a reference sequence is a substitution by a residue that is

physically or functionally similar to the corresponding reference residue, e.g., that has a similar size, shape, electric charge, chemical properties, including the ability to form covalent or hydrogen bonds, or the like. Thus, a "conservative substitution modified" sequence is one that differs from a reference sequence or a wild-type sequence in that one or more conservative substitutions are present. The "percentage similarity" between two sequences is a function of the number of positions that contain matching residues or conservative substitutions shared by the two sequences divided by the number of positions compared x 100. For instance, if 6 of 10 of the positions in two sequences are matched and 2 of 10 positions contain conservative substitutions, then the two sequences have 80% positive similarity.

**[0033]** As used herein, the term "conservative sequence modifications" is intended to refer to amino acid modifications that do not negatively affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative sequence modifications include nucleotide and amino acid substitutions, additions and deletions. For example, modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a human anti-VEGF antibody is preferably replaced with another amino acid residue from the same side chain family. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate antigen binding are well-known in the art (see, e.g., Brummell et al., *Biochem.* 32:1180-1187 (1993); Kobayashi et al. *Protein Eng.* 12(10):879-884 (1999); and Burks et al. *Proc. Natl. Acad. Sci. USA* 94:412-417 (1997))

**[0034]** "Amino acid consensus sequence" as used herein refers to an amino acid sequence that can be generated using a matrix of at least two, and preferably more, aligned amino acid sequences, and allowing for gaps in the alignment, such that it is possible to determine the most frequent amino acid residue at each position. The consensus sequence is that sequence which comprises the amino acids which are most frequently represented at each position. In the event that two or more amino acids are equally represented at a single position, the consensus sequence includes both or all of those amino acids.

**[0035]** The amino acid sequence of a protein can be analyzed at various levels. For example, conservation or variability can be exhibited at the single residue level, multiple residue level, multiple residue with gaps etc. Residues can exhibit conservation of the identical residue or can be conserved at the class level. Examples of amino acid classes include polar but uncharged R groups (Serine, Threonine, Asparagine and Glutamine); positively charged R groups (Lysine, Arginine, and Histidine); negatively charged R groups (Glutamic acid and Aspartic acid); hydrophobic R groups (Alanine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan, Valine and Tyrosine); and special amino acids (Cysteine, Glycine and Proline). Other classes are known to one of skill in the art and may be defined using structural determinations or other data to assess substitutability. In that sense, a substitutable amino acid can refer to any amino acid which can be substituted and maintain functional conservation at that position.

**[0036]** It will be recognized, however, that amino acids of the same class may vary in degree by their biophysical properties. For example, it will be recognized that certain hydrophobic R groups (e.g., Alanine, Serine, or Threonine) are more hydrophilic (*i.e.*, of higher hydrophilicity or lower hydrophobicity) than other hydrophobic R groups (e.g., Valine or Leucine). Relative hydrophilicity or hydrophobicity can be determined using art-recognized methods (see, e.g., Rose et al., *Science*, 229: 834-838 (1985) and Comette et al., *J. Mol. Biol.*, 195: 659-685 (1987)).

**[0037]** As used herein, when one amino acid sequence (e.g., a first V<sub>H</sub> or V<sub>L</sub> sequence) is aligned with one or more additional amino acid sequences (e.g., one or more VH or VL sequences in a database), an amino acid position in one sequence (e.g., the first V<sub>H</sub> or V<sub>L</sub> sequence) can be compared to a "corresponding position" in the one or more additional amino acid sequences. As used herein, the "corresponding position" represents the equivalent position in the sequence(s) being compared when the sequences are optimally aligned, *i.e.*, when the sequences are aligned to achieve the highest percent identity or percent similarity.

**[0038]** As used herein, the term "antibody database" refers to a collection of two or more antibody amino acid sequences (a "multiplicity" of sequences), and typically refers to a collection of tens, hundreds or even thousands of

antibody amino acid sequences. An antibody database can store amino acid sequences of, for example, a collection of antibody  $V_H$  regions, antibody  $V_L$  regions or both, or can store a collection of scFv sequences comprised of  $V_H$  and  $V_L$  regions. Preferably, the database is stored in a searchable, fixed medium, such as on a computer within a searchable computer program. In one embodiment, the antibody database is a database comprising or consisting of germline antibody sequences. In another embodiment, the antibody database is a database comprising or consisting of mature (i.e., expressed) antibody sequences (e.g., a Kabat database of mature antibody sequences, e.g., a KBD database). In yet another embodiment, the antibody database comprises or consists of functionally selected sequences (e.g., sequences selected from a QC assay).

**[0039]** The term "immunobinder" refers to a molecule that contains all or a part of the antigen binding site of an antibody, e.g., all or part of the heavy and/or light chain variable domain, such that the immunobinder specifically recognizes a target antigen. Non-limiting examples of immunobinders include full-length immunoglobulin molecules and scFvs, as well as antibody fragments, including but not limited to (i) a Fab fragment, a monovalent fragment consisting of the  $V_L$ ,  $V_H$ ,  $C_L$  and  $C_H1$  domains; (ii) a  $F(ab')_2$  fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fab' fragment, which is essentially a Fab with part of the hinge region (see, Fundamental Immunology (Paul ed., 3.sup.rd ed. 1993); (iv) a Fd fragment consisting of the  $V_H$  and  $C_H1$  domains; (v) a Fv fragment consisting of the  $V_L$  and  $V_H$  domains of a single arm of an antibody, (vi) a single domain antibody such as a Dab fragment (Ward et al., (1989) *Nature* 341:544-546), which consists of a  $V_H$  or  $V_L$  domain, a Camelid (see Hamers-Casterman, et al., *Nature* 363:446-448 (1993), and Dumoulin, et al., *Protein Science* 11:500-515 (2002)) or a Shark antibody (e.g., shark Ig-NARs Nanobodies<sup>®</sup>); and (vii) a nanobody, a heavy chain region containing the variable domain and two constant domains.

**[0040]** As used herein, the term "functional property" is a property of a polypeptide (e.g., an immunobinder) for which an improvement (e.g., relative to a conventional polypeptide) is desirable and/or advantageous to one of skill in the art, e.g., in order to improve the manufacturing properties or therapeutic efficacy of the polypeptide. In one embodiment, the functional property is stability (e.g., thermal stability). In another embodiment, the functional property is solubility (e.g., under cellular conditions). In yet another embodiment, the functional property is non-aggregation. In still another embodiment, the functional property is protein expression (e.g., in a prokaryotic cell). In yet another embodiment the functional property is a refolding efficiency following an inclusion body solubilization in a corresponding purification process. In certain embodiments, antigen binding affinity is not a functional property desired for improvement.

**[0041]** The term "epitope" or "antigenic determinant" refers to a site on an antigen (e.g., on VEGF) to which an immunoglobulin or antibody specifically binds. An epitope typically includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 consecutive or nonconsecutive amino acids in a unique spatial conformation. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, G. E. Morris, Ed. (1996).

**[0042]** The terms "specific binding," "selective binding," "selectively binds," and "specifically binds," refer to antibody binding to an epitope on a predetermined antigen. Typically, the antibody binds with an affinity ( $K_D$ ) of approximately less than  $10^{-7}$  M, such as approximately less than  $10^{-8}$  M,  $10^{-9}$  M or  $10^{-10}$  M or even lower.

**[0043]** The term " $K_D$ " or " $K_d$ " refers to the dissociation equilibrium constant of a particular antibody-antigen interaction. Typically, the antibodies of the invention bind to VEGF with a dissociation equilibrium constant ( $K_D$ ) of less than approximately  $10^{-7}$  M, such as less than approximately  $10^{-8}$  M,  $10^{-9}$  M or  $10^{-10}$  M or even lower, for example, as determined using surface plasmon resonance (SPR) technology in a BIACORE instrument.

**[0044]** The terms "neutralizes VEGF," "inhibits VEGF," and "blocks VEGF" are used interchangeably to refer to the ability of an antibody of the invention to prevent VEGF from interacting with one or more VEGF receptors such as VEGFR-1 and/or VEGFR-2, and, for example, triggering signal transduction.

**[0045]** A "recombinant immunobinder" as used herein refers to an immunobinder being produced by expression from recombinant DNA.

**[0046]** A "chimeric" immunobinder as used herein has a portion of the heavy and/or light chain identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular

antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies. Humanized antibody as used herein is a subset of chimeric antibodies.

**[0047]** "Humanized antibodies" as used herein are immunobinders that have been synthesized using recombinant DNA technology to circumvent immune response to foreign antigens. Humanization is a well-established technique for reducing the immunogenicity of monoclonal antibodies of xenogenic sources. This involves the choice of an acceptor framework, preferably a human acceptor framework, the extent of the CDRs from the donor immunobinder to be inserted into the acceptor framework and the substitution of residues from the donor framework into the acceptor framework. A general method for grafting CDRs into human acceptor frameworks has been disclosed by Winter in US Patent No. 5,225,539. US6,407,213, discloses a number of amino acid positions of the framework where a substitution from the donor immunobinder is preferred.

**[0048]** The term "nucleic acid molecule," refers to DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence.

**[0049]** The term "vector," refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome.

**[0050]** The term "host cell" refers to a cell into which an expression vector has been introduced. Host cells can include bacterial, microbial, plant or animal cells. Bacteria, which are susceptible to transformation, include members of the enterobacteriaceae, such as strains of *Escherichia coli* or *Salmonella*; *Bacillaceae*, such as *Bacillus subtilis*; *Pneumococcus*; *Streptococcus*, and *Haemophilus influenzae*. Suitable microbes include *Saccharomyces cerevisiae* and *Pichia pastoris*. Suitable animal host cell lines include CHO (Chinese Hamster Ovary lines) and NS0 cells.

**[0051]** The terms "treat," "treating," and "treatment," refer to therapeutic or preventative measures described herein. The methods of "treatment" employ administration to a subject, in need of such treatment, an antibody of the present invention, for example, a subject having a VEGF-mediated disorder or a subject who ultimately may acquire such a disorder, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of the disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

**[0052]** The term "VEGF-mediated disorder" refers to any disorder, the onset, progression or the persistence of the symptoms or disease states of which requires the participation of VEGF. Exemplary VEGF-mediated disorders include, but are not limited to, age-related macular degeneration, neovascular glaucoma, diabetic retinopathy, retinopathy of prematurity, retrolental fibroplasia, breast carcinomas, lung carcinomas, gastric carcinomas, esophageal carcinomas, colorectal carcinomas, liver carcinomas, ovarian carcinomas, the comas, arrhenoblastomas, cervical carcinomas, endometrial carcinoma, endometrial hyperplasia, endometriosis, fibrosarcomas, choriocarcinoma, head and neck cancer, nasopharyngeal carcinoma, laryngeal carcinomas, hepatoblastoma, Kaposi's sarcoma, melanoma, skin carcinomas, hemangioma, cavernous hemangioma, hemangioblastoma, pancreas carcinomas, retinoblastoma, astrocytoma, glioblastoma, Schwannoma, oligodendrogioma, medulloblastoma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, renal cell carcinoma, prostate carcinoma, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), Meigs' syndrome, rheumatoid arthritis, psoriasis and atherosclerosis.

**[0053]** The term "effective dose" or "effective dosage" refers to an amount sufficient to achieve or at least partially achieve the desired effect. The term "therapeutically effective dose" is defined as an amount sufficient to cure or at least partially arrest the disease and its complications in a patient already suffering from the disease. Amounts effective for this use will depend upon the severity of the disorder being treated and the general state of the patient's own immune

system.

**[0054]** The term "subject" refers to any human or non-human animal. For example, the methods and compositions of the present invention can be used to treat a subject with a VEGF-mediated disorder.

**[0055]** The term "Min-graft" or "min" as used herein refers to a humanized variable domain that was generated by grafting of rabbit CDRs from a rabbit variable domain into a naturally occurring human acceptor framework (FW 1.4, SEQ ID No. 172). No changes in the framework regions are made. The framework itself was preselected for desirable functional properties (solubility and stability).

**[0056]** The term "Max-graft" or "max" as used herein refers to a humanized variable domain that was generated by grafting of rabbit CDRs from a rabbit variable domain into the "rabbitized", human acceptor framework "RabTor" (rFW1.4, SEQ ID No. 173), or into a derivative thereof referred to as rFW1.4(v2) (SEQ ID No. 174). The "RabTor" framework was prepared by incorporating conserved rabbit residues (otherwise which are rather variable in other species) at framework positions generally involved in rabbit variable domain structure and stability, with the aim to generate a universally applicable framework that accepts virtually any set of rabbit CDRs without the need to graft donor framework residues other than at positions that are different in their presumable progenitor sequence, e.g. that were altered during somatic hypermutation and thus, possibly contribute to antigen binding. The presumable progenitor sequence is defined to be the closest rabbit germline counterpart and in case the closest germline counterpart could not be established, the rabbit subgroup consensus or the consensus of rabbit sequences with a high percentage of similarity.

**[0057]** The term "Min-Max" or "minmax" as used herein refers to a humanized variable domain comprising of a "Min-graft" variable light chain combined with a "Max-graft" variable heavy chain.

**[0058]** The term "Max-Min" or "maxmin" as used herein refers to a humanized variable domain comprising of a "Max-graft" variable light chain combined with a "Min-graft" variable heavy chain.

**[0059]** Different nomenclatures were used for the generated immunobinders. These are typically identified by a number (e.g. #578). In those cases where a prefix such as EP or Epi was used (e.g. EP 578 which is identical to Epi 578), the same immunobinder is thereby indicated. Occasionally, an immunobinder received a second designation which is identified by the prefix "ESBA". For example ESBA903 designates the same immunobinder as 578minmax or EP578minmax or Epi578minmax.

**[0060]** Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

**[0061]** Various aspects of the invention are described in further detail in the following subsections. It is understood that the various embodiments, preferences and ranges may be combined at will. Further, depending on the specific embodiment, selected definitions, embodiments or ranges may not apply.

#### ***Anti-VEGF Immunobinders***

**[0062]** The present invention provides immunobinders that bind VEGF and thus are suitable to block the function of VEGF *in vivo*. The CDRs of these immunobinders are derived from rabbit anti-VEGF monoclonal antibodies which were obtained from rabbits that were immunized with human VEGF and/or a fragment thereof (SEQ ID No.1). To our knowledge, this is the first time that monoclonal anti-VEGF antibodies were obtained from rabbits and characterized in detail. Surprisingly, the affinities (Kd) were found to be extraordinarily high.

**[0063]** In certain aspects, the unclaimed disclosure provides an immunobinder, which specifically binds VEGF, comprising at least one of a CDRH1, a CDRH2, a CDRH3, a CDRL1, a CDRL2, or a CDRL3 amino acid sequence. Exemplary CDR amino acid sequences for use in the immunobinders are set forth in SEQ ID Nos: 2-72 (Tables 1-6).

**Table 1.** CDR H1 amino acid sequences of anti-VEGF immunobinders

Sequence Identifier	CDR-H1	SEQ ID No.
60-11-4	GFPFSSGYWVC	2
60-11-6	GFSFSSGYWIC	3
435	GFSLNTNYWMC	4
453	GFSFSRSYYIY	5
375	GFSFTTGYMC	6
610	GIDFSGAYYMG	7
578	GFSLTDYYMT	8
534	GFSLSYYYMS	9
567	GFSLSDYYMC	10
509	GFSLSSYYMC	11
511	GFSLNTYYMN	12
509maxII	GFSLSSYYMS	13
Consensus	GFSLSSGYYMC	14

**Table 2.** CDR H2 amino acid sequences of antiVEGF immunobinders

Sequence Identifier	CDR-H2	SEQ ID No.
60	CIYAGSSGSTYYASWAKG	15
435	CMYTGSYNRAYYASWAKG	16
453	CIDAGSSGILVYANWAKG	17
375	CILAGDGSTYYANWAKG	18
610	YIDYDGDRYYASWAKG	19
578	FIDPDDDPYYATWAKG	20
534	IIGPGDYTDYASWAKG	21
567	CLDYFGSTDDASWAKG	22
509	CLDYVGDTDYASWAKG	23
511	IIAPDDTTYYASWAKS	24
509maxII	ILDYVGDTDYASWAKG	25
Consensus	CIDAGSDGDTYYASWAKG	26

**Table 3.** CDR H3 amino acid sequences of antiVEGF immunobinders.

Sequence Identifier	CDR-H3	SEQ ID No.
60	GNNYYIYTDGGYAYAGLEL	27
435	GSNWYSDL	28
453	GDASYGVDSFMLPL	29
375	SDPASSWSFAL	30
610	SDYSSGWGTDI	31
578	GDHNSGWGLDI	32
534	GDDNSGWGEDI	33
567	TDDSRGWGLNI	34
509	TDDSRGWGLNI	35
511	SGDTTAWGADI	36
Consensus	GDDSSGYTDGGYAYWGLDI	37

**Table 4.** CDR L1 amino acid sequences of anti-VEGF immunobinders.

Sequence Identifier	CDR-L1	SEQ ID No.
60	QASQSISSYLS	38
435	QASQSIGSSLA	39

Sequence Identifier	CDR-L1	SEQ ID No.
453	QSSQSVVNNNRLA	40
375	QASENINIWLS	41
610	QASQSISSWLS	42
578	QASEIIHSWUX	43
534	QASQSINIWLS	44
567	QADQSIYIWLS	45
509	QASQNIWIWLS	46
511	QASQSINIWCWS	47
511max	QASQSINIWLS	48
Consensus	QASQSININNWLS	49

**Table 5.** CDR L2 amino acid sequences of anti-VEGF immunobinders.

Sequence Identifier	CDR-L2	SEQ ID No.
60	KASTLAS	50
435	TAANLAS	51
453	YASTLAS	52
375	QASKLAS	53
610	QASTLAS	54
578	LASTLAS	55
534	KESTLAS	56
567	KASTLES	57
509	KASTLES	58
511	RASTLAS	59
Consensus	KASTLAS	60

**Table 6.** CDR L3 amino acid sequences of anti-VEGF immunobinders.

Sequence Identifier	CDR-L3	SEQ ID No.
60	QSNYGGSSSDYGNP	61
435	QNFATSDTVT	62
453	AGGYSSTSDNT	63
375	QNNYSYNRYGAP	64
610	QNNYGFRSYGGA	65
578	QNVYLASTNGAN	66
534	QNNYDSGNNGFP	67
567	QNNAHYSTNGGT	68
509	QNNAHYSTNGGT	69
511	QANYAYSAGYGAA	70
Consensus	QNNYHYSSSTNGGT	71

**[0064]** In one aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a consensus sequence of the group consisting of SEQ ID NO: 14, SEQ ID NO: 26, SEQ ID NO: 37, SEQ ID NO: 49, SEQ ID NO: 60 and SEQ ID NO: 71. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 14, SEQ ID NO: 26 and SEQ ID NO: 37 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 49, SEQ ID NO: 60 and SEQ ID NO: 71. Preferably, the CDR is selected from the group consisting of SEQ ID NO: 2 to SEQ ID NO: 13, SEQ ID NO: 15 to SEQ ID NO: 25, SEQ ID NO: 27 to SEQ ID NO: 36, SEQ ID NO: 38 to SEQ ID NO: 48, SEQ ID NO: 50 to SEQ ID NO: 59 and

SEQ ID NO: 61 to SEQ ID NO: 70.

**[0065]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 15, SEQ ID NO: 27, SEQ ID NO: 38, SEQ ID NO: 50 and SEQ ID NO: 61.

**[0066]** Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 2, SEQ ID NO: 15 and SEQ ID NO: 27 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 38, SEQ ID NO: 50 and SEQ ID NO: 61. In another preferred embodiment, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 3, SEQ ID NO: 15 and SEQ ID NO: 27 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 38, SEQ ID NO: 50 and SEQ ID NO: 61.

**[0067]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 4, SEQ ID NO: 16, SEQ ID NO: 28, SEQ ID NO: 39, SEQ ID NO: 51, and SEQ ID NO: 62. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 4, SEQ ID NO: 16, SEQ ID NO: 28 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 39, SEQ ID NO: 51, and SEQ ID NO: 62.

**[0068]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 5, SEQ ID NO: 17, SEQ ID NO: 29, SEQ ID NO: 40, SEQ ID NO: 52 and SEQ ID NO: 63. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 5, SEQ ID NO: 17, SEQ ID NO: 29 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 40, SEQ ID NO: 52 and SEQ ID NO: 63.

**[0069]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 6, SEQ ID NO: 18, SEQ ID NO: 30, SEQ ID NO: 41, SEQ ID NO: 53 and SEQ ID NO: 64. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 6, SEQ ID NO: 18 and SEQ ID NO: 30 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 41, SEQ ID NO: 53 and SEQ ID NO: 64.

**[0070]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 7, SEQ ID NO: 19, SEQ ID NO: 31, SEQ ID NO: 42, SEQ ID NO: 54 and SEQ ID NO: 65. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 7, SEQ ID NO: 19 and SEQ ID NO: 31 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 42, SEQ ID NO: 54 and SEQ ID NO: 65.

**[0071]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 8, SEQ ID NO: 20, SEQ ID NO: 32, SEQ ID NO: 43, SEQ ID NO: 55 and SEQ ID NO: 66. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 8, SEQ ID NO: 20 and SEQ ID NO: 32 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 43, SEQ ID NO: 55 and SEQ ID NO: 66.

**[0072]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 9, SEQ ID NO: 21, SEQ ID NO: 33, SEQ ID NO: 44, SEQ ID NO: 56 and SEQ ID NO: 67. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 9, SEQ ID NO: 21 and SEQ ID NO: 33 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 44, SEQ ID NO: 56 and SEQ ID NO: 67.

**[0073]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at

least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 10, SEQ ID NO: 22, SEQ ID NO: 34, SEQ ID NO: 45, SEQ ID NO: 57 and SEQ ID NO: 68. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 10, SEQ ID NO: 22 and SEQ ID NO: 34 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 45, SEQ ID NO: 57 and SEQ ID NO: 68

**[0074]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 35, SEQ ID NO: 46, SEQ ID NO: 58 and SEQ ID NO: 69. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 11, SEQ ID NO: 23 and SEQ ID NO: 35 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 46, SEQ ID NO: 58 and SEQ ID NO: 69. Alternatively, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 13, SEQ ID NO: 25 and SEQ ID NO: 35 and/or the CDRs of the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 46, SEQ ID NO: 58 and SEQ ID NO: 69.

**[0075]** In another aspect, the unclaimed disclosure provides an immunobinder comprising at least one CDR having at least 75% similarity, preferably at least 75% identity, more preferably at least 80%, 85%, 90% 95%, even more preferably 100% identity to a sequence of the group consisting of SEQ ID NO: 12, SEQ ID NO: 24, SEQ ID NO: 36, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 59, and SEQ ID NO: 70. Preferably, the VH of said immunobinder comprise the CDRs of the group consisting of SEQ ID NO: 12, SEQ ID NO: 24 and SEQ ID NO: 36. Additionally or alternatively, the VL of said immunobinder comprise CDRs of the group consisting of SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 59, and SEQ ID NO: 70, e.g. SEQ ID NO: 47, SEQ ID NO: 59, and SEQ ID NO: 70; or SEQ ID NO: 48, SEQ ID NO: 59, and SEQ ID NO: 70.

**[0076]** In a much preferred embodiment, the immunobinder disclosed herein neutralizes human VEGF and is cross-reactive with rat/mouse VEGF or a portion thereof.

**[0077]** The immunobinder can comprise an antibody or any alternative binding scaffold capable of accommodating CDRs. The CDRs set forth in SEQ ID Nos: 2-72 can be grafted onto any suitable binding scaffold using any art recognized methods (see, e.g., Riechmann, L. et al. (1998) *Nature* 332:323-327; Jones, P. et al. (1986) *Nature* 321:522-525; Queen, C. et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:10029-10033; U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen et al.). However, it is preferred that the immunobinders disclosed herein are humanized, and thus suitable for therapeutic applications.

**[0078]** In the case of antibodies, the rabbit CDRs set forth in SEQ ID Nos: 2-72 may be grafted into the framework regions of any antibody from any species. However, it has previously been discovered that antibodies or antibody derivatives comprising the frameworks identified in the so called "quality control" screen (WO0148017) are characterised by a generally high stability and/or solubility and thus may also be useful in the context of extracellular applications such as neutralizing human VEGF. Moreover, it has further been discovered that one particular combination of these VL (variable light chain) and VH (variable heavy chain) soluble and stable frameworks is particularly suited to accommodating rabbit CDRs. Accordingly, in one embodiment, the CDRs set forth in SEQ ID Nos: 2-72 are grafted into the human antibody frameworks derived by "quality control" screening disclosed in EP1479694. The amino acid sequences of exemplary frameworks are set forth in SEQ ID Nos: 172 to 174. It was surprisingly found that upon grafting into said framework or its derivatives, loop conformation of a large variety of rabbit CDRs could be fully maintained, largely independent of the sequence of the donor framework. Moreover, said framework or its derivatives containing different rabbit CDRs are well expressed and produced contrary to the rabbit wild type single chains and still almost fully retain the affinity of the original donor rabbit antibodies.

**[0079]** Thus, in a preferred embodiment, the CDRs and/or CDR motifs disclosed herein are present in a heavy chain variable region framework sequence having at least 80% sequence identity, more preferably at least 85%, 90% 95%, even more preferably 100% identity to the sequence of SEQ ID NO: 169. In a preferred embodiment, the heavy chain variable region framework sequence comprises SEQ ID NO: 170 or SEQ ID NO: 171.

**[0080]** In a preferred embodiment, the CDRs and/or CDR motifs disclosed herein are present in a light chain variable region framework sequence having at least 85% sequence identity, more preferably at least 90%, 95%, even more

preferably 100% identity to the sequence of SEQ ID NO: 167, more preferably comprising SEQ ID NO: 167 or SEQ ID NO: 168.

**[0081]** In rabbit antibodies, CDRs can contain cysteine residues that become disulphide linked to cysteine residues in the antibody framework. Accordingly, it may be necessary, when grafting rabbit CDRs containing cysteine residues into non-rabbit framework regions to introduce cysteine residues in the non-rabbit framework by, for example, mutagenesis to facilitate the stabilization of rabbit CDR through a disulphide linkage.

**[0082]** In other aspect, the unclaimed disclosure provides an immunobinder, which specifically binds VEGF, comprising at least one of a VL or a VH amino acid sequence.

**[0083]** Exemplary VH or VL amino acid sequences for use in the immunobinders set forth in SEQ ID Nos: 72-106 and 107-166, respectively.

**[0084]** In a preferred aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 130 and SEQ ID NO: 131 (VH 60-11-4, VH 60-11-6, VH 60-11-4min, VH 60-11-6min, VH 60-11-4max and VH 60-11-6max, respectively); and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 72, SEQ ID NO: 82 and SEQ ID NO: 93 (VL 60, VL 60min, VL 60max, respectively).

**[0085]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 109, SEQ ID NO: 120 and SEQ ID NO: 132 (VH 435, VH 435min and VH 435max, respectively);

and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 73, SEQ ID NO: 83 and SEQ ID NO: 94 (VL 435, VL 435min and VL 435max, respectively).

**[0086]** Preferably, said immunobinder has at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to SEQ ID NO: 175 (435max).

**[0087]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 110, SEQ ID NO: 121 and SEQ ID NO: 133 (VH 453, VH 453min and VH 453max, respectively);

and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 74, SEQ ID NO: 84 and SEQ ID NO: 95 (VL 453, VL 453min and VL 453max, respectively).

**[0088]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 111, SEQ ID NO: 122 and SEQ ID NO: 134 (VH 375, VH 375min and VH 375max, respectively);

and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 75, SEQ ID NO: 85 and SEQ ID NO: 96 (VL 375, VL 375min and VL 375max, respectively).

**[0089]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 112, SEQ ID NO: 123 and 135 (VH 610, VH 610min and VH 610max, respectively); and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 86 and SEQ ID NO: 97 (VL 610, VL 610min and VL 610max, respectively).

**[0090]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 113, SEQ ID NO: 124, SEQ ID NO: 129, SEQ ID NO: 136, SEQ ID NO: 142, SEQ ID NO: 144, SEQ ID NO: 146, SEQ ID NO: 147, SEQ ID NO: 148, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152, SEQ ID NO: 153, SEQ ID NO: 154, SEQ ID NO: 155, SEQ ID NO: 156, SEQ ID NO: 157, SEQ ID NO: 158, SEQ ID NO: 159, SEQ ID NO: 160, SEQ ID NO: 161, SEQ ID NO: 162, SEQ ID NO: 163, SEQ ID NO: 164, SEQ ID NO: 165 and SEQ ID NO: 166 (VH 578 and variants thereof); and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 77, SEQ ID NO: 87, SEQ ID NO: 92, SEQ ID NO: 98, SEQ ID NO: 103, SEQ ID NO: 104 and SEQ ID NO: 105 (VL 578 and variants thereof).

**[0091]** Preferably, said immunobinder has at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to SEQ ID NO: 178 (578min), SEQ ID NO: 179 (578max) or SEQ ID NO: 180 (578minmax).

**[0092]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 114, SEQ ID NO: 125 and SEQ ID NO: 137 (VH 534, VH 534min and VH 534max, respectively); and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 78, SEQ ID NO: 88 and SEQ ID NO: 99 (VL 534, VL 534min and VL 534max, respectively).

**[0093]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 115, SEQ ID NO: 126, SEQ ID NO: 138 and SEQ ID NO: 143 (VH 567, VH 567min and VH 567max, respectively); and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 79, SEQ ID NO: 89 and SEQ ID NO: 100 (VL 567, VL 567min and VL 567max, respectively).

**[0094]** Preferably, said immunobinder has at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to SEQ ID NO: 177 (567min).

**[0095]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 116, SEQ ID NO: 127, SEQ ID NO: 139 and SEQ ID NO: 140 (VH 509, VH 509min, VH 509max and VH 509maxII, respectively); and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 80, SEQ ID NO: 90 and SEQ ID NO: 101 (VL 509, VL 509min and VL 509max, respectively).

**[0096]** In another aspect, the unclaimed disclosure provides an immunobinder comprising a VH having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 117, SEQ ID NO: 128, SEQ ID NO: 141 and SEQ ID NO: 145 (VH 511, VH 511min, VH 511max and VH 511maxDHP, respectively); and/or a VL having at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 81, SEQ ID NO: 91, SEQ ID NO: 102 and SEQ ID NO: 106 (VL 511, VL 511min, VL 511max and VL 511minC41L, respectively).

**[0097]** Preferably, said immunobinder has at least 80%, more preferably at least 85%, 90%, 95%, most preferably 100% identity to SEQ ID NO: 176 (511\_max).

**[0098]** In certain aspects, the unclaimed disclosure further provides an immunobinder, which specifically binds VEGF, comprising an amino acid sequence with substantial similarity to an amino acid sequence set forth in SEQ ID Nos: 2-166 and in SEQ ID Nos: 175-180, and wherein the immunobinder essentially retains or improves the desired functional properties of the anti-VEGF immunobinder of the invention. Preferred percentage similarities include, but are not limited

to, at least 50%, 60%, 70%, 75%, 80%, 85%, 90% or 95% identity.

**[0099]** In certain aspects, the unclaimed disclosure further provides an immunobinder, which specifically binds VEGF, comprising an amino acid sequence with substantial identity to an amino acid sequence set forth in SEQ ID Nos: 2-166 and in SEQ ID Nos. 175-180, and wherein the immunobinder retains or improves the desired functional properties of the anti-VEGF immunobinder of the invention. Preferred percentage identities include, but are not limited to, at least 50%, 60%, 70%, 75%, 80%, 85%, 90% or 95% identity.

**[0100]** In certain aspects, the unclaimed disclosure further provides an immunobinder, which specifically binds VEGF, comprising an amino acid sequence with conservative substitutions relative to an amino acid sequence set forth in SEQ ID Nos: 2-166 and in SEQ ID Nos. 1-75-180, and wherein the immunobinder retains or improves the desired functional properties of the anti-VEGF immunobinder of the invention.

**[0101]** In some aspects, the unclaimed disclosure provides immunobinders that bind specifically to human VEGF and cross react with VEGF molecules of other species, for example, mouse VEGF, rat VEGF, rabbit VEGF or guinea pig VEGF. In a particular embodiment the anti-VEGF immunobinder can bind specifically to human and rat/mouse VEGF.

**[0102]** In some aspects, the unclaimed disclosure provides immunobinders that bind specifically to human VEGF and do not cross react with VEGF molecules of other species, for example, mouse VEGF, rat VEGF, rabbit VEGF or guinea pig VEGF.

**[0103]** In some aspects, the unclaimed disclosure immunobinders that bind specifically to human VEGF and wherein the immunobinders are affinity matured.

**[0104]** In one embodiment, antibodies and antibody fragments of the present invention are single chain antibodies (scFv) or Fab fragments. In the case of scFv antibodies, a selected VL domain can be linked to a selected VH domain in either orientation by a flexible linker. A suitable state of the art linker consists of repeated GGGGS amino acid sequences or variants thereof. In a preferred embodiment of the present invention a (GGGGS)<sub>4</sub> linker of the amino acid sequence set forth in SEQ ID NO: 181, but variants of 1-3 repeats are also possible (Holliger et al. (1993), Proc. Natl. Acad. Sci. USA 90:6444-6448). Other linkers that can be used for the present invention are described by Alftan et al. (1995), Protein Eng. 8:725-731, Choi et al. (2001), Eur. J. Immunol. 31:94-106, Hu et al. (1996), Cancer Res. 56:3055-3061, Kipriyanov et al. (1999), J. Mol. Biol. 293:41-56 and Roovers et al. (2001), Cancer Immunol. Immunother. 50:51-59.. The arrangement can be either VL-linker-VH or VH-linker-VL, with the former orientation being the preferred one. However, single VH or VL domain antibodies are also contemplated. In the case of Fab fragments, selected light chain variable domains VL are fused to the constant region of a human Ig kappa chain, while the suitable heavy chain variable domains VH are fused to the first (N-terminal) constant domain CH1 of a human IgG. At the C-terminus of the constant domain or at other sites of the variable or constant domain, an inter-chain disulfide bridge may be formed. Alternatively, the two chains may also be linked by a flexible linker resulting in a single chain Fab antibody.

**[0105]** The antibodies or antibody derivatives of the present invention can have affinities to human VEGF with dissociation constants  $K_d$  in a range of  $10^{-14}M$  to  $10^{-5}M$ . In a preferred embodiment of the present invention the  $K_d$  is  $\leq 1$  nM. The affinity of an antibody for an antigen can be determined experimentally using a suitable method (Berzofsky et al. "Antibody-Antigen Interactions", in Fundamental Immunology, Paul, W.E., Ed, Raven Press: New York, NY (1992); Kuby, J. Immunology, W.H. Freeman and Company: New York, NY ) and methods described therein.

**[0106]** The company Epitomics sells an anti-VEGF antibody which is a rabbit monoclonal antibody (VEGF (C-term) Rabbit Antibody, Cat.no. 1909-1). Said antibody is directed against residues on the C-terminus of human VEGF and therefore not able to neutralize VEGF. Hence, said antibody is not suitable for therapeutic applications. Moreover, said monoclonal IgG is not a humanized antibody but is a natural rabbit full-length immunoglobulin. In addition, it was shown that this antibody does not recognize the native form of VEGF.

#### *Immunobinders that bind the same epitopes on VEGF*

**[0107]** In another aspect, the unclaimed disclosure provides antibodies that bind to an epitope on VEGF recognized by

an antibody comprising any one of the amino acid sequences set forth in SEQ ID No 2-211. Such antibodies can be identified based upon their ability to cross-compete with an antibody comprising any one or more of the amino acid sequences set forth in SEQ ID No 2-211 in standard VEGF-binding assays including, but not limited, to ELISA. The ability of a test antibody to inhibit the binding to human VEGF of an antibody comprising any one or more of the amino acid sequences set forth in SEQ ID No 2-211 demonstrates that the test antibody can cross-compete thus interact with an overlapping epitope on human VEGF as an antibody comprising any one or more of the amino acid sequences set forth in SEQ ID No 2-211.

**[0108]** Additionaly or alternatively, such antibodies can be also identified using standard epitope mapping techniques to determine if they bind to the same peptide immunogens. Structural modelling techniques may also be employed to further define the precise molecular determinants to the antibody/VEGF interaction, including, but not limited to, NMR, X-ray crystallography, computer based modeling, or protein tomography (Banyay et al., 2004 ASSAY and Drug Development Technologies (2), 5, Page 516-567). Indeed, the crystal structure of VEGF has been solved and the surface amino acid residues involved in VEGFr binding are known (Fuh, et al., 2006, J. Biol. Chem., 281, 6625-6631). Accordingly, given the amino acid sequence of the peptide immunogen and the structural knowledge of VEGF available in the art, it is well within the skill in art to identify antibodies that bind to an epitope on VEGF recognized by the antibodies comprising any one or more of the amino acid sequences set forth in SEQ ID No 2-211.

**[0109]** In some embodiments, antibodies that bind to an epitope on VEGF recognized by an antibody comprising any one or more of the amino acid sequences set forth in SEQ ID No 2-211 bind to VEGF with an affinity of at least  $10^7 \text{ M}^{-1}$ , for example, at least  $10^7 \text{ M}^{-1}$ , at least  $10^8 \text{ M}^{-1}$ , at least  $10^9 \text{ M}^{-1}$ , at least  $10^{10} \text{ M}^{-1}$ , at least  $10^{11} \text{ M}^{-1}$ , at least  $10^{12} \text{ M}^{-1}$  or at least  $10^{13} \text{ M}^{-1}$ .

**[0110]** In some embodiments, antibodies that bind to an epitope on VEGF recognized by an antibody comprising any one or more of the amino acid sequences set forth in SEQ ID No 2-211 bind specifically to human VEGF and do not cross react with VEGF molecules of other species, for example, mouse VEGF, rat VGEF, rabbit VEGF, or guinea pig VEGF.

**[0111]** In some embodiments, antibodies that bind to an epitope on VEGF recognized by an antibody comprising any one or more of the amino acid sequences set forth in SEQ ID No 2-211 cross react with VEGF molecules of other species, for example, mouse VEGF, rat VEGF, or rabbit VEGF.

#### ***Optimized Variants***

**[0112]** The antibodies disclosed but not claimed may be further optimized for enhanced functional properties, e.g., for enhanced solubility and/or stability.

**[0113]** In certain embodiments, the antibodies of the invention are optimized according to the "functional consensus" methodology. For example, the VEGF immunobinders can be compared with a database of functionally-selected scFvs to identify amino acid residue positions that are either more or less tolerant of variability than the corresponding position(s) in the VEGF immunobinder, thereby indicating that such identified residue position(s) may be suitable for engineering to improve functionality such as stability and/or solubility. For example, one or more of the following substitutions may be introduced at an amino acid position (AHo numbering is referenced for each of the amino acid position listed below) in the heavy chain variable region of an immunobinder

1. (a) Q or E at amino acid position 1;
2. (b) Q or E at amino acid position 6;
3. (c) T, S or A at amino acid position 7, more preferably T or A, even more preferably T;
4. (d) A, T, P, V or D, more preferably T, P, V or D, at amino acid position 10,
5. (e) L or V, more preferably L, at amino acid position 12,
6. (f) V, R, Q, M or K, more preferably V, R, Q or M at amino acid position 13;
7. (g) R, M, E, Q or K, more preferably R, M, E or Q, even more preferably R or E, at amino acid position 14;
8. (h) L or V, more preferably L, at amino acid position 19;
9. (i) R, T, K or N, more preferably R, T or N, even more preferably N, at amino acid position 20;

10. (j) I, F, L or V, more preferably 1, F or L, even more preferably I or L, at amino acid position 21;
11. (k) R or K, more preferably K, at amino acid position 45;
12. (l) T, P, V, A or R, more preferably T, P, V or R, even more preferably R, at amino acid position 47;
13. (m) K, Q, H or E, more preferably K, H or E, even more preferably K, at amino acid position 50;
14. (n) M or I, more preferably 1, at amino acid position 55;
15. (o) K or R, more preferably K, at amino acid position 77;
16. (p) A, V, L or 1, more preferably A, L or I, even more preferably A, at amino acid position 78;
17. (q) E, R, T or A, more preferably E, T or A, even more preferably E, at amino acid position 82;
18. (r) T, S, I or L, more preferably T, S or L, even more preferably T, at amino acid position 86;
19. (s) D, S, N or G, more preferably D, N or G, even more preferably N, at amino acid position 87;
20. (t) A, V, L or F, more preferably A, V or F, even more preferably V, at amino acid position 89;
21. (u) F, S, H, D or Y, more preferably F, S, H or D, at amino acid position 90;
22. (v) D, Q or E, more preferably D or Q, even more preferably D, at amino acid position 92;
23. (w) G, N, T or S, more preferably G, N or T, even more preferably G, at amino acid position 95;
24. (x) T, A, P, F or S, more preferably T, A, P or F, even more preferably F, at amino acid position 98;
25. (y) R, Q, V, I, M, F, or L, more preferably R, Q, I, M, F or L, even more preferably Y, even more preferably L, at amino acid position 103; and
26. (z) N, S or A, more preferably N or S, even more preferably N, at amino acid position 107.

**[0114]** Additionally or alternatively, one or more of the following substitutions can be introduced into the light chain variable region of an immunobinder

- (aa) Q, D, L, E, S, or I, more preferably L, E, S or I, even more preferably L or E, at amino acid position 1;
- (bb) S, A, Y, I, P or T, more preferably A, Y, 1, P or T, even more preferably P or T at amino acid position 2;
- (cc) Q, V, T or I, more preferably V, T or I, even more preferably V or T, at amino acid position 3;
- (dd) V, L, I or M, more preferably V or L, at amino acid position 4;
- (ee) S, E or P, more preferably S or E, even more preferably S, at amino acid position 7;
- (ff) T or I, more preferably 1, at amino acid position 10;
- (gg) A or V, more preferably A, at amino acid position 11;
- (hh) S or Y, more preferably Y, at amino acid position 12;
- (ii) T, S or A, more preferably T or S, even more preferably T, at amino acid position 14;
- (jj) S or R, more preferably S, at amino acid position 18;
- (kk) T or R, more preferably R, at amino acid position 20;
- (ll) R or Q, more preferably Q, at amino acid position 24;
- (mm) H or Q, more preferably H, at amino acid position 46;
- (nn) K, R or I, more preferably R or I, even more preferably R, at amino acid position 47;
- (oo) R, Q, K, E, T, or M, more preferably Q, K, E, T or M, at amino acid position 50;
- (pp) K, T, S, N, Q or P, more preferably T, S, N, Q or P, at amino acid position 53;
- (qq) I or M, more preferably M, at amino acid position 56;
- (rr) H, S, F or Y, more preferably H, S or F, at amino acid position 57;
- (ss) I, V or T, more preferably V or T, R, even more preferably T, at amino acid position 74;
- (tt) R, Q or K, more preferably R or Q, even more preferably R, at amino acid position 82;

- (uu) L or F, more preferably F, at amino acid position 91;
- (w) G, D, T or A, more preferably G, D or T, even more preferably T, at amino acid position 92;
- (xx) S or N, more preferably N, at amino acid position 94;
- (yy) F, Y or S, more preferably Y or S, even more preferably S, at amino acid position 101; and
- (zz) D, F, H, E, L, A, T, V, S, G or I, more preferably H, E, L, A, T ,V, S, G or I, even more preferably A or V, at amino acid position 103.

**[0115]** The AHo numbering system is described further in Honegger, A. and Pluckthun, A. (2001) *J. Mol. Biol.* 309:657-670). Alternatively, the Kabat numbering system as described further in Kabat *et al.* (Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) may be used. Conversion tables for the two different numbering systems used to identify amino acid residue positions in antibody heavy and light chain variable regions are provided in A. Honegger, *J.Mol.Biol.* 309 (2001) 657-670.

**[0116]** In certain preferred embodiments, the immunobinder comprises a solubility enhancing mutation at an amino acid position selected from the group of heavy chain amino acid positions consisting of 12, 103 and 144 (AHo Numbering convention). In one preferred embodiment, the immunobinder comprises one or more substitutions selected from the group consisting of: (a) Serine (S) at heavy chain amino acid position 12; (b) Serine (S) or Threonine (T) at heavy chain amino acid position 103; and (c) Serine (S) or Threonine (T) at heavy chain amino acid position 144. In another embodiment, the immunobinder comprises the following substitutions: (a) Serine (S) at heavy chain amino acid position 12; (b) Serine (S) or Threonine (T) at heavy chain amino acid position 103; and (c) Serine (S) or Threonine (T) at heavy chain amino acid position 144.

***Hybridomas Expressing Rabbit Anti-VEGF Antibodies***

**[0117]** In another - aspect, the unclaimed disclosure provides a hybridoma expressing a monoclonal antibody comprising any one or more of the amino acid sequences set forth in SEQ ID Nos 72-81 and SEQ ID Nos I07-117. Methods for generating hybridomas from Rabbit B-cells are well known in the art and are disclosed, for example, in U.S. patent application 2005/0033031.

***Production of Anti-VEGF Immunobinders***

**[0118]** The antibodies or antibody derivatives may be generated using routine techniques in the field of recombinant genetics. Knowing the sequences of the polypeptides, the cDNAs encoding them can be generated by gene synthesis ([www.genscript.com](http://www.genscript.com)). These cDNAs can be cloned into suitable vector plasmids. Once the DNA encoding a VL and/or a VH domain are obtained, site directed mutagenesis, for example by PCR using mutagenic primers, can be performed to obtain various derivatives. The best "starting" sequence can be chosen depending on the number of alterations desired in the VL and/or VH sequences.

**[0119]** Methods for incorporating or grafting CDRs into framework regions include those set forth in, e.g., Riechmann, L. et al. (1998) *Nature* 332:323-327; Jones, P. et al. (1986) *Nature* 321:522-525; Queen, C. et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:10029-10033; U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen et al..

**[0120]** Standard cloning and mutagenesis techniques well known to the person skilled in the art can be used to attach linkers, shuffle domains or construct fusions for the production of Fab fragments. Basic protocols disclosing the general methods of this invention are described in Molecular Cloning, A Laboratory Manual (Sambrook & Russell, 3rd ed. 2001) and in Current Protocols in Molecular Biology (Ausubel et al., 1999).

**[0121]** The DNA sequence harboring a gene encoding a scFv polypeptide, or in the case of Fab fragments, encoding either two separate genes or a bi-cistronic operon comprising the two genes for the VL-C<sub>k</sub> and the VH-CH1 fusions are cloned in a suitable expression vector, preferably one with an inducible promoter. Care must be taken that in front of each gene an appropriate ribosome binding site is present that ensures translation. It is to be understood that the antibodies as disclosed but not claimed comprise the disclosed sequences rather than they consist of them. For example, cloning strategies may require that a construct is made from which an antibody with one or a few additional residues at the N-terminal end are present. Specifically, the methionine derived from the start codon may be present in the final protein in cases where it has not been cleaved posttranslationally. Most of the constructs for scFv antibodies give rise to an additional alanine at the N-terminal end. In a preferred aspect of the unclaimed disclosure an expression vector for periplasmic expression in *E. coli* is chosen (Krebber, 1997). Said vector comprises a promoter in front of a cleavable signal sequence. The coding sequence for the antibody peptide is then fused in frame to the cleavable signal sequence. This allows the targeting of the expressed polypeptide to the bacterial periplasm where the signal sequence is cleaved. The antibody is then folded. In the case of the Fab fragments, both the VL-C<sub>k</sub> and the VH-CH1 fusions peptides must be linked to an export signal. The covalent S-S bond is formed at the C-terminal cysteines after the peptides have reached the periplasm. If cytoplasmic expression of antibodies is preferred, said antibodies usually can be obtained at high yields from inclusion bodies, which can be easily separated from other cellular fragments and protein. In this case the inclusion bodies are solubilized in a denaturing agent such as, e.g., guanidine hydrochloride (GndHCl) and then refolded by renaturation procedures well known to those skilled in the art.

**[0122]** Plasmids expressing the scFv or Fab polypeptides are introduced into a suitable host, preferably a bacterial, yeast or mammalian cell, most preferably a suitable *E. coli* strain as for example JM83 for periplasmic expression or BL21 for expression in inclusion bodies. The polypeptide can be harvested either from the periplasm or from inclusion bodies and purified using standard techniques such as ion exchange chromatography, reversed phase chromatography, affinity chromatography and/or gel filtration known to the person skilled in the art.

**[0123]** The antibodies or antibody derivatives of the present disclosure can be characterized with respect to yield, solubility and stability *in vitro*. Binding capacities towards VEGF, preferably towards human VEGF, can be tested *in vitro* by ELISA or surface plasmon resonance (BIACore), using recombinant human VEGF as described in WO9729131, the latter method also allowing to determine the  $k_{off}$  rate constant, which should preferably be less than  $10^{-3}\text{s}^{-1}$ .  $K_d$  values of  $\leq 10\text{ nM}$  are preferred.

**[0124]** Aside from antibodies with strong binding affinity for human VEGF, it is also desirable to select anti-VEGF antibodies which have other beneficial properties from a therapeutic perspective. For example, the antibody may be one which inhibits HUVEC cell growth in response to VEGF (see Example 3). In one embodiment, the antibody may be able to inhibit HUVEC cell proliferation in response to a near maximally effective concentration of VEGF (0.08 nM). Preferably, the antibody has an effective dose 50 (ED50) value of no more than about 5 nM, preferably no more than about 1 nM, preferably no more than about 1 nM, preferably no more than about 0.5 nM and most preferably no more than about 0.06 nM, for inhibiting VEGF-induced proliferation of endothelial cells in this "endothelial cell growth assay", i.e., at these concentrations the antibody is able to inhibit VEGF-induced endothelial cell growth *in vitro* by, e.g., 50% or more.

#### ***Bispecific Molecules***

**[0125]** In another aspect, the unclaimed disclosure features bispecific molecules comprising an anti-VEGF antibody, or a fragment thereof. An antibody or antigen-binding portions thereof, can be derivatized or linked to another functional molecule, e.g., another peptide or protein (e.g., another antibody or ligand for a receptor) to generate a bispecific molecule that binds to at least two different binding sites or target molecules. The antibody may be derivatized or linked to more than one other functional molecule to generate multispecific molecules that bind to more than two different binding sites and/or target molecules; such multispecific molecules are also intended to be encompassed by the term "bispecific molecule" as used herein. To create a bispecific molecule, an antibody can be functionally linked (e.g., by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, antibody fragment, tumor specific or pathogen specific antigens, peptide or binding mimetic, such that a bispecific molecule results. Accordingly, the present unclaimed disclosure includes bispecific molecules comprising at least one first binding molecule having specificity for VEGF and a second binding molecule having specificity for one or more additional target epitope.

**[0126]** In one embodiment, the bispecific molecules comprise a binding specificity at least one antibody, or an antibody fragment thereof, including, e.g., an Fab, Fab', F(ab')<sub>2</sub>, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner et al. U.S. Patent No. 4,946,778.

**[0127]** While human monoclonal antibodies are preferred, other antibodies which can be employed in the bispecific molecules are murine, chimeric and humanized monoclonal antibodies.

**[0128]** The bispecific molecules can be prepared by conjugating the constituent binding specificities using methods known in the art. For example, each binding specificity of the bispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridylidithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (see e.g., Karpovsky et al. (1984) J. Exp. Med. 160:1686; Liu, MA et al. (1985) Proc. Natl. Acad. Sci. USA 82:8648). Other methods include those described in Paulus (1985) Behring Ins. Mitt. No. 78, 1 18-132; Brennan et al. (1985) Science 229:81-83), and Glennie et al. (1987) J. Immunol. 139: 2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

**[0129]** When the binding specificities are antibodies, they can be conjugated via sulfhydryl bonding, for example, via the C-terminus hinge regions of the two heavy chains or other sites, whether naturally occurring or introduced artificially. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, preferably one, prior to conjugation.

**[0130]** Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific molecule is a mAb x mAb, mAb x Fab, Fab x F(ab')<sub>2</sub> or ligand x Fab fusion protein. A bispecific molecule can be a single chain molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific molecules may comprise at least two single chain molecules. Further, a bispecific molecule may be a scFv that specifically binds to first target, wherein the VH and VL of said scFv are linked with a flexible linker comprising a domain providing specific binding to a second target. Suitable linkers are described in U.S. Provisional Application No. 60/937,820. Methods for preparing bispecific molecules are described for example in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

**[0131]** Binding of the bispecific molecules to their specific targets can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), FACS analysis, bioassay (e.g., growth inhibition), or by immunoblot assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (e.g., an antibody) specific for the complex of interest. For example, the VEGF-antibody complexes can be detected using e.g., an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-VEGF complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986.

**[0132]** The radioactive isotope can be detected by such means as the use of a  $\gamma$  counter or a scintillation counter or by autoradiography.

### ***Immunoconjugates***

**[0133]** In another aspect, the unclaimed disclosure features an anti-VEGF antibody, or a fragment thereof, conjugated to a therapeutic moiety, such as a cytotoxin, a drug (e.g., an immunosuppressant) or a radiotoxin. Such conjugates are

referred to herein as "immunoconjugates". Immunoconjugates that include one or more cytotoxins are referred to as "immunotoxins." A cytotoxin or cytotoxic agent includes any agent that is detrimental to (e.g., kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents also include, for example, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

**[0134]** Other preferred examples of therapeutic cytotoxins that can be conjugated to an antibody include duocarmycins, calicheamicins, maytansines and auristatins, and derivatives thereof. An example of a calicheamicin antibody conjugate is commercially available (Mylotarg™; Wyeth-Ayerst).

**[0135]** Cytotoxins can be conjugated to antibodies using linker technology available in the art. Examples of linker types that have been used to conjugate a cytotoxin to an antibody include, but are not limited to, hydrazones, thioethers, esters, disulfides and peptide-containing linkers. A linker can be chosen that is, for example, susceptible to cleavage by low pH within the lysosomal compartment or susceptible to cleavage by proteases, such as proteases preferentially expressed in tumor tissue such as cathepsins (e.g., cathepsins B, C, D).

**[0136]** For further discussion of types of cytotoxins, linkers and methods for conjugating therapeutic agents to antibodies, see also Saito, G. et al. (2003) *Adv. Drug Deliv. Rev.* 55:199-215; Trail, P.A. et al. (2003) *Cancer Immunol. Immunother.* 52:328-337; Payne, G. (2003) *Cancer Cell* 3:207-212; Allen, T.M. (2002) *Nat. Rev. Cancer* 2:750-763; Pastan, I. and Kreitman, R. J. (2002) *Curr. Opin. Investig. Drugs* 3:1089-1091; Senter, P.D. and Springer, C.J. (2001) *Adv. Drug Deliv. Rev.* 53:247-264.

**[0137]** Antibodies also can be conjugated to a radioactive isotope to generate cytotoxic radiopharmaceuticals, also referred to as radioimmunoconjugates. Examples of radioactive isotopes that can be conjugated to antibodies for being used diagnostically or therapeutically include, but are not limited to, iodine<sup>131</sup>, indium<sup>111</sup>, yttrium<sup>90</sup> and lutetium<sup>177</sup>. Methods for preparing radioimmunoconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including Zevalin™ (IDEC Pharmaceuticals) and Bexxar™ (Corixa Pharmaceuticals), and similar methods can be used to prepare radioimmunoconjugates using the antibodies disclosed but not claimed.

**[0138]** The antibody conjugates can be used to modify a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon- $\gamma$ ; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

**[0139]** Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.*, 62:119-58 (1982).

#### **Uses of Anti-VEGF antibodies**

**[0140]** For therapeutic applications, the anti-VEGF antibodies of the invention are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed herein, including those that may be administered to a human intravenously, as a bolus or by continuous infusion over a period of time, by topical, intraocular, intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, or inhalation routes. The antibodies also are suitably administered by intra tumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. The intraperitoneal route is expected to be particularly useful, for example, in the treatment of ovarian tumors.

**[0141]** For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

**[0142]** The anti-VEGF antibodies are useful in the treatment of VEGF-mediated diseases as described herein. For example, age-related macular degeneration (AMD) is a leading cause of severe visual loss in the elderly population. The exudative form of AMD is characterized by choroidal neovascularization and retinal pigment epithelial cell detachment. Because choroidal neovascularization is associated with a dramatic worsening in prognosis, the VEGF antibodies of the present invention are especially useful in reducing the severity of AMD. The progress of this therapy is easily monitored by conventional techniques including ophthalmoscopy, ocular fundus microscopy, and ocular computer tomography.

**[0143]** According to another embodiment of the invention, the effectiveness of the antibody in preventing or treating disease may be improved by administering the antibody serially or in combination with another agent that is effective for those purposes, such as tumor necrosis factor (TNF), an antibody capable of inhibiting or neutralizing the angiogenic activity of acidic or basic fibroblast growth factor (FGF) or hepatocyte growth factor (HGF), an antibody capable of inhibiting or neutralizing the coagulant activities of tissue factor, protein C, or protein S (see Esmon et al., PCT Patent Publication No. WO 91/01753, published 21 Feb. 1991), an antibody capable of binding to HER2 receptor (see Hudziak et al., PCT Patent Publication No. WO 89/06692, published 27 Jul. 1989), or one or more conventional therapeutic agents such as, for example, alkylating agents, photoocoagulants (such as verteporfin), folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Such other agents may be present in the composition being administered or may be administered separately. Also, the antibody is suitably administered serially or in combination with radiological treatments, whether involving irradiation or administration of radioactive substances.

**[0144]** The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody is contacted with a sample containing the VEGF protein (or fragment thereof) to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the VEGF protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the VEGF protein from the antibody.

**[0145]** Anti-VEGF antibodies may also be useful in diagnostic assays for VEGF protein, e.g., detecting its expression in specific cells, tissues, or serum. Such diagnostic methods may be useful in cancer diagnosis.

**[0146]** For diagnostic applications, the antibody typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

1. (a) Radioisotopes, such as  $^{111}\text{In}$ ,  $^{99}\text{Tc}$ ,  $^{14}\text{C}$ ,  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{3}\text{H}$ ,  $^{32}\text{P}$  or  $^{35}\text{S}$ . The antibody can be labeled with the radioisotope using the techniques described in Current Protocols in Immunology, Volumes 1 and 2, Coligen et al., Ed. Wiley-Interscience, New York, N.Y., Pubs. (1991), for example, and radioactivity can be measured using scintillation counting.
2. (b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in Current Protocols in Immunology, *supra*, for example. Fluorescence can be quantified using a fluorimeter.

3. (c) Various enzyme-substrate labels are available and U.S. Pat. No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (e.g., firefly luciferase and bacterial luciferase; U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan-et al., Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in Methods in Enzym. (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981). Examples of enzyme-substrate combinations include, for example:

1. (i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (e.g., orthophenylenediamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));
2. (ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and
3. (iii)  $\beta$ -D-galactosidase ( $\beta$ -D-Gal) with a chromogenic substrate (e.g., P-nitrophenyl- $\beta$ -D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl- $\beta$ -D-galactosidase.

**[0147]** In another embodiment of the invention, the anti-VEGF antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the VEGF antibody.

**[0148]** The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc. 1987).

**[0149]** Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of VEGF protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

**[0150]** Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, e.g., U.S. Pat. No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

**[0151]** For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

**[0152]** The antibodies may also be used for *in vivo* diagnostic assays. Generally, the antibody is labeled with a radio nuclide (such as  $^{111}\text{In}$ ,  $^{99}\text{Tc}$ ,  $^{14}\text{C}$ ,  $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{3}\text{H}$ ,  $^{32}\text{P}$  or  $^{35}\text{S}$ ) so that the tumor can be localized using immunoscintigraphy.

**[0153]** The antibody of the present invention can be provided in a kit, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (e.g., a substrate precursor which provides

the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (e.g., a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

#### ***Pharmaceutical Preparations***

**[0154]** In one aspect the invention provides pharmaceutical formulations comprising anti-VEGF antibodies for the treatment of VEGF-mediated diseases. The term "pharmaceutical formulation" refers to preparations which are in such form as to permit the biological activity of the antibody or antibody derivative to be unequivocally effective, and which contain no additional components which are toxic to the subjects to which the formulation would be administered. "Pharmaceutically acceptable" excipients (vehicles, additives) are those which can reasonably be administered to a subject mammal to provide an effective dose of the active ingredient employed.

**[0155]** A "stable" formulation is one in which the antibody or antibody derivative therein essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993), for example. Stability can be measured at a selected temperature for a selected time period. Preferably, the formulation is stable at room temperature (about 30° C) or at 40° C for at least 1 week and/or stable at about 2-8° C for at least 3 months to 2 years. Furthermore, the formulation is preferably stable following freezing (to, e.g., -70° C) and thawing of the formulation.

**[0156]** An antibody or antibody derivative "retains its physical stability" in a pharmaceutical formulation if it meets the defined release specifications for aggregation, degradation, precipitation and/or denaturation upon visual examination of color and/or clarity, or as measured by UV light scattering or by size exclusion chromatography, or other suitable art recognized methods.

**[0157]** An antibody or antibody derivative "retains its chemical stability" in a pharmaceutical formulation, if the chemical stability at a given time is such that the protein is considered to still retain its biological activity as defined below. Chemical stability can be assessed by detecting and quantifying chemically altered forms of the protein. Chemical alteration may involve size modification (e.g. clipping) which can be evaluated using size exclusion chromatography, SDS-PAGE and/or matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS), for example. Other types of chemical alteration include charge alteration (e.g. occurring as a result of deamidation) which can be evaluated by ion-exchange chromatography, for example.

**[0158]** An antibody or antibody derivative "retains its biological activity" in a pharmaceutical formulation, if the biological activity of the antibody at a given time is within about 10% (within the errors of the assay) of the biological activity exhibited at the time the pharmaceutical formulation was prepared as determined in an antigen binding assay, for example. Other "biological activity" assays for antibodies are elaborated herein below.

**[0159]** By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

**[0160]** A "polyol" is a substance with multiple hydroxyl groups, and includes sugars (reducing and non-reducing sugars), sugar alcohols and sugar acids. Preferred polyols herein have a molecular weight which is less than about 600 kD (e.g. in the range from about 120 to about 400 kD). A "reducing sugar" is one which contains a hemiacetal group that can reduce metal ions or react covalently with lysine and other amino groups in proteins and a "non-reducing sugar" is one which does not have these properties of a reducing sugar. Examples of reducing sugars are fructose, mannose, maltose, lactose, arabinose, xylose, ribose, rhamnose, galactose and glucose. Non-reducing sugars include sucrose, trehalose, sorbose, melezitose and raffinose. Mannitol, xylitol, erythritol, threitol, sorbitol and glycerol are examples of sugar alcohols. As to sugar acids, these include L-gluconate and metallic salts thereof. Where it is desired that the formulation is freeze-thaw stable, the polyol is preferably one which does not crystallize at freezing temperatures (e.g.

-20° C) such that it destabilizes the antibody in the formulation. Non-reducing sugars such as sucrose and trehalose are the preferred polyols herein, with trehalose being preferred over sucrose, because of the superior solution stability of trehalose.

**[0161]** As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components. The buffer of this invention has a pH in the range from about 4.5 to about 8.0; preferably from about 5.5 to about 7. Examples of buffers that will control the pH in this range include acetate (e.g. sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. Where a freeze-thaw stable formulation is desired, the buffer is preferably not phosphate.

**[0162]** In a pharmacological sense, in the context of the present invention, a "therapeutically effective amount" of an antibody or antibody derivative refers to an amount effective in the prevention or treatment of a disorder for the treatment of which the antibody or antibody derivative is effective. A "disease/disorder" is any condition that would benefit from treatment with the antibody or antibody derivative. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

**[0163]** A "preservative" is a compound which can be included in the formulation to essentially reduce bacterial action therein, thus facilitating the production of a multi-use formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

**[0164]** The present invention also provides pharmaceutical compositions comprising one or more antibodies or antibody derivative compounds, together with at least one physiologically acceptable carrier or excipient. Pharmaceutical compositions may comprise, for example, one or more of water, buffers (e.g., neutral buffered saline or phosphate buffered saline), ethanol, mineral oil, vegetable oil, dimethylsulfoxide, carbohydrates (e.g., glucose, mannose, sucrose or dextrans), mannitol, proteins, adjuvants, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione and/or preservatives. As noted above, other active ingredients may (but need not) be included in the pharmaceutical compositions provided herein.

**[0165]** A carrier is a substance that may be associated with an antibody or antibody derivative prior to administration to a patient, often for the purpose of controlling stability or bioavailability of the compound. Carriers for use within such formulations are generally biocompatible, and may also be biodegradable. Carriers include, for example, monovalent or multivalent molecules such as serum albumin (e.g., human or bovine), egg albumin, peptides, polylysine and polysaccharides such as aminodextran and polyamidoamines. Carriers also include solid support materials such as beads and microparticles comprising, for example, polylactate polyglycolate, poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose or dextran. A carrier may bear the compounds in a variety of ways, including covalent bonding (either directly or via a linker group), noncovalent interaction or admixture.

**[0166]** Pharmaceutical compositions may be formulated for any appropriate manner of administration, including, for example, topical, intraocular, oral, nasal, rectal or parenteral administration. In certain embodiments, compositions in a form suitable for topical use, for example, as eye drops, are preferred. Other forms include, for example, pills, tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs. Within yet other embodiments, compositions provided herein may be formulated as a lyophilizate. The term parenteral as used herein includes subcutaneous, intradermal, intravascular (e.g., intravenous), intramuscular, spinal, intracranial, intrathecal and intraperitoneal injection, as well as any similar injection or infusion technique.

**[0167]** The pharmaceutical composition may be prepared as a sterile injectible aqueous or oleaginous suspension in which the modulator, depending on the vehicle and concentration used, is either suspended or dissolved in the vehicle. Such a composition may be formulated according to the known art using suitable dispersing, wetting agents and/or suspending agents such as those mentioned above. Among the acceptable vehicles and solvents that may be employed are water, 1,3-butanediol, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils may be employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectible

compositions, and adjuvants such as local anesthetics, preservatives and/or buffering agents can be dissolved in the vehicle.

**[0168]** Pharmaceutical compositions may be formulated as sustained release formulations (*i.e.*, a formulation such as a capsule that effects a slow release of modulator following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal, or subcutaneous implantation, or by implantation at the desired target site. Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of modulator release. The amount of an antibody or antibody derivative contained within a sustained release formulation depends upon, for example, the site of implantation, the rate and expected duration of release and the nature of the disease/disorder to be treated or prevented.

**[0169]** Antibody or antibody derivatives provided herein are generally administered in an amount that achieves a concentration in a body fluid (*e.g.*, blood, plasma, serum, CSF, synovial fluid, lymph, cellular interstitial fluid, tears or urine) that is sufficient to detectably bind to VEGF and prevent or inhibit VEGF-mediated diseases/disorders. A dose is considered to be effective if it results in a discernible patient benefit as described herein. Preferred systemic doses range from about 0.1 mg to about 140 mg per kilogram of body weight per day (about 0.5 mg to about 7 g per patient per day), with oral doses generally being about 5-20 fold higher than intravenous doses. The amount of antibody or antibody derivative that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. Dosage unit forms will generally contain between from about 1 mg to about 500 mg of an active ingredient.

**[0170]** Pharmaceutical compositions may be packaged for treating conditions responsive to an antibody or antibody derivative directed to VEGF. Packaged pharmaceutical compositions may include a container holding an effective amount of at least one antibody or antibody derivative as described herein and instructions (*e.g.*, labeling) indicating that the contained composition is to be used for treating a disease/disorder responsive to one antibody or antibody derivative following administration in the patient.

**[0171]** The antibodies or antibody derivatives of the present invention can also be chemically modified. Preferred modifying groups are polymers, for example an optionally substituted straight or branched chain polyalkene, polyalkylene, or polyoxyalkylene polymer or a branched or unbranched polysaccharide. Such effector group may increase the half-life of the antibody *in vivo*. Particular examples of synthetic polymers include optionally substituted straight or branched chain poly(ethyleneglycol) (PEG), poly(propyleneglycol), poly(vinylalcohol) or derivatives thereof. Particular naturally occurring polymers include lactose, amylose, dextran, glycogen or derivatives thereof. The size of the polymer may be varied as desired, but will generally be in an average molecular weight range from 500Da to 50000Da. For local application where the antibody is designed to penetrate tissue, a preferred molecular weight of the polymer is around 5000Da. The polymer molecule can be attached to the antibody, in particular-to the C-terminal end of the Fab fragment heavy chain via a covalently linked hinge peptide as described in WO0194585. Regarding the attachment of PEG moieties, reference is made to "Poly(ethyleneglycol) Chemistry, Biotechnological and Biomedical Applications", 1992, J. Milton Harris (ed), Plenum Press, New York and "Bioconjugation Protein Coupling Techniques for the Biomedical Sciences", 1998, M. Aslam and A. Dent, Grove Publishers, New York.

**[0172]** After preparation of the antibody or antibody derivative of interest as described above, the pharmaceutical formulation comprising it is prepared. The antibody to be formulated has not been subjected to prior lyophilization and the formulation of interest herein is an aqueous formulation. Preferably the antibody or antibody derivative in the formulation is an antibody fragment, such as an scFv. The therapeutically effective amount of antibody present in the formulation is determined by taking into account the desired dose volumes and mode(s) of administration, for example. From about 0.1 mg/ml to about 50 mg/ml, preferably from about 0.5 mg/ml to about 40 mg/ml and most preferably from about 10 mg/ml to about 20 mg/ml is an exemplary antibody concentration in the formulation.

**[0173]** An aqueous formulation is prepared comprising the antibody or antibody derivative in a pH-buffered solution. The buffer of this invention has a pH in the range from about 4.5 to about 8.0, preferably from about 5.5 to about 7. Examples of buffers that will control the pH within this range include acetate (*e.g.* sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. The buffer concentration can be from about 1 mM to about 50 mM, preferably from about 5 mM to about 30 mM, depending, for example, on the buffer and the desired isotonicity of the formulation.

**[0174]** A polyol, which acts as a tonicifier and may stabilize the antibody, is included in the formulation. In preferred embodiments, the formulation does not contain a tonicifying amount of a salt such as sodium chloride, as this may cause the antibody or antibody derivative to precipitate and/or may result in oxidation at low pH. In preferred embodiments, the polyol is a non-reducing sugar, such as sucrose or trehalose. The polyol is added to the formulation in an amount which may vary with respect to the desired isotonicity of the formulation. Preferably the aqueous formulation is isotonic, in which case suitable concentrations of the polyol in the formulation are in the range from about 1% to about 15% w/v, preferably in the range from about 2% to about 10% w/v, for example. However, hypertonic or hypotonic formulations may also be suitable. The amount of polyol added may also alter with respect to the molecular weight of the polyol. For example, a lower amount of a monosaccharide (e.g. mannitol) may be added, compared to a disaccharide (such as trehalose).

**[0175]** A surfactant is also added to the antibody or antibody derivative formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20, 80 etc) or poloxamers (e.g. poloxamer 188). The amount of surfactant added is such that it reduces aggregation of the formulated antibody/antibody derivative and/or minimizes the formation of particulates in the formulation and/or reduces adsorption. For example, the surfactant may be present in the formulation in an amount from about 0.001% to about 0.5%, preferably from about 0.005% to about 0.2% and most preferably from about 0.01% to about 0.1%.

**[0176]** In one embodiment, the formulation contains the above-identified agents (i.e. antibody or antibody derivative, buffer, polyol and surfactant) and is essentially free of one or more preservatives, such as benzyl alcohol, phenol, m-cresol, chlorobutanol and benzethonium Cl. In another embodiment, a preservative may be included in the formulation, particularly where the formulation is a multidose formulation. The concentration of preservative may be in the range from about 0.1% to about 2%, most preferably from about 0.5% to about 1%. One or more other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in Remington's Pharmaceutical Sciences 21st edition, Osol, A. Ed. (2006) may be included in the formulation provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are non-toxic to recipients at the dosages and concentrations employed and include: additional buffering agents, co-solvents, antioxidants including ascorbic acid and methionine, chelating agents such as EDTA, metal complexes (e.g. Zn-protein complexes), biodegradable polymers such as polyesters, and/or salt-forming counterions such as sodium.

**[0177]** The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, preparation of the formulation.

**[0178]** The formulation is administered to a mammal in need of treatment with the antibody, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. In preferred embodiments, the formulation is administered to the mammal by topical application of eye drops to the ocular surface. For such purposes, the formulation may be applied using an eye drop applicator, for example.

**[0179]** The appropriate dosage ("therapeutically effective amount") of the antibody will depend, for example, on the condition to be treated, the severity and course of the condition, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, the type of antibody used, and the discretion of the attending physician. The antibody or antibody derivative is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The antibody or antibody derivative may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

**[0180]** As a general proposition, the therapeutically effective amount of the antibody or antibody derivative administered will be in the range of about 0.1 to about 50 mg/kg of patient body weight whether by one or more administrations, with the typical range of antibody used being about 0.3 to about 20 mg/kg, more preferably about 0.3 to about 15 mg/kg, administered daily, for example. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques.

#### **Articles of Manufacture**

**[0181]** In another aspect of the disclosure which is not claimed, an article of manufacture is provided comprising a container which holds the aqueous pharmaceutical formulation of the present invention and optionally provides instructions for its use. Suitable containers include, for example, bottles, vials, eye drop applicators and syringes. The container may be formed from a variety of materials such as glass or plastic. An exemplary container is a 3-20 cc single use glass or plastic vial. Alternatively, for a multidose formulation, the container may be 3-100 cc glass vial. The container holds the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

***Exemplification***

**[0182]** The present disclosure is further illustrated by the following examples, which should not be construed as further limiting.

**[0183]** Throughout the examples, the following materials and methods were used unless otherwise stated.

**General Materials and Methods**

**[0184]** In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA technology, immunology (especially, e.g., antibody technology), and standard techniques of polypeptide preparation. See, e.g., Sambrook, Fritsch and Maniatis, Molecular Cloning: Cold Spring Harbor Laboratory Press (1989); Antibody Engineering Protocols (Methods in Molecular Biology), 510, Paul, S., Humana Pr (1996); Antibody Engineering: A Practical Approach (Practical Approach Series, 169), McCafferty, Ed., Irl Pr (1996); Antibodies: A Laboratory Manual, Harlow et al., C.S.H.L. Press, Pub. (1999); and Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons (1992).

***Thermostability measurements***

**[0185]** Attenuated total reflectance Fourier transform IR (FTIR-ATR) spectra were obtained for various single chains and derivative molecules using the FT-IR Bio-ATR cell in a Tensor Bruker. The molecules were concentrated up to 3mg/ml and dialyzed overnight at 4°C against PBS, pH 6.5 and the buffer flow through was collected as blank. The denaturation profiles were obtained by thermo challenging the molecules with a broad range of temperatures in 5°C steps (25 to 95°C). All spectra manipulations were performed using OPUS software. The main buffer and transient atmospheric (CO<sub>2</sub> and H<sub>2</sub>O) background were subtracted from the protein spectrum. The resulting protein spectrum was then baseline corrected and the protein amide I spectra was determined from the width of the widest resolvable peak in the expected region. Second derivative spectra were obtained for the amide I band spectra using a third degree polynomial function with a smoothing function. Changes in protein structure were estimated by amide I second derivative analysis using a linear calibration curve for the initial curve-fit calculations assuming 0% denaturation for the 3 lower measurements and 100% denaturation for the 3 higher measurements. The denaturation profiles were used to approximate midpoints of the thermal unfolding transitions (TM) for every variant applying the Boltzmann sigmoidal model.

***Solubility measurements***

**[0186]** Relative solubility of various scFv molecules was measured after enhancing protein aggregation and precipitation in presence of ammonium sulfate. Ammonium sulfate was added to the protein in aqueous solutions to yield increments of 5% of saturation in the final mixture salt-protein. The precipitation in the dynamic range was determined empirically and the saturation intervals reduced in this range to 2.5% intervals saturation in the final mixture. After ammonium sulfate addition, samples were gently mixed and centrifuged 30 minutes at 6000rpm. The remaining protein in

supernatants was recovered for each ammonium sulfate percentage of saturation. Solubility curves were determined by measuring the protein concentration in the supernatant by UV-VIS measurements using NanoDropTM 1000 Spectrophotometer. Measurements of remaining soluble protein in supernatants were normalized and used to estimate midpoints of relative solubility for every variant applying the Boltzmann sigmoidal model.

#### **Short Term Stability test**

**[0187]** The scFv molecules were examined after two weeks incubation at 40°C for the presence of soluble aggregates and degradation products. Proteins with a concentration of 10 mg/ml were dialyzed overnight at 4°C against PBS with a broad range of pHs (3.5, 4.5, 5.5, 6.5, 7.0, 7.5 and 8.5). Control molecules with the same concentration in standard buffer PBS (pH 6.5) were stored at -80°C during the 2 weeks period. Determination of degradation bands by SDS-PAGE was done at t=0 and t=14d time points and soluble aggregates were assessed in the SEC-HPLC. Determination of remaining activity after 2 weeks at 40°C was done using Biacore.

#### **EXAMPLE 1**

##### **IMMUNIZATION STRATEGY FOR GENERATING ANTI-VEGF ANTIBODIES.**

**[0188]** In this example, an immunization strategy is described which used a novel antigenic VEGF-derived peptide, to generate antibodies capable of recognizing human, mouse and rabbit VEGFA.

**[0189]** From alanine-scanning mutagenesis studies performed at Genentech the residues of VEGFA that are crucial for high affinity interaction with VEGFr are known (Fuh, G. et al, (2006) J. Biol. Chem. 281, 6625-6631). Although the receptor-binding site probably represents a conformational epitope, most of the crucial residues lie on an alpha helix, on the first 10 amino acids of mature VEGFA.

**[0190]** Rabbit VEGFA contains three amino acids changes in this alpha helix, when compared to the human sequence; in contrast, mouse VEGFA is identical to human in this region. Thus, for the generation of mouse-human cross-reactive antibodies, rabbit presents a suitable species for immunization. In addition, rabbit immunization can lead to Abs with higher affinity than mouse immunization.

**[0191]** As outlined above, interaction with residues on the N-terminal alpha helix of VEGFA seems to be most crucial for binding to VEGFR1. Therefore, this 10 amino acid long stretch can be used as an epitope for immunization. Alternatively, full length VEGFA can be injected, however, other peptide stretches on VEGFA are more immunogenic, thus lowering the chance to raise neutralizing antibodies. This hypothesis is supported by the fact that two different peptides, both lying close to the C-terminus of VEGFA are potentially immunogenic as predicted by the method of Johnson and Wolf. This method predicts only minor immunogenic potential for the N-terminal alpha helix. Therefore, immunization with the peptide constituting the alpha helix only, can be more straightforward than immunization with full-length VEGFA. The probability to elicit a strong immune response can be further increased by fusion or chemical coupling of the peptide to Keyhole Limpet Hemocyanin (KLH).

**[0192]** Four immunization strategies were performed as follows

1. A. Pre-Immunization of rabbits with full-length human VEGFA<sub>165</sub> to enhance the probability to obtain conformational binders. Second boost with peptide from aa stretch 16-KFMDVYQRSYCHP-28 (underline: receptor interaction; double underline, divergent in rabbit, Cys is involved in disulfide bond according to crystal structure). The Cys contained in the peptided sequence could be used for coupling to KLH and would therefore not be exposed as free Cys. The final peptide would look as follows: KFMDVYQRSY-Cys-KLH.
2. B. Pre-Immunization of mice with full-length VEGFA<sub>165</sub> to enhance the probability to obtain conformational binders. Second boost with peptide from aa stretch 16-KFMDVYQRSYCHP-28 ( Cys is involved in disulfide bond according to crystal structure). The Cys contained in the peptided sequence can be used for coupling to KLH and would therefore not be exposed as free Cys. The final peptide would look as follows: KFMDVYQRSY-Cys-KLH.

3. C. Pre-immunization of rabbits/mice with peptide from aa stretch 16-KFMDVYQRSYCHP -28 (final peptide: KFMDVYQRSY-Cys-KLH). Second boost with full-length VEGFA<sub>165</sub> to enhance the probability to obtain conformational binders.
4. D. Immunization with full length VEGFA<sub>165</sub> in rabbits.

## EXAMPLE 2

### CDR Grafting and Functional Humanization of monoclonal rabbit anti-VEGF antibodies.

#### Grafting of Rabbit CDRs

**[0193]** Unlike traditional humanization methods which employ the human antibody acceptor framework that shares the greatest sequence homology with the non-human donor antibody, the rabbit CDRs were grafted into either framework FW1.4 (SEQ ID No. 172) to generate a Min-graft or into the "rabbitized" framework rFW1.4 (SEQ ID No. 173) or its variant rFW1.4(v2) (SEQ ID No. 174) to generate a Max-graft. Both frameworks were selected primarily for desirable functional properties (solubility and stability), structural suitability to accommodate a large variety of rabbit CDRs and reasonable homology to the rabbit variable domain consensus sequence. Framework rFW1.4 is a derivative of FW1.4 that was further engineered with the aim to serve as universal acceptor framework for virtually any set of rabbit CDRs. Although the stable and soluble framework sequence FW1.4 exhibits high homology to rabbit antibodies, it is not the most homologous sequence available.

#### Identification of residues potentially involved in binding

**[0194]** For each rabbit variable domain sequence, the nearest rabbit germline counterpart was identified. If the closest germline could not be established, the sequence was compared against the subgroup consensus or the consensus of rabbit sequences with a high percentage of similarity. Rare framework residues were considered as possible result of somatic hypermutation and therefore playing a role in antigen binding. Consequently, such residues were considered for grafting onto the acceptor framework rFW1.4 or rFW1.4(v2) to generate Max-grafts. Particularly, residues potentially implicated in direct antigen contact or influencing disposition of VL and VH were grafted. Further residues described to influence CDR structure were substituted if required. No framework substitutions were made when CDRs were grafted onto FW1.4 (Min-grafts). For example to generate 578minmax residue VH 94 (H94) of rFW1.4 was mutated to corresponding residue in the donor sequence. The rabbit antibody 578 contains Gly at H94 whereas both, the most homologous germline and the rabbit consensus contain Arg at position H94. Gly has an exceptional flexibility (*positive phi* angles) that is not found for other amino acids. This suggests a role in mainchain torsion angle and a possible strong influence of the loop conformation with implications on activity. Further examples of framework positions that were grafted to obtain the Max-grafts as disclosed herein can be identified by making a sequence alignment of the framework regions of rFW1.4, rFW1.4(v2) and the scFv sequences of interest provided herein. Webtools as known in the art may for example be used for said purpose (e.g. ClustalW as available on June 23, 2009 at <http://www.ebi.ac.uk/Tools/clustalw2/index.html> or MultiAlin as available on June 23, 2009 at <http://bioinfo.genotoul.fr/multalin>). All framework positions at which rFW1.4 and rFW1.4(v2) contain the same residue and at which the scFv of interest reveals a different residue, are framework positions that were grafted to obtain the Max-grafts.

#### Domain shuffling

**[0195]** Variable light chains of Min-grafts were combined with variable heavy chain Max-grafts to identify optimal combinations in terms of biophysical properties (solubility and stability) and activity.

### **Cloning and expression of scFvs**

**[0196]** The scFvs described and characterized herein were produced as follows. The humanized VL sequences (SEQ ID NOS:82-106) were connected to humanized VH sequences (SEQ ID NOS:118-166) via the linker of SEQ ID NO:181 to yield an scFv of the following orientation: NH<sub>2</sub>-VL-linker-VH-COOH. In many cases DNA sequences encoding for the various scFvs were de novo synthesized at the service provider Entelechon GmbH ([www.entelechon.com](http://www.entelechon.com)). The resulting DNA inserts were cloned into the bacterial expression vector pGMP002 via Ncol and HindIII restriction sites introduced at the 5' and 3' end of the scFv DNA sequence, respectively. Between the DNA sequence of the VL domain and the VH domain, a BamHI restriction site is located. In some cases the scFv encoding DNA was not de novo synthesized, but the scFv expressing constructs were cloned by domain shuffling. Accordingly, the VL domains were excised and introduced into the new constructs via Ncol and BamHI restriction sites, the VH domains via BamHI and HindIII restriction sites. In other cases, point mutations were introduced into the VH and/or VL domain using state of the art assembling PCR methods. The cloning of GMP002 is described in Example 1 of WO2008006235. The production of the scFvs was done analogue as for ESBA105 as described in Example1 of WO2008006235.

### **EXAMPLE3**

#### **BIACORE BINDING ANALYSIS OF ANTI-VEGF SCFVS**

**[0197]** In this example, the Biacore-binding ability of scFvs was tested and the binding affinity was measured using the exemplary surface plasmon resonance method with BIACore™ -T100. The VEGF proteins, tested for binding by these scFv candidates, in this example and later examples include purified *Escherichia coli*-expressed recombinant human VEGF<sub>165</sub>(PeproTech EC Ltd.), recombinant human VEGF<sub>121</sub>(PeproTech EC Ltd.), recombinant human VEGF<sub>110</sub>(ESBA Tech AG), recombinant murine VEGF<sub>164</sub>(PeproTech EC Ltd.), recombinant rat VEGF<sub>164</sub>(Biovision), recombinant rabbit VEGF<sub>110</sub>(ESBATech AG), and recombinant human PLGF (PeproTech EC Ltd.). For the surface plasmon resonance experiment, carboxymethylated dextran biosensor chips (CM4, GE Healthcare) were activated with N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide according to the supplier's instructions. Each of the 6 different VEGF forms, as exemplified above, was coupled to 1 of the 4 different flow cells on a CM4 sensor chip using a standard amine-coupling procedure. The range of responses obtained with these immobilized VEGF molecules after coupling and blocking were -250-500 response units (RU) for hVEGF<sub>165</sub>, -200 RU for hVEGF<sub>110</sub>, hVEGF<sub>121</sub>, murine VEGF<sub>164</sub>, rat VEGF<sub>164</sub> and rabbit VEGF<sub>110</sub> and ~400 RU for PLGF. The 4th flow cell of each chip was treated similarly except no proteins were immobilized prior to blocking, and the flow cell was used as in-line reference. Various concentrations of anti-VEGF scFvs (e.g., 90 nM, 30 nM, 10 nM, 3.33 nM, 1.11 nM, 0.37 nM, 0.12 nM and 0.04 nM) in HBS-EP buffer (0.01 M HEPES, pH 7.4 or 5, 0.15 M NaCl, 3 mM EDTA, 0.0005% surfactant P20) were injected into the flow cells at a flow rate of 30  $\mu$ l/min for 5 min. Dissociation of the anti-VEGF scFv from the VEGF on the CM4 chip was allowed to proceed for 10 min at 25 °C. Sensorgrams were generated for each anti-VEGF scFv sample after in-line reference cell correction followed by buffer sample subtraction. The apparent dissociation rate constant ( $k_d$ ), the apparent association rate constant ( $k_a$ ) and the apparent dissociation equilibrium constant ( $K_D$ ) were calculated using one-to-one Langmuir binding model with BIACore T100 evaluation Software version 1.1.

**[0198]** As one exemplary result, some lead anti-VEGF scFv candidates are listed in Table 7 showing their binding affinity to hVEGF<sub>165</sub>. Their potency as VEGF inhibitors, which is measured using VEGFR competition ELISA and/or HUVEC assay and described in latter examples, is also shown in Table 7. The kinetics curves of some exemplary lead candidates, e.g., 511max and 578max, for their binding to hVEGF<sub>165</sub> are illustrated in Figure 1. Their affinity constants ( $k_d$ ,  $k_a$  and  $K_D$ ) were also determined. Some lead candidates also display species specificity in their binding to various VEGF proteins of different sources. For example, some affinity data measured at pH5 using mouse and rat VEGF<sub>164</sub> as binding partner are shown in Tables 8 a and b. An exemplary lead scFv candidate, 578minmax, has a  $K_D$  of 5.76E-10 M and 7.48E-10 M in its binding to mouse and rat VEGF<sub>164</sub>, respectively at a pH of 5 (Tables 8 a and b) and 2,73E-11 and 2,19E-11 at a pH of 7,4 (data not shown). This species specificity is further illustrated in Figure 4 in the kinetics curves

and affinity data for the binding between 578minmax and human, mouse or rat VEGF proteins.

**[0199]** Besides the species specificity in their binding to VEGFs from different organisms, many lead scFv candidates also display differentiated binding affinities towards various VEGF isoforms. For example, the affinity data measured at pH 5,0 for some scFv candidates binding to human VEGF<sub>165</sub>, VEGF<sub>121</sub> and VEGF<sub>110</sub> are compared in Table 9. In the same experiments, PIGF protein was also used as a negative control without binding capacity to those scFv candidates. Also, the differentiated kinetics curves and affinity data for the binding between 578Max and VEGF isoforms, as an example, are illustrated in Figure 3.

**[0200]** The present invention also discloses derivatives originating from the lead anti-VEGF scFv candidates, which are mentioned above. Some lead derivatives of candidate 578 and 511, as listed in Table 10, are exemplified for their affinity and potency (measured at pH 5,0). In this experiment, Biacore measurement was used for the affinity of these derivatives towards hVEGF<sub>165</sub>, while hVEGFR2 competition ELISA and/or HUVEC assay were used to define their potency to inhibit VEGFs (Table 10). Three derivatives, 578max, 578minmax and 578 wt-His, are further exemplified in their kinetics curves and affinity data for binding to hVEGF<sub>165</sub> in Figure 4.

**[0201]** For derivatives of lead candidates, their biophysical characterizations were determined and exemplified in Figures 5-7 and table 11. These characteristics include, as exemplified in table 11, T<sub>m</sub> determined by FTIR, the percentage of β-sheet or protein loss after incubation at 60°C for 30 min, solubility determined by ammonium sulfate precipitation, refolding yield during the production process and expression levels in *E. coli*. Three derivatives, 578max, 578minmax and 578minmax\_DHP, were characterized for their thermal stability in their unfolding curves against different temperatures measured by FT-IR (Figure 5).

Table 7: overview of affinity and potency of lead candidates

ID	Protein Nr.	Rel. activity hVEGR2 comp. ELISA (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])	Rel. activity hVEGR1 comp. ELISA (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])	Rel. activity in HUVEC assay (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])
375-min	857	0.3	ND	ND
375-max	873	0.6	ND	ND
509-min	854	1.0	2.9	ND
509-max	855	4.1	13	0.003
509-maxII	856	0.6	0.09	0.0009
511-min	801	4.9	0.7	0.0011
511-max	802	8.7	8	0.0179
534-min C-His	807	0.1	ND	ND
534-max	793	1.1	ND	0.0014
567-min	884	9.7	14.9/57	ND
567-max	874	4.1	15.7/154.5	0.0086
578-min	820	4.1	4.8	0.1001
578-max	821	9.6	35.5/ 51.6	1.483
610-	882	0.1	ND	ND

ID	Protein Nr.	Rel. activity hVEGR2 comp. ELISA (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])	Rel. activity hVEGR1 comp. ELISA (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])	Rel. activity in HUVEC assay (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])			
min							
610-max	883	0.4	ND	ND			
435-min	944	0.03	ND	ND			
435-max	945	7.6	0.00039	ND			
ID		Biacore Measurements (pH 5)			Biacore Measurements (pH 7.4)		
		hVEGF <sub>165</sub>			hVEGF <sub>165</sub>		
		ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (M)
375-min		9.27E+05	5.01E-03	5.41E-09	> E+08	3.86E+00	NA
375-max		2.44E+06	6.55E-03	2.68E-09	5.09E+07	2.42E-01	4.74E-09
509-min		6.23E+05	1.14E-03	1.82E-09	3.52E+06	1.08E-02	3.06E-09
509-max		2.26E+06	2.72E-03	1.21E-09	1.42E+06	5.37E-04	3.78E-10
509-maxII		8.38E+05	2.82E-03	3.37E-09	7.59E+06	1.98E-02	2.61E-09
511-min		5.05E+05	1.28E-03	2.53E-09	6.75E+05	8.85E-04	1.31E-09
511-max		6.59E+05	4.40E-05	6.67E-11	8.00E+05	6.85E-05	8.56E-11
534-min C-His		2.71E+05	9.21E-03	3.41E-08	ND	ND	ND
534-max		1.88E+06	1.73E-02	9.21E-09	1.06E+06	2.62E-03	2.47E-09
567-min		2.01E+06	4.61E-04	2.30E-10	1.11E+06	7.00E-04	6.31E-10
567-max		1.20E+06	2.26E-04	1.88E-10	1.17E+06	1.67E-04	1.43E-10
578-min		1.14E+06	1.03E-02	9.01E-09	1.11E+06	2.02E-04	1.81E-10
578-max		7.00E+05	3.07E-04	4.39E-10	1.58E+06	3.76E-05	2.37E-11
610-min		2.51E+05	2.65E-03	1.06E-08	No binding	No binding	No binding
610-max		5.09E+05	6.01E-04	1.18E-09	> E+08	3.57E+01	NA
435-min		No binding	No binding	No binding	4.95E+05	1.43E-02	2.89E-08
435-max		1.67E+05	7.55E-04	4.53E-09	1.13E+06	1.04E-04	9.22E-11

Table 8a: species specificity of selected lead candidates (mouse and rat VEGF 164)

ID	Protein No.	mouse VEGF <sub>164</sub>			rat VEGF		
		ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (M)
509-min	854	6.14E+05	1.00E-03	1.63E-09	3.51E+05	8.44E-04	2.41E-09
509-max	855	4.09E+06	5.90E-03	1.45E-09	3.90E+06	6.45E-03	1.65E-09
<b>509-maxII</b>	<b>856</b>	<b>3.47E+07</b>	<b>6.01E-02</b>	<b>1.73E-09</b>	<b>1.47E+07</b>	<b>2.66E-02</b>	<b>1.81E-09</b>
511-min	801	6.25E+05	1.03E-03	1.64E-09	5.50E+05	1.12E-03	2.04E-09
511-max	802	7.53E+05	4.61E-05	6.13E-11	6.26E+05	6.63E-05	1.06E-10
567-min	884	2.06E+06	3.50E-04	1.70E-10	1.72E+06	4.80E-04	2.79E-10
567-max	874	1.64E+06	1.52E-04	9.29E-11	1.36E+06	2.03E-04	1.49E-10
578-min	820	1.40E+06	1.51E-02	1.07E-08	1.70E+06	1.82E-02	1.07E-08
578-max	821	1.03E+06	4.40E-04	4.29E-10	8.83E+05	5.28E-04	5.98E-10

Table 8b: species specificity of selected development candidates

ID	Protein No.	Biacore measurements mouse VEGF <sub>164</sub>			Relative values Mouse VEGF <sub>164</sub>	
		ka (1/Ms)	kd (1/s)	KD (M)	(kd hVEGF <sub>165</sub> /kd <sub>mV</sub> EGF164)	(Kd hVEGF <sub>165</sub> /Kd <sub>m</sub> VEGF164)
578minmax	903	1,14E+06	6,57E-04	5,67E-10	0,8	1,1
578 minmax_FW1.4:DHP	961	1,10E+06	6,69E-04	6,08E-10	0,6	0,9
578minmaxT84N_V89L	1008	1.23E+06	1,88E-03	1.53E-09	1,0	1,0
578min_max					1,4	1,8
T84N_V89L_DHP	1017	1,47E+06	2,16E-03	1.46E-09		
ID	Protein No.	Biacore measurements rat VEGF <sub>164</sub>			Relative values Mouse VEGF <sub>164</sub>	
		ka (1/Ms)	kd (1/s)	KD (M)	(kd hVEGF <sub>165</sub> /kd <sub>mV</sub> EGF164)	(Kd hVEGF <sub>165</sub> /Kd <sub>m</sub> VEGF164)
578minmax	903	8,58E+05	6,41E-04	7,48E-10	0,8	0,8
578 minmax_FW1.4:DHP	961	8,00E+05	6,76E-04	8,45E-10	0,6	0,7
578minmaxT84N_V89L	1008	8,02E+05	1,52E-03	1.89E-09	1,2	0,8
578min_max					1,6	1,5
T84N_V89L_DHP	1017	1,04E+05	1,90E-03	1.82E-09		

Table 9: Binding of selected lead candidates to VEGF isoforms (human VEGF121 and hVEGF110)

ID	Protein Nr.	hVEGF <sub>165</sub>			hVEGF <sub>110</sub>		
		ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (M)
509-min	854	6.23E+05	1.14E-03	1.82E-09	2.87E+05	4.74E-04	1.65E-09
509-max	855	2.26E+06	2.72E-03	1.21E-09	6.48E+05	2.35E-04	3.63E-10
509-maxII	856	8.38E+05	2.82E-03	3.37E-09	9.01E+05	1.33E-03	1.48E-09
511-min	801	5.05E+05	1.28E-03	2.53E-09	6.19E+05	8.98E-04	1.45E-09
511-max	802	6.59E+05	4.40E-05	6.67E-11	4.05E+05	7.96E-05	1.97E-10
567-min	884	2.01E+06	4.61E-04	2.30E-10	1.52E+06	3.82E-05	2.51E-11
567-max	874	1.20E+06	2.26E-04	1.88E-10	1.00E+06	3.27E-05	3.27E-11
578-min	820	1.14E+06	1.03E-02	9.01E-09	9.15E+05	1.04E-02	1.14E-08
578-max	821	7.00E+05	3.07E-04	4.39E-10	5.23E+05	7.22E-04	1.38E-09

(continued)

ID	hVEGF <sub>121</sub>			PIGF	
	ka (1/Ms)	kd (1/s)	KD (M)		
509-min	3.54E+05	4.53E-04	1.28E-09	no binding	
509-max	7.42E+05	2.49E-04	3.35E-10	no binding	
509-maxII	8.97E+05	1.23E-03	1.37E-09	no binding	
511-min	7.78E+05	9.63E-04	1.24E-09	no binding	
511-max	4.67E+05	9.97E-05	2.14E-10	no binding	
567-min	1.89E+06	4.54E-05	2.41E-11	no binding	
567-max	1.13E+06	5.76E-05	5.11E-11	no binding	
578-min	9.61E+05	8.80E-03	9.16E-09	no binding	
578-max	5.87E+05	5.58E-04	9.50E-10	no binding	

Table 10: Overview on affinity and potency of lead derivatives (578 and 511)

ID	Protein Nr.	Rel. activity hVEGR2 comp. ELISA (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])	Rel. activity in HUVEC assay (EC50 <sub>Luc</sub> [nM]/EC50 <sub>test</sub> [nM])	Biacore Measurements hVEGF <sub>165</sub>			
				hVEGF	ka (1/Ms)	kd (1/s)	KD (M)
578 wildtype C-His	798	ND	ND		8.34E+05	1.69E-04	2.00E-10
578-min	820	4.1	0.1001		1.14E+06	1.03E-02	9.01E-09
578-max	821	9.6	0.94/1.0/1.2/1.2 (new setup)		7.00E+05	3.07E-04	4.39E-10
578-max FW1.4_DHP	960	ND	ND		9.30E+05	2.48E-04	2.66E-10
578-minmax	903	8.4	1.6/1.4 (new setup)		8.06E+05	5.04E-04	6.25E-10
578minmax FW1.4_DHP	961	16.5	0.78/1.9		7.11E+05	4.09E-04	5.76E-10
578-max-min	902	6.5	ND		1.35E+06	8.83E-03	6.55E-09
578min_max T84N	991	ND	ND		7.21E+05	7.00E-04	9.71E-10
578min_max V89A	978	ND	ND		5.09E+05	6.12E-04	1.20E-09
578min_max V89L	980	ND	ND		8.75E+05	1.87E-03	2.13E-09
578min_max T84N_V89L	1008	8.4	ND		1.13E+06	1.80E-03	1.59E-09
578min_max T84N_V89A	1009	7.5	ND		8.01E+05	4.93E-04	6.15E-10
578min_max T84N_V89L_DHP	1017	ND	ND		ND	ND	ND
578min_max		ND	ND		ND	ND	ND
T84N_V89A_DHP 578max synth FW opt	950	ND	ND		1.35E+06	5.86E-04	4.33E-10
578min_max_synthFW	997	7.2	ND		1.23E+06	9.89E-04	8.03E-10
578max_min_synthFW	990	ND	ND		1.55E+06	5.31E-03	3.42E-09
578min_max_FW1. synth	1016	ND	ND		7.08E+05	7.02E-04	9.91E-10
511-min	801	4.9	0.0011		5.05E+05	1.28E-03	2.53E-09
511-max	802	8.7	0.0179		6.59E+05	4.40E-05	6.67E-11
511min_max	904	5.4	ND		3.66E+05	1.02E-04	2.78E-10
511max_min	905	ND	ND		5.11E+05	7.54E-04	1.48E-09

Table 11: Overview of biophysical characterization of lead derivatives (578 and 511)

ID	Protein Nr.	TM in Bio-ATR [°C]	% beta-sheet loss (Aquaspec 60°C)	% Protein loss (precipitation at 60°C)
578-min	820	66.85	ND	ND
578-max	821	70.36	-1.93%	16.20%
578-max FW1.4_DHP	960	ND	ND	ND

ID	Protein Nr.	TM in Bio-ATR [°C]	% beta-sheet loss (Aquaspec 60°C)	% Protein loss (precipitation at 60°C)
578-minmax	903	71.12	-0.52%	10.99%
578minmax FW1.4_DHP	961	70.18	-0.15%	14.82%
578-max-min	902	ND	ND	ND
578min_max T84N	991	70.78	0.11%	20.30%
578min_max V89A	978	63.23	-2.28%	48.22%
578min_max V89L	980	68.15	-0.79%	38.99%
578min_max T84N_V89L	1008	69	-0.80%	28.30%
578min_max T84N_V89A	1009	ND	ND	ND
578min_max T84N_V89L_DHP	1017	67.8	ND	ND
578min_max T84N_V89A_DHP	1080	66.3	ND	ND
578max synth FW opt	950	63.62	54.06%	97.85%
578min_max_synthFW	997	63.25	50.89%	98.02%
578max_min_synthFW	990	ND	ND	ND
578min_max_FW1. synth	1016	65.7	-0.20%	21.30%
511-min	801	ND	ND	ND
511-max	802	70.5	-1.53%	4.50%
511min_max	904	ND	ND	ND
511max_min	905	ND	ND	ND
567min	884	54	100.00%	100.00%

(continued)

ID	Solubility by ammonium sulfate precipitation [EC <sub>50</sub> in % of NH <sub>4</sub> (SO <sub>4</sub> ) <sub>2</sub> saturation,	Production: Refolding yield [mg/L]	Expression level in E. coli [arbitrary units]
578-min	ND	1.5	++
578-max	27.24	12.5	+
578-max FW1.4_DHP	ND	11.6	+
578-minmax	28.13	23.93	+++
578minmax FW1.4_DHP	32.36	50.5	+++
578-max-min	ND	4.5	+
578min_max T84N	ND	7.5	+++
578min_max V89A	ND	16	+++
578min_max V89L	ND	30	+++
578min_max T84N_V89L	27.88	24	+++
578min_max T84N_V89A	ND	22	+++
578min_max T84N_V89L_DHP	30.80	36	+++
578min_max T84N_V89A_DHP	30.70	30	+++

ID	Solubility by ammonium sulfate precipitation [EC <sub>50</sub> in % of NH <sub>4</sub> (SO <sub>4</sub> ) <sub>2</sub> saturation,	Production: Refolding yield [mg/L]	Expression level in E. coli [arbitrary units]
578max synth FW opt	28.30	19.4	++
578min_max_synthFW	30.05	24	+++
578max_min_synthFW	ND	0.5	++
578min_max_FW1. synth	25.10	28	+++
511-min	ND	13.5	+++
511-max	8.62	6.47	+++
511min_max	ND	3.75	+++
511max_min	ND	7	+++
567min	20.70	16.5	+++

**[0202]** Some derivatives, as listed in Figure 6, were compared for their denaturation and precipitation after thermal stress (e.g., under 50°C, 60°C, or 70°C) for 30 minutes. 578max, 578minmax and 578minmax\_DHP were further exemplified for their solubility, which was determined by ammonium sulfate precipitation. As in Figure 7, the percentage of soluble proteins of these derivatives under various concentrations of ammonium sulfate were compared.

Table 12a : anti-VEGF binders after incubation for 30 min at 50°C

Sample name	Beta sheet %	Nanodrop (mg/ml)
950	100,8	81,2
978	100,9	85,1
980	99,9	100,3
991	99,4	99,2
802	100,4	96,7
821	100,6	93,5
903	99,5	99,4
961	98,7	101,7
997	99,9	76,39

Table 12a : anti-VEGF binders after incubation for 30 min at 60°C

Sample name	Beta sheet %	Nanodrop (mg/ml)
950	45,9	2
978	102,3	52
980	100,8	61
991	99,9	80
802	101,5	96
821	101,9	84
903	100,5	89
961	100,1	85
997	49,1	2

Table 12a : anti-VEGF binders after incubation for 30 min at 70°C

Sample name	Beta sheet %	Nanodrop (mg/ml)
950	43,1	1,0
978	13,4	2,7
980	4,5	0,2
991	21,5	1,4

Sample name	Beta sheet %	Nanodrop (mg/ml)
802	100,4	80,8
821	58,4	3,3
903	81,9	0,7
961	46,3	1,1
997	0,0	0,3

#### EXAMPLE 4

##### VEGF RECEPTOR BLOCKING ASSAYS

**[0203]** For anti-VEGF scFv candidates or their derivatives, their potency as VEGF inhibitors was also measured besides their binding affinity to VEGFs in Example 3. The methods to measure their potency include, for example, the VEGFR competition ELISA, as exemplified in this example, and HUVEC assays (Figure 8).

**[0204]** The VEGFR competition ELISA assays include, for example, VEGFR2 Receptor blocking assays and VEGFR1 Receptor blocking assays. For VEGFR2 Receptor blocking assay, human VEGF<sub>165</sub> was coated on a 96-well Maxisorp ELISA plate (Nunc) at 0.05 µg/ml in PBS and blocked using PBS with 0.1% BSA and 0.2% Tween 20 (PBST). 500 ng/ml recombinant human VEGFR2/Fc chimera (R&D Systems Inc.), consisting of amino acid residues 1-764 of the extracellular domain of human VEGFR2 fused to a 6x histidine tagged Fc of human IgG<sub>1</sub>, was first incubated with 3-fold serially diluted anti-VEGF scFvs in PBST. After 30-60 min of incubation at room temperature, the mixtures were transferred to the human VEGF<sub>165</sub> immobilized plate and incubated for 90 min. Binding of the VEGFR2/Fc chimera to the immobilized VEGF<sub>165</sub> was detected with goat (Fab<sub>2</sub>) anti-human IgG Fcγ coupled to horseradish peroxidase (Jackson ImmunoResearch) followed by substrate (BM Blue POD substrate, Roche Diagnostics). Optical density at 450 nm (OD 450 nm) was measured using a Sunrise microplate reader (Tecan). Data were analyzed using a 4-parameter logistic curve fit, and EC<sub>50</sub> values were calculated from the dose-response curves of the scFvs. The exemplary potency of lead candidates or their derivatives, measured by VEGFR2 Receptor blocking assay, is listed in Table 7 and 9.

**[0205]** For VEGFR1 Receptor blocking assay, human VEGF<sub>165</sub> was coated on a 96-well Maxisorp ELISA plate (Nunc) at 0.0125 µg/ml in PBS and blocked using PBS with 0.4% BSA and 0.1% Tween 20. 100 ng/ml of recombinant human VEGFR1/Fc chimera (R&D Systems Inc.), consisting of amino acid residues 1-687 of the extracellular domain of human VEGFR1 fused to a 6x histidine tagged Fc of human IgG<sub>1</sub>, was first incubated with 3-fold serially diluted anti-VEGF scFvs in PBST. After 30-60 min of incubation at room temperature, the mixtures were transferred to the human VEGF<sub>165</sub> immobilized plate and incubated for 90 min. Binding of the VEGFR1/Fc chimera to the immobilized VEGF<sub>165</sub> was detected with goat (Fab<sub>2</sub>) anti-human IgG Fcγ coupled to horseradish peroxidase (Jackson ImmunoResearch) followed by substrate (BM Blue POD substrate, Roche Diagnostics). Optical density at 450 nm (OD 450 nm) was measured using a Sunrise microplate reader (Tecan). Data were analyzed as above, and EC<sub>50</sub> values were calculated from the dose-response curves of the scFvs. The exemplary potency of lead candidates, measured by VEGFR1 Receptor blocking assay, is listed in Table 7.

#### EXAMPLE 5

##### HUVEC ASSAY OF VEGF INHIBITION

**[0206]** This example exemplifies HUVEC assays as another method to measure the potency of the disclosed anti-VEGF scFv candidates, or their derivatives, as VEGF inhibitors.

**[0207]** Human umbilical vein endothelial cells (HUVECs) (Promocell), pooled from several donors, were used at passage 2 to passage 14. Cells were seeded at 1000 cells/well in 50  $\mu$ l complete endothelial cell growth medium (ECGM) (Promocell), that contained 0.4% ECGS/H, 2% Fetal Calf Serum, 0.1 ng/ml Epidermal Growth Factor, 1  $\mu$ g/ml Hydrocortison, 1 ng/ml basic Fibroblast Factor and 1% penicillin/streptomycin (Gibco). 7 to 8 h later, 50  $\mu$ l starving medium (ECGM without supplements containing 0.5% heat inactivated FCS and 1% penicillin/streptomycin) was added to the cells and the cells were starved for 1.5 to 16 hours. 3 fold Serial dilutions of anti-VEGF scFvs (0.023-150 nM) and one of the following -recombinant human VEGF<sub>165</sub> (0.08 nM), recombinant mouse VEGF<sub>164</sub> (0.08 nM), or recombinant rat VEGF<sub>164</sub> (0.3 nM)-were prepared in starving medium and preincubated for 30-60 min at room temperature. The different concentrations of VEGFs were used to compensate for their different relative biological activities. Concentrations that stimulate submaximal VEGF induced proliferation (EC<sub>90</sub>) were used. 100  $\mu$ l of the mixtures were added to the 96-well tissue-culture plates containing the HUVEC suspension and incubated for 4 days in a 37 °C/5% CO<sub>2</sub> humified incubator. Proliferation of HUVECs was assessed by measuring absorbance at 450 nm (620 nm used as reference wavelength) after addition of 20  $\mu$ l/well WST-1 cell proliferation reagent (Roche) using a Sunrise microplate reader (Tecan). Data were analyzed using a 4-parameter logistic curve-fit, and the concentration of anti-VEGF scFvs required to inhibit HUVEC proliferation by 50% (EC<sub>50</sub>) was derived from inhibition curves.

**[0208]** The exemplary potency of lead candidates or their derivatives, measured by HUVEC assays, is listed in Table 7. Further, the inhibition of hVEGF<sub>165</sub>-induced HUVEC proliferation by one derivative of lead candidates, 578minmax, is exemplified in Figure 9. EC<sub>50</sub> of 578minmax for inhibition of hVEGF<sub>165</sub>-induced cell-proliferation is determined to be 0.06 nM (Figure 9). The potency of 578minmax as a VEGF inhibitor is about 1.6 times better compared to Lucentis. The inhibition of mouse or rat VEGF<sub>164</sub>-induced HUVEC proliferation by 578minmax is also exemplified in Figure 10. EC<sub>50</sub> of 578minmax for inhibition of mouse and rat VEGF<sub>164</sub> induced cell-proliferation is 0.06 nM and 0.07 nM, respectively (Figure 10). Thus, mouse and rat VEGF are equipotent to human VEGF for being inhibited by the exemplary derivative (578minmax). Also in this experiment, Lucentis does not inhibit proliferation induced by rodent VEGF.

#### EXAMPLE 6

#### EFFECTS OF ANTI-VEGF SCFVS ON HVEGF<sub>165</sub> INDUCED VASCULAR PERMEABILITY IN HAIRLESS GUINEA PIGS

**[0209]** In this example, the effect of anti-VEGF scFvs on human VEGF<sub>165</sub> induced vascular permeability was assessed in guinea pigs using the Miles assay. Thirty application sites per animal were marked on the dorsum of hairless male guinea pigs using a permanent marker. On the treatment day each animal was administered intravenously with 1 ml of a 1% Evans blue dye solution under general anesthesia. One hour after dye injection, 0.1 ml of test solution containing 2.61 nM recombinant human VEGF<sub>165</sub> (PeproTech EC Ltd.) and various concentrations of anti-VEGF scFvs (0 nM, 0.085 nM, 0.256 nM, 0.767 nM, 2.3 nM, 6.9 nM, 20.7 nM, 62.1 nM; n = 7 animals per test item) was injected in triplicate into the marks on the dorsum (3 injections per concentration of test item). Injections of PBS served as a negative control in all animals. As an additional control, 6.9 nM Lucentis (Novartis) was injected in all animals.

**[0210]** One hour after injection of the test solutions, the animals were euthanized, and the pelts were collected, cleaned, and photographed digitally using incident and transmitted light. The area of Evans Blue dye that extravasated into the injection sites was evaluated using ImageJ. For each animal, anti-VEGF scFv concentration versus area of dye leakage was analyzed using a 4-parameter logistic curve fit. The concentration of anti-VEGF scFvs required to inhibit vascular leakage by 50% (EC<sub>50</sub>) was derived from inhibition curves.

**[0211]** The experiment protocol is exemplified in Figure 11. Also, the efficacy of scFv candidates, ESBA903 (578minmax) and 802 (51 lmax), in inhibiting the hVEGF was illustrated in Figure 11, represented by different sizes of areas containing the Evans Blue dye leaked from vascular system into skin. The efficacy data for 903 and 802 are shown in Figure 12. At 6.9 nM, 903 and 802 showed stronger inhibition of VEGF induced vascular leakage into the skin compared to Lucentis in all animals tested (Figure 12).

#### EXAMPLE 7

**EFFECTS OF TOPICAL ANTI-VEGF SCFVS TREATMENT ON hVEGF<sub>165</sub> INDUCED RETINAL VASCULAR LEAKAGE IN RATS**

**[0212]** In this example, topical efficacy of 578minmax is demonstrated using a modified Miles assay. These modifications include, for example, premixed study with intravitreal injections and topical application of scFvs.

**[0213]** Premixed different concentrations of anti-VEGF scFv (10, 3, and 1 fold molar excess over VEGF) and VEGF (500 ng) were applied via a single intravitreal injection. Avastin (Roche) (10, 3, and 1 fold molar excess over VEGF) was used as a positive control. Vehicle for 578minmax (Citrate Buffer, 20 mM Na-Citrate, 125 mM NaCl, pH 7) was used as negative control. As illustrated in Figure 13, premixing with hVEGF<sub>165</sub> facilitated 578minmax (ESBA903) to completely inhibit hVEGF-induced retinal vascular permeability. In this experiment, the inhibitory effect of 578minmax (ESBA903) was more significant compared to Avastin.

**[0214]** For topical application, five days before VEGF stimulation, adult Sprague-Dawley rats received 578minmax (1% = 10 mg/ml) via bilateral topical dosing qid (4 drops/day) till perfusion day (Day 6). Vehicle for 578minmax (topical dosing) and Alcon RTKi (10 mg/kg/d, oral gavage) were used as negative and positive controls. On Day 5, rats are anesthetized and their pupils are dilated. All animals receive intravitreal injections of 500 ng hrVEGF (10  $\mu$ l) in both eyes. Following 24 hours post-injection of VEGF, intravenous infusion of 3% Evans blue dye is performed on all animals during general anesthesia. After the dye has circulated for 90 minutes, the rats are euthanized. Blood samples are taken, then the rats are perfused with sterile saline solution, then both eyes of each rat are immediately enucleated and the retinas harvested using a surgical microscope. For both retina and plasma samples, 60  $\mu$ L of supernatant is used to measure the Evans blue dye absorbance (ABS) with a spectrophotometer at 620/740 nm. The blood-retinal barrier breakdown and subsequent retinal vascular permeability as measured by dye absorbance are calculated as means  $\pm$  s.e.m. of net ABS/wet weight/plasma ABS. One way ANOVA is used to determine an overall difference between treatment means, where  $P \leq 0.05$  is considered significant. As exemplified in Figure 14, the topical administration (5 days of pretreatment, 4 drops per day) of 578minmax (903) significantly inhibited hVEGF-induced retinal vascular permeability. This is the first demonstration of a topically effective antibody useful for the treatment of intraocular disease.

**SEQUENCE LISTING**

**[0215]**

SEQ ID NO:1  
Peptide Immunogen  
KFMDVYQRSYC

**VL sequences:**

**[0216]**

SEQ ID NO. 72: 60  
EVVMAQTPASVEAVGGTVTIKCQASQSISSYLSWYQQKPGQOPPKLLIYKASTLASGVPSRFKGSRS  
GTEYTLTISDLECADAAATYYCQSNYGGSSSDYGNPFGGGTEAVVVK

SEQ ID NO. 73: 435  
AFELTQTPSSVEAVGGTVTIKCQASQSIGSSLAWYQQKPGQRPKLLIYTAANLASGVPSRFRGSRS  
AAFTLTISDLECADAAATYYCQNFATSDTVTFGGGTEVVVK

SEQ ID NO. 74: 453  
AVVLTQTPSPVSAVGGTVSISCQSSQSVWNNNRLAWFQQKSGQPPKLLIYYASTLASGVPSRFKGS  
SGSGTEFTLTISDVQCDDAATYYCAGGYSSTSNTFGGGTEVVVK

SEQ ID NO. 75: 375

DVMTQTPASVEATVGGTITNCQASENINIWLSWYQQKPGQPPKLLIYQASKLASGVPSRFKGSGSGTQFTLTISDLECADAATYYCQNNYNSYNRYGAPFGGGTEVVVK

SEQ ID NO. 76: 610

DVMTQTPASVSEPVGGTVTICKCQASQSISSWLSWYQQKPGQPPKLLIYQASTLASGVPPRSSGSGSGTEYTLTISDLECADAATYFCQNNYGFRSYGGAFGGGTEVVVK

SEQ ID NO. 77: 578

DVMTQTPSSVSAAVGDTVTINCQASEIIHSWLAWSWYQQKPGQPPKLLIYLASTLASGVPSRFKGSGSGTQFTLTISDLECADAATYYCQNVYLASTNGANFGGGTEVVVK

SEQ ID NO. 78: 534

DVMTQTPSSVSAAVGDTVTICKCQASQSIWLSWYQQKSGQPPKLLVYKESTLASGVPSRFKGSGSGTQFTLTISDLECADAATYYCQNNYDSGNNGFPFGGGTEVVVK

SEQ ID NO. 79: 567

DVMTQTPSSVSAAVGDTVTINCQADQSIYIWLSDWYQQKPGQPPKLLIYKASTLESGVPSRFKGSGSGTQFTLTISDLECADAATYYCQNNAHYSTNGTFGGGTEVVVK

SEQ ID NO. 80: 509

DVMTQTPSSVSAAVGDTVTICKCQASQNIRIWLSWYQQKPGQPPKLLIYKASTLESGVPSRFKGSGSGTEFTLTISDLECADAATYYCQNNAHYSTNGTFGGGTEVVVK

SEQ ID NO. 81: 511

EVMTQTPASVEAVGGTITICKCQASQSIWCSWYQQKPGHPPKLLIYRASTLASGVSSRFKGSGSGTFTLTISDLECADAATYYCQANYAYSAGYGAFFGGTEVVVK

SEQ ID NO. 82: 60min

EIVMTQSPSTLSASVGDRVIITCQASQSISSYLSWYQQKPGKAPKLLIYKASTLASGVPSRFSGSGSGA  
EFTLTISLQPDDFATYYCQNFATSDTVTFGQGTKLTVLG

SEQ ID NO. 83: 435min

EIVMTQSPSTLSASVGDRVIITCQASQSIGSSLAWYQQKPGKAPKLLIYTAANLASGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQNFATSDTVTFGQGTKLTVLG

SEQ ID NO. 84: 453min

EIVMTQSPSTLSASVGDRVIITCQASQSSVWNNNRLAWYQQKPGKAPKLLIYASTLASGVPSRFSGSGSGSGAEFTLTISLQPDDFATYYCQNFATSDNTFGQGTKLTVLG

SEQ ID NO. 85: 375min

EIVMTQSPSTLSASVGDRVIITCQASENINIWLSWYQQKPGKAPKLLIYQASKLASGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQNNYNSYNRYGAPFGQGTKLTVLG

SEQ ID NO. 86: 610min

EIVMTQSPSTLSASVGDRVIITCQASQSISSWLSWYQQKPGKAPKLLIYQASTLASGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQNNYGFRSYGGAFGGTKLTVLG

SEQ ID NO. 87: 578min

EIVMTQSPSTLSASVGDRVIITCQASEIIHSWLAWSWYQQKPGKAPKLLIYLASTLASGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQNVYLASTNGANFGQGTKLTVLG

SEQ ID NO. 88: 534min

EIVMTQSPSTLSASVGDRVIITCQASQSIWLSWYQQKPGKAPKLLIYKESTLASGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQNNYDSGNNGFPFGQGTKLTVLG

SEQ ID NO. 89: 567min

EIVMTQSPSTLSASVGDRVIITCQADQSIYIWLSDWYQQKPGKAPKLLIYKASTLESGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQNNAHYSTNGTFQGTKLTVLG

SEQ ID NO. 90: 509min

EIVMTQSPSTLSASVGDRVIITCQASQNIRIWLSWYQQKPGKAPKLLIYKASTLESGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQNNAHYSTNGTFQGTKLTVLG

SEQ ID NO. 91: 511min

EIVMTQSPSTLSASVGDRVIITCQASQSIWCSWYQQKPGKAPKLLIYRASTLASGVPSRFSGSGSGAEFTLTISLQPDDFATYYCQANYAYSAGYGAFFGGTKLTVLG

SEQ ID NO. 92: 578min\_Pref subst

EIVLTQSPSSLSASVGDRVTITCQASEIIHSWLAWSWYQQRPGKAPKLLIYLASTLASGVPSRFSGSGSGDFTFTISLQPEDFAVYYCQNVYLASTNGANFGQGTKEIKR

SEQ ID NO. 93: 60max

EIVLTQSPSSLSASVGDRVTITCQASEIIHSWLAWSWYQQRPGKAPKLLIYLASTLASGVPSRFSGSGSGDFTFTISLQPEDFAVYYCQNVYLASTNGANFGQGTKEIKR

EIVMTQSPSTSASVGDRVIIITCQASQSISSYLSWYQQKPGKAPKLLIYKASTLASGVPSRFSGSGSGT  
EFTLTISLQPDDFATYYCQSNYGGSSDYGNPFGQGTKLTVLG

SEQ ID NO. 94: 435max  
EIVMTQSPSTSASVGDRVIIITCQASQSIGSSLAWYQQKPGKAPKLLIYTAANLASGVPSRFSGSGSG  
AEFTLTISLQPDDFATYYCQNFATSDTVTGFQGTKLTVLG

SEQ ID NO. 95: 453max  
EIVMTQSPSTSASVGDRVIIITCQSSQSVWNNRLAWYQQKPGKAPKLLIYASTLASGVPSRFSGSG  
GSGTEFTLTISLQPDDFATYYCAGGYSSTDNTFGQGTKLTVLG

SEQ ID NO. 96: 375max  
EIVMTQSPSTSASVGDRVIIITCQASENINIWLWYQQKPGKAPKLLIYQASKLASGVPSRFSGSGSG  
TQFTLTISLQPDDFATYYCQNNYSNRYGAPFGQGTKLTVLG

SEQ ID NO. 97: 610max  
EIVMTQSPSTSASVGDRVIIITCQASQSISSWLSWYQQKPGKAPKLLIYQASTLASGVPSRFSGSGSG  
TEFTLTISLQPDDFATYYCQNNYGRSYGGAFGQGTKLTVLG

SEQ ID NO. 98: 578max  
EIVMTQSPSTSASVGDRVIIITCQASEIIHSWLAWYQQKPGKAPKLLIYLASTLASGVPSRFSGSGSGT  
QFTLTISLQPDDFATYYCQNVYLASTNGANFGQGTKLTVLG

SEQ ID NO. 99: 534max  
EIVMTQSPSTSASVGDRVIIITCQASQSIWIWLWYQQKPGKAPKLLIYKESTLASGVPSRFSGSGSGT  
EFTLTISLQPDDFATYYCQNNYDGSNNGFPGQGTKLTVLG

SEQ ID NO. 100: 567max  
EIVMTQSPSTSASVGDRVIIITCQADQSIWYLWYQQKPGKAPKLLIYKASTLESGVPSRFSGSGSGT  
QFTLTISLQPDDFATYYCQNNAHYSTNGTFQGTKLTVLG

SEQ ID NO. 101: 509max  
EIVMTQSPSTSASVGDRVIIITCQASQNIWLSWYQQKPGKAPKLLIYKASTLESGVPSRFSGSGSGT  
EFTLTISLQPDDFATYYCQNNAHYSTNGTFQGTKLTVLG

SEQ ID NO. 102: 511max  
EIVMTQSPSTSASVGDRVIIITCQASQSIWIWLWYQQKPGKAPKLLIYRASTLASGVPSRFSGSGSGT  
EFTLTISLQPDDFATYYCQANYAYSAGYGAFFGQGTKLTVLG

SEQ ID NO. 103: 578max\_Pref subst  
EIVMTQSPSSLSASVGDRVITCQASEIIHSWLAWYQQRPGKAPKLLIYLASTLASGVPSRFSGSGSGT  
QFTLTISLQPEDFAVYQNVYLASTNGANFGQGTKLTVKEIKR

SEQ ID NO. 104: 578min VL: E1D  
DIVMTQSPSTSASVGDRVIIITCQASEIIHSWLAWYQQKPGKAPKLLIYLASTLASGVPSRFSGSGSGA  
EFTLTISLQPDDFATYYCQNVYLASTNGANFGQGTKLTVLG

SEQ ID NO. 105: 578min VL: I2V  
EVMTQSPSTSASVGDRVIIITCQASEIIHSWLAWYQQKPGKAPKLLIYLASTLASGVPSRFSGSGSG  
AEFTLTISLQPDDFATYYCQNVYLASTNGANFGQGTKLTVLG

SEQ ID NO. 106: 511min VL: C41L  
EIVMTQSPSTSASVGDRVIIITCQASQSIWIWLWYQQKPGKAPKLLIYRASTLASGVPSRFSGSGSG  
AEFTLTISLQPDDFATYYCQANYAYSAGYGAFFGQGTKLTVLG

#### VH sequences:

[0217]

SEQ ID NO. 107: 60-11-4  
QSLEESGGDLVKPGASLTCTASGFPFSSGYWVCWVRQAPGKGLEWIACIYAGSSGSTYYASWAK  
GRFTISKTSSTTVTLQMTSLTAADTATYFCARGNNYYIYTDGGYAYAGLELWGPGLTVSS

SEQ ID NO. 108: 60-11-6  
QSLEESGGDLVKPGASLTCTASGFSFGSGYWICWVRQAPGKGLEWIACIYAGSSGSTYYASWAK  
RFTISKTSSTTVTLQMTSLTAADTATYFCARGNNYYIYTDGGYAYAGLELWGPGLTVSS

SEQ ID NO. 109: 435  
QSLEESGGDLVQPGASLTLCVKVSGFSLNTNYWMCWVRQAPGKGLEWIGCMYTGSYNRAYYASW

AKGRFTSSKTSSTTVLEMTSLTAADTATYFCAKGSNWYSDLWGPGLTVSS

SEQ ID NO. 110: 453

QERLVESGGGLVQPEGSLTLCKASGFSRSYYIYWRQAPGKGLEWIACIDAGSSGILVYANWAK  
GRFTISKTSSTTVLQMTSLTAADTATYFCARGDASYGVDSFMLPLWGPGLTVSS

SEQ ID NO. 111: 375

QSLEESGGGLVQPEGSLTLCKASGFSFTTIDYMCWVRQAPGKGLEWIGCILAGDGSTYYANWAK  
GRFTGSKTSSTTVDLKMTGLTAADTATYFCARSDPASSWSFALWGPGLTVSS

SEQ ID NO. 112: 610

QSLEESGGRLVTPGPTPLTCTASGFSLTDDYYMTWVRLAPGKGLEWIGYIDYDGDRYYASWAKG  
RFTISKTSSTTVDLKITSPTTEDTATYFCARSDYSSGWGTDIWGPGLTVSS

SEQ ID NO. 113: 578

QSVEESGGRLVTPGPTPLTCTASGFSLSYYMSWVRQAPGKGLEWIGFIDPDDDPYYATWAKGRF  
TISRTSTTVNLKMTSPTTEDTATYFCAGGDHNSGWGLDIWGPGLTVSS

SEQ ID NO. 114: 534

QSLEESGGRLVTPGPTPLTCTASGFSLSYYMSWVRQAPGKGLEWIGIIGPFDYTDYASWAKGRF  
ISKTSTTVDLKITSPTTEDTATYFCRGDDNSGWGEDIWGPGLTVSS

SEQ ID NO. 115: 567

QSVEESGGRLVTPGAPLTLCVSFGFSLSDYYMCWVRQAPGKGLEWIGCLDYFGSTDASWAKGRF  
FTISKTSTAVDLKITSPTTEDTATYFCARTDDSRGWGLNIWGPGLTVSS

SEQ ID NO. 116: 509

QSVEESGGRLVTPGPTPLTCTASGFSLSYYMCWVRQAPGKGLEWIGCLDYFGSTDASWAKGRF  
TISKASTTVDLKITSLTTEDTATYFCARTDDSRGWGLNIWGPGLTVSS

SEQ ID NO. 117: 511

QSVEESGGRLVTPGPTPLTCTASGFSLSYYMCWVRQAPGKGLEWIGIAPDDTTYYASWAKSRST  
ITRDTNENTVTLKMTSLTEDTATYFCARSGDTTAWGADIWGPGLTVSS

SEQ ID NO. 118: 60-11-4min

EVQLVESGGGLVQPGGSLRLSCAASGFPSSGYWCWVRQAPGKGLEWVSCIYAGSSGTYASW  
AKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGNYYIYTDGGYAYAGLELGQGTIVTSS

SEQ ID NO. 119: 60-11-6min

EVQLVESGGGLVQPGGSLRLSCAASGFPSSGYWCWVRQAPGKGLEWVSCIYAGSSGTYASW  
AKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGNYYIYTDGGYAYAGLELGQGTIVTSS

SEQ ID NO. 120: 435min

EVQLVESGGGLVQPGGSLRLSCAASGFSLNTNYWMCWVRQAPGKGLEWVSCMYTGSYNRAYA  
SWAKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGSNWYSDLWQGTIVTSS

SEQ ID NO. 121: 453min

EVQLVESGGGLVQPGGSLRLSCAASGFSRSYYIYWRQAPGKGLEWVSCIDAGSSGILVYANWA  
KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGDASYGVDSFMLPLWQGTIVTSS

SEQ ID NO. 122: 375min

EVQLVESGGGLVQPGGSLRLSCAASGFSFTTIDYMCWVRQAPGKGLEWVSCILAGDGSTYYANW  
AKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSDPASSWSFALWQGTIVTSS

SEQ ID NO. 123: 610min

EVQLVESGGGLVQPGGSLRLSCAASGFSLTDDYYMTWVRLAPGKGLEWVSYIDYDGDRYYASWA  
KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSDYSSGWGTDIWQGTIVTSS

SEQ ID NO. 124: 578min

EVQLVESGGGLVQPGGSLRLSCAASGFSLSYYMSWVRQAPGKGLEWVSIIGPFDYTDYASWAKG  
RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGDASYGVDSFMLPLWQGTIVTSS

SEQ ID NO. 125: 534min

EVQLVESGGGLVQPGGSLRLSCAASGFSLSYYMSWVRQAPGKGLEWVSIIGPFDYTDYASWAKG  
RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGDASYGVDSFMLPLWQGTIVTSS

SEQ ID NO. 126: 567min

EVQLVESGGGLVQPGGSLRLSCAASGFSLSYYMCWVRQAPGKGLEWVSCLDYFGSTDASWAK  
GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKTDSSRGWGLNIWQGTIVTSS

SEQ ID NO. 127: 509min

EVQLVESGGGLVQPGGSLRLSCAASGFSLSYYMCWVRQAPGKGLEWVSCLDYFGSTDASWAK  
GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKTDSSRGWGLNIWQGTIVTSS

SEQ ID NO. 128: 511min

EVQLVESGGGLVQPGGSLRLSCAASGFSLNTYYMNWVRQAPGKGLEWVSIAPDDTTYYASWAKS  
RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSGDAAWGADIWGQGTLVTVSS

SEQ ID NO. 129: 578min\_Pref subst

substQVQLVQTGGGLVQPGGSLRLSCAASGFSLTDYYYYMTWVRQAPGKGLEWVFSFIDPDDDPYY  
ATWAKGRFTISRDNSKNTVYLQMNSLRAEDTALYYCAKGDHNSGWGLDIWGQGTLVTVSS

SEQ ID NO. 130: 60-11-4max

EVQLVESGGGLVQPGGSLRLSCTAGFPFSSGYWCWVRQAPGKGLEWVGCIYAGSSGSTYYASW  
AKGRFTISKDTSKNTVYLQMNSLRAEDTAVYYCARGNNYYIYTGGYAYAGLELWGQGTLVTVSS

SEQ ID NO. 131: 60-11-6max

EVQLVESGGGLVQPGGSLRLSCTAGFSFSSGYWICWVRQAPGKGLEWVGCIYAGSSGSTYYASW  
AKGRFTISKDTSKNTVYLQMNSLRAEDTAVYYCARGNNYYIYTGGYAYAGLELWGQGTLVTVSS

SEQ ID NO. 132: 435max

EVQLVESGGGLVQPGGSLRLSCVSGFSLNTNYWMCWVRQAPGKGLEWVGCMYTGSYNRAYYA  
SWAKGRFTSSKDTSKNTVYLQMNSLRAEDTAVYYCAKGSNWYSDLWGQGTLVTVSS

SEQ ID NO. 133: 453max

EVQLVESGGGLVQPGGSLRLSCAKGSFSRSYYIYWWVRQAPGKGLEWVGCIAGSSGILVYANWA  
KGRFTISKDTSKNTVYLQMNSLRAEDTAVYYCARGDASYGVDSFMLPLWGQGTLVTVSS

SEQ ID NO. 134: 375max

EVQLVESGGGLVQPGGSLRLSCAKGSFSFTTDYMCWVRQAPGKGLEWVGCIAGDGSTYYANW  
AKGRFTGSKDTSKNTVYLQMNSLRAEDTAVYYCARSDPASSWSFALWGQGTLVTVSS

SEQ ID NO. 135: 610max

EVQLVESGGGLVQPGGSLRLSCTAGFSLTDYYYYMTWVRQAPGKGLEWVGFIYDGDRYYASWA  
KGRFTISKDTSKNTVYLQMNSLRAEDTAVYYCARSDDSGWGTDIWGQGTLVTVSS

SEQ ID NO. 136: 578max

EVQLVESGGGLVQPGGSLRLSCTAGFSLTDYYYYMTWVRQAPGKGLEWVGFIYDGDRYYATWA  
KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTVSS

SEQ ID NO. 137: 534max

EVQLVESGGGLVQPGGSLRLSCTAGFSLSYYYMSWVRQAPGKGLEWVGCLDYFGSTDASWAKG  
RFTISKDTSKNTVYLQMNSLRAEDTAVYYCARGDDNSGWGEDIWGQGTLVTVSS

SEQ ID NO. 138: 567max

EVQLVESGGGLVQPGGSLRLSCSVGFSLSDDYYMCWVRQAPGKGLEWVGCLDYVGDTDASWAK  
GRFTISKDTSKNTVYLQMNSLRAEDTAVYYCARTDDSRGWGLNIWGQGTLVTVSS

SEQ ID NO. 139: 509max

EVQLVESGGGLVQPGGSLRLSCTAGFSLSSYYMCWVRQAPGKGLEWVGCLDYVGDTDASWAK  
GRFTISKDASKNTVYLQMNSLRAEDTAVYYCARTDDSRGWGLNIWGQGTLVTVSS

SEQ ID NO. 140: 509maxII

EVQLVESGGGLVQPGGSLRLSCTAGFSLSSYYMSWVRQAPGKGLEWVGILDYVGDTDASWAKG  
RFTISKDASKNTVYLQMNSLRAEDTAVYYCARTDDSRGWGLNIWGQGTLVTVSS

SEQ ID NO. 141: 511max

EVQLVESGGGLVQPGGSLRLSCTVGFSLNTYYMNWVRQAPGKGLEWVGIIAPDDTTYYASWAKS  
RSTISRDTSKNTVYLQMNSLRAEDTAVYYCARSDDSGDAAWGADIWGQGTLVTVSS

SEQ ID NO. 142: 578max\_Pref subst

substQVQLVQTGGGLVQPGGSLRLSCTAGFSLTDYYYYMTWVRQAPGKGLEWVGFIYDGDRYYATWA  
ATWAKGRFTISRDTSKNTVYLQMNSLRAEDTALYYCAGGDHNSGWGLDIWGQGTLVTVSS

SEQ ID NO. 143: 567minDHP

EVQLVESGGGSVQPGGSLRLSCAASGFSLDDYYMCWVRQAPGKGLEWVFSCLDYFGSTDASWAK  
GRFTISRDNSKNTLYLQMNSLRAEDTATYYCAKTDSSRGWGLNIWGQGTTVTVSS

SEQ ID NO. 144: 578maxDHP

EVQLVESGGGSVQPGGSLRLSCTAGFSLTDYYYYMTWVRQAPGKGLEWVGFIYDGDRYYATWA  
KGRFTISRDTSKNTVYLQMNSLRAEDTATYYCAGGDHNSGWGLDIWGQGTTVTVSS

SEQ ID NO. 145: 511maxDHP

EVQLVESGGGSVQPGGSLRLSCTVGFSLNTYYMNWVRQAPGKGLEWVGIIAPDDTTYYASWAKS  
RSTISRDTSKNTVYLQMNSLRAEDTATYYCARSDDSGDAAWGADIWGQGTTVTVSS

SEQ ID NO. 146: 578max\_Pref subst\_DHP  
 QVQLVQTGGGSVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTATYYCAGGDHNSGWGLDIWGQGTTVTSS

SEQ ID NO. 147: 578max VH: E1\_  
 VQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 148: 578max VH: V2Q  
 EQQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 149: 578max VH: Q46L  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 150: 578max VH: W54Y  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 151: 578max VH: V55I  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 152: 578max VH: D83A  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 153: 578max VH: N87A  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 154: 578max VH: Y105F  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYFCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 155: 578max VH: D83\_  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 156: 578max VH: N87\_  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDTSKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 157: 578max VH: T84N  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDN SKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 158: 578max VH: V89L  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDT SKNTVYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 159: 578max VH: V89A  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDT SKNTAYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 160: 578maxDHP VH: T84N  
 EVQLVESGGGSVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDN SKNTVYLQMNSLRAEDTATYYCAGGDHNSGWGLDIWGQGTTVTSS

SEQ ID NO. 161: 578maxDHP VH: V89L  
 EVQLVESGGGSVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDT SKNTLYLQMNSLRAEDTATYYCAGGDHNSGWGLDIWGQGTTVTSS

SEQ ID NO. 162: 578maxDHP VH: V89A  
 EVQLVESGGGSVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDT SKNTAYLQMNSLRAEDTATYYCAGGDHNSGWGLDIWGQGTTVTSS

SEQ ID NO. 163: 578max VH: T84N, V89A  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA  
 KGRFTISRDN SKNTAYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTSS

SEQ ID NO. 164: 578max VH: T84N, V89L  
 EVQLVESGGGLVQPGGSLRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIIDPDDDPYYATWA

LYQEVLSGGGLVQPGGSLRLSCTAS(X)<sub>n=3-50</sub> WYQQKPGKAPKLLIY(X)<sub>n=3-50</sub> GVKLWVSGVDFDSDTATW  
KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLTVSS

SEQ ID NO. 165: 578maxDHP VH: T84N, V89A  
EVQLVESGGGVQPGGSLRLSCTASGFLTDYYYYMTWVRQAPGKGLEWVGFDIDPDDDPYYATWA  
KGRFTISRDNSKNTAYLQMNSLRAEDTATYYCAGGDHNSGWGLDIWGQGTTVTSS

SEQ ID NO. 166: 578maxDHP VH: T84N, V89L  
EVQLVESGGGVQPGGSLRLSCTASGFLTDYYYYMTWVRQAPGKGLEWVGFDIDPDDDPYYATWA  
KGRFTISRDNSKNTLYLQMNSLRAEDTATYYCAGGDHNSGWGLDIWGQGTTVTSS

framework sequences (X residues are CDR insertion sites and may be any naturally occurring amino acid. At least 3 and up to 50 amino acids can be present):

SEQ ID NO. 167: Variable light chain FW1.4 and rFW1.4  
EIVMTQSPSTLSASVGDRVIITC(X)<sub>n=3-50</sub> WYQQKPGKAPKLLIY(X)<sub>n=3-50</sub>  
GVPSRFSGSGLSGAETLTISSLQPDDFATYYC(X)<sub>n=3-50</sub> FGQGTLKLT VLG

SEQ ID NO. 168: Variable light chain rFW1.4 variant 2 (v2)  
EIVMTQSPSTLSASVGDRVIITC(X)<sub>n=3-50</sub> WYQQKPGKAPKLLIY(X)<sub>n=3-50</sub>  
GVPSRFSGSGLSGAETLTISSLQPDDFATYYC(X)<sub>n=3-50</sub> FGQGTLKLT VLG

SEQ ID NO. 169: Variable heavy chain FW1.4  
EVQLVESGGGLVQPGGSLRLSCAAS(X)<sub>n=3-50</sub> WVRQAPGKGLEWVS (X)<sub>n=3-50</sub>  
RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK(X)<sub>n=3-50</sub> WGQGTL VTVSS

SEQ ID NO. 170: Variable heavy chain rFW1.4  
EVQLVESGGGLVQPGGSLRLSCTAS(X)<sub>n=3-50</sub> WVRQAPGKGLEWVG(X)<sub>n=3-50</sub>  
RFTISRDTSKNTVYLQMNSLRAEDTAVYYCAR(X)<sub>n=3-50</sub> WGQGTLV TVS

SEQ ID NO. 171: Variable heavy chain rFW1.4 variant 2 (v2)  
EVQLVESGGGLVQPGGSLRLSCTVS(X)<sub>n=3-50</sub> WVRQAPGKGLEWVG(X)<sub>n=3-50</sub>  
RFTISKDTSKNTVYLQMNSLRAEDTAVYYCAR(X)<sub>n=3-50</sub> WGQGTLVTVSS

#### ScFv framework sequences:

#### [0218]

SEQ ID NO. 172: FW1.4  
EIVMTQSPSTLSASVGDRVIITC(X)<sub>n=3-50</sub> WYQQKPGKAPKLLIY(X)<sub>n=3-50</sub>  
GVPSRFSGSGLSGAETLTISSLQPDDFATYYC(X)<sub>n=3-50</sub> FGQGTLKLT VLG  
GGGGSGGGGSGGGSGGGSEVOLVESGGGLVQPGGSLRLSCAAS(X)<sub>n=3-50</sub>  
WVRQAPGKGLEWVS(X)<sub>n=3-50</sub> RFTISRDNSKNTLYLQMNSLRAEDTA VYYCAK(X)<sub>n=3-50</sub>  
WGQGTLVTVSS

SEQ ID NO. 173: rFW1.4  
EIVMTQSPSTLSASVGDRVIITC(X)<sub>n=3-50</sub> WYQQKPGKAPKLLIY(X)<sub>n=3-50</sub>  
GVPSRFSGSGLSGTETFTLTISLQPDDFATYYC(X)<sub>n=3-50</sub> FGQGTLKLT VLG  
GGGGSGGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCTVAS(X)<sub>n=3-50</sub>  
WVRQAPGKGLEWVG(X)<sub>n=3-50</sub> RFTISRDTSKNTVYLQMNSLRAEDTAVYYCAR(X)<sub>n=3-50</sub>  
WGQGTLVTVSS

SEQ ID NO. 174: rFW1.4 variant 2 (v2)  
EIVMTQSPSTLSASVGDRVIITC(X)<sub>n=3-50</sub> WYQQKPGKAPKLLIY(X)<sub>n=3-50</sub>  
GVPSRFSGSGLSGTETFTLTISLQPDDFATYYC(X)<sub>n=3-50</sub> FGQGTLKLT VLG  
GGGGSGGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCTVS(X)<sub>n=3-50</sub>  
WVRQAPGKGLEWVG(X)<sub>n=3-50</sub> RFTISKDTSKNTVYLQMNSLRAEDTAVYYCAR(X)<sub>n=3-50</sub>  
WGQGTLVTVSS

#### ScFv anti-VEGF sequences:

#### [0219]

SEQ ID NO. 175: 435 max

EIVMTQSPSTLSASVGDRVIICTQASQSIGSSLWYQQKPGKAPKLLIYTAAANLASGVPSRSFGSRSG  
AEFTLTISSLCPDDFATYYCQNFATSDTVFGQGTKLTVLGGGGGGGGGGGGGGGGGSEVQL  
VESGGGLVQPGGSLRLSCKASGFSLNTNYWCWVRQAPGKLEWVGCMYTGSYNRAYASWA  
KGRFTSSKDTSKNTVYQLQMNSLRAEDTAVYYCAGGSNWYSDLWQGQTLTVSS

SEQ ID NO. 176: 511\_max

EIVMTQSPSTLSASVGDRVIICTQASQSINIWLWYQQKPGKAPKLLIYRASLASGVPSRFSGGSGT  
EFTLTISSLQPDFATYYCQANYAASAGYGAFAQGQGTKLTVLGGGGGSGGGGGSGGGGGSGGGSEV  
QLVESGGGLVQPGGSLRLSTCVSGFSLNYYMNWVRQAPGKGLEWVGHIAFPDTTYYASWAKRS  
TISRDTSKNTVYLMQNSLRAEDTAVYYCARSQDTTAWGADIWQGQTLTVSS

SEQ ID NO. 177: 567\_min

EIVMTQSPSTLSASVGDRVIITCQADQSYIWLWSWYQCKPGKAPKLLIYKASTLESGVPSRFGSGSGA  
EFTLTISLQLPDDFATYYCONNAHYSTNGTFFGQGTKLTVLGGGGGGSGGGGGSGGGGGSGGGGSEVQ  
LVESGGGGLVQPGGSLRLSCAASGFSLSDYYMCWVRQAPGKGLEWVSCLDYFGSTDASWAKGRFT  
ISRDNSKNTLYLQMNSLRAEDTAVYYCAKTDDSRGWLNIWQGQTLTVSS

SEQ ID NO. 178: 578min

EIVMTQSPSTLSASVGDRVITCQASEIIHSWLAWSYQQKPGKAPKLLIYLASTLASGVPSRFSGSGSGA  
EFTLTISSLQPPDFATYQCNVYLASTNGANFGQGTKLTVLGGGGGSGGGGSGGGGSGGGGSEVQ  
LVESGGGLVLQPGGSLRLSCAASGFSLTDYYMMTWRQAPGKGLEWVFSIDPDDPYATWAKGRF  
TISRDNSKNTLYLQMNSLRAEDTAVVYCAKGDHNSGWLIDWQGQTLTVSS

SEQ ID NO. 179: 578max

EIVMTQPSTLSASVGRDVIITCQASEIIHSLWYQQKPGKAPKILLIYLASTLASGVPSRSGSGSGT  
QFTLTLISLQQPDDFATYYCQNVIYLASTNGANFGQGTKLTVLGGGGSGGGGGGGGGGGSEVQ  
LVESGGGLVQPGSRLSCTASGFSLTDDYYMTWVRQAPGKGLEWVGFIDPDDPPYATWAKGRF  
TISRDTSKNTVYLOMNSLRAEDTAVYYCAGGDHNSGWGLDIWQGQTLTVSS

SEQ ID NO. 180: 578minmax (ESBA903)

EIVMTQSPLSASVGDRVIITCQASEIHSWLAWYQCKPGKAKPLLIYLASTLASGVPSRFGSGSGA  
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LVESGGGLVQPGSRLSCTASGFSLTDYMMTWRQAPGKLEWVGFIDPDDDPYATWAKRFT  
TISRDTSKNTVYQMNSLRAEDTAVYYCAGGDHNSGWGLDIWGQGTLVTVSS

SEQ ID No. 181 linker

GGGGSGGGGSGGGGSGGGGS

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**Patentkrav**

1. Immunglobulin eller fragment deraf, der omfatter en variabel tungkæde (VH) og en variabel letkæde (VL), hvor den variable tungkæde (VH) omfatter aminosyresekvensen ifølge SEQ ID NO: 164, og den variable letkæde (VL) omfatter aminosyresekvensen ifølge SEQ ID NO: 87.  
5
2. Immunglobulin eller fragment deraf ifølge krav 1, hvor immunglobulinet eller fragmentet deraf er et scFv, et Fab-fragment, et Fab'-fragment eller et F(ab')<sub>2</sub>-fragment.  
10
3. Immunglobulin eller fragment deraf ifølge krav 1 eller 2, hvor den variable letkæde af immunglobulinet eller fragmentet deraf har en N-terminal methioninrest i det sidst afledte protein i forhold til startkodonen.  
15
4. Immunglobulin eller fragment deraf ifølge et hvilket som helst af kravene 1 til 3, hvor immunglobulinet eller fragmentet deraf er et scFv, og den variable tungkæde og den variable letkæde er forbundet af aminosyresekvensen ifølge SEQ ID NO: 181.  
20
5. Farmaceutisk sammensætning, der omfatter immunglobulinet eller fragmentet deraf ifølge et hvilket som helst af kravene 1 til 4.  
25
6. Farmaceutisk sammensætning ifølge krav 5 til anvendelse ved behandling af en VEGF-medieret sygdom.  
30
7. Anvendelse af den farmaceutiske sammensætning ifølge krav 5 til fremstilling af et lægemiddel til behandling af en VEGF-medieret sygdom.  
8. Farmaceutisk sammensætning til anvendelse ifølge krav 6 eller anvendelse ifølge krav 7, hvor den VEGF-medierede sygdom er valgt fra gruppen bestående af aldersrelateret makuladegeneration, neovaskulært glaukom, diabetisk retinopati og præmatur retinopati.

9. Farmaceutisk sammensætning til anvendelse ifølge krav 6 eller anvendelse ifølge krav 7, hvor den VEGF-medierede sygdom er valgt fra gruppen bestående af retrorental fibroplasi, brystkarcinomer, lungekarcinomer, gastriske karcinomer, øsofageale karcinomer, kolorektale karcinomer, leverkarcinomer, ovariekarcinomer, sarkomer, arrhenoblastomer, cervixkarcinomer, endometrialt karcinom, endometrial hyperplasi, endometriose, fibrosarkomer, choriokarcinom, hoved-og-hals-cancer, nasofaryngalt karcinom, laryngale karcinomer, hepatoblastom, Kaposis sarkom, melanom, hudkarcinomer, hæmangiom, kavernøst hæmangiom, hæmangioblastom, pankreaskarcinomer, retinoblastom, astrocytom, glioblastom, schwannom, oligodendrogiom, medulloblastom, neuroblastomer, rhabdomyosarkom, osteogent sarkom, leiomyosarkomer, urinvejskarcinomer, thyreoideakarcinomer, Wilms tumor, nyrecellekarcinom, prostatakarcinom, abnorm vaskulær proliferation i forbindelse med fakomatoser, ødem (såsom ødem i forbindelse med hjernetumorer), Meigs' syndrom, rheumatoid arthritis, psoriasis og aterosklerose.

15

10. Farmaceutisk sammensætning til anvendelse ifølge et hvilket som helst af kravene 6, 8 og 9, eller anvendelse ifølge et hvilket som helst af kravene 7 til 9, hvor immunglobulinet eller fragmentet deraf er formuleret med henblik på topisk, intraokulær, oral, nasal, rektal eller parenteral administration.

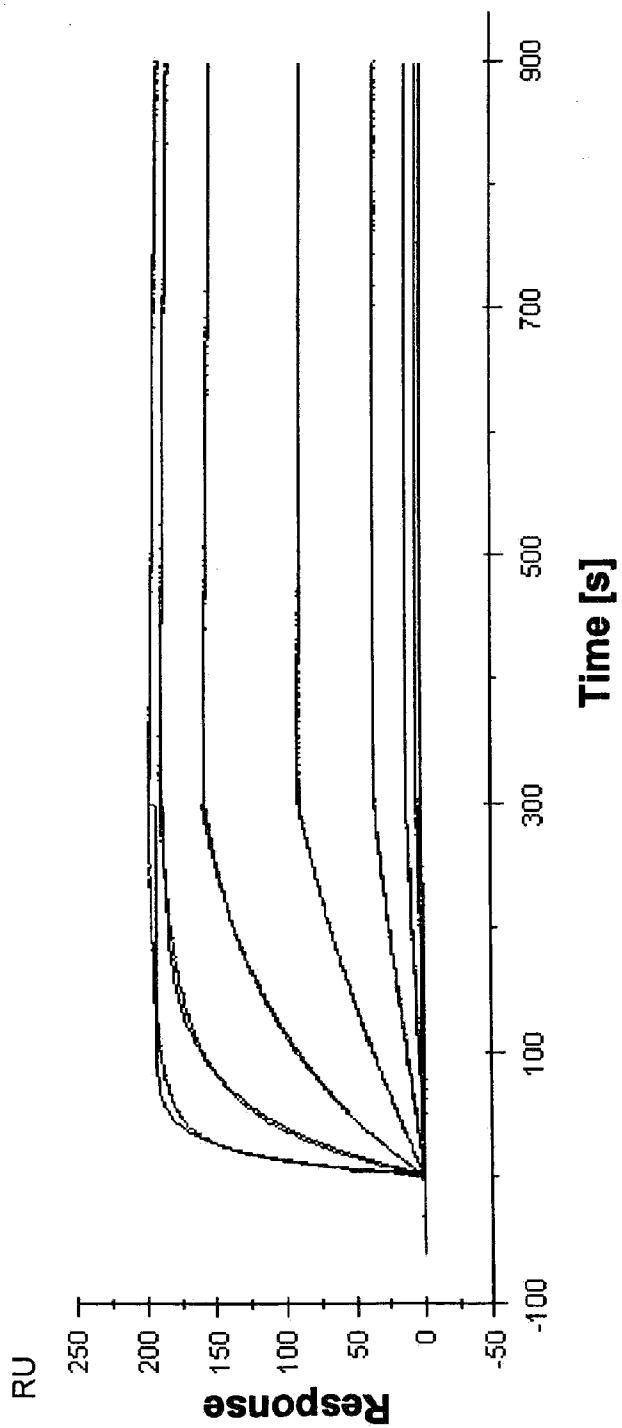
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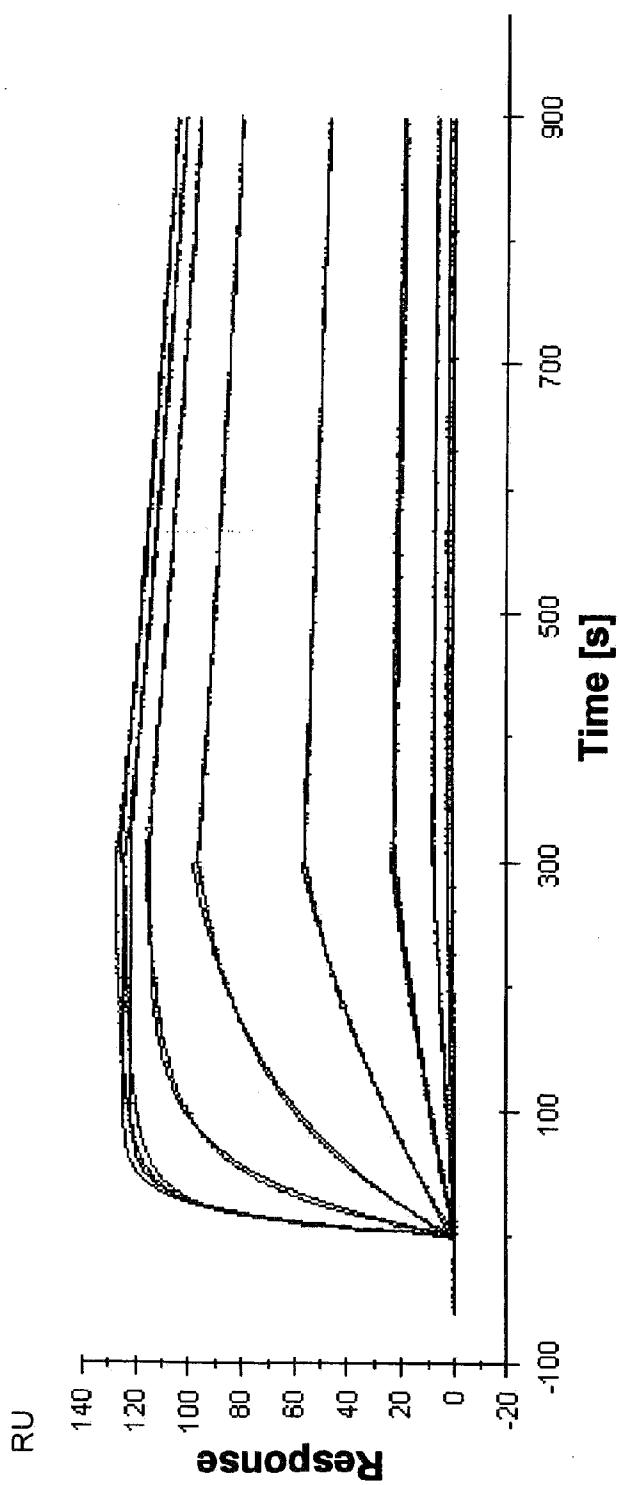
11. Kit til anvendelse i en fremgangsmåde til diagnosticering af en VEGF-medieret sygdom, hvilket kit omfatter immunglobulinet eller fragmentet deraf ifølge et hvilket som helst af kravene 1 til 4 eller den farmaceutiske sammensætning ifølge krav 5.

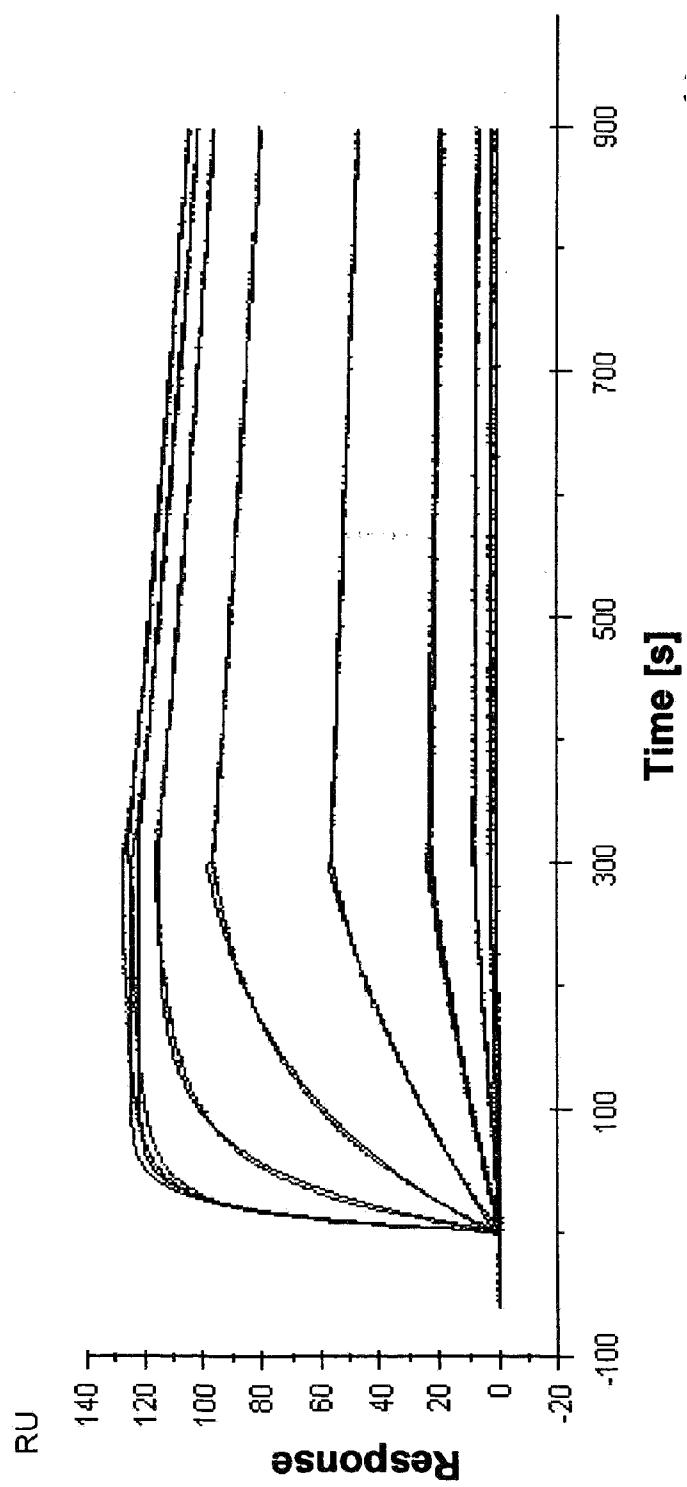
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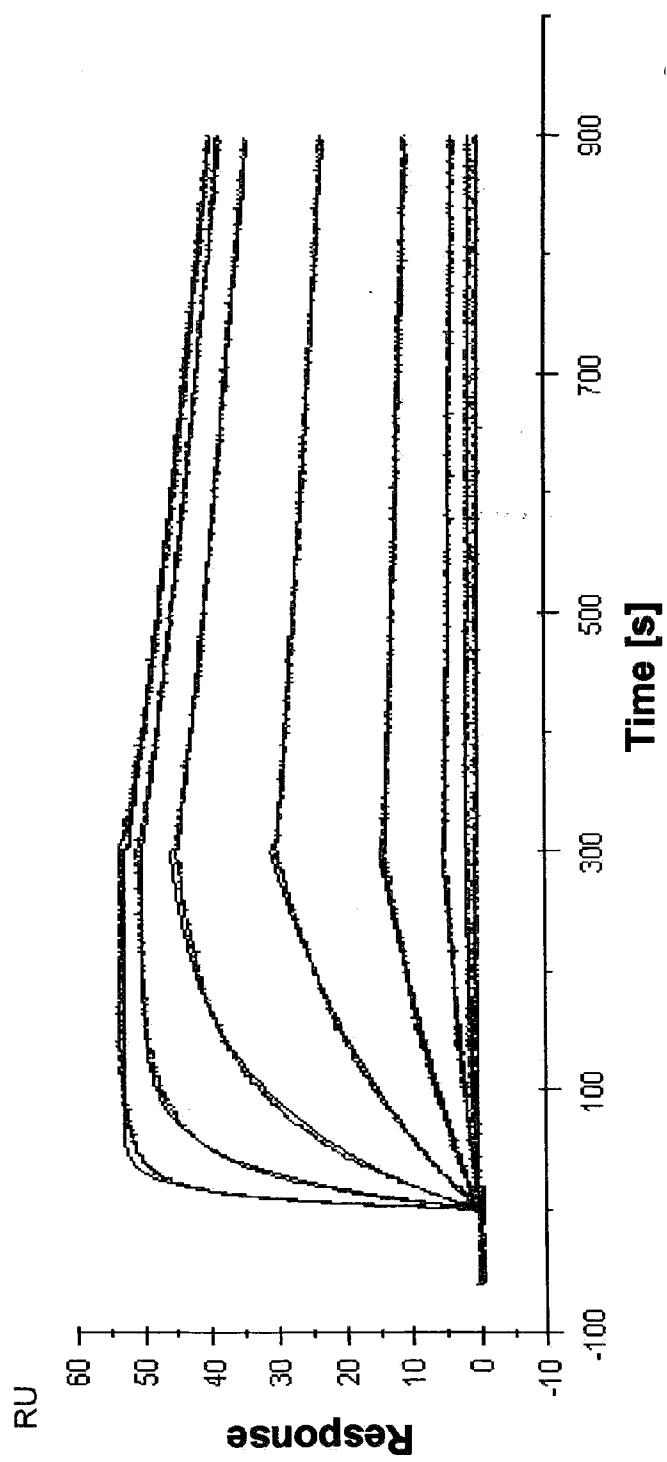
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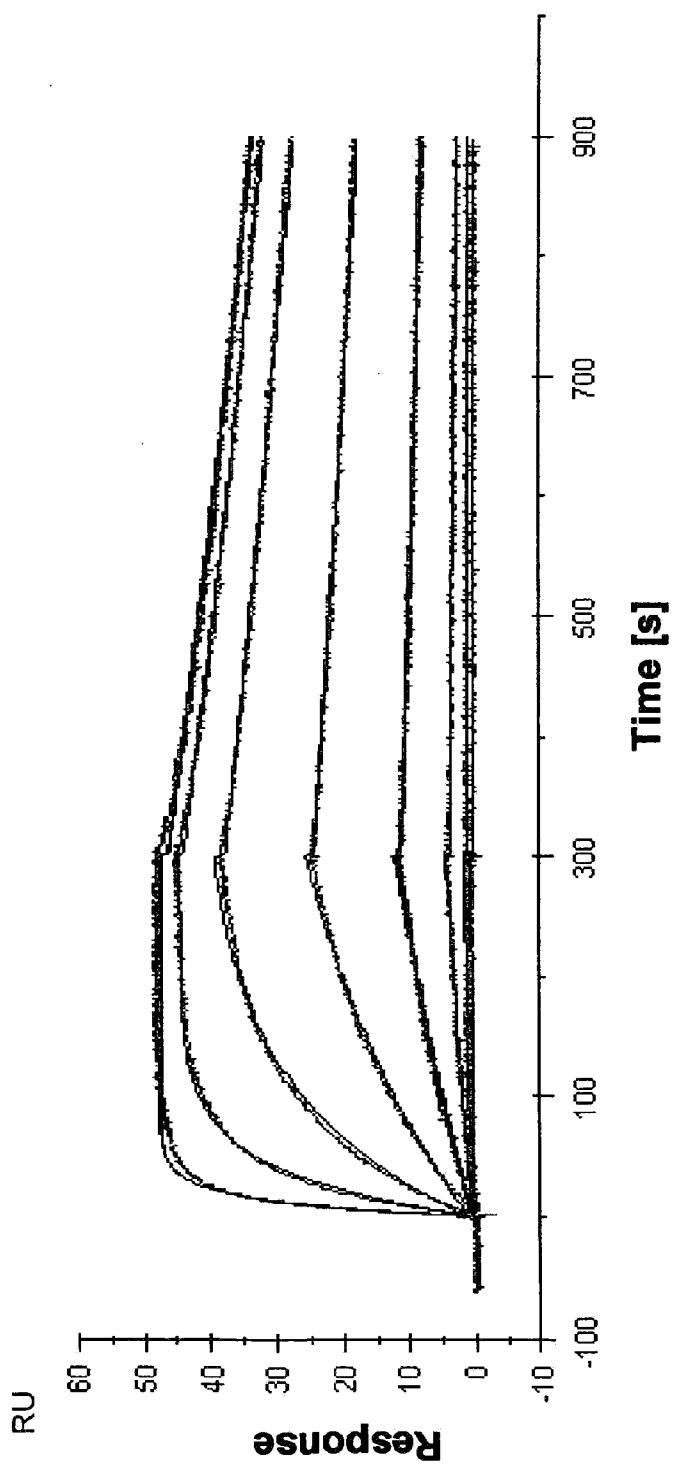
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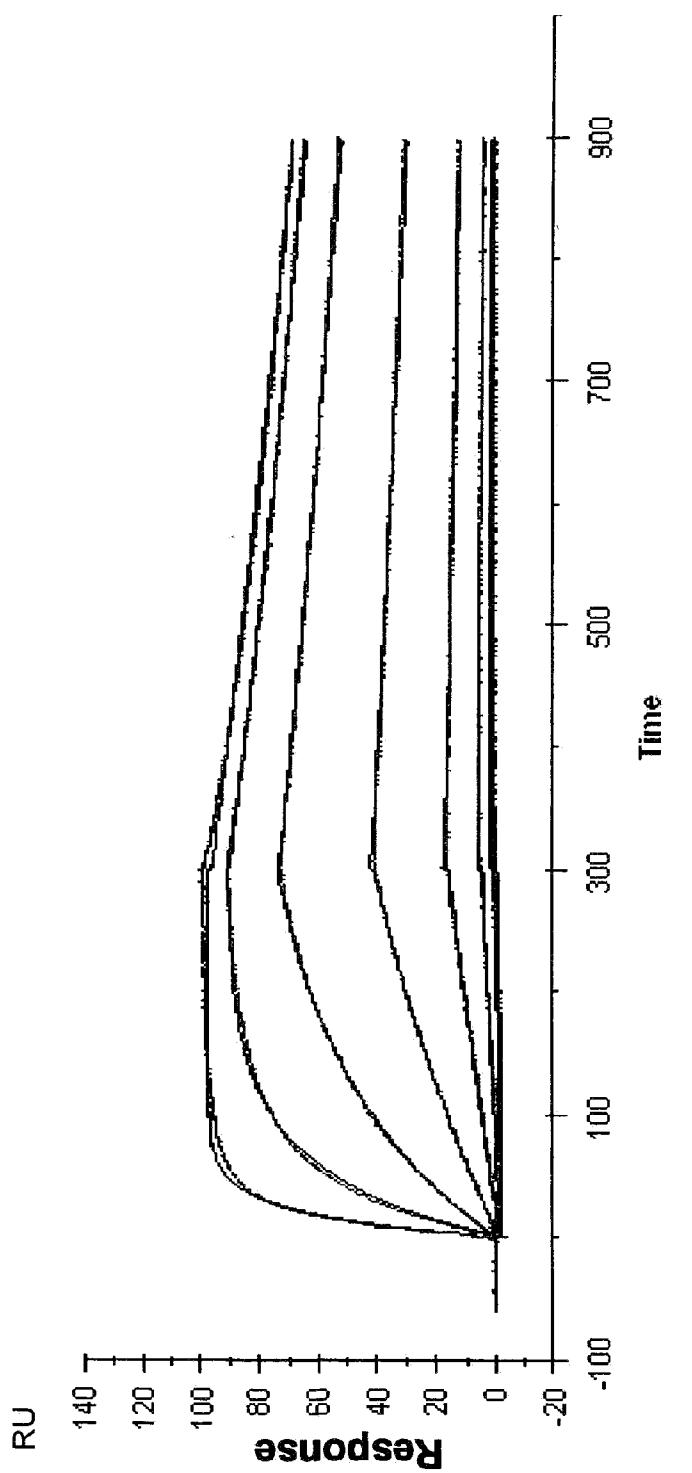


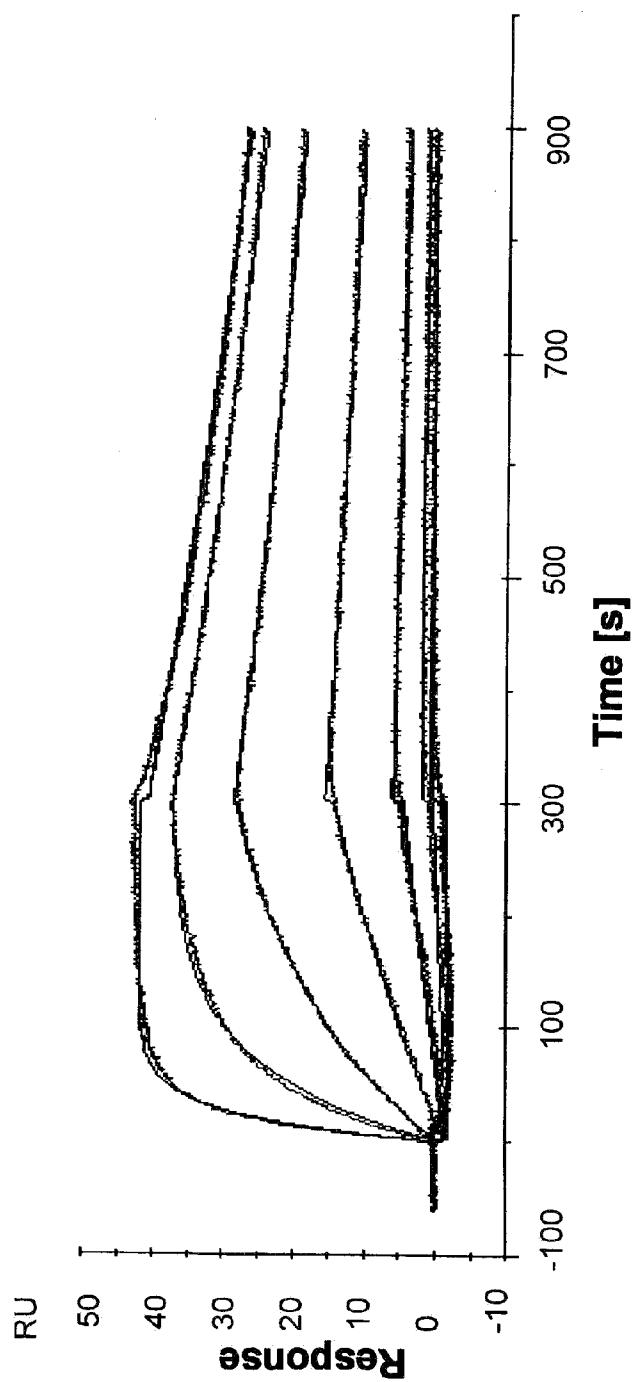
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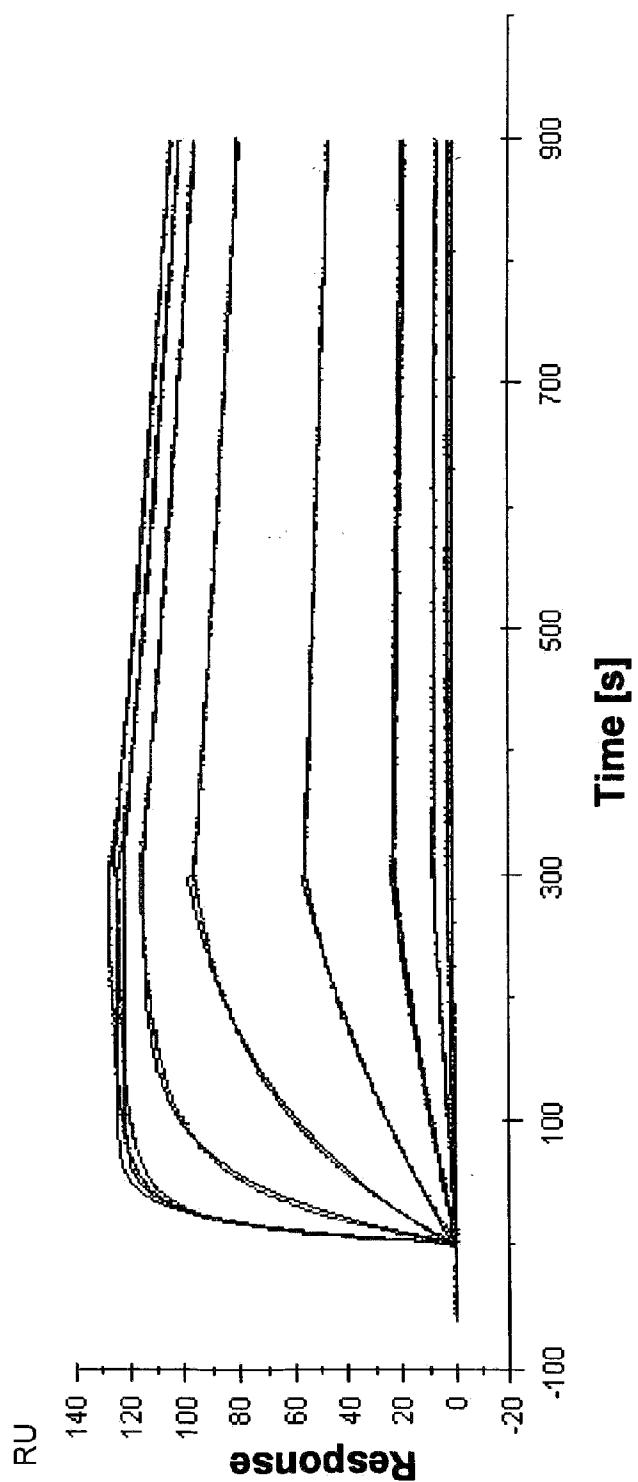
**Fig. 2a**

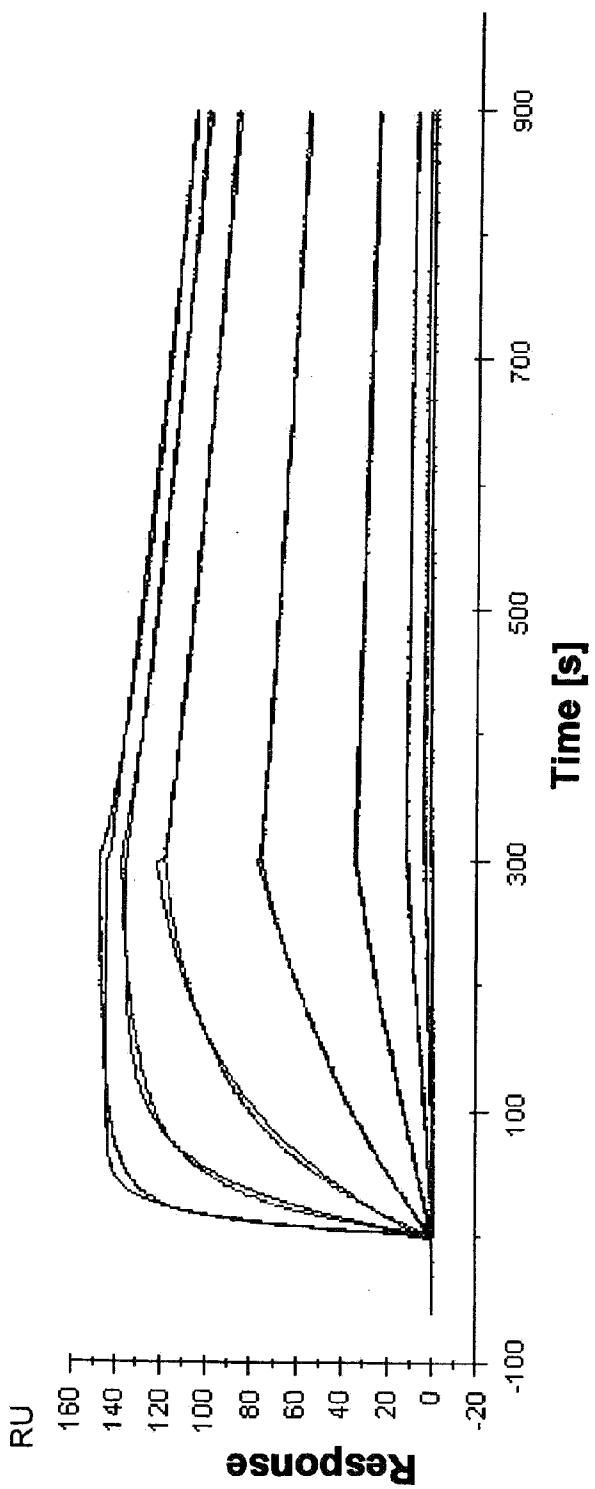
**Fig. 2b**

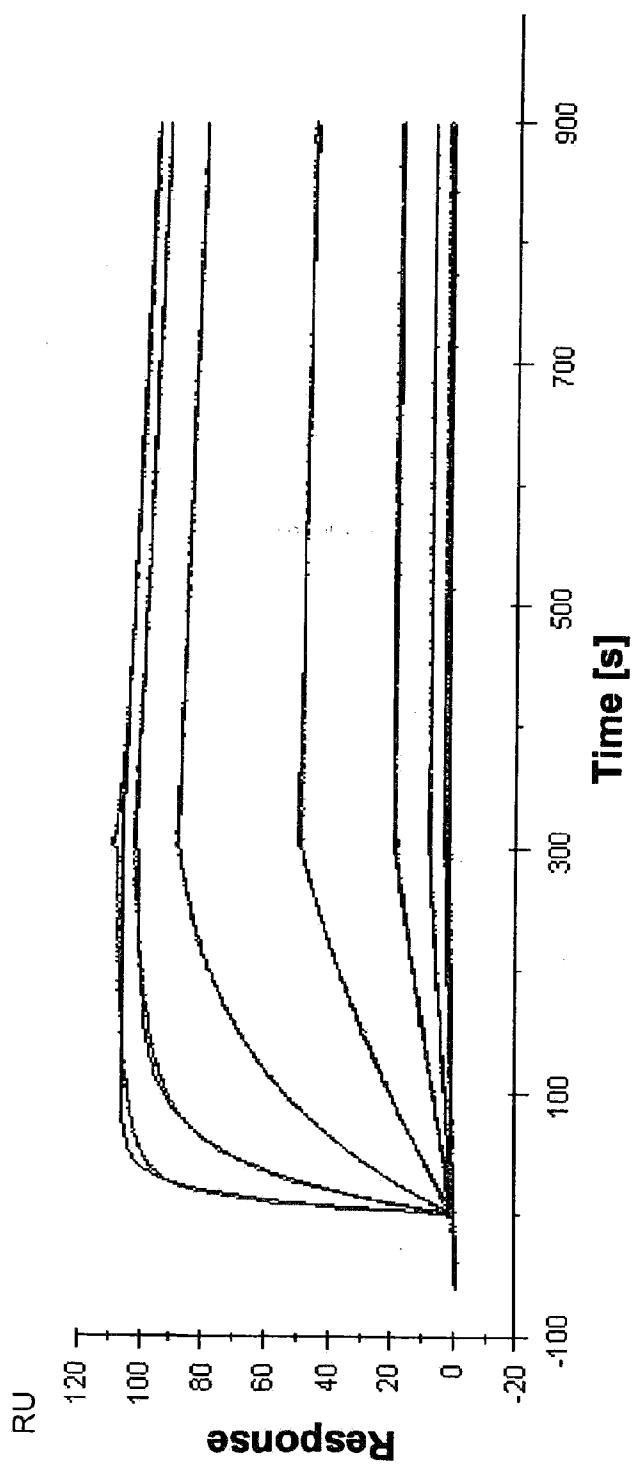
**Fig. 2c**

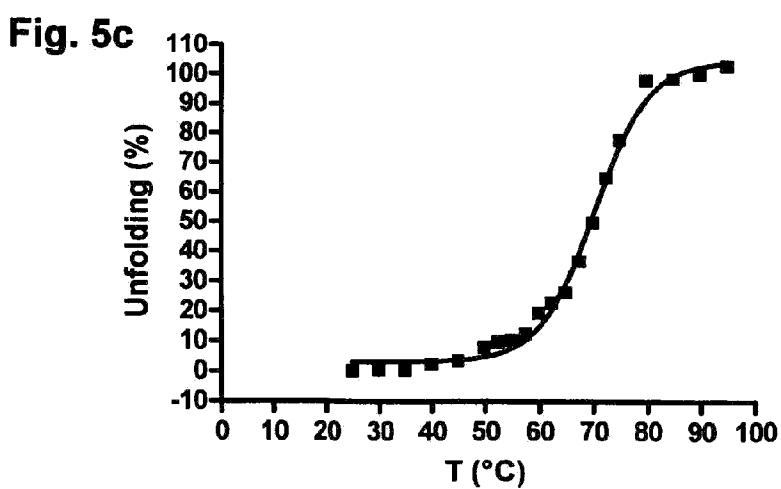
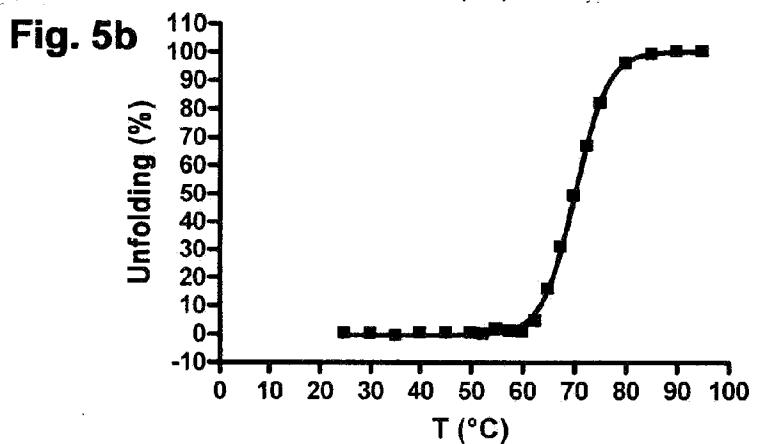
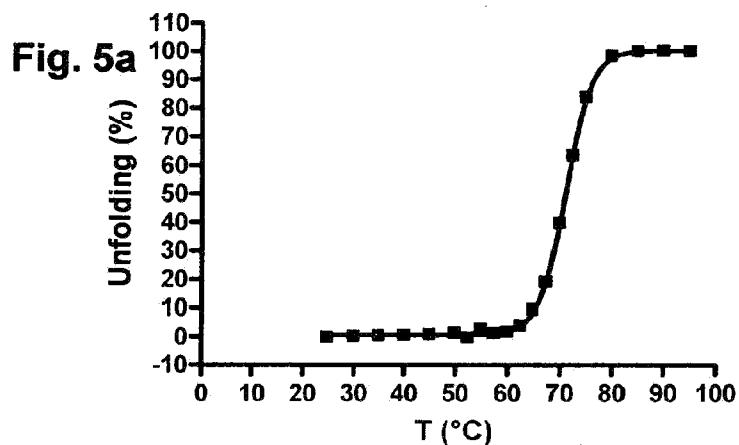
**Fig. 3a**

**Fig. 3b**

**Fig. 4a**

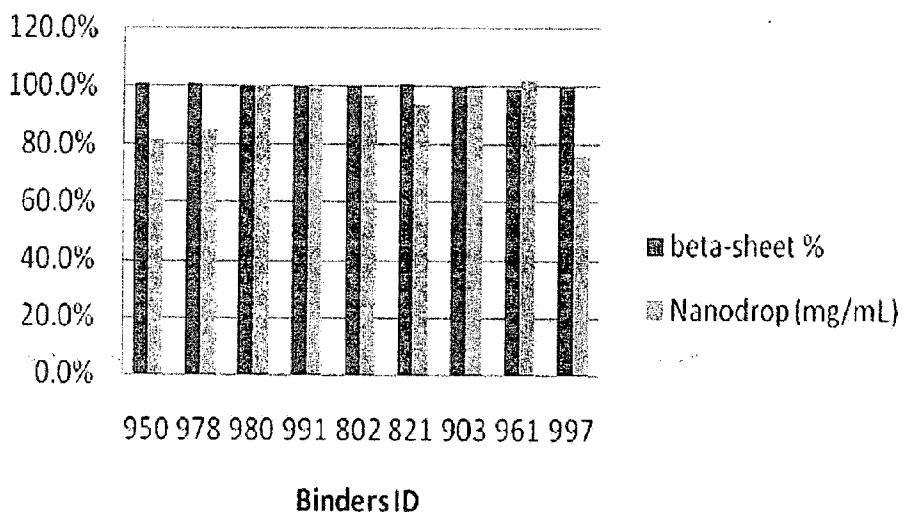
**Fig. 4b**

**Fig. 4c**



Values normalized to the native prot  
(%)

Fig. 6a



Values normalized to the native prot  
(%)

Fig. 6b

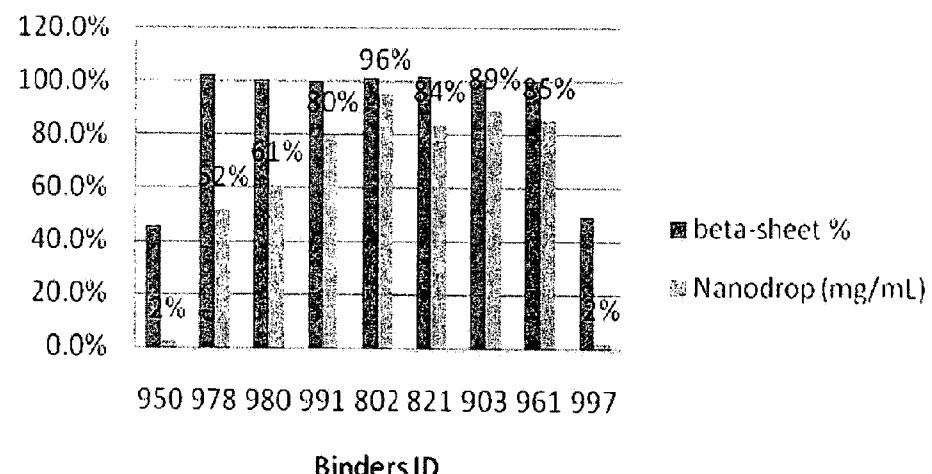


Fig. 6c

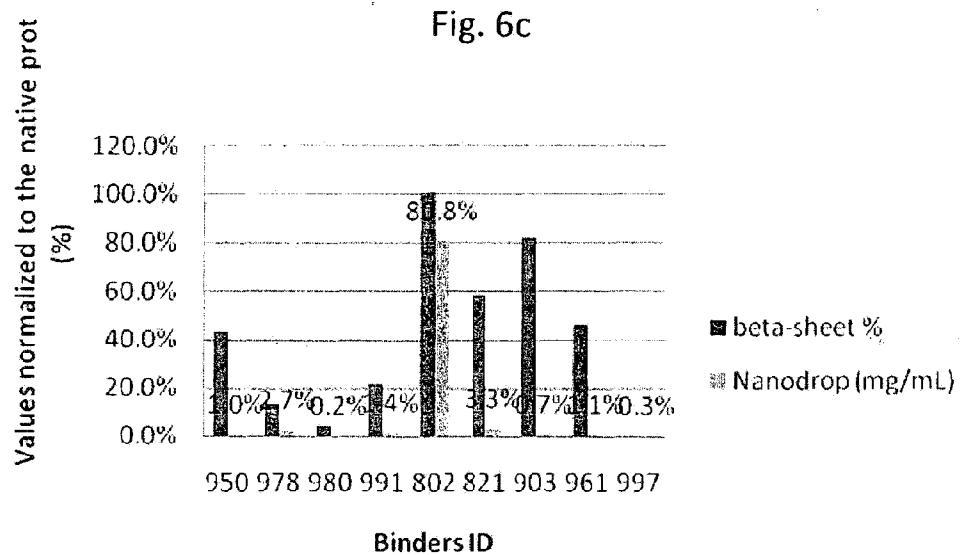
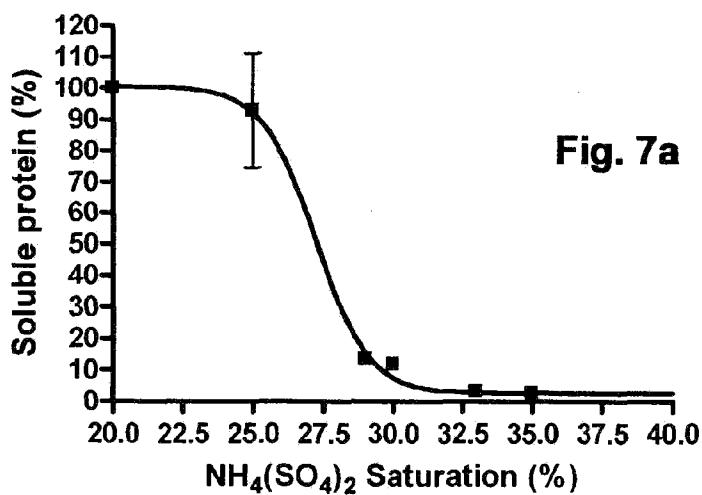
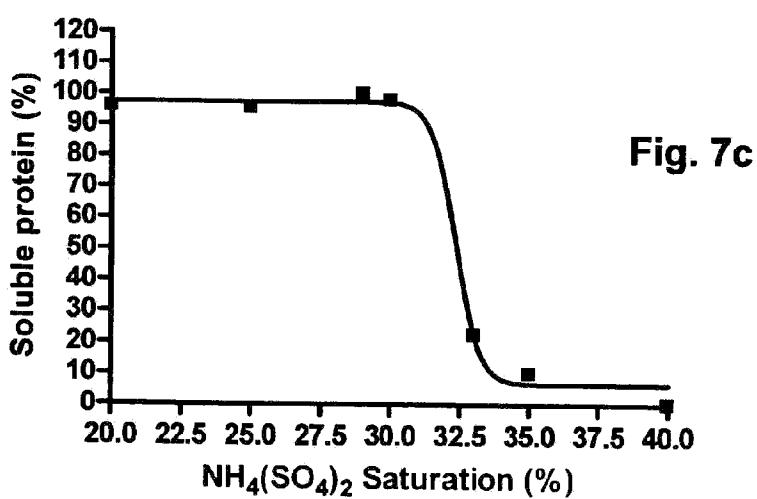
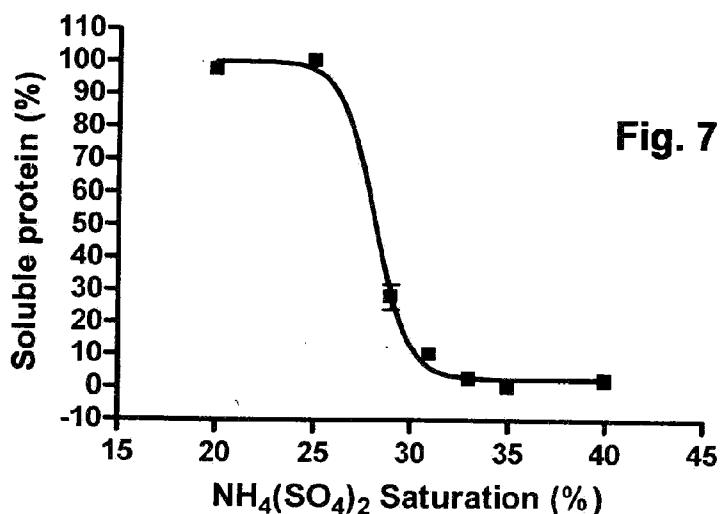


Fig. 7a





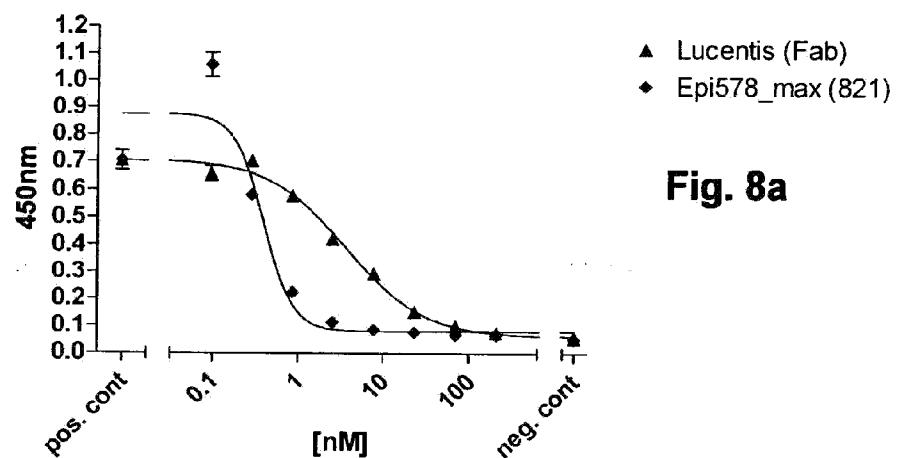


Fig. 8a

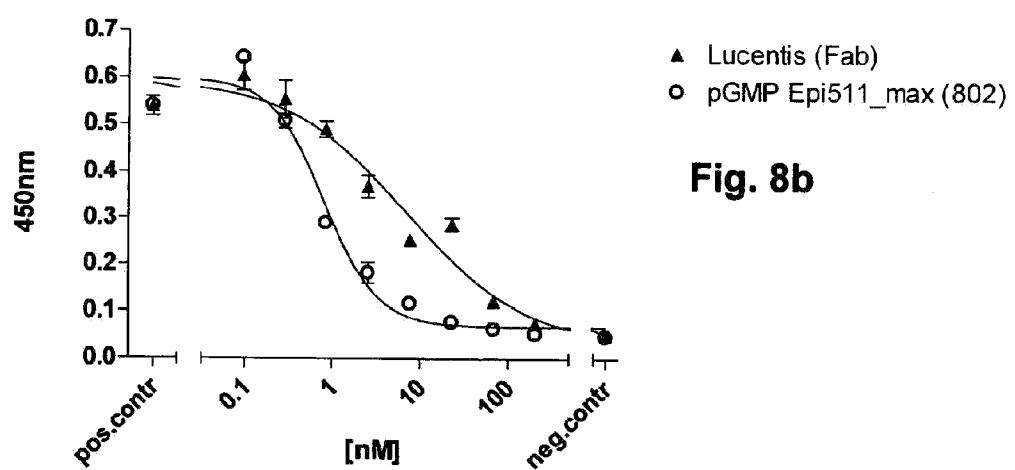
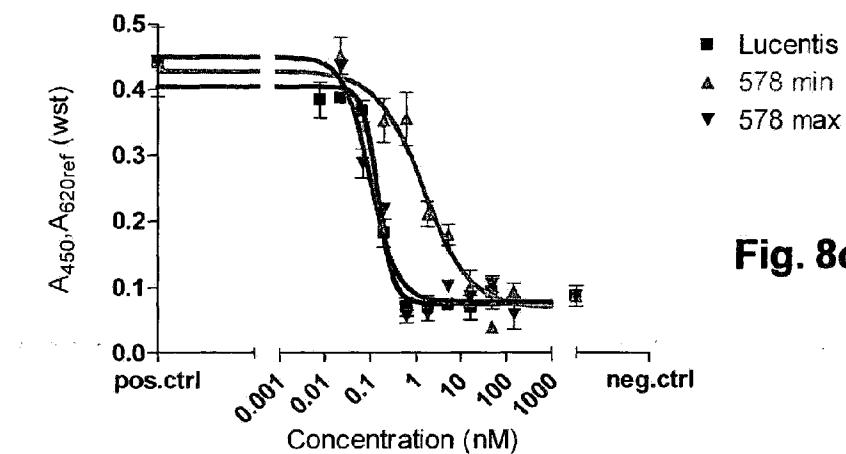
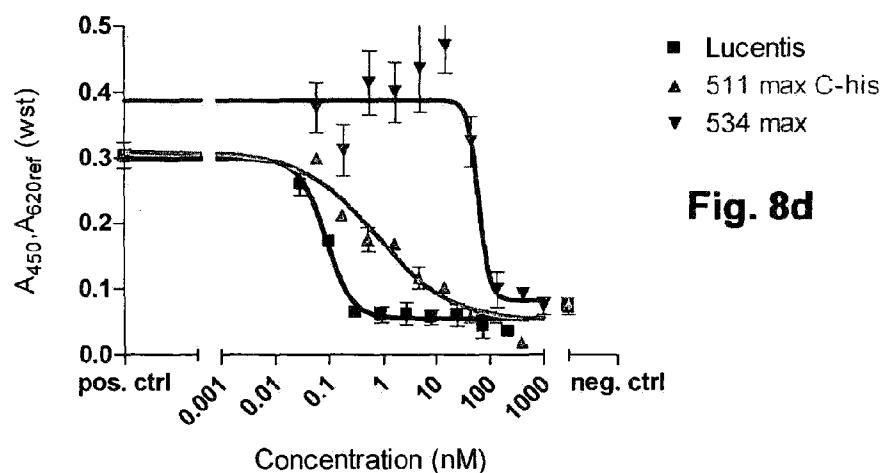
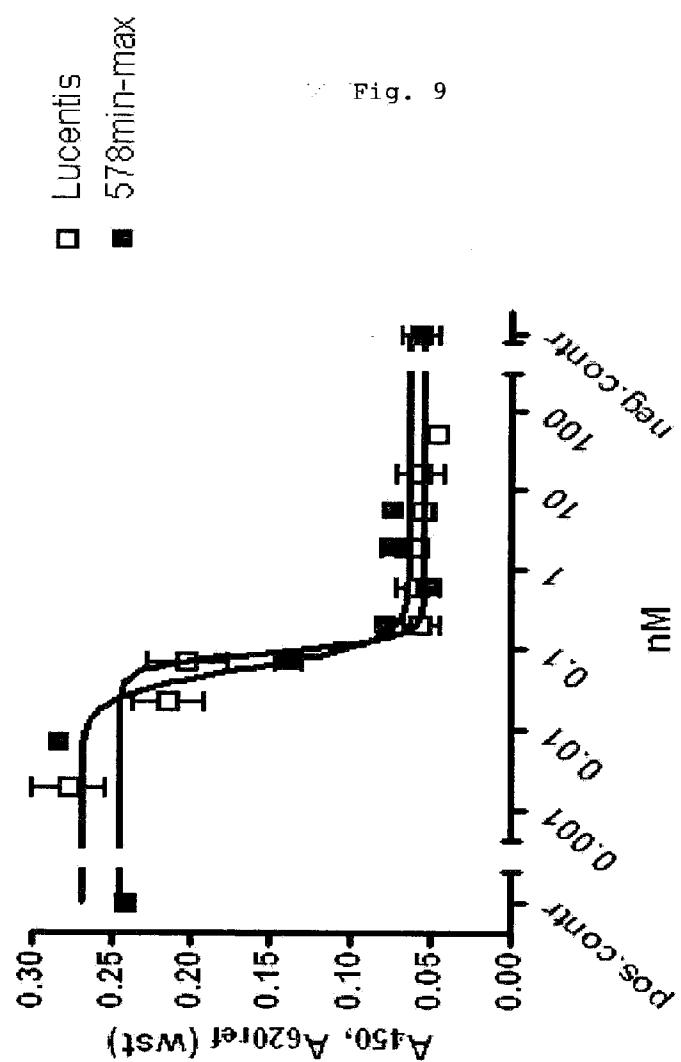


Fig. 8b

**Fig. 8c****Fig. 8d**



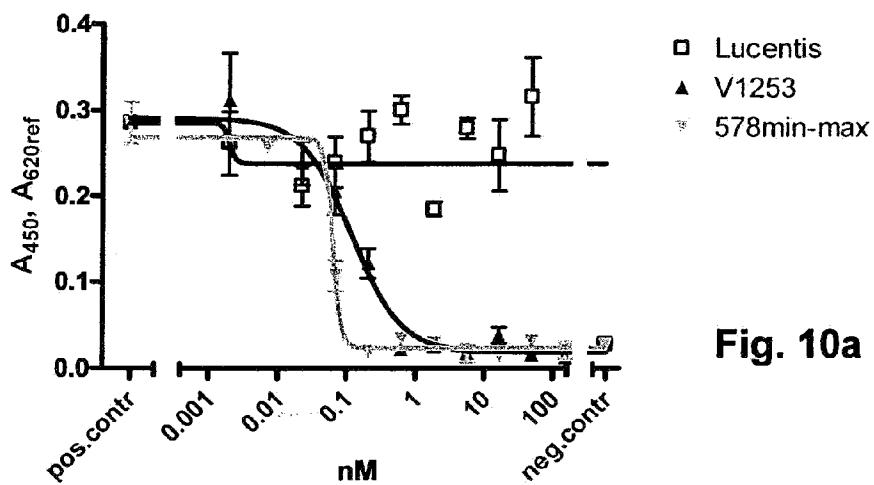


Fig. 10a

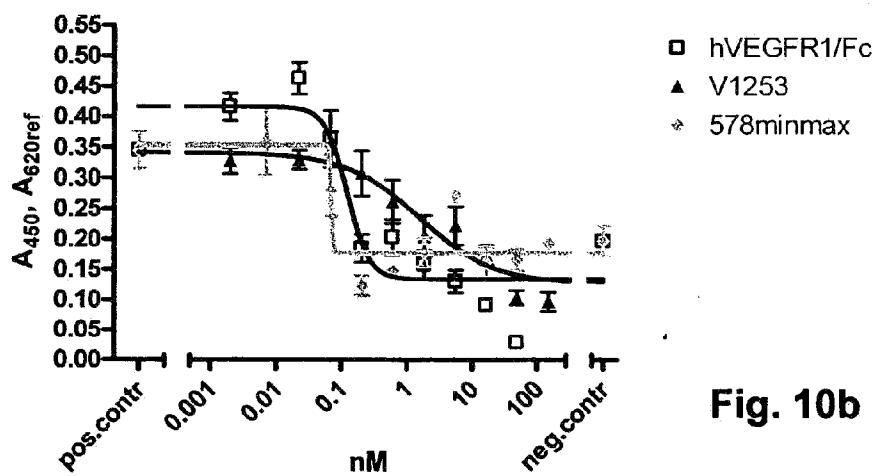
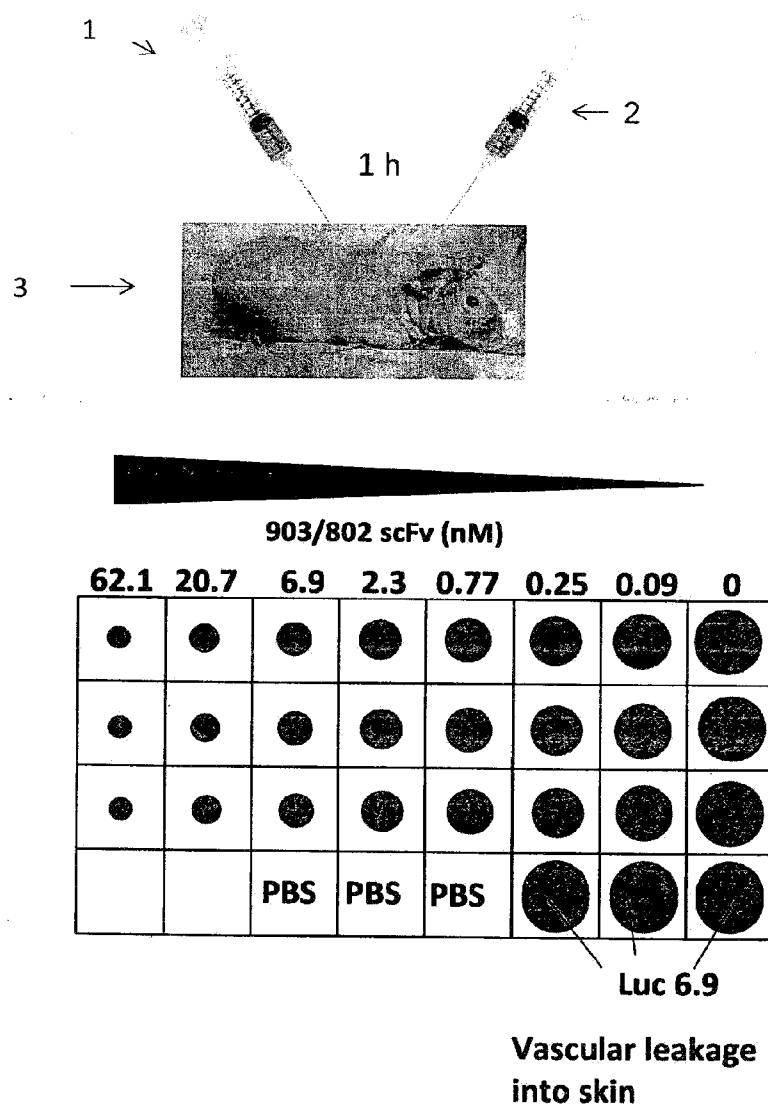
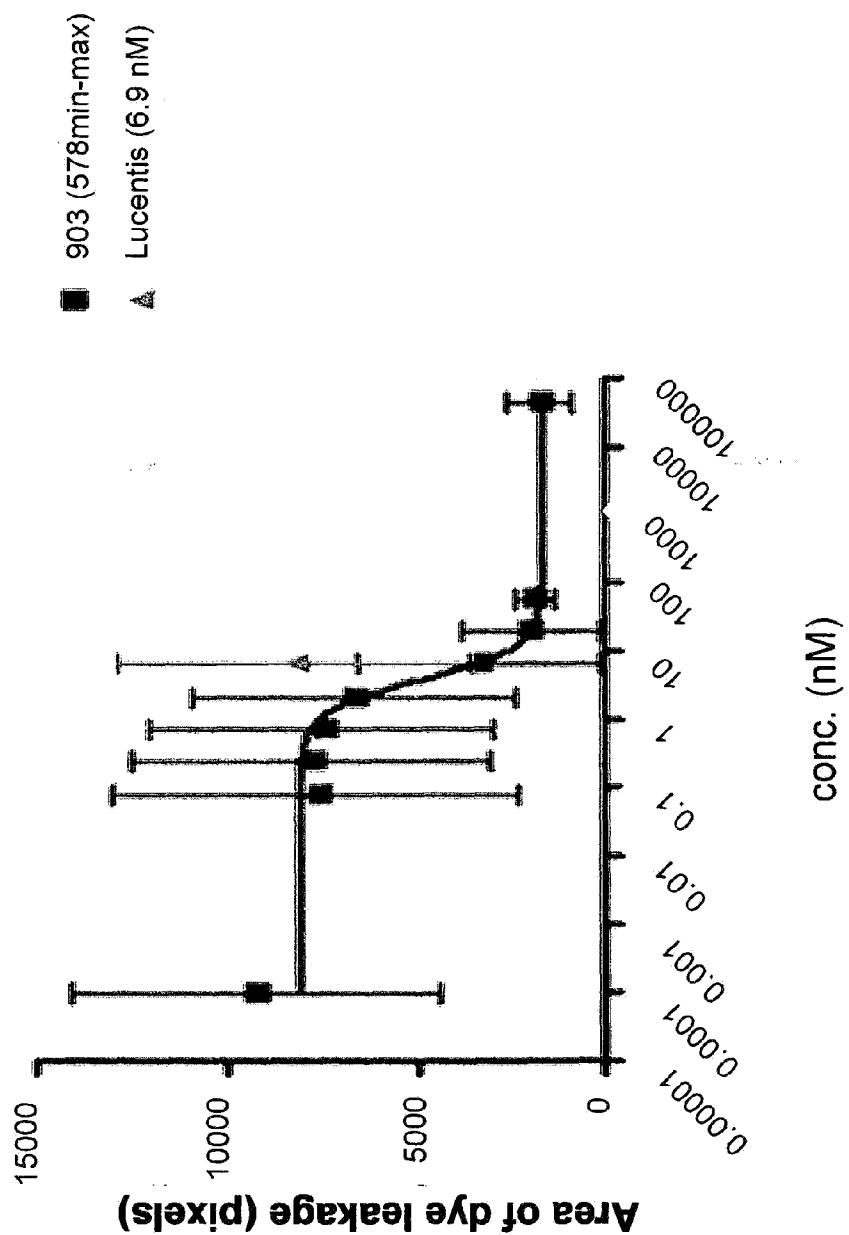


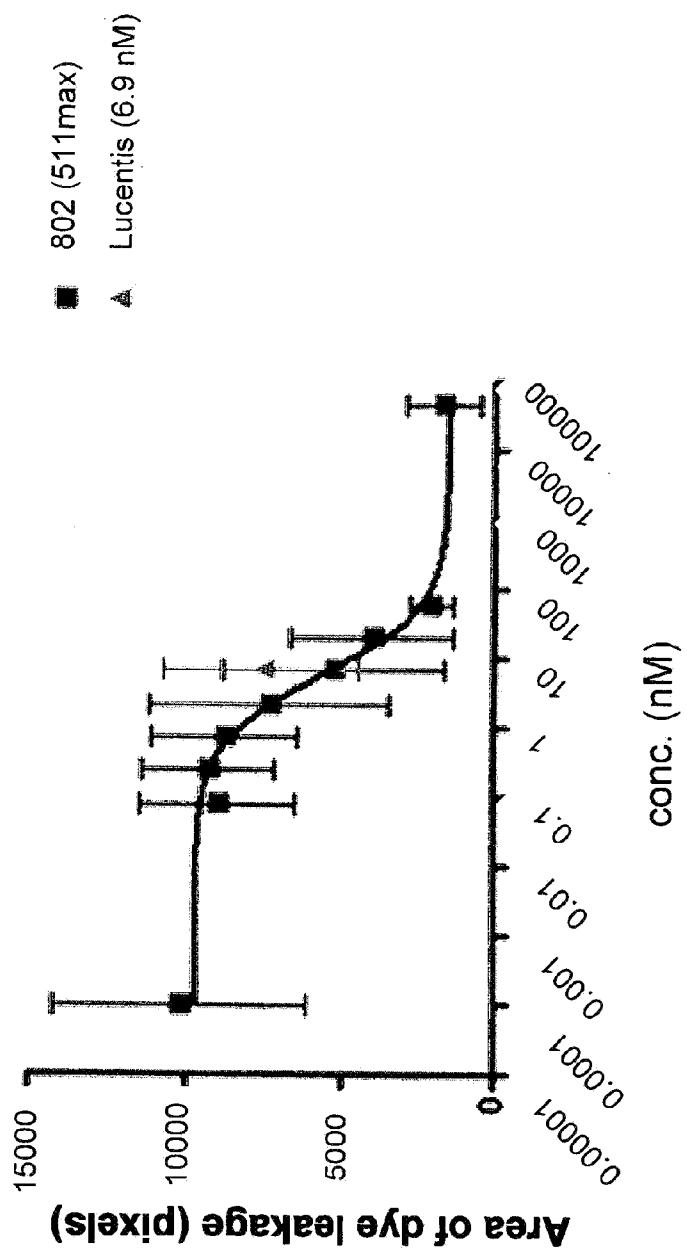
Fig. 10b

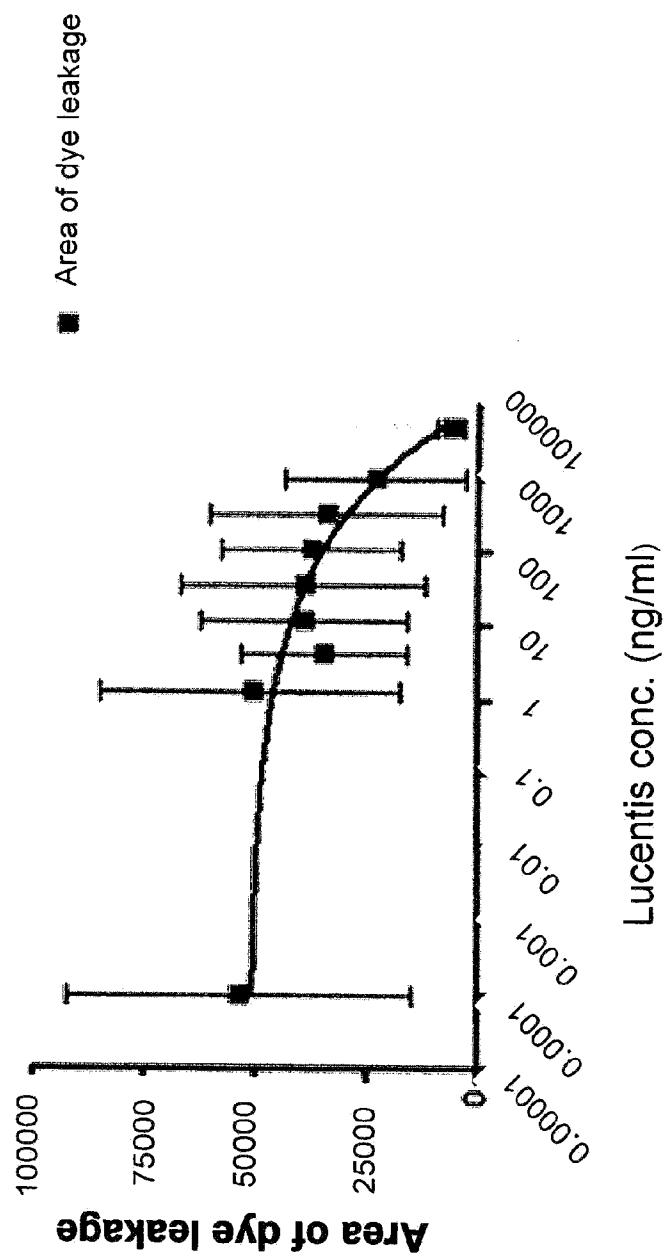
Fig. 10

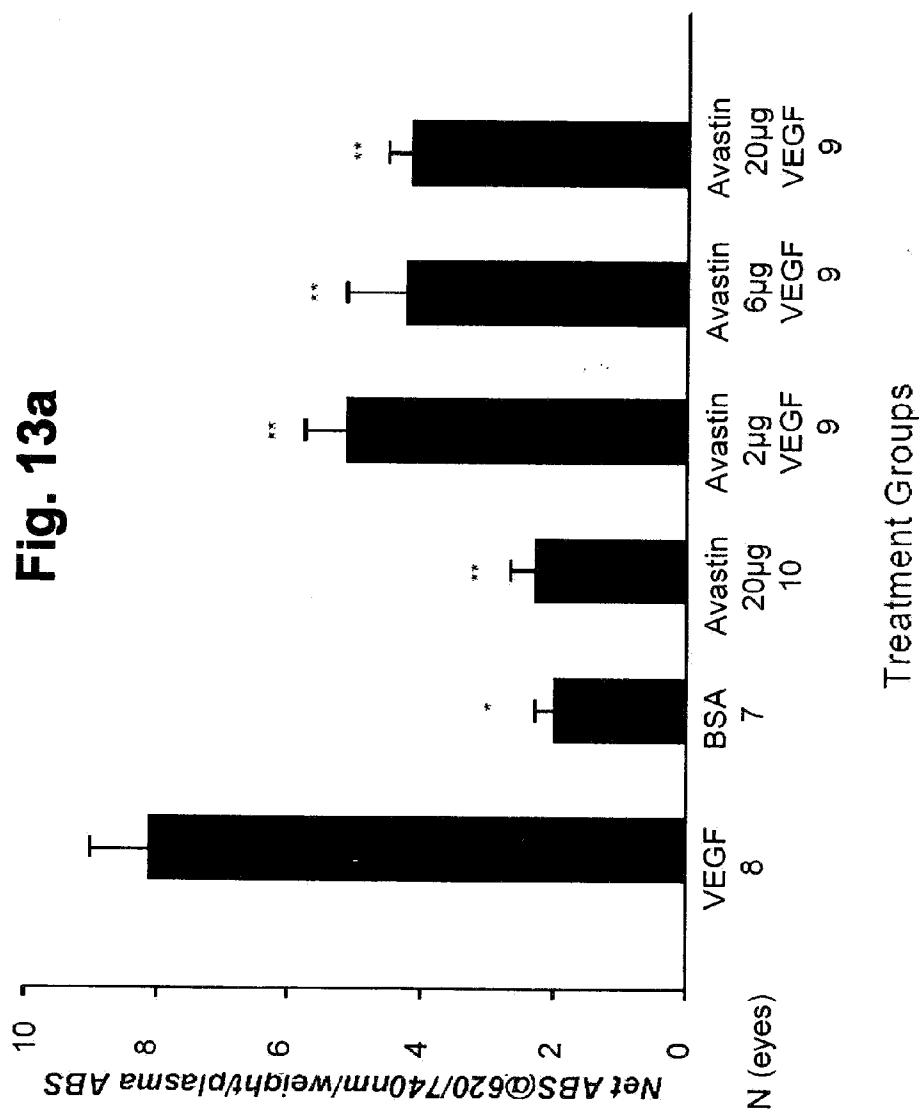


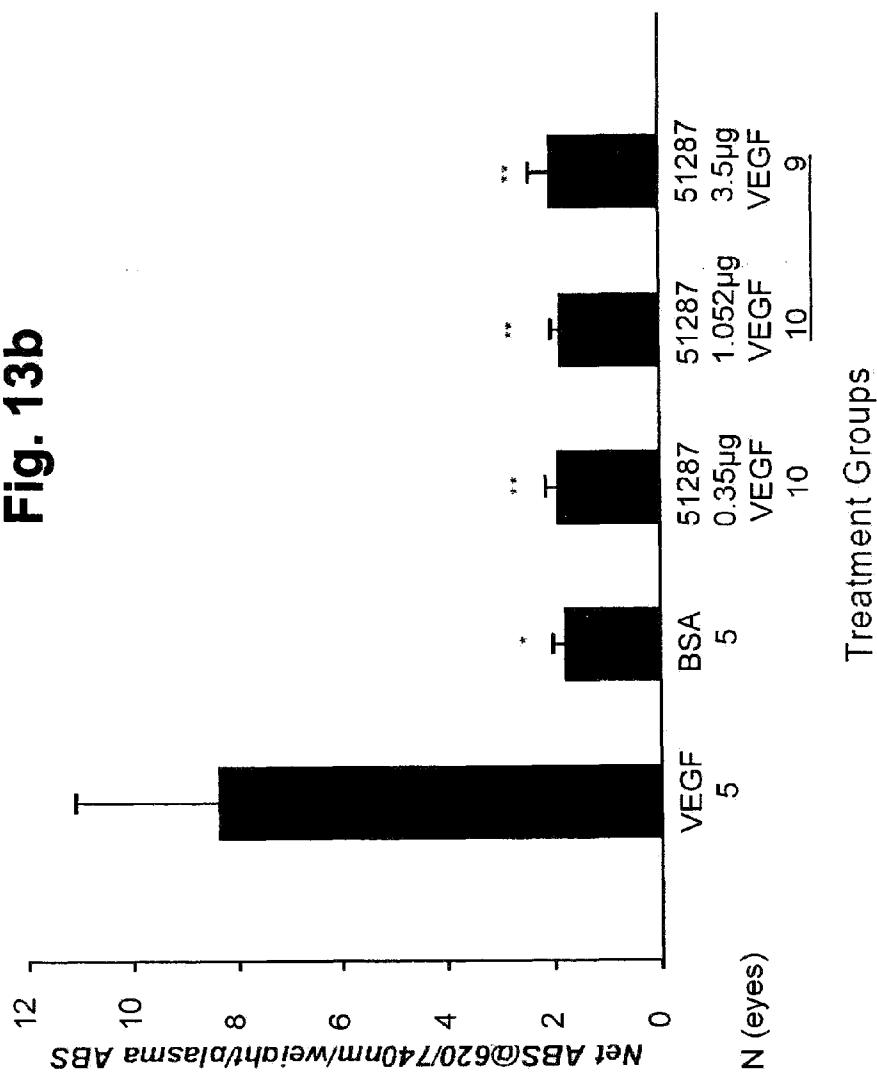
**Fig. 11**

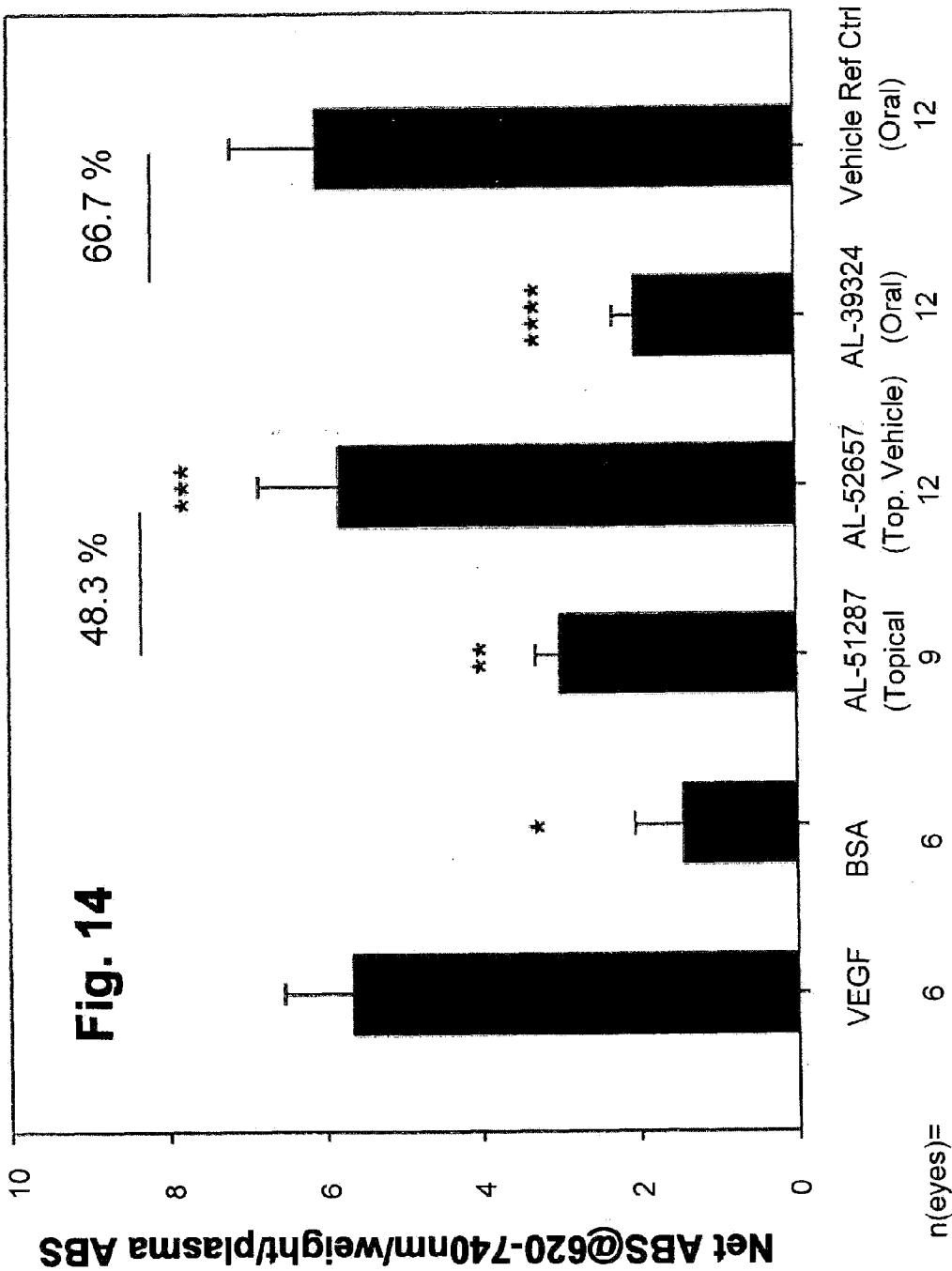
**Fig. 12a**

**Fig. 12b**

**Fig. 12c**

**Fig. 13a**

**Fig. 13b**



ESBATech's definition		Kabat										Chothia										
		Chothia					Kabat					Chothia					Kabat					
25	26	27	28	29	30	31	*	*	*	*	*	32	33	34	35	35a	35b	36				
25	26	27	28	29	30	31	31a	31b	32	33	34	35	*	*	*	*	*	36				

Figure 15

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.

